An analysis of the proteolytic system in *Aspergillus* in order to improve protein production

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Stellingen

1. Er wordt bij het optimaliseren van eiwitproduktie in *Aspergillus* stammen ten onrechte te weinig aandacht besteed aan de mate waarin een dergelijke stam in staat is tot verzuring van het cultuurmedium in relatie tot proteolyse.

2. De door Caddick (1994) beschreven indeling van mutaties in het bij de ammonium metabolietrepressie betrokken *are*A gen in *Aspergillus* is arbitrair omdat een dergelijke indeling in sterke mate afhankelijk is van de keuze van de targetgenen die hiervoor worden gebruikt.
Caddick (1994) Progress in Industrial Microbiol. **29**:347-353

3. Bij het tijdens autoregulatie negatief moduleren van de functionaliteit van het PacC eiwit is de C-terminale QED repeat niet betrokken.

4. De extracellulaire serinecarboxypeptidases in *Aspergillus niger* hebben mogelijk een functie bij de autokatalytisch geïnduceerde maturatie van het extracellulaire pepstatine-rembare zure protease PEPA.
Inoue et al. (1996) Eur. J. Biochem. **237**:719-725

5. Bij het verklaren van de verschillen in opbrengsten tussen homologe en heterologe eiwitproduktie in filamenteuze schimmels wordt de vaak afwijkende aminozuursamenstelling van heterologe eiwitten en de invloed van de veranderde aminozuurbehoefte veelal over het hoofd gezien.

6. Omdat de door Tilburn et al. (1995) waargenomen pH regulatie van het *prt*A gen in *Aspergillus nidulans* door Katz et al. (1996) niet gereproduceerd kan worden is het niet uitgesloten dat de *xpr*E mutaties en *xpr*F,G suppressoren in *Aspergillus nidulans* betrokken zijn bij pH regulatie.
Tilburn et al. (1995) EMBO J. **14**:779-790; Katz et al. (1996) Mol. Gen. Genet. **250**:715-724

7. Niet-functionerende gemeentesecretarissen, procureurs generaal, ABP functionarissen en besturen van toezichthoudende organen blijken bij niet functioneren alleen vergezeld van forse afkoopremies (de gouden handdruk) te kunnen vertrekken. Opmerkelijk hierbij is verder dat de hierover verontwaardigde Tweede-Kamerleden zelf bij vertrek gebruik maken van een zogenaamde 'aanvulling op hun nieuwe inkomen'.

8. Het voorgenomen teruggeven aan de natuur van grote stukken cultuurgrond in de provincie Zeeland vereist een aanpassing van de Zeeuwse lijfspreuk in 'Luctor et Submergo'.

9. De vertaling van het Latijnse woord doctorandus suggereert ten onrechte dat een ingenieur niet zou kunnen promoveren.

10. Het beursale AIO systeem is een stap in de richting van het creëren van een wetenschapsproletariaat.

11. Bij het uit aerodynamisch oogpunt inklapbaar maken van de verlichting van bepaalde sportauto's hebben de constructeurs over het hoofd gezien dat de wetten van de aerodynamica ook 's nachts van toepassing zijn.

12. Het aanbieden van walkman-vrije coupés zal meer mensen overhalen tot het gebruik van de trein dan de uitgebreide 'We gaan ervoor' reclamecampagnes.

Stellingen behorende bij het proefschrift

An analysis of the proteolytic system in *Aspergillus* in order to improve protein production
Wageningen, 11 juni 1996
J.P.T.W. van den Hombergh

aan Caroline en mijn ouders

Contents

1 General Introduction; fungal proteases, structure, characteristics, function and regulation[1]

J.P.T.W. van den Hombergh and J. Visser

1.1 Introduction

Traditionally the proteases have been regarded as degradative enzymes, capable of cleaving proteins into small peptides and/or amino acids, and whose role it is to digest nutrient protein or to participate in the turnover of cellular proteins. However, although the best characterized proteases have this elementary function, it has also been shown that proteases play key roles in a wide range of cellular processes, via mechanisms of selective modification by limited proteolysis, and thus can have essential regulatory functions (Holzer and Tschensche 1979; Holzer and Heinrich, 1980).

There is a number of reasons why proteases of filamentous fungi are of particular interest. The basic process of hydrolytic cleavage of peptide bonds in proteins appears costly and potentially detrimental to an organism if not properly controlled. The desired limits to proteolytic action are achieved through the specificity of proteinases, by compartmentalization of proteases and substrates within the cell, through modification of the substrates allowing recognition by the respective proteases, by regulation via zymogen activation, and the presence or absence of specific inhibitors, and of course also by regulation of protease gene expression. Studying proteases in fungi will help to understand fundamental cellular functions involving proteolysis, including intracellular protein turnover, processing, translocation, sporulation, germination and differentiation. Filamentous fungi provide interesting possibilities to study proteases as it is relatively easy to generate mutants, and powerful genetical, biochemical and molecular biological techniques are available for analysis. In fact, *Aspergillus nidulans* and *Neurospora crassa* are used as model organisms for analyzing the molecular basis of a range of physiological and developmental processes. Their well-developed genetics enable direct access to biochemical and genetical studies, under defined nutritional and cultivation conditions. Furthermore, a large group of fungi pathogenic to humans, live-stock and crop, have been isolated and proteolysis could play a role in their pathogenicity (host penetration, countering host defense mechanisms and/or nutrition during infection). Proteases are also frequently used in laboratory and clinical practice and in industrial processes; proteases both microbial and non-microbial are in wide-spread use in the food industry (baking, brewing, cheese manufacturing, meat tenderizing), in the

[1] Accepted for publication in Applied Molecular and Cellular Biology of Filamentous Fungi. S. Brown and R.M. Berka (Eds.)

tanning industry and in the manufacture of biological detergents (Aunstrup, 1980). The commercial interest in exploiting certain filamentous fungi, especially the *Aspergilli*, as hosts for the production of both homologous and heterologous proteins, has also more recently renewed interests in fungal proteases (van Brunt, 1986ab). Especially heterologous expression is often hampered by the proteolytic degradation of the expressed products by homologous proteases. These commercial interests have resulted in detailed studies of proteolytic spectra and construction of protease deficient strains and have improved the knowledge about protease expression and regulation in these organisms.

Eukaryotic microbial proteases have been reviewed by North (1982). More recently, Suarez Rendueles and Wolf (1988) have reviewed the *S. cerevisiae* proteases and their function. Here we concentrate on proteases in filamentous fungi and focus on *Aspergillus* proteases in particular.

1.2 Properties and evolution of proteases

Proteases differ from almost all other enzymes in that their substrate specificities are invariably extremely difficult to define, and certainly do not represent an acceptable basis on which to name and classify the enzymes. In literature the *in vitro* properties of proteases are classified in a number of ways, for example on the basis of the pH range over which they are active (acid, neutral or alkaline), on the basis of endo- or exo-peptidase activity, on the basis of their ability to hydrolyze specific proteins or amino acid bonds (keratinase, collagenase, elastase etc.) or on the basis of their similarities to other well-characterized proteases such as trypsin and pepsin (aspergillopepsin). A solution to this problem was put forward by Hartley (1960), who observed that the proteases seemed to act through four distinct catalytic mechanisms, and hence could be regarded as 'serine', 'thiol', 'acid' or 'metal' proteases. Now that much more is known about the chemistry of the catalytic sites and that for every type a different sensitivity to various inhibitors has been described, the concept of distinguishing these four groups of enzymes remains completely valid, although three of the four names have been amended, so that we now speak of 'serine', 'cysteine', 'aspartic' and 'metallo' proteases (Barrett, 1980). These form subclasses 21, 22, 23 and 24, respectively, of the peptide bond hydrolases (class 3.4) in the enzyme nomenclature scheme (IUB Nomenclature Committee). There is some overlap between the latter classification and the classification based on pH dependence as aspartic proteases are all active at acidic pH, metallo proteases have in general pH optima around neutrality and alkaline proteases are often serine proteases. It is however very difficult to draw any conclusions about the physiological role of a protease directly from its *in vitro* properties, since physiological substrates are not often used. The pH dependence may indicate something about the localization of a particular protease (for example an intracellular acid protease is probably located in the lysosome or the vacuole), but with non-physiological substrates considerable differences in pH

optima are observed for the hydrolysis of different proteins by the same enzyme. A pH optimum may be as much a feature of the substrate as one for the protease. Thus, substrate specificity indicates a range of proteolytic events in which a protease might be able to participate, but it cannot be always related directly to activity on the physiological substrates.

Proteases are presumed to have arisen from the earliest phases of biological evolution since even the most primitive organisms must have required them for digestion and for the metabolism of their own proteins (Neurath, 1984). In fact, todays mammalian digestive proteases can be shown to have a common ancestry with those of microbial origin. In proteolytic enzymes, certain amino acids fulfil the functions of nucleophiles, others as proton donors and each family of proteases has its characteristic set of such functional amino acid residues arranged in a particular formation to form the active site. Members of a family are generally believed to have evolved from a common ancestor (Walsh, 1975). For the serine, aspartic and cysteine proteases it appears that evolution has occurred on at least two separate occasions (Barrett, 1986); the serine proteases include two distinct families and these families presumably have evolved independently from each other; for example, the mammalian serine proteases (such as chymotrypsin) differ from certain bacterial serine proteases, the subtilisins, in amino acid sequence and three-dimensional conformation to the extent that common ancestry is not likely (Matthews, 1977). Despite these multiple origins, the serine, cysteine and aspartic proteases, at least, have one superfamily that has been overwhelmingly more successful than the others. As far as we know only this most succesful group of each catalytic class of proteases occurs in mammals - it is perfectly possible that the metallo proteases also conform to these generalizations .

1.2.1 The serine proteases

The serine-dependent mechanism of peptide bond hydrolysis may be the most successful that has evolved for endopeptidases, in that the serine proteases (EC 3.4.21) seem to be the most numerous group, and are extremely widespread and diverse. Two superfamilies can be distinguished, the chymotrypsin superfamily and the subtilisin superfamily (Hartley, 1970; James, 1976). Some serine proteases, especially in higher animals, are synthesized as zymogens, which can be very complex in some cases and can confer new properties to the enzyme, because they are sometimes not lost after cleavage.

Most commonly used serine protease inhibitors modify the serine active site residue; phenylmethylsulphonyl fluoride (PMSF); (Hartley, 1960), diisopropylfluorophosphate (DIFP) or the histidine active site residue, N-α-p-tosyl-L-lysinechloromethylketone (TLCK) and L-1-tosylamide-2-

(a)

```
Serine                                                     *
Bovin Chymotrypsin A    F H F C G G S L I s e N W V V T A A H C g v t t s d
Bovin Trypsin           y H F C G G S L I n s q W V V s A A H C y k s g i q
Pig Elastase            a H t C G G t L I r q N W V m T A A H C v d r e l t

Sutilisin                                                  *
B. subtilis Subtilisin  g g a s f v p s e t n p y q D G s s H G T H V A G T
A. niger PEPC           a t w g k t i p t n d e d l D G n g H G T H c s G T
A. niger PEPD           r a s l a y n a a g g e h v D G v g H G T H V A G T

Ser CPD                                       *
Wheat CPDIII            A L S F L K V H N A G H M V P M D Q P K A A L E M L
Rice CPD                A L S F L K V H N A G H M V P M D Q P K A A L E M L
S. cerevisiae CPY       h f t y l r V f n g G H M V P f D v P e n A L s M v
A. niger PEPF           g L m w e a t f q s G H M q P a f q P r v s y r h L
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(b)

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Serine                                                     *
Beef Chymotrypsin A     v M i C A G - a s G V - S S C M G D S G G P L V C q
Beef Trypsin            n M f C A G y l e G g k D S C q G D S G G P v V C s
Hog Elastase            s M v C A G - g n G V r S g C q G D S G G P L H C l

Subtilisin                                                 *
B. subtilis Subtilisin  V s i q s T l p g g t y a a y n G T S M A S P H V A
A. niger PEPC           i l s t w i g s n y a t n i i s G T S M A S P H i A
A. niger PEPD           V l s a w T g s n s a t n t i s G T S M A t P H V t

Serine CPD                                    *
Wheat CPDIII            K N . . D F F I T G E S Y A G H Y I P A F A S R V H
Rice CPD                K N . . D F F I T G E S Y A G H Y I P A F A S R V H
S. cerevisiae CPY       n K g q D F h I a G E S Y A G H Y I P v F A S e i l
A. niger PEPF           K N . f k i y v T G E S Y A G r Y v P y i s a a f l
```

Fig. 1.1. Serine protease catalytic sites. For the serine endo proteases, the bold residues are identical to those in beef chymotrypsin A, in the sequences around the (starred) catalytically active residue **(a)** His_{60} and **(b)** Ser_{227} (chymotrypsin A numbering). For the serine subtilisin-type endo proteases, the bold residues are identical to those in *Bacillus subtilis* subtilisin, in the sequences around the (starred) catalytically active residue **(a)** His_{170} and **(b)** Ser_{327} (*B. subtilis* subtilisin numbering). For the serine carboxypeptidases (CPDs), the bold residues are identical to those in Wheat serine carboxypeptidase III in the sequences around the (starred) catalytically active residue **(a)** Ser_{218} and **(b)** His_{472} (Wheat carboxypeptidase III numbering). The homologies around the active site Asp residues is not shown. The amino acid sequences are from Acc. No. P00767, for beef chymotrypsin A, from Acc. No. P00760 for beef trypsin, from P00772 for hog pancreatic elastase, from Acc. No. P04189 for *B. subtilis* subtilisin, from Frederick et al. (1993) for *A. niger* PEPC, from Jarai et al. (1994a) for *A. niger* PEPD, from Baulcombe et al. (1987) for wheat carboxypeptidase III, from Washio and Ishikawa, (1992) for rice CPD, from Valls et al. (1987) for *S. cerevisiae* CPY and from van den Hombergh et al. (1994) for *A. niger* PEPF.

phenylethylchloromethylketone (TPCK) (Schoelmann and Shaw, 1963). In addition, several serine protease specific microbial inhibitors, antipain, leupeptin and chemostatin, inhibit most serine proteases.

The active site of a serine protease consists of a serine, a histidine and an aspartic acid residue and especially the Ser and His residues are strongly conserved among these proteases (Fig. 1.1).The three active site residues (Ser, His and Asp) form a hydrogen bonding system often referred to as 'catalytic triad'. The catalytic mechanism is depicted in Fig. 1.2 (see Kraut, 1977 and Steitz and Shulman, 1982 for reviews on the structure and mechanisms of serine proteases). Initially the hydroxyl group of the active site serine residue attacks the carbonyl carbon atom of the scissile bond of the substrate, with general base catalysis by histidine, to give a tetrahedral intermediate adduct and an imidazolium ion. Decomposition of the tetrahedral adducts results in the release of the amino portion of the substrate and the formation of an acyl serine derivative. The leaving amino group receives a proton from the imidazole of the His-residue. The acyl enzyme is hydrolyzed to yield the active enzyme and the carboxylic acid product through the reverse reaction pathway, with the hydroxyl group of water acting as the nucleophile instead of the hydroxyl group of serine. It is well possible that the actual serine protease mechanism is more concerted than that depicted in Fig. 1.2, since it has been proposed that the His-residue acts as a proton shuttle and may never become fully protonated as shown in Fig. 1.2 (Kraut, 1977). In addition, it is possible that the enzyme distorts or strains the substrate either geometrically or electronically towards the structure of the tetrahedral intermediate (Steitz and Shulman, 1982; Delbaere and Brayer, 1985). Thus, formation of the enzyme-substrate complex and the tetrahedral intermediate could be more continuous than shown in Fig. 1.2.

In general, the substrate specificities of serine proteases are exceedingly diverse. However, classified by their primary substrate specificity, three major types of serine proteases can be identified: trypsin-like, chymotrypsin-like and elastase-like. Trypsin-like proteases cleave in general substrates with positively charged amino acid residues (Lys and Arg) in the P_1 position of the substrate whereas chymotrypsin-like and elastase-like proteases prefer substrates with aromatic or large aliphatic, and small aliphatic side chains, respectively. These specificities can to a large extent be explained by the structure of the S_1 subsites (Barrett, 1986) in these serine proteases (Kraut, 1977) but also extended substrate binding regions have been described (although they are less restrictive than S_1). Apart from the substrate binding sites on the carboxyl (P) side of the scissible bond, most serine proteases have also extended substrate binding sites on the leaving group (P') side, which, although these subsites have not been studied extensively with either substrates and/or inhibitors, are likely to be less important than the P side binding sites. It is remarkable that the enzymes of the subtilisin superfamily have acquired a mechanism similar to that of the chymotrypsin superfamily by convergent evolution. Activity of the serine proteases is most commonly maximal at slightly alkaline pH. No

absolute activator requirements are known, but Ca^{2+} ions are required in the activation reaction of some of the proenzymes, and to stabilize some of the enzymes.

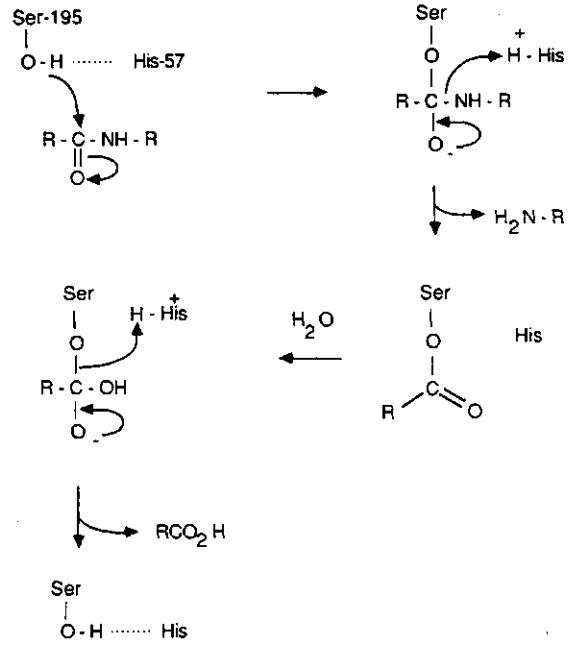

Fig. 1.2. Catalytic mechanism of a serine protease. The catalytic residues are Ser_{195}, His_{57} and Asp_{102} (not shown). Residues are numbered according to beef chymotrypsin numbering.

Fungal serine proteases

Serine endo proteases. Non acid producing fungi tend to secrete high levels of serine protease (Matsushima et al., 1981; Hänzi et al., 1993). Virtually all of the described *Aspergillus* alkaline proteases (Table 1.1) were characterized as serine proteases as they were inhibited by PMSF, antipain and chymostatin. In addition to these reagents which directly interact with the active site serine residue, some of the listed serine proteases are also inhibited by some thiol reagents such as *p*-chloromercuribenzoate. This could indicate the presence of a cysteine residue in the close proximity of the active site, which is proposed for the *yeast* carboxypeptidase Y (see **serine carboxypeptidases**). Serine protease inhibitors based on chloromethylketones, such as TLCK and TPCK, have no inhibitory effect on these fungal serine proteases. Inhibition through the modification of the active site His residues has been shown for the *A. melleus* serine protease (Kobayashi et al., 1984).

Table 1.1 *Aspergillus* serine proteases

Organism	Type[1]	Protein	Protease gene	Mw	pH_{opt}	pI	Origin	Reference
				Serine endo-proteases				
A. candidus	Ser			23.0 (?)	11.0/11.5	4.9	S	
A. flavus	Ser			18.0 (nd)			S	Turkova et al., 1969
A. flavus	Ser			19.5/22.0	8.5/10.0		S	
A. flavus								Rhodes et al., 1990
A. flavus								Ramesh et al., 1994
A. flavus var. *column.*	Ser	type PI			8.5/9.0		CF	Impoolsup et al., 1981
A. fumigatus								Monod et al., 1991
A. fumigatus								Frosco et al., 1992
A. fumigatus								Larcher et al., 1992
A. fumigatus								Jaton-Ogay et al., 1992
A. melleus	Ser			32.0 (?)	7.5/8.5		S	
A. nidulans	Ser	type PI type PII		30.9 (nd) 30.0 (nd)	6.5/7.5 6.5/7.5	4.6 4.3	ME ME	Ansari and Stevens, 1983b
A. nidulans	Ser	type PI_i type PI_e type PII_i type PII_e		39 (nd) 24 (nd) >45 (nd) 40 (nd)	6.5 6.5 6.5 6.5		ME CF ME CF	Stevens and Stevens, 1980
A. nidulans	Ser	type PI type PII type PIII?		31 (d) 30 (d)			ME ME/CF ME/CF	Stevens, 1985
A. nidulans	Ser	type α type ß type type					ME ME/CF ME/CF ME/CF	Cohen, 1973ab, 1977

Table 1.1 (continued)

Organism	Type[1]	Protein	Protease gene	Mw[2]	pH_{opt}[3]	pI	Origin	Reference
Serine endo-proteases								
A. niger	Ser			68 (d)	7.4	6.2	ME	Bosmann, 1973
A. niger	Ser			30 (nd)	7.0/10.0	3.7	CF	Sakka et al., 1985
A. niger	Subt	PEPC	*pep*C					Frederick et al., 1993
A. niger	Subt	PEPD	*pep*D					Jarai et al., 1994a
A. oryzae	Ser			23 (nd)	10.5		CF	Nakadai, 1972ab, 1973d
A. oryzae	Ser	Type PII		35 (nd)			CF	Kundu and Manna, 1975
A. oryzae								Tatsumi et al., 1989
A. oryzae var. *microsp.*	Ser			22.0 (?)	9.3/9.5	6.0	S	
A. sojae	Ser			22.5(?)	11.0	5.1	S	
A. sulphureus	Ser			23.0 (nd)	7.0/10.0		S	Turkova et al., 1972
Serine exo-proteases								
A. niger	Ser CPD Ser CPD						CP	Krishnan and Vijayalakshimi, 1985, 1986
A. niger	Ser CPD Ser CPD	CPDI CPDII		81.0 (d) 81.0 (d)			CF CF	Dal Degan et al., 1992
A. niger	Ser CPD	PEPF/CPDII	*pep*F					van den Hombergh et al., 1994

Table 1.1 (continued)

Organism	Type[1]	Protein	Prot gene	Mw[2]	pH_{opt}[3]	pI	Origin	Reference
				Serine exo-proteases				
A. niger	Ser CPD	CPY	*cpy*					Yaver et al., 1995
A. niger var. *macrosporus*	Ser CPD			136.0 (nd) 60.0 (d)	3.0	4.1	CF	Kumagai et al., 1981 Kumagai and Yamasaki, 1981
A. oryzae	Ser CPD	type CPI		120.0 (nd)	4.0		CF	Nakadai, 1972ab
	Ser CPD	type CPII		105.0 (nd)	3.0		CF	
	Ser CPD	type CPIII		61.0 (nd)	3.0		CF	
	Ser CPD	type CPIV		45.0 (nd)	4.0		CF	
A. oryzae	Ser CPD	type CPIV		130.0 (nd)			CF	Nakadai and Nasuno, 1977a
A. oryzae	Ser CPD	type CPO-1		63.0 (nd)	3.7/4.0	4.08	CF	Takeuchi and Ichishima, 1981 Takeuchi et al., 1982
		type CPO-2		63.0 (nd)	3.7/4.0	4.18	CF	
A. oryzae	Ser APD	Amino type IV		130.0 (nd)	7.0		CF	Nakadai and Nasuno, 1977b
A. oryzae	Ser CPD							Nomi et al., 1978
A. satoi	Ser CPD			125.0 (nd)			CF	Takeuchi and Ichishima, 1986
	Ser CPD			72.0 (nd)			CF	
A. satoi	Ser CPD	type CPI_{ab}		130.0 (nd)	3.0/3.5	4.08/4.22	ME	Ichishima et al., 1980
	Ser CPD	type CPII		130.0 (nd)	3.0/3.5	4.16	ME	
	Ser CPD			130.0 (nd)	3.0/3.5	4.17	CF	
A. satoi	Ser CPD	150.0 (nd)					S	Ichishima et al., 1972
	Ser CPD	51,0 (nd)						

[1] Ser, serine endoprotease; Subt, subtilisin; Ser CPD, serine carboxypeptidase; Ser APD, serine aminopeptidase
[2] nd, non dissociating conditions; d, dissociating conditions
[3] S, soluble fraction; CS, culture supernatant; CP, commercial preparation; ME, mycelial extract

All characterized fungal serine proteases are monomeric and have molecular masses between 18.5 and 35 kDa, usually around 25 kDa (Table 1.1) although, in some species, including *A. niger* (Bosmann, 1973) and *A. nidulans* (Stevens and Stevens, 1980) larger enzymes have been reported. From some *Aspergillus* species more than one serine protease has been characterized (Cohen, 1973ab; Stevens and Stevens, 1980; Stevens, 1985). Serine proteases can be glycosylated and their isoelectric points (IEP) are often low, between 4.4 and 6.2, but in a few cases high IEPs have been reported. For example *Fusarium* species (Tomoda et al., 1979) and *N. crassa* extracellular protease have IEPs of 8.9 and higher. In general fungal serine proteases have broad substrate specificities; most enzymes hydrolyse the Leu(15)-Tyr(16) and Phe(25)-Tyr(26) bonds of oxidized ß-insulin. In addition, the Gln(4)-His(5), Glu(13)-Ala(14), Tyr(16)-Leu(17) and Tyr(26)-Thr(27) bonds are cleaved by many of these fungal serine endo proteases. Klapper and coworkers (1973a) reported in addition to the peptidase activity, an esterase activity for some *A. oryzae* alkaline proteases. Substrate competition experiments have shown that this activity uses the same active site as the peptidase activity (Klapper et al. 1973b). In *Aspergillus* a correlation has been postulated between the presence of an active alkaline protease and the ability to grow on media containing collagen as sole carbon and nitrogen source. However, *A. niger* failed to show any substantial growth and proteolytic activity on collagen, although a subtilisin-type gene, *pep*D, was isolated using a polymerase chain reaction (PCR) approach with degenerate primers based on conserved regions of subtilisin-type proteases (Jarai et al., 1994a). The other cloned *A. niger* endo-serine protease *pep*C is homologous to the yeast *ysc*B gene. Thus the PEPC protein will probably also be located in the vacuole, similar to the situation in yeast (see also **1.4.1.3**). The *pep*D exon-intron structure and the PEPD deduced amino acid sequence show similarities to the genes and their encoded secreted alkaline proteases of *A. oryzae*, *A. fumigatus* and *A. nidulans* (Cheevadhanarak et al., 1991; Jaton-Ogay et al., 1992; Katz et al., 1995). Furthermore, alkaline serine proteases have been isolated from *A. niger*; a serine protease with a molecular weight of 30 kDa was previously purified from an *A. niger* mutant (Sakka et al., 1985) and Barthomeuf and co-workers (1989) describe a serine protease, isolated from an *A. niger* strain grown on defatted soy cakes. The latter enzyme has a molecular weight of 21 kDa and has the capacity to hydrolyse a synthetic peptide with a sequence that is commonly found in type I collagen, but is also present in casein and hemoglobin.

Serine carboxypeptidases. The molecular mass of serine carboxypeptidases, Ser CPDs (EC 3.4.16), isolated from individual filamentous fungi varies from 45 to 150 kDa under native conditions. Molecular masses identified under dissociating conditions and inhibition studies with ^{14}C-DIFP indicate that many of these enzymes are active as dimers or trimers (Kumagai et al. 1981; Kumagai

and Yamasaki, 1981). Whereas the fungal Ser CPDs appear to be homo-subunit enzymes and formation of the enzyme complex does not involve covalent binding, the plant Ser CPDs contain non-identical subunits and disulphide bridges are involved in complexation of the individual subunits. All characterized fungal Ser CPDs have IEPs between 4.1 and 4.2 and have pH optima between 3.0 and 4.0. Little is known about the degree of glycosylation of the individual enzymes. Both the *A. niger* CPDI and CPDII and the Ser CPD from *A. niger* var. *awamori* are heavily glycosylated (approx. 25% of the M_w; Kumagai and Yamasaki, 1981; Dal Degan et al., 1992).

Protein hydrolysis can be considered as a transacylation reaction with a peptide as acyldonor, H_2O as acylacceptor and an amino acid as leaving group (see Fig. 1.3). From studies with synthetic substrates it is known that Ser CPDs can also catalyse reactions with other acyldonors and acylreceptors. The Ser CPD from *A. satoi* and CPY from *S. cerevisiae* have both peptidase- and amidase- activities (Takeuchi and Ichishima, 1986; Breddam, 1986). This phenomenon has been studied in detail for a vacuolar Ser CPD of *S. cerevisiae*, yscY or CPY. In addition to the peptidase and amidase activities, yscY has also esterase activity, indicating that yscY does not need a carboxylgroup as leaving group (Breddam, 1986). From hydrolysis studies with synthetic substrates a model for the substrate binding site was deduced (Fig. 1.3). The typical dead-end structure in this model can possibly explain the exo-peptidase character of the activity. Two substrate binding sites are present for the side-chains at the P1 and the P_1' position of the substrate. Substrate specificity is largely determined by the specificity towards these side chains.

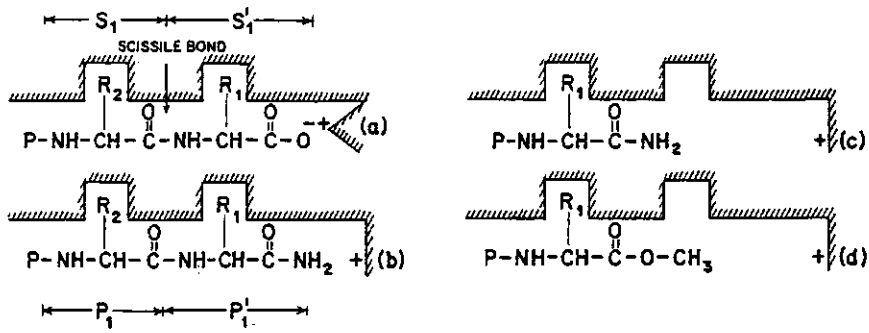

Fig. 1.3. Schematic representation of the substrate binding in the active site of serine carboxypeptidase Y (as modified from Breddam, 1986). The figure demonstrates conceivable binding modes for peptides, peptide amides, and peptide esters, accounting for peptidase (**a**), peptidyl amino acid amide hydrolase (**b**), amidase (**c**), and esterase (**d**) activities of the enzyme. The subsites and the substrate positions are numbered S_1, S_2, etc. and P_1, P_2, etc., respectively, away from the catalytic site towards the N-terminus of the substrate, and S'_1, S'_2, etc. and P'_1, P'_2, etc., respectively, towards the C-terminus.

The active site mechanisms of serine endo- and serine carboxypeptidases are probably very similar. Ser CPDs are also inhibited by PMSF, DIPF, TLCK and TPCK, indicating the presence of active site Ser and His residues. Furthermore the presence of free sulphydryl groups and inhibition by mono iodoacetate and p-chloromercuribenzoate have been described for Ser CPDs from *A. oryzae* and *A. satoi* (Ichishima et al., 1980ab; Takeuchi and Ichishima, 1981; Takeuchi et al., 1982).

Serine aminopeptidases. Literature concerning filamentous fungal serine aminopeptidases (EC 3.4.11) is very limited. Most aminopeptidases produced by *Aspergilli* are metallo-type amino peptidases (see **1.2.4**). In *A. oryzae* three (metallo-)amino peptidases (I, II and III) and one (serine)amino peptidase (IV) have been characterized (Nakadai et al., 1973abc, 1977b). The amino-peptidase IV from *A. oryzae* is inhibited by DIFP and N-bromosuccinimide but is insensitive to sulphydryl reagents. The enzyme was isolated from a culture filtrate, has a molecular mass of 130 kDa (native conditions) and a pH optimum of 7.0. It catalyses preferentially the hydrolysis of oligopeptides with an N-terminal leucine, although some other oligopeptides are also hydrolysed.

1.2.2 The Cysteine proteases

Cysteine proteases (EC 3.4.22) are momentarily divided into at least five distinct families or classes; the papain and calpain family, the strepcoccal cysteine proteases, the clostripain family and the viral cysteine proteases (Barrett, 1986). The best characterized is the papain family and it is now clear that the papain superfamily contains most of the known cysteine proteases. Amino acid sequence data (Fig. 1.4) show that cysteine proteases of higher plants (such as papain from *Carica papaya*) are members of the papain superfamily, as are human cathepsin B and rat cathepsin H. Another large group of cysteine proteases requires Ca^{2+} for activity and these enzymes are therefore called calpains. Calpains differ from other cysteine proteases in substrate specificity and have a highly conserved molecular mass comprising two subunits of 80 and 30 kDa. The essential Cys residue is located in the 80 kDa subunit, in the part that is homologous with papain (Ohno et al., 1984) (Fig. 1.4). C-terminal segments of the 80 kDa subunit contain calmodulin-like calcium-binding sites that are undoubtedly responsible for the effects of Ca^{2+} on the activity of calpains. In contrast to the role of the zinc atom in many metallo proteases, which participates directly in the catalytic mechanisms, the role of Ca^{2+} in the calpains seems to be to mediate masking or unmasking of the whole active site by a conformational change, since binding of protein inhibitors is also affected. After the discovery of the evolutionary relationship of the calpains to papain, it was concluded that all known mammalian cysteine proteases belong to a single evolutionary superfamily, as is also the case for serine and aspartic proteases. In analogy with subtilisin amongst the serine proteases, bacteria but also eukaryotic

(a)

```
Papain                                                          *
C. papaya Papain                G A V T - P V K N Q G S C G S C W A F S A V V T I E
Human Cathepsin B               p t i k - e i r d Q G S C G S C W A F G A V e a I s
Rat Cathepsin H                 G n V v s P V K N Q G A C G S C W t F S t t g a l E

Calpain                                                         *
Human Calpain                   d G A T R T D I C Q G A L G D C W L L A A I a S L T
Chicken Calpain                 g G A T R T D I C Q G A L G D C W L L A A I g S L T
A. nidulans PalB                t v s g n T D l v Q d v L t D C s v v A s l c a t T

Streptococcus cysteine protease                                 *
S. pyrogenes protease           k p g e q s f v g q a a t G h C v a t a t a q i m k

Clostripain                                                     *
C. histolyticum Clostripain     t l t e x q s v d l l a f d a C m

Viral cysteine protease                                         *
Polio Virus protease            t a k t L m Y n f P T r a g q C G g v i t c t - G -
Cowpea Musaic Virus (CPMV)      - - r y L e Y e a P T i p e d C G s l v i a h i G v
```

(b)

```
Papain                            *
C. papaya Papain                D H A V a A V G Y G p n - - - - Y I L I K N S W G T
Human Cathepsin B               g H A i r i l G w G v e n g t p Y w L v a N S W n T
Rat Cathepsin H                 n H A V l A V G Y e - q n g l l Y w i v K N S W G s

Calpain                           *
Human Calpain                   G H A Y S V T g a K q V N Y R G Q v v s L I R m R N
Chicken Calpain                 G H A Y S V T a f K d V N Y R G Q q e q L I R i R N
A. nidulans PalB                e H d Y A i l d m K e l k g R r Q f - - L I - - k N

Streptococcus cysteine protease   *
S. pyogenes protease            g H A f v i d d g a g r N f y h v d w g w g g v s d

Viral cysteine protease           *
Polio Virus protease            m H V g G - n G s h G f a a a L k r - s y f t Q s Q
Cowpea Musaic Virus (CPMV)      v H V a G i q G k i G c A s l L p p l e p i A Q a Q
```

Fig. 1.4. Cysteine protease catalytic sites. For the papain-type proteases, the bold residues are identical to those in *Carica papaya* papain in the sequences around the (starred) catalytically active residue **(a)** Cys_{25} and **(b)** His_{159} (*Carica papaya* papain numbering). For the calpain-type proteases the bold residues are identical to those in human calpain in the sequences around the (starred) catalytically active Cys and His residues. For the *Streptococcus* cysteine protease, the clostripain and the viral cysteine protease, the bold residues are identical to those in either *Carica papaya* papain or human calpain. The putative catalytic histidine residue of clostripain has not been identified. Whereas the calpains are clearly related to the papains, the *Streptococcus* protease is related distantly, if at all, and the clostripain and the polio virus enzyme are unrelated (see text). The amino acid sequences are from Dayhoff (1972) for *C. papaya* Papain, from Ritonja et al. (1985) for human cathepsin B, from (P07384) for human calpain, from Takio et al. (1983) for rat cathepsin H, from Ohno et al. (1984) for chicken calpain, from Denison et al. (1995) for *A. nidulans* PalB, from Dayhoff et al. (1978) for the *Streptococcus pyrogenes* protease, from Gilles et al. (1983) for *Clostridium histolyticum* clostripain and from Argos et al. (1984) for the polio virus protease.

microorganisms may contain cysteine proteases that have evolved independently. These are represented by the proteases from *Streptococcus* species (Tai et al., 1976) and clostripain from *Clostridium histolyticum* (Siffert et al., 1976). The *Streptococcus* proteinase is unusual amongst bacterial proteases in being synthesized as a zymogen with an N-terminal activation peptide (Yonaha et al., 1982). The locations in the polypeptide chain of the cysteine and histidine residues involved in catalytic activity are not very different from those of the papain-like cysteine proteases, but the amino acid sequences around them are different (Fig. 1.4). Like papain and many of it relatives the streptococcal protease has a S_2 specificity subsite with a strong preference for binding side chains resembling that of phenylalanine (Kortt and Liu, 1973). This unusual characteristic is consistent with the hypothesis that the streptococcal enzyme is a very distant relative of papain rather than a product of convergent evolution. X-ray crystallographic data can perhaps give a more definite conclusion on this possible relationship.

Clostripain is a cysteine protease highly specific for substrates with an arginyl residue at P_1 like some serine proteases (Siffert et al., 1976). The enzyme is, like calpains, calcium-activated but the sequence around the essential cysteine is different from any other known cysteine protease (Gilles et al., 1983) (Fig. 1.4).

Finally, some viral cysteine proteases like the polio virus proteinase (see Fig. 1.4) and a type of cysteine proteases found in germinating beans may also be representatives of separate cysteine protease classes, as they show no homology with any other known cysteine protease.

The catalytic mechanism of papain-type cysteine proteases is shown in Fig. 1.5 (reviewed in 1982 by Polgar and Halasz). Cysteine proteases contain a catalytically active cysteine sulphydryl group and a histidine imidazole group within the active site of the enzyme (Polgar, 1973; Lowe, 1976; Polgar and Halasz, 1982; Gilles and Keil, 1984). The thiolate-imidazolium ion pair between Cys and His is the reactive nucleophile. An acyl enzyme is formed with the thiol ester of Cys and hydrolyzed similarly as described for the serine proteases (see **1.2.1**). The enzymes function via an attack of the Cys sulphydryl group on an amide or ester carbonyl group to form a covalent intermediate, the thioester with the release of the first product (Lowe and Williams, 1965). The acyl group of the thio ester is transferred to a water molecule in a slow process to liberate the carboxylic acid and to regenerate the enzyme. Attack of water on the thioester in the deacylation step is catalyzed by the imidazole group of His.

Alkylation of the cysteine sulphydryl group by a variety of reagents, e.g. *N*-ethylmaleimide, iodo- and bromoacetates, *p*-chloromercuric benzoate (Means and Feeney, 1971), or pyridyldisulphides (Brocklehurst and Little, 1973; Brocklehurst, 1979) renders a cysteine protease catalytically inactive. Thus sensitivity to thiol-blocking reagents, and their activation by reagents expected to regenerate

thiol groups from disulphides specifically identifies a cysteine protease.

Papain and the related plant cysteine proteases generally show maximal activity at neutral or slightly alkaline pH (Zucker et al., 1985), and the calpains are virtually all most active at about pH 7.8. Lysosomal cysteine proteases are unstable above neutral pH and this contributes certainly to the observation that they have acidic pH optima in most assay systems. Under favourable conditions, assays with synthetic substrates can reveal pH optima above neutral pH however, suggesting that the catalytic mechanisms may not be fundamentally different from those of the plant enzymes (Willenbrock and Brocklehurst, 1984; Mason et al., 1985). In general, assays with protein substrates tend to show low pH optima for the additional reason that a substrate often becomes unfolded and as a consequence is more susceptible to hydrolysis under acidic conditions.

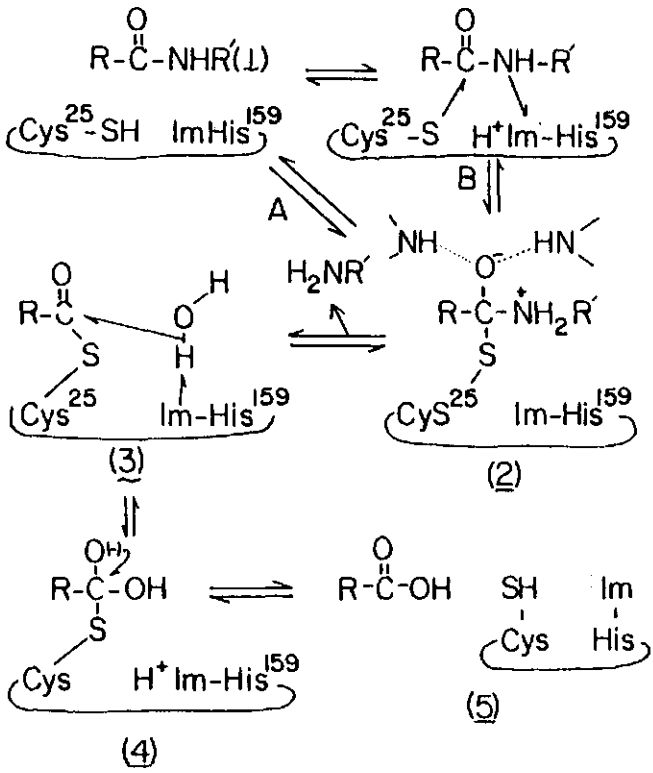

Fig. 1.5. Mechanism of hydrolyis of peptide substrates by cysteine proteases. The catalytic residues are Cys_{25} and His_{159} (numbered according to papain numbering).

Fungal cysteine proteases

The occurence of cysteine proteases in fungi is very limited. Since *p*-chloromercuribenzoate inhibits a number of serine proteases, proof that an enzyme is a cysteine protease must depend on the demonstration that it is sensitive to additional cysteine protease inhibitors, such as iodoacetate, and preferably that its activity can be enhanced by reducing agents, such as cysteine and dithiothreitol (DTT). An extracellular enzyme that was inhibited by thiol reagents and enhanced by reducing agents has been reported in *A. oryzae*. (Kandu and Manna, 1975). Furthermore, an extracellular collagenase of *A. sclerotionum* may also be a cysteine protease, even though it was inhibited by EDTA (Kandu et al., 1974). Very recently, Denison and coworkers (1995) showed that one of the factors involved in the signalling of ambient pH, PalB, has amino acid sequence similarity to the catalytic domain of the calpain family of calcium-activated cysteine proteases (see also **1.5.1** pH regulation).

1.2.3 The Aspartic proteases

Aspartic proteases (EC 3.4.23) seem to be confined to the eukaryotes and thus they appear to be one of the younger classes of proteases. In general aspartic proteases have molecular masses ranging from 30-45 kD and isoelectric points below pH 5.1, although exceptions have been reported. From X-ray crystallography and amino acid sequence data it is concluded that these proteases have evolved from each other by duplication of genetic material (Tang, 1979). Moreover, each of the two halves of the molecule is itself a duplicated structure, so that the enzymes are constructed of four copies of a primitive unit. At what stage these duplications have taken place is not known, but there is a possibility that the *Scytalidium lignicolum* acid protease represents such a primitive molecule (Barrett, 1986; Maita et al., 1984). Most aspartic proteases are produced as zymogens, auto-activated by limited proteolysis at acid pH. Most zymogens appear to be stable at neutral pH, whereas activated aspartic proteases are irreversibly inactivated above neutral pH.

Most pepsin-type aspartic proteases are subject to tight-binding inhibition by pepstatin and various other 'statin'-containing inhibitors. Additionally it was demonstrated that the pro-peptides of several aspartic proteases show strong inhibiton. The very low pH optima of aspartic proteases implicated the involvement of carboxyl groups in the active site mechanism, which was proven by the specific inhibition of aspartyl protease activity by blocking aspartic acid residues either by diazo-acetyl-norleucine methyl ester (DAN) or by 1,2-epoxy-3-(*p*-nitro-phenoxy)propane (EPNP) (Tang et al., 1973; Tang, 1979). However, the pepsin inhibitor *p*-bromophenacyl bromide does not inhibit fungal aspartic proteases. A number of aspartic proteases are sensitive to *N*-bromosuccinimide, I_2 and potassium permanganate, suggesting the possible involvement of a tyrosine. Not every aspartic

protease is inhibited by pepstatin and a small but significant group of insensitive fungal enzymes have now been reported.

(a)

Pepsin																									
														*											
Pig Pepsin	**I**	**G**	**I**	**G**	**T**	**P**	a	**Q**	d	**F**	**T**	**V**	**I**	**F**	**D**	**T**	-	**G**	**S**	**S**	**N**	**L**	**W**	**V**	**P**	**S**
Hig Cathepsin D	**I**	**G**	**I**	**G**	**T**	**P**	p	**Q**	c	**F**	**T**	**V**	v	**F**	**D**	**T**	-	**G**	**S**	**S**	**N**	**L**	**W**	**V**	**P**	**S**
Mouse Rennin	**I**	**G**	**I**	**G**	**T**	**P**	p	**Q**	t	**F**	k	**V**	**I**	**F**	**D**	**T**	-	**G**	**S**	a	**N**	**L**	**W**	**V**	**P**	**S**
Bovine Chymosin	**I**	y	l	**G**	**T**	**P**	p	**Q**	e	**F**	**T**	**V**	l	**F**	**D**	**T**	-	**G**	**S**	**S**	d	f	**W**	**V**	**P**	**S**
A. niger Aspergillipepsin E	**I**	e	l	**G**	**T**	**P**	p	**Q**	k	**F**	k	**V**	v	l	**D**	**T**	-	**G**	**S**	**S**	**N**	**L**	**W**	**V**	**P**	**S**
A. niger Aspergillopepsin A	v	t	v	**G**	k	s	t	.	.	l	h	l	d	**F**	**D**	**T**	-	**G**	**S**	a	d	**L**	**W**	**V**	f	**S**
P. janthinellum Penicillopepsin	t	p	v	t	i	g	g	t	t	l	n	l	n	**F**	**D**	**T**	-	**G**	**S**	a	d	**L**	**W**	**V**	f	**S**
Non pepsin																										
Scytalidium acid protease B	s	e	g	d	s	i	q	m	s	v	**T**	a	t	s	**D**	**T**	s	**G**	**S**	a	t	**L**	e	n	l	t
A. niger var. *macrosporus* PEPA	s	e	g	d	s	i	k	v	t	v	e	a	t	s	k	s	s	**G**	**S**	a	t	v	e	n	l	t

(b)

Pepsin																									
														*											
Pig Pepsin	**G**	e	**T**	i	**A**	**C**	s	**G**	**G**	**C**	**Q**	**A**	**I**	**V**	**D**	**T**	**G**	**T**	**S**	**L**	**L**	t	**G**	**P**	**T**	**S**
Pig Cathepsin D	s	s	l	t	l	**C**	k	**G**	**G**	**C**	**E**	**A**	**I**	**V**	**D**	**T**	**G**	**T**	**S**	**L**	i	v	**G**	q	p	e
Mouse Rennin	s	s	**T**	l	l	**C**	e	e	**G**	**C**	e	v	v	**V**	**D**	**T**	**G**	s	**S**	f	i	s	a	**P**	**T**	**S**
Bovine Chymosin	**G**	v	v	v	**A**	**C**	e	**G**	**G**	**C**	**Q**	**A**	**I**	l	**D**	**T**	**G**	**T**	**S**	k	**L**	v	**G**	**P**	s	**S**
A. niger Aspergillopepsin E	**G**	d	d	v	**A**	e	m	e	n	t	g	v	**I**	l	**D**	**T**	**G**	**T**	**S**	**L**	i	a	l	**P**	a	d
A. niger Aspergillopepsin A	**G**	d	g	s	s	s	s	s	**G**	f	s	**A**	**I**	a	**D**	**T**	**G**	**T**	t	**L**	i	l	l	d	d	e
P. janthinellum Penicillopepsin	t	a	g	s	q	s	g	d	**G**	f	s	g	**I**	a	**D**	**T**	**G**	**T**	t	**L**	**L**	l	l	d	d	**S**

Fig. 1.6. Aspartic protease catalytic sites. The bold residues are identical to those in pig pepsin A, in sequences around the (starred) catalytically active residue **(a)** Asp_{32} and **(b)** Asp_{215} (pepsin numbering). The amino acid sequences of the pepsin-type aspartic proteases are from Tang et al. (1973) for pig pepsin A, Shewale and Tang (1984) for pig cathepsin D, Misono et al. (1982) for mouse rennin, Dayhoff (1978) for bovine chymosin, Berka et al. (1990) for Aspergillopesin A, Jarai et al. (1994b) for Aspergillopepsin E and Dayhoff (1978) for *Penicillium janthinellum* Penicillopepsin, respectively. The *Scytalidium* acid protease B (Maita et al., 1984) and the *A. niger* var. *macrosporus* proteinase A (Inoue et al., 1991) represent non pepsin-type proteases, as also shown by the limited homology with pepsin-type proteases.

Remarkably, the general features of the mechanism by which pepsin-type aspartic proteases catalyze the hydrolysis of peptide bonds are not completely established, although purified pepsins have been available for more than 60 years and detailed investigations of the catalytic mechanism have been pursued for over 25 years. Structural studies have shown that pepsin-type aspartic proteases contain two active-site aspartic acid residues and that they are remarkably similar to each other with respect to their active sites (Subramanian et al., 1977) as shown in Fig. 1.6. The active site in these bilobal

enzymes is located in a deep groove situated between the lobes. In the active form of the enzyme, the two carboxyl groups are hydrogen bonded to each other and probably to water (Bott et al., 1982; James and Sielecki, 1983). An additional and apparently essential amino acid found in all aspartic proteases sequenced to date, is positioned on a flap region of the enzyme which can open and close upon binding of substrates or inhibitors. This Tyr residue is also involved in the binding and inhibition of pepsin-type proteases by pepstatin-derived inhibitors. In spite of the detailed information about the active sites of aspartic proteases, both in the native enzyme and with pepstatin-derived inhibitors bound, the molecular details of how aspartic proteases catalyse the hydrolysis of amide bonds is still not known for certain. In Fig. 1.7 a schematic representation of the relationships between proposed catalytic mechanisms for action of aspartic proteases is depicted. Nucleophilic catalysis (right) leads to covalent intermediates whereas direct addition of water (left) leads to a tetrahedral intermediate. At this moment, the experimental data is most consistent with a general acid-base mechanism (Antonov et al., 1978, 1981). However, all possibilities consistent with covalent catalysis are not rigorously excluded by these data (Hoffmann et al., 1984). Strong evidence for direct addition of water via a general acid-base mechanism was obtained by Rich and co-workers (1984). They showed that highly stereoselective addition and elimination takes place within the active site of pepsin, using ketone analogues of pepstatin-derived peptides. Given the reasonable assumption that pepstatin-catalyzed hydration of peptide substrate to form the tetrahedral intermediate occurs in a similar process parallel to the observed pepsin-catalyzed hydration of pseudosubstrate ketones, then pepsin catalyzes the hydrolysis of peptides by a direct addition of water and not by a nucleophilic mechanism. This also suggests that a slow, ordered release of products more reasonably accounts for data which have earlier been interpreted as evidence for covalent intermediates. The simplest view of the pH dependence of the aspartic proteases is that the pH optimum lies between the pK values of the two catalytic carboxyl groups, but of course, other factors are involved as well. Typical pH optima for aspartic proteases range from pH 3.5-5.5.

In general, aspartic pepsin-type proteases act best on peptide bonds between bulky hydrophobic amino acid residues. Analyses of the specificity of proteases A1 and A2 of *Scytalidium lignicolum* suggests that the nature of the amino acid at the P'_1 is less important than in the pepsin-type proteases (Oda and Murao, 1976). Aspartic proteases (as well as metallo proteases) tend to have functionally important subsites in the active site cleft which bind amino acid residues on both sides of the catalytic site. Unlike the serine and cysteine proteases, they will generally not cleave non-peptide bonds, e.g., to liberate chromogenic C-terminal blocking groups from synthetic substrates. Lengths of peptide chains to form a suitable substrate and specificity towards substrates vary with the individual enzymes.

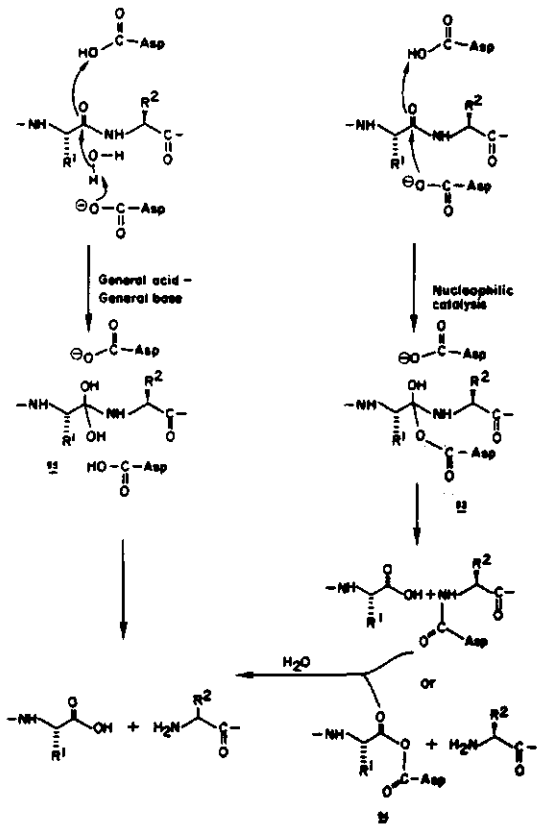

Fig. 1.7. Schematic representation of the relationships between proposed catalytic mechanisms for aspartic proteases. Direct addition of water (left) leads to a tetrahedral intermediate. Nucleophilic catalysis (right) leads to covalent intermediates. The catalytic residues are Asp_{32} and Asp_{215} (hog pepsin numbering).

Fungal aspartic proteases

Pepsin-type aspartic endo proteases. Many fungi, especially those which acidify their environment, produce proteases which are active at acidic pHs, and a large portion of these have been shown to have properties consistent with aspartic endo-proteases. Amino acid sequence analysis and X-ray crystallographic analysis (Hsu et al., 1977; Subramanian et al., 1977; Tang et al., 1978) revealed considerable homologies between fungal aspartic proteases and mammalian aspartic proteases (such as pepsin and rennin), and suggest a common ancestry (Tang, 1979), although clear differences have also been observed. The pepsin inhibitor *p*-bromophenyl bromide for instance does not inhibit fungal acid proteases. Many aspartic pepsin type endo-proteases have been isolated and characterized in *Aspergillus* (Table 1.2). In *A. niger* two pepsin type proteases have been isolated and characterized. One of these proteases, PEPA, appears to be secreted (Berka et al., 1990) whereas the other protease,

PEPE, is probably located intracellularly, based on the strong homologies to the *S. cerevisiae* vacuolar protease yscA (Jarai et al., 1994b).

Most of the pepsin-type aspartic proteases are monomeric, are glycosylated and have molecular masses in the range of 30 to 45 kDa, although higher molecular weights have also been described (North, 1982).

Non pepsin-type aspartyl endo proteases. Apart from the pepsin-type proteases, which are strongly inhibited by pepstatin, a second class of aspartic endo-proteases, insensitive to pepstatin inhibition, has been reported (see also Fig. 1.6). To this second class belong the *A. niger* var. *macrosporus* protease A (Chang et al., 1976; Iio and Yamasaki 1976), the *A. niger* protease B, proteases A1, A2 and B of *Scytalidium lignicolum* (Oda and Murao, 1974) and the extracellular proteases from the basidiomycete *Lentinus edodus*. Aspartic proteases of this class, which are considerably smaller than pepsin-type proteases, have an unusual two-chain structure (Inoue et al., 1991; Takahashi et al., 1991ab) (see also **1.4.2**). The homology (approx. 50%) between the non-pepsin-type *A. niger* and *Scytalidium lignicolum* aspartic proteases indicates that these proteases belong to the same protease family. However, also interesting differences have been observed; most notably, the *Scytalidium* protease has no two-chain structure and the assumed active site residues in the *Scytalidium* protease (Glu_{53} and Asp_{98}) are not conserved in the *A. niger* protease (Inoue et al., 1991; Takahashi et al., 1991ab). These results suggest that both enzymes have diverged to a considerable extent from each other.

The fungal aspartyl endo proteases have a pH optimum between pH 2.5 and 4.5. The experimentally determined pH-optimum (pH_{opt}) is, as already emphasized, partially dependent on the substrate used and in general one observes the pH_{opt} (cytochrome c) < the pH_{opt} (haemoglobin) < the pH_{opt} (casein). The isoelectric points of all reported aspartic proteases are below 5.1 and the majority have IEPs between 3.4 and 4.6. (Table 1.2).

Table 1.2. *Aspergillus* aspartic proteases

Organism	Type	Protein	Prot. gene	Mw[1]	pH_{opt}	pI	Origin[2]	Reference
				Aspartic endo proteases				
A. awamori	pepsin	Aspergillopepsin A	*pep*A				CF	Berka et al., 1990 Ostoslavskaya et al., 1979
A. awamori	non-pepsin							
A. foetidus	pepsin	Aspergillopepsin F			2.5	4.1	S	Ostoslavskaya et al.,1986
A. fumigatus					2.8/3.2	3.7	CF	Panneerselvam and Dhar, 1980, 1981a,b
A. kawachi				35 (d)	2.8/3.4	3.9	CF	Yagi et al., 1986
A. nidulans	pepsin	Aspergillopepsin A PEPA	*pep*A					van den Hombergh et al., 1996c
A. niger	pepsin			41 (d)	3.4	4.1	ME	Bosmann, 1973
A. niger				56 (d)	3.4	4.5	ME	Idem
A. niger	pepsin	Aspergillopepsin A PEPA	*pep*A					
A. niger				31 (?)	3.2		S	Turk, 1981
A. niger	pepsin	Aspergillopepsin E PEPE	*pep*E					Jarai et al., 1994b
A. niger	non-pepsin	PEPB						Buxton pers. commun.
A. niger var. *macrosp.*	non-pepsin (type A)			19 (nd)	3.0		CP	Horiuchi et al., 1969 Chang et al., 1976 Iio and Yamasaki, 1976
A. niger var. *macrosp.*	pepsin (type B)			34 (nd)	3.0		CP	Idem
A. oryzae								Tsujita and Endo, 1976, 1977abcd, 1978ab, 1980
A. oryzae	pepsin			39 (nd)	3.7		CF	Nakadai et al., 1977a
A. oryzae	pepsin	Aspergillopepsin O	*pep*O					Berka et al. 1993

[1] nd, non dissociating conditions; d, dissociating conditions

[2] S, soluble fraction; CS, culture supernatant; CP, commercial preparation; ME, mycelial extract

Fungal aspartic proteases are usually capable of hydrolyzing a range of native proteins but the majority of them have little or no activity against small synthetic substrates. The substrate specificities of a number of aspartic proteases from fungi (including *A. satoi*, *A. fumigatus*, *P. janthinellum*, *Candida albicans* and *Rhizopus chinensis*) have been assessed by characterizing the hydrolysis of the oxidized ß chain of insulin and some other small polypeptides and were shown to be strikingly similar (Hwang and Hseu, 1980; Ichishima et al., 1980b; Ichishima et al., 1981; Kimura et al., 1979; Panneerselvam and Dhar, 1981ab; Tanaka et al., 1977). Although aspartic proteases appear to have broad substrate specificities *in vitro*, they preferentially hydrolyze peptide bonds between two bulky amino acids. In contrast to the *A. niger* var. *macrosporus* PEPA, the pepsin type proteases in general cleave the Tyr(16)-Leu(17) and Phe(24)-Phe(25) bonds in oxidized ß-insulin. Most of the aspartic proteases also cleave the Leu(15)-Tyr(16) bond and to a lesser extent the Ala(14)-Leu(15) and Phe(25)-Tyr(26) bonds. Many of the aspartic proteases are synthesized as zymogens and are capable of autocatalytically removing the pro-peptide in order to activate the enzyme. Interestingly, many fungal proteases are also able to activate bovine trypsinogen (Belyauskaite et al., 1980; Panneerselvam and Dhar, 1980; Rüchel, 1981; Rudenskaya et al., 1980). This activation process was already described by Kunitz in 1938 for a *Penicillium* protease. Fungal activation of trypsinogen proceeds via Lys(6)-Ile(7) bond cleavage, which is also cleaved during autocatalytic activation. It has been shown that *A. oryzae* proteases are capable of activating trypsinogen, chymotrypsinogen (Abita et al., 1969; Robinson et al., 1973) and pepsinogen (Stepanov et al., 1969). Many fungal aspartic proteases have milk clotting activities, as they are capable of cleaving the Phe(105)-Met(106) bond of κ-casein, which is also cleaved by mammalian rennin. The pH-optimum for milk-clotting is normally closer to neutrality than that for protease activity. A synthetic rennin substrate based on the sequence around the Phe(105)-Met(106) bond is also hydrolyzed by some milk-clotting fungal proteases (Houmard and Raymon, 1979; Martin et al., 1980).

1.2.4 The Metallo proteases

The metallo proteases are a very ancient type of proteases, being wide-spread in bacteria, fungi, as well as in higher organism. Both metallo exoproteases (carboxypeptidases, aminopeptidases and dipeptidases), which fall into several EC catagories according to their specificities, as well as metallo endoproteases (EC 3.4.24) have been described. Although active sites appear to have significant similarities in properties the metallo proteases appear to differ widely. In many cases zinc (Zn) is the catalytically active metal in the natural forms of the enzyme. However, some of them retain activity when the Zn ion is replaced by the (also) divalent metal ion cobalt.

(a)

Thermolysin	* * *
B. thermoproteolyticus	H E l T H a v T d Y t A G l i y q n E S G a i n E a f S D
A. fumigatus MEP	H E y T H g r T y Y i A G ----#---- E S G g m g E g w S D
A. niger PEPH	H E y T H g k p l p l p l ----#---- E S G g m g E g w g D
Astacin	* * *
Astacin	H E L m H a I G F y H E H t R m D R D n y v t I N y q N v
Drosophila Tolloid	H E L g H t I G F h H E H a R g D R D k h i v I N k g N i
Rat meprin-beta	H E f l H a l G F w H E q s R a D R D d y i t I v w d r i
Serratia	* * *
Serratia Protease	H E I G H A L G L S H P G D Y N A G E G N P t Y r D V T Y
Protease B (B374)	H E I G H A L G L S H P a e Y N A G E G d i s Y k n s a a
Protease C (EC16)	H E I G H A L G L n H P G D Y N A G E G N P s Y s D V T Y
Matrixin	* * *
Human MMP	H E L G H S L G L s H S t d i g a L M Y P s Y t F S g d v
Rabbit MMP	H E f G H a L G L d H S s v p e r L M Y P m Y r y l e g s
Rat MMP	H E L G H S L G L f H S n n k e s L M Y P v Y r F S t s q
Snake Venom	* * *
Ht-d	H E L G H N L G M E H D G K D - C L R G A S L C I M R P G
HT-2	H E L G H N L G M E H D G K D - C L R G A S L C I M R P G
H2	H E L G H N L G M E H D d K D k C k c e A - - C I M s d v
Acid	* *
A. nidulans PEPJ	H E F T H A P G V Y S P G T D D L G Y G Y D A A T r L S A
A. oryzae NPII	H E F T H A P G V Y q P G T e D L G Y G Y D A A T q L S A
A. nidulans PEPI	H E F T H A P G V Y S P G T D D L G Y G Y D A A T a L d t

(b)

Astacin	*
Astacin	V - - - g e d Y q Y y S I M H Y g K y S F S i q w g v l e
D. melanogaster Tolloid	V d l p l l p Y d l n S I M H Y a K n S F S k s p y l d t
Rat meprin-beta	s d s l n v p Y d Y t S v M H Y s K t a F q n g t e s t i
Serratia	*
Serratia Serralysin	- V T Y A E D T R Q F S l M S Y W S e t N T G G D n g G H
Protease B (B374)	s a a Y A E D s R Q F S i M S Y W e v e N T G G D f k G H
Protease C (EC16)	- V T Y A E D T R Q F S i M S Y W S E k N T G G D f k G H
Matrixin	*
Human MMP	d v q L a Q D D I d G I Q a i Y G r s q N P v q P i g P q
Rabbit MMP	g s p L h e D D v r G I Q h l Y G p n p N P q p P a t t t
Rat MMP	n n r L s Q D D I e G I Q s l Y G a r p s s d a t v v P v
Snake Venom	*
Ht-d	T k G R S Y E F S D d S M h Y Y e r F L k Q Y K P Q C I L
HT-2	T p G R S Y E F S D a S M r Y Y q k F L d Q Y K P Q C I L
H2	s d k p S k l F S D c S k n d Y q t F L t k Y n P Q C I L
Acid	*?
A. nidulans PEPJ	L S A s q A L N N A D S Y A L Y A N G V Y L G C
A. oryzae NPII	L S A q d A L N N A D S Y A L Y A N G V Y L G C
A. nidulans PEPI	L d t d d A L N N A D S Y A L Y A N a i n L G C

Fig. 1.8. Metallo endoprotease catalytic sites. The bold residues are identical to those in *B. thermoproteolyticus* Thermolysin, Astacin, Serratia protease, Human Matrixin metallo protease (MMP), hemorrhagic toxin (Ht-d) and *A. nidulans* PEPI for the Thermolysin, Astacin, Matrixin, Snake Venom and Acid metalloprotease families, respectively. The starred residues indicate the four (putative) metal ion chelating residues; the first three metal ligands **(a)** and the fourth metal ligand **(b)**. For the thermolysin-type proteases the fourth ligand is H_2O, and for the Acid metallo proteases the third and fourth metal chelating ligands are unknown (a region containing a highly conserved Tyr residue is indicated as putative fourth metal ligand); --#--, the putative third ligand in the *Aspergillus* thermolysin-type proteases is located at longer distance (23 amino acids) in the primary amino acid sequence (compared to bacterial thermolysins). The amino acid sequences are from Colman et al. (1972) for *B. thermoproteolyticus* Thermolysin, from Jaton-Ogay et al. (1994) for *A. fumigatus* MEP, from van den Hombergh et al., (1996g) for *A. niger* PEPH, from Jiang and Bond (1992) for the Astacin proteases, from Dahler et al. (1990) for the Serratia proteases, from Takeya et al. (1990) for the Snake Venom metallo proteases, from Acc. No. P23694 for the *Serratia* Serralysin, from Acc. No. P03956 for human MMP, from Acc. No. P for rabbit MMP, from Acc. No. P07152 for Rat MMP, from van den Hombergh et al. (1996d) for *A. nidulans* PEPI and PEPJ, from Tatsumi et al. (1991) for *A. oryzae* NPII; MMP, matrix metallo protease; Ht, hemorrhagic toxin.

Two histidine residues, present either in a HEXXH or a HXXEH motif are involved in chelating the Zn ion, essential for catalytic activity in metallo proteases. The glutamic acid present within these motifs has been shown to act as a catalytic base. Apart from the two His ligands the third and fourth ligand involved in positioning the Zn ion are only known for a few metallo proteases. In some closely related bacterial metallo endoproteases the third and fourth ligands are a remote glutamic acid residue and water, respectively. Within another class of metallo proteases, containing a histidine and a tyrosine as third and fourth Zn binding ligand respectively, several subclasses of metallo proteases have been identified, based on amino acid sequence homologies in the close proximity of these ligands (i.e. thermolysin, astacin, serratia, matrixin and the snake venom family). In Fig. 1.8 the zinc-binding regions for the HEXXH-type metallo protease families are shown. Recent sequence data on fungal metallo proteases indicate that an additional metallo protease family can be identified (van den Hombergh et al., 1996d; see also *metallo endo proteases*).

Although the metallo-exopeptidases and metallo-endopeptidases do not have the same origin, the structures of their active sites have converged to such an extent that one can draw similar conclusions from the extensively studied carboxypeptidase A metallo exoprotease and thermolysin (metallo endo protease). Thus, in general all metallo proteases appear to use a similar mechanism which is shown in Fig. 1.9. In the native structures of the carboxypeptidase A and the thermolysin, the four ligands (two His residues, a glutamic acid residue and a water molecule) are arranged in essentially the same tetrahedral geometry around the zinc atom (Kester and Matthews, 1977). While the exact mechanism of these enzymes is still controversial, much of the evidence is consistent with the mechanism shown in Fig. 1.9 (see also Vallee et al., 1983). In the resting state of the enzyme, water is bound as the

fourth zinc ligand and also hydrogen bonded to the carboxylate of Glu-270. During the formation of the enzyme-peptide substrate complex, the water is displaced from the metal by the carbonyl group of the peptide substrate and probably remains trapped in the active site. This process likely occurs through a five-coordinate transition state. The trapped water molecule then attacks the carbonyl of the scissile bond of the substrate to give a tetrahedral-like intermediate. This process is facilitated by the carboxylate of Glu-270 which acts as a base to deprotonate the attacking water molecule. The zinc atom polarizes the carbonyl making it more susceptable to nucleophilic attack and also stabilizes the presumed tetrahedral intermediate. The displacement of the metal bound water and its subsequent attack at the carboxyl group are likely to be concerted processes. Decomposition of the tetrahedral intermediate results in bond cleavage.

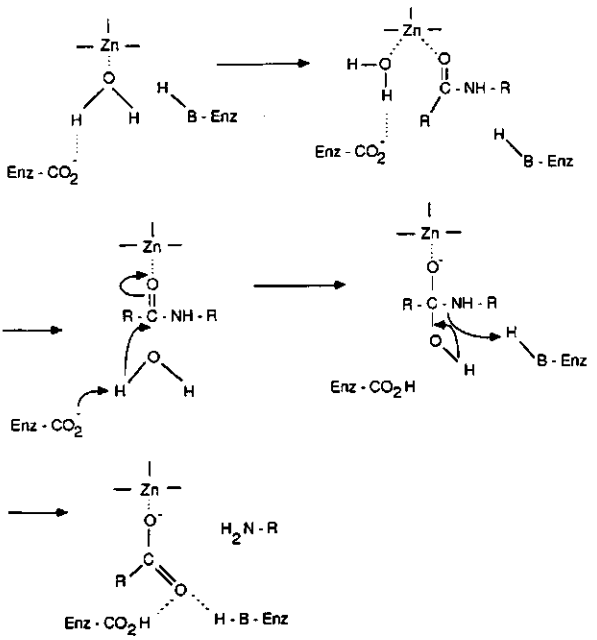

Fig. 1.9. Catalytic mechanism of a metallo protease. H-B-Enz represents a proton donor.

Like carboxypeptidase A, thermolysin contains an essential glutamic acid residue (Glu-143) and a zinc atom at the active site (Kester and Matthews, 1977). However, thermolysin also contains an active site histidine residue (His-231) which was originally proposed to function as a proton donor during catalysis. A novel mechanism has been proposed for thermolysin based on crystallographic data obtained with a transition state analogue inhibitor of the enzyme (Mozingo and Matthews, 1984; Hangaurer et al., 1984) According to this mechanism (Fig. 1.10) the carboxylate of Glu-143 acts as 'proton shuttle' between the attacking water molecule (ligated to the zinc atom) and the nitrogen of the scissile peptide bond. This process also results in a tetrahedral intermediate which then collapses to products with Glu-143 again acting to shuttle the remaining proton from the oxygen derived from the nucleophilic water and delivering it to the free amino group as it is being generated. In carboxypeptidase A a similar 'proton shuttle' function has been proposed for Glu-270. The actual mechanism for a newly isolated metallo protease could well be a further refinement or a hybrid of the mechanisms presented (Figs. 1.9 and 1.10). The rather unique specificities of metallo proteases, which allow them to catalyze certain peptide bond cleavages much more efficiently than other types of proteases are probably related to specific active site residues involved in substrate binding (Powers and Harper, 1986).

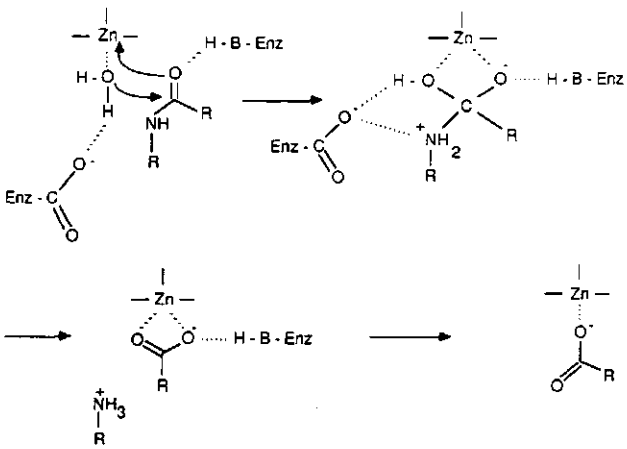

Fig. 1.10. Alternative catalytic mechanism of a metallo protease. H-B-Enz represents a hydrogen bonding group.

Virtually all metallo protease inhibitors reported to date contain a moiety capable of interacting with the active site metal ion. Commonly used metallo protease inhibitors are metal chelators such as EDTA (ethylenediamine tetraacetic acid), 2,2'-bipyridine and 1,10-phenanthroline. Inertness to serine protease activity and sensitivity to metal chelating inhibitors is a good indicator for a metallo protease. Many proteases, including serine proteases require calcium for activity and as EDTA is an excellent calcium chelating agent one needs additional inhibitors to unambiguously prove metallo protease activity.

Fungal metallo proteases

Metallo endo proteases. There are only a few examples of cloned metallo protease genes in filamentous fungi (Table 1.3). It has been suggested that in filamentous fungi two types of metallo proteases exist. One group of metallo proteases is characterized by a low molecular mass (19-20 kDa), has a slightly acidic pH optimum (pH 5-6) and different specificity towards the oxidixed insulin-ß chain as compared to another group of metallo proteases which operate best at neutrality, have a molecular weight around 41 kDa and have specificities towards oxidized insulin-ß comparable to thermolysin-like proteases (Gripon et al., 1980). Gripon and co-workers propose to call the enzymes in the first sub-class, which are in contrast to the thermolysin-like metallo proteases insensitive to phosphoramidon, acid metallo proteases because of their slightly acidic pH optima. Whereas the metallo proteases from *P. caseicolum*, *P. roqueforti* and *P. citrinum* together with the neutral proteases II from *A. flavus*, *A. fumigatus*, *A. oryzae*, *A. sojae* and PEPH and PEPI from *A. nidulans* are acid metallo proteases, the cloned metallo protease from *A. fumigatus* (MEP) and *A. niger* (PEPH), the neutral protease I from *A. oryzae* and *A. sojae* and the metallo protease from *Fusarium oxysporum* are thermolysin-like neutral metallo proteases (Table 1.3). Isoelectric points of members of both sub-groups of metallo proteases appear to be quite similar (i.e. ranging from 4.1 to 5.0).

For members of both proposed sub-classes, metallo protease genes have been characterized (Tatsumi et al., 1991). The *A. oryzae* neutral (acid metallo) protease II gene was cloned and expressed in yeast. From *A. nidulans* two similar acid metallo proteases were isolated by van den Hombergh and co-workers (1996d) who also showed that in *A. oryzae* a second acid metallo protease is present, similar to the situation in *A. nidulans*. Thermolysin-like metallo proteases were cloned from *A. fumigatus* (Jaton-Ogay et al., 1994), *Fusarium oxysporum* (Moyer at al., 1995) and *A. niger* (van den Hombergh et al., 1996g). Both the amino acid and nucleotide sequence data show, apart from the conserved HEXXH motifs, no significant homologies between the two fungal metallo protease subclasses.

Table 1.3. *Aspergillus* metallo proteases

Organism	Type[1]	Protein	Prot gene	Mw[2]	pH_{opt}[3]	pI	Origin	Reference
				Metallo endo proteases				
A. flavus	acid	type PII			7.0/7.5		CF	Impoolsup et al., 1981
A. flavus	acid	MEP20	*mep*20	23 (d)			CF	Ramesh et al., 1995
A. fumigatus	thermolysin	MEP	*MEP*	44 (d)		5.0	CF	Monod et al., 1993b Jaton-Ogay et al., 1994
A. fumigatus				83 (d)			ME	Ibrahim-Granet et al., 1994
A. fumigatus	acid	MEP20	*mep*20	23 (d)			CF	Ramesh et al., 1995
A. nidulans		type α					ME/CF	Cohen, 1973ab
A. nidulans	acid acid	PEPI PEPJ	*pep*I *pep*J					van den Hombergh et al., 1996d
A. niger	thermolysin	PEPH	*pep*H				CF	van den Hombergh et al., 1996g
A. oryzae	thermolysin acid	type NPI type NPII		41 (nd) 19.2 (nd)	7.0 5.5/6.0		CF CF	Nakadai et al., 1973e Nakadai et al., 1973f
A. oryzae	acid	type NPII	*npII*					Tatsumi et al., 1991
A. sojae	thermolysin acid	type NPI type NPII		42.2 (nd) 16.8 (nd)		4.7 4.2	CF CF	Sekine, 1972, 1973
				Metallo exo proteases				
A. oryzae	Met APD Met APD Met APD	leucine APD I leucine APD II leucine APD III		26.5 (nd) 61.0 (nd) 56.0 (nd)	8.0 8.0 8.0			Nakadai et al., 1972ab Nakadai et al., 1973abc

[1] Met APD, Metallo amino peptidase
[2] nd, non dissociating conditions; d, dissociating conditions
[3] S, soluble fraction; CS, culture supernatant; CP, commercial preparation; ME, mycelial extract

Moreover, the acid metallo proteases shows no sequence homology to any of the previously described metallo protease families, indicating that a novel family of metallo proteases has been identified (van den Hombergh et al., 1996d).

Metallo exo peptidases. Little is known about occurrence of metallo amino peptidases (E.C. 3.4.11) and metallo carboxypeptidases (E.C. 3.4.17) in filamentous fungi (Table 1.3). From an *A. oryzae* culture filtrate three (metallo-) amino peptidases - called leucine aminopeptidase I, II and III because of their substrate preference for oligopeptides with N-terminal leucine residues - have been purified (Nakadai et al., 1973abc). Inhibition of these enzymes by 1,10-phenanthroline proves metallo-type activity and additional inhibition by DIFP suggests the presence of an active site Ser residue. The pH_{opt} of the enzymes is neutral to alkaline. Also in *A. nidulans* a metallo aminopeptidase activity has been described (Stevens, 1985).

1.2.5 Unclassified proteinases

Not all proteases have been assigned to one of the four protease groups presented in this chapter. For this reason, section 3.4.99 of the Enzyme Nomenclature is reserved for proteases of unidentified catalytic mechanism. To a large extent this section serves as a waiting list for the enzymes that, when thorougly purified and characterized, will emerge as members of one of the other groups. However, still the possibility exists that proteases truly representing a novel fifth type of catalytic mechanism are in this catagory. There are some observations that some exo-proteases cleave peptide bonds by mechanisms that are different form those of the known endopeptidases, and there is no obvious reason why endopeptidases acting by such mechanisms may not yet be found (Barrett, 1986). A possible candidate with a putative different catalytic mechanism is the digestive protease from crayfish, as the enzyme has no homology with any other protease and the activity is not inhibited by any of the known naturally occuring or synthetic protease inhibitors (Zwilling and Neurath, 1981; Kraush et al., 1982). These observations could be an indication that proteases with an as yet unknown catalytic mechanism exist.

1.3 Functions of proteases

The specificity of a proteinase is assumed to be closely related to its physiological function and its mode of expression. With respect to the function of a particular protease, its location is often very important; for example, most of the vacuolar and periplasmic proteases are involved in protein degradation, while many of the membrane-bound proteases are important in protein processing (Suarez Rendueles and Wolf, 1988). The different roles of proteases in many cellular processes can

be divided into four main functions: protein degradation, posttranslational processing and (in)activation of specific proteins, morphogenesis, and pathogenesis.

Protein degradation (turnover and nutrition)

An obvious role for proteases in organisms which utilise protein as a nutrient source is in the hydrolysis of nutrients. In fungi, this would involve the degradation outside the cells by extracellular broad specificity proteases. Protein degradation is also important for rapid turnover of cellular proteins and allows the cell to remove abnormal proteins and to adapt their complement of protein to changing physiological conditions. Generally, proteases of rather broad specificity should be extremely well-controlled in order to protect the cell from random degradation of other than correct target proteins.

Posttranslational processing and (in)activation of specific proteins

Primary translation products are often longer than the final functional products, and proteolysis must be involved in the subsequent posttranscriptional processing. Apart from direct involvement in the processing, selective activation and inactivation of individual proteins are also well-known phenomena catalyzed by specific proteases. The selectivity of limited proteolysis appears to reside more directly in the proteinase-substrate interaction. Specificity may be derived from the proteolytic enzyme which recognizes only specific amino acid target sequences. On the other hand, it may also be the result of selective exposure of the 'processing site' under certain conditions such as pH, ionic strength or secondary modifications, thus allowing an otherwise non-specific protease to catalyze a highly specific event. The activation of vacuolar zymogens by limited proteolysis gives an example of the latter kind.

Morphogenesis

Morphogenesis or differentiation can be defined as a regulated series of events leading to changes from one state to another in an organism. Although direct relationships between proteases and morphological effects could not be established in many cases, the present (circumstantial) evidence suggests a significant involvement of proteases in fungal morphogenesis; apart form the observed extensive protein turnover during differentiation, sporulation and spore germination, proteases appear also to be directly involved in growth-related processes as hyphal tip branching and septum formation (Deshpande, 1992).

Pathogenesis

Species of *Aspergillus*, in particular *A. fumigatus* and *A. flavus*, have been implicated as the causative agents of a number of diseases in humans and animals called aspergillosis (Bodey and Vartivarian, 1989). It has been repeatedly suggested that proteases are involved in virulence of *A. fumigatus* and *A. flavus* like there are many studies linking secreted proteases and virulence of bacteria. In fact, most human infections due to *Aspergillus* species are characterized by an extensive degradation of the parenchyma of the lung which is mainly composed of collagen and elastin (Campbell, 1994). Consequently research has been focussed on the putative role of the secreted proteases in virulence of *A. fumigatus* and *A. flavus* which are the main human pathogens and are known to possess elastinolytic and collagenic activities (Kolattukudy et al., 1993). These elastinolytic activities were shown to correlate *in vitro* with infectivity in mice (Kothary et al., 1984). Two secreted proteases are known to be produced by *A. fumigatus* and *A. flavus*, an alkaline serine protease (ALP) and a neutral metallo protease (MEP). In *A. fumigatus* both the genes encoding these proteases were isolated, characterized and disrupted (Reichard et al., 1990; Tang et al, 1992, 1993; Jaton-Ogay et al., 1994). However, *alp mep* double mutants showed no differences in pathogenicity when compared with wild type strains. Therefore, it must be concluded that the secreted *A. fumigatus* proteases identified *in vitro* are not essential factors for the invasion of tissue (Jaton-Ogay et al., 1994). Although *A. fumigatus* accounts for only a small proportion of the airborne mould spores, it is the most frequently isolated fungus from lung and sputem (Schmitt et al., 1991). Other explanations for the virulence of the fungus could be that the conditions in the bronchia (temperature and nutrients) are favourable for the saprophytic growth of *A. fumigatus* (Latgé et al., 1994). As a consequence, invasive aspergillosis could be a circumstantial event, when the host pathogenic defences have been weakened by immunosuppressive treatments or diseases like AIDS.

1.4 Cellular localization and characterization of proteolytic processing and degradation

In the past, proteolysis was often divided into intra- and extracellular proteolysis, where intracellular proteolytic events were thought to be exclusively associated with lysosomal-like compartments of the cell. Today, it is realised that proteolytic events take place in every cellular compartment, including cytoplasm, vacuole, mitochondria, secretory pathway and nucleus. Many of the organelles in the eukaryotic cell have their own specific set of proteolytic activities, optimized for their particular function, as will be discussed below.

1.4.1 Intracellular proteolysis

The dynamic state of cellular proteins has more recently been linked to mechanisms responsible for intracellular selective proteolytic degradation. Intracellular proteolytic degradation has important roles in the modulation of the levels of specific proteins, the elimination of damaged proteins and the maintenance of amino acid pools in cells affected by stress inducing situations such as starvation. Finally, another role for intracellular proteolytic pathways is to confer short half-lives on proteins whose concentrations must vary with time and to confer alterations in the state of the cell. Usually, regulatory proteins or enzymes have fast turnover rates, in order to ensure that their levels are rapidly changed in response to appropriate stimuli. Amino acid sequences, conformational determinants or chemically modified protein structures that confer metabolic instability are called degradation signals, or degrons (Varshavsky, 1991). The N-end rule (Bachmair et al., 1986) is in fact the manifestation of a specific degradation signal called the N-degron, resulting in rapid ubiquitin mediated proteolysis of proteins. Since its discovery in 1986, distinct versions of the N-end rule have been shown to operate in all organisms examined thusfar.

1.4.1.1 Cytoplasmic proteolysis. The ubiquitin system for proteolytic degradation

The major proteolytic process in the cytoplasm, the ubiquitin pathway, is highly selective and controlled. The elucidation of this pathway started in 1978 when Ciechanover and coworkers described a heat-stable polypeptide required for the activity of an ATP-dependent proteolytic system in reticulocytes (Ciechanover et al., 1978). This polypeptide was subsequently identified as ubiquitin (Ub), a 76-amino-acid protein, which is highly conserved and present in all eukaryotes. Proteins destined for degradation are covalently ligated to Ub in an ATP-dependent reaction and are subsequently degraded in a process (involving eight distinct steps) by a specific protease complex that acts on ubiquitinated proteins. An outline of the system and the enzymatic reactions involved is summarized in Fig. 1.11. In yeast, the Ub encoding genes are either fusions of Ub to itself (*UBI4*) or to specific ribosomal proteins (*UBI1-3*). These fusions are processed by Ub-specific, functionally overlapping proteases encoded by *YUH1*, *UBP1-UBP3*, and at least one other gene, yielding mature Ub. Ligation of Ub to proteins is initiated by the ATP-dependent activation of its C-terminal Gly residue, catalyzed by a specific Ub-activating enzyme, E_1S (Step 1). Deletion of the yeast E_1 gene, *UBA1*, is lethal, indicating the vital nature of Ub conjugation for cell viability. Next, activated Ub is further transferred by transacylation to thiol groups of a family of Ub-carrier proteins, E_2S (Step 2). The large and probably still incomplete family of E_2 genes may reflect the requirement for recognition of different subsets of proteins or the recognition of an equally large number of E_3 components that act on specific proteins. E_2-Ub thiol esters are the donors of Ub for isopeptide bond

formation between the C-terminal Gly residue of Ub and ϵ-amino groups of Lys residues of proteins. Ub-protein ligation may occur by direct transfer from E_2 (Step 3), or by a process in which target proteins are first bound to specific sites of Ub-protein ligases, E_3S (Step 4), and then Ub is transferred from E_2 (Step 5). *In vitro* substrates of E_3-independent Ub ligation are mainly basic proteins such as histones. In all cases of E_3-dependent ligation and in some E_3-independent reactions, multiple Ub units are linked to proteins. Some of these are arranged in polyubiquitin chains in which a major point of linkage was shown to be at Lys_{48} of Ub. Proteins ligated to multiple Ub units are degraded by a 26S protease complex. The exact function of Ub attachment is still under investigation, but a possibble explanation could be that the multi-Ub chain formation on a targetted substrate may produce an additional binding site (or sites) for components of the proteolytic machinery, the 26S complex, involved in final degradation. The 26S complex is formed by the assembly of three components, designated CF-1, CF-2 and CF-3, by an ATP-dependent process (Step 6). CF-3 has been identified as the 20S 'multicatalytic' protease complex, a particle observed in eukaryotic cells and also referred to as proteasome or prosome. The proteasome is a cylindrical particle organized as stacks of four rings of subunits, which are mostly non-identical. The proteasome has ATP-independent proteolytic activity and three distinct types of endoprotease activity have been identified cleaving peptide bonds on the carboxyl side of basic, hydrophobic-neutral and acidic amino-acids residues. Although the genes of several subunits of the proteasome complex from rat, human and *Drosophila* have been cloned, the deduced amino acid sequences do not resemble any of the known proteases (Hershko and Ciechanover, 1992). In yeast, disruption of genes encoding different subunits is lethal (Fujiwara et al., 1990; Emori et al., 1991; Heinemeyer et al., 1991), indicating essential functions of the 20S protease complex. Recently, a subunit of the *A. niger* proteasome has been cloned and characterized (Jarai, pers. commun.).

The cumulative evidence suggests that the 20S protease complex is the 'catalytic core' of the 26S complex that degrades ubiquitinated proteins. It is not clear, however, whether the 20S protease complex is the only source of proteolytic catalytic sites for the 26S complex. Another question which remains to be solved is whether the 20S complex has roles other than those associated with the proteasome. Indications for other functions are the observation by Driscoll and Goldberg (1989) that when isolated from certain tissues and under certain conditions the 20S complex has ATP-dependent (but ubiquitination-independent) protease activity. The assembled 26S protease complex degrades the protein moiety of Ub-protein conjugates to small peptides, in a process that requires the hydrolysis of ATP (Step 7). However, the role of ATP in the assembly of the 26S complex and its proteolytic action remains to be elucidated.

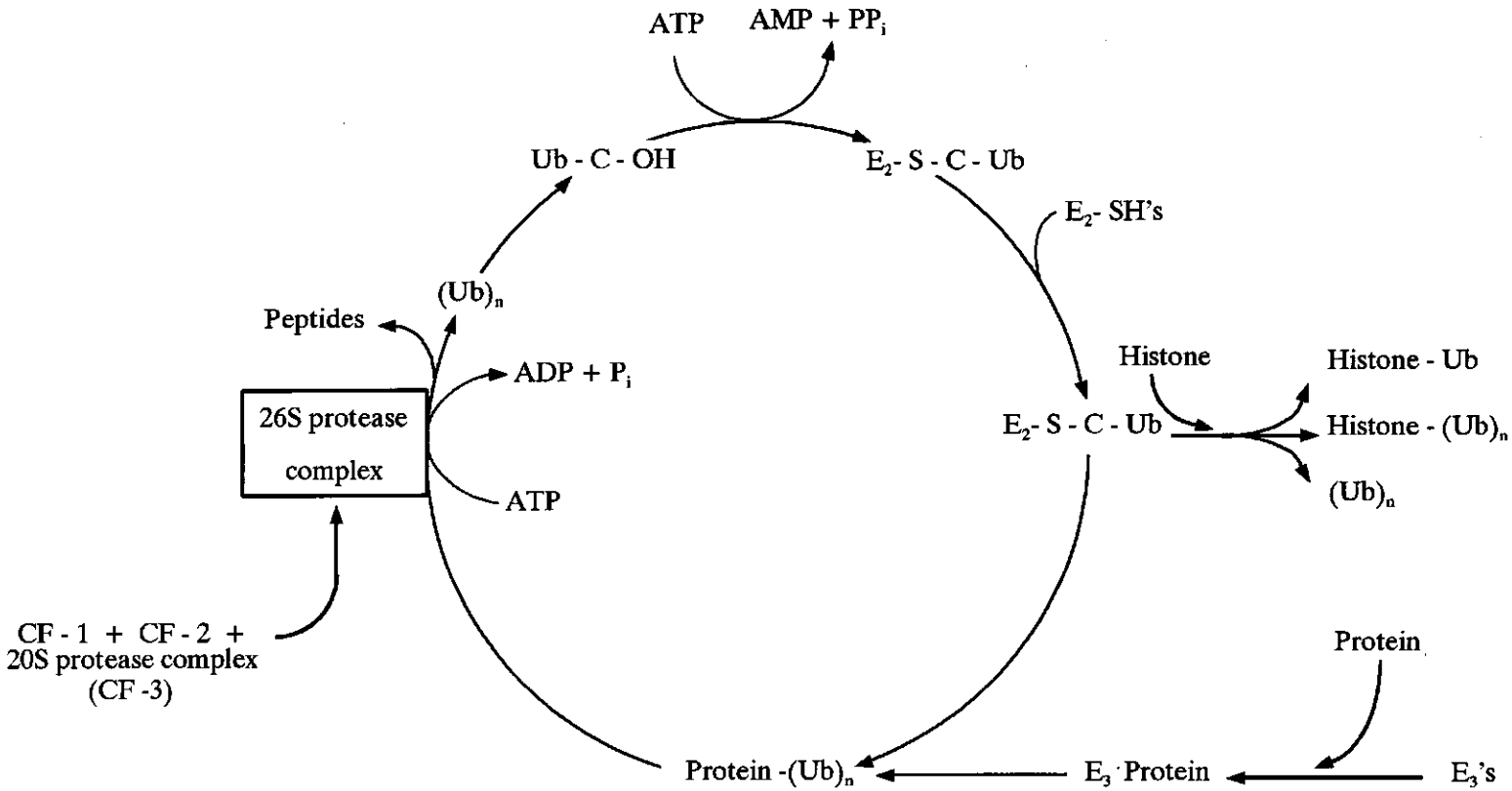

Fig. 1.11. The ubiquitin degradation pathway. Sequence of events as postulated by Hershko and Ciechanover (1992). Individual steps discussed in the text are numbered 1-8.

Following proteolysis, the Ub polypeptides are recycled, presumably by the action of several Ub C-terminal hydrolases. One of these enzymes acts on polyUb chains (Step 8).

A key problem is which specific structural features of a protein are recognized by the Ub system in order to target them for degradation. Bachmair et al. (1986) demonstrated that the identity of the N-terminal residues determined the half-live of proteins. As summarized in Fig. 1.12, N-terminal R,K,H,L,F,Y,I,W residues are recognised as primary destabilizing residues in *S. cerevisiae* and bound by E_3 (also referred to as 'N-recognin') which subsequently ensures ubiquitination of Lys residues. Apart from these primary destabilizing residues, both bacteria and eukaryotes contain aminoacyl tRNA-protein transferases (aa-transferases), which conjugate specific amino acids to N-termini of receptor proteins (reviewed by Soffer, 1980). Therefore, Varshavsky (1992) proposes the existence of secondary destabilizing N-terminal residues that function through their conjugation, by an aa-transferase, to other, primary destabilizing residues (Bachmair et al., 1986). Ferber and Ciechanover (1987) confirmed this hypothesis showing that Ub-dependent degradation of certain N-end rule substrates requires the conjugation of Arg, a primary destabilizing residue, to their N-termini. Subsequent studies (Gonda et al., 1989; Balzi et al., 1990; Baker and Varshavsky, 1991) have determined that the secondary destabilizing residues in yeast are Asp and Glu, while in mammalian reticulocytes they are Asp, Glu and Cys. The third level in the hierarchical organization of the N-end rule was revealed by the finding that in both yeast and mammals, N-terminal Asn and Gln are tertiary destabilizing residues - they function through their conversion, by a specific deamidase, into secondary destabilizing Asp and Glu. Thus in general the N-degron, manifestated in the N-end rule, comprises two distinct determinants: a destabilizing N-terminal residue and specific internal Lys residue(s). It furthermore has been shown that in multisubunit proteins these determinants can reside in different subunits and can be recognized in trans (Varshavsky, 1992).

The twelve destabilizing residues (Fig. 1.13) are often absent from the mature N-termini in noncompartmentalized proteins. It has been demonstrated that a specific methionine (Met) aminopeptidase removes only the N-terminal residue if the second residue is stabilizing in the yeast N-end rule (Arfin and Bradshaw, 1988; Hirel et al., 1989; Sherman et al., 1985). In particular, Ala, Ser and Thr, which are stabilizing in yeast, do not prevent the removal of N-terminal methionine by Met aminopeptidase and therefore can be exposed at the N-termini of proteins that initially bear these residues at position two. However, most non-compartmentalized proteins lack destabilizing residues at their N-termini (Bachmair et al., 1986). While this bias is largely due to the properties of the already mentioned Met aminopeptidase, it may also stem in part from the underrepresentation of short-lived proteins in the presently known set of N-terminal sequences.

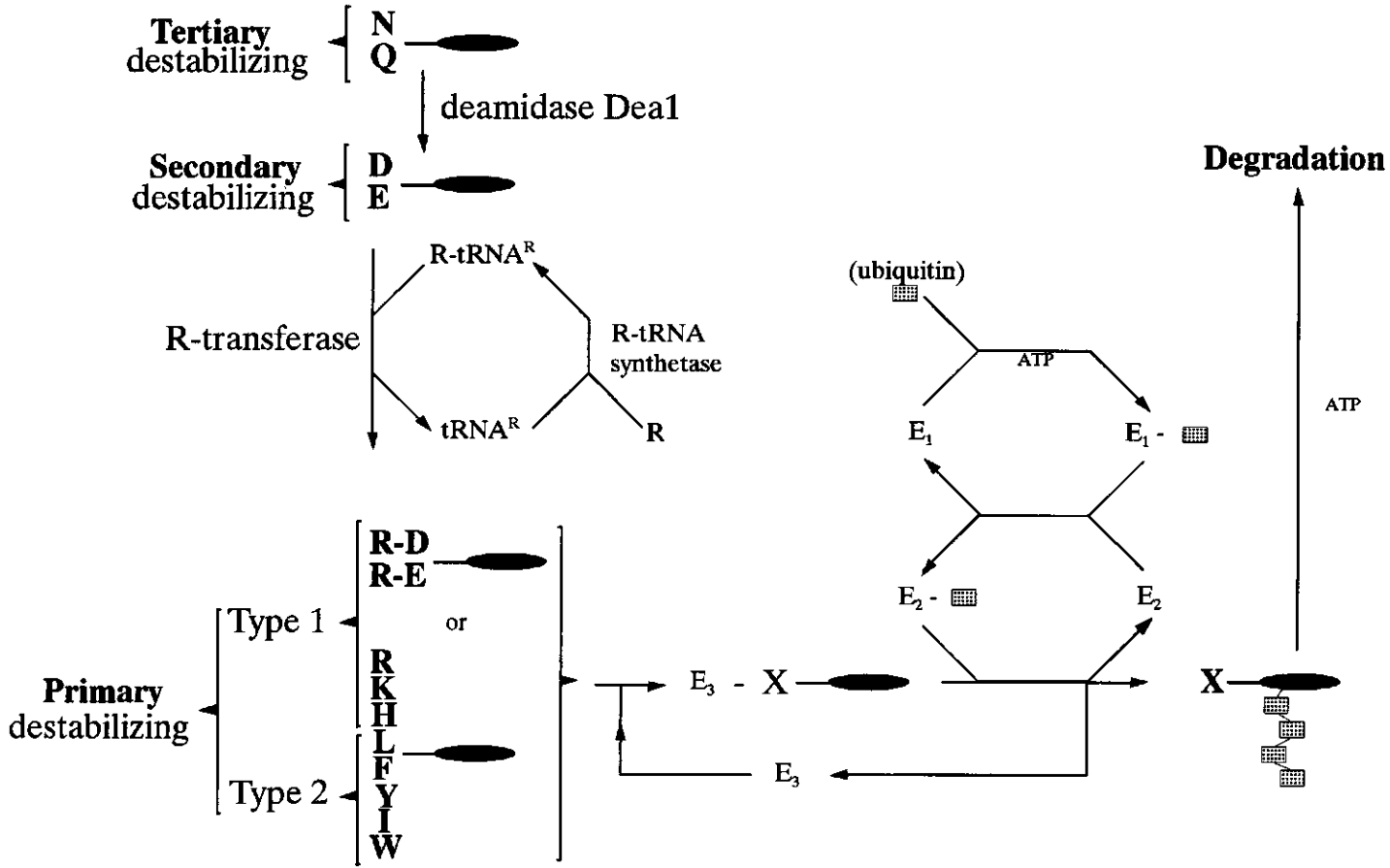

Fig. 1.12. The *S. cerevisiae* N-end rule pathway. Conversion of N-terminal N and Q to D and E is carried out by a deamidase encoded by the *DEA1* gene. Conjugation of the primary destablizing residue R to the secondary destabilizing N-terminal residues D and E is catalyzed by Arg tRNA-protein transferase (R-transferase). Type I and Type II primary destabilizing N-terminal residues are bound by two distinct sites (interacting either with basic residues (I) or with bulky hydrophobic residues (II) of E3 (also referred to as N-recognin). E3 forms a complex with one of the E2-Ub-conjugated enzymes. If a substrate is marked with both the determinants of the N-degron it is subsequently ubiquitinated, which then targets the substrate for degradation by the proteasome.

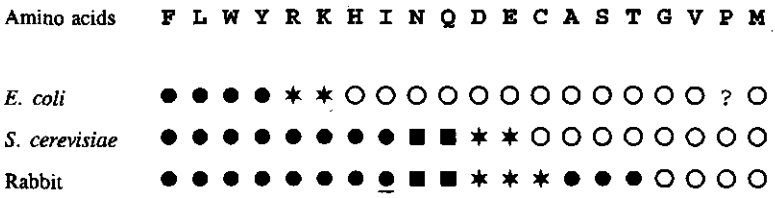

Fig. 1.13. Comparison of N-end rules. Open circles: stabilizing residues; filled circles, stars and squares denote primary, secondary and tertiary destabilizing residues in the N-end rules of *E. coli*, *S. cerevisiae* and rabbit reticulocytes. The underlined I(soleucine) indicates that it is a weakly destabilizing residue in yeast and a borderline destabilizing residue in reticulocytes. A question mark at P(roline) indicates that the status is unknown in the *E. coli* N-end rule. Modified from Tobias et al. (1991)

1.4.1.2 Proteolysis in the secretory pathway

There is no firm evidence for a general protein degradation system in the secretory pathway. However, there are a number of well-defined proteolytic activities either residing in the secretory system or transported through the system. In addition, all vacuolar proteases will travel via endoplasmic reticulum and parts of the Golgi apparatus to their final destination.

As a first step in secretion, the polypeptide chain of a protein destined for secretion has to enter the endoplasmic reticulum during which its signal peptide is proteolytically removed by a signal peptidase. The 'canonical' signal peptides share a common design, characterized by a short, positively charged amino-terminal region (n-region) followed by a central hydrophobic region (h-region) and a more polar carboxy-terminal region (c-region) that contains the cleavage site. The signal peptidase apparently recognizes a '(-3, -1)-pattern' with small uncharged residues in position -3 and -1 relative to the cleavage site (von Heijne, 1981, 1983, 1984ab, 1985, 1986).

Other proteases residing in the secretory pathway include processing peptidases. In yeast two processing proteases have been cloned, *KEX*1 and *KEX*2. These membrane-associated proteases are amongst others involved in the maturation of the α-factor and the killer factor. The *KEX2*-encoded protease, yscF, is a membrane-bound Ca^{2+}-dependent serine protease, located in the late Golgi apparatus or the secretory vesicles (Achstetter and Wolf, 1985). It cleaves at the C-terminal side of a pair of basic amino acid residues. A large number of secreted filamentous fungal proteins have also been found to be synthesized as prepro-proteins, processing of which often involves cleavage after one or two basic amino acids in conjugation with other amino acids (previously reviewed by Calmels et al., 1991). The pair Lys-Arg is the most frequently observed fungal dibasic cleavage site, and in those cases it is generally assumed that the cleavage is catalyzed by a processing protease related to the yeast yscF protease. Processing at monobasic sites is also often found for peptide precursors in higher eukaryotes, and a series of rules and tendencies that govern monobasic cleavages has been

proposed (Devi, 1991). Devi found that these rules were also applicable to a large number of dibasic cleavage sites. Indeed, in those fungal prepro-peptides where there is second basic amino acid present in the region preceeding the dibasic cleavage site (LiP2, KP6α, Proteinase A and PGI) it is found at one of the positions predicted. However, virtually all reported fungal monobasic cleavage sites appear to conflict with one of Devi's four rules. This holds particularly for the most stringent, positively formulated first rule, the presence of a (second) basic amino acid either at the -3, -5, or the -7 position with respect to the basic residue at the monobasic cleavage site (Visser et al., 1994). In general, at position -3 with respect to the basic amino acid at the cleavage site there is a preference for a hydrophobic aliphatic amino acid, as noted by Tao et al. (1990). Little is known about the specificities of the processing enzymes that cleave protein precursors in filamentous fungi. It is thus not clear if processing is performed by one single enzyme of relaxed substrate specificity, or by two or more different enzymes, similar to the situation observed for mammalian subtilisin-like prohormone convertases (Benjannet et al., 1991; Thomas et al., 1991; Edgington, 1992). Bourbonnais and coworkers (1991) have presented some evidence for the existence of a distinct *S. cerevisiae* monobasic-specific protease whose activity is independent of the KEX2 enzyme. However, the cleavage at a monobasic site in yeast K_1 killer pretoxin is dependent on KEX2 (Zhu et al., 1992).

yscα, also referred to as KEX1, is a membrane-associated yeast carboxypeptidase, with high specificity for basic amino acids (Cooper and Bussey, 1989). Its biological role is the removal of the two basic amino acids after cleavage by yscF. Apart from the KEX1 processing at the C-terminus, processing at the N-terminus, mediated by STE13, a dipeptidyl aminopeptidase (Kreil, 1990), is required for obtaining active α-factor in yeast. Also in *A. nidulans* a STE13-like aminopeptidase activity has been observed, as the N-terminal Ala-Pro dipeptide from human Interleukin-6 (hIL-6) was removed when expressed in this fungus (Contreras et al., 1991). Clearly, the high evolutionary conservation of protein-processing systems suggests that filamentous fungi will have many of the enzymes previously identified in yeast or mammalian cells. However, some interesting differences have also been observed. For instance, certain *A. oryzae* strains cannot cleave the KEX2 site in glucoamylase-IL6 fusion constructs as is observed in *A. nidulans* (Davies, 1994).

For production of heterologous proteins, there is a growing interest in the use of processing enzymes for site-specific cleavage of recombinant fusion proteins (Nagai and Thorgersen, 1987; Seeboth and Heim, 1991). Improved chymosin production was obtained when it was expressed from a glucoamylase/chymosin fusion gene, in which case chymosin was probably autocatalytically released from the fusion protein after secretion (Ward et al., 1990). Contreras et al. (1991) have reported 250-fold higher production of hIL-6 in *A. nidulans* when it was synthesized as a glucoamylase/hIL-6 fusion protein and subsequently processed *in vivo*. The mature IL-6 was fused through a short KEX-2

site containing spacer peptide, Arg-Met-Asp-Lys-Arg, to the C-terminus of the entire *A. niger* glucoamylase sequence. This is in contrast to previous constructs which contained only the glucoamylase pre- or prepro-secretion signals.

While many of the signal- and processing proteases serve specific functions and as such exhibit a narrow specificity range, they might become crucial in situations where large amounts of foreign proteins are being produced and secreted. Correct processing may become rate-limiting when secretion rates are high or yscF-like endoproteases may recognise exposed dibasic cleavage sites within heterologous proteins, thus reducing expression yields. Calmels et al. (1991) have shown that the typical yeast and mammalian dibasic propeptide cleavage sites (Lys-Arg, Arg-Lys and Arg-Arg) are rare in secreted filamentous fungal proteins. In addition, it is certainly possible that high-level production of proteins may represent a stress signal for the cell which leads to the induction of a variety of proteolytic activities as previously proposed by Hirsch et al. (1989).

1.4.1.3 Vacuolar proteolysis

Vacuoles in filamentous fungi and yeast are considered to have an important function in degradation. Several proteolytic activities which reside in the vacuole have been characterized in yeast (Suarez Renduelez and Wolf, 1988). The more recent cloning in *A. niger* of at least three genes which are homologous to yeast vacuolar proteases (Frederick et al., 1993; Jarai et al., 1994b; Yaver et al. 1995) suggests evolutionary conservation of the so-called activation cascade of vacuolar proteases. The three proteases involved in this cascade in yeast (yscA, yscB and yscY) are synthesized as inactive precursors. Upon arrival in the acidic environment of the vacuole the inactive acid protease precursor (pro-yscA) is probably autocatalytically activated by removal of its pro-region (Ammerer et al., 1986; van den Hazel et al., 1992). Active yscA proteolytically activates the precursors of several other vacuolar enzymes, including those of protease B (yscB) and carboxypeptidase Y (yscY or CPY). Active yscB is also capable of activation of pro-yscB and pro-yscY. The PEPE protease, the *A. niger* homologue of the yeast yscA protease has been disrupted (van den Hombergh et al., 1996f). In the Δ*pep*E disruptant, reduced carboxypeptidase activity was observed, although Northern analyses showed no changes in the levels of *cpy* mRNAs in the disruptant compared to wild type, indicating that in *A. niger* a similar vacuolar proteolytic cascade is present and that disruption of the *pep*E protease prevents maturation of CPY. Reduction of serine protease activities in the Δ*pep*E strain indicated similar effects for the PEPC protease.

An enzyme similar to the yeast carboxypeptidase S (yscS) has not been described until now, but it would be an interesting activity to study in relation to protein secretion and degradation in the secretory pathway. This protease does not need activation by yscA and yscB, and thus could travel

through the secretory pathway as an active enzyme, putatively capable of degrading gene products during secretion.

Apart from their highly specific function in maturation of several vacuolar proteins, Wolf and Ehmann (1981) showed that in *S. cerevisiae* several vacuolar proteases are needed for the differentiation process leading to sporulation, which is initiated upon nitrogen limitation. These proteases most likely provide the amino acids for protein biosynthesis related to spore formation at the expense of unneeded (vegetative) cell protein . However, it is also possible that vacuolar proteases are involved in activating other vacuolar enzymes needed for the sporulation events. The direct correlation between the presence of active yscA and endo-spore formation has been demonstrated in *S. cerevisiae*. In *A. niger* no Δ*pep*E-related sporulation defects are observed as expected since formation of endospores is absent in *A. niger*. Protease yscA activity in yeast is furthermore necessary for cell survival under nitrogen starvation conditions. Again, this could also be a secondary effect as essential activities normally activated by yscA are missing when yscA is disrupted which might be incompatible with cell survival. In support of the idea that vacuolar proteases are strongly involved in non-specific protein degradation are observations both in yeast and in *A. niger* that mutants lacking the vacuolar aspartyl endoprotease are severely hampered in the degradation of 'unspecific' substrates such as casein and BSA (Suarez Rendueles and Wolf, 1988; van den Hombergh et al., 1996f). Thus, one can assume that these enzymes represent major unspecific protease activities.

In yeast, the activities of many vacuolar proteases is cryptic in whole cell extracts, due to specific cytoplasmic inhibitor proteins. Incubation of cell extracts at low pH releases protease activity. A similar phenomenon has been observed in *A. niger* indicating that similar protease inhibitors are present (van den Hombergh, unpublished results). In yeast, for yscA, yscB and yscY specific inhibitor proteins have been isolated, and it has been shown that release of yscA activity at low pH is caused by specific yscB-dependent digestion of the yscA inhibitor protein I^{a3}.

1.4.1.4 Mitochondrial proteolysis

With the exception of a small number of proteins that are encoded by the mitochondrial genome, the vast majority of the mitochondrial proteins are encoded by nuclear genes, synthesized in the cytosol and imported into mitochondria. Several specific mitochondrial proteases have been identified which have a function during or after the actual import of mitochondrial proteins. A precursor protein is targetted to the mitochondrion by virtue of a specific presequence which is proteolytically removed by a specific metal-dependent mitochondrial processing peptidase, MPP, present in the mitochondrial

site containing spacer peptide, Arg-Met-Asp-Lys-Arg, to the C-terminus of the entire *A. niger* glucoamylase sequence. This is in contrast to previous constructs which contained only the glucoamylase pre- or prepro-secretion signals.

While many of the signal- and processing proteases serve specific functions and as such exhibit a narrow specificity range, they might become crucial in situations where large amounts of foreign proteins are being produced and secreted. Correct processing may become rate-limiting when secretion rates are high or yscF-like endoproteases may recognise exposed dibasic cleavage sites within heterologous proteins, thus reducing expression yields. Calmels et al. (1991) have shown that the typical yeast and mammalian dibasic propeptide cleavage sites (Lys-Arg, Arg-Lys and Arg-Arg) are rare in secreted filamentous fungal proteins. In addition, it is certainly possible that high-level production of proteins may represent a stress signal for the cell which leads to the induction of a variety of proteolytic activities as previously proposed by Hirsch et al. (1989).

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crassa) or *MAS1*/*MIF*1 (*S. cerevisiae*), containing the processing enhancing protein that stimulates the activity of MPP about 50-fold (Arretz et al., 1991; Hawlitschek et al., 1988; Schulte et al., 1989; Yang et al., 1988). The highly specific activity of this peptidase seems hard to reconcile with the absence of any cleavage recognition motif in the presequences. Analysis of a large number of known presequences and site-directed mutagenesis studies have only revealed that one or two positively charged residues, ususally arginine, at positions -3 and -2 relative to the cleavage site, are necessary for recognition and cleavage by MPP (Hartl et al., 1989; Niidome et al., 1994). Some data however, suggest that certain structures in the presequence and/or the mature part of the precursor proteins are important for efficient processing of precursor protein by MPP (Hartl et al., 1989).

In addition to mitochondrial targetting signals precursor proteins can contain sorting signals for intramitochondrial protein transport. Precursors of a group of intermembrane space proteins possess a bipartite presequence; the first half of the presequence resembles a typical mitochondrial targetting sequence that directs the import into the matrix and is cleaved off by the matrix processing peptidase. The second half shares similarities with prokaryotic leader sequences, consisting of a hydrophobic stretch preceded by basic residues, and this sequence is responsible for the intramitochondrial sorting of preproteins. In yeast, the second half of these targetting sequences is removed by inner membrane protease I (IMP1), a heterodimer composed of one 21.4 kDa Imp1p subunit and one 19 kDa Imp2p subunit (Nunnari et al., 1993; Schneider et al., 1991, 1994) present at the intermembrane space site of the inner membrane. Both IMP1 and IMP2 have a significant sequence similarity to the *E. coli* leader peptidase (Behrens et al., 1991). The processing of these proteins thus seems to follow a mechanism conserved from bacteria to mitochondria of eukaryotes. A number of proteins with bipartite presequences undergo a second cleavage by a matrix protease termed mitochondrial intermediate peptidase (MIP) or octapeptidyl peptidase (Isaya et al., 1991, 1994). This protease specifically removes an octapeptide from the amino terminal end that is generated after the cleavage by MPP.

1.4.2 Extracellular proteolysis

Many *Aspergilli* have a broad spectrum of secreted proteases and members of several classes of proteases have been described. As shown in Tables 1, 2, 3 and 4 many of the characterized fungal proteases are extracellular proteases. More recently, a lot of molecular genetic data has been generated with respect to cloning, characterization and expression of proteases from different filamentous fungi. In *A. niger* 5 genes (*pep*A, *pep*B, *pep*D, *pep*F and *pep*H) encoding extracellular proteolytic activities have been cloned and characterized. *A. niger* acidifies its medium very rapidly which explains the abundance of especially acid proteases, but also a neutral metallo protease and an

alkaline serine protease have been characterized. In contrast, the *A.nidulans* and *A. oryzae* protease spectra have a more neutral pH optimum for degradation of 'aspecific' proteins. Apart from their direct application, many extracellular proteases have been identified as one of the main reasons for low expression yields in filamentous fungi, especially when heterologous proteins are expressed. Recently, Bartling and coworkers (1996) have shown that the different protease spectra in *A. niger*, *A. awamori* and *A. nidulans* result in large differences of the expression yields of a heterologous protein, indicating that the choice of the expression host is very important and needs to be optimized for the particular protein of interest. The extracellular proteases in *Aspergillus* species have been extensively studied with respect to their expression and their effects on the degradation of target proteins. Reducing extracellular proteolysis has been shown to be a very useful means to improve expression in filamentous fungi (*vide infra*).

Some of the extracelllular proteases, for example the *A. niger* var. *macrosporus* protease A, are processed in a very complex way. The mature protease consists of two non-covalently associated polypeptide chains (Takahashi et al., 1991ab), a light (L) and heavy (H) chain, which are encoded by a single gene (Inoue et al., 1991). Processing probably involves four proteolytic steps: (I) cleavage at a signal peptidase cleavage site, (II) behind the asparagine residue preceding the N-terminus of the L-chain, (III) after the Tyr-residue at the C-terminus of the L-chain and (IV) behind the dibasic site preceding the N-terminus of the H-chain.

Apart from the proteases destined for secretion small amounts of mislocated proteases can also be present extracellularly, as shown for vacuolar yscY protease in yeast. In fact a significant part of the observed C-terminal degradation of heterologously expressed hirudin in yeast could be attributed to extracellularly mis-localized yscY protease (Hinnen et al., 1995). Recently, van den Hombergh and coworkers (1995g) described the disruption of the vacuolar acid protease *pep*E and as a result of this also a small reduction of extracellular pepstatin-inhibitable protease activity in the disruptant. Apart from the possible mislocalisation of intracellular proteases, cell lysis can also contribute to these observations.

1.5 Regulation of protease expression

Considering the variety of proteases and processes which involve proteolytic steps, the observation that some of these proteases show very complex regulation is not surprising. Regulation of proteases takes place at different levels. Many extracellular proteases show complex regulation at the level of transcription. Additionally, proteases can also be regulated at the translational level, or are synthesized as zymogens to prevent proteolysis before arriving at the correct location or are activated via cascade proteolysis.

1.5.1 Regulation at the transcriptional level

Wide domain regulation.

Wide domain regulatory mechanisms of particular importance for protease expression are carbon catabolite repression, nitrogen metabolite regulation, pH regulation and probably sulphur metabolite repression. These wide domain regulatory systems provide a means to adapt overall metabolism to use preferentially those nutrients that can be metabolized most efficiently. Carbon and nitrogen wide domain regulatory can affect simultaneously the expression of some genes involved in the metabolism of compounds that can supply the cell with sources of both nitrogen and carbon, and relief of either one leads to derepression. The wide domain pH regulatory system enables cells to synthesize proteins, which function at or outside the permeability barrier of the cell, only at pH values where they can function efficiently.

Cohen and co-workers have described in the early seventies that depletion of low molecular weight sources of nitrogen, carbon, phosphate and sulphur resulted in elevation of protease activities in *A. nidulans*, presumably caused by release from repression (Cohen, 1972, 1973a). At the same time it was shown that there is a reciprocal relationship between the release of extracellular protease and the concentrations of glucose present in the medium in *A. oryzae* (Klapper et al., 1973b). Clearly, these observations indicate the involvement of several wide domain regulatory systems in the overall regulation of protease expression in *Aspergillus*.

Carbon catabolite repression.

In the past a considerable amount of literature on carbon catabolite repression in *A. nidulans* has accumulated as reviewed by Arst and Bailey (1977) and more recently by Kelly (1994). A few model systems have been of most use in the elucidation of carbon catabolite repression at the molecular level: alcohol metabolism, the proline utilization cluster and the acetamidase gene (Felenbok and Sealy-Lewis, 1994; Scazzocchio, 1994; Hynes, 1994). The *cre*A genes cloned from *A. nidulans* (Dowzer and Kelly 1991) and *A. niger* (Drysdale et al., 1993), encode zinc finger proteins which bind to specific sites, SYGGRG, in target genes (Kulmburg et al., 1993). Many *cre*A mutant alleles have been isolated in *A. nidulans* and more recently in *A. niger* (Vanhanen et al., unpublished results), as suppressors of *are*A mutations. In *A. niger* wild type it has been shown that the cloned extracellular proteases (*pep*A, *pep*B and *pep*F) are regulated by carbon catabolite repression at the level of transcription (Jarai and Buxton, 1994; van den Hombergh et al., 1994). However, differences between the different extracellular proteases in relation to the specific carbon source used were also observed. Whereas the *pep*A gene was not repressed by glycerol, both the *pep*B and *pep*F genes showed severe repression after transfer to this carbon source. For the intracellular proteases, *pep*C and *pep*E, no

regulation at the level of transcription with respect to the carbon source was observed in *A. niger* wild type (Jarai et al., 1994b). In yeast however, activity of yscA, yscB and yscY increases several-fold *in vivo* when cells enter diauxic growth and stationary phase when they are grown on acetate or are transferred to nitrogen-free medium (Betz and Weiser, 1976).

*A. niger cre*A mutants (Vanhanen et al., 1996) have been used to prove the direct involvement of CREA in the observed carbon catabolite repression effects for the extracellular proteases (Fraissinet-Tachet et al., 1996). For all four cloned extracellular proteases in *A. nidulans* carbon catabolite repressive effects have been observed; the serine protease *prt*A has been studied in wild type (Katz et al., 1995), whereas for both the metallo proteases (*pep*I and *pep*J) and the aspartic protease (*pep*A) both wild type and a *cre*A^{d}2 mutant were used. Also in the *A. niger cre*A mutants the carbon catabolite repression of all extracellular proteases is reduced (Fraissinet-Tachet et al., 1996).

Interestingly, the most severe *cre*A mutant alleles described sofar in *A. niger*, *cre*A^{d}2 and *cre*A^{d}4, appeat to show increased expression of vacuolar proteases (Fraissinet-Tachet et al., 1996). However, this is probably not a direct *cre*A effect as no carbon regulation has been observed in wild type. As many *cre*A controlled systems are derepressed, synthesis of a large number of proteins is expected, which could trigger an increased need for protein turnover resulting in higher expression of vacuolar proteases.

Nitrogen metabolite repression

Filamentous fungi are capable of utilizing a wide range of compounds as sole nitrogen source and a large array of genes are dedicated to this aspect of metabolism. Nitrogen metabolite repression or 'ammonium' repression ensures expression of these genes only when a suitable substrate is present, leading to repression of enzymes and permeases involved in nitrogen sources other than ammonium or L-glutamine (Pateman et al., 1973; for a recent review see Caddick 1994). A single regulatory gene, called *are*A in *Aspergilli* and *nit*-2 in *N. crassa*, its product being required to alleviate nitrogen metabolite repression, has been cloned from *N. crassa* (Fu and Marzluf, 1990), *A. nidulans* (Caddick et al., 1986ab; Kudla et al., 1990) and more recently from *A. niger* (MacCabe et al., 1996b). The AREA regulator protein contains a putative $CX_2CX_{17}CX_2C$ zinc finger which enables it to bind to a specific target sequence (GATA) in the promoters of genes under AREA control. Many mutations in the *N. crassa nit*-2 and the *A. nidulans are*A genes, which have been designated *are*A^{r}, *are*A^{d} or altered function mutations, have been characterized (Caddick, 1994). The *are*A^{r} have been characterized as loss of function mutants, whereas the gene products of *are*A^{d} mutants seem to be functionally equivalent to the *are*A^{+} product (AREA) under de-repressing growth conditions, which

is the 'active' form. In wild type the level of 'active' AREA must reflect the nitrogen state of the organism, which appears to relate directly to the intracellular glutamine concentration (Wiame et al., 1985). The ability to perceive the nitrogen state of the cell, whether directly of indirectly, requires a negatively acting function within the gene, which is non functional in the *are*A^{d} alleles. The 'altered function' *are*A mutants lead to another hierarchy of expression for various activities, when compared to the wild type or other mutant strains. The heterogeneity of the cis-acting regulatory regions for the various target genes subject to *are*A control explains the altered specific interactions in the derepression of individual genes as observed for the 'altered function' class of *are*A mutants (Caddick, 1994).

The first observation of nitrogen-metabolite repression of protease activity was described by Shinmyo and coworkers (1971) who showed that synthesis of the *A. niger* acid protease was ammonium repressed. In the early seventies, during the analysis of nitrogen metabolite repression in *A. nidulans* one of the best characterized mutants, designated *xpr*D1, was isolated on the basis of derepressed proteolytic activities. Later it was demonstrated that this mutation in fact was an *are*A^{d} allele associated with a pericentric inversion (Arst, 1982; Arst and Scazzocchio, 1985).

The transcriptional regulation of several intra- and extracellular proteases in relation to nitrogen metabolite repression has been studied in *A. niger* and in *A. nidulans*. Jarai and Buxton (1994) show clear ammonium repression for the two extracellular acid proteases, *pep*A and *pep*B. Whereas both acid proteases are severely repressed by ammonium, urea represses *pep*B expression more severely than *pep*A. An explanation could be that some proteases are more tightly controlled by *are*A and conversion of a 'non-repressing' nitrogen source (urea) into a repressing nitrogen source could generate the low level of ammonium repression needed for repressing *pep*B expression. Similarly urea and glycerol effects have been observed for an extracellular serine carboxypeptidase (*pep*F) from *A. niger* (van den Hombergh et al., 1994). Fraissinet Tachet et al. (1996) demonstrated the direct involvement of the *are*A gene in the observed nitrogen related regulatory phenomena by using several *are*A mutants. For the intracellular *A. niger* proteases no direct nitrogen metabolite repressive effects have been observed.

pH regulation

Many filamentous fungi are able to grow over a wide pH range - seven to eight pH units for the wild type and extendable by mutations (Rossi and Arst, 1990). Despite of the widespread occurrence in the microbial world, the pH regulatory system has only been characterized in detail in *A. nidulans* and more recently in *A. niger*. The elucidation of the pH regulatory system in *A. nidulans* was started by Dorn (1965ab), who identified large numbers of mutants affected in the regulation of phosphatase

expression. Later, it was shown that several of these mutants were indeed affected in pH regulation (Arst and Cove, 1970; Arst et al., 1980). Two classes of pH regulatory mutants were identified and designated as *pac* (mimicking growth at alkaline pH) and *pal* (mimicking growth at acid pH). It was shown that the *pac* mutants can be divided into two classes, *pac*C^{c} and *pac*C$^{+/-}$, respectively. Subsequently it was hypothesised that the *pal* genes are involved in a pathway that generates an effector molecule for PacC. The pH regulatory gene, *pac*C, which has been cloned from *A. nidulans* (Tilburn et al., 1995) and *A. niger* (MacCabe et al., 1996a), encodes a triple zinc finger protein of the C_2H_2 class, which binds to specific target sequences (GCCARG) in the promoter of target genes. In the present model of pH regulation, presented by Tilburn and coworkers (1995), the *pac*C gene is highly expressed at alkaline pH. However, the complete primary translation product, PacC, expressed at high pH is not functional as shown by Orejas et al. (1995). For activation of PacC specific limited proteolysis is needed in order to generate a truncated functional PacC protein which acts both as an activator for alkaline expressed genes and as a repressor for acid expressed genes. The proteolytic step is not encoded by the *pal* 'pathway' and it has been postulated that the C-terminal domain of PacC which is removed during activation serves as a masking domain. Proteolysis cannot take place before an effector produced by the *pal* genes (expressed at an alkaline pH) distorts the masking and enables the limited proteolysis to take place. This model explains why *pac*C mutants are epistatic to the *pal* mutants and also explains the two classes of *pac*C mutant phenotypes which have been identified. Detailed molecular analysis of *pac*C mutants showed that the *pac*C^{c} mutations all rendered a demasked protein that was C-terminally truncated (Tilburn et al., 1995; Orejas et al., 1995). As the *pac*C^{c} mutants are demasked and therefore express *pac*C constitutively, apparently the need for signalling the external pH is absent. This leads to the conclusion that the C-terminal part of PacC is probably involved in negatively modulating the function of *pac*C in response to pH. *pac*C$^{+/-}$ mutants, which do result from mutations upstream of the postulated proteolytic cleavage site and are therefore mutated within the functional active protein, are loss of function mutations.

Interestingly, one of the *pal* genes involved in signalling the ambient pH in *A. nidulans*, *pal*B, encodes a cysteine protease. Apparently, part of the *pal* encoded signal transduction pathway also involves a proteolytic step (Denison et al., 1995). However, this proteolytic step is probably not the final proteolytic step to generate the functional PacC protein. It has been postulated that the *pal* pathway introduces a modification of PacC at alkaline pH, thereby disrupting intramolecular interactions to allow activating proteolysis (Orejas et al., 1995). The type of PacC modification mediated by the *pal* pathway in response to alkaline pH is not known, but an earlier (and more C-terminal) proteolytic cleavage of PacC resulting in susceptibility to further proteolysis might be mediated by PalB. Alternatively, PalB might cleave one of the *pal* gene products in a signalling

cascade (Denison et al., 1995). Recently we cloned the *pal*B equivalent of *A. niger* suggesting that a signal transduction pathway is present in *A. niger* similar to the *A. nidulans pal* pathway (van den Hombergh, unpublished results).

Apart from proteolytic steps involved in the pH regulatory system itself, several proteases appear to be regulated in response to ambient pH. Except for the cloned *A. niger* and *A. nidulans* protease genes, little is known about pH regulation of proteases in other filamentous fungi. During the characterization of the *pac*C gene in *A. nidulans* Tilburn and coworkers (1995) have used the alkaline serine protease from *A. nidulans* (*prt*A; Katz et al., 1995) as an alkaline target gene. Clearly they show that this gene is only expressed at high pH in wild type, repressed in a *pac*C$^{+/-}$ mutant and constitutively expressed in a *pac*C^{c} mutant. Since *A. niger* acidifies its medium very rapidly, its acid proteases are ideal candidates for studying pH regulation of acid target genes. The extracellular acid proteases, *pep*A and *pep*B, were shown to be pH regulated in *A. niger* wild type (Jarai and Buxton, 1994). The direct involvement of *pac*C was later shown by Fraissinet-Tachet et al. (1996). They used a specific *A. niger pac*2C^{c} disruption strain, which has an alkaline mimicking phenotype similar to the *A. nidulans pac*C^{c} mutants (van den Hombergh et al., 1996b), to show the direct involvement of *pac*C in the observed pH regulation of the extracellular proteases (*pep*A, *pep*B and *pep*F). Due to the constitutive expression of *pac*C in the disruption strains, genes normally expressed at an acidic ambient pH, are down regulated as described by Fraissinet-Tachet et al. (1996). Surprisingly however, the expression of both extracellular acid proteases was elevated at pH 8. As the *pac*2C^{c} disruptant is phenotypically similar to the *A. nidulans pac*C^{c} mutants in which *pal* signalling is absent, it appears that the *pal* genes are not involved in these observed differences for acid protease expression. Absence of pH signalling in *A. niger pac*2C^{c} has been clearly demonstrated by van den Hombergh and co-workers (1996b) as the *pac*2C^{c} mRNA is highly expressed at every pH tested. The altered regulation of acid proteases in the *pac*2C^{c} strain can be the result of the disturbed interaction between cis-acting regulatory functions upon binding of a truncated PacC^{c} protein. However, this observation could also indicate that with respect to the acid part of the pH regulation the present model has to be optimized further or that not all the components of the pH regulatory system involved in acid pH regulation have yet been identified.

Sulphur metabolite repression

Addition of reduced sulphur sources such as cysteine or methionine to the growth medium leads to repression of a number of enzymes needed for utilization of less preferable poorer sulphur sources like sulphate. These include first of all enzymes of the sulphate assimilation pathway and sulphohydrolases and to a lesser extent, enzymes of the alternative pathway of cysteine synthesis

(Paszewski et al., 1994). Sulphur metabolite repression is turned on most efficiently by addition of L-methionine. Whether this amino acid itself is the regulatory effector is unknown as it can be readily converted into other sulphur-containing compounds. Four *scon* (sulphur controller) complementation groups (*scon*A, B, C, D) have been identified in *A. nidulans* mutants defective in sulphur metabolite repression (Fu et al., 1990; Natorff et al., 1993). Little is known about sulphur metabolite repression in *Aspergilli* and putative involvement in protease regulation. However, already in 1967, Yanagita and Nomachi described de-novo synthesis of an *A. niger* acid protease upon sulphur deficiency in the culture medium. A similar phenomenon has been observed for some of the neutral-alkaline endopeptidases from *A. nidulans* (Cohen, 1973b; Stevens and Stevens, 1980; Stevens, 1985).

Additional regulatory systems

Specific induction

In *A. niger* external addition of proteins induces protease gene expression at the transcriptional level (Jarai and Buxton, 1994; van den Hombergh et al., 1994). However, little is known about the actual inducers and the mechanisms involved. How protein degradation products are imported into the cell and what the actual inducers are remain still unanswered questions. The isolation of *prt* (protease deficient) mutants in *A. niger* which are affected in the induction by protein could prove to be a valuable tool to clone some of the genes involved in protein induction by complementation.

Protease mutants; identification of additional regulatory systems

Several mutagenesis studies were done to generate strains in which proteolytic degradation of both homologous and heterologous products was reduced. In *A. niger* several proteolytic mutants have been characterized (Table 1.4). Mattern et al. (1992) described the isolation of several UV induced protease mutants, using a halo-screening method based on skimmilk. More recently, at least seven protease complementation groups have been isolated by van den Hombergh et al. (1995). After improving a conventional halo-screening method using a dual substrate (gelatin and casein), they have characterized, both genetically and biochemically, fourteen *prt* mutants, representing a range of reduced proteolytic activities compared to wild type. Allocation of the mutations to one of the eight linkage groups in *A. niger* showed that one mutation is located on linkage group I (*prt*C), three mutations are located on linkage group III (*prt*B, *prt*E and *prt*G), one mutation is located on linkage group V (*prt*F) and two mutations are located on linkage group VIII (*prt*A and *prt*D). Although for many of the *A. niger prt* mutants the precise nature of the mutation is unknown, several of these mutations must be regulatory or cascade activating mutations since the residual activities observed in these mutants indicate that these mutations must have affected more than one protease.

Table 1.4. Protease mutants in *Aspergillus*

Species	Mutant	Mutation	Linkage group	Residual activity (%)	Part of the protease spectrum affected by mutation(s)	Reference
A. awamori var. *kawachi*	HF-15	-	nd	7-50%[1]	nd	Hayashida and Flor, 1981
	HF-10	-	nd	500%	nd	
A. ficum	M-33	-	nd	decr.[2]	nd	Hayashida and Teramoto, 1986
	M-72	-	nd	incr.[2]	nd	
A. nidulans	-	*xpr*D1	III	incr.[2]	AREA regulated proteases	Cohen, 1972
A. niger	AB1.13	*prt*-13	VI	1-2[3]	acid proteases	Mattern et al., 1992
	AB1.18	*prt*-18	I	15-20[3]	PEPA	
	AB1.33	*prt*-33	?	nd	nd	
	AB1.39	*prt*-39	VIII	nd	nd	
	AB1.18-25	*prt*-18 *prt*-25	I nd	nd	nd	
	NW220	*prt*-3	III	80[4]	nd	van den Hombergh et al., 1995
	NW221	*prt*C7	I	52[4]	PMSF inh. act.	
	NW222	*prt*A10	VIII	21[4]	all act.	
	NW223	*prt*-11		69[4]	nd	
	NW224	*prt*C14	I	32[4]	PMSF inh. act.	
	NW225	*prt*E15	III	63[4]	pepst. inh. act. PMSF inh. act.	
	NW226	*prt*G24	III	7[4]	all act.	
	NW227	*prt*B26	III	2[4]	all act.	
	NW228	*prt*F28	V	3[4]	all act.	
	NW229	*prt*F29	V	3[4]	all. act.	
	NW230	*prt*E30	III	20[4]	pepst. inh. act. PMSF inh. act.	
	NW231	*prt*C31	I	63[4]	PMSF inh. act.	
	NW232	*prt*D32	VIII	4[4]	all act.	
	NW233	*prt*C7 *prt*A33	I VIII	1[4]	nd	
	NW234	*prt*C7 *prt*-35	I ?	3[4]	nd	
	NW237	*prt*A10 *prt*C14	VIII I	4[4]	nd	
	NW238	*prt*A10 *prt*E15	VIII III	4[4]	nd	
	NW239	*prt*A10 *prt*F28	VIII V	2[4]	nd	

[1], variation depending on the culture conditions; [2], residual protease activities were not presented; incr, increased protease activity compared to wild type; decr, decreased protease activity compared to wild type; [3], activities against sperm whale myoglobin; [4], activities against BSA.

Detailed biochemical analysis of residual protease spectra in *prt* mutants revealed in many cases mutation specific changes in the overall protease spectrum. Both reduced and elevated expression of certain parts of the proteolytic spectrum have been found. The latter possibly resulting from a compensatory effect of non-mutated proteases to compensate for the loss of other proteolytic activities, caused by the specific mutation. In some *prt* mutants elevated levels of an extracellular metallo protease, *pep*H, have been identified (van den Hombergh et al., 1996g). Based on the allocation of the *cre*A, *are*A and *pac*C genes in *A. niger* to linkage groups IV, III and IV, respectively, with the possible exception of the *prt*-3 and the *prt*-35 mutations, the *prt* mutations are not allelic to any of the three mentioned wide domain regulatory genes. Thus, several additional factors, involved in protease expression have been identified, proving the complexity of protease expression. Although transcriptional regulation still appears to be one of the main levels of regulation of gene expression, in several *prt* mutants the protease mRNA levels are nearly identical or similar to wild type transcript levels, possibly indicating that post-transcriptional regulatory mechanisms are affected (van den Hombergh et al. 1996e).

1.5.2 Posttranscriptional regulation

Zymogen regulation

Apart from the transcriptional regulation many organisms have additional post-transcriptional regulation systems. An important mechanism to ensure that a protease becomes only active after having arrived at its correct location is called zymogen activation. Many of the proteases characterized contain pro-sequences to which several functions have been ascribed. In addition to shielding proteolytic activity they can be involved in routing and specific targetting within the cell, as described for the PEP4 protease in yeast (Suarez Rendueles and Wolf, 1988).

Protease inhibitors

In fresh mycelial extracts from *A. nidulans* (Cohen, 1973ab; Stevens and McLennan, 1983; Ansari and Stevens, 1983a; Stevens, 1985; Stevens et al., 1985), *A. niger* (Walker and Chaplin, 1983), *A. oryzae* (Matsushima, 1979), *A. japonicus* (Hanada et al., 1978) an increase of protease activity with time has been observed, Zymogen activation and inactivation of specific protease inhibitors similar to the situation in *S. cerevisiae* (Wolf, 1980) are probably responsible for these observations. In *S. cerevisiae* several inhibitors specific for vacuolar proteases have been characterized. These inhibitors are being inactivated via proteolysis, but the vacuolar proteases are not capable of hydrolyzing their own specific inhibitors. In *S. cerevisiae* these inhibitors are cytoplasmic proteins, thus separated from their target proteins and probably involved in protection of cytoplasmic proteins against vacuolar

hydrolases, if vacuolar damage occurs.

1.6 The protease problem

The main reasons for the renewed interest in microbial proteases are the protease related expression problems observed in several expression hosts used in the bioprocess industry. The increasing use of heterologous hosts for the production of proteins by recombinant DNA technology, has recently brought this problem into focus, since it seems that heterologous proteins are much more prone to proteolysis (Archer et al., 1992; van den Hombergh et al., 1996a).

In *S. cerevisiae* the protease problem and the involvement of several proteases, which complicates targetted gene disruption approaches to overcome this problem, was already recognised in the early eighties (Wolf, 1980). During secretion a protein is exposed to several proteolytic activities residing in the secretory pathway. Additionally, in a saprophytic microorganism like *Aspergillus* secreted proteins can be exposed to several extracellular proteolytic activities. In *S. cerevisae* already more than 40 independent proteolytic activities have been described (Suarez Rendueles and Wolf, 1988). Many proteins expressed in *S. cerevisiae* and *Aspergillus* undergo, in addition to the removal of the signal peptide, other proteolytic events during secretion.

From Table 1.5, which lists a number of heterologous proteins which are expressed in *Aspergillus*, it is obvious that in many reports (partial) degradation of the product has been described. Attemps to control the problem of proteolysis may be approached at several stages in the protein production process. Bioprocess engineering steps to handle the problem of proteolysis (e.g. by downstream processing at low temperatures, by early separation of product and protease(s) or by the use of protease inhibitors) reduce the problem. However, it is certainly not eliminated because much of the degradation occurs *in vivo* during the production of the protein.

In understanding how proteolysis is controlled inside the cell, a major question concerns the recognition mechanism by which proteolysis is triggered. To what extent are proteolytically susceptable (heterologous) proteins recognized as aberrant because of misfolding or, if correctly folded, as 'foreign', because they do not possess features essential for stability which are specific to the host? Various types of stress can also cause the overall proteolysis in a cell to increase significantly. Factors known to increase the rate of proteolysis include nutrient starvation and various other types of stress (i.e. elevation of temperature, osmotic stress, toxic substances and expression of certain heterologous proteins). To deal with proteolysis-related expression problems *in vivo*, severalapproaches have been proven succesful as will be discussed below. However, we have to keep in mind that true 'non-proteolytic cells' cannot exist, since proteolysis by intracellular proteases is involved in many essential metabolic and 'housekeeping' reactions. Reducing proteolysis will

Table 1.5. Heterologous proteins expressed in *Aspergillus*

Protein[1]	Heterologous source	Expression Host	Promoter	Reference
Vertebrate				
prochymosin	cow	*A. nidulans*	*gla*A	Cullen et al. (1987)
		A. nidulans	*oli*C	Ward et al. (1991)
		A. oryzae	*gla*A	Ward et al. (1990)
interferon α2	human	*A. nidulans*	*alc*A *gla*A	Gwynne et al. (1987)
		A. nidulans *A. niger*	*aph*A *aph*A	MacRae et al. (1993)
tPA	human	*A. nidulans*	*alc*C *tpi*A *adh*A	Upshall et al. (1987)
EGF	human	*A. nidulans*	*alc*A	Gwynne et al. (1987)
GMCSF	human	*A. nidulans*	*adh*A	Upshall et al. (1991)
CBG	human	*A. nidulans*	*alc*A	Devchand and Gwynne (1991)
SOD	human	*A. nidulans*	*alc*A	Devchand and Gwynne (1991)
GH	human	*A. nidulans*	*alc*A	Devchand and Gwynne (1991)
IL6	human	*A. nidulans*	*alc*A	Devchand and Gwynne (1991)
		A. nidulans	*gla*A	Carrez et al. (1990)
		A. niger	*gla*A *gpd*A	Contreras et al. (1991)
egg-white lysozyme	chicken	*A. niger*	*gla*A *gpd*A	Archer et al. (1990, 1992)
PLA_2	porcine	*A. niger*	*gla*A *gpd*A	Archer et al. (1992)
lactoferrin	human	*A. oryzae*	*amy*A	Ward et al. (1992)

Table 1.5 (continued)

Insect				
Bm86	*Boophilus microplus*	*A. nidulans* *A. niger*	*amd*S	Turnbull et al. (1990)
Filamentous fungal				
aspartyl-protease	*Mucor miehei*	*A. oryzae*	*amy*A	Christensen et al. (1988)
glucoamylase	*A. niger*	*A. nidulans*	*gla*A	Punt et al. (1991)
pectin lyase B	*A. niger*	*A. nidulans*	*pki*A	Kusters-van Someren et al. (1992)
lipase		*A. oryzae*	*amy*A	Davies (1994)
Bacterial				
endoglucanase	*Cellulomonas fimi*	*A. nidulans*	*alc*A *gla*A	Gwynne et al. (1987)
enterotoxin subunit B	*E. coli*	*A. nidulans*	*amd*S	Turnbull et al. (1989)
ß-galactosidase	*E. coli*	*A. niger*	*gpd*A	Punt et al. (1991)
ß-glucuronidase	*E. coli*	*A. niger*	*gpd*A	Roberts et al. (1989)
pectate lyase 3	*Erwinia carotovora*	*A. nidulans* *A. awamori* *A. niger*	*pki*A	Bartling et al. (1996)

[1], abbreviations: CBG, Corticosteroid Binding Globulin; EGF, Epidermal Growth Factor; GH, Growth Hormone; GMCSF, Granulocyte-Macrophage Colony Stimulating Factor; IL6, Interleukin 6; PLA_2, PhosphoLipase A_2; SOD, SuperOxide Dismutase; tPA, tissue Plasminogen Activator. When proteolytic degradation by *Aspergillus* proteases has been reported, the protein is underlined. Modified from Davies (1994)

therefore always be a process in which the changed genetic background which results in decreased proteolysis has to be analysed for potential secondary effects which could lead to reduced protein production (such as reduced growth rate or sporulation).

Disruption of proteases in filamentous fungal expression hosts

Berka and coworkers (1990) were the first to describe the cloning and disruption of the *A. awamori pep*A gene. More recently, we have disrupted three aspartyl proteases in *A. niger*. Both the major extracellular aspartyl proteases and the major vacuolar aspartyl protease were disrupted. Double and triple disruptants were generated by recombination and tested for residual protease activities and expression and secretion of the *A. niger* pectin lyase PELB protein, which is very susceptable for proteolytic degradation (van den Hombergh et al., 1995). Disruption of *pep*A and *pep*B both resulted in reduction of extracellular protease activities with 80% and 6%, respectively. In the Δ*pep*E disruptant also other (vacuolar) protease activities were severely affected which is caused by inactivation of the proteolytic cascade for other vacuolar proteases. Reduced extracellular activities correlated well with reduced *in vitro* degradation of PELB (van den Hombergh et al., 1996f).

Protease deficient (prt) mutants

Several *Aspergillus* protease deficient mutants have been studied to see whether protein production is improved in these strains. Archer and coworkers (1992) describe the reduced proteolysis of hen egg white lysozyme in supernatants of an *A. niger* double *prt* mutant generated by Mattern and coworkers (1992) and conclude that although the degradation is not absent, it is significantly reduced. Van den Hombergh et al. (1995) showed that the *in vitro* degradation of *A. niger* PELB is reduced in all seven *prt* complementation groups they have isolated. Virtually no degradation was observed in the *prt*B, *prt*D and the *prt*F mutants. Recently, the expression of the *pel*B gene was shown to be improved in several of the complementation groups tested (*prt*A-F). The highest expression levels were observed in the *prt*A, *prt*B, *prt*D and *prt*F mutants which is in agreement with the *in vitro* data. In addition to the single mutants, which contained residual extracellular proteolytic activities varying from 2-80 % compared to wild type activity, double mutants were generated both by recombination and by additional rounds of mutagenesis. Via this approach several double *prt* mutants were selected and further characterized (Table 1.4), which showed a further reduction of PELB degradation compared to the parental strains.

Down regulation of protease activities

Instead of elimination of protease activities via disruption or mutagenesis, which could result in impaired growth and/or expression, reduced proteolysis can also be achieved by down-regulating interfering proteolytic activities. The complex regulation of several proteases allows optimization of culture conditions to reduce protease expression. As shown by Fraissinet-Tachet and coworkers (1996) the extracellular proteases in *A. niger* are all regulated by carbon catabolite repression and nitrogen metabolite repression. Nutrient starvation also causes the overall proteolysis rate in a cell to increase strongly. In expression strategies which allow high expression on media containing high glucose and ammonium concentrations reduced proteolysis has been reported. Several constitutive glycolytic promoters (*gpd* and *pki*A) are highly expressed under these conditions and can also be used to drive (heterologous) gene expression in continuous fermentations. The type of nutrient starvation imposed can influence different proteases to varying extent, which means that the importance of the nutrient conditions in a given process depends on the type of proteolysis that is involved. Therefore, the effects observed after nutrient starvation also raises the question whether proteolysis is induced by conditions of substrate limitation which are frequently used in many large-scale fermentation processes.

Protective protein fusions and modifications

Finally, the expressed protein can also be stabilized and thus to some extent be protected against proteolytic degradation. The improved expression results for some heterologous proteins upon fusion to glucoamylase are maybe also partially due to reduced proteolysis via shielding. A possible mechanism behind this protection could be steric hindrance for the protease to reach the susceptable peptide bond(s). However, remote parts of the protein sequence may function not only to prevent proteolysis, but also to change the site of proteolysis. Other types of protein protection one can think of are specific amino acid changes if certain peptide bonds are very susceptable to proteolysis, provided the functional properties of the product allow these changes. Futhermore, glycosylation has also shown to stabilize some proteins, probably by similar hindrance effects as described for protective fusions. Little is known about glycosylation in filamentous fungi and considering the possibility to protect by over-glycosylation is not one of the more obvious choices.

Increasing knowledge about the intra- and extracellular proteolytic mechanisms, in which in addition to the proteases themselves several others factors such as chaperone functions, folding, modification and protein structure seem to play key roles, new strategies for control of proteolysis can be developed.

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2 Aim and outline of the thesis

This thesis describes the results of studies on the genetics, biochemistry and physiology of proteases in *A. nidulans* and *A. niger*. The work presented here was done as part of a project to study the possibilities for strain improvement for protein production in *Aspergillus niger*. As postulated in chapter I degradation of protein products still forms a major obstacle in improving gene expression and protein secretion in *Aspergillus*. The aim of this thesis was to obtain more information about which proteases in *A. nidulans* and *A. niger* cause the major expression problems, how protease-related problems can be solved and whether it was possible to construct specific strains which can be used for overexpression of both homologous and heterologous genes. This involved characterization of the protease spectrum, the cloning and disruption of specific protease activities, detailed protease regulation studies, classical mutagenesis to generate *prt* mutations and expression studies of both homologous and heterologous genes under optimized physiological conditions.

In Chapter III three fungal expression hosts, *A. niger*, *A. awamori* and *A. nidulans*, have been compared with respect to the expression of the heterologous *Erwinia carotovora* pectin lyase 3 protein, which is very susceptible to proteolytic degradation. Large differences in expression yields, the proteolytic spectrum of the host and glycosylation of the target protein were observed, indicating the importance of the choice of the expression host. Chapter IV describes the cloning of an intracellular aspartic protease, *pep*E, from *A. niger*. It was shown that *pep*E and *pep*C (another intracellular *A. niger* protease) are constitutively expressed. Chapter V describes the cloning and characterization of a serine carboxypeptidase, *pep*F, from *A. niger*. This extracellular exoprotease is C-, N-, and pH regulated and also induced by the addition of protein to the growth medium. During the generation and subsequent analysis of a set of protease (*prt*) mutants (see chapter VIII), it was observed that some *prt* mutants expressed (elevated levels of) metallo protease activities. In chapter VI the cloning and characterization of *pep*H, a thermolysin-type metallo protease from *A. niger*, is described. Furthermore, the observed elevated metallo protease activities in some of the *prt* mutants were shown to be correlated with increased *pep*H mRNA levels. Disruption of three acid proteases, which cause the major degradation problems observed in *A. niger*, is described in chapter VII. Two extracellular proteases (*pep*A and *pep*B) and one intracellular protease (*pep*E) were disrupted and effects on the residual protease spectrum, the cascade activating mechanism for vacuolar proteases, and *in vitro* degradation of tester proteins were investigated. The generation and characterization of seven protease mutant complementation groups is presented in chapter VIII. In the seven different complementation groups identified, the residual activities varied from 2-80%. Degradation of proteins in culture supernatants of some of these *prt* mutants decreased 500-1000 fold. Construction of multiple

prt mutants, either by recombination or by additional mutagenesis, further reduced degradation. After the generation of transformable *prt* mutant strains, expression of the *pkipel*B expression secretion cassette was shown to be improved in several *prt* complementation groups tested (Chapter IX). Northern analysis of the *prt* mutants with all cloned protease genes and the allocation of these protease genes to linkage groups, using CHEF-Southern analysis, are also presented in this chapter. Cloning and analysis of the pH wide domain regulatory gene, *pac*C, is described in Chapter X. In chapter XI we report the disruption of the *A. niger* pH regulatory gene *pac*C, the effects of this on auto-regulation of this gene and on the expression of acid and alkaline phosphatase target genes. The major phosphatases in *A. niger*, their localization and the effects of pH and phosphate on their biosynthesis are presented. The direct involvement of the *cre*A, *are*A and *pac*C genes in the observed carbon catabolite repression, nitrogen metabolite repression and pH regulation of extracellular proteases is demonstrated in chapter XII. Specific *cre*A and *are*A mutants and the *pac*C^{c} disruption strain were used in a Northern analysis to show direct involvement of these wide domain regulatory genes on the level of transcription of the *pep*A, *pep*B and *pep*D genes.

The effects of pH regulatory mutations on protein production in *A. niger* and *A. nidulans* are described in chapter XIII. The reduced proteolysis in *pac*C^{c} alkaline mimicking mutants initiated the cloning and characterization of an aspartic pepstatin repressible protease (*pep*A) from *A. nidulans* (Chapter XIII). Two novel metallo proteases from *A. nidulans* are presented in chapter XIV. These metallo proteases show no significant homology with any of the known metallo protease families (apart from the two zinc (Zn) chelating His residues), which could indicate that they belong to a novel metallo protease family. As *A. nidulans* has a predominant neutral to alkaline protease spectrum these metallo proteases could significantly contribute to the observed proteolytic degradation of expressed proteins, also observed in this organism. Finally, in Chapter XV a general discussion of the results presented in this thesis is given.

3 Expression of an *Erwinia* pectate lyase in three species of *Aspergillus*[1]

S. Bartling, J.P.T.W. van den Hombergh, O. Olsen, D. von Wettstein, J. Visser

Abstract

Transgenic filamentous fungi of the species *Aspergillus niger*, *A. nidulans* and *A. awamori* expressing and secreting *Erwinia carotovora* (*Eca*) subsp. *atroseptica* pectate lyase 3 (PL3) were generated. Correct processing of the pre-enzyme was achieved using the *A. niger* pectin lyase A (PEL A) signal peptide. With the prepro-peptide of *A. niger* polygalacturonase II, secreted enzymes still possessed the six amino acid (aa) pro-sequence, indicating the importance of the conformation of the precursor protein for correct cleavage of the signal sequence. PL3 expression was markedly increased in media optimized for limited protease activity, and reached 0.4, 0.8 and 2.0 mg/l for expression in *A. niger*, *A. awamori* and *A. nidulans*, respectively. Glycans attached to the PL3 enzymes exhibited species-specific differences, and an increase of molecular mass coincided with reduced specific activities of the enzymes.

Introduction

Among filamentous fungi, *Aspergillus* species such as *A. niger*, *A. awamori*, *A. nidulans* and *A. oryzae* are widely used as hosts for the expression of homologous as well as heterologous genes (reviewed by Devchand and Gwynne, 1991; Ward, 1991; Davies, 1994). These lower eukaryotes, of which several have GRAS status, provide some advantages for heterologous expression such as correct post-translational modifications as observed in many cases and less over-glycosylation than observed in yeast. However, host proteases may markedly reduce the yield of recombinant products. Although individual strains and species exhibit marked differences in growth behavior and the production of proteases, experimental data that directly compare yields of a heterologous protein expressed in different host species is scarce.

Aspergilli synthesize and secrete various pectolytic enzymes such as polygalacturonases (Bussink, 1992) and pectin lyases (Kusters-van Someren et al., 1991). The pectate lyase (PEL A) has only been identified in *A. nidulans*. This enzyme is more closely related to PLs from plants

[1] Curr. Genet. **29**:474-481 (1996)

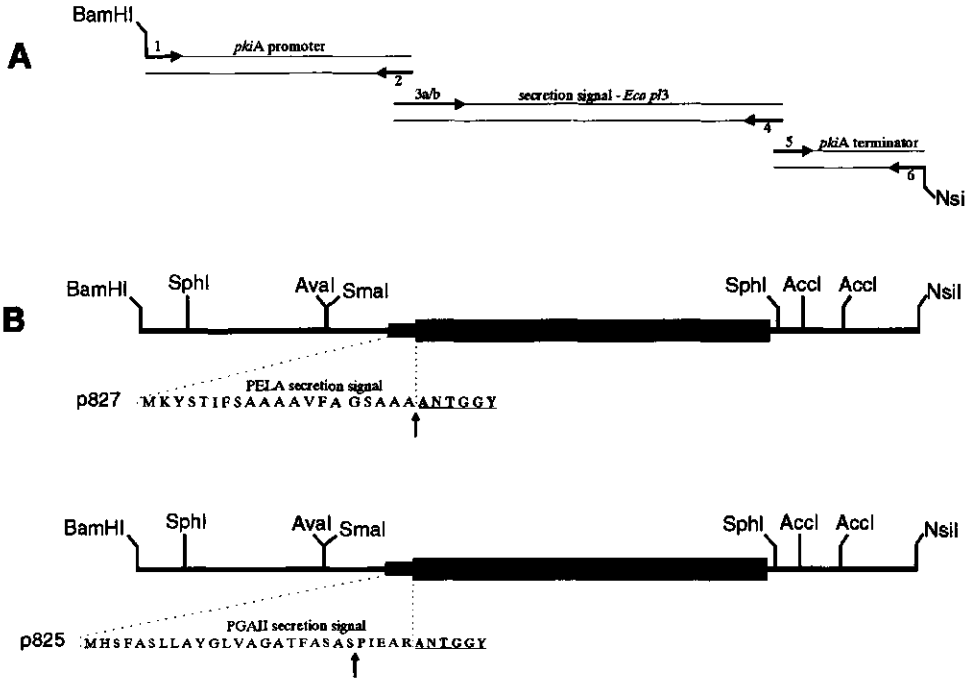
nidulans). Two micrograms of pure recombinant PL3 (Bartling et al. 1995) were added to 10 µl serial dilutions of the adjusted supernatants and incubated for 15 min at 37°C. The samples were either directly analysed by SDS-PAGE or adjusted to contain 1 µM pepstatin, 1 mM phenylmethanesulfonyl fluoride, 2 mM phenanthroline, pH 8.5, to measure the residual activity of PL3 without concurrent enzymatic proteolysis.

Results and discussion

Aspergillus transformants

In addition to the use of a strong promotor, to ensure high-level expression, a signal sequence is required to target the expressed protein to the secretory pathway. Although several different secretion signals have been used successfully, it is at present not possible yet to predict the secretion efficiency in individual cases by using a specific signal sequence. Accordingly, two expression vectors (p825 and p827, Fig. 3.1) were constructed to direct synthesis and secretion of PL3 in *Aspergillus*. The PCR technique involving splicing by overlap extension was used to combine the *A. niger pki*A promotor and terminator sequences directly with sequences encoding pre-PL3. While this allowed gene expression in media supplemented with glucose, secretion was realized by using either the *A. niger* PGAII (p825) or PEL A signal sequence (p827). By cotransforming *A. niger*, *A. nidulans* and *A. awamori* protoplasts either with p825 or p827 using the *A. niger pyr*A gene, encoding orotidine 5'-phosphate decarboxylase, for selection towards uridine auxotrophy (Goosen et al., 1987), around 1800 transformants were obtained from each strain. All transformants were covered with top agarose supplemented with PGA, and subsequent staining with ruthenium red revealed that for approximately 600 colonies clear zones were detected, indicating the secretion of active PL3. Distinctly larger halos were observed in about 30% of these transformants. Moreover, immunoblot analyses, employing polyclonal antibodies raised against PL3, confirmed the presence of PL3 in culture samples obtained from four transformants of each strain (data not shown). No abnormal phenotypes at either vegetative or sporulation stages were detected.

Since plasmid-mediated transformation of *Aspergillus* cells results in insertion into the genome, Southern analysis of genomic DNA from individual transformants showed several fragments hybridizing to the probe encompassing sequences encoding PL3. Strongly hybridizing 5.4-kb fragments, corresponding to *Bam*HI-linearized plasmids, were observed in most of the transformants (Fig. 3.2). This result may reflect events of tandem integrations. In several cases of heterologous expression, the introduction of multiple copies of a target gene has resulted in a considerable increase in protein production (for a review see Verdoes et al., 1995).



C

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primer 1    CGCGGATCCGCGACGATGTGGGGTCAACT
primer 2a   GTAGGCGAGAAGAGAAGCAAACGAGTGCATCTTGACGGATGATTGATCTCTACTGAACCG
primer 2b   AGCAGCGCTGAAGATAGTAGAGTACTTCATCTTGACGGATGATTGATCTCTACTGAACCG
primer 3a   ATGCACTCGTTTGCTTCTCTTCTCGCCTACGGCCTGGTCGCCGGCGCCACCTTCGCTTCT
            GCCTCTCCTATCGAAGCTCGAGCAAATACGGGGGGTTATGCCACC
primer 3b   ATGAAGTACTCTACTATCTTCAGCGCTGCTGCCGCTGTTTTCGCTGGTTCCGCCGCTGCA
            GCAAATACGGGGGGTTATGCCACCACTGACGGTGGTGAC
primer 4    TGGCATGCCAGACTGCTTTTGCATTTAGTTGCAATTTGCTGCTGTCAGCACGG
primer 5    TAAATGCAAAAGCAGTCTGGCATGCCA
primer 6    ATTCGGAATGCATTTCCACTAACAAA
```

Fig. 3.1. Construction of expression cassettes with the *Erwinia* gene encoding PL3 flanked by the *A. niger* *pki*A promoter and terminator. Either the sequence encoding the *A. niger* PEL A signal sequence or PGAII prepro sequence is included in the cassette. Three primary amplification products **(A)** were fused using splicing by overlap extension to generate the inserts in plasmids p825 and p827 **(B)**. Vertical arrows indicate processing sites of the protein precursor as determined by N-terminal sequencing. The sequences of PCR primers 1-6 used for construction of the illustrated expression cassettes are indicated 5' to 3' **(C)**. Underlined sequences are identical to the nucleotides encoding the signal sequences of *A. niger* PGAII (primers 2a and 3a) and PEL A (primers 2b and 3b). Primer 1 contains a *Bam*HI restriction site upstream of a sequence identical to bases 306-321 of the *pki*A promoter (de Graaff et al., 1992). Two sets of primers 2 and 3 were used to engineer fusions of the *pki*A sequence upstream of the *pki*A translational start codon with the sequence encoding the secretion signal of PGAII (primers 2a and 3a; Bussink et al., 1990) or that of PEL A (primers 2b and 3b; Kusters-van Someren et al., 1991). Primer 4 spans the 3' end of the Pl3 encoding sequence and the 5' end of the pkiA terminator. Primer 5 joins the 3' end of the PL3 encoding sequence with the *pki*A terminator, while primer 6 is identical to the sequences of the pkiA terminator but is extended to incorporate a *Nsi*I site in the PCR product.

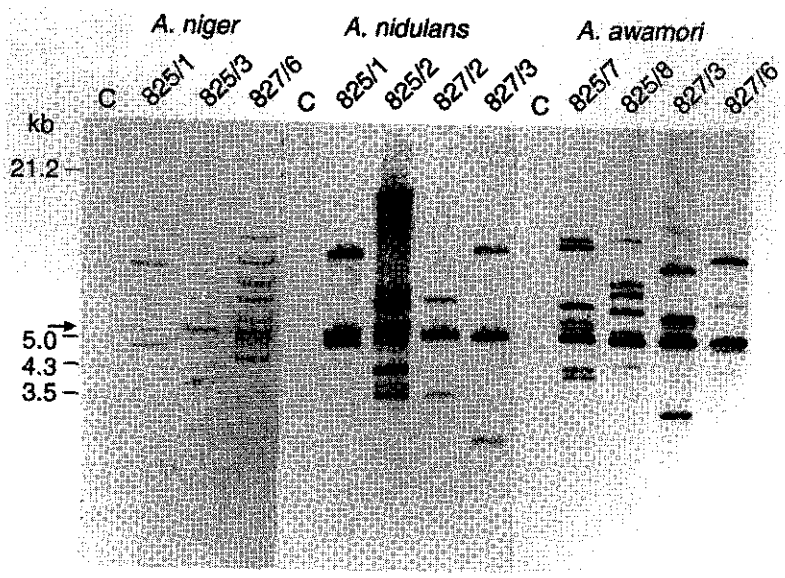

Fig. 3.2. Southern analyses of *Aspergillus transformants* expressing PL3. Genomic DNA from untransformed (C) and various fungal isolates transformed with either p825 (825) or p827 (827) was digested with *Bam*HI prior to electrophoresis. The arrow at 5.2 kbp corresponds to migration of linearized p825 or p827. Sizes of DNA fragment standards are given in kilobases.

Proteolysis and secretion of PL3

Depending on the growth conditions, liquid cultures of *Aspergillus* can contain high concentrations of proteases that may reduce the yield of a secreted heterologous product. For *A. niger* and *A. nidulans* it has been previously demonstrated that upon de-repression with respect to the carbon and/or the nitrogen source extracellular protease expression increases significantly (Cohen, 1973; Jarai and Buxton, 1994; van den Hombergh et al., 1994). In addition, under de-repressed conditions the expression of extracellullar proteases is high in *A. niger*, only at an acidic external pH . By assaying the total extracellular protease activity over a pH range from pH 2 to pH 10 is was shown that culturing *A. niger* and *A. awamori* in acidic environments of around pH 4.0 generated the highest potential for the degradation of BSA, whereas pH 7.0 was optimal for *A. nidulans* (Fig. 3.3). Accordingly, alterations in pH and proteolytic activity were monitored during the growth of *A. niger*, *A. awamori* and *A. nidulans*. Upon cultivation for 60 h, cultures of *A. niger* reached an external pH of 3.0 (Fig. 3.3B) and exhibited the highest proteolytic activity towards BSA (Fig. 3.3C). In contrast, acidification to pH 4.5 in supernatants of *A. awamori* reduced the relative protease activities to 60% of that measured for *A. niger* (Fig. 3.3C). Due to the highest protease activity at neutral to alkaline pH in cultures of *A. nidulans*, acidification of

the growth medium after 60 h of growth resulted in the lowest proteolysis measured (Fig. 3.3ABC). Despite species-specific differences, these results emphasize that acidification of the media during cultivation is an important factor with respect to the action of proteases.

As an approach to assess the stability of a heterologous protein in *Aspergillus* cultures, purified recombinant PL3 (Bartling et al., 1995) was added to serial dilutions of cleared supernatants. SDS-PAGE analyses (Fig. 3.4) combined with measurements of residual PL3 activities (Table 3.1) established that proteolytic activities were highest in supernatants of *A. niger*, intermediate in *A. awamori* and lowest in *A. nidulans*.

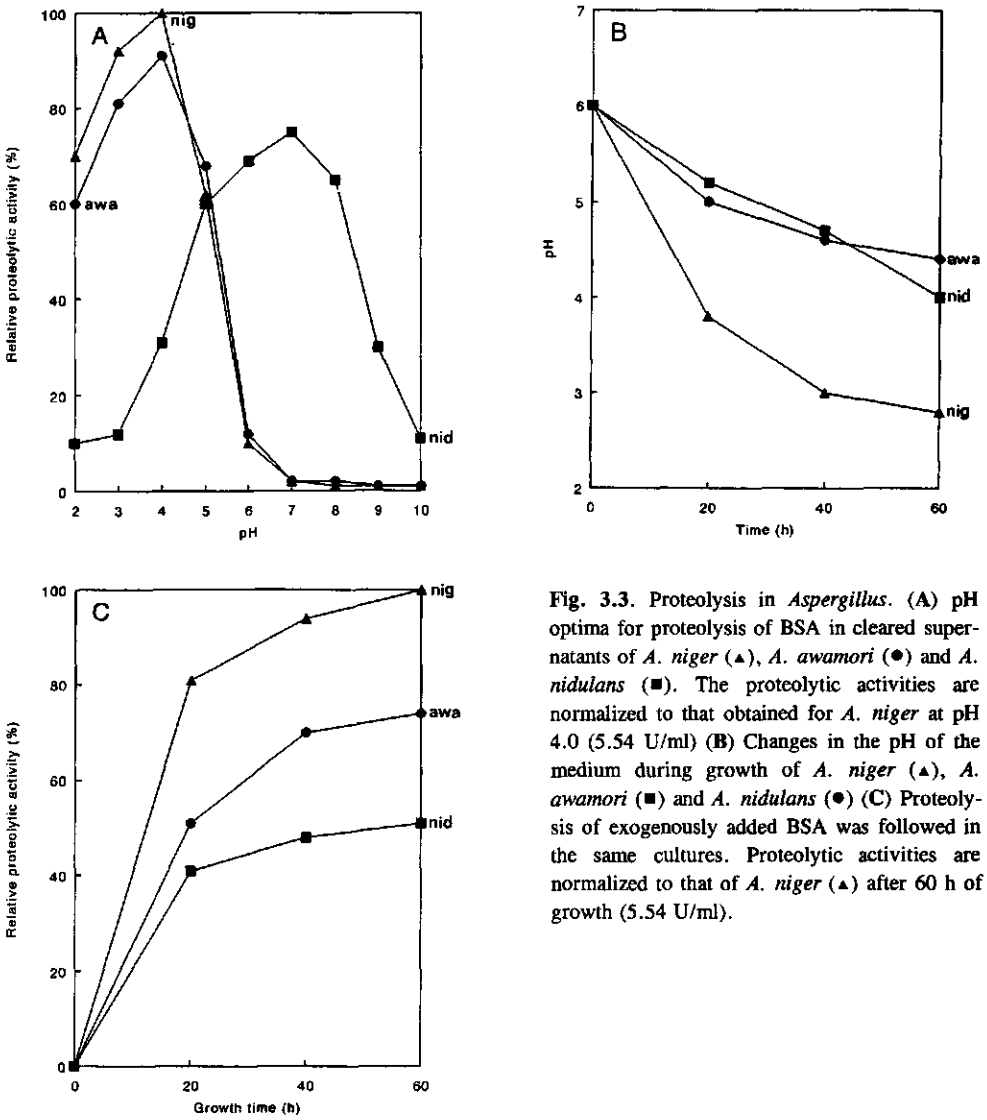

Fig. 3.3. Proteolysis in *Aspergillus*. (**A**) pH optima for proteolysis of BSA in cleared supernatants of *A. niger* (▲), *A. awamori* (●) and *A. nidulans* (■). The proteolytic activities are normalized to that obtained for *A. niger* at pH 4.0 (5.54 U/ml) (**B**) Changes in the pH of the medium during growth of *A. niger* (▲), *A. awamori* (■) and *A. nidulans* (●) (**C**) Proteolysis of exogenously added BSA was followed in the same cultures. Proteolytic activities are normalized to that of *A. niger* (▲) after 60 h of growth (5.54 U/ml).

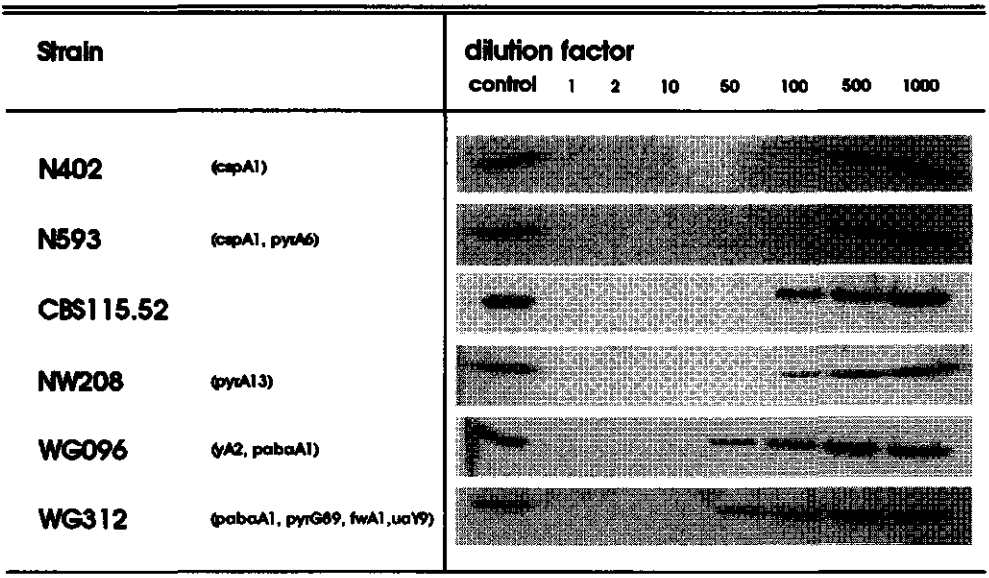

Fig. 3.4. Degradation of PL3 upon addition to cleared supernatants of *Aspergillus*. 2 μg of PL3 was incubated for 15 min in serial dilutions of culture fluids from *A. niger*, *A. awamori* and *A. nidulans*. Incubation in heat-inactivated supernatant served as a control. Proteins were analyzed using SDS-PAGE and Coomassie Brilliant Blue staining.

The findings described in Fig. 3.3 and 3.4 confirm that the major extracellular proteolytic activities in cultures of *A. niger* are confined to acidic proteases (Mattern et al., 1992; van den Hombergh et al., 1995). It has been demonstrated that aspergillopepsin A accounted for 80-85% of the total proteolysis in *A. niger*. For *A. nidulans*, at least three neutral or alkaline extracellular proteases have been reported (Cohen, 1973; Katz et al., 1994). Since in *A. niger* the expression of a number of proteases was affected by the presence of repressing carbon and/or nitrogen sources (Jarai and Buxton, 1994; van den Hombergh et al., 1994), we consequently approached heterologous expression in *Aspergillus* by growing the cultures in buffered media containing glucose at high concentrations as the carbon source.

In addition to a possible reduction in proteolytic activity, high carbon concentrations in principle allow high-level constitutive expression directed by the *pki*A promotor, thus increasing the yield of PL3 in *Aspergillus* cultures. To preserve PL3 integrity, selected *Aspergillus* transformants were grown in 5-ml batch cultures of LPM and HPM, providing low and high buffering capacities, respectively, and culture aliquots were taken at 20, 40 and 60 h. For all transformants, immunodetection revealed higher quantities of PL3 in HPM-containing cultures (Fig. 3.5).

Table 3.1 Residual activities of PL3 after incubation for 15 min in serial dilutions of the cleared supernatant of *Aspergillus* cultures

Strain	Cont	Dilution Factor [1]						
		1	2	10	50	100	500	1000
A. niger N402	**100**	0	0	0.9	6	12	47	**102**
A. niger N593	**96**	0	0	0.8	3	5	40	**98**
A. awamori 115.52	**95**	0	0	4.9	17	21	88	**99**
A. awamori NW208	**102**	0.3	0	3.0	13	27	87	**96**
A. nidulans WG096	**98**	0	1.7	14	53	92	**104**	**99**
A. nidulans WG312	**98**	0	1.9	11	43	92	**100**	**102**

[1]: Incubations of serial dilutions with PL3 protein were repeated as descibed in Fig. 3.4. The PL3 activity was measured and expressed as percentage of residual activity compared to the control. Non-affected activities are indicated in bold. Activities in heat-inactivated supernatants served as control samples. Data are normalized to that obtained from a heat-inactivated supernatant of *A. niger* strain N402.

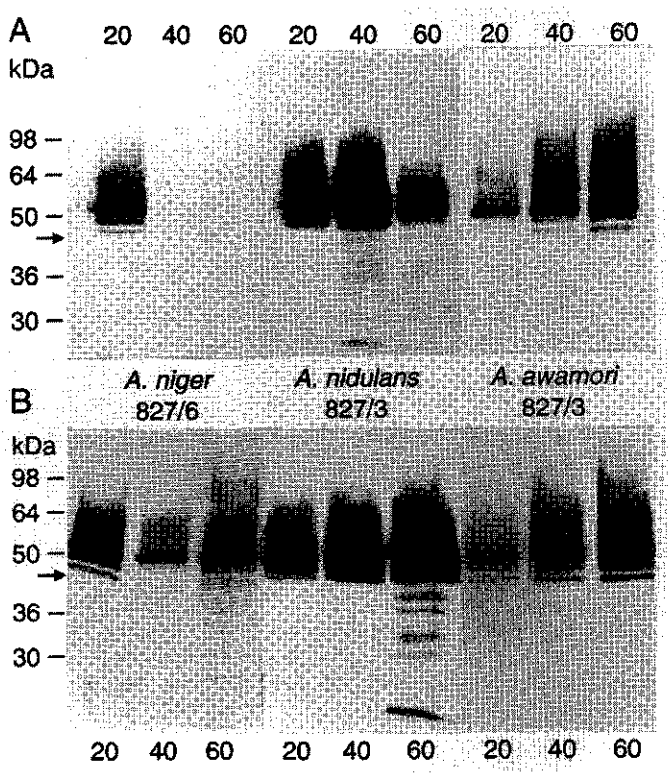

Fig. 3.5. Western blot analysis of heterologously produced PL3 in *Aspergillus*. Transformants were grown in 5-ml cultures of LPM **(A)** or HPM **(B)**. Growth time is given in h, the position of unglycosylated PL3 (arrow) and molecular mass standards are indicated.

Increasing levels of PL3 were measured during the first 60 h of incubation with *A. nidulans* and *A. awamori*; both cultures reached a pH of 5.5 at the end of incubation. In contrast, cultures of *A. niger* already contained maximal quantities of PL3 after 20 h, after which the yield declined. This coincided with an acidification of the medium, reaching pH 4.5 after 60 h of growth, thus demonstrating that extracellular acid proteases contribute substantially to the degradation of PL3 under acidic conditions. Growth in LPM resulted in complete proteolysis of *A. niger*-synthesized PL3 after 20 h (Fig. 3.5). Complete degradation of PL3 in the LPM after 20 h coincided with the observed de-repression of the transcription of extracellular proteases, indicating that *de novo* synthesis of acid proteases generates the proteolytic activity. At 60 h of cultivation a pH of 3.5 was measured. Unpurified, immunostained PL3 secreted by all *Aspergillus* transformants revealed a molecular mass larger than 50 kDa. Compared with a value of 38 kDa for the recombinant *E. coli*-synthesized enzyme, the heterogeneous glycosylation which occurs in *Aspergillus* seems to cause a smear of PL3 on Western blots.

Purification and analyses of PL3 from *Aspergillus* transformants

In order to reduce proteolysis and to obtain high yields of PL3, 1-l cultures of isolate 6 of *A. niger* transformed with p827 (*A. niger* 827/6), and isolate 3 of *A. awamori* (*A. awamori* 827/3) and also of *A. nidulans* (*A. nidulans* 827/3) were propagated in HPM for 20, 40 and 60 h, respectively. In parallel, *A. nidulans* transformant 825/2 was cultivated for 60 h in order to examine for possible influences of the signal sequence on the secretion and yield of heterologous PL3. Following concentration of the individual cleared supernatants, PL3 was purified by employing sequential ion-exchange chromatography on columns containing DE53 and S-Sepharose (Bartling et al., 1995). This yielded 0.4 mg of PL3 from *A. niger*, 0.8 mg from *A. awamori* and around 2.0 mg of PL3 from either *A. nidulans* transformant.

As in Western blots (Fig. 3.5), SDS-PAGE and subsequent Coomassie staining of the purified PL3 also showed several protein forms with major protein bands migrating at approx. 50 kDa (Fig. 3.6). Differences in the electrophoretic mobilities may reflect structural species-specific variations in attached glycans. Analysis of the deduced amino acid sequence for PL3 revealed the presence of three Asn-X-Thr/Ser sequons for potential *N*-glycosylation, spanning the residues 118-120, 198-200 and 315-317. Hence, enzymatic removal of *N*-linked glycans was attempted by using Endo H_f. This resulted in different banding patterns on Western blots (data not shown), from which the release of *A. nidulans*, *A. niger* and *A. awamori* PL3-specific glycans was estimated to be 90, 60 and 40% complete, respectively. These differences seem to be species-specific since Endo H_f specifically cleaves the chitobiose core of high mannose oligosaccharides from

N-linked glycoproteins (Maley et al., 1989). Whether the unremoved glycans were resistant to digestion with Endo Hf_f or were *O*-linked remains unclear. Despite glycosylation, each purified PL3 exhibited a pI of 10, similar to that of the prokaryotic enzyme (Bartling et al., 1995).

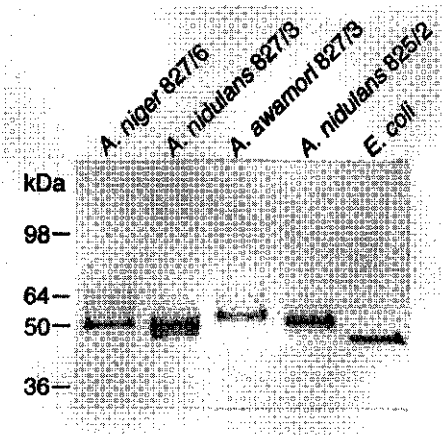

Fig. 3.6. Purified recombinant PL3. SDS-PAGE was performed on samples containing enzyme purified from the indicated species. Proteins were visualized with Coomassie Brilliant Blue.

The first 15 amino acids of recombinant PL3 from *Aspergillus* transformed with p827 and *E. coli* were identical. This indicated correct signal peptidase cleavage of the 20-residue-long PELA pre-sequence fused to PL3. For *A. nidulans* transformed with p825, 21 amino acids of the PGAII pre-sequence were removed by the signal peptidase, leaving an N-terminal extension of six residues on PL3 (Fig. 3.1 and Table 3.2). However, incomplete processing of the prepro-sequence did not affect the yield of secreted protein. It is notable that aberrant processing of the PGAII prepro-sequence had no effect on the enzymic activity. Given the three-dimensional structure of the *Erwinia chrysanthemi* PEL C isoenzyme (Yoder et al., 1993), the PGAII-derived hexapeptide extension on PL3 is unlikely to interfere with the catalytic site or alter other structural properties. Thus, our results provide no evidence for essential functional implications of the unprocessed peptide extension regarding secretion or enzyme activity.

Over-expression of PGAII, both under the control of its homologous *pga*II promotor (Bussink et al., 1991) or under the control of the *A. niger pki*A promotor and containing its homologous secretion signal (data not shown), exhibited correct processing. This implies that not only the properties of the pro-peptides influence signal cleavage, but also that sequence characteristics in the N-terminal part of the mature protein are important. Possibly, endogenous proteins expose epitopes that are essential for cleavage by *Aspergillus* peptidases (Visser at al., 1994). The presence of residues Glu at -3, Arg at -1 and Ala at +1 with respect to the N-terminus of mature PL3 is not in accordance with the (-1, -3) rule of von Heijne (1984), which predicts the absence of Glu and Arg at these positions. However, a number of processed fungal proteins contain Arg at position -1 (Table 3.2).

Table 3.2 Comparison of fungal pre-pro sequences and their putative processing sites

↑-pos. [a]	Partial pre-pro sequence	Enzyme	Expression host	Reference
27 aa	v a g a t f a s a S P I E A R ↑ D S C T F	PGII	*A. niger* N400	Bussink et al., 1990
27 aa	a a s a t l a s a S P I E A R ↑ G S C T F	Polygalacturonase	*A. niger* RH5344	Ruttkowski et al., 1990
27 aa	a a s a t l a s a S P I E A R ↑ G S C T F	PGII	*A. tubingensis* NW756	Bussink et al., 1991
27 aa	A G A L V A A A P S G L D A R ↑ D G C T F	Polygalacturonase	*C. carbonum*	Scott-Craig et al., 1990
21 aa	v a g a t f a s a ↑ S P I E A R **A N T** G G	PL3	*A. niger* N400	*vide intra*
27 aa	T A V T A L A V P S P L E A R ↑ **A** V **T** **W** T	α-Sarcin	*A. giganteus*	Oka et al., 1990
27 aa	T A V S V L A A P S P L D A R ↑ **A** T W T C	Restrictotin	*A. restricteus*	Lamy and Davies, 1991
22 aa	S L V V S L A A A L P H Y I R ↑ S **N** G I E	Glucose oxidase	*A. niger*	Frederick et al., 1990
27 aa	L L A G A S A L P N G L S P R ↑ N **N** A F C	KP6 (α N-terminus)	*U. maydis*	Tao et al., 1990
24 aa	A T L A T L A A S V P L E E R ↑ Q A C S S	Cellobiohydrolase II	*T. reesei*	Teeri et al., 1987
27 aa	T A F A A P A P E P D L V S R ↑ S A G I N	Xylanase A	*A. tubingensis*	de Graaff et al., 1994
41 aa	G T G I P E G S L Q F L S L R ↑ A S A P I	Galactose oxidase	*D. dendroides*	McPherson et al., 1992

[a]: the figures indicate the number of amino acid residues from the translation initiation point to the (putative) mono-basic processing site; ↑: (putative) processing site. If the actual signal cleavage site has been experimentally determined, the pre-peptide is shown in lower case and the pro-peptide and N-terminal sequence of the mature protein are shown in upper case. Identical amino acid residues compared to the N-terminal sequence of PL3 are shown in bold.

Following signal peptidase action, processing of N-terminal extensions have been speculated to occur by proteases with relaxed sequence specificities (McPherson et al., 1992). Upon expression of an *Aspergillus* glucoamylase in yeast, Innis et al. (1985) demonstrated that an N-terminal extension comprising Lys at +2 and Arg at -1 was cleaved by the endopeptidase KEX-2. Because a functional KEX-2 like dipeptidyl-aminopeptidase has been identified in *A. niger* (Contreras et al., 1991), it remains to be determined whether the combined action of this protease and a signal peptidase is required for proper processing of prepro-sequences in *Aspergillus*.

Kinetic analyses for the de-polymerization of PGA using the four purified PL3 enzymes displayed K_ms of about 0.27 mg/ml, similar to the value for the enzyme produced in *E. coli*. The V_{max} values measured were 130 (*A. awamori*), 150 (*A. nidulans*) and 190 μmol/(min·mg protein) (*A. niger*), and thus were only about 20-30% of that of the *E. coli*-secreted enzyme. Despite species-specific differences in the glycosylation patterns of PL3, all enzymes exhibited reduced activities. It is plausible that the glycans alter the enzyme conformations which are necessary for cleavage of the substrate, or else prevent correct protein folding. Although little is known about glycosylation in *Aspergillus*, Ward (1989) suggested that glycosyl residues attached to proteins may enhance transit through the secretory pathway. Moreover, protein stability and folding properties may be improved by attached glycans. Since thermodynamic measurements require mono-molecular species, it has not been possible to analyse whether specific glycoforms of PL3 enzymes exhibit differences in stability.

The results presented in the present paper show that the use of optimized expression casssettes is not sufficient for obtaining high yields of a secreted recombinant protein in *Aspergillus*. More important, apparently, is the diminished secretion of species-specific proteases which drastically contribute to reduce the yield of a produced protein. Accordingly, it will be of interest to characterize the production of PL3 in protease-deficient *Aspergilli*.

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4 Cloning and characterization of the *pep*E gene of *Aspergillus niger* encoding a new aspartic protease and regulation of *pep*E and *pep*C[1]

G. Jarai, J.P.T.W. van den Hombergh and F.P. Buxton

Abstract

We have cloned the *pep*E gene of *Aspergillus niger* encoding an aspartic protease by screening a λ genomic DNA library with a heterologous probe, the *Neurospora crassa* gene coding for a vacuolar proteinase. Sequencing of the *pep*E genomic and cDNA clones revealed that the gene contains three introns, which are 91, 56 and 58-bp long. The deduced protein consists of 398 amino acids, has a putative signal sequence to allow transport into the endoplasmic reticulum and probably undergoes a second proteolytic processing step at its N-terminus to yield the mature enzyme. The putative mature part of the PEPE protein has extensive homology with other aspartic proteinases such as pepsins, cathepsins and, in particular, with proteinase A of *S. cerevisiae* and pepsin 1 of *Candida albicans*. Northern blot analyses revealed that cells contain an abundant *pep*E transcript whose amount does not change upon carbon or nitrogen limitation, the presence of proteins in the medium or changes in pH of the medium. We also show that *pep*C, the *A. niger* homologue of the yeast protease B is also expressed constitutively under these conditions.

Introduction

Recently, there has been a growing interest in studying filamentous fungal proteases and, in particular, proteases of *Aspergilli*. These proteases are of importance for a number of reasons. Their activities seem to be primarily responsible for difficulties in achieving high levels of heterologous protein expression and secretion in *A. niger* (Dunn-Coleman et al., 1991; Roberts et al., 1992), an expression host with proven abilities (Archer et al., 1990; Ward et al., 1990; Roberts et al., 1992). Proteases are also important, for example, for their role in soy sauce fermentation in *A. oryzae* (Tatsumi et al., 1989) and for their suggested role in the pathogenicity of *A. fumigatus* (Tang et al., 1982; Jaton-Ogay et al., 1992; Monod et al., 1993). Proteases are

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also useful as target genes to study gene regulation in this economically and medically important group of fungi, since some members of the protease family appear to be under complex regulatory control (G.J. and F.P.B., unpublished data).

A number of proteases have been purified and studied from different species of the *Aspergilli*. In *A. niger* a wide range of protease activities such as acidic proteases, alkaline and semi-alkaline serine proteases and serine carboxypeptidases (Takahashi et al., 1991; Barthomeuf et al., 1989; Sakka et al., 1985; Krishnan and Vijayalakshi et al., 1985, 1986) have been described. Recently, some of the genes coding for the proteases of *A. niger* have been isolated. Two extracellular acid proteases, *pep*A and *pep*B have been cloned from *A. niger* var. *awamori* (Berka et al., 1990) and from *A. niger* var. *macrosporus* (Inoue et al., 1991), respectively. The *pep*A encodes aspergillopepsin A and seems to be responsible for the majority of extracellular acidic activities while the *pep*B gene codes for a non-pepsin type acidic endopeptidase with an unusual two-chain structure. Two subtilisins, members of another important group of proteases, the serine proteases, have also been cloned from *A. niger*. The *pep*C gene is a homologue of the yeast *PRB1* gene (Frederick et al., 1993) whereas the *pep*D gene encodes a member of the fungal extracellular alkaline protease family (Jarai et al., 1994).

In the yeast *Saccharomyces cerevisiae*, proteinase A encoded by the *PEP4* gene, is an aspartic proteinase that is localized in the vacuole (Ammerer et al., 1986). The yeast proteinase A has been implicated in the *in vivo* maturation process of many zymogens, such as carboxypeptidase Y and proteinase B (Ammerer et al., 1986) and has also been often suggested to play a role in heterologous protein expression in yeast (Hirsch et al., 1989). Recently, a *Neurospora crassa* vacuolar protease gene has been cloned and shown to be highly homologus to the yeast *PEP4* gene (Bowman, 1993). We were interested in cloning the *A. niger* homologue of this vacuolar protease-encoding gene for two reasons. Firstly, we have shown that the *pep*C gene product of *A. niger*, a subtilisin closely related to the yeast vacuolar proteinase B, is localized intracellularly (unpublished data). Thus the cloning of the *A. niger* homologue of the yeast *PEP4* gene encoding proteinase A which is implicated in the processing of the yeast proteinase B zymogen, could make the detailed characterization of the *in vivo* maturation pathway in *A. niger* possible. Secondly, this putative proteinase, may play an important role in the heterologous protein expression and secretion in this filamentous fungus.

In the present paper we describe the cloning of the *pep*E gene of *A. niger*, using a heterologous *N. crassa* probe. We show that the *pep*E gene consists of four exons and three introns, encodes a putative protein that is homologous to the yeast proteinase A and other aspartic proteases. We also show, that the *pep*E and the *pep*C genes, both encoding putative vacuolar

proteases are expressed in a constitutive manner under carbon and nitrogen repression and derepression and alkaline or acid pH conditions.

Materials and Methods

Strains and growth conditions. *A. niger* N400 (CBS 120.49) has been previously described (Goosen et al., 1987). Minimal culture media contained per liter: 6 g $NaNO_3$ / 1.5 g KH_2PO_4 / 0.5 g KCl / 0.5 g $MgSO_4$ / trace elements and supplemented with 2% (w/v) sucrose as carbon sourse. Liquid cultures were inoculated with 10^6 spores/ml and incubated at 30°C on an orbital shaker at 220 rpm. Agar was added at 1.5% (w/v) for solid medium. *Escherichia coli* DH5αF' was used as host for plasmids and LE392 for bacteriophage λ. They were grown in 2 x YT (Sambrook et al., 1989) medium supplemented with 50 μg Amp/ml when required. Plasmids and phage λ were propagated by standard techniques (Sambrook et al., 1989).

Library construction and probing. The construction of the genomic library from *A. niger* N400 in λ phage EMBL4 was described by Harmsen et al. (1990). The library was screened with the *N. crassa* probe using 6 x SSC / 0.1% (w/v) SDS at 60°C for hybridization and 2 x SSC / 0.1% (w/v) SDS at 60°C for the most stringent wash.

Nucleotide sequence of *pep*E. The *pep*E gene was subcloned as a 2.9-kb *Bam*HI-*BglII* fragment into the pTZ18R vector and both strands were completely sequenced by the dideoxy-chain termination method (Sanger et al., 1977) using synthetic oligo primers and Sequenase (US Biochemical, Cleveland, OH, USA).

Transcription and intron mapping. Cells were grown in minimal medium with 1% (w/v) BSA as the sole carbon and nitrogen source. Total RNA was prepared from freeze dried mycelia by lysis in buffer containing 10 mM Tris pH 7.5 / 100 mM LiCl / 2% (w/v) SDS, several phenol extractions and ethanol precipitation. Poly A^+ was isolated by oligo-dT chromatography (Aviv and Leder, 1972). The 5' end of the message was mapped by runoff transcription. Poly A^+ RNA was hybridized with ^{32}P-end-labeled oligo **5** (see arrow **5**; Fig. 4.2). The transcripts produced by reverse transcriptase were run in an 8 % sequencing gel together with sequencing reactions produced by dideoxy sequencing with the same oligo. The splice sites of the introns were identified by cloning and sequencing partial cDNA copies of the *pep*E transcript. First strand synthesis was performed by standard methods (Sambrook et al., 1989). The priming oligo for intron 1 and 2 was oligo **2** and for intron 3 oligo **4** (see arrows **2** and **4**, respectively; Fig 4.2). The cDNAs were then amplified by PCR using oligos **1** and **2** for intron 1 and 2 and oligo **3** and **4** for intron 3 and cloned into pTZ18R. Two independent clones were sequenced for each region.

Northern analysis. Wild-type *A. niger* conidia were inoculated into complete YPD (1% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) D-glucose) medium and grown for 16-24 h at 30°C. Mycelia was filtered, washed three times with distilled water and transferred to minimal medium (see strains and growth conditions), supplemented as described above. The supplements were: ammonium chloride: 100 mM; glucose: 1% (w/v); glycerol: 1% (w/v); BSA 1% (w/v); Cells were grown for an additional 5-6 h. Mycelia were harvested by filtration, freeze dried and used for RNA isolation (see transcription and intron mapping).

Blotting and hybridization. 10 μg of total RNA was loaded on formaldehyde agarose gels (Sambrook et al., 1989), transferred to Hybond-N membranes (Amersham) and UV-cross linked. Hybridization and washing of the membranes were carried out essentially as described (Sambrook et al., 1989).

Results and Discussion

Cloning of the *pep*E gene

A 0.65-kb *Sal*I fragment, derived from the coding region of the *N. crassa* gene homologous to the yeast *PEP4* gene was used as a probe in a series of genomic Southern analyses. Restriction digests of total *A. niger* genomic DNA were hybridized with the probe and washed under various conditions. The most stringent washing conditions, allowing easily detectable and specific hybridization was found to be 2 x SSC at 60°C. Using these conditions, the same fragment was used to probe a genomic library in bacteriophage λ. Three positive clones were identified and subsequently purified. The location of the hybridizing fragment within the λ clones was established by restriction mapping, hybridization and sequencing. Several subclones spanning that region were then constructed in pTZ18R and two of them are shown in Fig. 4.1.

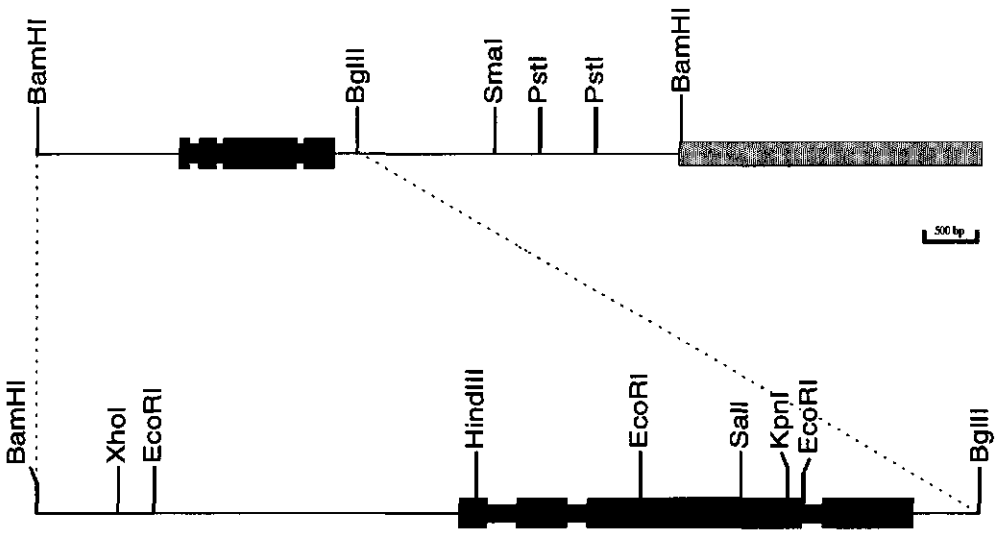


Fig. 4.1. Physical map of the *pep*E gene of *A. niger*. The restriction map of two subclones derived from the isolated λ clones containing the *pep*E gene are shown. The lower part shows pPEPE10, a 2.9-kb subclone, whose complete nucleotide sequence was determined and contains the *pep*E gene coding region (filled bar) and 1.2 kb upstream and 0.2 kb downstream non-coding sequences (thin lines). The little restrictions in the coding region show the position of the introns.

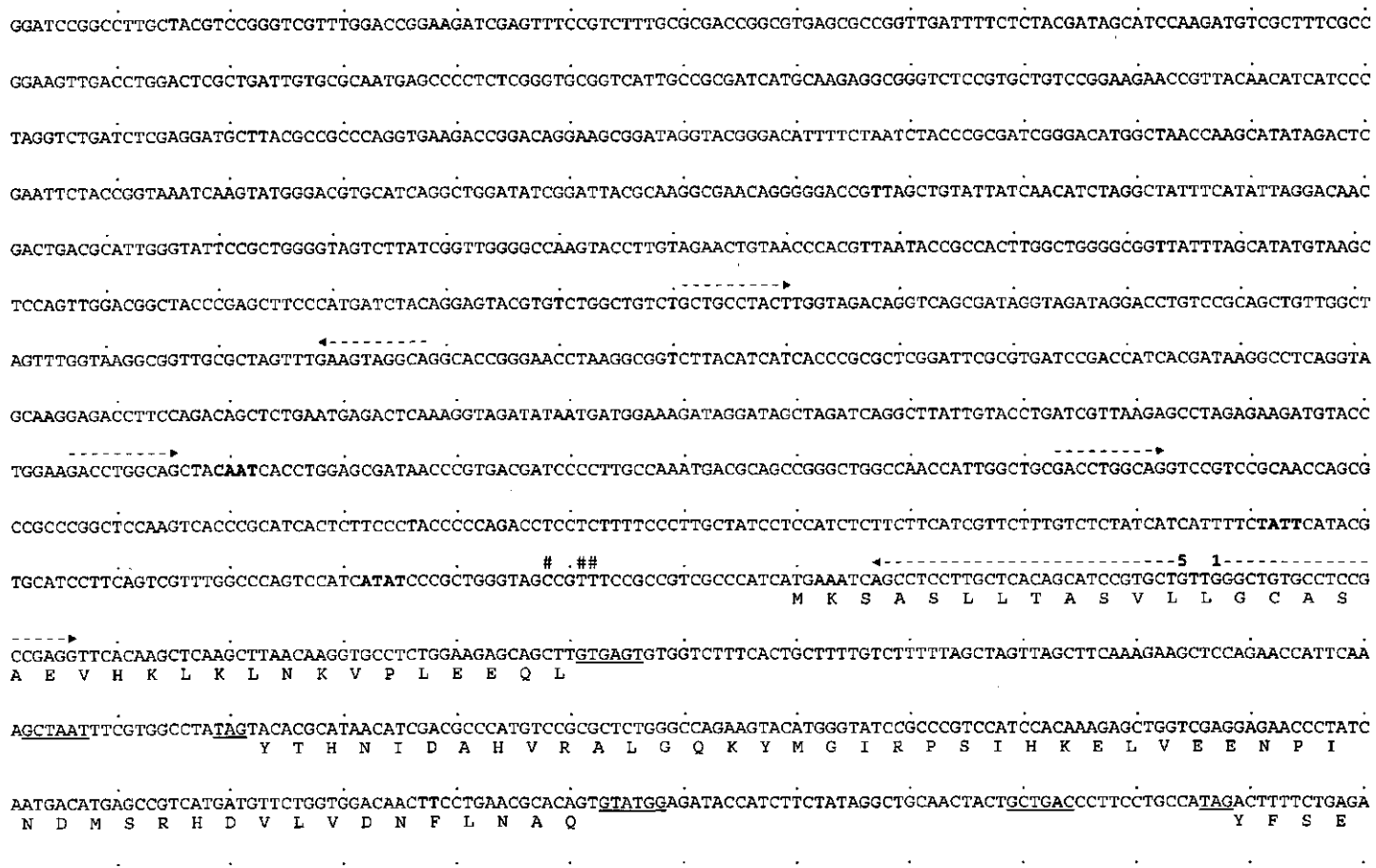
Nucleotide sequence and intron mapping of the *pep*E gene

The nucleotide sequence of a 2.9-kb *Bam*HI-*Bgl*II subclone, pPEPE10, was then determined on both strands by the dideoxy chain termination method using synthetic oligo primers (Sanger et al., 1977). The nucleotide sequence region is shown in Fig. 4.2 and contains a 1194-bp long ORF, 1268-bp upstream and 208-bp downstream non-coding sequences.

The DNA and the deduced amino acid sequence of the *pep*E gene indicated that the ORF is interrupted by several introns. We have mapped the exact location of the introns by sequencing cDNAs that were created by reverse transcriptase PCR using oligos 1-4 shown in Fig. 4.2. Three introns were identified whose lengths are 91-, 56- and 58-bp, respectively. The three introns contain the *Aspergillus* (and other fungal) consensus sites, the 5' splice donor site (GTRNGT), the 3' splice acceptor site (YAG) and the internal element (RCTRAC), possibly needed for lariat formation during splicing, except that the 5' acceptor site has one mismatch in intron 2 and the internal element has one mismatch in intron 1.

Transcript mapping of the *pep*E gene

The transcription start site (tsp) was mapped by primer extension using oligo 5 (Fig. 4.3). There appeared to be three tsp 21, 18 and 17 nucleotides upstream of the translation start site, the 1st and 3rd being particularly strong. It is quite common that promoters of the *Aspergilli* and other fungal genes have multiple tsp (Gurr et al., 1987; Unkles, 1992) and their location is often within 100 nucleotides upstream of the ATG. The 17-21 nucleotide long 5' untranslated sequence found in *pep*E is relatively short although there are genes for which even shorter such sequences have been reported in *A. niger* (Kos et al., 1988). There is no TATAAA-box present upstream of tsp within the first 200 nucleotides, although two TATAAA-like elements ATAT and TATT are found at 16 and 57 nucleotides upstream, respectively. The 5'non-coding region also contains a perfect copy of the CAAT element at 269 nucleotides upstream of the tsp and an approximately 90 nucleotides long sequence, centered around 110 nucleotides upstream of the tsp which is very rich in pyrimidines, a feature of many fungal promoters. The functional significance of all these elements is yet to be determined since litte is known about their role in fungal promoters (Gurr et al., 1987; Unkles, 1992). The 5' region also contains a number of 7-10 nt long direct and inverted repeats, the two 10 nucleotides long ones are shown in Fig. 4.2. These repeats are also believed to play a role in controlling gene expression in eukaryotes (Johnson and McKnight, 1989). Translation in fungal genes usually starts with the first Met of the ORF and a consensus has been found at the translation start site. The -3 position preceding the first ATG codon is generally an A (83%) and the -1 position is frequently a C (Gurr et al., 1987) as also found at the



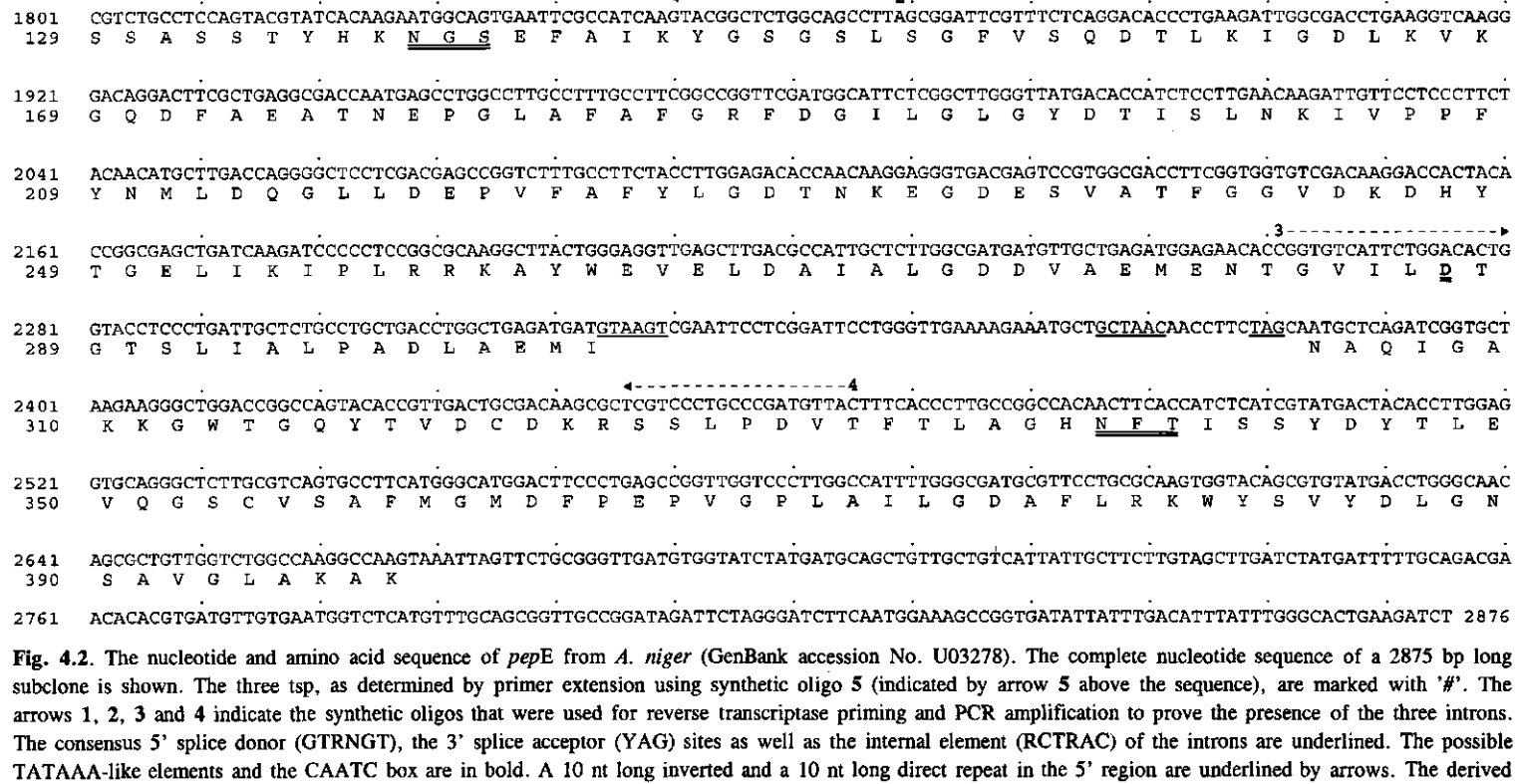


Fig. 4.2. The nucleotide and amino acid sequence of *pep*E from *A. niger* (GenBank accession No. U03278). The complete nucleotide sequence of a 2875 bp long subclone is shown. The three tsp, as determined by primer extension using synthetic oligo **5** (indicated by arrow **5** above the sequence), are marked with '**#**'. The arrows **1**, **2**, **3** and **4** indicate the synthetic oligos that were used for reverse transcriptase priming and PCR amplification to prove the presence of the three introns. The consensus 5' splice donor (GTRNGT), the 3' splice acceptor (YAG) sites as well as the internal element (RCTRAC) of the introns are underlined. The possible TATAAA-like elements and the CAATC box are in bold. A 10 nt long inverted and a 10 nt long direct repeat in the 5' region are underlined by arrows. The derived amino acid sequence is shown under the ORF. The putative signal sequence cleavage site is indicated by the vertical arrow. The two Asp active site residues are in bold and underlined. Putative N-linked glycosylation sites are double underlined.

translation start of the *pep*E gene. The translation stops with a TAA stop codon, followed by an A. Only little bias has been shown for stop codons in fungal genes, with TAA being slightly more frequent (42%) than the other two nonsense codons (Unkles, 1992). The position after the stop codon is often (70%) an A in fungal genes as in *pep*E.

The 3' non-translated region contains no obvious polyadenylation signal within the first 200 nucleotides after the translation stop codon as also observed for a number of other genes of the *Aspergilli*.

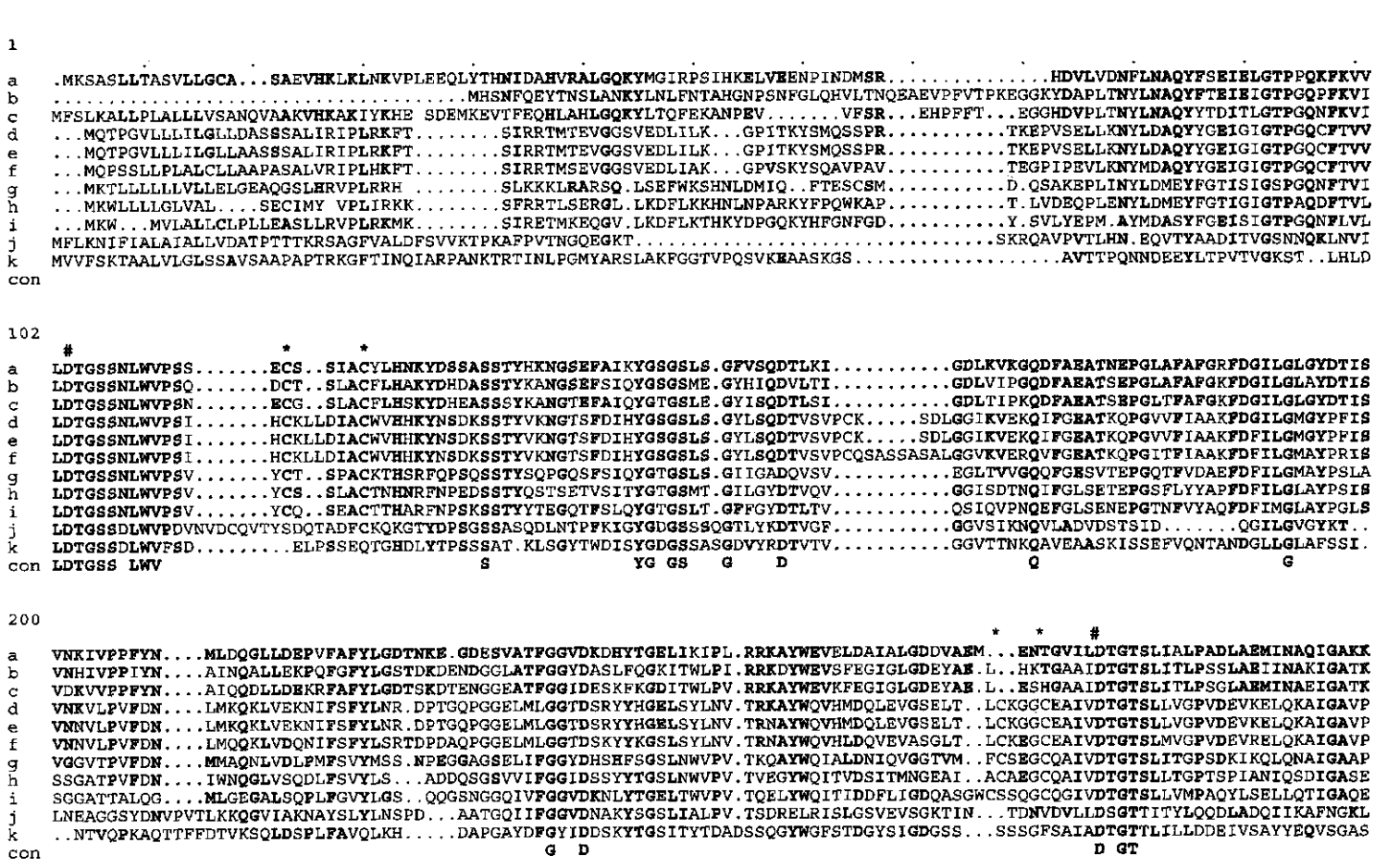
The PEPE protein

The ORF in the *pep*E genes encodes a protein comprising 398 amino acids with a calculated molecular weight of 43.4 kDa. A sequence comparison of the deduced PEPE protein sequence to other aspartic proteases is shown in Fig. 4.3. As it is obvious from the data in Fig. 4.3, other amino acids sequences of the aligned proteins can be divided into two parts based on similarities among them. There is little homology at the N-terminus of these proteases, whereas they are remarkably similar between approximately the 70th amino acid and the C-terminus. The PEPE protein has not yet been purified but several of the other proteases, such as the mature mammalian pepsins and cathepsins, the yeast proteinase A and aspergillopepsin A have been purified and N-terminal aa have been determined. Those data indicate that the region with the pronounced similarity corresponds to the mature parts of the zymogens. It is very likely that the PEPE, as all known aspartic proteases, is also synthesized as a precursor and is then processed further to yield the mature enzyme. Based on the aa sequence data it is not possible to establish exactly the putative N-terminus of the mature PEPE. However, one can predict that it starts shortly before aa residue 73. The yeast prepro-proteinase A, which is cleaved after Glu_{76} (Rothman et al., 1986) and the PEPE protein share a sequence of amino acids showing significant homology between His_{79} and Tyr_{91} (yeast proteinase A) and His_{73} and Tyr_{85} (PEPE) with 9 identical amino acids and 2 conservative substitutions out of 13 amino acid residues, indicating that this part of the PEPE polypeptide is likely to be part of the mature enzyme.

The structure of the very N-terminus of the PEPE polypeptide resembles the structure of signal peptides (von Heijne, 1986) in that there is a positively charged amino acid after the first Met followed by a hydrophobic core. We have carried out a signal-peptide cleavage site prediction for the PEPE protein (von Heijne, 1986; not shown). This prediction strongly suggests that there is indeed a signal sequence at the N-terminus of PEPE with a very likely cleavage site after Ala_{18} suggesting that the PEPE protein is translocated into the endoplasmic reticulum where its N-terminal signal peptide is cleaved off. These structural features of the PEPE polypeptide suggest

that it is synthesized as a precursor and then possibly undergoes two processing events to yield the mature enzyme consisting of approximately 325 amino acids. Proteins entering the secretory pathway are often glycosylated and the PEPE contains two potential N-glycosylation sites, Asn_{138}GlySer and Asn_{338}PheTyr, although their functional significance is as yet unknown. The amino acid sequence of the mature parts of aspartic proteases (shown in Fig. 4.3) and the putative mature part of PEPE of *A. niger* are strongly conserved. Not surprisingly, the most extensive homology was found between PEPE and the proteinase A of *S. cerevisiae* with 69.2% identical amino acids and the pepsin 1 of *C. albicans* with 65.2% identical amino acids (in the absence of the complete *N. crassa* sequence we could not carry out that comparison). Mammalian pepsins and cathepsins are two related families of aspartic proteases and the PEPE protein has approximately 47-49% identical amino acid residues with the mammalian cathepsins and approximately 40-41% identical amino acid residues with mammalian pepsins. Interestingly, two fungal pepsins, both extracellular, aspergillopepsin A of *A. niger* and pepsin 2 of *C. albicans* are not closely related to PEPE (27% and 32% identity, respectively).

The region around the active site residues Asp_{103} and Asp_{287} are extremely well conserved among all these proteases. There are 9 consecutive amino acids following Asp_{103} and 6 consecutive amino acids following Asp_{287} with 100% identity among all the listed proteases but Aspergillopepsin A and candidapepsin 2, both contain a few substitutions in these regions. The mature mammalian pepsins and cathepsins also contain 6 conserved Cys residues which form intrachain disulfide bonds (Azuma et al., 1989). The putative mature part of PEPE contains 4 Cys residues whose location corresponds exactly to the 1st, 2nd, 5th and 6th conserved Cys residues in the mammalian proteins (Fig. 4.3). These 4 Cys residues are also conserved in the yeast proteinase A and candidapepsin 1, but are absent from Aspergillopepsin A and candidapepsin 2. An interesting possibility is that the 4 Cys residues form 2 intrachain disulfide bonds thus stabilizing PEPE in a similar conformation to the higher eukaryotic enzymes. These data indicate that the PEPE and PEPA, the two pepsin-type proteases of *A. niger* studied so far are of distant evolutionary origin. The cellular localization of the PEPE protein is yet to be determined. However, its close similarity with the yeast proteinase A, a vacuolar enzyme, suggests that PEPE may also be localized in the vacuole. This hypothesis is further supported by PEPE being more closely related to mammalian cathepsins that are found intracellularly in lysosomes than to the secreted mammalian pepsins.



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312              *                                  *
a    GWT..GQYTVDCDKRSSLP.DVTFTLA.GHNFTISSYDYTLEV....QGSCVSAFMGMDFPEPVG.PLAILGDAFLRKWYSVYDLGNSAVGLAKAK..........
b    SWS..GQYQVDCAKRDSLP.DLTLTFA.GYNFTLTPYDY.YEV....SGSCISVFTPMDFFPPIG.DLAIVGDAFLRKYYSIYDLDKNAVGLAPSKV..........
c    GWT..GQYTLDCNTRDNLP.DLIFNFN.GYNFTIGPYDYTLEV....SGSCISVITPMDFPEPVG.DLAIVGDAFLRKYYSIYDLGNNAVGLAKAI..........
d    LIQ..GEYMIPCEKVSSLP.IITFKLG.GQNYELHPEKYILKVSQAGKTICLSGFMGMDIPPPSG.DLWILGDVFIGCYYTVFDREYNRVGFAKAATL.........
e    LIQ..GEYMIPCEKVSSLP.IITFKLG.GQNYELHPEKYILKVSQAGKTICLSGFMGMDIPPPSG.DLWILGDVFIGCYYTVFDREYNRVGFAKAATL.........
f    LIQ..GEYMIPCEKVSTLP.AITLKLG.GKGYKLSPEDYTLKVSQAGKTLCLSGFMGMDIPPPSG.DLWILGDVFIGRYYTVFDRDNNRVGFAEAARL.........
g    .VD..GEYAVECANLNVMP.DVTFTIN.GVPYTLSPTAYTLLDFVDGMQFCSSGIQGLDIHPPAG.DLWILGDVFIRQFYSVFDRGNNRVGLAPAVP.........
h    NSD..GDMVVSCSAISSLP.DIVFTIN.GVQYPVPPSAYIL....QSEGSCISGIQGMNLPTESG.ELWILGDVFIRQYFTIFERANNQVGLAPVA..........
i    GEY..GEYFVSCDSVSSLP.TLSFVLN.GVQFPLSPSSYII....QEDNFCMVGLESISLTSESGQDLWILGDVFLRSYYAIFDMGNNKVGLATSV..........
j    TQD..SNGNSFYEVDCNLSGDVVFNFSKNAKISVPASEFAASLQGDDGQPYDKCQLLFDVNDAN.....ILGDNFLRSAYIIYDLDDNEISLAQVKYTSASSISALT
k    GETEAGGYVFSCSTNPP.....DFTVVIGDYKAVVPGKYINYAPISTGSSTCFG....GIQSNSGLGLSILGDVFLKSQYVIFNSEGPKLGFAAQA..........
con                                                                I GD F                 A
```

Fig 4.3. The aa sequence comparison of PEPE with other aspartic proteases. The aa sequences were aligned using the program PILEUP available in the GCG package (Genetics Computer Group, Madison, WI, USA). The aligned sequences are as follows: a, *A. niger* PEPE; b, *C. albicans* pepsin 1 (Lott et al. 1989); c, *S. cerevisiae* proteinase A (Moehle et al., 1987); d, rat cathepsin E (Fujita et al., 1991); e, rat cathepsin D (Birch and Loh, 1990); f, human cathepsin D (Faust et al., 1985); g, human cathepsin E (Azuma et al., 1989); h, human pepsin A (Evers et al., 1989); i, rat pepsin A (Ichihara et al., 1986); j, *C. albicans* pepsin 2 (Hube et al., 1991); k, *A. niger* PEPA (Berka et al., 1990). The active site residues are indicated with a '#' above the sequence. Conserved Cys residues are indicated with a '*' above the sequence.

Genomic Southerns

We established the copy number of the *pep*E gene in *A. niger* by genomic Southern experiments. Total DNA of *A. niger* was digested with *Bam*HI, *Eco*RI, *Hin*dIII and *Sal*I, blotted and hybridized with the 2.9-kb *Bam*HI-*Bgl*II *pep*E fragment as probe (hybridization conditions: 6 x SSC / 0.1% (w/v) SDS at 65°C overnight; most stringent wash: 0.1 x SSC /0.1% (w/v) SDS, 65°C, 2 x 30 min). The results indicated that the *pep*E gene was present in a single copy in the genome of *A. niger* (data not shown).

Regulation of *pep*E and *pep*C expression

It was of further interest to determine how the expression of the pepE gene is regulated. We have also included the previously cloned *pep*C gene encoding a subtilisin into this study because yeast homologues of these genes are both localized in the vacuole and our preliminary data also suggests that the PEPC and PEPE proteins are intracellular proteases.

Using Northern analysis, we studied the regulation of *pep*E and *pep*C gene expression at the level of mRNA content because transcriptional regulation is believed to be the predominant regulatory phenomenon in filamentous fungi. First, we established sizes of the *pep*E and *pep*C mRNAs using commercially available RNA size markers (data not shown). We found that the *pep*E probe, a 1.6-kb *Bgl*II fragment (Fig. 4.1), hybridized to a 1.5 kb, and the *pep*C probe, a 1.2-kb *Bgl*II fragment (Frederick et al., 1993) to a 1.7kb RNA species (data not shown) which agree well with lengths of these two genes.

In the absence of a true constitutive marker gene from *A. niger* we used the *pki*A (encoding pyruvate kinase) gene as a control in the experiments described below to show that approximately equal amounts of RNAs have been loaded on the gels. The promoter of the *pki*A gene has been referred to as strong and constitutive although the amount of *pki*A encoded RNA shows some increase in cells grown on glucose (de Graaff et al., 1992).

The regulation of gene expression by nitrogen source is an important phenomenon in filamentous fungi affecting a number of genes involved in nitrogen metabolism. First we investigated whether the expression of *pep*E and *pep*C is influenced by nitrogen limitation. Cells were grown overnight in complete liquid medium, then washed three times with distilled water, divided into two and transferred into minimal medium supplemented with glucose with no nitrogen source or with ammonia and then grown for an additional 5-6 hours. RNA was isolated from the harvested cells and hybridized to the *pep*E, *pep*C and *pki*A probes, stripping the membranes between each probing. As shown in Fig. 4.4A, whether cells were grown in medium supplemented with ammonia or not, both *pep*E and *pep*C transcripts were abundantly present indicating that nitrogen repression does not effect their expression.

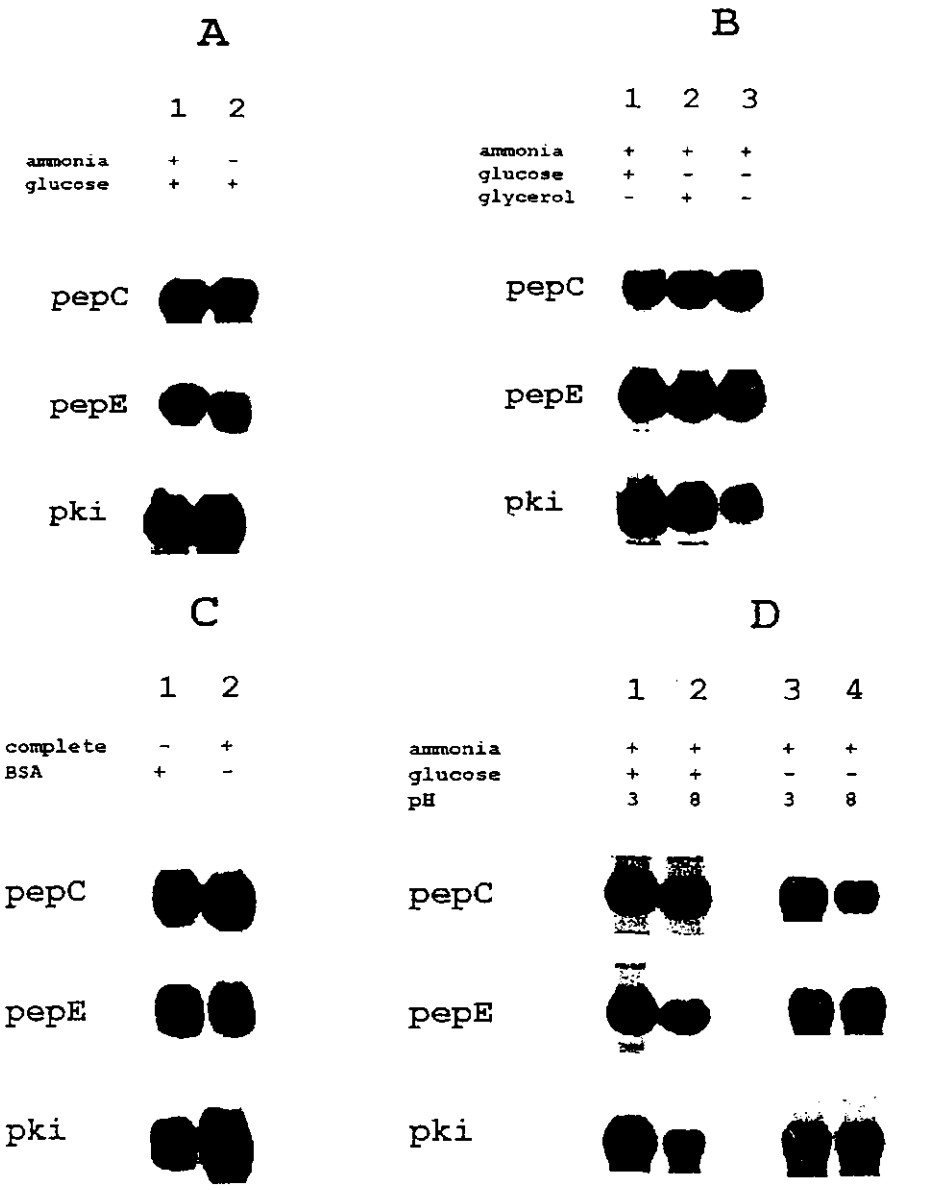
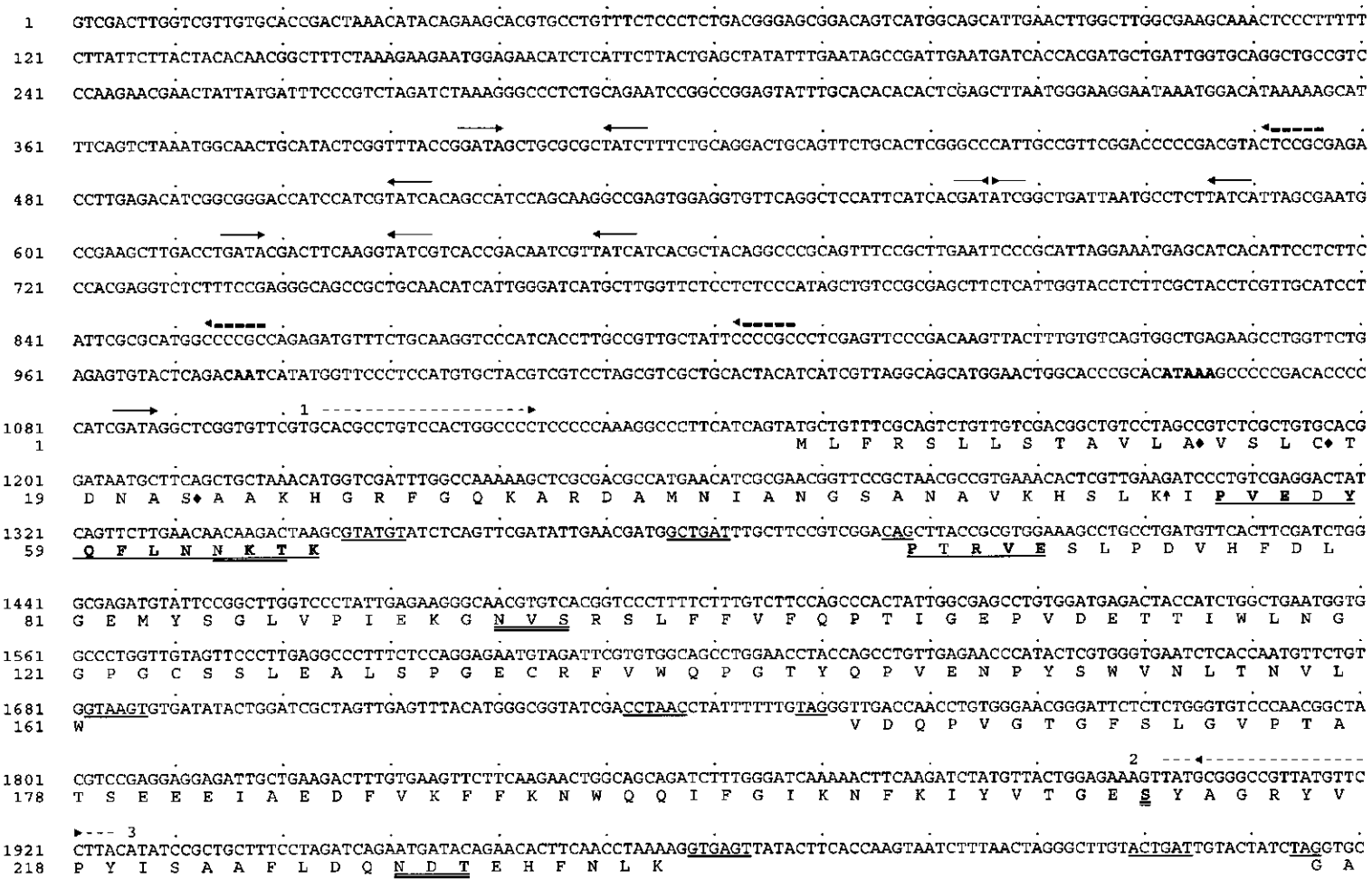


Fig 4.4. Northern blot analyses of the regulation of the *pep*E and *pep*C gene expression. (**A**) Effect of nitrogen repression. Total RNA was isolated from cells that were grown in minimal medium supplemented with glucose as carbon source and with ammonia as nitrogen source (lane 1) or with no nitrogen source (lane 2). RNA samples were separated in agarose gels, blotted and hybridized separately with the *pep*C, *pep*E and *pki* probes. The blots were stripped between each hybridization. (**B**) Effect of carbon repression. Experiments were carried out as for A. Lanes: 1, RNA from cells grown with ammonia and glucose; 2, with ammonia and glycerol; 3, with ammonia and no carbon source. (**C**) Effects of simultaneous carbon and nitrogen derepression and protein induction. Lanes: 1, RNA from cells grown with BSA as the sole carbon and nitrogen source; 2, from cells grown in complete medium. (**D**) Effect of pH. RNA was isolated from cells grown in glucose and ammonia (lanes 1 and 2) or with ammonia and no carbon sources (lanes 3 and 4) at pH 3 (lanes 1 and 3) or at pH 8 (lanes 2 and 4).



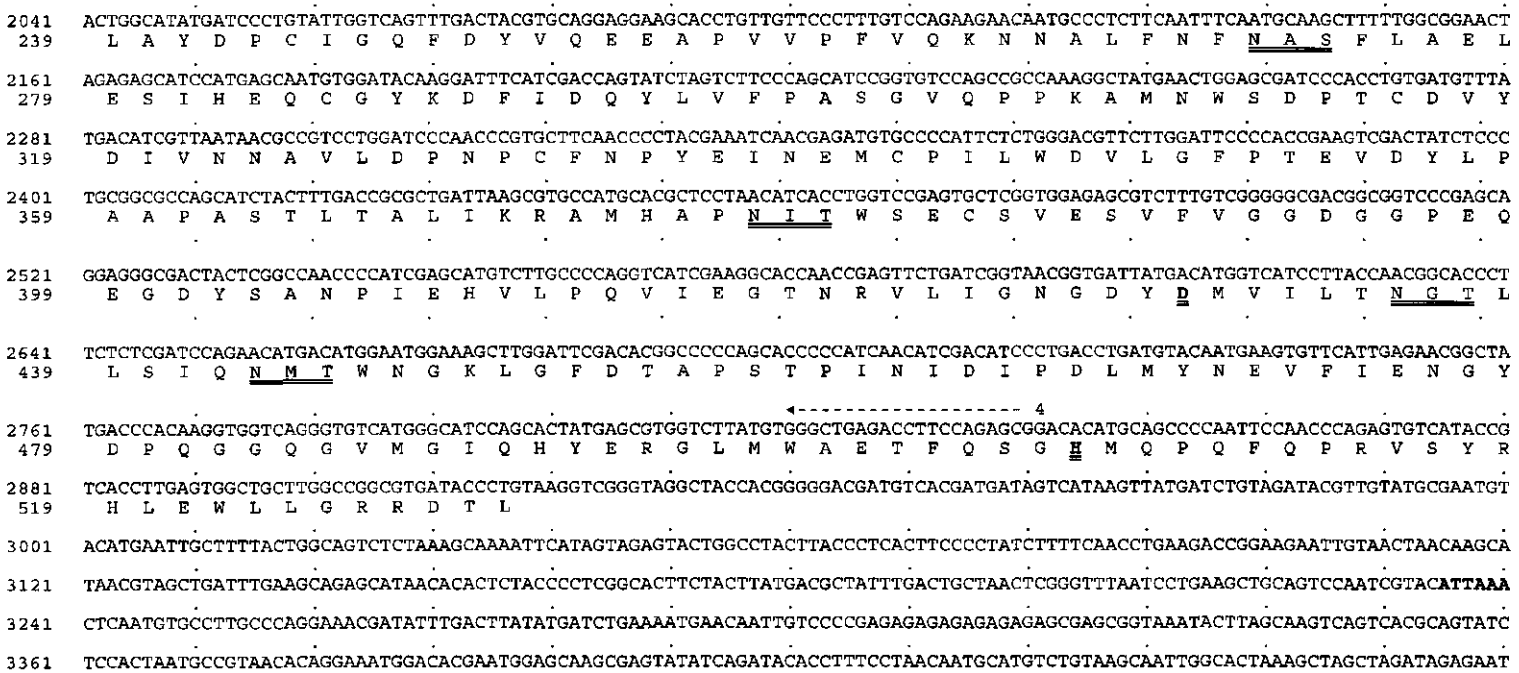


Fig. 5.2. The sequence of *pep*F from *A. niger* (GenBank accession No. X79541) and deduced aa sequence. The dashed arrows above the sequence labelled **1**, **2**, **3** and **4** indicate the oligos that were used for reverse transcriptase, PCR, cloning and sequencing to prove the presence of the three introns. The consensus 5' splice donor (GTRNGT), the 3' splice acceptor (YAG) and the internal element (RCTRAC) of the three introns are underlined. Possible TATAAA-like element, CAAT box and possible AATAAA-like element are in bold. Three putative binding sites for the wide domain regulatory glucose-responsive repressor protein CREA are indicated with '◄- - - - -'. Putative binding sites for the wide domain regulatory ammonium-responsive protein AREA are indicated with '————►'. The start of the mature protein is indicated with a '↑'. The deduced N-terminal sequence of the mature PEPF protein compared to the sequenced N-terminus (Dal Degan et al., 1992) is underlined (corresponding aa are in bold). Three putative signal-peptide cleavage sites predicted for the PEPF protein (von Heijne, 1986) are indicated with '◆'. The three putative active site residues (S_{212}, D_{430} and H_{507}) are in bold and double underlined. The seven putative *N*-linked glycosylation sites (N-X-T/S) in the mature PEPF protein are double underlined.

Most Ser-CPDs from plants are dimeric, with each monomer consisting of two different peptide chains linked by disulfide bridges (Breddam, 1986), while fungal Ser-CPDs are either monomeric or homodimeric but, in all instances, their subunits are made of a single peptide chain. PEPF is monomeric and approximately 60 aa longer than all known Ser-CPDs.

Genomic Southern analysis

Genomic Southern analysis was carried out to confirm full length cloning and to establish the copy number of the *pep*F gene. Total DNA of *A. niger* N400 was digested with *Hin*dIII, *Bam*HI, *Eco*RI and *Sal*I, blotted and hybridized with the 1.2-kb *Sal*I fragment as probe (hybridization conditions: 6 x SSC / 0.1% (w/v) SDS at 68°C over night; most stringent wash: 0.2 x SSC / 0.1% (w/v) SDS, 68°C, 3x30 min). This Southern blot analysis indicated that the *pep*F gene is present in a single copy in the genome of *A. niger* (data not shown).

Regulation of *pep*F expression

It was of interest to determine how the expression of the *pep*F gene is regulated and we studied the regulation of *pep*F at the level of mRNA content, using Northern analysis. We have used the *pep*C gene, encoding a putative vacuolar subtilisin-type protease (Frederick et al., 1993) as control since we have previously shown, that the *pep*C gene is expressed at high levels under all growth conditions tested, with only very limited, growth related variations (Jarai et al., 1994b).

First we investigated the effect of nitrogen and carbon repression upon the expression of *pep*F (Fig. 5.4). As shown in Fig. 5.4A cells grown in the presence of glucose and ammonia contained no detectable *pep*F message. On the other hand, the absence of either the nitrogen or the carbon source resulted in elevated levels of the *pep*F message indicating that both nitrogen and carbon repression affect the expression of *pep*F. This is supported by the fact that the promoter region of *pep*F contains several binding sites for both the wide domain regulatory proteins CREA and AREA involved in carbon catabolite and ammonium repression respectively (Fig. 5.2); the 5' region of *pep*F contains several copies of 5'-GATA-'3 in both directions. This is the recognition site for the ammonium-responsive regulator protein AREA in *A. nidulans* (Kudla et al., 1990). Also two perfect copies of the recognition sequence of the glucose-responsive CREA repressor protein of *A. nidulans*, 5'-G/CPyGGGG-3' (Kulmburg et al., 1993), mediating carbon catabolite repression are present in the 5' non-coding region of *pep*F. Additionally, *S. cerevisiae* MIG1 and *A. nidulans* CREA bind to sequences in which an A is present at the fifth position of the consensus, provided a G is present at the first position (Cubero and Scazzochio, 1994; Lundin, et al., 1994).

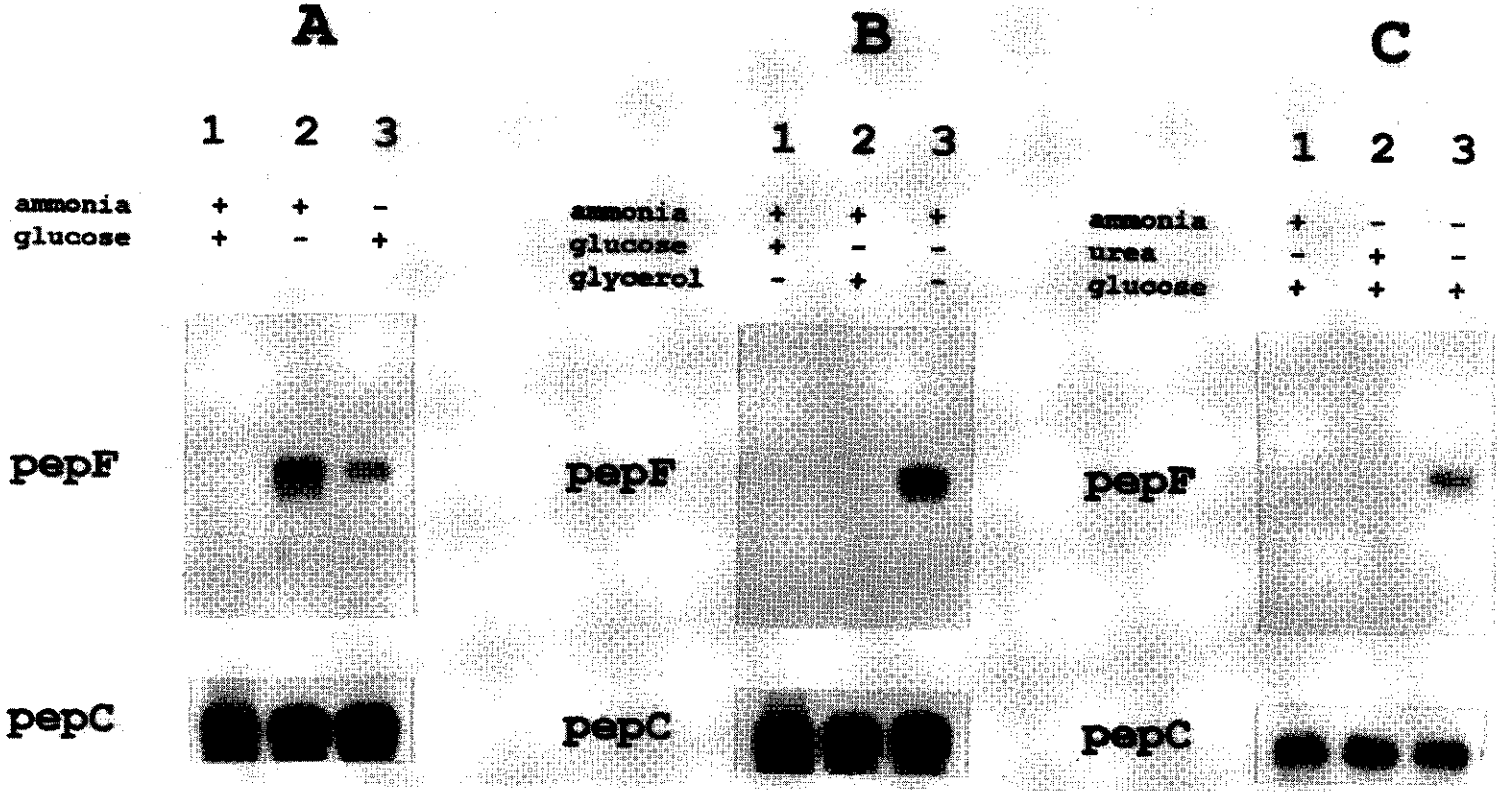


Fig. 5.4. Northern blot analyses of the carbon and nitrogen regulation of the *pep*F gene. **(A)** Effect of nitrogen and carbon derepression. Cells were grown overnight in complete liquid medium pH 6.0, then washed three times with distilled water, divided into three aliquots and transferred into minimal medium pH 6.0 supplemented as indicated in the figure and grown for an additional 6-8 h before RNA was isolated and hybridized to the *pep*F and *pep*C probes, stripping the membranes between the two probings. **(B)** Carbon repression of *pep*F by glucose and glycerol. Experiments were carried out as for **A**. **(C)** Nitrogen repression of *pep*F by ammonium and urea. Experiments were carried out as for **A**.

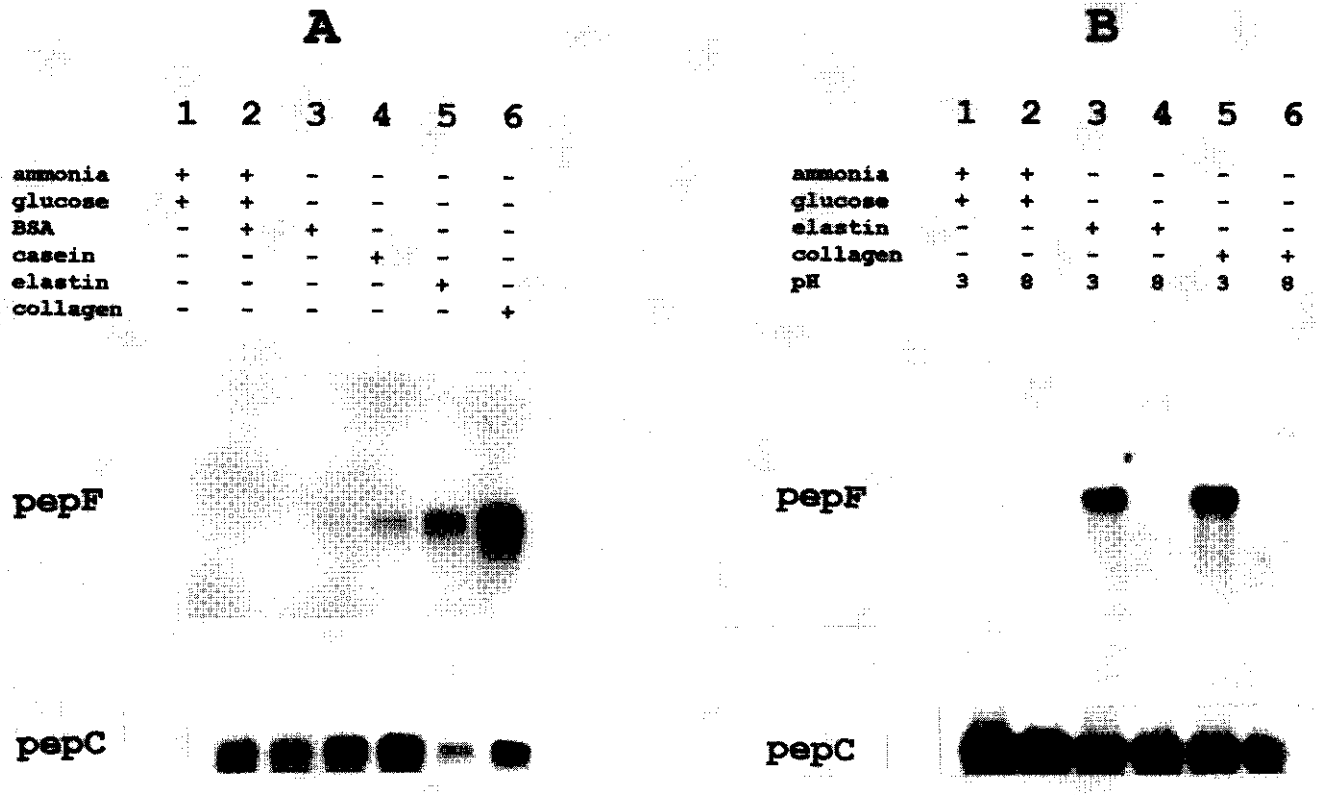


Fig. 5.5. Northern blot analyses of protein induction and pH regulation of *pep*F. **(A)** Comparison of different external protein inducers. Each protein inducer was used at 1% (w/v). **(B)** Effect of pH. Alkaline growth conditions were achieved by continuous adjustment of the pH using NaOH. Experiments were carries out as described in Fig 5.4.

At position 471 in the *pep*F promoter (Fig. 5.2) such a 5'-GCGGAG-3' putative CREA binding site is present. Whether any of these binding sites are involved in the observed carbon and nitrogen repression awaits further analysis. Even glycerol can effectively, although not entirely, repress the expression of *pep*F (Fig. 5.4B). Urea, as sole N source, also represses *pep*F expression completely (Fig. 5.4C).

When we compared the *pep*F message levels in nitrogen and carbon derepressed cells grown on different proteins as sole carbon and nitrogen sources, it was clear that the expression level of *pep*F changed significantly (Fig. 5.5A). Whereas only little message could be detected in cells grown on casein (lane 4) and hardly any in cells grown on BSA (lane 3), elastin (lane 5) and collagen (lane 6) appeared to induce the expression of *pep*F to highly elevated levels. When we compared the transcript levels of *pep*F in cells which were grown at pH 8.0 to that in cells grown at pH 3.0 under both repressing and inducing conditions (Fig. 5.5B), we found that when cells were de-repressed-induced and grown at acidic pH, high message levels were detected (lanes 3 and 5), whereas cells grown on identical derepressing-inducing media whose pH was adjusted to 8.0, showed no *pep*F message.

Conclusions

(1) The *pep*F gene of *A. niger* has been isolated using a degenerate oligo based on the N-terminal sequence of PEPF.

(2) The *pep*F gene encodes a protein consisting of 530 aa, and contains three introns of 53, 69 and 59 bp, which were confirmed by generating cDNA with reverse transcriptase PCR and sequencing.

(3) Signal sequence cleavage prediction analysis suggests that PEPF contains a 17 aa long signal peptide at its N-terminus which directs transport into the endoplasmic reticulum. A second proteolytic processing event at a monobasic cleavage site (Lys_{52}) removes another 35 aa propeptide thus yielding the 478-aa mature protease.

(4) The deduced aa sequence of PEPF shows homology to yeast and plant Ser-CPDs.

(5) Southern analysis revealed that *pep*F is present in a single copy in *A. niger*.

(6) Northern analysis showed that the *pep*F gene is regulated by carbon catabolite and nitrogen metabolite repression, under pH control and is inducible by protein at the level of RNA content. However, additional regulatory mechanisms cannot be ruled out.

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6 Residual protease spectra in *A. niger prt* mutants contain elevated metallo protease activities; identification, cloning, characterization and expression of *pep*H, a novel *A. niger* metallo protease[1]

J.P.T.W. van den Hombergh, L. Fraissinet-Tachet and J. Visser.

Abstract

In the past, several protease deficient (*prt*) mutants have been isolated from *A. niger*. Characterization of the residual protease spectra in *A. niger prt*D and *prt*F protease mutants revealed increased metallo protease activities compared to wild type. The need for a further reduction of the proteolytic activities in these *prt* mutants initiated the screening for metallo proteases in *A. niger*, resulting in the isolation of *pep*H. The *pep*H gene contains three putative introns of 46 bp, 43 bp and 71 bp, respectively, and encodes a 70 kDa prepro protein, which after secretion is processed to a 44 kDa functional metallo protease. FASTA analysis revealed homologies with thermolysin-type metallo proteases in the putative active site regions. RNA slot-blot analyses showed increased *pep*H transcript levels in a *prt*F28 mutant which corresponded with the increased metallo protease activities in this strain. Immunological detection, using an antibody raised against an *A. fumigatus* metallo protease, confirmed increased expression of a 45 kDa protein in this mutant.

Introduction

Several filamentous fungi have been developed as expression-secretion hosts for both homologous and heterologous proteins (Davies, 1994). However, although production of heterologous proteins has sometimes been successful in filamentous fungi, partial or complete proteolytic degradation of secreted products has also often been described (e.g. Kusters-van Someren et al., 1992; Mackenzie et al., 1993).

Characterization of the protease spectrum in *A. niger* identified proteolytic activities possibly interfering with high level expression and resulted in combined molecular and genetic

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approaches to generate protease deficient strains which can be used to improve homologous and heterologous protein production. Up to now, seven protease genes have been cloned in *A. niger*. They include two subtilisin-type serine proteases, *pep*C (Frederick et al., 1993) and *pep*D (Jarai et al., 1994a), two serine carboxypeptidases, *pep*F (van den Hombergh et al., 1994) and *cpy* (Yaver et al., 1995) and three acid proteases, *pep*A (Berka et al., 1990), *pep*B (Inoue et al., 1991) and *pep*E (Jarai et al., 1994b). As the major proteolytic activities in *A. niger* are acid proteases (Jarai and Buxton, 1994), the three acid protease genes cloned in *A. niger* have been disrupted (van den Hombergh et al., unpublished results). In culture supernatants of double and triple disruptants, obtained by recombination, strong reduction of protein degradation has been observed (van den Hombergh et al. unpublished results). Furthermore, a large set of UV-induced protease deficient (*prt*) mutants has been isolated and characterized (van den Hombergh et al., 1995). Genetical analysis identified seven *prt* complementation groups and initial *in vitro* results showed that in culture supernatants of some *prt* mutants the protein degradation was drastically reduced, down to 2% compared to wild type. After isolation of transformable *prt* recombinants, several *pyr*A, *prt* mutants were co-transformed with an expression cassette for the *A. niger pel*B gene, encoding a proteolytically very susceptible protein (Kusters-van Someren et al., 1992). Initial expression results in these *prt* mutants indicate that protein production is significantly improved through the reduction of product degradation (van den Hombergh et al., unpublished results). However, degradation is not absent, which indicates the need for further reduction of proteolytic activities in these expression strains.

In this manuscript we describe how analysis of residual protease spectra in *A. niger prt* mutants has resulted in the identication of residual metallo-type protease activities and the subsequent cloning of the *A. niger pep*H gene, encoding a putative thermolysin-type metallo protease.

Materials and methods

Strains, media and culture conditions. All *A. niger* strains were derived from the wild-type strain N400 (CBS 120.49). N402 has short conidiophores (*csp*A1) while N573 contains an additional *bio*A1 mutation. All *prt* mutants were described by van den Hombergh et al. (1995). *A. oryzae* wild type strain (ATCC 20386) was used to isolate a fragment of the *A. oryzae* NPII-encoding gene. *Escherichia coli* DH5α (Life Technologies) was used as host for routine plasmid propagation. Minimal medium (MM) contained per liter: 6.0 g $NaNO_3$ / 1.5 g KH_2PO_4 / 0.5 g KCl / 0.5 g $MgSO_4$ / trace elements and 1% (w/v) glucose. For complete medium (CM) minimal medium was supplemented with 2% (w/v) trypton / 1% (w/v) yeast extract / 1% (w/v) casamino acids / 0.5% (w/v) ribonucleic acids. Medium optimized for protease expression was described by van den Hombergh et al. (1995). Liquid cultures were inoculated with 10^6 spores/ml and incubated at 30 °C on an orbital shaker at 250 rpm. Agar was added at 1.2 % (w/v) for solid medium.

Biochemical analyses.
a) Enzyme assays Enzyme assays were performed as detailed in van den Hombergh et al. (1995). *b) Western analysis* Cleared culture filtrates from strains grown on medium optimized for protease expression were used for SDS polyacrylamide gelelectrophoresis and Western blotted onto nitrocellulose membrane. Following incubation with alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma), proteins cross-reacting with polyclonal antibodies raised against purified *A. fumigatus* MEP protein [kindly provided by Dr. M. Monod] were visualized using the recommendations supplied with the alkaline phosphatase (Bio-Rad).
c) Degradation of PELB protein The *in vitro* degradation method for PELB by van den Hombergh et al. (1995) was slightly modified. The incubations were in this case performed for longer time periods (2.0 h) and at pH 6.0, which is optimal for *A. fumigatus* MEP activity. After SDS polyacrylamide electrophoresis and Western analysis, degradation products were visualized by immunological detection, using polyclonal antibodies against PELB raised in rabbits and the alkaline phosphatase-conjugated goat anti-rabbit antibodies as second antibody.

Cloning of the *pep*H gene. In a series of heterologous Southern experiments performed as detailed by Sambrook et al. (1989). *A. niger* chromosomal DNA was hybridized with (I) the *A. fumigatus* MEP 1.3 kb *Eco*RI fragment, encoding the mature part of MEP [kindly provided by Dr. M. Monod] and (II) a 0.41 kb fragment generated by PCR from an *A. oryzae* wild type strain (encoding the mature part of the *A. oryzae* NPII gene), to establish conditions for cloning putative metalloprotease(s). An *A. niger* N400 genomic library (Harmsen et al., 1990) was screened with the *A. fumigatus mep* probe. Standard molecular methods, including DNA purification, restriction analysis, ^{32}P random hexanucleotide primed labeling, ligation and bacterial transformation were performed as described by Sambrook et al. (1989). Plasmid vectors pUC (Yanisch-Perron et al., 1985) and pBluescript (Short et al., 1988) were used for subcloning. *Aspergillus* chromosomal DNA was isolated according to de Graaff et al.(1988).

RNA dot-blot analysis. The *A. niger* protease mutants and a wild type strain were pre-grown on liquid CM containing 2% (w/v) glucose and appropriate supplements. After 20 h mycelia were harvested, washed and transferred to a medium containing 1.5 g KH_2PO_4 / 0.5 g KCl / 0.5 g $MgSO_4$ and either 0.5% (w/v) elastin (Fluka) or 0.5% BSA (Boehringer, fraction V) as sole carbon and nitrogen source. After an additional 6 h of incubation at 30 °C, mycelia were harvested, washed and frozen in liquid nitrogen. Total RNA was isolated using TRIzol (GibcoBRL) according to the supplier's instructions. For slot-blot experiments 20 μg of total RNA were transferred to Hybond-N membranes (Amersham) and UV-crosslinked. (Pre-)hybridization of membranes was performed at 42°C in (pre-)hybridization buffer containing 0.75 M NaCl / 50 mM NaH_2PO_4 pH 7.4 / 10 mM EDTA / 0.1% (w/v) SDS / 2 x Denhardt's / 10% (w/v) dextran sulphate / 50% (w/v) formamide supplemented with 200 μg/ml denatured herring sperm DNA. Specific ^{32}P-labelled probes were generated by random priming (Sambrook et al., 1989). Membranes were washed down to 0.2 x SSC / 0.5% (w/v) SDS at 65°C.

Results and discussion

Metallo protease related protein degradation in *A. niger*

Recently, we have described the isolation and characterization of a large set of protease mutants comprising at least seven complementation groups (van den Hombergh et al., 1995). All *prt*

mutations result in reduced extracellular proteolytic activities and have been tested *in vitro* for reduced degradation of proteins which are very susceptable to proteolytic degradation. As the degradation in some *prt* mutants was severely impaired, we have also tested *in vivo* degradation of PELB protein produced in a *prt* genetic background. Especially in a *prt*A, *prt*B, *prt*D and a *prt*F genetic background the degradation of PELB was considerably reduced compared to the wild type situation (van den Hombergh et al., unpublished results). However, proteolytic degradation was not absent, even in the *prt*B mutant, which has only 2% residual protease activity. Identification of residual proteolytic activities in these strains is important as this might enable us to further reduce proteolytic degradation in the expression strains we are developing.

When characterizing the residual protease spectra in the *prt* mutants, increased neutral protease activities were observed in some *prt* mutants (van den Hombergh et al., 1995). Enzyme assays in combination with specific inhibitors demonstrated increased EDTA inhibitable protease activities in cleared culture supernatants of NW228 (*bio*A1, *prt*F28), NW229 (*bio*A1, *prt*F29) and NW232 (*bio*A1, *prt*D32). EDTA inhibitory effects on protease activities indicated the involvement of proteases which need metal ions for activity, such as metallo proteases or some serine and cysteine proteases which require calcium. Protease assays were repeated in the presence of 1,10 phenanthroline, which is a more specific metallo protease inhibitor as it does not chelate calcium. The enzyme assays in the presence of 1,10 phenanthroline demonstrated that in these three *prt* mutants (*prt*F28, *prt*F29 and *prt*D32) increased metallo protease activities were present (Fig. 6.1).

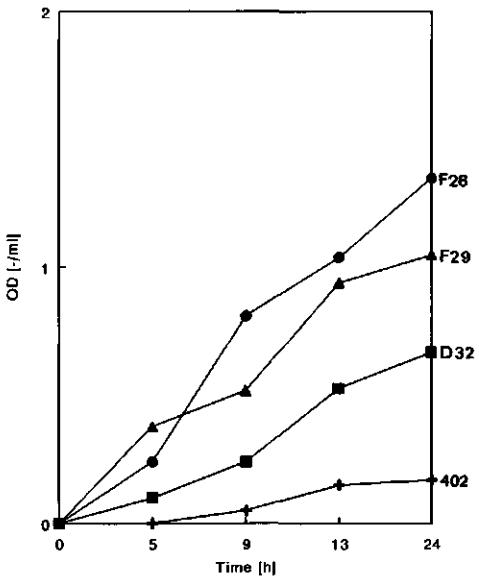

Fig. 6.1. Increased metallo protease activities in *prt* mutants. Cleared supernatants from 72 h wheat bran cultures (van den Hombergh et al., 1995) were assayed for protease activity against BSA (fraction V) either in the presence or in the absence of 2 mM 1,10 phenanthroline. 1,10 phenanthroline-inhibited activities were obtained from the difference between the 1,10 phenanthroline-inhibited and non inhibited enyzme assays. Activities are expressed as the OD_{280} change per h per 1 ml culture supernatant. +, N402; ●, NW228 (*prt*F28); ▲, NW229 (*prt*F-29); ■, NW232 (*prt*D32)

Previously we have described an *in vitro* degradation system based on the homologous *A. niger* pectin lyase B (PELB) protein and we could show that virtually no PELB degradation was observed when this protein was incubated for 15 min in culture supernatants of the *prt*F mutants (van den Hombergh et al., 1995). After modification of the system as described in the *Material and Methods* section we repeated *in vitro* PELB degradation in N573 and NW228 culture supernatants, both in the presence and absence of EDTA or 1,10 phenanthroline, and visualized degradation products by Western analysis and immunological detection (Fig. 6.2). In the control, in which N573 culture filtrate was boiled prior to incubation with PELB protein, no degradation products were observed below the major 40 kDa protein band. The additional band above the major PELB band is probably the result of differences in glycosylation as has been proposed previously (Kusters-van Someren et al., 1992). The *in vitro* results demonstrate the complete degradation of PELB protein in the wild type supernatant and only limited degradation in the *prt*F28 mutant supernatant. An EDTA inhibitory effect upon degradation is observed both in wild type and in NW228. The effects of EDTA and 1,10 phenanthroline on the degradation pattern for PELB in NW228 culture filtrates, are comparable. For both inhibitors the pattern of the PELB degradation products is reduced to one major band. The differences in the number of observed degradation products between EDTA- and 1,10 phenanthroline-inhibited culture supernatants of the wild type could indicate an additional EDTA-mediated chelating effect on non-metallo-type proteases. However, the reduction in PELB degradation both in the presence of 1,10 phenanthroline and of EDTA, clearly indicates the involvement of metallo protease activities in residual PELB degradation.

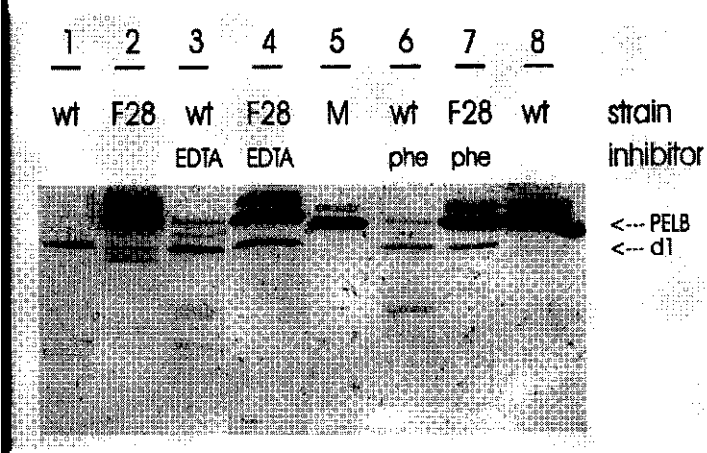

Fig 6.2. Metallo protease inhibition affects degradation in cleared culture supernatants of *prt*F28. Two micrograms of purified PELB protein were incubated in undiluted cleared culture supernatant derived from wheat bran cultures (van den Hombergh et al., 1995). After incubation the degradation products were separated via SDS PAGE, Western blotted and visualized with specific PELB antibodies. Track order; 1, N573; 2, NW228; 3, N573 (EDTA); 4, NW228 (EDTA); 5, 1 μg pure PELB; 6, N573 (1,10 phenanthroline); 7, NW228 (1,10 phenanthroline); 8, N573 (culture supernatant boiled prior to incubation); ← **PELB**, 40 kDa PELB protein; ← **d1**, major PELB degradation product

Since the *in vitro* degradation experiments clearly demonstrated that inhibition of metallo protease activities still further reduced proteolytic degradation, we decided to screen for metallo protease genes in *A. niger*.

Cloning and characterization of *pep*H

When we started to screen for *A. niger* metallo protease genes, two metallo proteases had been cloned from *Aspergillus* species, the neutral protease II of *A. oryzae* (Tatsumi et al., 1991) and the metallo protease (MEP) of *A. fumigatus* (Jaton-Ogay et al., 1994). FASTA analysis showed very low homologies between these two proteases and therefore both metallo protease genes were used as probes in heterologous hybridization experiments with *A. niger* chromosomal DNA. For the *A. oryzae* probe no hybridizing signals were detected with *A. niger*. In control experiments using *A. oryzae* and *A. nidulans* chromosomal DNA we clearly observed hybridizing fragments for both species, which for *A. nidulans* resulted in the cloning of two NPII type metallo protease genes (van den Hombergh et al., unpublished results). With the *A. fumigatus* probe specific hybridizing signals were detected at 60°C and 2 x SSC / 0.5% (w/v) SDS (data not shown). Under identical conditions, a genomic library in phage λ (Harmsen et al., 1990) was screened. Ten clonally pure hybridizing phages were obtained. Restriction mapping and hybridization studies of isolated DNA identified a hybridizing 3.8 kb *Hin*dIII fragment which was subcloned into pUC18 to generate plasmid pIM675. As shown in Fig. 6.3, approximately 1.7 kb of the *Hin*dIII insert in pIM675 was sequenced. Alignment of the *A. niger* putative translation product and the *A. fumigatus* MEP protein showed that the coding sequence for approximately 200 amino acids at the N-terminus and the *pep*H promoter were lacking in the *Hin*dIII fragment. Therefore a large *pep*H containing *Eco*RI fragment was subcloned in pUC18, resulting in plasmid pIM676 which was partially sequenced. The DNA sequence of the *pep*H gene and the deduced amino acid sequence shown in Fig. 6.3

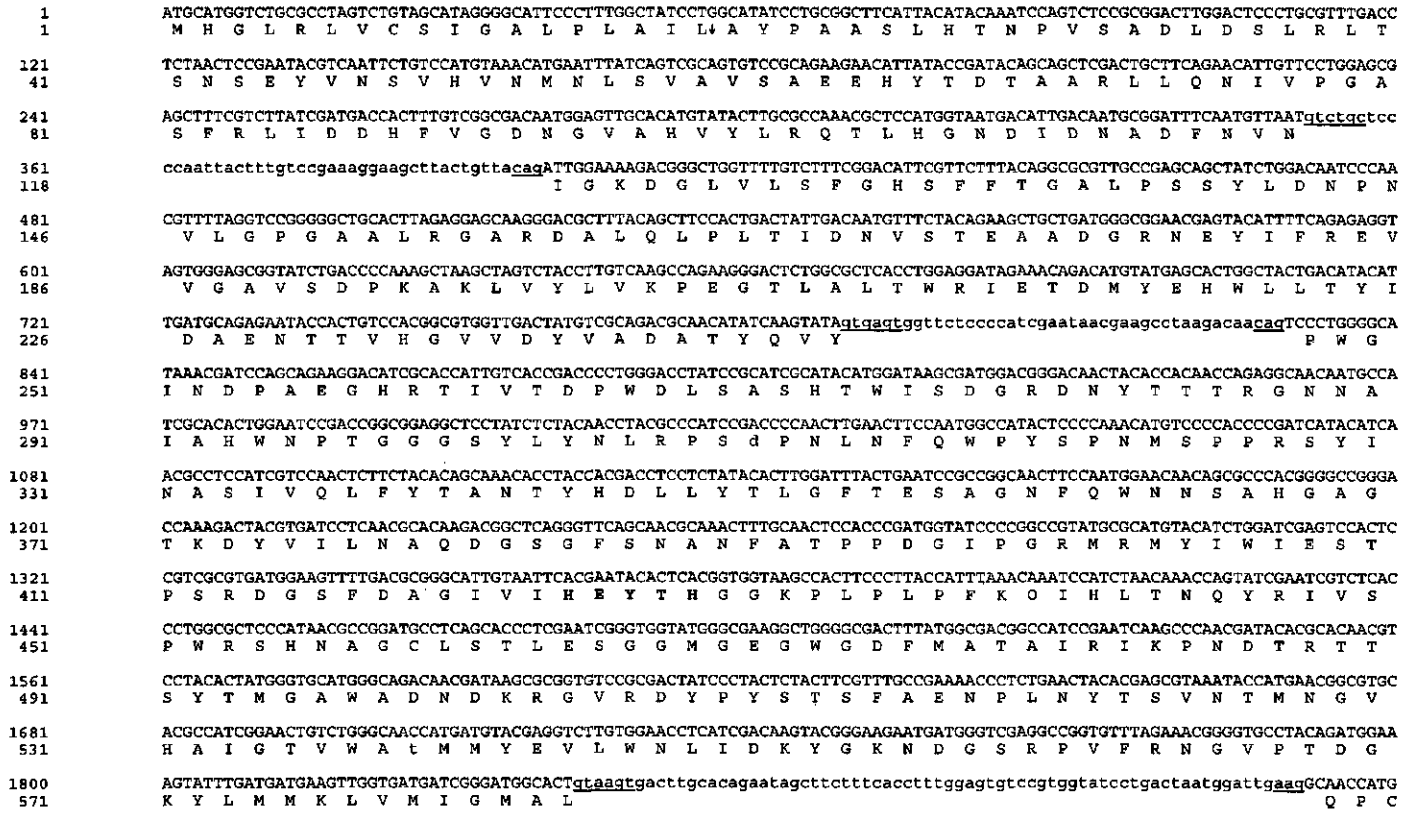

Fig. 6.3. Partial *A. niger pep*H DNA nucleotide sequence and deduced aa sequence. Exons are indicated in upper case, whereas putative introns are in lower case letters. The putative 5' splice donor and the 3' splice acceptor of the three introns are underlined. The putative signal peptide cleavage site predicted for the PEPH protein (von Heijne, 1986) is indicated with '↓'. The HEXXH-motif containing two putative zinc ligands (H_{414} and H_{418}) is indicated in bold.

The PEPH translation product

Analysis of the deduced amino acid sequence of PEPH reveals that the protein is probably synthesized as a zymogen, containing a prepro-region of approximately 241 amino acids, similar to the situation in *A. fumigatus* (Jaton-Ogay et al., 1994). Signal cleavage analysis according to von Heijne (1986) predicted a putative signal peptidase cleavage site after Leu_{18} (Fig. 4). Based on the homologies with the *A. fumigatus* MEP which was N-terminally sequenced (Ibrahim-Granet et al., 1994), we predict that the mature PEPH starts at or around Ala_{241} (Fig. 6.4). Thus, removal of the 223 aa propeptide results in a mature protein with a predicted molecular mass of 44 kDa. The *A. fumigatus* MEP zymogen is probably capable of autocatalytically processing the precursor to yield mature MEP (Jaton-Ogay et al., 1994). Recently, it has been shown by Moyer and co-workers (1995) that a similar metallo protease of *Fusarium* is capable of processing a *Fusarium* serine protease precursor during co-expression of both *Fusarium* proteases in *A. oryzae*. PEPH contains the consensus motif for putative active site residues in metallo proteases (HEXXH) which is located between hydrophobic amino acids (Fig 6.4). The two histidines in the motif ($\mathbf{H}_{424}$EYT-$\mathbf{H}_{428}$) are involved in coordinating the cation Zn^{2+} in the active center and $\mathbf{E}_{425}$ is involved in the bond-breaking process (Jongeneel et al., 1989).

Based on the homologies with the *A. fumigatus* MEP, the *A. niger* PEPH belongs to the class of metallo proteases to which the *A. sojae* and *A. oryzae* neutral protease I (Sekine et al., 1972; Nakadai et al., 1973), the *Bacillus thermoproteolyticus* thermolysin and related enzymes of other bacteria belong (for a review, see Hälse and Finkelstein, 1993). FASTA analyses for the PEPH protein showed only a high degree of homology with the *A. fumigatus* MEP. The mature parts of the metallo proteases show the highest conservation. As expected, the prepro-regions show reduced homologies, which confirms the observation that the prepro-region of MEP did not hybridize to *pep*H, under the conditions used for screening the genomic library (data not shown). Apart from the zinc-binding motif, no significant homologies with other metallo proteases were identified in the databases.

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PEPH    1    MhGLrLvcsigalplailaypaaSlhtNpvsaDLdslRLtSnseYVN-svhVnmnlSvav
MEP     1    MrGLrLagalalpasvfahpahqSyglNrrtvDLnafRLkslakYVNatetVieapSsfa

PEPH   60    saeeh-YtdtAarllqnIvPgAsFRliDDHfVGDNGVAHVylRQTlhGnDIDNADFNVNi
MEP    61    pfkpqsYvevAtqhvkmIaPdAtFRvvDDHyVGDNGVAHVhfRQTanGlDIDNADFNVNv

PEPH  119    GKDGlVlSfGhSFfTGalPSS-yLdnpnvlgPgaALrGardaLQLPlTiDnvStEaadgr
MEP   121    GKDGkVfSyGnSFyTGqiPSSaaLtkrdfsdPvtALkGttntLQLPITvDreSsEsseek

PEPH  178    neYiFreVvGaVSDPKAKLVYlVKpeGTLALtWRiETDmyehWLLTYIDAentt-vHGVV
MEP   181    esYvFkgVsGtVSDOKAKLVYfVKddGTLALaWRvETDidsnWLLTYIDAksgeeiHGVV

PEPH  237    DYVAdAtYQVYpWGINDPaEGhRTivtDPWDlsashTWISDGrdNYTTtRGNNaIAhwNP
MEP   241    DYVAeADYQVYaWGINDPtEGeRTvikDPWDsvtefTWISDGstNYTTsRGNNgIAqsNP
                    ↑

PEPH  297    tGGgSYLyNlRPSdpnLnFqwPYSpnmSPPrSYInASIvQLFYTANtYHDLLYTLGFTEs
MEP   301    sGGpSYLnNyRPSsssLsFkyPYSvssSPPsSYIdASIiQLFYTANiYHDLLYTLGFTEk

PEPH  357    AGNFqwNnsahGagtkDYVILNAQDGSGfsNANFATPPDGiPGRMRMYiWiESTPsRDGS
MEP   361    AGNFeyNtngqGglgnDYVILNAQDGSGtnNANFATPPDGqPGRMRMYvWtESTPyRDGS

                  ●  ■                                          ■
PEPH  417    FdAGIVIHEYTHGgkplplpFkoihLTnqyrivsp-WRshNagCLstLESGGMGEGWgDF
MEP   421    FeAGIVIHEYTHGrTyylAGFIdtrLTpffsfqpthWRpaNsnCLnaLESGGMGEGWSDF
Thermolysin       HExTHGvTxxtAGlI----------------------yxnqSGaxnEafSD

PEPH  476    MATAIRiKPnDtRtTsYTMGaWAdNdkrGvRdYPYSTSfaeNPLnYTSVNtmNgVHAIGT
MEP   481    MATAIRlKPgDkRsTdYTMGeWAsNragGiRqYPYSTSlstNPLtYTSVNslNaVHAIGT

PEPH  536    VWAtMmYEVLWNLIDKyGKNDgsrPvfRnGVPTDGKYLmMKLVMiGMALQPCNPNFVQAR
MEP   541    VWAsMlYEVLWNLIDKhGKNDapkPtlRdGVPTDGKYLaMKLVMdGMALQPCNPNFVQAR
```

Fig. 6.4. Partial aa sequence comparison of *A. niger* PEPH and *A. fumigatus* MEP. The aa sequences were aligned using the program PILEUP available in the GCG package. Part of the thermolysin consensus sequence for three zinc ligands is aligned to demonstrate some homology around the E_{470} (PEPH numbering) residue. Putative zinc ligands are indicated with '■'. The E_{425} probably involved in the bond-breaking process is indicated with '●'. The N-terminus of the mature MEP is indicated with ↑. -, no aa present at that position. Conserved putative N-linked glycosylation sites are double underlined. Non-conserved putative N-glycosylation sites are underlined.

The cloning of a new metallo protease gene from *A. niger* confirmed the presence of this family of protease (functions) in *A. niger*. To determine whether the observed correlation between the presence of metallo protease activity and *in vitro* degradation of the PELB tester protein in some *prt* mutants was related to expression of the *pep*H gene, Western and Northern analysis of the *prt* mutants and the parental strain N573 were performed. The wheat bran culture supernatants of a set of *prt* mutants, previously used to assay metallo protease activities, were centrifuged and than

screened for a putative thermolysin-type metallo protease using polyclonal antibodies raised against pure *A. fumigatus* MEP (Fig. 6.5). In NW228, NW229 and NW232 proteins cross-reacting with the anti-MEP antibodies were observed. The size of these cross-reactive proteins is approximately 48 kDa, which corresponds with the size of the mature *A. fumigatus* MEP (45 kDa; Jaton-Ogay et al., 1994). In N573 only a very faint band is observed whereas in NW228, NW229 and NW232 increased levels are detected. Moreover, the presence of several cross-reactive bands in the 45-50 kDa range could indicate differences in glycosylation. The Western blot demonstrates that the increased metallo protease activities observed in the culture supernatants of NW228, NW229 and NW232 correlates with the presence of these ~45 kDa cross-reactive bands. Although this is not absolute proof, these observations strongly suggest that the metallo protease related degradation is caused by the PEPH gene product. Interestingly, in the NW228 and NW229 supernatants a ~70 kDa cross-reactive band is observed (see Fig. 6.5). Whether this is an aspecific band awaits further analysis. However, it could be an interesting observation as the molecular mass of this band corresponds to the size of a putative pro-PEPH protein which could indicate partially incorrect processing. This might implicate the involvement of yet another protease in PEPH maturation and an effect of the *prt*F mutation upon this PEPH-maturating protease.

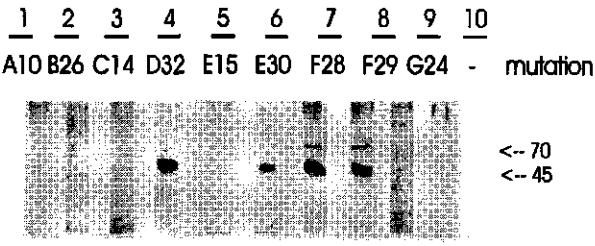

Fig. 6.5. Western analysis of *A. niger prt* mutants grown on medium optimized for protease expression. Cleared culture supernatants from *A. niger prt* mutants and N573 were subjected to SDS PAGE, Western blotted and analysed using *A. fumigatus* anti-MEP antibodies. Track order: 1, NW222 [*prt*A10]; 2, NW227 [*prt*B26]; 3, NW224 [*prt*C14]; 4, NW232 [*prt*D32]; 5, NW225 [*prt*E15]; 6, NW230 [*prt*E-30]; 7, NW228 [*prt*F28]; 8, NW229 [*prt*F29] 9, NW226 [*prt*G24]; 10, N573;← **~45**, indicates the position of the ~45 kDa cross-reactive bands; ← **~70**, indicates the position of the ~70 kDa cross-reactive band in NW228 and NW229 culture supernatants.

_pep_H expression in _prt_ mutants

The *prt* mutants used in the Western analysis were pre-grown on complete medium, after which mycelium was harvested, washed and tranferred to a medium containing either 0.5% (w/v) BSA or 0.5% (w/v) elastin as sole carbon and nitrogen source, in order to induce protease expression. After an additional 6 h of growth on this medium, expression of *pep*H was visualised with slot-blot Northern hybridizations using total RNA extracted from mycelium recovered from the transfer cultures (Fig. 6.6). As a loading control the blots were probed with the 28S rDNA probe from *Agaricus bisporus* (Acc. No. X91812).

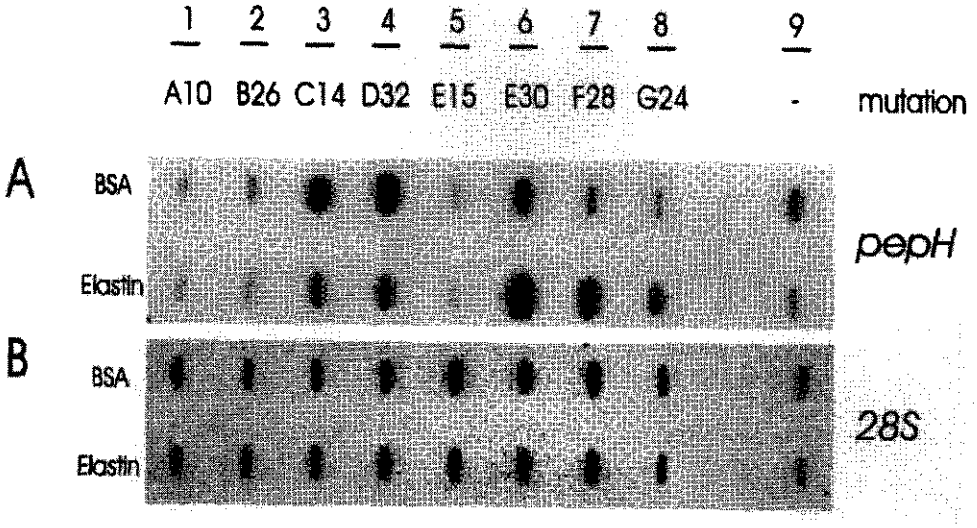

Fig. 6.6. Slot-blot Northern analysis of *pep*H expression in *prt* mutants. Total RNA extracted from protein-induced transfer cultures was slot-blotted and hybridized with (**A**) a *pep*H probe and (after stripping the blot) with (**B**) a 28S rDNA derived probe. BSA, RNA isolated from BSA-induced transfer cultures; Elastin, RNA isolated from elastin-induced transfer cultures. Track order: 1, NW222 [*prt*A10]; 2, NW227 [*prt*B26]; 3, NW224 [*prt*C14]; 4, NW232 [*prt*D32]; 5, NW225 [*prt*E15]; 6, NW230 [*prt*E30]; 7, NW228 [*prt*F28]; 8, NW226 [*prt*G24]; 9, N573.

Whereas the 28S rDNA probe shows similar hybridization intensities, indicative for correct loading and blotting of the RNA preparations, clear differences are observed with the *pep*H probe. For the BSA induced transfer cultures higher *pep*H expression is observed in NW224, NW230 and NW232, whereas elastin induction results in elevated expression in NW230 and NW228. Comparing the Northern data with the Western data and the protease activities measured in culture supernatants, for NW228 and NW232 the increased *pep*H mRNA levels correlate with increased extracellular metallo protease levels. Interestingly, large differences in *pep*H mRNA levels are

observed between the NW225 and NW230, which carry allelic mutations. The Western blot data however, correlates with the Northern data as no expression is observed in NW225 and higher expression is observed in NW230. The increased expression levels in NW224 do not correlate with the Western blot data. Whether this is due to the different growth conditions (i.e. wheat bran cultures for the Western analysis versus protein induced cultures for the Northern analysis) is unknown. However, in a *prt*C14 genetic background the transcriptional levels of several extracellular proteases is increased, although the total extracellular proteolytic activity is reduced to 20%. Furthermore, for the extracellular *A. niger* PEPA protease also no relation between the presence of a PEPA protein and *pep*A mRNA levels has been observed in several *prt* mutants(van den Hombergh et al., unpublished results).

Heterologous Southern analysis

Homologous and heterologous Southern analyses were performed using the *A. niger pep*H gene, the *A. fumigatus MEP* gene and the *A. oryzae* NPII (metallo protease) gene as probes. These Southern analyses of *A. niger* N402 chromosomal DNA demonstrated that *pep*H is present in one copy in the *A. niger* genome and that a second metallo protease closely related to *A. oryzae* NPII does not exist in *A. niger* (data not shown).

In summary, the results presented strongly indicate that the observed metallo protease activity and the correlated degradation problems in *prt*F strains are to some extent related to the *pep*H gene product. However, involvement of other metallo protease activities cannot be excluded. It is also possible that the metallo protease-related residual degradation in some *prt* mutants is caused by a protease which is activated by PEPH, as Moyer et al. (1995) have described processing mediated by the PEPH equivalent from *Fusarium oxysporum*. Definite proof of PEPH involvement in degradation awaits the construction of a *pep*H gene disruption, preferably in a *prt* genetic background. Recently a *prt*F28, *pyr*A6, *arg*B13 recombinant has been isolated which, after disruption of *pep*H with a construct containing the *A. nidulans arg*B gene as selection marker, will generate a *prt*F, Δ*pep*H disruptant which can be co-transformed using the *A. niger pyr*A gene and thus allows *in vivo* analysis of PELB degradation (as described in chapter 9; van den Hombergh et al., unpublished results). The observation that PELB degradation is still not absent in the presence of metallo protease inhibitors demonstrates that other (non metallo-type) residual proteases are present and consequently could indicate the need for additional modifications of the residual protease spectrum, either by identification and inactivation of these activities or by introducing by recombination other *prt* mutations which reduce these activities.

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7 Disruption of three acid proteases in *Aspergillus niger*: effects on protease spectrum, cascade activation and degradation of target proteins[1]

J.P.T.W. van den Hombergh, M.D. Sollewijn Gelpke[2], P.J.I. van de Vondervoort, F.P. Buxton[3] and J. Visser

Summary

Three acid protease genes encoding two extracellular proteases (PEPA and PEPB) and one intracellular protease (PEPE) were disrupted in *Aspergillus niger*. Northern analysis showed the absence of wild type protease mRNAs in the disruptants while Western analysis proved the absence of the encoded proteases. Characterization of the residual proteolytic spectra in the disruptants indicated that the extracellular protease activity was reduced to 16% and 94% for the Δ*pep*A and the Δ*pep*B disruptants, repectively. In the Δ*pep*E disruptant the total intracellular proteolytic activity was reduced to 32%. Apart from the reduced intracellular pepstatin-inhibitable aspartyl protease activity, also serine and serine carboxypeptidase activities were significantly reduced in the Δ*pep*E strain. This may indicate for the presence of a cascade activation mechanism for several vacuolar proteases, triggered by the PEPE protein, similar to the situation in *Saccharomyces cerevisiae*. Disruption of a single protease gene had no effects on the transcription of other non-disrupted protease genes in *A. niger*. In supernatants of all three disruptants a proteolytically very susceptible tester protein (PELB) was degraded at a reduced rate, even in the Δ*pep*E disruptant. By recombination we also constructed Δ*pep*AΔ*pep*B, Δ*pep*BΔ*pep*E and Δ*pep*AΔ*pep*E double disruptants as well as a Δ*pep*AΔ*pep*BΔ*pep*E triple disruptant, lacking all three acid protease activities.

Introduction

Aspergillus niger produces a broad spectrum of proteases, both specific and non-specific, which is recognized to be one of the major reasons for the low protein expression yields which are

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frequently observed, especially when producing heterologous proteins. The genetics of proteolytic degradation in *Aspergilli* is very complex due the involvement of many regulatory factors in protease expression. Cohen (1972, 1973) showed in a series of pioneering studies that in *A. nidulans* extracelllular proteases were repressed by low molecular weight sources of carbon, nitrogen and sulphur. Jarai and Buxton (1994) and van den Hombergh et al. (1994) have recently shown that in *A. niger* both pathway specific induction by proteins and wide domain regulatory mechanisms (carbon catabolite repression, nitrogen metabolite repression and pH regulation) are involved in the overall regulation of extracellular proteases. Several intracellular (vacuolar) proteases (*pep*C and *pep*E) appear to be constitutively expressed in *A. niger* (Jarai et al., 1994).

Attempts to control the problem of proteolysis have targetted various stages in the protein production process. However, much of the degradation occurs *in vivo* thus indicating the need for modification of the production hosts. Deletion of the major extracellular aspartic protease from *A. awamori* (Berka et al., 1990) significantly improved heterologous expression of chymosin (Bodie et al., 1994). The use of (multiple) protease-deficient strains for the expression of recombinant proteins has been described in other expression-secretion systems such as *Escherichia coli* (Gottesman, 1990), *Bacillus* (He et al., 1991) and yeast (Hinnen et al., 1994). Studies in these expression hosts have shown that not only the extracellular proteases but also intracellular proteases are involved in the overall degradation of expressed proteins. Deletion of proteases present in the secretory pathway and in the vacuole in yeast reduced (partial) degradation of hirudin (Hinnen et al., 1994). In *Saccharomyces cerevisiae* the vacuolar proteases are activated in a cascade; the *ysc*A encoded PrA is synthesized as an inactive precursor (proPrA), which is autocatalytically activated by removal of its pro-region after arriving in the vacuole (van den Hazel et al., 1992). PrA proteolytically activates the precursors of several other vacuolar enzymes, including those of proteinase B (PrB) and carboxypeptidase Y (CPY). Thus, by inactivating the first protease within the cascade (PrA), two additional proteases (PrB and CPY) are inactivated as these proteases are not capable of autocatalytic (self-)activation.

A. niger has an acidic pH optimum for degradation of extracellular proteins and the aspartyl proteases have been identified as causing the major problems in expression. Therefore we have disrupted three *A. niger* aspartic proteases, two pepsin type proteases, *pep*A and *pep*E, and one non-pepsin type protease, *pep*B. In these strains we have studied the effects on residual protease activities and degradation of target proteins, both *in vitro* and *in vivo*. *Pep*A and *pep*B are the major extracellular acid proteolytic activities whereas *pep*E is the *A. niger* homologue of *ysc*A and therefore probably located intracellularly in the vacuole. Disruption of *pep*E should inactivate a putative proteolytic cascade in the vacuole and result in additional loss of the *pep*C and *cpy*

encoded proteolytic functions which are the *A. niger* homologous of yeast *ysc*B and *ysc*Y. Finally, the single disruptants allowed a controlled construction of multiple protease-deficient strains, which can be used for further improving both homologous and heterologous expression in *A. niger*.

Materials and Methods

Strains, media and growth conditions. All *A. niger* strains were derived from the wild-type strain N400 (CBS 120.49). The morphological mutant N402 has short conidiophores (*csp*A1). Strain N647, which has white spores (*fwn*A6) and which is arginine (*arg*B13) and nicotinamide (*nic*A1) deficient, was used for transformation. Disruptants NW157, NW158 and NW159 were derived from N647 after transformation with the *pep*A, *pep*B and *pep*E disruption constructs (see also Table 1). The strains used for and generated during the genetic analysis are listed in Table 1. *Escherichia coli* DH5α (Life Technologies) and JM101 were used as hosts for routine plasmid propagation. Minimal medium (MM) for *Aspergillus* contained 10 mM NH_4Cl, 11 mM KH_2PO_4, 6.7 mM KCl, 2 mM $MgSO_4$, 1% (w/v) glucose and essential salts (Vishniac and Santer, 1957). Complete Medium (CM) contained MM supplemented with 2% (w/v) peptone, 1% (w/v) yeast extract, 0.5% (w/v) ribonucleic acids, 0.5% (w/v) casamino acids. Induction medium (IM) contained MM in which NH_4Cl was replaced by 10 mM $NaNO_3$, without carbon source and supplemented with 0.5% (w/v) BSA (Boehringer, fraction V) and 0.5% (w/v) elastin (Fluka). Glucose Repression Medium (GRM) contained MM in which NH_4Cl was replaced by 10 mM $NaNO_3$ and glucose was added to a final concentration of 2% (w/v).

Molecular methods. Standard methods for DNA purification, restriction enzyme digestion, agarose gel electrophoresis, DNA fragment isolation, ligation and bacterial transformations of *E. coli* were described by Sambrook et al. (1989). *A. niger* chromosomal DNA was isolated according to de Graaff et al. (1988), digested with restriction enzymes, separated by agarose gel electrophoresis on 0.8% (w/v) agarose and blotted to a nylon membrane (Hybond N^+) via capillary elution according to Sambrook et al. (1989). Hybridizations were performed overnight using hybridization buffer containing 6 x SSC [0.15 M NaCl, 0.015 M Na_3citrate pH 7.6] / 5 x Denhardt's / 0.5% (w/v) SDS / 10 mM EDTA / 50 mM Tris pH 7.5. Homologous hybridizations were performed at 65°C and washed with 0.2 x SSC / 0.5% (w/v) SDS. *A. niger* strain N647 was transformed as described by Tilburn et al. (1983), using 5 μg (linearized) DNA.

Construction of disruption plasmids. The construction of the disruption plasmids for *pep*A (pIM644), *pep*B (pIM645) and *pep*E (pIM646) is described in Fig. 7.1. All disruption vectors contained the *A. nidulans arg*B gene as selection marker (Johnstone et al., 1985) and, after transformation of the arginine requiring *A. niger* strain N647, transformants were selected and purified by re-plating at low spore densities on selective medium. Putative disruptants were identified using a PCR screening technique; spores were inoculated in 2 ml complete medium and the cultures were incubated overnight at 30°C. The mycelial mat was harvested, freeze dried and dissolved in an extraction buffer described by de Graaff et al. (1988). After phase separation 10 μl of the liquid phase was used for PCR reactions using a Sensa 949E PCR apparatus and PCR conditions were those recommended for Taq polymerase (Pharmacia). Primers PEPA1 and PEPA2, PEPB1 and PEPB2 and PEPE1 and PEPE2 were specific for the *pep*A, *pep*B and *pep*E genes, respectively. The primers numbered 1 are the upstream primers and the primers numbered 2 are the downstream primers (located within the DNA fragment that is deleted in the disruption construct). The ARG5 and the ARG6 primers are located at the borders of the *A. nidulans arg*B fragment (Fig. 7.2ABC).

Northern analysis.

Cultures. Spores from the three single disruptants, NW157, NW158 and NW159, were inoculated in 250 ml CM supplemented with 10 mM NH_4Cl and 2% glucose (w/v). The cultures were incubated in an orbital shaker at 30°C for 20 h at 220 rpm. Mycelia were harvested by gentle vacuum filtration, washed briefly, re-harvested, divided into three portions of which one was frozen in liquid nitrogen. The two others were transferred to 70 ml volumes of (A) Induction Medium (IM) containing elastin and BSA to induce protease expression in the absence of any repressing carbon and nitrogen source, and (B) Glucose Repression Medium (GRM) containing glucose to impose carbon catabolite repression on the extracellular proteases. After their transfer, the cultures were incubated for a further period of 6 h under the same conditions as before, mycelia were recovered by vacuum filtration and frozen immediately in liquid nitrogen.

RNA extraction and hybridization. 0.5 g amounts of frozen mycelium were pulverised for 2 min in a Braun dismembrator. RNA was extracted using the TRIzol method (GibcoBRL). RNA was run on formaldehyde containing gels loaded with 10 μg per track as described by Sambrook et al. (1989). After electrophoresis, RNA was transferred to Nylon membranes by capillary elution in 10 x SSC. Pre-hybridization and hybridization were performed at 42°C in hybridization buffer containing 50% (w/v) formamide / 0.75 M NaCl / 50 mM NaH_2PO_4 (pH 7.4) / 10 mM EDTA / 2 x Denhardt's / 0.1% (w/v) SDS / 10% (w/v) dextran sulphate. Northern blots were washed at 65°C in 0.2 x SSC / 0.1% (w/v) SDS. All blots were autoradiographed wet to facilitate stripping and re-probing, which was necessary as sizes of the hybridizing mRNAs were all in the 1.3-1.8 kb range.

DNA probes. DNA fragments were labelled with ^{32}P using the random priming method (Sambrook et al., 1989). *pep*A: a 0.49 kb *Cla*I-*Hin*dIII fragment from plasmid ptz18A400, which contains the complete *A. niger* Aspergillopepsin A gene; *pep*B: a 0.73 kb *Bgl*II-*Sal*I fragment encoding the 5' part of the *A. niger pep*B gene; *pep*C: 0.56 kb *Hpa*I-*Hin*dIII fragment from plasmid pANB1 (Frederick et al., 1993); *pep*E: 0.5 kb *Eco*RI fragment from plasmid pIM623, containing the complete *pep*E gene; *A. nidulans arg*B probes were all derived from plasmid pIJ16 (Johnstone et al., 1985): complete *arg*B probe: 2.8 kb *Pst*I-*Eco*RI fragment from pIJ16; 5'*arg*B probe: 0.48 kb *Hin*dIII fragment; 3'*arg*B probe: 0.7 kb *Sal*I-*Hin*dIII fragment; *cpy*: a 2.0 kb PCR fragment containing the complete *A. niger cpy* coding region as described by Fraissinet-Tachet et al. unpublished results); 28S rDNA probe: the 0.9 *Eco*RI fragment from the *Agaricus bisporus* 28S rDNA gene (Acc. No. X91812).

Genetic analysis. The procedures for genetic analysis have been described previously (Bos et al., 1988): after isolation of heterozygous somatic diploids from the disruptants and tester strains (as described in Fig. 7.4) haploidization was induced via addition of benomyl to the growth medium. Haploid segregants were isolated, purified and tested for genetic markers and protease expression.

Biochemical analysis. The selected disruptants and wild type *A. niger* N402 were grown on medium optimized for protease production as described by van den Hombergh et al. (1995). Media and mycelium samples were collected after 72 h of growth and frozen at -70°C. Part of the mycelium was freeze-dried to compare growth of individual strains by dry weight measurements. Furthermore, disruptants were grown on CM and mycelium was harvested after 20 h. Cell free extracts were prepared from mycelium from both cultures.

Enzyme assays. Both in culture supernatants and in cell free extracts proteolytic activities were measured using BSA (Boehringer, fraction V) as a substrate (van den Hombergh et al., 1995). CPY activity was measured using modified coupled peptidase assay as described by Stevens et al., (1986). Protein concentrations in cell-free extracts were determined with the Bicinchonic-acid protein kit (Sigma) according to the suppliers instructions and using BSA (Boehringer, fraction V) as a standard.

Western analysis. Cleared supernatants from cultures optimized for protease expression and cell free extracts from CM-grown mycelium were subjected to SDS-polyacrylamide gelelectrophoresis (PAGE), the proteins were electroblotted onto nitrocellulose membranes (Probind, Pharmacia) and incubated with polyclonal antibodies raised against purified *A. niger* PEPA and recombinant PEPE, produced in *E. coli* (van den Hombergh et al., unpublished results). Following incubation with alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma), protein bands were visualized following the recommendations supplied with the alkaline phosphatase kit (Bio-rad).

In vitro protein-degradation experiments. Proteolytic degradation in cleared supernatants of *Aspergillus* cultures was quantified using *A. niger* PELB as a tester protein according to van den Hombergh et al. (1995).

Results

Disruption of *pep*A, *pep*B and *pep*E

The disruption plasmids pIM644 (*pep*A), pIM645 (*pep*B) and pIM646 (*pep*E) were all designed to function as a one-step gene-disruption vector as described in Fig. 7.1. All constructs contained the protease genes disrupted via partial (*pep*A and *pep*E) or complete deletion (*pep*B) of the coding region and insertion of the *A. nidulans arg*B gene (Johnstone et al., 1985). The deletions in the protease coding regions of *pep*A and *pep*E removed essential active site amino acid residues (D_{281}, *pep*A and D_{287}, *pep*E), ensuring inactivation of the protease locus after integration of the construct at the homologous locus. Transformation of strain N647 with the 9.4 kb *Eco*RI Δ*pep*A fragment of pIM644, the 5.2 kb *Not*I Δ*pep*B fragment of pIM645 and the 7.9 kb Δ*pep*E *Bam*HI fragment of pIM646 generated $argB^+$ transformants which were purified on selection plates (MM without arginine). After purification, the *arg*B^+ transformants were grown in 2 ml cultures. Mycelia were freeze dried, DNA was extracted in a quick small scale procedure and used in three primer PCR reactions. The upstream primer was chosen in the 5' region of the disruption construct and the two downstream primers were located in the *arg*B gene and in the protease gene fragment that is deleted in the disruption construct, respectively (as shown in Fig. 7.2). This PCR approach enabled the direct selection of a putative disruptant as only the upstream primer and the *arg*B primer result in a (disruption) PCR fragment whereas integration at a heterologous locus leaves the protease gene intact and thus results in an additional wild type PCR fragment. Using this PCR method two out of five N647/Δ*pep*A transformants, one out of twenty N647/Δ*pep*B transformants and two out of ten N647/Δ*pep*E transformants were selected as putative disruptants for the *pep*A, *pep*B and *pep*E genes, respectively (data not shown).

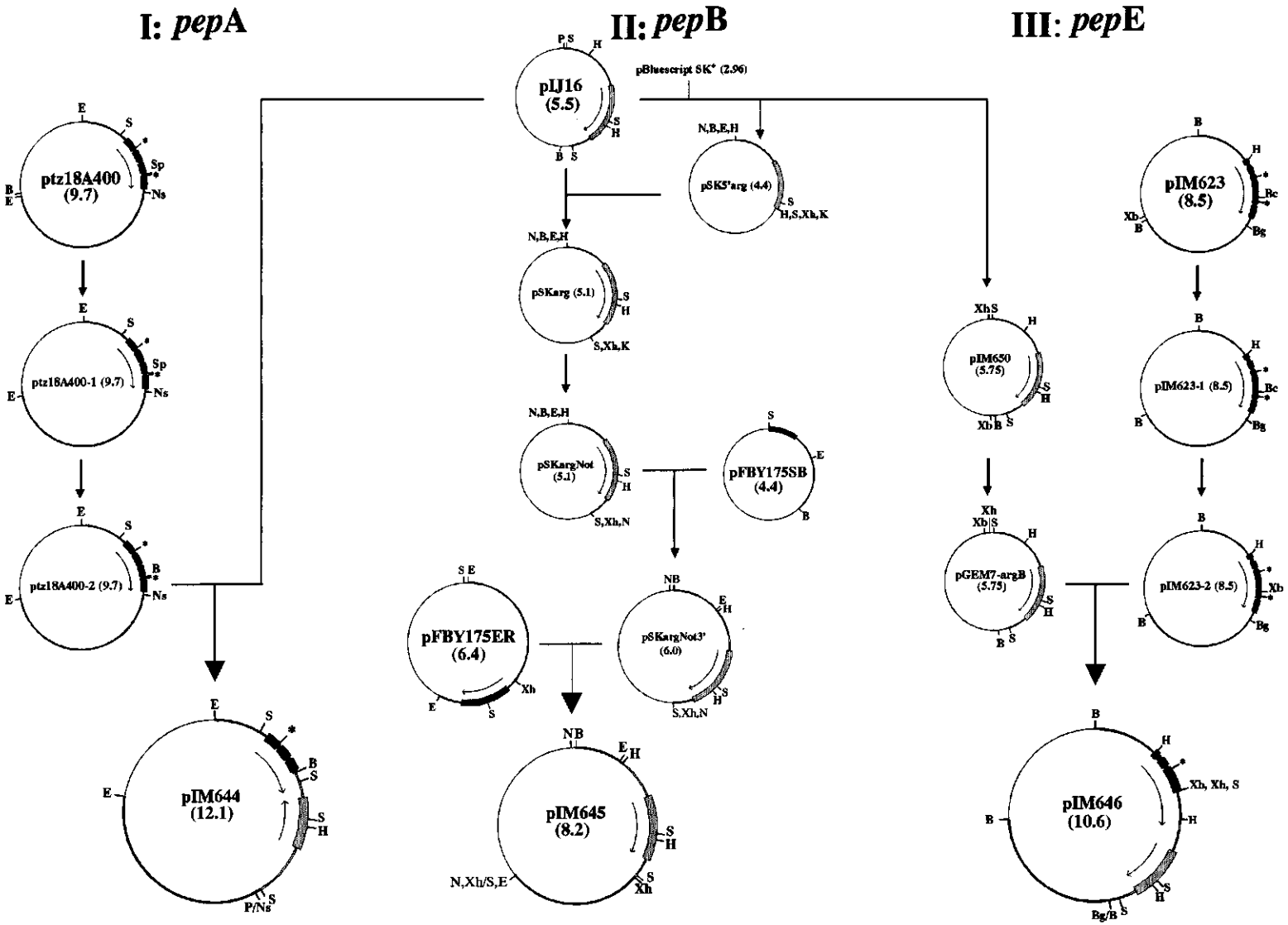

I: pepA
II: pepB
III: pepE
pIJ16 (5.5)
pBluescript SK+ (2.96)
ptz18A400 (9.7)
ptz18A400-1 (9.7)
ptz18A400-2 (9.7)
pSKS'arg (4.4)
pSKarg (5.1)
pSKargNot (5.1)
pFBY175SB (4.4)
pFBY175ER (6.4)
pSKargNot3' (6.0)
pIM644 (12.1)
pIM645 (8.2)
pIM650 (5.75)
pGEM7-argB (5.75)
pIM623 (8.5)
pIM623-1 (8.5)
pIM623-2 (8.5)
pIM646 (10.6)

Fig. 7.1. Construction of the disruption plasmids. Coding regions of protease genes are indicated in black boxes; the *A. nidulans arg*B gene is indicated with a grey box; constrictions in the coding regions indicate the positions of the introns; arrows indicate the orientation of transcription. (I) construction of pIM644 (*pep*A disruption construct). Plasmid ptz18a400, containing a 7.1 kb *Eco*RI insert on which the *A. niger pep*A gene is located was digested with *Bam*HI. After fill-in of cohesive ends, the linear plasmid was religated (plasmid ptz18a400-1) in order to remove the *Bam*HI site. Digestion of ptz18a400-1 with *Spe*I and religation in the presence of a specific 10-mer oligo removed the *Spe*I restriction site and introduced a *Bam*HI restriction site, resulting in plasmid ptz18a400-2. Plasmid ptz18a400-2 was digested with *Bam*HI and *Nsi*I, thus removing one of the two active site Asp residues from the *pep*A gene. Finally, the 2.7 kb *A. nidulans arg*B containing *Bam*HI-*Pst*I fragment was ligated into the 6.7 kb *Bam*HI-*Nsi*I fragment from ptz18a400-2, resulting in plasmid pIM644. pIM644 (12.1 kb) was digested with *Eco*RI to generate the 9.4 kb Δ*pep*A disruption fragment which was used for transformation. (II) construction of pIM645 (*pep*B disruption construct). The 1.4 kb *Hind*III fragment from plasmid pIJ16 was subcloned into pBluescript SK$^+$, resulting in plasmid pSK5'*arg*. After digestion with *Sal*I, the 0.8 kb *Sal*I fragment of pIJ16 was inserted into plasmid pSK5'*arg*, generating pSK*arg*, containing a functional *A. nidulans arg*B gene. With a specific oligomer the *Kpn*I restriction site in pSKarg was changed into a *Not*I restriction site. The 0.9 kb *Bam*HI-*Eco*RI fragment from pFBY175SB (containing the 3' flanking region of *pep*B) was inserted in pSKargNot to generate plasmid pSKargNot'. Finally, the 2.2 kb *Xho*I-*Sal*I fragment from pFBY175ER (containing the 5' flanking region of *pep*B) is inserted in XhoI-digested pSKargNot3' to generate the disruption plasmid pIM645. After *Not*I digestion the linear 5.2 kb Δ*pep*B disruption fragment was used for transformation. (III) construction of pIM646 (*pep*E disruption construct). Plasmid pIM623, containing a 5.9 kb *Bam*HI insert on which the *pep*E gene is located, was digested with *Xba*I and, after fill in of cohesive ends, religated to generate plasmid pIM623-1 in which the the *Xba*I (polylinker-) restriction site is removed. Plasmid pIM623-1 was digested with *Bcl*I and religated with a specific 10-mer oligo containing an *Xba*I restriction site. In the resulting plasmid (pIM623-2) the *Bcl*I restriction site was replaced with a *Xba*I restriction site. pIM650, containing the *A. nidulans arg*B gene, was digested with *Bam*HI and *Xho*I and the 2.7 kb *arg*B containing fragment was subcloned into *Bam*HI and *Xho*I digested pGEM7 (Promega), resulting in plasmid pGEM7-*arg*B. Finally, the 2.7 kb *Bam*HI-*Xba*I *arg*B containing fragment from pGEM7-*arg*B was subcloned into *Xba*I-*Bgl*II digested pIM623-2 (thus removing the 0.7 kb *Xba*I-*Bgl*II fragment, which contains the second active site Asp residue). The resulting 10.6 kb plasmid (pIM646) was digested with *Bam*HI and the 7.9 kb Δ*pep*E disruption fragment was used for transformation.

All other transformants tested resulted in PCR fragments consistent with an ectopic heterologous integration pattern of the disruption constructs. Genomic DNA was isolated from transformants N647/pIM644-1, N647/pIM645-3 and N647/pIM646-2 and restriction enzyme digested, electrophoresed and Southern blotted. The N647-pIM644-1 DNA containing Southern blot was probed with the 2.4 kb *Eco*RI-*Spe*I *pep*A fragment and, after stripping, reprobed with the 2.7 kb *Bam*HI-*Pst*I *A. nidulans arg*B fragment from pIJ16. As shown in Fig. 7.2 the hybridization patterns of the transformant selected in the PCR screening is consistent with a correct replacement of the homologous *pep*A locus by the disruption construct. Similar Southern blot hybridizations confirmed disruption of the *pep*B and *pep*E locus in N647/pIM645-3 and N647/pIM646-2, respectively (data not shown).

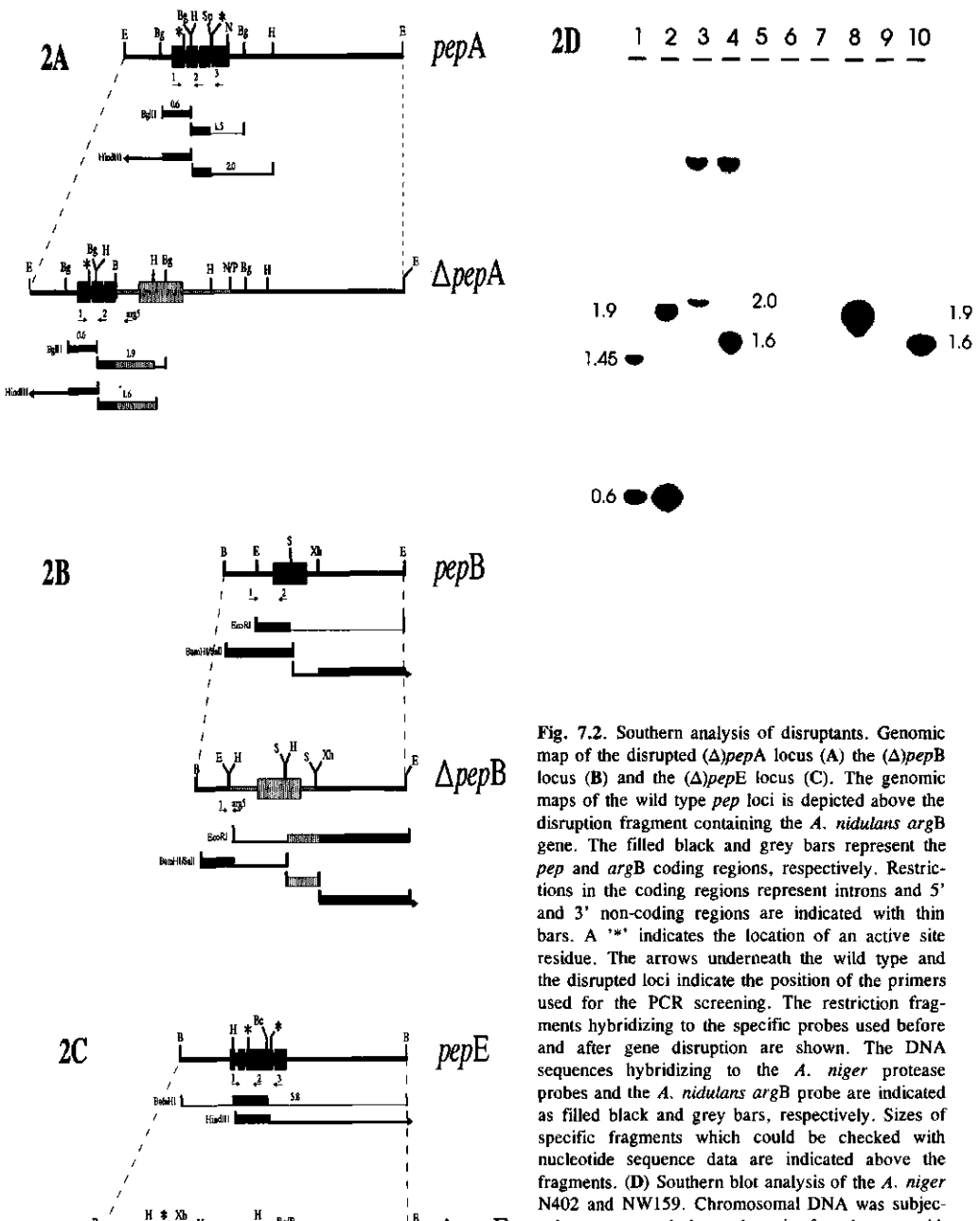


Fig. 7.2. Southern analysis of disruptants. Genomic map of the disrupted (Δ)*pep*A locus (**A**) the (Δ)*pep*B locus (**B**) and the (Δ)*pep*E locus (**C**). The genomic maps of the wild type *pep* loci is depicted above the disruption fragment containing the *A. nidulans arg*B gene. The filled black and grey bars represent the *pep* and *arg*B coding regions, respectively. Restrictions in the coding regions represent introns and 5' and 3' non-coding regions are indicated with thin bars. A '*' indicates the location of an active site residue. The arrows underneath the wild type and the disrupted loci indicate the position of the primers used for the PCR screening. The restriction fragments hybridizing to the specific probes used before and after gene disruption are shown. The DNA sequences hybridizing to the *A. niger* protease probes and the *A. nidulans arg*B probe are indicated as filled black and grey bars, respectively. Sizes of specific fragments which could be checked with nucleotide sequence data are indicated above the fragments. (**D**) Southern blot analysis of the *A. niger* N402 and NW159. Chromosomal DNA was subjected to agarose gel electrophoresis after cleavage with *Bgl*II or *Hin*dIII and Southern blotted. The filters were hybridized with the labelled 1.2 kb *Sal*I-*Spe*I fragment of *pep*A (lanes 1-4) and the 0.9 kb *Bam*HI-*Hin*dIII fragment from *A. nidulans arg*B (7-10). Track order: 1,7) N402, *Bgl*II; 2,8) NW157, *Bgl*II; 3,9) N402, *Hin*dIII; 4,10) NW157, *Hin*dIII.

Northern analysis of disruptants

The disruptants shown to have resulted from a single copy integration at their respective homologous loci were studied via Northern analyses. Three disruptants (Δ*pep*A, Δ*pep*B and Δ*pep*E) and a wild type strain N402 were pre-grown on CM containing 10 mM NH_4Cl, 2% (w/v) glucose and appropriate supplements. After o/n growth the cultures were harvested, washed and aliquots of mycelium were grown for another 6 h in IM to determine whether the disrupted protease loci produced stable mRNAs, and in GRM to study whether the protease mRNAs were still regulated at the transcriptional level compared to wild type. After harvesting, the mycelia were frozen in liquid nitrogen, pulverised and total RNA was isolated. Total RNA was electrophoresed in formaldehyde containing gels and blotted to Hybond N^+ filters. These Northern blots were hybridized with the *pep*A, *pep*B and *pep*E probes, respectively (Fig. 7.3). Clearly, the Δ*pep*B produces no *pep*B mRNA (the complete coding region is deleted from the *pep*B disruptant, as shown in Fig. 7.2), whereas the Δ*pep*A and Δ*pep*E loci produce stable mRNAs of 1.4 and 1.6 kb, repectively. Using the *pep*A and the *pep*E fragments which are deleted from the Δ*pep*A and the Δ*pep*E disruption constructs, no hybridization was observed in the corresponding disruption strains, demonstrating the correct 3' truncation of the *pep*A and *pep*E genes, respectively (data not shown). Both the Δ*pep*A and the Δ*pep*E disruption loci produce a hybrid mRNA as in the disruption strains both the specific protease probes and the 2.7 kb *A. nidulans arg*B probe encompassing the complete coding region, hybridize to the mRNA produced by the disrupted locus. Using specific fragments of the *A. nidulans arg*B gene as probes enables specific detection of either the Δ*pep*A or the Δ*pep*E locus by virtue of its *arg*B containing fragment since the transcription of the hybrid messenger proceeds into either the 5' (Δ*pep*A) or the 3' (Δ*pep*E) region of *A. nidulans arg*B (Fig. 7.3).

Both the Δ*pep*A and Δ*pep*E hybrid mRNAs appear to be regulated similarly as the wild-type mRNAs (Jarai and Buxton, 1994). (Δ)*pep*A mRNA levels drop significantly in the presence of ammonium and glucose due to *are*A and *cre*A mediated repression whereas de-repression and induction with protein results in strong increase of transcription. Wild type *pep*E expression is constitutive (Jarai et al., 1994) as is the expression of the hybrid Δ*pep*E mRNA. Disruption of protease *pep*A or *pep*B has no effect upon the transcription of other extracellular proteases as wild type expression is observed for all extracellular proteases tested (data not shown). Disruption of the *pep*E protease also had no effects on *pep*C and *cpy* transcript levels, of which the gene products are postulated to be cascade activated by PEPE (*vide infra*). As control the blots were probed with the *Agaricus bisporus* 28S rDNA probe, demonstrating that equal amounts of RNA were loaded.

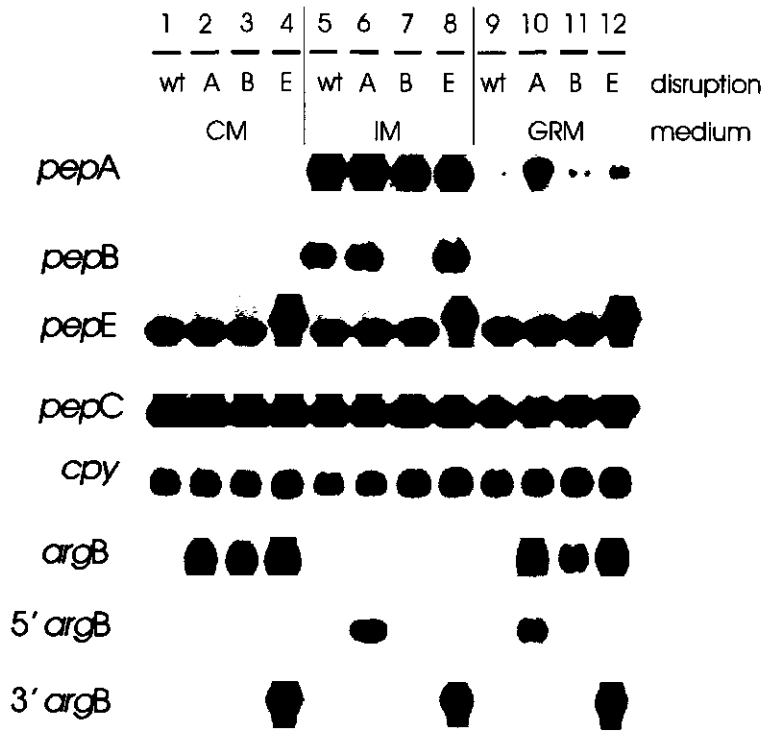


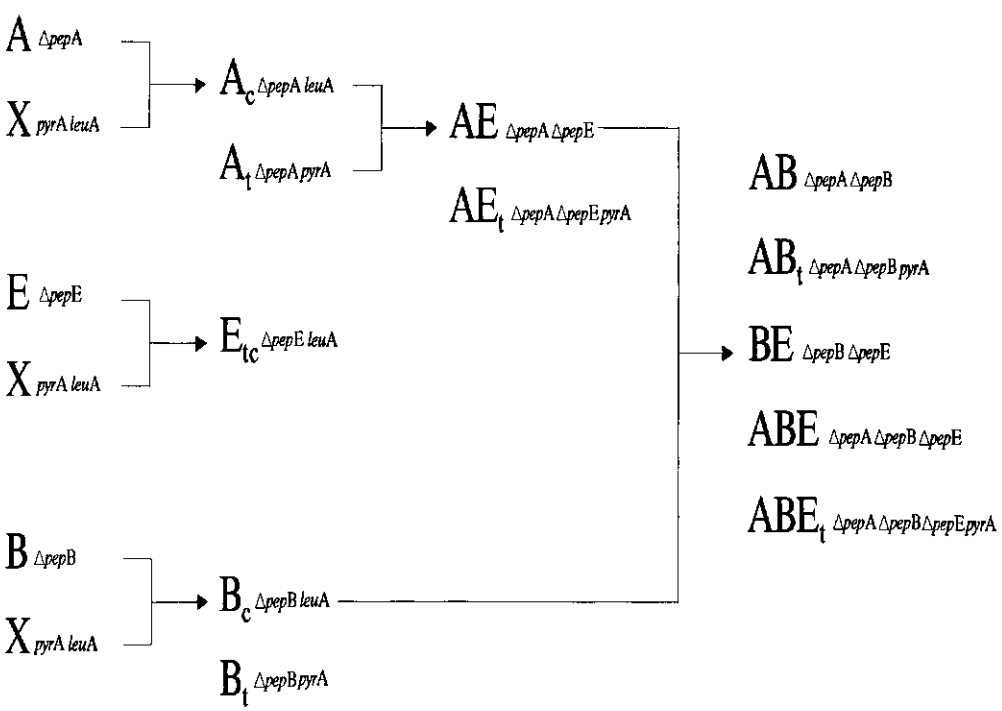
Fig. 7.3. Northern analyses of protease expression in protease disruptants. Total RNA was isolated from wild type strain N402 and disruptants NW157, NW158 and NW159 after transfer to either Induction Medium (IM) or to Glucose Repression Medium (GRM). Track order: 1, N402 (prior to transfer); 2, NW157 (prior to transfer); 3, NW158 (prior to transfer); 4, NW159 (prior to transfer); 5, N402 (IM); 6, NW157 (IM); 7, NW158 (IM); 8, NW159 (IM); 9, N402 (GRM); 10, NW157 (GRM); 11, NW158 (GRM); 12, NW159 (GRM); (*pep*A) Northern blot probed with the *A. niger pep*A probe. (*pep*B) Northern blot probed with the *A. niger pep*B probe. (*pep*E) Northern blot probed with the *A. niger pep*E probe. (*pep*C) Northern blot probed with the *A. niger pep*C probe. (*cpy*) Northern blot probed with the *cpy* probe. (*arg*B) Northern blot probed with the *arg*B probe. (5'*arg*B) Northern blot probed with the *A. nidulans* 5'*arg*B probe (3'argB) Northern blot probed with *A. nidulans* 3'*arg*B probe.

Clearly, integration of the disruption constructs at their homologous locus is either correlated with the disappearance of the mRNA (*pep*B) or with expression of a hybrid mRNA (*pep*A and *pep*E) as shown by hybridization with an *A. nidulans arg*B probe. Transformants N647/pIM644-1, N647/pIM645-3 and N647/pIM646-2, henceforth designated NW157 (Δ*pep*A), NW158 (Δ*pep*B) and NW159 (Δ*pep*E), were chosen for further analyses. *A. nidulans arg*B expression also appears to be regulated in *A. niger* as expression is high in glucose and ammonium containing medium whereas expression is low in medium lacking both glucose and ammonium and also in the presence of protein.

Controlled construction of double and triple protease disruptants

From allocation experiments by Verdoes et al. (1994) it was known that in *A. niger* the *pep*A gene is located on linkage group I. In CHEF-Southern experiments the *A. niger pep*B and *pep*E genes have been allocated to linkage group II and IV, respectively (van den Hombergh et al., unpublished results). The genomic location on three different linkage groups of the disrupted proteases enabled recombination strategies as shown in Fig. 7.4, to combine the individually disrupted protease loci within one expression strain. As the acid proteases were disrupted in an identical genetic background to enable direct comparisons of the phenotypic effects, recombinants had to be generated which could be used in crosses to generate double mutants. All the disruptants were crossed with the NW133 (*gox*C17, *pyr*A6, *leu*A1) strain and a *fwn*A6, Δ*pep*A, *leu*A1 ($\mathbf{A}_c$, Fig. 7.4A), a Δ*pep*E, *pyr*A6 ($\mathbf{E}_c$, Fig. 7.4A) and a *leu*A1, Δ*pep*B ($\mathbf{B}_c$, Fig. 7.4A) were isolated. A cross between strains $\mathbf{A}_c$ and $\mathbf{E}_c$ resulted in isolation of the Δ*pep*A, Δ*pep*E double disruptant (**AE**) from which, in a subsequent cross with strain $\mathbf{B}_c$, the two other double disruptants (**AB**: Δ*pep*A, Δ*pep*B; **BE**: Δ*pep*B, Δ*pep*E) and the triple disruptant **ABE** (Δ*pep*A, Δ*pep*B, Δ*pep*E) were isolated.

A



B

Strain	Code in Fig **4A**	Linkage groups							
		I	II	III	IV	V	VI	VII	VIII
NW157 (Δ*pep*A)	**A**	*fwn*A6 Δ*pep*A *arg*B$^+$				*nic*A1 *arg*B13			
NW160 (Δ*pep*A)	**A$_t$**	*fwn*A6 Δ*pep*A *arg*B$^+$		*pyr*A6	*leu*A1	*nic*A1 *arg*B13			
NW158 (Δ*pep*B)	**B**	*fwn*A6	Δ*pep*B *arg*B$^+$			*nic*A1 *arg*B13			
NW163 (Δ*pep*B)	**B$_t$**		Δ*pep*B *arg*B$^+$	*pyr*A6	*leu*A1	*nic*A1 *arg*B13			
NW159 (Δ*pep*E)	**E**	*fwn*A6			Δ*pep*E *arg*B$^+$	*nic*A1 *arg*B13			
NW133	**X**		*gox*C17	*pyr*A6	*leu*A1				
NW161 (Δ*pep*A)	**A$_c$**	*fwn*A6 Δ*pep*A *arg*B$^+$	*gox*C17		*leu*A1	*nic*A1 *arg*B13			
NW164 (Δ*pep*E)	**E$_{tc}$**			*pyr*A6	Δ*pep*E *arg*B$^+$	*nic*A1 *arg*B13			
NW162 (Δ*pep*B)	**B$_c$**		Δ*pep*B *arg*B$^+$		*leu*A1	*nic*A1 *arg*B13			
NW166 (Δ*pep*A Δ*pep*E)	**AE**	*fwn*A6 Δ*pep*A *arg*B$^+$			Δ*pep*E *arg*B$^+$	*nic*A1 *arg*B13			
NW165 (Δ*pep*A Δ*pep*E)	**AE$_t$**	*fwn*A6 Δ*pep*A *arg*B$^+$		*pyr*A6	Δ*pep*E *arg*B$^+$	*nic*A1 *arg*B13			
NW168 (Δ*pep*A Δ*pep*E)	**AB**	*fwn*A6 Δ*pep*A *arg*B$^+$	Δ*pep*B *arg*B$^+$			*nic*A1 *arg*B13			
NW167 (Δ*pep*A Δ*pep*E)	**AB$_t$**	*fwn*A6 Δ*pep*A *arg*B$^+$	Δ*pep*B *arg*B$^+$	*pyr*A6		*nic*A1 *arg*B13			
NW170 (Δ*pep*B Δ*pep*E)	**BE**		Δ*pep*B *arg*B$^+$		Δ*pep*E *arg*B$^+$	*nic*A1 *arg*B13			
NW172 (Δ*pep*A Δ*pep*B Δ*pep*E)	**ABE**	*fwn*A6 Δ*pep*A *arg*B$^+$	Δ*pep*B *arg*B$^+$		Δ*pep*E *arg*B$^+$	*nic*A1 *arg*B13			
NW171 (Δ*pep*A Δ*pep*B Δ*pep*E)	**ABE$_t$**	*fwn*A6 Δ*pep*A *arg*B$^+$	Δ*pep*B *arg*B$^+$	*pyr*A6	Δ*pep*E *arg*B$^+$	*nic*A1 *arg*B13			

Fig. 7.4. The controlled construction of *A. niger* disruption strains. **(A)** Schematic representation of the individual crosses which resulted in the isolation of the double and triple disruptants. **(B)** List of the genotypes of the individual disruptants generated via recombination. [1], allocation of *pep*A to linkage group I was concluded from the 3.4 % recombination frequency between the *fwn*A6 and the *arg*B$^+$ marker; [2], allocation of *pep*B to linkage group II was concluded from the 5.0 % recombination frequency between the *gox*C17 and the *arg*B$^+$ marker. [3], allocation of *pep*E to linkage group IV was concluded from the 0 % recombination frequency between the *leu*A1 and the *arg*B$^+$ marker; c, isolated recombinant strain used in the controlled construction; t, transformable strain; tc, transformable recombinant strain used in the controlled construction.

Western analysis and enzyme assays of disruptants

The selected disruptants were grown on medium optimized for protease expression to induce the complete residual protease spectrum. Medium samples were taken after approximately three days of growth. The disruptants were also grown on CM and after one day mycelium was harvested and used to prepare cell free extracts. Both the medium samples and cell free extracts derived from CM-grown cultures were analysed on SDS PAGE gels, Western blotted and specific proteases were visualized with polyclonal antibodies raised against purified *A. niger* PEPA and (recombinant) PEPE (unpublished results). The *pep*E gene is not repressed on CM, in contrast to *pep*A and *pep*B. As PEPA and PEPE are both aspergillopepsins the repression of *pep*A expression on CM will prevent any putative problems of cross-reactivity between the antibodies raised against PEPE and the PEPA protease. In Fig. 7.5 it is shown that the NW157 (Δ*pep*A) and NW159 (Δ*pep*E) single disruptants produce no PEPA and PEPE protease, respectively. Expression of non-disrupted proteases in these disruptants is not affected.

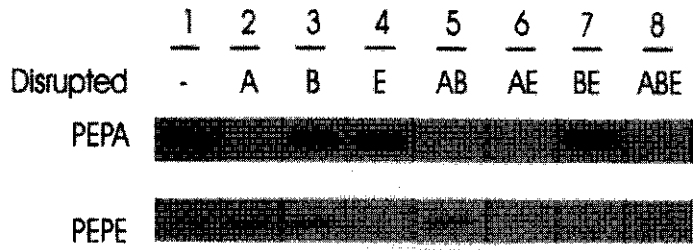


Fig. 7.5. Western analysis of protease disruptants. Strains were grown both on medium optimized for protease expression and on CM. Presence of protease PEPA in cleared 72 h supernatants of cultures (cs) optimized for protease expression and in cell free extracts (cfe) generated from mycelium harvested from 20 h CM cultures was visualized after SDS PAGE using specific polyclonal antibodies. Track order: 1, N402 (wild type); 2, NW157 (Δ*pep*A); 3, NW158 (Δ*pep*B); 4, NW159 (Δ*pep*E); 5, NW168 (Δ*pep*A, Δ*pep*B); 6, NW166 (Δ*pep*A, Δ*pep*E); 7, NW170 (Δ*pep*B, Δ*pep*E); 8, NW172 (Δ*pep*A, *pep*B, Δ*pep*E).

Sequencing the disruption fragments showed that both the Δ*pep*A and Δ*pep*E mRNAs should result upon translation in C-terminally truncated hybrid proteins, ΔPEPA and ΔPEPE. Probably because of their aspecific 'tail', ΔPEPA and ΔPEPE are very unstable proteins, which are readily degraded. Furthermore, the Western analysis results for the double and triple disruptants correspond with the expected phenotypes for the individual disruptants.

The culture supernatants were also used to measure residual proteolytic activities compared to wild type (Fig. 7.6). The overall acid protease activities in the supernatants were reduced down to 16% and 94% for the Δ*pep*A and Δ*pep*B disruptants, respectively (Fig. 7.6A). Interestingly, also for the Δ*pep*E disruptant a small but reproducible reduction (approx. 3% compared to wild type) was observed in the culture supernatant (Fig. 7.6A). For the Δ*pep*A and Δ*pep*B disruptants no significant changes in intracellular proteolytic activities were observed, whereas the protease activity in the Δ*pep*E cell free extract was reduced to 32% of the level of N402. To test whether a vacuolar PEPE initiated cascade activation mechanism is present in *A. niger*, the overall PMSF-inhibitable activity and serine carboxypeptidase activities were measured in the Δ*pep*E strain. Both the PMSF-inhibitable activity (Fig. 7.6C) and the serine carboxypeptidase activity (data not shown) were significantly reduced, demonstrating that also serine type activity and serine carboxypeptidase activity was affected and thus indicating the presence of a (vacuolar) cascade activation mechanism in which protease PEPE is involved. The extracellular activities in both the Δ*pep*A-Δ*pep*B and the Δ*pep*A-Δ*pep*E double disruptants appear to be reduced compared to the Δ*pep*A single disruptant, indicative for an effect of both the Δ*pep*B and the Δ*pep*E disruption on extracellular degradation. Combining all three disruptions in the triple disruptant results in a further small reduction compared to the double Δ*pep*A-Δ*pep*B disruptant (Fig. 7.6D). Intracellular activities in double and triple disruptants were similar as observed in wild type or the Δ*pep*E, depending on whether the strain carried a disrupted *pep*E locus or not (data not shown).

Improved stability of target proteins in disruptants

Recently, we have described the use of the homologous *A. niger* pectin lyase B (PELB) protein as a sensitive *in vitro* target protein to quantify the reduced proteolytic activities in culture supernatants of protease (*prt*) deficient mutants (van den Hombergh et al., 1995). Using this *in vitro* system, the degradation of PELB in culture supernatants of the single, double and triple mutants was quantified (Table 7.1). Clearly it is shown that in addition to the considerably reduced degradation in the Δ*pep*A strain, also in the Δ*pep*B and Δ*pep*E disruptants small but significant reduction of PELB inactivation is observed. Combination of the individual disruptions results in an

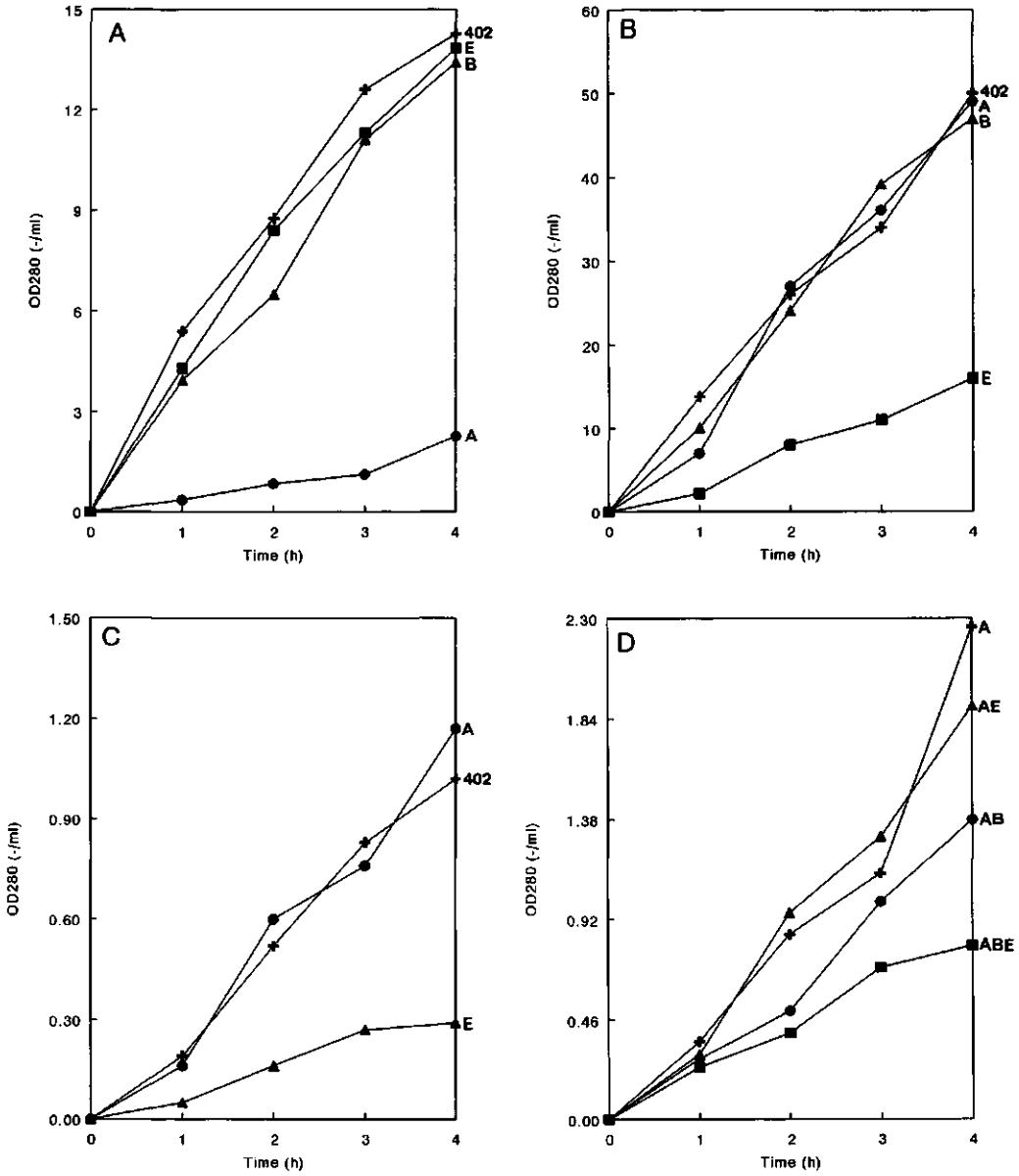


Fig. 7.6. Residual proteolytic activities (at pH 4.0) in protease disruptants. Activities are expressed as the OD_{280} change per h per 1 ml culture supernatant (extracellular activities) or per mg protein (intracellular activities). (**A**) Total extracellular protease activities in cleared supernatants of +, *A. niger* N402; ●, NW157 (Δ*pep*A); ▲, NW158 (Δ*pep*B); ■, NW159 (Δ*pep*E); (**B**) Total protease activities (at pH 4.0) in cell free extracts of +, *A. niger* N402; ●, NW157 (Δ*pep*A); ▲, NW158 (Δ*pep*B); ■, NW159 (Δ*pep*E); (**C**) Total PMSF inhibitable activities (at pH 6.0) in cell free extracts of +, N402; ●, NW157 (Δ*pep*A); ▲, NW159 (Δ*pep*E). PMSF-inhibitable activities were calculated from the difference between inhibited and non-inhibited enzyme assays. (**D**) Extracellular protease activities in cleared supernatants of +, *A. niger* NW157 (Δ*pep*A); ●, NW168 (Δ*pep*A, Δ*pep*B); ▲, NW166 (Δ*pep*A, Δ*pep*E); ■, NW172 (Δ*pep*A, Δ*pep*B, Δ*pep*E).

additional improvement as shown by a further reduction of PELB inactivation. Finally, in the triple disruptant the proteolytic inactivation of PELB is reduced with a factor 100-250.

Table 1. PELB degradation in culture supernatants of disruptants

Strain	Disruption	Residual proteolytic activity[1]	Residual PELB activity[2] dilution factor							
			cont.	1	2	10	50	100	500	1000
N402	-	100	**98**	0	0.3	1.2	7	12	49	**91**
NW157	Δ*pep*A	16	**99**	0.6	2	8	49	**91**	**103**	**100**
NW158	Δ*pep*B	94	**100**	0.2	0.5	3	15	23	82	**102**
NW159	Δ*pep*E	97	**97**	0.2	0.7	4	17	28	88	**97**
NW165	Δ*pep*A Δ*pep*B	9	**102**	7	15	70	**96**	**98**	**96**	**103**
NW168	Δ*pep*A Δ*pep*E	16	**97**	0	3	10	61	**100**	**97**	**106**
NW170	Δ*pep*B Δ*pep*E	92	**103**	0	1	5	18	30	**92**	**100**
NW172	Δ*pep*A Δ*pep*B Δ*pep*E	7	**98**	14	32	**92**	**101**	**106**	**95**	**102**

[1], percentage residual proteolytic activity against BSA at pH 4.0 compared to wild type. [2], incubations of serial dilutions with PELB protein. The PELB activity was measured and expressed as the percentage residual acitvity compared to the control. Non-affected activities are indicated in bold; cont, as a control an undiluted medium sample was boiled prior to incubation with PELB

To allow isolation of transformable multiple disruptants, an *A. niger* (*pyr*A6, *leu*A1) strain was chosen for the initial crosses. As demonstrated in Fig 7.4, this has resulted for two out of the three double disruptants (NW165 and NW167) and for the triple disruptant (NW171) in the isolation of *pyr*A6-containing recombinants. These transformable disruptants will allow co-transformation of a plasmid containing the *pkipel*B expression cassette (Kusters-van Someren et al., 1992) and plasmid pGW635, containing the *A. niger pyr*A gene (Goosen et al., 1987), as the selection marker. This expression cassette, in which the *A. niger pel*B gene is inserted between the *A. niger pki*A promoter and terminator, ensures high constitutive expression of *pel*B and thus enables *in vivo* analysis of reduced PELB degradation.

Discussion

In many microorganisms currently in use for production of proteins, improving production involved reducing the proteolytic degradation of the final products at some stage. Both classical and molecular genetic approaches have been shown to be very useful in generating protease reduced or deficient expression strains (Gottesman, 1990; He et al., 1991; Hinnen et al., 1994). In *A. niger* we have already shown that the classical genetic approach resulted in the isolation of at least seven *prt* (protease mutant) complementation groups (van den Hombergh et al., 1995). Proteolytic degradation in some of these mutants is reduced to 2% compared to wild type, however, extracellular protease activities are not completely absent. The molecular genetic approach to generate protease deficient strains described in this manuscript also proves to be a powerful tool in improving protein production in *A. niger*. Characterization of the extracellular protease spectrum in *A. niger* enabled cloning and disruption of those activities which were shown to interfere with high level protein production. Disruption of several of these interfering activities allowed *in vivo* testing whether these activities were involved in the observed proteolysis of specific target proteins and resulted in the controlled construction of (multiple) disruptant strains. Although mutagenesis has been shown to be very succesful with respect to reducing protease expression, repeated mutagenesis could lead to accumulation of background damage. Combining different disrupted protease loci within one strain avoids these problems. Additionally, specific disruption constructs can be used to eliminate residual activities from *prt* mutants to further reduce protein degradation.

Extracellular proteolysis was severely reduced in the Δ*pep*A strain and the reduction of extracellular proteolytic activity corresponds with the percentage of pepstatin inhibitable activity in *A. niger* wild type. Also for the Δ*pep*E disruptant the reduction of intracellular acid protease activity corresponds to the level of pepstatin inhibitable intracellular acid activity. Thus, the *pep*A and *pep*E genes encode the major extracellular and intracellular pepsin-type proteases in *A. niger*. Surprisingly, also in the Δ*pep*E a small reduction of overall extracellular proteolytic activity was also observed. This could indicate that similar to the situation described for *S. cerevisiae* CPY, a small portion of the PEPE protease is either mislocalized and secreted or appears in the culture medium upon cell lysis. As *A. niger* acidifies its medium very rapidly, it could well be that similar to the situation in the vacuole, the ProPEPE protease is autocatalytically activated upon secretion in the acidified medium, or another extracellular protease is involved in maturation. The Δ*pep*B disruption also reduces extracellular protease activities, and approximately 6% of total extracellular proteolytic activity appears to be PEPB.

Asuming that the PEPE protease is located in the vacuole (based on homologous with *S. cerevisiae*), the intracellular enzyme data may indicate a vacuolar cascade mechanism.

Apart from the possible conservation of vacuolar proteases and their cascade-like activation between *S. cerevisiae* and *Aspergillus*, also differences have been observed. In *S. cerevisiae* it has been shown by Wolf and Ehmann (1981) that several vacuolar proteases are needed for the differentiation process of sporulation. They most likely provide the amino acids at the expense of unneeded (vegetative) cell protein for new protein synthesis for the endospores to be formed. However it is also possible that vacuolar proteases are involved in activating other vacuolar enzymes needed for sporulation events. As *A. niger* does not produce endospores, it is not surprising that a relation with vacuolar proteases is absent. Furthermore, yscA activity in yeast appears to be necessary for cell survival under nitrogen starvation conditons. Whether this is also a secondary effect is unknown, but a similar effect is absent in *A. niger*. Vacuolar proteases are probably mainly involved in highly non-specific protein degradation, demonstrated by the Δ*pep*E disruptant in which the degradation of 'unspecific' substrates as BSA and casein is severely hampered. Thus, one is led to assume that in *A. niger* like in yeast these enzymes represent the major unspecific protease activities.

No severe effects on sporulation or growth were observed in the disruptants. However, complementation of the *arg*B13 mutation in the *A. niger* strain N647 with a single copy of the *A. nidulans arg*B gene did not render the resulting transformant completely arginine prototrophic; growth of single copy *arg*B$^+$ transformants was slightly reduced compared to arginine supplemented *A. niger* N647 and wild type. Similar effects were observed in the controls (N647 transformed with the *arg*B gene from *A. nidulans*), indicating that the effect was not caused by the disruption of a protease gene. Interestingly, the *A. nidulans arg*B gene appears to be regulated in *A. niger* and the observed low expression on protein rich medium could indicate repression as arginine can be generated from degradation of extracellular proteins.

For all three single disruptants the proteolytic degradation of the *A. niger* tester protein PELB was reduced and the reduction could be correlated with reduction of proteolytic activity in the supernatants. Apparently PEPA, PEPB and PEPE (present in the culture supernatant because of mislocalization and/or cell lysis) can all contribute to the observed rapid degradation of PELB in wild type culture fluids. In double and triple disruptants the degradation was further reduced, down to 7% for the triple disruptant. Definite proof that all three acid proteases are involved in the *in vivo* degradation of PELB during expression in wild type awaits the *in vivo* PELB degradation data in disruptants expressing the *pel*B gene.

In *S. cerevisiae* it has been shown that, apart from extracellular and (partially) mislocalized proteases, also some degradation can be caused by intracellular proteases. Part of this degradation was shown to be caused by proteases that are residing in the secretory pathway but also elimination of proteases which are transported through the secretory system resulted in higher expression levels (Hinnen et al., 1994). In *S. cerevisiae* the PEP4 protease is only active when the propeptide is removed (probably autocatalytically upon arrival in the more acidic environment of the vacuole). Similarly, one might speculate that the PEPE protein will not be active during routing through the secretory pathway and will therefore probably not be involved in degradation in the secretion route. Little is known about intracellular proteolysis in *A. niger* but the Δ*pep*E data demonstrates that it will be important to study intracellular proteases and especially those which are present in or travel through the secretory pathway. Detailed knowledge about intracellular proteolytic activities will allow a further controlled optimization of protein production either via cloning or modification of the genes coding for interfering intracellular proteases.

The present study demonstrates that gene disruption of protease genes interfering with high level expression and subsequent recombination allows controlled construction of improved expression strains. Combining disruptions and *prt* (protease) mutations will lead to a set of expression strains even more reduced in proteolytic degradation which can be used as a starting point to further optimize both homologous and heterologous gene expression.

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8 New protease mutants in *Aspergillus niger* result in strongly reduced *in vitro* degradation of target proteins; genetic and biochemical characterization of seven complementation groups[1]

J.P.T.W. van den Hombergh, P.J.I. van de Vondervoort, N.C.B.A. van der Heijden and J. Visser

Abstract

Several mutants of *Aspergillus niger*, deficient in extracellular protease expression, have been isolated and characterized both genetically and biochemically. The mutant strains, obtained after *in vivo* UV-mutagenesis of conidiospores and selected by halo-screening on a new dual-substrate plate assay, belong to at least seven different complementation groups. These seven *prt* loci were assigned to linkage groups using master strains with marked chromosomes. One *prt* locus (*prt*C) could be assigned to linkage group I, three (*prt*B, *prt*E and *prt*G) to linkage group III, one (*prt*F) to linkage group V and the two remaining (*prt*A and *prt*D) were assigned to linkage group VIII. Extracellular proteolytic activities varied from 2-3% up to 80% of the protease activity of the parental strain. Assigning the different *prt* mutants to structural or regulatory genes is difficult since only one structural gene, *pep*A, has been mapped unambiguously on linkage group I but is not identical to *prt*C. All *prt* mutants except for *prt*C are likely to be regulatory mutants or else belong to a proteolytic cascade because residual activities showed that more proteolytic activities were affected simultaneously. Double mutants were constructed both by recombination and by a second round of mutagenesis. In both cases mutants with further reduced extracellular proteolytic acitivities were isolated. A sensitive *in vitro* degradation assay, based on the homologous pectin lyase B (PELB) protein to analyze proteolytic degradation in *A. niger*, was developed and used to show extremely reduced proteolytic PELB degradation in the culture media of some of these mutants.

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Introduction

The ability of filamentous fungi of the genus *Aspergillus* to secrete large quantities of proteins, and the development of molecular genetic tools for these fungi, has resulted in the exploitation of this group of organisms as expression-secretion hosts for heterologous as well as homologous proteins (Berka et al., 1991). Several eukaryotic proteins of both therapeutic and technological interest have been expressed and secreted in *Aspergilli* (Upshall et al., 1987; Devchand and Gwynne, 1991; Contreras et al., 1991; Davies, 1994). The eukaryotic protein secretion process often involves a range of posttranslational modifications, such as proteolytic processing, disulphide bond formation and glycosylation, that are required to obtain the correct protein structure and biological activity. Many of these post-translational processes occur in *Aspergillus* (Devchand and Gwynne, 1991). Despite the high potential expression rates, yields of the target proteins vary considerably. One cause of low yields is proteolytic degradation of the target proteins by host proteases (Kusters-van Someren et al., 1992; Mackenzie et al., 1993). In some cases yields were improved by deleting the major aspartyl protease (*pep*A) of the host (Berka et al., 1990; Archer et al., 1992). Proteases also reduce the expression levels of homologous proteins and in some cases losses were reduced by the use of protease mutants (Hayashida and Flor, 1981; Flor and Hayashida, 1983; Fiedurek et al., 1985; Hayashida and Teramoto, 1986). Protease mutants isolated by Mattern et al., (1992) have also been used to improve expression of heterologous proteins in *A. niger* (Archer et al., 1992). They showed that mutations affecting both the extracellular proteases *pep*A and *pep*B from *A. niger* resulted in a considerable decrease of extracellular protease activity correlated with an improvement of heterologous protein production. However, residual proteolytic activities, causing degradation of various heterologous proteins, are still present within these mutants.

In this paper we describe the isolation of mutants from seven different protease complementation groups in *A. niger*. These mutants have been genetically and biochemically characterized respectively by complementation tests in diploids and by protease enzyme assays in the presence or absence of specific protease inhibitors.

Materials and methods

Strains. All *A. niger* strains used were derived from the wild-type strain N400 (CBS 120.49). The *prt* mutants which have reduced extracellular protease levels were obtained from the auxotrophic and morphological mutant N573 which is biotin deficient (*bio*A1) and has short conidiophores (*csp*A1). For genetic analysis we used NW136, carrying a colour marker and auxotrophic markers on different linkage groups, i.e.: *fwn*A1 (I), *arg*H12 (II), *pyr*A6, (III) *csp*A1 (III), *lys*A7 (III), *his*C3 (IV), *phe*A1 (V), *pdx*A2 (VI), *nic*B2 (VII), *trp*B2 (VIII) (Bos et al., 1988), as a master strain.

Media and growth conditions. *A. niger* was grown on complete medium (CM) and on supplemented minimal medium (SM) with a composition according to Pontecorvo et al. (1953). All media contained traces of $CuSO_4$, $FeSO_4$, $MnCl_2$ and $ZnSO_4$. The carbon sources used were autoclaved separately and then added to the media. Usually this was 1% (w/v) glucose; other carbon sources used are indicated in the text. SM was prepared by adding supplements to minimal medium (MM) at a concentration of 200 $mg \cdot l^{-1}$ (amino acids and nucleotides) and 2 $mg \cdot l^{-1}$ (vitamins). Optimized medium for protease expression contained SM supplemented with 0.05 M glucose and 3% (w/v) wheat bran. Casein-gelatin media contained SM supplemented with 50 mM glucose, 0.5% (w/v) casamino acids, 1% (w/v) gelatin, 1% (w/v) casein. Starch plates contained SM supplemented with 0.05 % (w/v) glucose and 1% (w/v) corn starch (BDH). Conidial suspensions were made in saline-Tween 80 (0.05% w/v) and then vigorously shaken to break conidial chains and reduce conidial aggregation. These conidial suspensions were filtered through a glasswool plug to remove mycelial fragments. Spores were inoculated at $10^6 \cdot ml^{-1}$ and all cultures were grown at 30°C.

Mutagenesis and mutant selection. A suspension of freshly harvested spores of *A. niger* N573 (*csp*A1, *bio*A1), grown on CM, was exposed to 200 $J \cdot m^{-2}$ UV light from a Philips TUV lamp emitting UV-C light. UV-treated spores (survival rate 37-75%) were plated for single colonies on gelatin-casein medium containing 0.01% (w/v) Triton X-100 to restrict colony diameter. After 72 h of incubation a turbid halo is formed around the colonies due to extracellular proteolytic activities. Initial proteolysis of the casein yields peptide fragments which aggregate with the gelatin and produce a turbid halo. Further incubation at 30°C for another 24 h causes complete clearance of the halo due to additional digestion of the casein-gelatin complexes. After 72 h of growth, followed by a 12-h incubation period at 4°C to further improve contrast, the plates were scored and colonies with a smaller halo, or not showing a halo at all, were reanalyzed on casein-gelatin medium, as well as on starch plates, to distinguish between specific protease mutants and secretion-deficient mutants.

Complementation tests. Since in all tests the *prt* mutations were recessive (see also *Genetic analysis*), we were able to compare the *prt* mutations by complementation analysis. However, preliminary complementation tests showed that some of the heterokaryons were not stable on casein-gelatin plates implicating that no conclusive evidence could be obtained concerning these *prt* mutations. Therefore, tests for complementation of different *prt* mutations were exclusively done in diploids. Diploids were isolated from heterokaryons of different *prt* mutants which carried different auxotrophic markers in a way described previously (Bos et al., 1988). Conidiospores of diploids were inocculated on casein-gelatin plates and halo formation was compared to the halo size obtained for mutants, wild type and heterozygous diploids.

Genetic analysis. The procedures for genetic analysis have been previously described (Bos et al., 1988): somatic diploids from a mutant and a master strain were isolated and the heterozygous diploid was haploidized on CM using benomyl. Segregants were isolated, purified, and tested for genetic markers and proteolytic activity using the plate assay. Diploids of *prt* mutations and master strains were tested for the dominance of *prt* mutations.

Biochemical analysis of the *prt* mutants and protease activity measurements.
Enzyme assays. Mutants were grown on optimized medium for protease production at 30°C using a Gallenkamp orbital shaker at 220 rpm. Media and mycelium samples were collected after 72 h of growth and frozen at -70 °C. Part of the mycelium was freeze-dried to compare growth of individual strains by dry weight measurements. Both extracellular and intracellular proteolytic activities were measured using BSA (Boehringer, fraction V) as a substrate. Intracellular proteolytic activities were measured in cell-free extracts. To obtain cell-free extracts mycelium was disrupted in a Braun dismembrator, homogenized in 50 mM sodium phosphate buffer pH 6.0 and cellular debris was then removed by centrifugation. Proteolytic activity in culture media and cell-free extracts were determined by incubating 50 μl samples with 450 μl 1% (w/v) BSA in either 0.1 M sodium acetate buffer pH 4.0, 0.1 M sodium phosphate buffer pH 6.0 or 0.1 M Tris-HCl buffer pH 8.0 at 37°C. All buffers and substrate solutions contained 5 mM sodium azide to

prevent microbial growth. At different time points the reactions were stopped with 500 μl of 10% (w/v) TCA. After incubation at 0°C for 0.5 h the precipitated proteins were removed by centrifugation and the optical density of the TCA-soluble fraction was measured at 280 nm. Under these conditions good linearity was observed up to an optical density of 1.00. One unit of protease activity is defined as a change of one absorbance unit per h at 280 nm for the 1 ml reaction-precipitation mixture as described above (Anson, 1939). Extracellular protease activities are expressed in units per ml of culture fluid. Intracellular (specific) protease activities are expressed in units per mg of protein in the cell-free extract. Protein concentrations in cell-free extracts were determined with the Bicinchonic-acid protein-assay kit (Sigma) according to the supplier's instructions and using BSA (fraction V) as a standard. In order to accurately measure very low residual proteolytic activities a tryptophan ninhydrin method (Samejima et al., 1971ab), adjusted from a manual for the Hitachi f-4500 fluorescence spectrophotometer, was used; 100 μl medium sample was incubated with 400 μl of 0.1 M sodium acetate buffer, 0.6% (w/v) BSA pH 4.0 for 70 h at 37°C. Non-digested protein was again removed by TCA precipitation and after microcentrifugation 1 volume of 0.25 M NaOH was added to 1 volume of the TCA-soluble fraction. Finally 2.5 volumes of 0.2 M sodium phosphate buffer pH 8.0, 1 volume of 50 mM ninhydrin and 0.5 volume of 10 mM phenylacetaldehyde were added to the mixture. After incubation for 15 min at 60°C the samples were cooled and their fluorescence was measured during 5 s (excitation wavelength, 390 nm; emission wavelength, 490 nm). Activities were expressed as the percentage residual activity compared to wild-type activity, which was measured under the same conditions and taken as 100%.

***In vitro* protein degradation experiments.** The effect of residual proteolytic activity within these medium samples was tested on the homologous pectinolytic protein PELB produced and described before (Kusters-van Someren et al., 1992; Kester and Visser, 1994). Two μg of pure protein was incubated with serial dilutions of culture media of these protease mutants in 0.1 M sodium acetate buffer pH 4.0. All incubations were interrupted after 15 min either by adding SDS PAGE loading buffer and boiling the samples or by adjusting the pH to 8.5. For the incubations stopped with SDS PAGE loading buffer, protein fragments were separated by a SDS-polyacrylamide gel electrophoresis according to Laemmli (1970) and visualized by Coomassie Brilliant Blue staining. The incubations stopped by adjusting the pH to pH 8.5 were used to determine the amount of active PELB protein present after the *in vitro* digestions by measuring the pectin lyase activity against highly esterified pectin (DE 94.6 %) as described by Kester and Visser (1994). At pH 8.5 no proteolytic activity can be detected in the culture fluids and the acitivity of the PELB protein is maximal. Enzyme activities of pectin lyase B were expressed in international units using a molar extinction coefficient of 5500 $M^{-1}cm^{-1}$ for unsaturated product (Van Houdenhoven, 1975).

Results

Isolation and phenotypic description of prt mutants

The only well defined media described in literature used to screen for protease activities on plates are based on skimmed milk or casein (Davies, 1994). On this medium a clear halo can result not only from proteolytic degradation of the milk or casein but also from concomittant acidification of the medium, a usual occurence in the proximity of *A. niger* colonies. To improve halo-screening for extracellular proteinases in *A. niger* several protein-containing media were tested. A medium based on a substrate used in a protease assay described by Montville (1983) was selected to screen for residual proteolytic activities of UV-induced mutants on plates. On this gelatin/casein containing medium large-sized striking halos were observed due to complexation of casein and

gelatin after initial proteolytic cleavages. The pH of the medium did not seem to interfere with halo formation.

After screenig 10^4 mutants 153 initial '*prt*' mutants with reduced proteolytic activity were isolated, purified, and retested. Possible secretion mutations were recognized through their effects on starch hydrolysis. Twenty six mutants representing a range of reduced proteolytic activity compared to wild type were further studied. By assaying the residual extracellular proteolytic activity at pH 4.0 using BSA as a substrate, 14 mutants having 80% or less activity compared to wild-type were characterized further.

Complementation tests

The selected mutants were further characterized in complementation tests which were exclusively done in diploids due to stability problems observed for some of the heterokaryons. This heterokaryon instability was probably caused by small amounts of biotin present in these media and the fact that one of the strains used in the complementation studies contained only the *bio*A1 mutation in addititon to the protease mutation. The results of these complementation tests are compiled in Table 8.1. Complementation analysis required the introduction by somatic recombination of an auxotrophic marker other than *bio*A1 in one of the two *prt* mutants in order to be able to make a heterokaryon and to isolate a heterozygous diploid from two mutants which had originally the same background. Initially, from a cross between NW222 (*bio*A1, *prt*A10) and tester strain NW136 during the allocation of the *prt*A10 mutation a *prt*A10, *bio*A$^+$ recombinant was isolated. This strain NW239 (*fwn*A1, *pdx*A2, *nic*B5, *prt*A10) was used in complementation analysis with all other *prt* mutants. All other *prt* mutations showed complementation with NW239. Since also activity measurements indicated that different classes of protease mutants were isolated we decided to allocate a series of *prt* mutations (see below). These allocation experiments resulted for almost all mutants in the isolation of *bio*A$^+$ recombinants which were used to complete the complementation analysis, as summarized in Table 8.1. Only for the *prt*E mutants we did fail to isolate *bio*A$^+$ recombinants, due to close linkage to the *bio*A1 and *prt*E15 markers. Complementation analysis between the *prt*E mutants was done with recombinants having other additional markers and the *bio*A1 homozogous diploid was supplemented with biotin during complementation analysis. The allocation data and initial biochemical characterization of the *prt* mutations provided sufficient data to select a limited set of complementation tests to finish the complementation analysis (see Table 8.1). Complementation tests gave clearcut results since all *prt* mutations were completely recessive. In Table 8.1 the mutations are given a capital letter for locus identification and a numerical code for allele identification according to the general rules used in *Aspergillus* genetics

(Clutterbuck, 1973).

Table 8.1. Complementation tests of *A. niger prt* mutants.

Mutant linkage group	*prt*C7 (I)	*prt*B26 (III)	*prt*E15 (III)	*prt*E30 (III)	*prt*G24 (III)	*prt*F28 (V)	*prt*A10 (VIII)	*prt*D32 (VIII)
*prt*C7 (I)	-						+	
*prt*A10 (VIII)	+	+	+		+	+	-	+
*prt*C14 (I)	-						+	
*prt*E15 (III)		+	-	-	+		+	
*prt*G24 (III)		+	+		-		+	
*prt*B26 (III)		-	+	+	+		+	
*prt*F28 (V)				+		-	+	+
*prt*F29 (V)						-		
*prt*E30 (III)		+	-	-		+		
*prt*C31 (I)	-							
*prt*D32 (VIII)						+	+	-
*prt*A33 (VIII)		+				+	-	+
prt-35							+	+

Complementation is indicated with +; no complementation is indicated with -

Allocation of *prt* genes to linkage groups

From every complementation group identified at least one mutant was used to allocate the relevant *prt* gene to one of the eight linkage groups of *A. niger* (Debets et al., 1990). The results of these allocation experiments expressed as the frequency of recombination between each of the *prt* loci and the linked marker(s) of the master strain are compiled in Table 8.2. Seven *prt* genes were mapped unambiguously: *prt*A on linkage group VIII, *prt*B on linkage group III, *prt*C on linkage group I, *prt*D on linkage group VIII, *prt*E on linkage group III, *prt*F on linkage group V and *prt*G on linkage group III. The observed recombination frequencies for the three mutations located on linkage group III (*prt*B, *prt*E and *prt*G) and both mutations located on linkage group VIII (*prt*A and *prt*D) indicated that on both linkage groups independent *prt* complementation groups, were identified. Complementation tests between *prt*A10 and *prt*D32 and between *prt*B26, both *prt*E mutants and *prt*G (Table 8.1) were carried out to show that these mutations were not allelic. It is however difficult to determine the linear order of genes on the same linkage group since the

frequency at which recombinants are found is partly dependent on clonal segregation (Debets et al., 1989).

Table 8.2. Assignment of *A. niger prt* genes to linkage groups.

Diploid strain	*prt* mutations	Linked markers [1]	% Recombinants	Linkage group
NW222//NW136	*prt*A10	*trp*B2	4.3	VIII
NW227//NW136	*prt*B26	*pyr*A6	13.7	III
NW221//NW136	*prt*C7	*fwn*A1	18.7	I
NW231//NW136	*prt*C31	*fwn*A1	5.9	I
NW232//NW136	*prt*D32	*trp*B2	21.8	VIII
NW225//NW136	*prt*E15	*bio*A1	10.8	III
NW230//NW136	*prt*E30	*bio*A1	12.1	III
NW228//NW136	*prt*F28	*phe*A1	20.2	V
NW226//NW136	*prt*G24	*bio*A1	19.8	III
NW220//NW136	*prt*-3	*bio*A1	28.0	(III)[2]
NW223//NW136	*prt*-11	*trp*B2	29.0	(VIII)[2]

The frequencies of recombination between the various *prt* loci and linked markers in the tester strain NW136 (*csp*A1, *fwn*A1, *arg*H12, *pyr*A6, *lys*A7, *his*C3, *phe*A1, *pdx*A2, *nic*B2, *trp*B2, with *arg*H, *pyr*A, *lys*A, *his*C, *phe*A, *pdx*A, *nic*B, *trp*B being requirements for arginine, uridine, lysine, histidine, phenylalanine, pyridoxine, nicotinic acid, tryptophan respectively) are given; [1], Markers of different linkage groups give free recombination (35-65% recombinants); [2], linkage has not been investigated further because of poor contrast between mutation and wild-type on casein gelatin medium. Complementation has not been tested in complementation experiments due to similar problems

Construction of strains with several *prt* mutations

To obtain strains which carry several *prt* mutations we used two approaches. In the first we combined several of the characterized *prt* mutations in one strain via recombination. In the gene allocation experiments several recombinants which had a particular *prt* mutation on one linkage group and auxotrophic markers on several other linkage groups were isolated. By constructing diploids between such a *prt*A10 recombinant, NW236 (*fwn*A1, *pdx*A2, *nic*B5, *prt*A10), with other *prt* mutants from other complementation groups, strains were isolated which had replaced a linkage group with a marker gene for a linkage group with a *prt* mutation. Via this approach

strains NW237 (*fwn*A1, *prt*C14, *pdx*A2, *nic*B2, *prt*A10), NW238 (*fwn*A1, *bio*A1, *prt*E15, *pdx*A2, *prt*A10) and NW239 (*fwn*A1, *prt*F28, *nic*B2, *prt*A10) were constructed which contained in addition to the *prt*A10 mutation the *prt*C14, *prt*E15 or *prt*F28 mutation respectively. The presence of both mutations in these recombinants was confirmed by activity measurements and/or complementation experiments in diploids between the recombinant strain and parental mutant strains.

In the second approach we used the characterized *prt*C7 mutant which was used in a second mutagenesis experiment in order to induce additional *prt* mutations in the *prt*C7 background. This *prt*C7 mutant was selected because the residual extracellular activity present in this mutant was almost completely inhibited by pepstatin as shown in Table 8.3 (see also *Biochemical characterization*). A second mutation affecting pepstatin inhibitable proteases would be expected to generate a double mutant with severely reduced extracellular protease activities. After three independent mutagenesis experiments (in which 10^4 mutants were screened) 5 *prt* mutants were selected out of which two, NW233 (*prt*C7, *bio*A1, *prt*-33) and NW234 (*prt*C7, *bio*A1 *prt*-35), having very low residual proteolytic activities, were further characterized. Initial complementation analysis showed that the *prt*-33 mutation was allelic with the *prt*A10 mutation and that the *prt*-35 mutation was non allelic with the *prt*D32 mutation. Further complementation analysis awaits the construction of new recombinants of the other protease mutations that can be crossed with (NW233 and) NW234.

Since some protease mutations in yeast affect sporulation and/or growth (especially when combined in one strain) all protease mutant strains were compared with wild-type for sporulation and for growth on solid and liquid medium. Almost all mutants which have less than 50% residual activity sporulated less than wild-type and showed slightly reduced growth on solid medium. Only NW227 [*prt*B26] showed significantly reduced growth and sporulation whereas NW228 [*prt*F28] and NW229 [*prt*F29] were indistinguishable from wild-type in these respects. Growth in liquid medium resulted in biomass yields comparable to wild-type for all mutants, as judged from comparisons of freeze dried mycelium samples.

Biochemical characterization of *prt* mutants; enzyme assays

All mutants were grown on a medium optimized for protease production to determine which proteolytic activities were still present in the individual mutants. *A. niger* N400 has a pH optimum of approximately 4.0 for the degradation of several proteins such as hemoglobin, casein, BSA (data not shown) and myoglobin (van Noort et al., 1991). Protease activity in the wild-type strain N400 and in the protease mutants was determined by measuring the degradation of BSA, under various conditions; activity (both intracellular and extracellular) was determined at pH 4.0 and

Table 8.3. Extracellular protease activities of the *A. niger prt* mutants.

Strain	*prt* mutation(s) (linkage group)	Act. [1]	Act. % res. [2]	Act. [3]	Act. % res. [4]	Act. [5]	Act. % res. [6]
				pepst.	pepst.	PMSF	PMSF
NW220	*prt*-3 (III)	3.68	80	0.16	17	3.53	80
NW221	*prt*C7 (I)	2.42	52	0.06	6	2.44	55
NW222	*prt*A10 (VIII)	0.98	21	0.31	34	0.67	15
NW223	*prt*-11 (VIII)	3.16	69	0.29	31	2.80	63
NW224	*prt*C14 (I)	1.48	32	0.08	8	1.52	34
NW225	*prt*E15 (III)	2.88	63	0.85	92	2.76	62
NW226	*prt*G24 (III)	0.32	7	0.02	nd	nd	nd
NW227	*prt*B26 (III)	0.11	2	nd	nd	nd	nd
NW228	*prt*F28 (V)	0.12	3	nd	nd	nd	nd
NW229	*prt*F29 (V)	0.12	3	nd	nd	nd	nd
NW230	*prt*E30 (III)	0.94	20	0.40	43	0.91	21
NW231	*prt*C31 (I)	2.88	63	0.29	32	1.86	65
NW232	*prt*D32 (VIII)	0.16	4	nd	nd	nd	nd
NW233 [7]	*prt*C7 (I) *prt*A33 (VIII)	0.04	1	nd	nd	nd	nd
NW234 [7]	*prt*C7 (I) *prt*-35	0.13	3	nd	nd	nd	nd
NW237 [8]	*prt*A10 (VIII) *prt*C14 (I)	0.17	4	nd	nd	nd	nd
NW238 [8]	*prt*A10 (VIII) *prt*E15 (III)	0.18	4	nd	nd	nd	nd
NW239 [8]	*prt*A10 (VIII) *prt*F28 (V)	0.11	2	nd	nd	nd	nd
N573	-	4.60	100	0.92	100	4.42	100

Activities were measured at pH 4.0 with BSA (fraction V) as a substrate. Activity measurements were done in quadruplicate from two independent cultures grown under the same conditions. Results showed good linearity and standard deviations varied from 1-4%; [1], extracellular activities were expressed as U/ml culture fluid; [2], percentage residual activity compared to N573; [3], extracellular activity in the presence of 2 μg/ml pepstatin; [4], percentage residual activity compared to N573 in the presence of 2 μg/ml pepstatin; [5], extracellular activity in the presence of 100 μg/ml PMSF; [6], percentage residual activity compared to N573 in the presence of 100 μg/ml PMSF; [7], double mutants obtained via second round of mutagenesis in strain NW221; [8], double mutants obtained via recombination of characterized mutations; nd, not determined

6.0. Furthermore several protease inhibitors (pepstatin, PMSF and EDTA) were used to identify which class(es) of protease(s) were still present in the mutant strains. Extracellular activities were expressed as U/ml since freeze dried mycelium samples showed that the amounts of biomass formed by the protease mutants and wild-type on the medium optimized for protease expression were very similar (differences $\leq$ 4%).

Indeed, in all mutants the extracellular activity was highest at pH 4.0 (see Table 8.3) and above pH 6.0 virtually no extracellular activity was measured. The residual activities in the mutants at pH 4.0 ranged from 80% (*prt*-3) down to 2.3% (*prt*B26) compared to N573. Combining mutations within one strain resulted in strains with severely reduced proteolytic activities; combining the *prt*A10 mutation with respectively the *prt*C14, *prt*E15 and the *prt*F28 mutation all resulted in strains with 4% residual proteolytic activity or less. Especially for the relatively weak *prt*C14 and *prt*E15 mutations the double mutants have considerably reduced proteolytic activities compared to their parental strains. If and to what extent the *prt*A10 mutation contributes to the low activity in NW239 is not clear, as NW228 has comparable residual activities. Also via a second round of mutagenesis in strain NW221 double mutants with very low residual (NW233 and NW234) activities were isolated. Because the *prt*C7 mutation was not crossed out in strains NW233 and NW234 the individual effects of the *prt*A33 and *prt*-35 mutations have not been determined.

Inhibition studies with specific protease inhibitors were only performed with mutants which had more than 5% residual activity. From inhibition studies with N573 it is clear that 77% of the total acidic activity is pepstatin inhibitable and 4% is PMSF inhibitable. Pepstatin inhibition studies indicate that in all mutants still (some) pepstatin inhibitable proteases are expressed. Both the strong *prt*C mutations (*prt*C7 and *prt*C14) result in very low residual activities in the presence of pepstatin and the relative amounts of pepstatin inhibitable activity are increased with a factor 4 (*prt*C14) and 8.3 (*prt*C7) compared to N573. In the *prt*A and *prt*E mutants the relative amounts of pepstatin repressible activity are lower than in N573, in contrast to *prt*C mutations.

Clear PMSF inhibition is only observed in N573 and mutants NW222, NW223 and NW231. The stronger *prt*C mutations (*prt*C7 and *prt*C14) show no PMSF inhibition, corresponding with the relatively high percentages of pepstatin-repressible activities. For the *prt*E mutations the percentages of non-PMSF repressible activities are identical to N573 (approx 4% of total activity).

Table 8.4. Intracellular protease activities of the *A. niger prt* mutants.

Strain	*prt* mut.	4A activity pH 4 [1]	% res. act. [2]	res. act. pH 4 BSA pepst. [3]	% res act. pH 4 BSA pepst. [4]	4B act. pH 6 BSA [5]	% res. act. [6]	res. act. pH 6 BSA PMSF [7]	% res act. pH 6 [8]
NW220	*prt*-3	5.40	43	2.16	84	0.37	100	0.073	61
NW221	*prt*C7	4.36	35	1.08	42	0.34	92	0.005	4
NW222	*prt*A10	1.84	15	1.05	41	0.30	81	0.096	80
NW223	*prt*-11	19.00	152	5.23	205	0.36	97	0.100	83
NW224	*prt*C14	2.45	20	1.22	48	0.32	87	0.073	61
NW225	*prt*E15	2.80	22	1.53	60	0.36	98	0.110	89
NW226	*prt*G24	2.54	20	1.08	43	0.40	108	0.150	117
NW227	*prt*B26	1.39	11	0.54	21	0.34	91	0.110	89
NW228	*prt*F28	1.44	12	0.76	30	0.58	157	0.240	199
NW229	*prt*F29	1.28	10	0.71	28	0.54	146	0.230	192
NW230	*prt*E30	4.51	36	1.19	47	0.34	91	0.082	69
NW231	*prt*C31	1.44	12	0.89	35	0.26	70	0.120	100
NW232	*prt*D32	1.09	9	0.43	17	0.20	54	0.013	106
N573	-	12.52	100	2.56	100	0.37	100	0.120	100

Activities were measured at pH 4.0 (A) and pH 6.0 (B) with BSA (fraction V as a substrate. Activity was measured in triplicate from mycelium derived from two independent cultures grown under identical conditions (Standard deviations for all activity measurements varied from 3-7%).[1], intracellular activities were expressed as U/mg protein; [2], percentage residual activity compared to N573; [3], intracellular activity in the presence of 2 μg/ml pepstatin; [4], percentage residual activity compared to N573 in the presence of 2 μg/ml pepstatin; [5], intracellular activities were expressed as U/mg protein; [6], percentage residual activity compared to N573; [7], intracellular activity in the presence of 100 μg/ml PMSF; [8], percentage residual activity compared to N573 in the presence of 100 μg/ml PMSF

Intracellular proteolytic activities were measured in cell-free extracts under identical conditions as the extracellular activities. Protein concentrations in cell-free extracts were measured and activities were expressed in U/mg protein. In all mutants the intracellular activities at pH 4.0 were reduced compared to N573, except for NW223, which showed elevated protease activities. All acidic activities were to some extent pepstatin inhibitable (Table 8.4A) and virtually no PMSF inhibition was measured (data not shown). In all mutants the relative amounts of intracellular non-pepstatin

inhibitable activities were increased compared to N573.

Activities measured at pH 6.0 are not pepstatin inhibitable (data not shown) but as found for N573 and all mutants to some extent PMSF inhibitable (Table 8.4B). Intracellular residual activities varied from 54 - 156%. Only both *prt*F mutations resulted in levels higher than N573. The PMSF inhibition studies show again for the *prt*F mutations very high activities. Biochemical characterization of the prt mutants.

in vitro protein degradation

To test whether the independent *prt* mutants showed lower protein degradation compared to wild-type the degradation of the homologous *A. niger* pectinolytic enzyme, PELB (Kusters-van Someren, 1992) was tested *in vitro* by incubating purified protein in culture filtrates of *prt* mutants and N400. The strains were grown under the same conditions as described for the residual protease activity enzyme assays. Purified proteins were incubated in serial dilutions of culture filtrates and degradation was visualized with CBB staining after polyacrylamide gel electrophoresis.

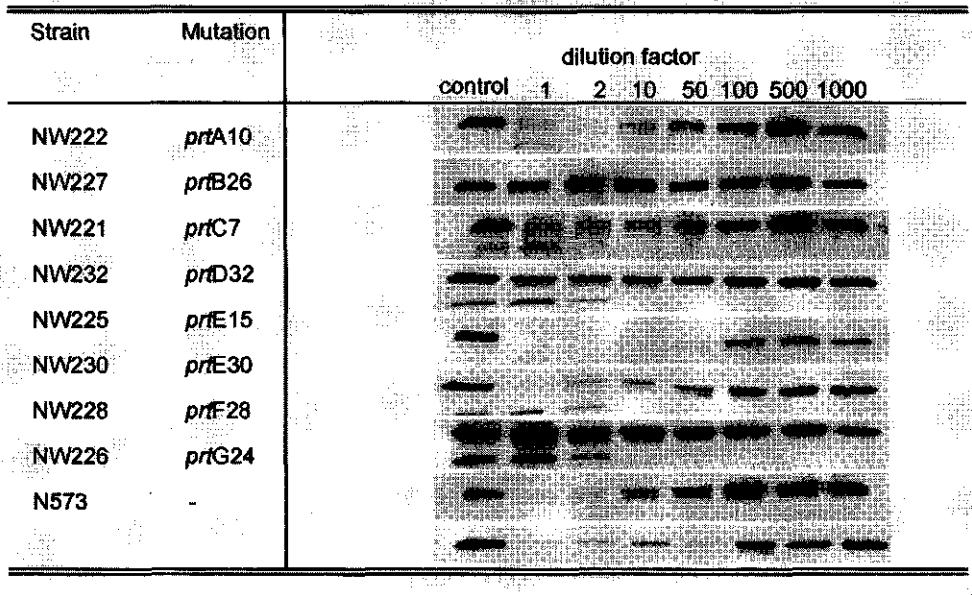

Fig. 8.1. *In vitro* digestion of PELB in *A. niger* culture fluids. [1], One μg of PELB protein was incubated with serial dilutions of culture fluids from various *prt* mutants; cont, as a control the medium sample was boiled prior to incubation with PELB. Products were separated on a 10 % SDS PAA gel and visualized with CBB.

The PELB protein, which has been purified recently (Kester and Visser, 1994) proved to be very unstable in culture media of wild-type *A. niger*. An *in vitro* degradation assay, based on the PELB protein was developed and *in vitro* PELB degradation in at least one member of all seven complementation groups was compared to wild-type. As shown in Fig. 8.1 the rate of PELB degradation was lower in all mutants and virtually no degradation was observed in mutants NW227, NW228 and NW232. The inactivation of the PELB protein was quantified by measuring the residual pectinolytic acitvity of PELB during the *in vitro* assay (see Table 8.5). From these *in vitro* degradation studies one can notice differences in the degree of *in vitro* PELB degradation in culture filtrates of the various mutants. Furthermore, the degree of degradation observed in the mutants correlated in most cases with the residual proteolytic acitvity, but seemed also to be dependent on the specific protein investigated. PELB is more stable in culture fluids of NW230 when compared with NW222 although these strains have identical residual activities against BSA, which was used in the enzyme assays (Table 8.3).

Table 8.5. Quantification of residual PELB activity

Strain	Mutation	Residual proteo-lytic activity[1]	Residual PELB activity[2] dilution factor							
			cont.	1	2	10	50	100	500	1000
NW222	*prt*A10	21	96	0.5	2	6	21	74	**91**	**100**
NW227	*prt*B26	2	95	**99**	**103**	**94**	**98**	**100**	**100**	**102**
NW221	*prt*C7	53	97	0	0.5	5	12	30	74	**98**
NW232	*prt*D32	4	98	**97**	**100**	**98**	**97**	**104**	**101**	**103**
NW225	*prt*E15	63	100	0.2	0.3	1.8	9	12	40	**100**
NW230	*prt*E30	20	99	1.2	3	26	42	84	**95**	**96**
NW228	*prt*F28	3	97	**97**	**97**	**100**	**102**	**98**	**99**	**101**
NW226	*prt*G24	7	96	2.0	5	21	68	**101**	**98**	**100**
N573	-	100	98	0	0.5	2	9	28	59	**97**

[1], percentage residual proteolytic activity agaist BSA at pH 4.0 compared to N573. [2], incubations of serial dilutions with PELB protein were repeated as described in Fig. 1. The PELB activity was measured and expressed as the percentage of residual activity compared to the control. Non-affected activities are indicated in bold; cont., as a control an undiluted medium sample was boiled prior to incubtion with PELB.

Discussion

Fungal strain improvement often involves several rounds of random mutagenesis and subsequent selection of mutant strains with increased productivity. Although quite valuable, this approach has also disadvantages and limitations. Repeated mutagenic treatment may lead to an increase in initially undetected mutations (genetic background damage), which can result in the accumulation of background mutations and thus the loss of potentially good producers. All the mutants described here were induced at low mutagen dose to minimize genetic background damage. Basically all mutants were obtained from nearly wild-type strains thus minimizing the negative effects of repeated mutagenesis. This approach provides the tools for a succesful breeding program by constructing strains with multiple *prt* mutations without background damage.

As *A. niger prt* mutations are found at a rather high frequency, this indicates that many genetic loci influence protease expression levels. This is in agreement with the observed complex regulation of the two extracellular acid proteases *pep*A and *pep*B (Jarai and Buxton, 1994) and the extracellular serine carboxypeptidase, *pep*F, from *A. niger* (Hombergh van den et al., 1994). These studies showed that carbon and nitrogen metabolite repression, pH regulation and certain protein inducers are involved in protease gene regulation.

In Table 8.6 the main characteristics of the seven *prt* complementation groups are presented. The locations of the allocated *prt* mutations on linkage groups combined with the recombination percentages observed show that at least six new *prt* complementation groups were identified. The *prt*C and *prt*D mutations show significantly different recombination patterns than the *prt*-18 and *prt*-39 mutations described by Mattern et al., 1992. However, both the *prt*A mutations and the *prt*-39 mutation (Mattern et al., 1992) show close linkage to the *trp*B2 marker on linkage group VIII. Therefore, these mutants could be allelic.

Extracellular enzyme activities and inhibition studies in wild type show that overall extracellular proteolytic activity has an acidic pH optimum which correponds to the pH optima of the three cloned extracellular proteases (PEPA; Berka et al., 1991; PEPB: Inoue et al., 1991; PEPF: Hombergh van den et al., 1994). *A. niger* is known for its strong acidification of culture media thus explaining that these acidic activities cause the major problems during homologous and heterologous expression. All mutants isolated have reduced extracellular proteolytic activities varying from 2-80%. Interestingly the relative amounts of certain classes of activities have changed (elevated as well as reduced) compared to wild-type as shown in Table 8.6. The *prt*C mutants have relatively high pepstatin inhibitable activities whereas other activities are low. The *prt*E mutants show relatively high residual activities and the *prt*A mutants have high PMSF inhibitable activities. Due to these specific mutations the spectrum of protease(s) can be changed

Table 8.6. Genetical and biochemical characteristics of *prt* mutants

compl. group	non-allelic linked mutations	Activity most affected	Residual protease spectrum [1]	Characteristics residual extracellular protease spectrum	Nature of the mutation
*prt*A10 (VIII) *prt*A33 (VIII)	*prt*D *prt*-11 *prt*-39 [2]	pepstatin inh. act. rest act.	pepstatin 68 % PMSF 32 % residual 0 %	rel. high PMSF inh. neutral activity no residual activity	regulatory / cascade [4]
*prt*B26 (III)	*prt*E15 *prt*E30 *prt*G24 *prt*-3 (?)	all act.	[3]	[3]	regulatory / cascade
*prt*C7 (I) *prt*C14 (I) *prt*C31 (I)	*prt*-18 [2]	PMSF inh. act	pepstatin 98 % PMSF 0 % residual 2 %	rel. high pepstatin inh. acid activity no PMSF inh. neutral activity rel. low residual activity	unknown
*prt*D (VIII)	*prt*A10 *prt*A33 *prt*-11 *prt*-39 [2]	all act.	[3]	[3]	regulatory / cascade
*prt*E15 *prt*E30	*prt*B26 *prt*G24 *prt*-3 (?)	pepstatin inh. act. PMSF inh. act.	pepstatin 57 % PMSF 3 % residual 40 %	decreased pepstatin inh. acid activity decreased PMSF inh. neutral activity increased residual activity	regulatory / cascade
*prt*F28 *prt*F29			[3]	[3]	regulatory / cascade
*prt*G24	*prt*B26 *prt*E15 *prt*E30 *prt*-3 (?)	all act.	pepstatin 94 % residual 6 %	rel. high pepstatin inh. acid activity overall low residual activity	regulatory / cascade

[1]: residual activities are presented as percentage of total non-inhibited activity; [2]: *prt* mutant described by Mattern *et al.* (1992); [3]: residual activities were to low to accurately measure inhibition by pepstatin and PMSF; [4]: regulatory nature of the mutation was proven by generation of multicopy *pep*A transformants in *prt*A10 background

but also compensatory effects due to elevated expression of not mutated proteases have been observed (Suarez Rendueles and Wolf, 1988). The acidic activities measured in the mycelium extracts can probably to a large extent be ascribed to the proteases located in the vacuole, presumably involved in protein degradation similar to the situation in yeast (Suarez Rendueles and Wolf, 1988). Cell membrane bound and periplasmic acid proteinases can also contribute to these activities. The pH 6.0 activity measurements show for the *prt*F mutations very high intracellular activities. This mutant class is especially interesting since it shows very low extracellular residual activities and it shows phenotipically no differences compared to wild-type. All other *prt* mutants show reduced sporulation effects except for the *prt*F mutations. Whether the higher intracellular activities at pH 6.0 and normal sporulation in the *prt*F mutations are correlated awaits further research.

Assigning the *prt* mutants to structural or regulatory genes is difficult primarily because, except for *pep*A, the cloned genes have not been assigned to linkage groups (Verdoes et al., 1994), but also because a distinction between structural and regulatory mutations is not strict. It is known from yeast that some proteases regulate expression of several other proteases as they process and/or activate in a cascade other proteases (Suarez Rendueles and Wolf, 1988). The *prt*B, *prt*D, *prt*F and *prt*G mutations are probably regulatory/cascade mutations since the residual extracellular protease activities are much lower than those of a strain in which the major aspartic protease *pep*A has been disrupted (20 % residual activity; Mattern et al., 1992) which can only be explained if more proteases are affected by these mutations. Furthermore, the *prt*A and *prt*E mutations are probably also regulatory/cascade mutations since in some of these mutants the residual activity is approx. 20 % compared to wild-type. The *pep*A gene is still intact as the *prt*A and *prt*E mutations are not located on linkage group I. For the *prt*A mutation the regulatory function of the locus is confirmed by multicopy *pep*A transformants which do not have elevated protease levels in a *prt*A10 background (data not shown).

The development of a very sensitive *in vitro* system to analyze protein digestion enabled us to accurately quantify the reduced protein degradation within the culture fluids of these mutants. Although the amount of degradation in general corresponded with the measured overall extracellular proteolytic activities some differences were observed as well. Strain NW222 shows higher PELB degradation compared to strain NW230 although overall extracellular proteolytic activities are identical. Probably the specific protease(s) affected by the *prt*A mutation can more effectively degrade PELB as compared to protease(s) affected by the *prt*E mutations.

Considering the decrease of extracellular protease activities and the increased *in vitro*

stability of the PELB protein during incubations in mutant culture samples, classical mutagenesis provides a means for improving protein production. Which protease(s) need to be mutated or disrupted depends mainly on the individual protein to be expressed. Constructing a specific set of characterized strains in which different protease(s) are not expressed will facilitate screening for a strain which shows improved expression in a minimized *prt* background, since combining of multiple mutations will eventually cause reduced growth and/or production.

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9 Production of the homologous pectin lyase B protein in six genetically defined protease deficient *Aspergillus niger* mutant strains[1]

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Abstract

Transformable recombinants have been isolated for six protease deficient (*prt*) mutants in *Aspergillus niger*. These *pyr*A *prt* recombinants were co-transformed with a plasmid containing an expression cassette for the *A. niger pel*B gene and a second plasmid, pGW625, containing the *pyr*A selection marker containing plasmid. Transformants were tested for improved expression of the *pel*B gene, encoding the proteolytically susceptable PELB tester protein. In four complementation groups (*prt*A, B, D and F) distinct improvement of *pel*B expression was observed. Western analysis demonstrated in all *prt* mutants reduced extracellular PEPA levels, although in several *prt* mutants no significant reduction in transcript levels of extracellular proteases was observed, probably indicative for posttranscriptional effects of the *prt* mutations. The *in vivo* experiments in single *prt* mutants and the *in vitro* experiments in *prt* double mutants demonstrated the usefulness of protease deficient mutants to improve protein production in *A. niger*. Allocation of the protease genes *pep*A [I], B [II], C [IV], D [I] and E [IV] indicated that all *prt* mutations identified do not represent alleles of the *pep* genes cloned in *A. niger*. Furthermore, this indicates that several new genes involved in extracellular protease expression have been identified.

Introduction

Filamentous fungi become increasingly interesting as a host for the production of both homologous and heterologous proteins (van den Hondel et al., 1991; Davies, 1994; van den Hombergh et al., 1996a). These reviews list a number of proteins which have been produced by filamentous fungi. Especially *Aspergilli* have proved to be potentially very useful microorganisms for the expression of a variety of eukaryotic gene products (Davies, 1994). In particular *A. niger* is an

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attractive expression host as this GRAS species is capable of secreting large amounts of rather different proteins (up to 40 g/l have been reported; Montenecourt and Eveleigh, 1985; Nevalainen et al., 1991). However, yields of the produced proteins vary considerably, especially for heterologous proteins. One major cause of low yields has been recognised to be proteolytic degradation of the proteins produced by host proteases, either during secretion or in the extracellular environment. In *A. niger* a wide range of proteolytic activities, both extracellular and intracellular including aspartyl, serine and serine carboxypeptidases have been described (Sakka et al., 1985; Krishnan and Vijayalakshmi, 1985, 1986; Barthomeuf et al., 1989; Takahashi et al., 1991). Several protease encoding genes have been cloned, as recently reviewed by van den Hombergh et al. (1996a). Reduction of proteolytic degradation has been achieved in different ways. Removal of specific proteolytic activities using gene disruption techniques has been described for the *A. awamori pep*A gene (Berka et al., 1990). Protease deficient mutants selected for reduced extracellular proteolysis (Hayashida and Teramoto, 1986; Hayashida and Flor, 1981, Flor and Hayashida, 1983; Fiedurek et al., 1985; van Noort et al., 1991; van den Hombergh et al., 1995) also result in improved expression levels as has been shown for the heterologous hen egg-white lysozyme (HEWL) and porcine pancreatic phospholipase A_2 (PLA_2) (Archer et al., 1992). In addition to inactivation of proteolytic activities by mutagenesis and/or gene disruption, some protease genes can also be down-regulated in the growth phase when expression and secretion of the (heterologous) proteins has to be achieved. The expression of all the extracellular proteases characterized in *A. niger* is controlled by wide domain regulatory mechanisms, and can be affected by the use of specific repressing carbon and nitrogen sources and by the pH of the culture medium (Jarai and Buxton,1994; van den Hombergh et al., 1994; Bartling et al., 1996). Furthermore, the introduction of specific wide domain regulatory mutations which reduce protease expression can also improve protein production (van den Hombergh et al., unpublished results).

Recently, we have described the isolation of a large number of *prt* (protease deficient) mutants, which by genetic analysis, were divided into at least seven complementation groups (van den Hombergh et al., 1995). Degradation experiments using these mutants have clearly shown that *in vitro* the degradation of a proteolytically very susceptable *A. niger* protein, pectin lyase B, is strongly reduced in several *prt* mutants. In the present paper we report the *in vivo* expression studies in these *prt* deficient mutants using an expression cassette based on a *pki-pel*B fusion construct to investigate the correlation between *in vitro* and *in vivo* data. Furthermore, the residual protease spectrum in the *prt* mutants was studied at the transcriptional level. Finally, the protease genes cloned thusfar were allocated to one of the eight linkage groups in *A. niger* and their linkage was compared with the *prt* loci previously established.

Materials and methods

Strains, media and culture conditions. All *A. niger* strains were derived from wild type strain N400 (CBS 120.49). The morphological mutant N402 (*csp*A1) has short conidiophores. NW219 (*csp*A1, *pyr*A6, *leu*A1, *nic*A1) was used to generate a *pkipel*B transformant with a wild type protease background. All protease (*prt*) mutations were generated in the auxotrophic mutant N573 (*csp*A1, *bio*A1) which is biotin deficient (van den Hombergh et al., 1995). The single prt mutants used were: NW222 (*csp*A1, *bio*A1, *prt*A10); NW227 (*csp*A1, *bio*A1, *prt*B26); NW221 (*csp*A1, *prt*C7, *bio*A1); NW224 (*csp*A1, *prt*C14, *bio*A1); NW232 (*csp*A1, *bio*A1, *prt*D32); NW230 (*csp*A1, *bio*A1, *prt*E30); NW225 (*csp*A1, *bio*A1, *prt*E15); NW228 (*csp*A1, *bio*A1, *prt*F28); NW226 (*csp*A1, *bio*A1, *prt*G24). The double prt mutants used were: NW233 (*csp*A1, *prt*C7, *bio*A1, *prt*-33); NW234 (*csp*A1, *prt*C7, *bio*A1, *prt*-35); NW237 (*csp*A1, *fwn*A1, *prt*C14, *pdx*A2, *nic*B2, *prt*A10); NW238 (*csp*A1, *fwn*A1, *bio*A1, *prt*E15, *pdx*A2, *prt*A10); NW239 (*csp*A1, *fwn*A1, *prt*F28, *nic*B2, *prt*A10); The transformable prt strains constructed were: NW207 (*csp*A1, *pyr*A6, *lys*A7, *phe*A1, *pdx*A2, *prt*A10); NW150 (*fwn*A1, *pyr*A5, *prt*B26, *pdx*a2, *nic*B2); NW151 (*prt*C7, *arg*H12, *pyr*A6, *lys*A7, *bio*A1, *trp*B2); NW152 (*bio*A1, *pyr*A5, *nic*B5, *prt*D32); NW153 (*prt*E15, *pyr*A6, *lys*A7, *bio*A1, *his*C3); NW154 (*pyr*A6, *prt*F28). *Media.* Minimal medium (MM) contained 6.0 g $NaNO_3$ / 1.5 g KH_2PO_4 / 0.5 g KCl / 0.5 g $MgSO_4$ / trace elements (Vishniac and Santer, 1957) at pH 6.0. Complete medium contained MM supplemented with 1% (w/v) yeast extract / 2% (w/v) peptone / 0.5% (w/v) ribonucleic acids / 0.5% (w/v) casamino acids at pH 6.0. Low phosphate medium (LPM) contained 4.0 g NH_4Cl / 1.5 g KH_2PO_4 / 0.5 g KCl / 0.5 g $MgSO_4$ / 0.1% (w/v) yeast extract and spore elements (Vishniac and Santer, 1957) at pH 6.0. Glucose or fructose, autoclaved separately, were used as carbon sources in these media. Solid media contained 1.2% (w/v) agar. *Culture conditions.* All *A. niger* strains were grown at 30 °C. Liquid cultures were inoculated with 10^6 spores per ml and grown in orbital shakers at 220 rpm.

Genetic analysis. The procedures for genetic analysis have been described previously by Bos et al. (1988): after heterokaryon formation between a mutant and a master strain, somatic diploids were isolated. The heterozygous diploids were haploidized on CM using benomyl. Segregants were isolated, purified and subsequently tested for genetic markers and proteolytic activities using a dual substrate protease plate assay (van den Hombergh et al. 1995).

Transformation of *prt* strains. Transformable protease deficient recombinants containing the *pyr*A6 mutation were selected and purified, as described in *Genetic analysis*. After re-testing the genetic markers and the *prt* phenotype these strains were co-transformed with 1 μg of the plasmid pGW635, containing the functional *A. niger* orotidine-5'-phosphate-decarboxylase (*pyr*A) gene, and 20 μg of the *pkipel*B plasmid, containing an expression casette for the *A. niger* pectin lyase B gene, *pel*B (Kusters-van Someren et al. 1992). Transformants were randomly selected and subsequently purified by replating at low spore densities. Purified transformants were grown on LPM containing appropriate supplements and 2% (w/v) glucose as sole carbon source and at 20 h both culture samples and mycelium samples were taken. Expression and secretion of active PELB in culture supernatants was visualized using Western analysis and enzyme assays. Mycelia were used in a Northern blot analysis to compare *pel*B transcript levels.

Expression of PELB. Selected PELB expressing transformants were grown in 200 ml LPM cultures as described above. Culture samples were taken at 20, 40 and 60 h and analyzed by Western blotting and pectin lyase enzyme assays. *Western analysis.* Aliquots of supernatants were subjected to SDS-PAGE, the protein was electroblotted onto a nitrocellulose membrane (Hybond-C, Amersham) and incubated with polyclonal mouse antibodies raised against purified PELB. Following incubation with alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma), PELB bands were visualized following the recommendations supplied with the alkaline phosphatase. *Enzyme assays.* Active pectin lyase B activity was measured using highly esterified pectin (DE 94.6%) as described by Kester and Visser (1994). Enzyme activities of pectin lyase B were expressed in international units using a molar extinction coefficient of 5500 M^{-1} cm^2 for the unsaturated product (Van Houdenhoven, 1975).

Northern analysis.
Culture conditions I: prt:pkipelB transformants. All selected *prt:pkipelB* transformants were grown on LPM. After 20 h mycelium was harvested and used for Northern analysis. *II: prt mutants*. All *prt* mutants were grown on liquid CM containing 2% (w/v) glucose and appropriate supplements. After 20 h, mycelia were harvested, washed and transferred to MM also containing the appropriate supplements and either 0.5% (w/v) BSA (Boehringer, fraction V) or 0.5% (w/v) elastin (Fluka) but no carbon source. Transfer cultures were harvested after 6-8 h and mycelium was immediately frozen in liquid nitrogen. *RNA isolation and Northern analysis*. Total RNA was prepared from mycelium, pulverised under liquid nitrogen, using TRIzol (GibcoBRL) according to the instructions of the supplier. For Northern analyses total RNA was loaded on formaldehyde-agarose gels (Sambrook et al., 1989), transferred to Hybond-N membranes (Amersham) and UV-cross linked. Hybridization and washing of the membranes was performed essentially as described by Sambrook et al. (1989). Membranes were (pre-)hybridized in (pre-)hybridization buffer containing 0.75 M NaCl / 50 mM NaH_2PO_4 pH 7.4 / 10 mM EDTA / 0.1% (w/v) SDS / 2xDenhardt's / 10% (w/v) dextran sulphate / 50% (w/v) formamide at 42 °C. Membranes were washed down to 0.2xSSC / 0.5% (w/v) SDS at 65 °C. *Probes*. Specific DNA fragments were labelled with ^{32}P using random hexanucleotide priming (Sambrook et al., 1989). *pel*B: a 0.87 kb *Sph*I-*Sma*I fragment from plasmid pGW830 (Kusters-van Someren et al., 1992); *pep*A: a 0.49 kb *Cla*I-*Hin*dIII fragment from plasmid ptz18A400, containing the complete *A. niger pep*A gene (kindly provided by Dr. Buxton); *pep*B: a 0.73 kb *Bgl*II-*Sal*I fragment from plasmid pFBY175ER, containing the the *A. niger pep*B gene (kindly provided by Dr. Buxton); *pep*C: a 0.56 kb *Hpa*I-*Hin*dIII fragment from plasmid pANB1 (Frederick et al., 1993); *pep*D: a 0.38 kb *Kpn*I-*Eco*RV fragment from plasmid PEPD12 (Jarai et al., 1994a); *pep*E: a 0.5 kb *Eco*RI fragment from plasmid pIM623, containing the complete *pep*E gene (Jarai et al., 1994b); *cpy*: a 2.0 kb product containing the complete *cpy* coding region (Yaver et al., 1995) generated by PCR as described by (Fraissinet-Tachet et al. unpublished results); 28S rDNA: the 0.9 kb *Eco*RI fragment of the *Agaricus bisporus* 28S gene (Acc. No. X91812)

Southern analysis. Strains were grown on CM containing appropriate supplements and 2% (w/v) glucose. After 20 h of growth mycelium was harvested, frozen in liquid nitrogen, pulverised and used for chromosomal DNA extraction as detailed by de Graaff et al. (1988). Chromosomal DNA was restriction digested, separated by agarose gel electrophoresis, blotted to Hybond N membranes (Amersham) and hybridized under stringent condtions (Sambrook et al., 1989) with the *pel*B probe.

CHEF-Southern analysis. CHEF-southern analysis was performed according to Verdoes et al. (1994), using a CHEF CRII apparatus and a Model 1000 Mini Chiller (Bio-rad). Conditions for preparation of intact chromosomal DNA and CHEF electrophoresis were as described by Debets et al. (1990) with minor modifications as detailed by van den Hombergh et al. (1996b).

Results

Transformation of *prt* mutants

In previous experiments carried out to allocate the *prt* mutations on one of the eight linkage groups in *A. niger*, the individual *prt* mutants were crossed with tester strain NW136 which contains the *pyr*A6 mutation. Recombinants containing both the *prt* and the *pyr*A6 mutation were isolated for the crosses with the *prt*A10, *prt*B26, *prt*C7, *prt*E15 and *prt*F28. Recombinants were purified, retested and those containing a limited number of additional genetic markers were selected. These *prt*, *pyr*A6 recombinants were then co-transformed with plasmids pGW635

(Goosen et al., 1987) and *pkipel*B (Kusters-van Someren et al., 1992) containing the *A. niger pyr*A selection marker and an expression cassette for the *A. niger* pectin lyase B (*pel*B) gene, respectively. Transformants expressing the *pel*B gene enable analysis of the *in vivo* degradation and comparisons with the *in vitro* degradation data. In the *pkipel*B expression cassette the *pel*B coding region is inserted between the *A. niger* pyruvate kinase (*pki*A) promoter and terminator, allowing high constitutive gene expression on media containing glucose. For all six *prt* strains transformants were recovered, although in some of the *prt* mutants the transformation frequencies were low compared to wild type. Subsequently, randomly selected transformants were purified and screened for expression and secretion of functional PELB.

Selection of *prt-pkipel*B transformants

In *Aspergilli*, the transformants obtained are usually stable as the DNA which is introduced readily integrates into the genome upon transformation. However, often a large variation in site of integration and production levels between transformants is observed (Davies, 1994), indicating that apart from (functional) copy number, the site of integration affects production levels. Initially, all selected *prt*:*pkipel*B transformants and a multicopy *pkipel*B transformant in a wild type protease background (NW219:*pkipel*B/4), were grown on LPM. After 20 h of growth both the mycelium and the culture filtrates were harvested and the presence of PELB protein in the culture filtrates was visualized by Western analysis and subsequent immunological staining of PELB. Pectin lyase enzyme assays proved that all *prt-pkipel*B transformants expressed and secreted functionally active PELB (data not shown). Total RNA was isolated from the harvested mycelia and Northern analysis (*vide intra*) allowed selection of transformants which expressed *pel*B mRNA levels comparable to the NW219:*pkipel*B/3 multicopy transformant, thus correcting for differences in site of integration and copy number (Fig. 9.1).

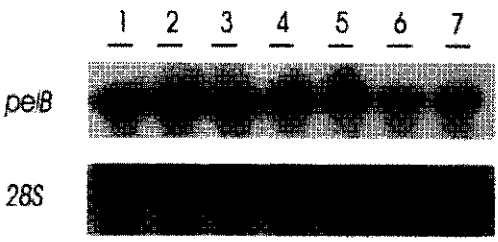

Fig. 9.1. Northern analysis of *pel*B expression in the selected *prt* transformants. Northern analysis of total RNA (10 μg/track) from N219-*pkipel*B/4 and selected *prt-pkipel*B transformants after growth on LPM medium. (**A**) Northern blot probed with *pel*B; (**B**) Northern blot probed with 28S rDNA; track order: 1, NW219:*pkipel*B/3 ; 2, NW207:*pkipel*B/6 ; 3, NW150:*pkipel*B/4 ; 4, NW151:*pkipel*B/1 ; 5, NW152:*pkipel*B/3 ; 6, NW153:*pkipel*B/6 ; 7, NW154:*pkipel*B/9.

PELB expression

The six selected *pkipel*B transformants, one for each of the *prt* mutants, and the N219:*pkipel*B/3 control strain were grown in 200 ml LPM cultures and *pel*B expression was examined after 20, 40 and 60 h (Table 9.1). This experimental setup ensured that due to depletion of the carbon source after 20 h (residual) protease expression increased and degradation of produced PELB can be studied *in vivo*. Enzyme assays showed that the expression had increased in several *prt* complementation groups. The highest expression was observed in the *prt*A10 and *prt*F28 protease mutant background. Furthermore, the amount of protein formed in several *prt* mutants increased for at least 60 h compared to the wild type protease background in which case PELB is completely degraded between 20 and 40 h.

Table 9.1. Expression and secretion of active PELB from *prt-pkipel*B transformants

Strain	Mutation	Residual protease act.[1]	PELB activity[2]		
		%	20 h	40 h	60 h
NW219:*pkipel*B/3	-	100	1.0	d	d
NW207:*pkipel*B/6	*prt*A10	21	2.5	7.0	11.0
NW150:*pkipel*B/4	*prt*B26	2	1.1	5.2	8.8
NW151:*pkipel*B/1	*prt*C7	52	1.0	0.2	d
NW152:*pkipel*B/3	*prt*D32	4	1.4	5.0	8.0
NW153:*pkipel*B/6	*prt*E15	63	1.0	d	d
NW154:*pkipel*B/9	*prt*F28	3	1.7	7.6	12.8

[1], van den Hombergh et al. (1995)
[2], PELB activity in LPM cultures is expressed as the ratio PELB activity compared to the activity of NW219/*pkipel*B/3 at 20 h in cleared culture supernatant. d, no PELB activity present due to complete degradation.

The *pel*B expression data correlated well with the *in vitro* degradation data (van den Hombergh et al., 1995). In a *prt*A, *prt*B, *prt*D and *prt*F background improved *pel*B expression was observed, whereas in the *prt*C7 and *prt*E15 mutants no significant improvement was seen (see Table 9.1). This correlation between *in vivo* and *in vitro* results led us to test the five double *prt* mutants as previously described (van den Hombergh et al., 1995, 1996a) using the *in vitro* assay, to screen for further improvements compared to the single *prt* mutants (Table 9.2). These experiments indicate that further reduction of PELB degradation was achieved, to some extent in the *prt*C7/-*prt*35 double mutant but especially in the *prt*A10/*prt*F28 and the *prt*C7/*prt*A33 double mutants.

Table 9.2. Reduced PELB degradation in *prt* double mutants

Strain	*prt* mutation	Res. act.[1]	Residual PELB activity[2]							
		%	serial dilution							
			control	1	2	10	50	100	500	1000
N402	-	100	**103**	0	0	0	0	0.5	3	5
NW222	*prt*A10	21	**101**	0	0	0	0.2	1	6	20
NW221	*prt*C7	53	**97**	0	0	0	0	0.1	1	4
NW228	*prt*F28	3	**98**	4	9	59	**103**	**100**	**98**	**110**
NW233	*prt*C7 *prt*A33	1	**98**	42	**94**	**101**	**106**	**101**	**99**	**104**
NW234	*prt*C7 *prt*-35	3	**106**	9	14	76	**98**	**102**	**98**	**101**
NW237	*prt*A10 *prt*C14	4	**100**	3	8	11	46	**94**	**103**	**100**
NW238	*prt*A10 *prt*E15	4	**103**	0.1	1	5	21	68	**106**	**101**
NW239	*prt*A10 *prt*F28	2	**97**	36	82	**106**	**103**	**100**	**104**	**98**

[1], percentage residual protease activity compared to wild type strain N402.

[2], *in vitro* degradation of PL3 was performed and monitored as described by van den Hombergh et al., 1995. Pure PELB was incubated in serial dilutions of supernatants from wheat bran cultures. After incubation (5 h) the residual PELB acitivity was measured and expressed as the percentage of residual activity compared to the control. Non-affected activities are indicated in bold; cont, as a control the medium sample was boiled prior to incubation with PELB.

Northern analysis *prt* strains; single mutants

Although biochemical characterization of the residual extracellular protease spectrum in *prt* mutants demonstrated reduction of extracellular acid protease activity varying from 2-80% compared to wild type, the actual function of the individual *prt* genes is unknown. It has been postulated that several *prt* mutations are regulatory or cascade activation mutations (van den Hombergh et al., 1995). To determine whether some of these mutations have indeed a (regulatory) effect on the level of transcription, the expression of all cloned *A. niger* protease genes was studied at the mRNA level. From all seven complementation groups the most severe alleles (*prt*A10, *prt*B26, *prt*C14, *prt*D32, *prt*E30, *prt*F28 and *prt*G24) were selected and analyzed in a series of Northern experiments. All cloned extracellular protease genes were found to be regulated by carbon catabolite repression, nitrogen metabolite repression, pH regulation and induction at the

level of protein (Jarai and Buxton, 1994; van den Hombergh et al., 1994). Therefore, a growth transfer experiment was performed in which pregrown mycelium was transferred to media without any repressing carbon and nitrogen source and which contained different proteins, i.e. BSA or elastin, to induce protease biosynthesis. For *A. niger* wild type it was shown before that these transfer conditions result in high expression of proteases (Jarai and Buxton, 1994; Jarai et al., 1994b; van den Hombergh et al., 1994). In order to have the complete residual protease spectrum expressed, all seven single *prt* mutants were grown for 20 h, together with the wild type strain N402, on MM containing appropriate supplements, yeast extract and glucose. Harvested mycelium was transferred to MM cultures in which the ammonium chloride was replaced by sodium nitrate and which were supplemented with either BSA or elastin as protein inducer. After 6-8 h of growth mycelia and culture supernatants were collected. Total RNA was isolated from the mycelia and analyzed by Northern blotting (Fig. 9.2).

The Northern analysis demonstrated that in the wild type strain, the expression of the genes encoding extracellular proteases (*pep*A, *pep*B, *pep*D and *pep*F), was higher on media containing elastin as protein inducer compared to BSA. In the *prt*A10 mutant the elastin-induced transcript levels of *pep*A were hardly affected compared to wild type, whereas the BSA-induced transcript levels were not detectable. The expression of *pep*D was severely reduced whereas no expression of *pep*B was observed. The *pep*F transcript levels however, appeared to be increased. The *prt*B mutant was affected in the transcript levels of all the extracellular proteases tested; *pep*A and (elastin induced) *pep*D mRNA levels were strongly reduced whereas *pep*B and *pep*F transcripts could not be detected. Surprisingly, the *pep*D expression in the *prt*B26 background was higher when BSA was used as an inducer, this in contrast to the situation in wild type, where elastin induction resulted in the highest protease expression. In the *prt*C14 mutant the expression of *pep*D and *pep*F and especially of *pep*B, did increase. Although no large effect was observed in the elastin-induced culture on the expression of *pep*A, the BSA-induced culture resulted in this case in higher expression levels. In the *prt*D32 mutant the *pep*B and *pep*F transcript levels were absent and hardly detectable, respectively. However, *pep*A was only affected when induced with BSA whereas *pep*D expression compared to wild type increased on elastin. In the *prt*E30 mutant the *pep*A and *pep*F transcripts were virtually identical to or just slightly higher than wild type levels. For *pep*B and *pep*D the elastin-induced transfer cultures resulted in clearly elevated mRNA levels. The *prt*F28 mutant showed transcript levels comparable to those in the *prt*D32 mutant (i.e. no detectable *pep*B and *pep*F mRNAs and elevated levels of elastin induced *pep*D transcripts, respectively). For *pep*A only the BSA-induced transcript levels decreased, similar to the situation in the *prt*A10 mutant. Finally, the *prt*G24 strain was not or only slightly affected in *pep*A and

*pep*D expression, it showed strongly elevated levels of *pep*B mRNA whereas little *pep*F mRNA could be detected. This analysis indicates that under the conditions tested the reduced protease activities in several *prt* mutants are not reflected by reduced transcription levels for these extracellular proteases.

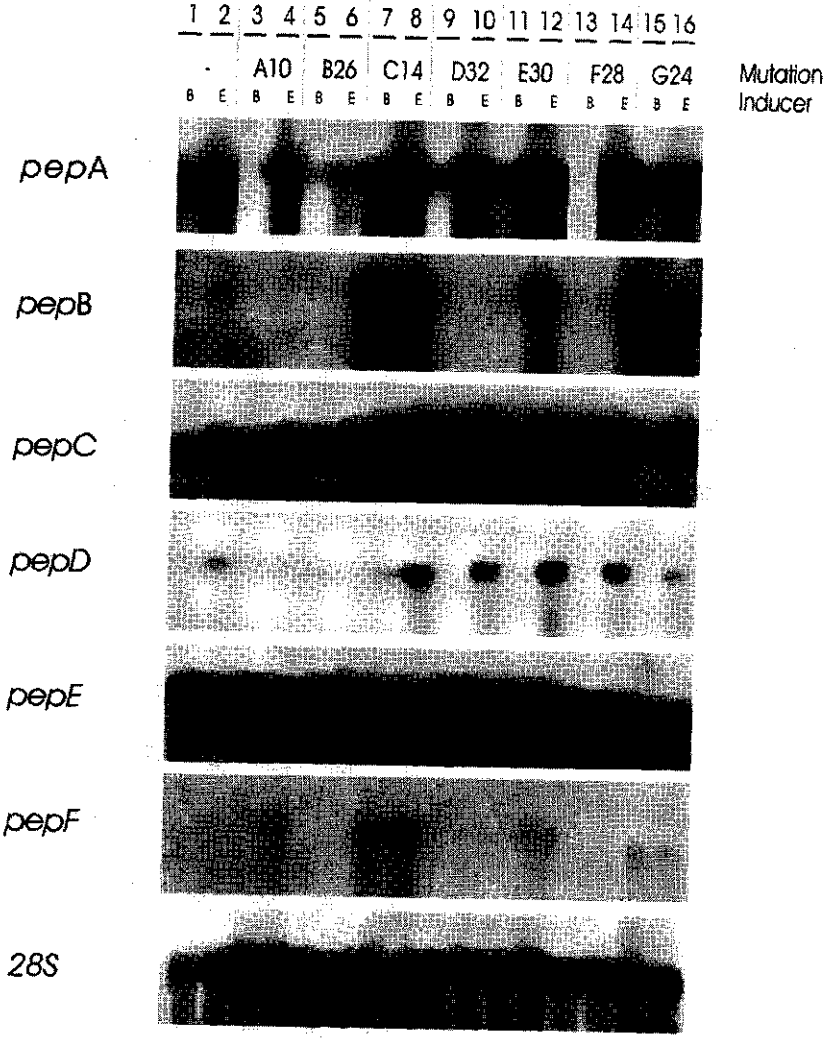

Fig. 9.2. Protease expression in single *prt* mutants. Northern analysis of single *prt* mutants. Total RNA samples extracted from BSA-induced (B) and elastin-induced (E) transfer cultures were separated in agarose gels, blotted and hybridized separately with the *pep*A, *pep*B, *pep*C, *pep*D, *pep*E, *pep*F, *cpy* and *28S* rDNA probes. Track order; 1,2: N402 [-]; 3,4: NW222 [*prt*A10]; 5,6: NW227 [*prt*B26]; 7,8: NW224 [*prt*C14]; 9,10: NW232 [*prt*D32]; 11, 12: NW230 [*prt*E30]; 13,14: NW228 [*prt*F28]; 15,16: NW226 [*prt*G24]. Due to differences in track order for the *pep*A Northern this blot is a composition.

The *pep*C, *pep*E and *cpy* encoded proteases are the homologues of *S. cerevisiae ysc*B, *ysc*A and *cpy* genes and therefore probably also located in the vacuole (see also chapter 7). In wild type the expression of the *pep*E and *pep*C genes has been shown to be constitutive, with only very limited, growth-related variations (Jarai et al., 1994b) and a very similar regulation has been observed for the *cpy* gene (data not shown). For these three intracellular proteases no significant effects compared to the 28S rDNA loading control have been observed in the protease mutants. A summary of all the effects observed is given in Table 9.3.

Double prt mutants

To test whether a combination of *prt* mutations has more pronounced effects on the expression of the various proteases, double mutants were analyzed in similar transfer experiments as described for the single *prt* mutants (Fig. 9.3 and Table 9.3). Since the less severe *prt*C7 and *prt*E15 alleles were used to make strains NW233 (*prt*C7, *prt*A33), NW234 (*prt*C7, *prt-35*) and NW238 (*prt*A10, *prt*E15), the single *prt*E15 and *prt*C7 mutants were also included in this analysis.

In the *prt*E15 mutant no differences between BSA and elastin induction were observed for the *pep*A, *pep*B and *pep*D genes. Only for the *pep*F gene increased transcription is observed after elastin-induction. In the *prt*A10-*prt*C14 double mutant the transcription levels of several extracellular protease encoding genes did increase similar to the situation in the *prt*C14 single mutant. Also in the *prt*E15-*prt*A10 and the *prt*A10-*prt*F28 double mutants, higher levels of *pep*A, *pep*B and *pep*D were observed after elastin induction, similar to wild type. However, also differences were found. On elastin containing transfer cultures the expression levels of *pep*A and *pep*B are higher compared to the *prt*E15 single mutant. The expression of *pep*F increased in a *prt*E15-*prt*A10 genetic background, whereas in the *prt*A10-*prt*F28 double mutant no *pep*F mRNA was detected. In the *prt*C7 mutant the transcription of *pep*A is not significantly changed although similar to the situation in *prt*E15 the differences between elastin and BSA induction are less; *pep*B transcription is reduced and *pep*F and *pep*D transcription were not detected. The introduction of a second mutation *prt*-35 did increase transcriptional levels of *pep*A, *pep*B and *pep*D. No significant changes in transcriptional levels of the *pep*C, *pep*E and *cpy* genes were observed in the double mutants, similar to the situation in the single *prt* mutants.

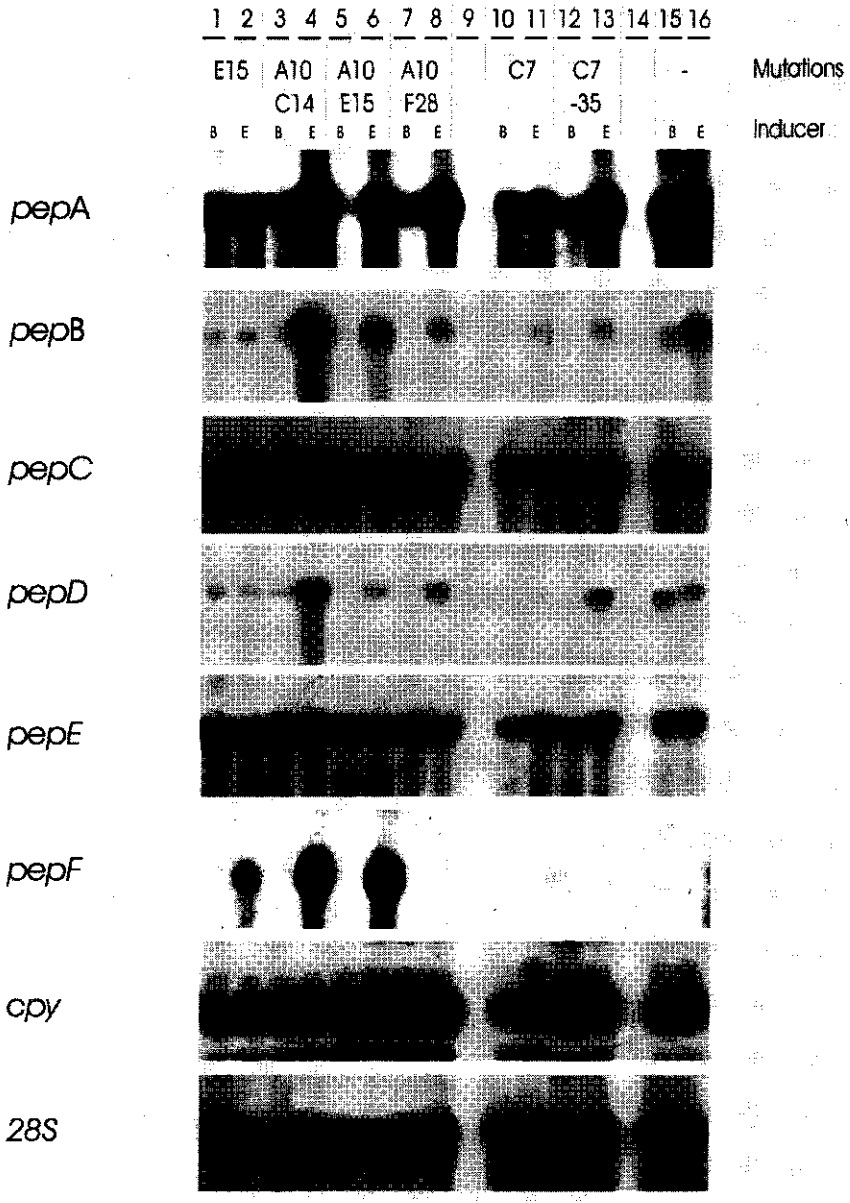

Fig. 9.3 Northern-blot analysis of double *prt* mutants. Northern analysis of single *prt* mutants. Experiments were carried out as described in Fig. 9.2. Track order; 1, 2: NW225 [*prt*E15]; 3, 4: NW237 [*prt*A10 *prt*C14]; 5, 6: NW238 [*prt*A10 *prt*E15]; 7, 8: NW239 [*prt*A10 *prt*F28]; 9, -; 10, 11: NW221 [*prt*C7]; 12, 13: NW234 [*prt*C7 *prt*-35]; 14: - ; 15, 16: wild type [-].

Table 9.3 Protease gene expression in *prt* strains

	*pep*A		*pep*B		*pep*C		*pep*D		*pep*E		*pep*F		*cpy*	
	BSA	Elastin	BSA	Elastin	BSA	Elastin	BSA	Elastin	BSA	Elastin	BSA	Elastin	BSA	Elastin
wild type	+	+++	+/-	+	+++	+++	-	+	+++	+++	+/-	+	+++	+++
*prt*A10	<	.	.	<	.	.	.	<	.	.	.	./>	.	.
*prt*B26	<	<	.	<	.	.	>	<	.	.	.	<	.	.
*prt*C7	.	<	<	<	.	.	.	<	.	.	.	<	.	.
*prt*C14	./>	.	>	>	.	.	>	>	.	.	>	>	.	.
*prt*D32	<	.	./<	<	.	.	.	>	.	.	.	<	.	.
*prt*E15	.	<	./<	<	.	.	>	<	.	.	.	>	.	.
*prt*E30	.	.	.	>	.	.	.	>	.	.	.	.	.	.
*prt*F28	<	.	.	<	.	.	.	>	.	.	.	<	.	.
*prt*G24	.	.	>	>	.	.	.	.	.	.	.	<	.	.
*prt*A10 *prt*E15	<	.	<	.	.	.	<	.	.	.	.	>	.	.
*prt*A10 *prt*C14	.	./>	.	>	.	.	.	>	.	.	.	>	.	.
*prt*A10 *prt*F28	.	.	<	<	.	.	./<	.	.	.	.	<	.	.
*prt*C7 *prt*-35	.	.	<	<	.	.	.	.	.	.	.	<	.	.

Expression in *prt* strains is compared to wild type (N402); -, no expression detected; +, expression detected; ., expression unchanged; <, reduced expression; >, increased expression.

CHEF-Southern analysis

In CHEF-Southern experiments using the strains with introduced electrophoretic mobility variation of chromosomes described by Verdoes et al. (1995), the protease genes *pep*A, *pep*B, *pep*C, *pep*D, *pep*E and *cpy* were allocated to one of the eight linkage groups in *A. niger*. In Table 9.4 these results are summarized and compared to the linkage data for all the seven *prt* complementation groups. For the *pep*A, *pep*B and *pep*E genes, the CHEF-Southern allocation results were confirmed via crosses between strains carrying individual disruptions for these three proteases and tester strains containing markers on every linkage group (van den Hombergh et al., unpublished results).

Table 9.4. CHEF-Southern allocation of the cloned protease (*pep*) genes to one of the eight linkage groups in *A. niger*

linkage group	I	II	III	IV	V	VI	VII	VIII
genetic markers	*fwn*A	*gox*C *arg*H	*bio*A *lys*A *pyr*A *csp*A	*leu*A *his*C	*phe*A	*pdx*A	*nic*B	*trp*A
protease genes, mutations	*pep*A[1] *pep*D *prt*C	*pep*B[2]	*prt*B *prt*E *prt*G *are*A[3]	*pep*C *pep*E[4] *cpy* *cre*A[5] *pac*C[6]	*prt*F			*prt*A *prt*D

The *pep*F gene is omitted from the table as CHEF-Southern analysis was not conclusive. [1], independently demonstrated by the recombination frequency (3.4%) found between the *fwn*A6 mutation and a disruption of the *pep*A locus (van den Hombergh et al., unpublished results); [2], independently demonstrated by the recombination frequency (5.0%) found between the *gox*C17 mutation and a disruption of the *pep*B locus (van den Hombergh et al., unpublished results); [3], (van de Vondervoort, unpublished results); [4], independently demonstrated by the recombination frequency (0%) found between the *leu*A1 mutation and a disruption of the *pep*E locus. (van den Hombergh et al., unpublished results); [5], (Vanhanen et al., unpublished results); [6], van den Hombergh et al. (1996b)

The results from Table 9.4 demonstrate that the *pep*A, *pep*D and the *prt*C genes are located on linkage group I. Based on the reduced extracellular protease activities in *prt*C mutants it cannot be excluded that the *prt*C mutations are allelic to *pep*A or *pep*D. However, it appears unlikely as Northern analysis demonstrates effects in *prt*C mutants at the transcriptional level of the *pep*A,

*pep*D and even the *pep*B gene which is located on linkage group II. Furthermore, no *pep*D-encoded alkaline protease activity was detected upon the biochemical characterization of *A. niger* wild type protease spectrum, excluding that a reduction to 32% residual extracellular protease activity is caused by a mutation (*prt*C14) in the *pep*D gene. The *pep*B gene is located on linkage group II and no *prt* mutations have been isolated which also reside on linkage group II, probably because even a *pep*B gene disruption only reduces the extracellular protease activity with less than 10% (van den Hombergh et al., unpublished results) and only *prt* mutants with 80% or less extracellular protease activity were selected. The situation on linkage group III is more complicated since three individual *prt* genes (*prt*B, E and G) and the wide domain nitrogen metabolite repressor gene *are*A are allocated to this linkage group. Several extracellular proteases are regulated by AREA implicating that one of the *prt* genes on linkage group III could be allelic to *are*A. Therefore we have analyzed all the recombination data avalaible for the three *prt* genes and the *are*A gene (see Fig. 9.4).

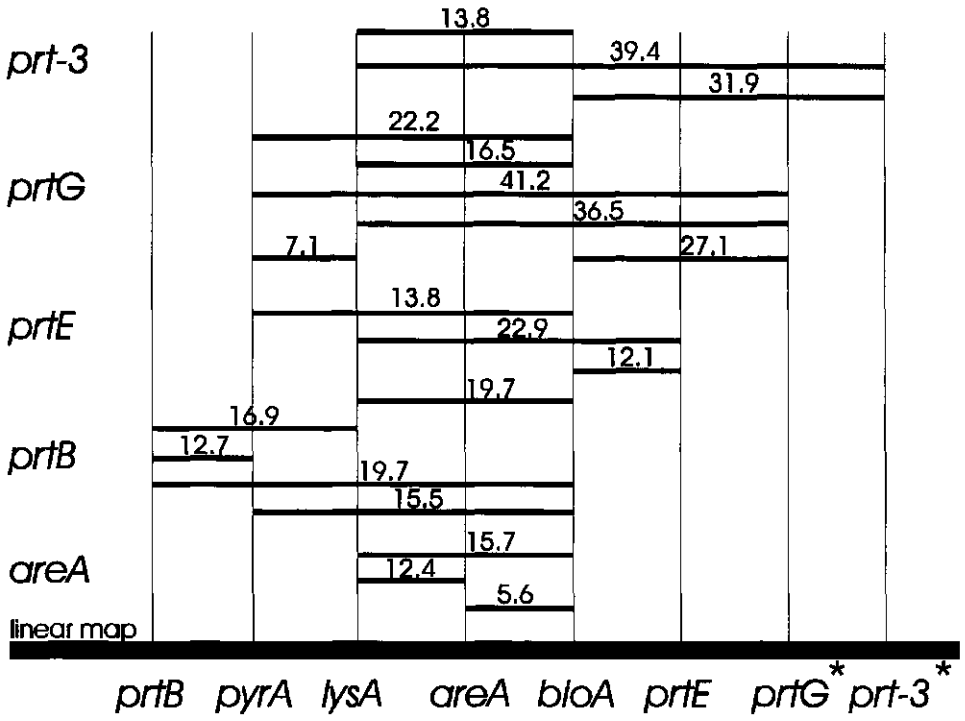

Fig. 9.4 Linear order of genes on linkage group III. Recombination percentages between two genes are indicated above the solid line which connects the two genes. Markers on different linkage groups give free recombination, 35-65%, (data not shown). Linkage for the *prt*-3 has not been investigated further because of poor contrast between this *prt* mutation and wild type on casein gelatin plates (van den Hombergh et al., 1995).*, another cross clearly gave better linkage with *bio*A1 or *thi*A1 (< 25%).

From the results presented in this figure it appears that the linear map of the individual genes determined for linkage group III excludes *prt*B, *prt*E and *prt*G from being allelic to *are*A. Both the *prt*E and *prt*G genes are located more distal from the centromere than the *bio*A marker on the left arm of linkage group III, in contrast to *are*A. The *prt*B gene is probably located more distal from the centromere than the *lys*A marker on the right arm of linkage group III.

All genes encoding the intracellular (vacuolar) proteases are located on linkage group IV, which could indicate clustering. No structural protease genes have been assigned yet to the other two linkage groups, V and VIII, on which protease mutations have been localised. The CHEF Southern results also demonstrate that several *prt* mutations are not allelic to any of the cloned structural protease genes or any of the wide domain regulatory genes, *are*A, *cre*A and *pac*C.

Discussion

Comparison of the *in vitro* degradation data and the *in vivo* PELB expression data for all six *prt* complementation groups tested shows good correlation. If such a correlation between *in vitro* and *in vivo* data will also be observed for other proteins which are momentarily tested, the *in vitro* degradation assay will provide a rapid and reliable screening method to select the *prt* mutant best suited for the production of a particular protein. The degradation in the single *prt* mutants is not absent, indicating the need for additional reduction of proteolytic activities either via construction of multiple *prt* deficient mutants and/or *pep* disruptants or via additional down-regulation of proteases during the expression of the genes of interest. Reduced degradation of PELB in double *prt* mutants demonstrates that combination of several characterised *prt* mutations can further improve expression. Considering the improved expression in several *prt* genetic backgrounds, a detailed characterization of the *prt* mutants was necessary.

The Northern analysis showed that several *prt* mutations have posttranscriptional effects on the expression of the extracellular proteases. Only for the *prt*B mutation a large effect is observed at the transcriptional level (complete absence of extracellular protease gene transcription). It has already been shown that all extracellular *A. niger* proteases show complex regulation, in which several wide domain regulatory mechanisms are involved. Carbon catabolite repression, nitrogen metabolite repression and pH regulation, mediated via *cre*A, *pac*C and *are*A genes, respectively, have been demonstrated for the *pep*A, *pep*B and *pep*F genes. Both the *cre*A and *pac*C genes have been allocated to linkage group IV (van de Vondervoort, unpublished results; van den Hombergh et al., 1995), excluding that *prt* mutations are allelic to *cre*A or *pac*C. The *are*A gene is allocated to linkage group III, where also the *prt*B, *prt*E and *prt*G mutations are located. From the recombination frequencies between the *pyr*A, *lys*A and *bio*A markers and the

prt mutations it is concluded that probably all three *prt* genes are not allelic to *are*A. However, the linear order is not absolute since the frequency at which recombinants are isolated is partly dependent on clonal segregation (Debets et al., 1989). For the *prt*-3 mutation it is unclear whether this gene is located on linkage group III, but if this is true, the high recombination percentages with the *lys*A and the *bio*A markers indicate that this mutation is probably located at a large distance from the *bio*A marker and is therefore probably not allelic to *prt*E or *prt*G. However, complete proof that *prt*-3 belongs to a novel eighth *prt* complementation group awaits further analysis.

Cohen (1973) has reported differences in protease expression in relation to changes in sulphur and phosphate sources used in the culture media, possibly indicating that sulphur metabolite repression and phosphate repression, other wide domain regulatory systems, are also involved in overall protease regulation. Whether any of the *A. niger* proteases is regulated by sulphur and/or phosphate sources and whether any of the *prt* genes is involved in sulphur or phosphate regulation is unknown, but similar to the situation for *are*A, certain mutations in a positive regulator could result in decreased gene expression of the genes under its control. In addition to the wide domain regulatory systems the specific induction by protein is clearly demonstrated by the effects of addition of BSA or elastin to the culture media. In some of the *prt* mutants the induction is changed compared to wild type. As six of the *prt* mutants (*prt*A,B,D,E,F-,G) have earlier been classified as regulatory or cascade activating mutations (- i.e. their residual activities indicate that several proteolytic functions are affected) combined with the fact that although several mutants have *pep*A mRNA levels comparable to wild type, no or strongly reduced PEPA levels are observed in culture supernatants, these regulatory effects are probably operating at a post-transcriptional level. The isolation and characterisation of at least five new genetic loci involved in protease expression, in addition to the wide domain regulatory functions already mentioned, demonstrates the complexity of protease expression in *A. niger*.

The fact that no significant changes were observed in the mRNA levels of all three intracellular proteases is probably a result of the screening strategy as all the *prt* mutants were selected for reduced extracellular proteolytic activities. The CHEF-Southern analysis has assigned all presumably vacuolar proteases to linkage group IV for which no *prt* genes have been isolated. Thus the *prt* genes do not encode any of the three cloned intracellular proteases and judged from the Northern analysis the *prt* genes are not involved in transcriptional regulation of these genes. If the reduced intracellular acid protease activities in several *prt* mutants (van den Hombergh et al., 1995) can be attributed to reduced vacuolar protease actvities, the effects of these *prt* mutations on vacuolar proteases have also to be posttranscriptional. However, reduced levels of cell membrane-

bound and periplasmic proteases could also be the cause of the reduced intracellular protease activities as they were measured in cell free extracts. Now that we have seen how mutations in these *prt* loci affect the regulation of protease expression and improve protein production, it will be interesting to clone these genes and to identify their function.

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10 Identification, cloning and analysis of the *Aspergillus niger* *pac*C wide domain pH regulatory gene[1]

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Abstract

The wide domain regulatory gene implicated in modulating gene expression in response to ambient pH has been cloned and sequenced from the industrially useful filamentous fungus *Aspergillus niger*. This gene, *pac*C, is able to restore a *pac*C$^+$ phenotype to *A. nidulans* *pac*C^{c}11 and *pac*C^{c}14 mutants with respect to extent of conidiation, conidial pigment intensity and acid phosphatase regulation. The *pac*C gene of *A. niger* comprises three exons, encodes a three zinc finger protein of 667 amino acids, and shows external-pH-dependant regulation of expression, mRNA levels being elevated under alkaline conditions and considerably reduced under acidic conditions. The occurrence of PacC consensus binding targets within the sequences upstream of *pac*C might indicate autoregulation.

Introduction

Classical genetic analysis of pH regulation in the filamentous fungus *Aspergillus nidulans* has previously indicated the existence of six genes involved in the pH dependant modulation of expression of specific structural genes encoding certain extracellular enzymes (e.g. acid and alkaline phosphatases) and permeases such as the γ-amino-*n*-butyrate permease encoded by *gab*A (Caddick and Arst, 1986; Caddick et al., 1986). These loci were divided into two distinct groups based on the phenotypes of mutations. One group of mutations, designated *pal*, were shown to mimic the effects of growth under acidic conditions whereas mutations at a single locus, designated *pac*C, resulted in the phenotypic mimicry of growth under alkaline conditions (Caddick et al., 1986). More recently, the existence of six discrete *pal* genes, namely *pal*A, B, C, F, H and I has been established (Arst et al., 1994) and it has also been noted that *pac*C mutations themselves can be divided into two classes defined by the phenotype when cultured at neutral pH: those that mimic growth under alkaline conditions - the type originally observed (Caddick et al., 1986) -

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and those, including a null allele, that mimic growth under acidic conditions (Tilburn et al., 1995). The epistasis of mutations in *pac*C to all acidity-mimicking *pal* mutations and the heterogeneity of *pac*C mutant phenotypes led to the hypothesis that *pac*C is a wide domain regulatory gene (Caddick et al., 1986), which is strongly supported by the finding that it encodes a zinc-finger-containing sequence-specific DNA binding protein (Tilburn et al., 1995). The importance of pH regulation in the production of secondary metabolites has been highlighted by the observation that the pH of the growth medium and mutations leading to altered reponses to ambient pH affect penicillin G levels (Shah et al., 1991; Espeso et al., 1993) and modulate expression of at least one of the genes involved in penicillin biosynthesis in *A. nidulans* i.e. isopenicillin N synthetase (*ipn*A) (Espeso et al., 1993).

Aspergillus niger is a commercially important filamentous fungus related to the genetically amenable model organism *A. nidulans*. Given the industrial importance of extracellular enzyme production in *A. niger* and its related variants, the potential role of the *A. niger* equivalent to the *A. nidulans pac*C gene ($pacC_{nid}$) as a regulator of gene expression is of considerable interest, particularly as several extracellular proteases are pH regulated (Jarai and Buxton, 1994; van den Hombergh et al., 1994). Whereas *A. nidulans* is only a modest producer of organic acids, *A. niger* produces citric acid prolifically, resulting in considerable acidification of its environment (Kubicek et al., 1994). Cloning of the *A. niger pac*C gene ($pacC_{nig}$) gene is an essential first step for the detailed molecular genetic investigation of the regulation of pH response in *A. niger*. We report here the isolation and functional identification of this gene, its DNA sequence and pattern of expression.

Materials and Methods

Southern hybridization. Genomic restriction enzyme digests were run on a 1% (w/v) agarose gel which was UV irradiated and subsequently vacuum blotted to Hybond-N membrane (Amersham). Pre-hybridization was done overnight at 56°C in 4 x SET [20 x SET: 3 M NaCl / 0.4 M Tris pH 7.8 / 20 mM EDTA] / 5 x Denhardt's [0.1% (w/v) Ficol 400 / 0.1% (w/v) polyvinylpyrrolidone / 0.1% (w/v) bovine serum albumin-fraction V] / 0.1% (w/v) sodium dodecylsulphate (SDS) / 0.1% (w/v) $Na_4P_2O_7 \cdot 10H_2O$ / 100 μg/ml denatured herring sperm DNA. Hybridization was done overnight under the same conditions with a labelled probe generated by random hexanucleotide priming (Feinberg and Vogelstein, 1983). Washing was carried out at the hybridization temperature in 4 x SET / 0.1% (w/v) SDS / 0.1% (w/v) $Na_4P_2O_7 \cdot 10H_2O$.

Cloning of *A. niger pac*C. The genomic library of *A. niger* N400 wild type strain was constructed by ligating partially *Sau*3AI-digested genomic DNA fragments into the λ replacement vector λEMBL4 cut with *Bam*HI (Harmsen et al., 1990). This genomic library was screened (total number of plaques equaled eight times genome size) under low stringent conditions as mentioned in 'Southern hybridization', using a 3.0 kb *Bam*HI/*Eco*RI fragment of plasmid p5 (containing $pacC_{nid}$) as a probe. Phage DNA mini-preps were performed as detailed in Sambrook et al. (1989). Isolated DNA was restriction enzyme digested, separated on 1% (w/v) agarose gels, alkaline vacuum blotted to Hybond-N membrane (Amersham) and probed at low

stringency (56°C in 4 x SET). Hybridizing bands were isolated by gel purification and electro-elution and subcloned into suitably restricted pEMBL18.

***Aspergillus* strains and transformation.** The strain numbers and genotypes of the *A. nidulans* strains used were: 407.17: *paba*A1, *arg*B2, *pac*C11; 407.18: *paba*A1, *y*A2, *arg*B2, *pac*C14, *cho*A1; WG164: *y*A2, *w*A3, *arg*B2; WG096: *paba*A1, *y*A2. See Clutterbuck (1993) for definition of gene symbols. *A. niger* strain N402 is derived from CBS 120.49 and carries the mutation *csp*A1 (Bos et al., 1988). Protoplasts were prepared and transformed by a modification of the method of Tilburn et al. (1983) as detailed in MacCabe et al. (1989).

DNA sequencing. DNA sequencing was performed using the dideoxynucleotide chain termination procedure (Sanger et al. 1977) employing universal and reverse primers and also dedicated 17-mer primers. Analyses of nucleotide and amino acid sequences were done using PC/Gene 6.60 (IntelliGenetics Inc.) and the Wisconsin Sequence Analysis Package (Genetics Computer Group).

Acid phosphatase analysis. Mycelia for acid phosphatase analyses were grown in minimal, phosphate-limited, citrate-buffered medium: 10 mM ammonium tartrate, 1% (w/v) glucose, 18.1 mM KCl, essential salts (Vishniac and Santer, 1957), 50 mM citrate pH 4.5, 100 μM sodium phosphate (pH 7) and appropriate supplements. 50 ml volumes were seeded to a spore density of $3x10^6$/ml and incubated in an orbital shaker at 37°C, 220 rpm for 25 h. Mycelia were harvested by vacuum filtration and frozen immediately in liquid nitrogen and ~0.5 g amounts pulverised for 2 minutes in a Braun dismembrator. The resulting powder was resuspended in 2 ml ice cold 50 mM Tris pH 8.2 and stored on ice for 0.5-2 h. Mycelial debris was removed by microcentrifugation for 5 min at 4°C. Supernatants were recovered and concentrated to final volumes of ~300 μl by Speedvac lyophilization after re-freezing in liquid nitrogen. 15 μl aliquots were run in the absence of SDS and ß-mercaptoethanol on non-denaturing 10% (w/v) polyacrylamide gels. After electrophoresis the gels were immersed in 0.6 M sodium acetate pH 4.8 for 30 min and then transferred to staining solution (0.6 M sodium acetate pH 4.8, 0.05% (w/v) sodium naphthyl acid phosphate, 0.5% (w/v) fast garnet GBC salt (Dorn, 1965).

Northern analyses. Spores of *A. niger* strain N402 were inoculated in 250 ml minimal medium (MM) [1% (w/v) glucose, 10 mM ammonium tartrate, 11 mM KH_2PO_4, 6.5 mM KCl, 2 mM $MgSO_4.7H_2O$, essential salts (Vishniac and Santer, 1957), pH 6] supplemented with 0.05% (w/v) yeast extract and incubated in an orbital shaker at 30°C for 20 h at 220 rpm. Mycelia were harvested by gentle vacuum filtration, washed briefly in MM, re-harvested, divided into three portions and transferred to 50 ml volumes of MM lacking yeast extract and buffered to pH 4.5, 6.5 and 8 with citrate, MES and TRIS, respectively, at 100 mM; in all three media the sodium ion concentration was 180 mM. After their transfer the cultures were incubated for a further period of 6 h under the same conditions as before, mycelia recovered by vacuum filtration to form a compacted mat, frozen immediately in liquid nitrogen and ~0.5 g amounts pulverised for 2 minutes in a Braun dismembrator. RNA was extracted according to the method of Cathala et al. (1983). The concentrations of all RNA preparations were estimated spectrophotometrically. RNA was glyoxalated and analysed on 1.4% (w/v) agarose gels run in 10 mM sodium phosphate pH 7.0 loaded with 10 μg per track as described by Sambrook et al. (1989). RNA was transferred to GeneBind membrane (Pharmacia) by vacuum blotting in the presence of 7.5 mM NaOH. Pre-hybridization and hybridization were done at 65°C in the same buffer described in 'Southern hybridization'; washing was done at the hybridization temperature for 20 min periods with 2 x SET / 0.1% (w/v) SDS / 0.1% (w/v) $Na_4P_2O_7.10H_2O$ followed by 0.5 x SET / 0.1% (w/v) SDS / 0.1% (w/v) $Na_4P_2O_7.10H_2O$. All blots were autoradiographed wet to facilitate re-probing.

Reverse transcription (RT)/PCR analyses. RT/PCR was performed on polyA$^+$-selected RNA using the enzyme Tth polymerase (Boehringer) according to the manufacturer's instructions. For primers PACFWD and PACREV the RT reaction was run using 250 ng RNA (polyA$^+$) at 68°C for 25 min followed by 35 cycles of PCR using a program comprising 95°C 1 min, 60°C 1 min, 72°C 1 min.

Results and Discussion

Identification and cloning of *A. niger pac*C

In order to identify an *A. niger* gene equivalent to *pac*C_{nid}, a Southern blot of restriction enzyme digested *A. niger* DNA was probed at low stringency with a fragment from plasmid p5 which carries *pac*C_{nid} (Tilburn et al., 1995). Weakly hybridizing bands were detected in *A. niger* genomic digests, indicating the existence of *pac*C-related sequences in *A. niger* (data not shown). *pac*C-related sequences in *A. niger* were recovered by screening a genomic λ-phage library with the same probe under the same low stringency conditions used for the Southern blot analysis. Nine clonally pure hybridizing phages were obtained. Restriction mapping and hybridization studies of isolated phage DNA identified a hybridizing *Bam*HI fragment of 5.4 kb which was subcloned into pEMBL18 to generate plasmid pPAC001. The restriction map of this fragment is presented in Fig. 10.1.

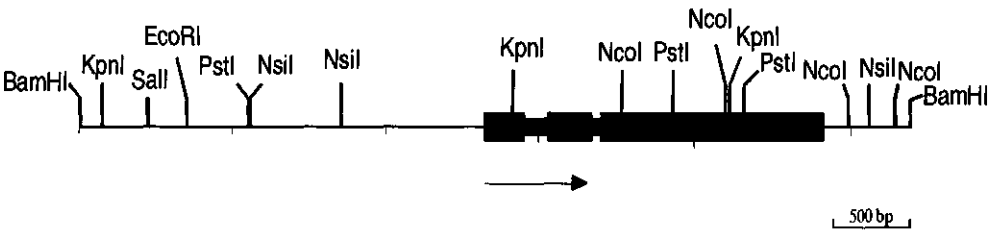

Fig. 10.1. Restriction map of the *A. niger* 5.4 kb genomic *Bam*HI DNA segment encoding *pac*C. The coding region of *pac*C is shown as filled bars, while non-coding 5' and 3' regions are shown as thin bars. The constrictions in the coding region show the positions of the introns. The direction of transcription is indicated by an arrow.

Restoration of *pac*C^+ phenotype in *A. nidulans pac*C mutant strains

To assess the functional relatedness of the *A. niger* DNA sequences cloned to *pac*C_{nid}, *A. nidulans* *pac*C^c11 and *pac*C^c14 mutants were transformed with pPAC001. Two readily observable phenotypic characteristics of these *A. nidulans* *pac*C^c mutants are sparse conidiation and poor conidial pigment colour development when grown on pH 6.0 plates; growth on pH 8.0 plates ameliorates both of these mutant characteristics. To provide a positive selection for transformants, the *pac*C_{nid} hybridizing *Bam*HI fragment present in pPAC001 was subcloned into the *arg*B carrying plasmid pIJ16 (Johnstone et al., 1985) resulting in plasmid pPAC003. Similarly, the *A. nidulans* *Bam*H1/*Eco*R1 recombinant insert present in p5 was also subcloned into pIJ16, producing

plasmid pPAC004. These constructs were transformed into an *A. nidulans pac*C^{c}11 *arg*B2 strain (407.17) and arginine prototrophs were selected directly on minimal medium plates (pH 6.0); a control transformation was performed with pIJ16. In the case of *arg*$^+$ transformants arising from plasmids pPAC003 and pPAC004, the following phenotypes were apparent: i) strongly conidiating, dark green (wild type conidiation and colour), ii) sparsely conidiating, light green, and iii) sparsely conidiating, (rather) white. All pIJ16 derived transformants were sparsely conidiating and (rather) white. Upon purification from the selection plates the light green and (rather) white transformants from both pPAC plasmids fully conidiated with dark green pigment, possibly indicating stabilization of the transforming sequences. pIJ16 transformants remained sparsely conidiating and (rather) white. The *A. nidulans pac*C^{c}14 phenotype (strain 407.18) was similarly repaired by pPAC003 and pPAC004 (conidial pigmentation being yellow in this case).

The successful restoration of *pac*C$^+$ phenotype in the *A. nidulans pac*C^c mutants by the cloned *A. niger* sequence indicates functional equivalence of *pac*C $_{nig}$ to *pac*C$_{nid}$.

Staining for acid phosphatase activity

To assess further the functional equivalence of the *A. niger* putative *pac*C sequences, the three types of transformants obtained from the *A. nidulans pac*C^{c}11 mutant were stained for phosphatase activities regulated by external pH. As a primary screen, colony staining according to the method of Dorn (1965) was performed using strains 407.17 (*pac*C^{c}11) and WG096 (*pac*C$^+$) as controls. This analysis indicated the qualitative restoration of acid phosphatase production in those transformants carrying *pac*C$_{nid}$ and *pac*C$_{nig}$ in contrast to the recipient *pac*C^{c}11 mutant and a pIJ16 transformant neither of which stained for acid phosphatase activity (data not shown).

Acid phosphatase production was also examined by polyacrylamide gel electrophoresis. The same transformant and recipient strains were grown in phosphate-limited, citrate-buffered pH 4.5 minimal medium. Mycelial extracts were prepared and electrophoresed on non-denaturing 10% polyacrylamide gels which were subsequently stained for acid phosphatase activity (Dorn, 1965). Fig. 10.2 shows that the qualitative restoration of acid phosphatase expression under conditions of phosphate limitation at pH 4.5 is similar between transformants carrying either the *A. nidulans pac*C plasmid (pPAC004) or the *A. niger* sequences (pPAC003). Transformation with pIJ16, as expected, failed to restore acid phosphatase production.

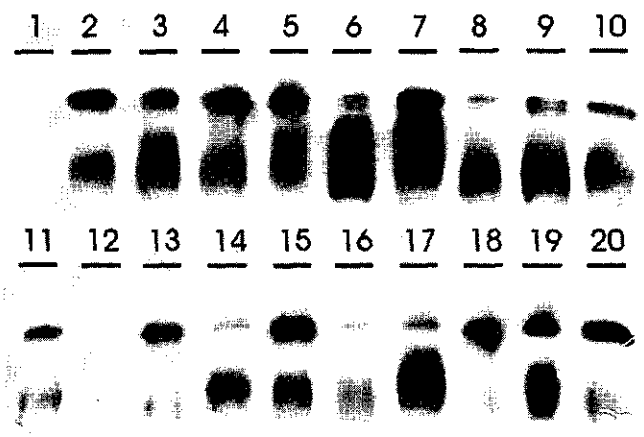

Fig. 10.2. Non-denaturing 10% polyacrylamide gels of mycelial extracts stained for acid phosphatase activity by the method of Dorn (1965). Lane 1, strain 407.17 (*pacC*^{c}11); lanes 2-11, transformants of strain 407.17 transformed with pPAC003 (*A. niger pacC*); lane 12, Strain 407.17 transformed with pILJ16; lanes 13-19, transformants of strain 407.17 transformed with pPAC004 (*A. nidulans pacC*); lane 20, strain WG096 (*pacC*$^{+}$).

DNA sequence of the *A. niger pacC* gene

The complete DNA sequence of the 5.4 kb *Bam*HI insert in pPAC001 was determined and computer analysis revealed that a deduced translation product has considerable levels of identity to the deduced amino acid sequence of the *pacC*$_{nid}$. Alignment of the *A. niger* and *A. nidulans* putative translation products and extrapolation to the DNA sequence indicated that the *A. niger pacC* structural gene (*pacC*$_{nig}$) spans 2195 bp, comprises three exons, separated by introns of 139 bp and 52 bp, and encodes a zinc finger protein of 667 amino acids with a predicted molecular mass of 70.9 kDa. The coding sequence of *pacC*$_{nig}$ and its flanking regions is shown in Fig. 10.3. The presence of the two putative introns located towards the 5' end of the *A. niger* gene was confirmed by reverse transcription(RT)/PCR using two oppositely oriented 20-mer primers (PACFWD and PACREV; Fig. 10.3) spanning all of exon 2 and its flanking putative introns. PolyA^{+} selected RNA isolated from mycelium (N402) grown under alkaline conditions (resulting in the elevated production of *pacC* mRNA; *vide infra*) was reverse transcribed with the enzyme Tth using the downstream primer and the product amplified with Tth in the presence of both upstream and downstream primers. Comparison of the size of the resulting fragment with a control generated by PCR using the cloned gene (i.e. pPAC001) as a template showed a reduction in size corresponding to the combined length of the putative introns (data not shown). The DNA fragment generated by RT/PCR was cloned into pGEM-T (Promega) and sequenced using universal and reverse primers. The sequence confirmed exactly the intron/exon boundaries shown in Fig. 10.3A originally deduced from the alignment of the putative translation products of both *pacC*$_{nid}$ and *pacC*$_{nig}$.

Both *pac*C_{nig} and *pac*C_{nid} have the same intron/exon organisation, though the first intron of the *A. nidulans* gene (85 bp) is approximately half the size of that of *pac*C_{nig}. Comparison of the coding sequences (minus introns) reveals 72.1% identity (at the DNA level) between the two. With regard to the upstream regions (1 to -1200), no meaningful sequence alignments could be obtained. Several pyrimidine-rich regions, a feature of many fungal promoters (Unkles, 1992), are present in these regions in both genes and each also contains four consensus PacC target sites (GCCARG - Tilburn et al., 1995; positions -8, -531, -579 and -1158). Although the positions and orientations of these PacC target sites appear not to be highly conserved, the overall distribution of target sites shows similarities with *A. nidulans*. Furthermore, both in *A. niger* and in *A. nidulans* two imperfect copies of a PacC target site (each containing one mismatch in comparison to the consensus sequence) are present at comparable distances from and in the close proximity to three of the consensus PacC sites (as shown in Fig. 10.3B). Whether these similarities have any functional relevance is unknown. Neither a clear TATA element nor a polyadenylation signal are evident in pacC_{nig} (Fig. 10.3A).

The *A. niger pac*C translation product

Computer analysis of the deduced primary structure of the *A. niger* PacC protein (PacC_{nig}) shows that it possesses three polypeptide elements which resemble, zinc fingers (Zf) of the C_2H_2 class and which are encoded within exons 1 and 2. Each of the Zf elements (numbering from the most amino-terminal finger) displays different C-C H-H spacing: (Zf1) 4-4, (Zf2) 4-3 and (Zf3) 2-3. Comparisons to the consensus (see below) derived from analysis of proteins possessing adjacent, repeated zinc fingers of the $\mathbf{CX_2CX_{12}HX_3H}$ type (Jacobs, 1992) show that of the three PacC_{nig} zinc fingers only Zf3 possesses the conserved phenylalanine residue located at position s1 (nomenclature as defined by Jacobs, 1992) and that the m1 leucine (present in Zf1 and Zf3) is conservatively replaced by isoleucine in Zf2. No f1 tyrosine or phenylalanine residue preceeds any of the three Zf elements, although histidine, a somewhat less frequent alternative (Jacobs, 1992), is present at this location in Zf3. The primary structure of Zf3 shows considerable similarity to the consensus of the 2-3 spaced zinc fingers (Jacobs, 1992) as shown in Fig. 10.4A. Both the consensus 2-3 spaced zinc finger and Zf3 are examples of the so-called TFIIIA/Krüppel type of C_2H_2 Zfs (Miller et al., 1985; Rosenberg et al., 1986). TFIIIA-like zinc fingers are distinct from those of the cysteine-rich motifs in steroid receptors, the cysteine-rich motif in the yeast transcription factor GAL4 or the Cys-Cys-His-Cys motif in a set of retroviral proteins (Pavletich and Pabo, 1991). FASTA analyses for PacC_{nig} identified with high frequency zinc finger proteins, mainly by virtue of the Zf3 region and also several proteins containing alanine rich regions, mainly through the N-

A:

```
-1200 GTTGTGACCTTCCAAGACTTCATCCACGGAGCGGGAAAGGTGCCAAGAGGGGGAGGAGGGTGAAAGAAGAGAAGAACGTGAAGAAGCGGGTGGAGGAGATTTATTGGCACTTCAAGAAGC
                                          ==4==▸

-1080 CAATGTGGCAGTTCCCCCGAGCAAAGAAGGAGTCTACCATATGGCTTCTTACCGATCTATGTGCCTGAAAATTGAGGACAGACGGGCAGGAGAGTGAGACATTTGAAATGCCATGATGGG

 -960 GGATGACTAACAAAGGATTCACCAAAAATGCATCGGTCCGCGCGGGGTTTTGAGTGGGTGACAGGGGAACTGTGGTGTATGGTTACCGTATTGAGACGGTCGCTAACTTACTAGTAGCCC

 -840 ACTGCGTTATTTGACGGCCTGTTCTTGCACTACTAGTGAGACTAGTAGGTTACTGACTGGGGTTGAGTGAGGGGAACCATGCACCGTGTGTGCAGGGACTTCTCTTGCAACGGTTTGACT

 -720 TGTACTTGTAGATACTGAGTGGACTGAGATGTAGCAAATAACAGCCATTGTAGTAACCGCAGTGATGGTTAGCAGCCGAGTGAAGGGCCAAAAAGAGGGCAGGCAGACATTTACACACAC

 -600 GCCACCAGCCCCGCACGGCCTCTTGGCCCGGTCGGTGCCCAGGCCTCCAGGCTTGTCGTGTCTTGTCATCCTGGCCAGTCTTGTCCCCGTCTTCCGCCCGGCTGTGCAAGCGGCCTTTTC
                          ◂==3==                                                ◂==2==

 -480 TAGGCAAGGCTCGGCTCTGAACCCAAGCCTCCCCCTCTCGGCCCAAGACTTCCTCCCTTCCCCTTCTCCCCCCCTGATTCTCTGCTGTTCCTCTTCCTCTGGTCCGGCAATCTGTTAGTT

 -360 TCCCTTATTACTTTCACATCCCCCCCCATATTTCTCTTTGCTCATATCTGCCATTTGTGTTTGTGATCTTAACACTCTTGCTGTCCTTTCCTTTCCCTTTTCTTTCCCCTCCTCTTTGGC

 -240 CAATTCTCTCCCTTTTTTTCAGATATCCGGTGTCGGTGTGCGTATCGTCGATTTTCTTGTCCGCCACTCCCTCACCGCGCCATCTGTCGTAGTCGATCCTCTTGTTTTCCCTTCATTAGC

 -120 ACCAAGAGTCTTGGGTCTCCCCATCTTTTCCTCCCCTTGCACGCTTGAGGCGGACTTGTGTTGAGTCGCTTTCTACTTCCGCTGCTCCCAAGAAAGGGACAAGAAAGATTTTTCTTGGCA
                                                                                                                    ◂==1==

   1 ATGTCGGAACCCCAAGACACCACGACGGCCCCTTCGACGACGGCCGCTCCCATGCCTACCTCAACTTCCCAGGATTCTCCTTCAGCTCAGCAACCTGCCCAGGTATCCTCAGCGACCGCT
   1 M  S  E  P  Q  D  T  T  T  A  P  S  T  T  A  A  P  M  P  T  S  T  S  Q  D  S  P  S  A  Q  Q  P  A  Q  V  S  S  A  T  A

                                                                                                  pacfwd: ------------------▸
 121 GCTTCTGCGGCCGCGACGGCTGCGGCTGCCTCGGCTGCCGTGGCAAATCCCCCCATGAACGGTACCACCACTCGTCCATCTGAGGAGTTGTCCTGCCTTTGGCAGGGCTGCTCGGAAAAG
  41  A  S  A  A  A  T  A  A  A  A  S  A  A  V  A  N  P  P  M  N  G  T  T  T  R  P  S  E  E  L  S  C  L  W  Q  G  C  S  E  K

 241 TGCCCTTCCCCTGAAGCTCTTTATgtgagttgtccttctttttcttaaatgaaaatcattctatattctatactgtgatgagacagtccaccggcaatgggctacgtgttggcttgactca
  81  C  P  S  P  E  A  L  Y

 361 tcacatcctcgtttggatggggtagctgaccgcctcggcttagGAACATGTGTGCGAGCGTCACGTCGGCCGCAAGAGCACAAACAACCTCAACCTGACCTGCCAATGGGGCAGCTGCCG
  89                                              E  H  V  C  E  R  H  V  G  R  K  S  T  N  N  L  N  L  T  C  Q  W  G  S  C  R

 481 CACTACTACTGTCAAGCGCGACCACATCACTTCTCATATTCGCGTGCATGTACCGCTAAAACCACACAAGTGTGATTTCTGCGGCAAAGCCTTCAAGCGTCCCCAGGACTTGAAGAAGCA
 115   T  T  T  V  K  R  D  H  I  T  S  H  I  R  V  H  V  P  L  K  P  H  K  C  D  F  C  G  K  A  F  K  R  P  Q  D  L  K  K  H

 601 CGTCAAGACCCATGCCGACGATTCAGTCCTGGTCCGGTCGCCGGAGCCCGGAGCCCGGAATCCGGACATGATGTTCGGTGGAGGTGCCAAGGgtaagtgtttcccaggctggctgtcttg
 155   V  K  T  H  A  D  D  S  V  L  V  R  S  P  E  P  G  A  R  N  P  D  M  M  F  G  G  G  A  K

                                                                                ◂------------------ :pacrev
 721 cactgccggctgacatgagatgtaqGCTATGCGACCGCTGCGCACTATTTTGAGCCCGCGTTGAACGCGGTCCCGAGCCAGGGCTATGCGCATGGCGCTCCCCAGTATTACCAGTCGCAC
 185                                 G  Y  A  T  A  A  H  Y  F  E  P  A  L  N  A  V  P  S  Q  G  Y  A  H  G  A  P  Q  Y  Y  Q  S  H
```

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841 CCTCCCCCTCAGCCGGCCAACCCGTCCTACGGAAATGTCTACTACGCCCTGAACCATGGCCCAGAGGCTGGTCACGCTTCCTATGAATCCAAGAAGCGCGGTTACGATGCCCTGAATGAG
217  P  P  P  Q  P  A  N  P  S  Y  G  N  V  Y  Y  A  L  N  H  G  P  E  A  G  H  A  S  Y  E  S  K  K  R  G  Y  D  A  L  N  E

961 TTCTTTGGCGACCTGAAACGGCGTCAGTTCGACCCGAACTCCTATGCTGCCGTTGGCCAGCGCCTGCTGGGCTTGCAGAGCCTGTCGCTCCCTGTGTTGAGCAGCGGGCCCTTGCCTGAG
257  F  F  G  D  L  K  R  R  Q  F  D  P  N  S  Y  A  A  V  G  Q  R  L  L  G  L  Q  S  L  S  L  P  V  L  S  S  G  P  L  P  E

1081 TACCAGCCGATGCCTGCACCGGTGGCCGTTGGTGGTGGCGGATACAGCCCCGGGGGTGCTCCCTCCGCACCGGCCTACCACCTGCCGCCTATGAGTAACGTGCGGACCAAGAACGACTTG
297  Y  Q  P  M  P  A  P  V  A  V  G  G  G  G  Y  S  P  G  G  A  P  S  A  P  A  Y  H  L  P  P  M  S  N  V  R  T  K  N  D  L

1201 ATCAACATTGATCAGTTCCTGCAGCAGATGCAGGATACTATATATGAGAACGATGACAATGTGGCTGCTGCTGGTGTTGCCCAGCCGGGCGCTCATTACGTTCATGGTGGCATGAGCTAC
337  I  N  I  D  Q  F  L  Q  Q  M  Q  D  T  I  Y  E  N  D  D  N  V  A  A  A  G  V  A  Q  P  G  A  H  Y  V  H  G  G  M  S  Y

1321 CGCACCACCCACTCGCCTCCCACCCAGTTGCCCCCCAGCCATGCGACAGCGACCAGCTCTGCCTCAATGATGCCAAACCCCGCCACCCACTCGCCTTCCACAGGCACTCCCGCCCTTACG
377  R  T  T  H  S  P  P  T  Q  L  P  P  S  H  A  T  A  T  S  S  A  S  M  M  P  N  P  A  T  H  S  P  S  T  G  T  P  A  L  T

1441 CCGCCGTCTAGCGCTCAGTCATACACCTCCGGTCGCTCGCCCGTTTCCTTGCCGTCGGCGACAAGGGTCTCGCCGCCTCACCATGAAGGAGGCAGCATGTACCCTCGTCTGCCGTCAGCT
417  P  P  S  S  A  Q  S  Y  T  S  G  R  S  P  V  S  L  P  S  A  T  R  V  S  P  P  H  H  E  G  G  S  M  Y  P  R  L  P  S  A

1561 ACCATGGCTGACAGCATGGCGGCCGGGTACCCGACCGCGTCGAGTACCGCACCGCCGTCTACCCTCGGTGGCATCTTCGACCACGATGACCGCCGCCGGTACACGGGTGGCACGCTGCAG
457  T  M  A  D  S  M  A  A  G  Y  P  T  A  S  S  T  A  P  P  S  T  L  G  G  I  F  D  H  D  D  R  R  R  Y  T  G  G  T  L  Q

1681 CGCGCCCGTCCAGAGACTCGCCAGTTGAGCGAGGAAATGGATCTTACCCAGGACAGCAAGGATGAGGGTGAGCGTACTCCTAAGGCCAAGGAGCACTCTTCGCCATCCTCACCTGAGCGC
497  R  A  R  P  E  T  R  Q  L  S  E  E  M  D  L  T  Q  D  S  K  D  E  G  E  R  T  P  K  A  K  E  H  S  S  P  S  S  P  E  R

1801 ATCTCTGCTAGCTTGATCGACCCGGCTCTTTCGGGCACTGCCGCGGAAGCTGAAGCAACTCTGCGGACCGCCCAGGCGGCGACGGAGGTGGCCGAACGGGCAGATGTACAGTGGGTGGAG
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1921 AAGGTGCGGCTGATTGAGTATCTGCGCAACTACATTGCATCCCGGCTGGAACGGGGCGAGTTCGAGAATAATGAGTCTGGCGGCGGCAACAGCAGTAGCAACGGCAGCAGTCATGAGCAG
577  K  V  R  L  I  E  Y  L  R  N  Y  I  A  S  R  L  E  R  G  E  F  E  N  N  E  S  G  G  G  N  S  S  S  N  G  S  S  H  E  Q

2041 ACACCTGAGGCCAGCCCGGACACTCATATGGAAGGAGTGGAAAGCGAGGTTCCAAGTAAGGCCGAGGAACCGGCTGTGAAGCCGGAGGCAGGTGATGTGGTGATGTACCCGACGCTACGC
617  T  P  E  A  S  P  D  T  H  M  E  G  V  E  S  E  V  P  S  K  A  E  E  P  A  V  K  P  E  A  G  D  V  V  M  Y  P  T  L  R

2161 GCGGTGGATGAAGATGGTGATTCAAAAATGCCTTAGGTTGTTTGCTGGTCACTATCTCTTTTTTTCTTTTCTCTTTCTCTGGGCTTATATTCGATTCTTCCGGAGCGAGTTCTTCCGGCT
657  A  V  D  E  D  G  D  S  K  M  P  *

2281 TGCTACGAACTGTCTGGGTGCTAGTTTGATTCCCTGTCCACGTGCACGTGGTAACGCAGTGTGATTACGCCCATGAGCCATGGGCCGTGTATATGTGGGCCCGGCCCTGGGATTTACTTT

2401 CTTTCATTTACGTCTCCTCTCTCATTCCTTGCTATTTCTTTTACACGGAGTTCTGTATGAGGTGTGTATATGGATGGCGCCGCGGAATGCATGGGACACGGCGCCGAATCCAAATGGCGT
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B:

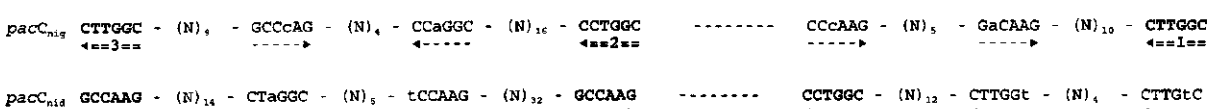

Fig. 10.3. (**A**) The coding sequence, flanking regions and putative translation product of the *A. niger pac*C gene. Start and stop codons are in bold; exon sequences are in upper case while intron sequences are in lower case. The dashed arrows above the sequence labelled **pacfwd** and **pacrev** indicate the oligonucleotides that were used for reverse transcriptase, PCR, cloning and sequencing to identify the two introns. Consensus sequences for the 5' splice donor (GTRNGT), the 3' splice acceptor (YAG), and the putative lariat sequence (RCTRAC) of the two introns are underlined. The four putative bindingsites for the wide domain pH regulatory protein PacC (GCCARG: Tilburn et al., 1995) and their orientation are indicated in bold and are double underlined. (**B**) Location, orientation and spacing of imperfect PacC binding sites in the close proximity of perfect PacC binding sites in *A. niger* and *A. nidulans pac*C promoters; PacC binding sites are in bold and double underlined; single mis-matches in imperfect PacC binding sites are in lower case.

terminal Ala-rich region (residues 38-55). The $PacC_{nig}$ zinc finger region shows considerable similarities and has identical C-C and H-H spacing to zinc finger regions in $PacC_{nid}$ (Tilburn et al., 1995) and the yeast meiotic regulatory protein RIM1 (Su and Mitchell, 1993) as shown in Fig. 10.4B. It is noteworthy that the greatest sequence divergence for these three DNA binding regions occurs in Zf1. This might indicate that Zf1, like Zf1 of GLI, does not contact DNA (Pavletich and Pabo, 1993). Alignment of the translation products of the *A. niger* and *A. nidulans* (Tilburn et al., 1995) *pac*C genes (Fig. 10.4) revealed very high levels of identity throughout the central regions of the two proteins. In this central part the conserved zinc finger region (residues 70-166) and also a putative bipartite nuclear localization signal, SKKR....KRRQ (residues 245-248 and 261-264) (Boulikas, 1994), are located. Furthermore two proline-glycine-rich and two serine-threonine rich regions are present in the central region (Fig. 10.3B), similar to the situation in *A. nidulans*. Variability between $PacC_{nig}$ and $PacC_{nid}$ is observed at the amino terminal end but greater differences are seen at the carboxyl terminus. Computer analysis of the sequences of the two proteins showed the presence of a predominantly acidic region in $PacC_{nid}$ between residues 593 to 618 which is represented in $PacC_{nig}$ at a considerably lower charge density (residues 598 to 632). The sequence of amino acids responsible for the acidic region in $PacC_{nid}$ is an alternating stretch of glutamine and aspartic/glutamic acid residues which is absent in the $PacC_{nig}$. Structure prediction indicates that this difference may result in the absence of an extensive α-helix in the C-terminal region of $PacC_{nig}$.

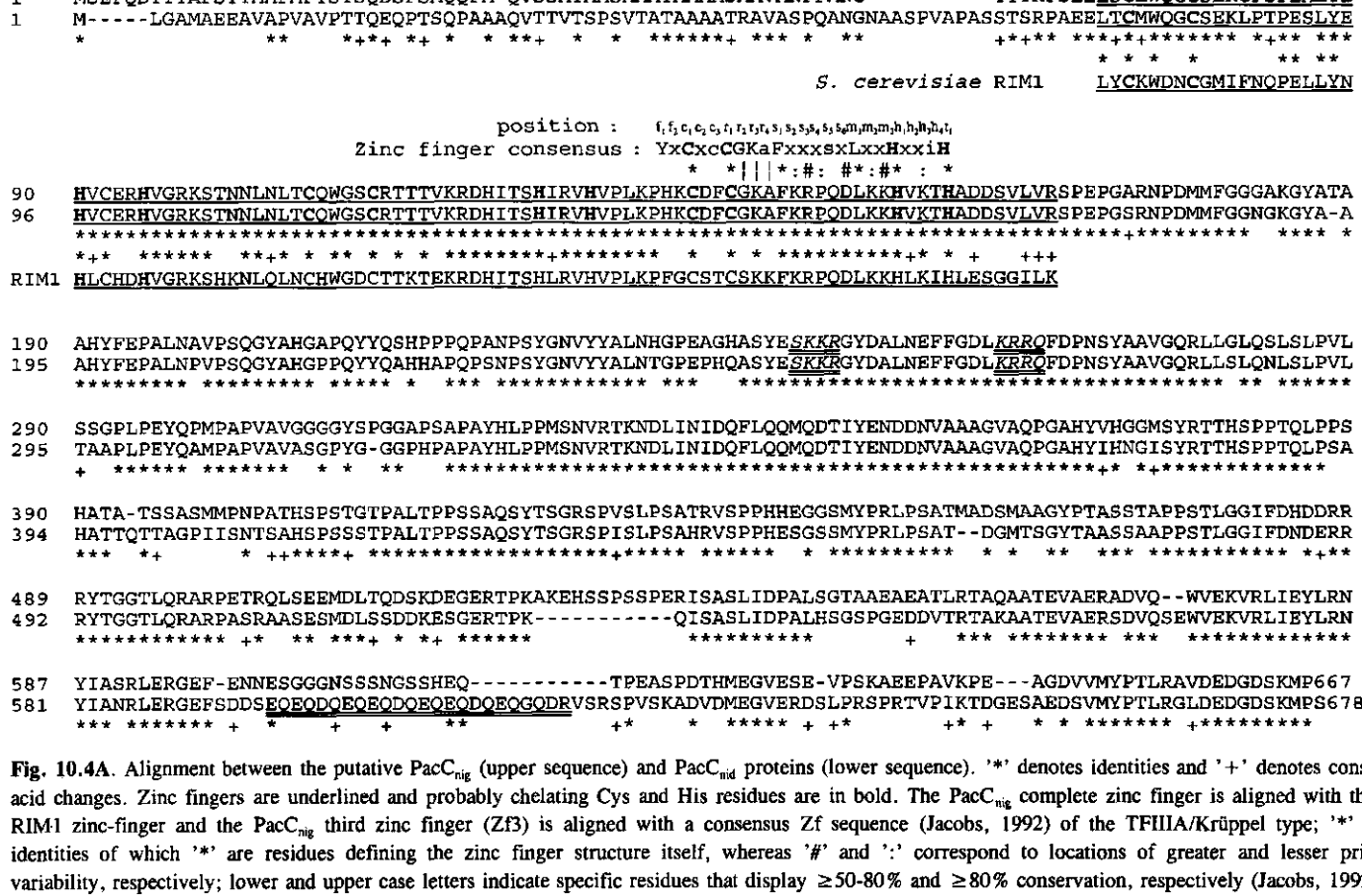

Fig. 10.4A. Alignment between the putative $PacC_{nig}$ (upper sequence) and $PacC_{nid}$ proteins (lower sequence). '*' denotes identities and '+' denotes conservative amino acid changes. Zinc fingers are underlined and probably chelating Cys and His residues are in bold. The $PacC_{nig}$ complete zinc finger is aligned with the *S. cerevisiae* RIM1 zinc-finger and the $PacC_{nig}$ third zinc finger (Zf3) is aligned with a consensus Zf sequence (Jacobs, 1992) of the TFIIIA/Krüppel type; '*' and '|' denote identities of which '*' are residues defining the zinc finger structure itself, whereas '#' and ':' correspond to locations of greater and lesser primary structure variability, respectively; lower and upper case letters indicate specific residues that display ≥50-80% and ≥80% conservation, respectively (Jacobs, 1992).

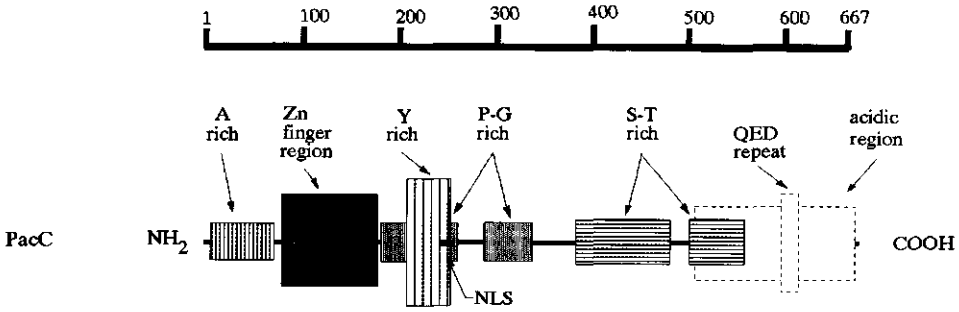

Fig. 10.4B. Schematic presentation of the $PacC_{nig}$ protein. A rich, Y rich, P-G rich and S-T rich indicate alanine, tyrosine, proline/glycine and serine/threonine rich regions in the $PacC_{nig}$; NLS: putative nuclear localization signal. Positions of the QED-repeat and the acidic region found in $PacC_{nid}$ are indicated in $PacC_{nig}$ (open boxes). The dashed lines around the open boxes indicate the absence of the QED-repeat in $PacC_{nig}$ and the considerable reduced charge density of the region which is referred to as acidic in *A. nidulans*.

pH regulation of *A. niger pac*C gene expression

In view of the role of the *A. nidulans pac*C gene as a modulator of gene expression in response to ambient pH (Tilburn et al., 1995) and the presence of possible PacC binding sites in the $pacC_{nig}$ promoter, we have investigated the expression of the *A. niger pac*C gene itself. For the first experiment equal wet weights (2.5 g) of N402 biomass were transferred from minimal medium to minimal media (50 ml) buffered at pH 4.5 (citrate), 6.5 (MES) and 8.0 (TRIS). The cultures were incubated for a further 6 h, after which time the pH of each transfer culture was measured and total RNA isolated, northern blotted and probed with the 360 bp *Pst*I/*Kpn*I fragment of exon 3. A control blot from a gel loaded with the same amount of RNA was probed with actin. The results are shown in Fig. 10.5.

The *pac*C gene produces a mRNA of approximately 2.8 kb, the expression of which is greatest under alkaline conditions and decreases as the pH is lowered. This is particularly striking when the relative intensities of the actin and *pac*C signals are compared (Fig. 10.5). To exclude possible buffer effects, similar transfer experiments were performed in the presence of potassium phosphate in which the culture pH values were similar to those in the above buffered media and gave identical results to those shown in Fig. 10.5 (data not shown). Thus it seems likely that *pac*C is autogenously regulated in a similar fashion in the two *Aspergillus* species. Constitutive expression of *pac*C in alkaline mimicing $pacC^{c}$ mutants of *A. nidulans* has led to the suggestion that the C-terminal portion of PacC may act in a negative fashion and that alkaline mimicing

*pac*C^c mutations are gain of function alleles (Tillburn et al., 1995). Analogous observations have recently been made in *A. niger* (van den Hombergh et al. unpublished results). The close similarities both of structure and expression of $PacC_{nig}$ and $PacC_{nid}$ may indicate a critical role for $PacC_{nig}$ in regulating the expression of genes involved in the synthesis of extracellular enzymes. Since, in contrast to *A. nidulans*, *A. niger* acidifies its medium very rapidly and several *pac*C-regulated acid target genes have been identified, *A. niger* is a good candidate for studying wide domain pH regulation at acid pH. If and to what extent the differences between $PacC_{nig}$ and $PacC_{nid}$ are functionally relevant and linked to differences in physiology is currently being studied.

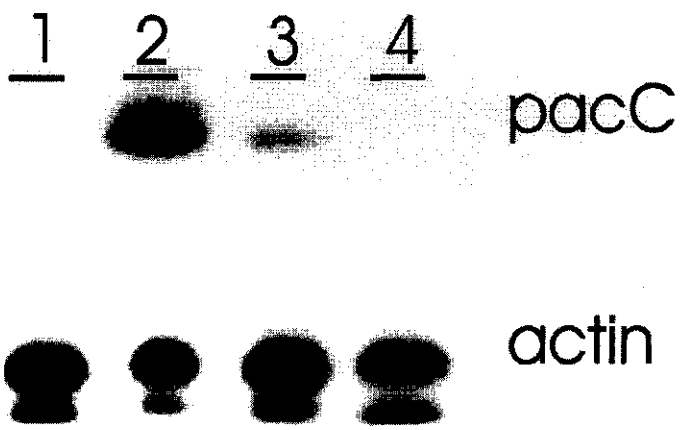

Fig. 10.5. Northern analysis of total RNA (10 μg/track) isolated from *A. niger* strain N402 after transfer to media buffered at different pH values. Track order: 1, immediately prior to transfer; 2, TRIS-buffered medium pH 8.0; 3, MES-buffered medium pH 6.5; 4, citrate-buffered medium pH 4.5. Northern blot probed with 360 bp *Pst*I-*Kpn*I *pac*C fragment (top). Northern blot probed with the actin probe, Fidel et al., 1988, (bottom).

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Cathala, G., Savouret, J-F., Mendez, B., West, B., Karin, M., Martial, J.A. and Baxter, J.D. (1983) DNA **2**:329-335

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11 Regulation of acid phosphatases in an *Aspergillus niger* *pac*2C^c disruption strain[1]

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Summary

An *Aspergillus niger* strain has been constructed in which the pH regulatory gene, *pac*C, was disrupted. The *pac*C gene of *A. niger*, like that of *A. nidulans*, is involved in the regulation of acid phosphatase expression. Disruptants were identified by reduced acid phosphatase colony staining. Southern analysis demonstrated integration of the disruption plasmid at the *pac*C locus and Northern analysis showed that the disruption strain produced a truncated *pac*C messenger of 2.2 kb (as compared to 2.8 kb in the wild type). The strain carrying the *pac*C disruption was used to assign the *pac*C gene to linkage group IV which was further confirmed by CHEF-Southern analysis. This strain further allowed analysis to determine which extracellular enzyme and transport systems are under control of *pac*C in *A. niger*. Expression of the *A. niger pac*C wild-type gene and the truncated *pac*C gene showed that in contrast to the auto-regulated wild-type expression, which was elevated only at alkaline pH, the truncated *pac*C gene was deregulated, as high expression occurred regardless the pH of the culture medium. Analysis of the phosphatase spectrum by isoelectric focusing and enzyme activity staining both in the wild-type and the *pac*C disruptant showed that at least three acid phosphatases are regulated by the *pac*C gene. For the single alkaline phosphatase no pH regulation was observed.

Introduction

Many microorganisms encounter variations in pH in their environment. Although they probably all have a pH homeostatic mechanism that restricts the range of intracellular pH variation, molecules located at or beyond the permeability barrier of the cell can only be protected from extremes of pH by ensuring that synthesis takes place at an appropriate pH. It would therefore appear advantageous to possess a regulatory system for sensing the environmental pH, thus

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providing a means by which pH regulated gene expression can be mediated (Arst, 1994).

In *A. nidulans* several mutations have been described which modulate the expression of specific structural genes encoding various extracellular enzymes and transport systems such as acid and alkaline phosphatases and the γ-amino-n-butyrate permease (Caddick and Arst, 1986; Caddick et al., 1986b). Based on phenotypic effects two classes of mutations were identified. One class, involving 6 independent genetic loci designated *pal* genes, was shown to mimic the effects of growth under acidic conditions whereas the second class, involving a single locus (*pac*C), was shown to mimic the effects of growth under alkaline conditions. The subsequent isolation of more *pac*C mutants has since shown that this gene can mutate to both phenotypic classes. In addition to the type initially observed which resulted in mimicry of growth under alkaline conditions (*pac*C^{c}), a second class (*pac*C$^{+/-}$), which to some extent mimics growth under acidic conditions was also isolated (Tilburn et al., 1995). Since *pac*C mutations are epistatic to all *pal* mutations and regulatory effects of *pac*C affect several independent functions, it has been suggested that *pac*C is a wide domain regulatory gene (Caddick et al., 1986b). Tilburn and co-workers (1995) have reported strong evidence in favour of that hypothesis and they have presented a model for pH regulation in *A. nidulans*. More recently, it was demonstrated that proteolytic conversion of PacC to its functional form is an essential and pH-sensitive step in the regulation of gene expression by ambient pH (Orejas et al., 1995).

The importance of the *pac*C gene has been emphasized by the finding that it regulates the production of secondary metabolites. *Pac*C has been shown to be involved in the biosynthesis of penicillin in *A. nidulans* via transcriptional regulation of at least one of the genes involved (*ipn*A) (Espeso et al., 1993). Furthermore, the expression of all three cloned extracellular protease genes in *A. niger*, *viz. pep*A, *pep*B and *pep*F is under pH control, exhibiting high expression at low pH and no expression at high pH, which suggests involvement of PacC in their regulation (Jarai and Buxton, 1994; van den Hombergh et al., 1994). Considering the industrial importance of *A. niger* as an expression system for both homologous and heterologous gene products and the serious problems encountered due to proteolysis by endogenous proteases it is of special interest to study wide domain pH regulation in this organism.

Recently the *pac*C genes from both *A. nidulans* (Tilburn et al., 1995) and *A. niger* have been cloned and both were shown to restore wild type phenotype in transformants arising from two different *A. nidulans pac*C^{c} mutants (MacCabe et al., 1996). The availability of the cloned *A. niger* gene opens the possibility to investigate pH regulation of gene expression through the use of *pac*C disruption strains. Caddick et al. (1986b) showed that in *A. nidulans* the expression of acid phosphatase PV, phosphate-repressible phosphodiesterase and alkaline phosphatase PI are all under

*pac*C control. In *A.niger* one acid phosphatase gene (*aph*A) has been cloned (MacRae et al., 1988). The promoter of this gene seems to respond to pH regulation and has recently been used for heterologous expression of human interferon α2 (MacRae et al., 1993). Here we report the construction of an *A. niger pac*C disruption strain having an alkalinity mimicking *pac*C^c phenotype and the effects of this disruption on the regulation of both the *pac*C gene itself and of pH regulated phosphatases.

Materials and Methods

Strains and media. All *A. niger* strains were derived from the wild-type strain N400 (CBS 120.49). N402 has short conidiophores (*csp*A1). N647 (*csp*A1, *fwn*A6, *nic*A1, *arg*B13) was used for transformation. NW138 (*csp*A1, *fwn*A6, *nic*A1, *pac*2C^c *arg*B$^+$, *arg*B13) was derived from N647 and contained a single copy of the disruption plasmid pPAC020 at the *pac*C locus. *A. niger* NW148 (*his*D4, *bio*A1, *lys*A7, *leu*A1, *met*B10, *arg*B15, *pab*A1, *cnx*C5, *trp*B2) was used as a master strain for the allocation of the *pac*2C^c mutation to one of the eight linkage groups. *A. niger* strains B3, B13, B17, B38 and B39 were used for CHEF-Southern analyses. These strains, in which linkage groups VIII, I, VII, VI and III, respectively, have altered electrophoretic mobilities due to introduced chromosomal size variation (Verdoes et al., 1994), were kindly provided by Dr. J.C. Verdoes (TNO Medical Biological Laboratory, Rijswijk, The Netherlands). Minimal medium (MM) contained per litre 6.0 g $NaNO_3$ / 1.5 g KH_2PO_4 / 0.5 g KCl / 0.5 g $MgSO_4$ and trace elements according to Vishniac and Santer (1957) and was adjusted to pH 6.0. Complete medium (CM) contained MM supplemented with 2% (w/v) peptone / 1% (w/v) yeast extract / 1% (w/v) casamino acids / 0.5% (w/v) ribonucleic acids.

Construction of a *pac*C^c disruption strain. For common DNA manipulations such as plasmid isolation, random priming, Southern blotting analysis and normal subcloning to generate the disruption plasmid, standard methods were applied (Sambrook et al., 1989). For transformation, the *A. niger* arginine auxotrophic strain N647 (*csp*A1, *nic*A1, *fwn*A6, *arg*B13) was used and transformants were selected for arginine prototrophy on MM stabilized with 0.95 M sucrose and supplemented with nicotinic acid. Transformants were purified several times by re-screening at low spore density. Purified transformants were grown on MM in which phosphate salts were substituted by chloride salts at equimolar concentrations (-P_i medium) and which contained appropriate supplements and 1% (w/v) glucose as sole carbon source. After two days of growth at 30°C acid phosphatase activity was visualized using an acid phophatase colony staining method described by Dorn (1965). The colonies were submerged in 0.6 M sodium acetate buffer containing the reagents α-napthylphosphate and the diazonium salt Fast Garnet GBC. In the presense of most phosphatases the substrate (α-naphthylphosphate) is hydrolyzed. The α-naphthol reacts with the diazonium salt to form an insoluble coloured precipitate. Putative *pac*C disruption strains were selected on the basis of decreased colony staining.

Genetic analysis. The procedures used for genetic analysis of *A. niger* have been described in detail by Bos et al. (1988). Somatic diploids between NW138 and master strain NW148 were isolated and the heterozygous diploid was haploidized on CM supplemented with histidine, biotin, lysine, leucine, methionine, arginine, *p*-aminobenzoic acid, niacin, tryptophan and nicotinic acid required by the *his*D4, *bio*A1, *lys*A7, *leu*A1, *met*B10, *arg*B15, *pab*A1, *cnx*C5, *trp*B2 and *nic*A1 mutations master strain and disruptant, respectively. Haploidization was induced by adding benomyl, dissolved in acetone and used at a final concentration of 0.25 mg·ml^{-1}.

CHEF-Southern analysis. Localization of the *pac*C gene to one of the *A. niger* linkage groups was also achieved using CHEF-Southern analysis according to the method of Verdoes et al. (1994). CHEF gel electrophoresis was performed using a CHEF CRII apparatus and a Model 1000 Mini Chiller (Bio-rad). The preparation of intact chromosomal DNA and CHEF electrophoresis were as described by Debets et al. (1990), with minor modifications. Gels were prepared using chromosomal grade agarose (0.7 % (w/v); Bio-rad) in 1 x TAE buffer and were electrophoresed at 4°C. Pulse duration was for 24 h, 48 h and 72 h with a pulse switch of 55, 47 and 37 min, respectively. Gels were blotted and hybridized with the 1.6 kb *Sall-Nsi*I fragment of *A. niger pep*A [kindly provided by Dr. F.P. Buxton, Ciba Geigy AG, Basel] and the 1.2 kb *Kpn*I fragment of the *A. niger pac*C gene. All blots were autoradiographed wet to facilitate re-probing.

Northern analysis of *pac*C gene expression. N402 and NW138 were grown in MM supplemented with 1% (w/v) glucose / 0.05% (w/v) yeast extract and appropriate supplements at 30°C for 20 h in a rotary shaker at 220 rpm after seeding at a spore density of 1 x 10^6 ml^{-1}. After harvesting and washing in minimal medium, mycelia were transferred to MM lacking yeast extract, buffered to pH 4.5, 6.5 and 8.0 with citrate, MES and Tris, respectively at 100 mM concentration in each case. The transfer cultures were incubated for a further period of 6 h under identical conditions after which mycelia were recovered, washed several times with distilled water, frozen and pulverised as detailed below. Total RNA was isolated as described by Cathala et al. (1983). Concentrations of all RNA preparations were estimated spectrophotometrically. RNA was either glyoxylated or treated with formamide and analysed on 1.4% (w/v) agarose gels as described by Sambrook et al. (1989), loaded with 10 μg per track. Transfer of RNA to GeneBind membranes (Pharmacia) and hybridization at 65°C were performed according to Sambrook et al. (1989). Blots were washed for 20 min periods against 0.3 M NaCl / 2 mM EDTA / 40 mM Tris pH 7.8 / 0.1% (w/v) SDS / 0.1% (w/v) $Na_4P_2O_7{\cdot}10H_2O$ followed by 75 mM NaCl / 0.5 mM EDTA / 10 mM Tris pH 7.8 / 0.1% (w/v) SDS / 0.1% (w/v) $Na_4P_2O_7{\cdot}10H_2O$ and autoradiographed wet when reprobing was necessary.

Localization of phosphatases. *A. niger* N402 was grown in a fermenter on MM without phosphate at pH 6.0 for 20 h. Mycelium was harvested after 24 h of growth and used to prepare cell-free extracts and to isolate protoplasts. Protoplasts were released from the mycelium using 1-3 mg of Novozyme 234 (Novo) ml^{-1} in protoplast stabilizing buffer containing 10 mM Tris HCl (pH 7.5) and 1.33 M sorbitol. Protoplasts and the cell wall fraction were separated via filtration through myracloth. Protoplasts were pelleted via centrifugation and subsequently lysed in 20 mM sodium phosphate buffer (pH 6.0). The supernatant, obtained after 5 min centrifugation at 10,000 x g, was used for further analyses. When needed, extracts and supernatants were concentrated via speedvac lyophilization after re-freezing in liquid nitrogen. Phosphatases present in the individual fractions were measured as described in *Biochemical analyses*.

Biochemical analyses. Strains were inoculated at spore densities of 1 x 10^6 ml^{-1} and grown in a rotary shaker at 30°C on MM containing 1% (w/v) glucose as carbon source and appropriate supplements. Cultures were buffered at pH 8.0 with 200 mM Tris, at pH 6.0 with 200 mM MES and at pH 4.0 with 200 mM sodium citrate. After 24 h, mycelia were collected by vacuum filtration and frozen immediately in liquid nitrogen. For the preparation of cell free extracts mycelia were pulverized (Braun dismembrator) and resuspended in ice cold 50 mM Tris buffer pH 8.2 and stored on ice for 0.5 h. Mycelial debris was removed by centrifugation for 5 min at 4°C. Isoelectric focussing was performed over a pH range of pH 3.0 - 7.0 on a FBE-3000 isoelectric focussing apparatus 7(Pharmacia). Protein concentrations in extracts and supernatants were determined using the Bicinchonic acid protein assay kit (Sigma) according to the suppliers instructions using BSA (fraction V) as a standard. Alkaline phosphatase activity was visualized after isoelectric focussing using 0.05 % (w/v) α-naphtyl phosphate and Fast Violet B salt (Sigma) according to Dorn (1965) in 0.5 M Tris buffer pH 8.0. Acid phosphatase activity was visualized after isoelectric focussing in 0.5 M NaAc buffer pH 4.0 using 0.05% (w/v) α-naphtyl-phosphate and 0.5% (w/v) Fast Garnet GBC salt (Sigma). For phosphatase enzyme assays the *p*-nitrophenylphosphate method described by Caddick and Arst (1986) was used. Specific activities are expressed in nmoles *p*-nitrophenol liberated per mg soluble protein in extract/culture fluid. Glucose oxidase (GOX) and glucose 6-phosphate dehydrogenase

(G6Pdh) activities were measured spectrophotometrically as described previously by Witteveen et al. (1990) and Bruinenberg et al. (1983), respectively, and expressed as μmol min^{-1} mg^{-1}.

Results

Construction of a *pac*C^c disruption strain

As *A. niger* acidifies its medium very rapidly (Visser et al., 1994) we were especially interested in generating a *pac*C disruption strain in which pH regulatory effects of *pac*C on genes expressed under acidic conditions would be strong. Based on the data presented by Tilburn et al. (1995), we decided to construct a *pac*C^c disruption construct which would result in a truncation of the PacC protein similar to truncations in *A. nidulans pac*C^c alkaline mimicking mutants. Generating a single *pac*C^c disruptant would allow us to test which enzyme systems are under *pac*C control, as, based on the model presented by Tilburn et al. (1995), an acid target gene would be down regulated in a *pac*C^c mutant. An internal 1.0 kb *Kpn*I-*Pst*I fragment from the *pac*C gene was cloned into a vector containing a 2.8 kb *Bam*HI-*Pst*I fragment on which the *arg*B gene from *A. nidulans* was located. The resulting plasmid (pPAC020; Fig. 11.1A) was used to transform *A. niger* N647. As a control N647 was also transformed with plasmid pIJ16 (Johnstone et al., 1985) which contains the *A. nidulans arg*B gene. Fifty randomly isolated transformants were purified by re-plating for single colonies on selective media. It has been previously shown in *A. nidulans* that several phosphatases are *pac*C regulated and can be used as tester enzymes for pH regulatory effects (Caddick and Arst, 1986; Caddick et al., 1986b). However, both in *A. nidulans* (Caddick et al., 1986a) and in *A. niger* (MacRae et al., 1988), phosphate repression of some phosphatases has also been observed. Thus for screening of putative *A. niger pac*C disruptants both minimal and phosphate free media were used. From the originally purified transformants 25 were grown together with controls (untransformed N647 and two N647-pIJ16 transformants) for two days on both MM and -P_i medium. Acid phosphatase activity was examined using the colony staining method of Dorn (1965). This analysis indicated that two transformants N647-pPAC020/2 and N647-pPAC020/3, showed reduced acid phosphatase staining on both media as compared to the controls.

Southern analysis

Total genomic DNA was isolated according to the method of de Graaff et al. (1988) from the *A. niger* wild type strain (N402), from the two putative *pac*C disruptants (N647-pPAC020/2 and N647-pPAC020/3) and from a set of randomly selected N647-pPAC020 transformants. DNA was

digested with *Pst*I or *Kpn*I and Southern blotted. Blots were probed with the 1.4 kb *Kpn*I fragment from the *A. niger pac*C gene (Fig 11.1D1) and, after stripping, re-probed with the 1.0 kb *Hin*dIII fragment from the *A. nidulans arg*B gene (Fig 11.1D2).

As is shown in Fig. 11.1B/D1 in N402 the *pac*C probe hybridizes to 2.8 kb and 0.5 kb *Pst*I fragments and a 1.4 kb *Kpn*I fragment. Due to integration of the disruption construct at the homologous *pac*C locus, 6.8 kb *Pst*I and 6.8 kb *Kpn*I fragments hybridize to the *pac*C probe in addition to the wild-type bands, as shown in Fig. 11.1C/D1. As a control the same digests were hybridized with an *arg*B probe. In all cases the largest fragment hybridizing to the *pac*C gene also hybridized to the *arg*B gene (Fig. 11.1C/D2). Since the *arg*B gene originates from *A. nidulans* no hybridizing fragments are observed in wild-type. From the Southern analysis it can be concluded that indeed one copy of the disruption construct has integrated at the homologous *pac*C locus in N647-pPAC020/2. In N647-pPAC020/3 a similar integration event has taken place but in addition a second copy of the pPAC020 plasmid has probably integrated elsewhere into the genome, giving rise to an additional hybridizing band observed in the Southern analysis for the N647-pPAC020/3 transformant. The other N647-pPAC020 transformants analyzed had resulted from integration events of the pPAC020 plasmid elswhere into the genome, as they contained wild-type *pac*C loci (data not shown), correlating with their wild-type phosphatase production as judged by the colony staining. Therefore, Southern analysis confirms the homologous integration of the *pac*C disruption construct and shows that the observed effects on phosphatase activities are solely linked to integration at the *pac*C locus. For further analysis, only the N647-pPAC020/2 transformant, henceforth designated NW138, was used.

Genetic allocation of the *pac*C locus

Strain NW138 was used to allocate genetically the *pac*C locus to one of the eight linkage groups in *A. niger* (Bos et al., 1988). The results of these allocation experiments expressed as the percentage of recombination between the disrupted *pac*C locus and the markers of the master strain are compiled in Table 11.1. Both the *pac*C^c phenotype and the *arg*B$^+$ phenotype, used to select the transformants, were allocated to linkage group IV.

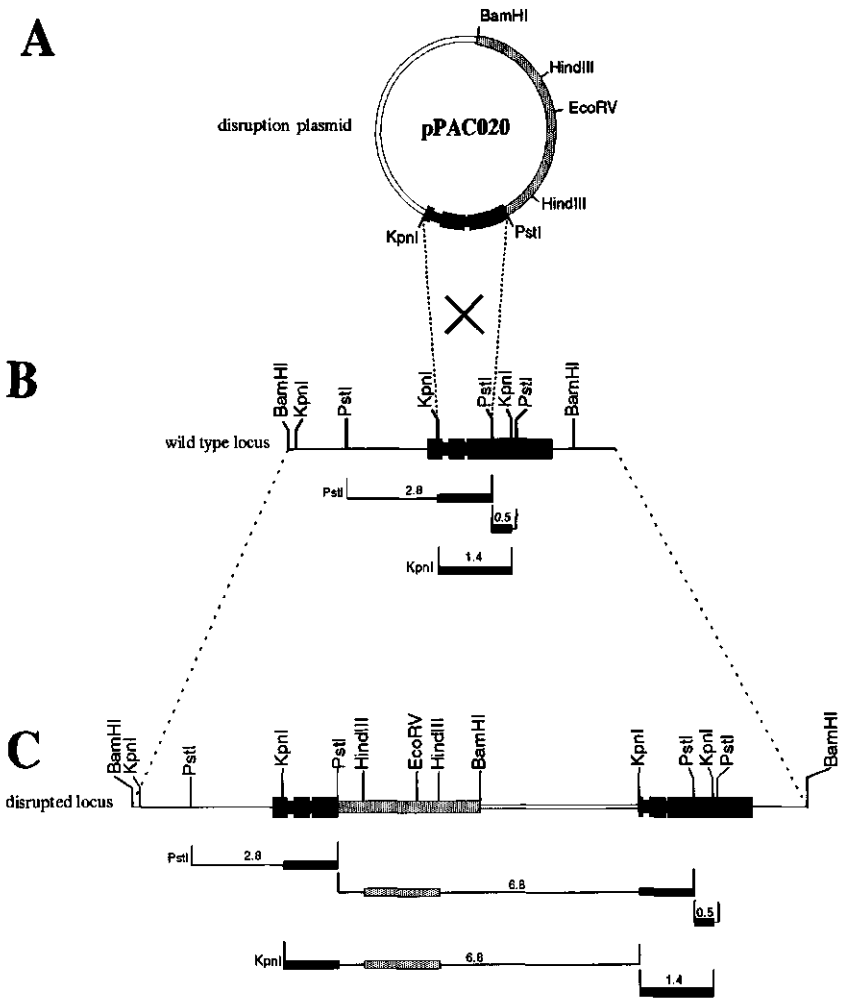

Fig. 11.1ABC. Disruption of the *pac*C locus in *A. niger*. **(A)** Plasmid pPAC020 containing the internal 1.0 kb *Kpn*I-*Pst*I of *pac*C was constructed from plasmids pPAC002 (MacCabe et al., 1996) and pIJ16 (Johnstone et al., 1985): from plasmid pPAC002 a 1.4 kb *Kpn*I fragment was subcloned into pBluescript SK+ resulting in plasmid pPAC014; plasmid pPAC014 was digested with *Pst*I and *Bam*HI which together removed an additonal 360 bp from the *pac*C fragment; into the *Pst*I-*Bam*HI digested pPAC014 plasmid a 2.7 kb *Pst*I-*Bam*HI fragment from plasmid pIJ16 containing the functional *A. nidulans arg*B gene was cloned, resulting in plasmid pPAC020. **(B)** Genomic DNA map of the *pac*C locus of the wild type strain N402. **(C)** Genomic DNA map of the disrupted *pac*C locus in NW138. The coding region of *pac*C is shown as a filled bar, while non-coding 5' and 3' regions are shown as thin bars. Constrictions in the coding region show the positions of the introns. The filled grey bar represents the *arg*B gene from *A. nidulans*. The single cross-over event with pPAC020 produces the desired disruption. The number and sizes of the *Pst*I and *Kpn*I restriction fragments before **(B)** and after **(C)** gene disruption are shown. Within these restriction fragments the DNA sequences hybridizing to the *A. niger pac*C and the *A. nidulans arg*B probes are indicated as filled black bars and filled grey bars, respectively.

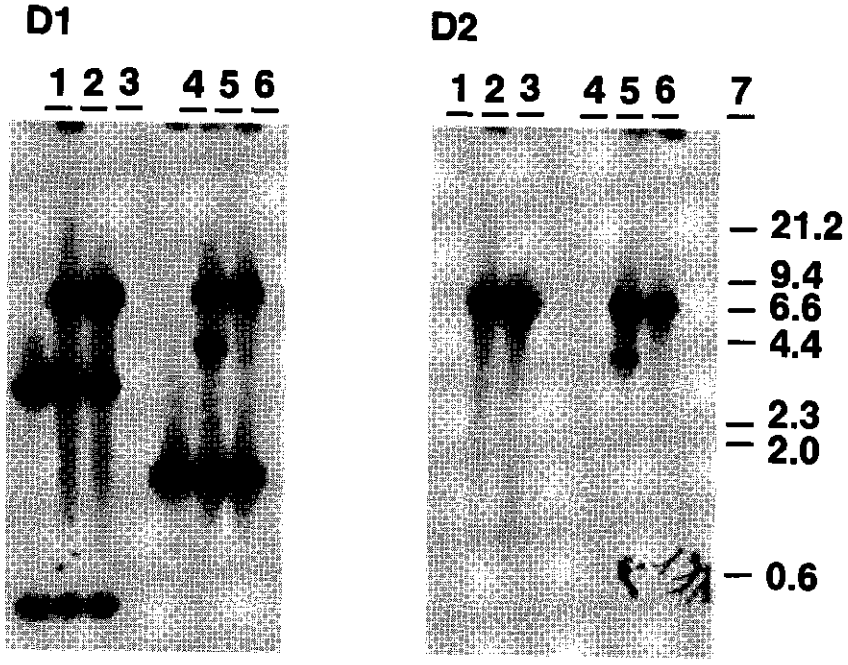

Fig 11.1D. Southern blot analysis of *A. niger* N402, N647-pPAC020/2 and N647-pPAC020/3 (=NW138). Chromosomal DNA was subjected to agarose gel electrophoresis after cleavage with *Pst*I or *Kpn*I and Southern blotted. The filters were hybridized with the labelled 1.4 kb *Kpn*I fragment from *A. niger pac*C (**D1**) and, after stripping, with the labelled 1.0 *Hind*III fragment from *A. nidulans arg*B (**D2**). Track order: 1, N402, *Pst*I; 2, N647-pPAC020/3, *Pst*I; 3, N647-pPAC020/2, *Pst*I; 4, N402, *Kpn*I; 5, N647-pPAC020/3, *Kpn*I; 6, N647-pPAC020/2, *Kpn*I; 7, size markers (kb).

The genetically determined location of the *pac*C gene was confirmed by CHEF-Southern analysis performed according to Verdoes et al. (1994). Using the five master strains described, all eight linkage groups of *A. niger* were separated electrophoretically, blotted and probed with the 1.4 kb *Kpn*I fragment from the *pac*C gene. The *pac*C gene could be unambiguously allocated to linkage group IV, both in N402 and in the five master strains the 6.6 Mb linkage group hybridized to the *pac*C probe (Fig. 11.2). As a control, the blot was stripped and reprobed with the 1.6 kb *Sal*I-*Nsi*I fragment of *A. niger pep*A. Linkage group I clearly hybridized to this probe (data not shown) as was previously shown by Debets et al. (1990), indicating correct separation of linkage groups.

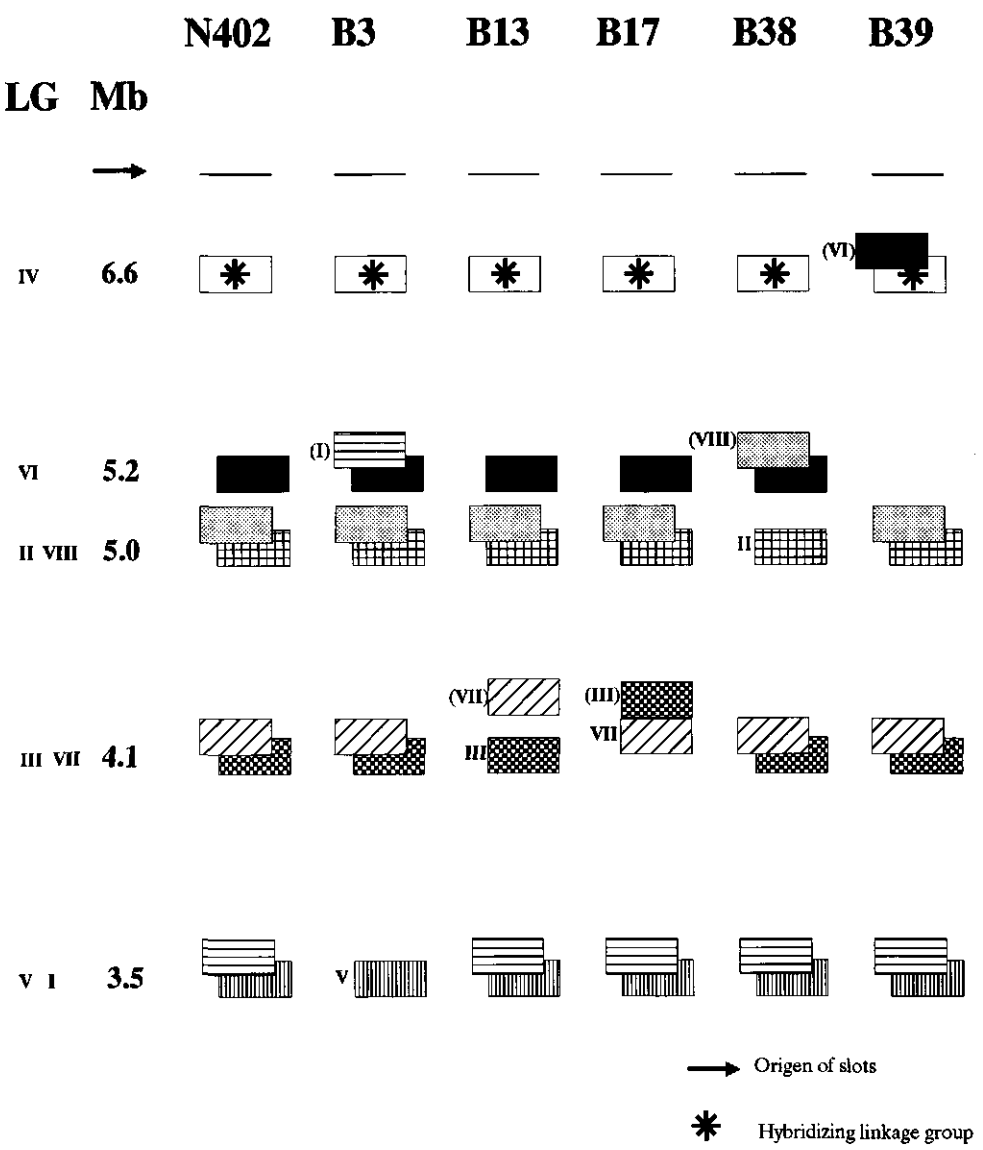

Fig. 11.2. Assignment of *A. niger pac*C to linkage group IV. Schematic representation of CHEF Southern analysis of *pac*C. The electrophoretic karyotype of *A. niger* N402 and the set of five *A. niger* strains with introduced variations in chromosomal electrophoretic mobility are schematically indicated as described by Verdoes et al. (1994). The linkage groups of N402 are indicated by roman numerals. Sizes are indicated in Mb. The position of the chromosomes in the (tester) strains is indicated by differently marked boxes. The linkage group with an introduced mobility variation is indicated in parentheses. The chromosome hybridizing to the 1.4-kb *Kpn*I fragment of *pac*C is indicated by '*'. Track order: lane 1, N402; lane 2, strain B3 (I); lane 3, strain B13 (VII); lane 4, strain B17 (III); lane 5, strain B38 (VIII); lane 6, strain B39 (VI). Modified from Verdoes et al. (1994).

Table 11.1. Genetic allocation of the *A. niger pac*C gene to linkage group IV.

Linkage group	Genetic markers		% Recombination	
	NW138	NW148	with *arg*B$^+$,*pac*2C^c [1]	linked mutation [2]
I	*fwn*A6		49.4	
II		*his*D4	67.9	
III		*bio*A1	45.7	16.0
		*lys*A7	46.9	
IV	*pac*2C^c *arg*B$^+$	*leu*A1	16.0	
V	*arg*B13	*met*B10	44.4	11.1
	*nic*A1	*arg*B15	50.6	
VI		*pab*A1	43.0	
VII		*cnx*C5	63.0	
VIII		*trp*B2	34.6	

For the description of all the markers present in the tester strain NW148 see *Material and methods*. [1], the percentages of recombination between the *arg*B$^+$ marker and the underlined genetic markers in the tester strain and *pac*C disruptant are presented; [2], linked markers on linkage groups III (*bio*A1 and *lys*A7) and V (*met*B10 and *nic*A1), serving as controls during genetical analysis, show clear linkage similar to that between *arg*B$^+$ and *leu*A1.

Analysis of *pac*C expression

Since truncation of the *pac*C gene has pronounced effects on phosphatase expression and both in *A. nidulans* and in *A. niger* there is evidence that *pac*C is autoregulated (Tilburn et al., 1995; MacCabe et al., 1996) we have investigated the expression of the *A. niger pac*C gene by Northern analysis. For this experiment *A. niger* N402 and NW138 were pre-grown on minimal media and equal wet weights were transferred to pH-stabilized minimal media and incubated for a further 6 h. Total RNA was isolated from the mycelia, Northern blotted and probed with the 1.0 kb *Kpn*I-*Pst*I fragment of *pac*C (Fig. 11.3A). Two control blots loaded with equal amounts of RNA were probed with a 0.9 kb *Eco*RI fragment from the *A. bisporus* 28S r DNA gene (Fig. 11.3B) and the 0.45 kb *Pst*I fragment of *pac*C which is located immediately 3' to the site of truncation of *pac*C in NW138. The 0.45 kb *Pst*I fragment of *pac*C did not give any signal in total RNA preparations from NW138 but yielded the same signals as the 1.0 kb *Kpn*I-*Pst*I probe in RNA preparations from N402 (data not shown). As shown in Fig. 11.2, NW138 contains both a 3' truncated and a

5' truncated *pac*C gene within the disrupted locus. The promotorless, 5'-truncated copy of *pac*C is not transcribed as is revealed with the 0.45 kb *Pst*I probe. In contrast, the 3'-truncated copy of *pac*C drives transcription of a truncated 2.2 kb mRNA. The extent of the truncation (approx. 0.6 kb) indicates that transcription terminates considerably downstream the *Pst*I site used in the disruption construct and that the mRNA formed is stable.

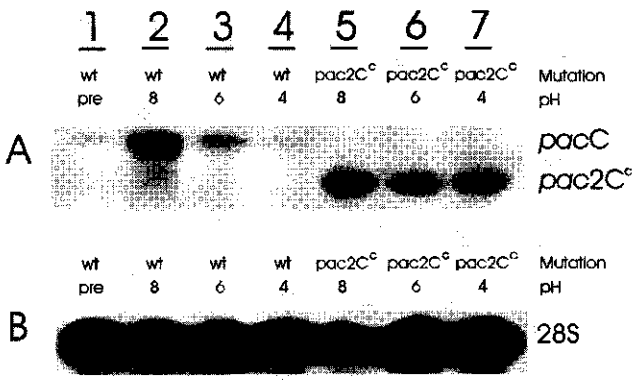

Fig. 11.3. Northern analysis of *pac*C expression in *A. niger* N402 and NW138. Total RNA (10 μg/track) was isolated after transfer to media buffered at different pH values and analyzed. Northern blot probed with the 1.0 kb *Kpn*I-*Pst*I *pac*C fragment (top). Northern blot probed with the 0.9 kb fragment from the *A. bisporus* 28S rDNA gene ,Acc. No. X91812, (bottem). Track order: lane 1, N402, immediately prior to transfer; lane 2, N402, Tris pH 8.0; lane 3, N402, MES pH 6.5; lane 4, N402, citrate pH 4.5; lane 5, NW138, Tris pH 8.0; lane 6, NW138, MES pH 6.5; lane 7, NW138, citrate pH 4.5.

DNA sequencing of the disruption plasmid (pPAC020) showed that several in frame stop codons are present downstream the *Pst*I site, the first of which is located at 140 bp from the *Pst*I site (within the *A. nidulans arg*B fragment). This stop codon would result in a mutant PacC protein (Pac2C) containing first 344 amino acids of PacC followed by the 48 amino acid sequence VDGDGGQALNVASNQIGFEVKVIHMAVANANLDGRQGCRSNKGIFVFR at its C-terminus. Fig. 11.4 shows a schematic representation of the *A. niger* wild-type PacC and truncated Pac2C proteins.

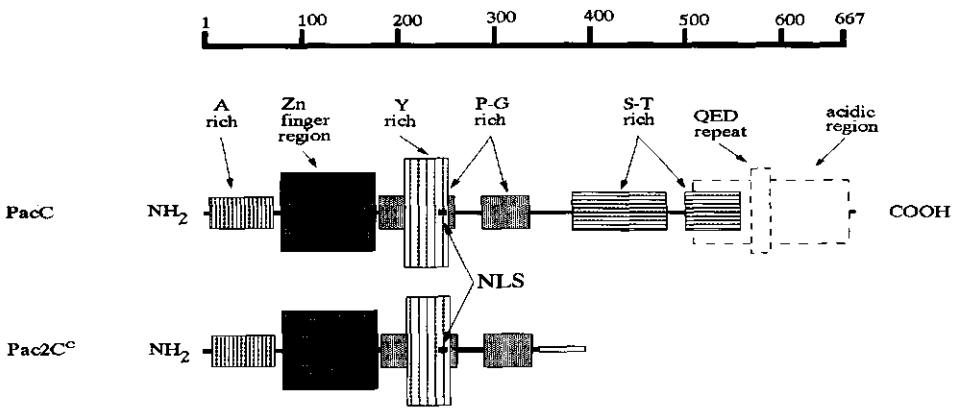

Fig. 11.4. Schematic represesentation of wildtype PacC and Pac2C proteins. A rich, Y rich, P-G rich and S-T rich indicate alanine, tyrosine, proline/glycine and serine/threonine rich regions in the $PacC_{nig}$; NLS: putative nuclear localization signal. The 48 amino acid protein tail in $Pac2C^c$ is indicated by an open box.

From the northern analyses shown in Fig. 11.3, it is apparent that irrespective of the pH of the culture medium, expression of the truncated *pac*C gene is high and comparable to the wild type expression observed at pH 8.0. Furthermore, the autoradiograph shows that transcription of the wild-type 2.8-kb *pac*C mRNA is high under alkaline conditions and low under acidic conditions, similar to the situation in *A. nidulans* (Tilburn et al., 1995).

Identification and localization of phosphatases

To analyze phosphatase expression in relation to pH and phosphate in the growth medium, information about the localization of the individual phosphatase activities is required. Since both acid and alkaline phosphatases are expressed at pH 6.0, the wild type strain N402 was grown at this pH on -P_i medium. Mycelium was used to generate both cell free extracts and protoplasts. After protoplasting, the cell wall fraction and the protoplasts were separated. The protoplasts were lysed and the distribution of acid and alkaline phosphatases in cell free extracts, cell wall fractions, protoplasts and culture fluids was studied using isoelectric focussing combined with alkaline and acid phosphatase activity staining and enzyme assays (Fig. 11.5). An isoelectric focussing gel stained for acid phosphatase activity (Fig. 11.5A) clearly showed that in *A. niger* three major acid phosphatase activities could be identified (PI, PII and PIII), whereas a similar isoelectric focussing gel stained for alkaline phosphatase activity (Fig. 11.5B) identified only one alkaline phosphatase (PIV). In these cell-free extracts the acid phosphatases showed (in contrast to

the alkaline phosphatase) heterogeneity, as for every major acid activity several individual activities with slightly different isoelectric focussing-points are observed. Whether independently encoded activities, (partial) degradation or differences in post-translational modification (e.g. glycosylation) cause this heterogeneity is unknown. As internal controls for the localization experiments, glucose oxidase (GOX) and glucose 6-phophate dehydrogenase (G6Pdh) were used as a cell wall associated and a cytoplasmic marker, respectively (Witteveen et al., 1992). To determine whether phosphatases and control enzymes were stable in Novozyme containing solutions, cell free extracts were incubated with Novozyme. The results on the activity staining of the fractions after isoelectric focussing and the enzyme activity data indicate that the majority of the acid phosphatase activity is located in the cell wall and that the alkaline phosphatase is located intracellularly. The PI activity in the cell wall fraction shows an additional third band compared to the cell free extracts, which could be some (partial) degradation of PI activity but this is probably not caused by Novozyme as no such additional PI band is observed in cell free extracts incubated with Novozyme. The results with the control enzymes used in the localization experiments prove that little cross contamination of the independent fractions has occurred and that Novozyme contains no alkaline and virtually no acid phosphatase activity. The results presented in Fig. 11.5 also show that cell free extracts can be used to analyze phosphatase expression as such extracts contain all the phosphatases identified in the individual fractions. Virtually no phosphatase activity was observed in the medium, in contrast to the situation in *A. nidulans* (Caddick et al., 1986b).

Analysis of phosphatase expression

It has been shown by Caddick and Arst (1986) and Caddick et al. (1986a, 1986b) that certain *A. nidulans* phosphatases are regulated by the phosphate concentration and/or the pH of the culture medium. Since in *A. nidulans*, the *A. niger pac*C gene also appears to be involved in pH regulation of phosphatase expression as MacCabe et al. (1996) showed that the *A. niger* gene was able to restore wild type acid phosphatase expression in an *A. nidulans pac*C^{c}11 mutant. To assess further pH regulation of the phosphatases in *A. niger* we have analyzed phosphatase expression both in N402 and in the *pac*C disruption strain NW138. Both strains were grown in MM and -P_i media buffered at pH 4.0, 6.5 and 8.0 with citrate, MES and Tris, respectively, at concentrations of 200 mM. Mycelial extracts were analyzed using phosphatase enzyme assays and isoelectric focussing with subsequent staining for acid and alkaline phosphatase activity (Fig. 11.6).

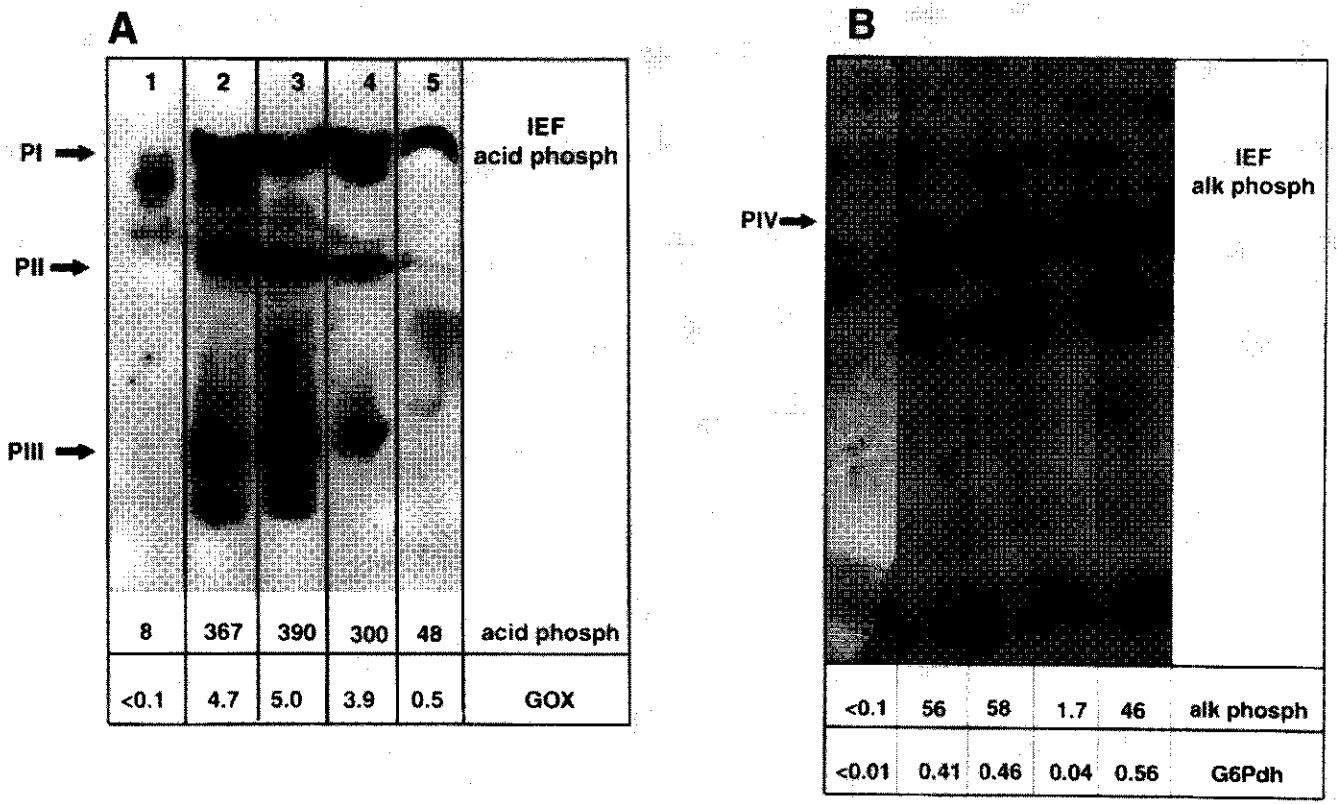

Fig. 11.5. Localization of phosphatases in *A. niger*. Samples containing one mg of protein were loaded on isoelectric focussing gels and after isoelectric focussing stained for acid phosphatase activity (**A**) and alkaline phosphatase acitivity (**B**). Track order: 1, Novozyme solution; 2, cell free extract incubated with Novozyme; 3, cell free extract; 4, Cell wall fraction; 5, Lysed protoplasts. Phosphatase activities are expressed in nmoles *p*-nitrophenol liberated per mg soluble protein in fraction. GOX and G6Pdh activities are expressed as μmol min^{-1} mg^{-1} protein in fraction.

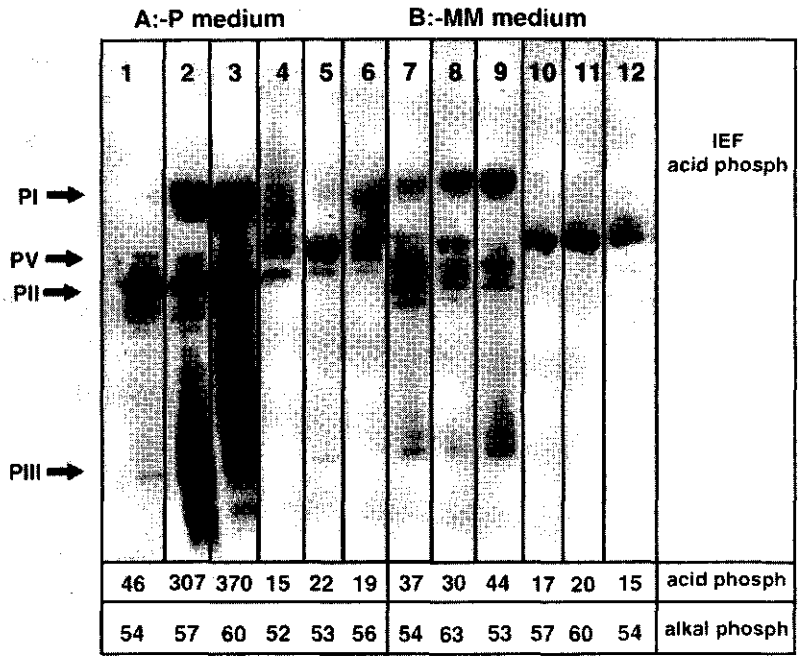

Fig. 11.6. Analysis of phosphatase expression in *A. niger*. Both N402 and NW138 were grown on -P_i (**A**) and MM (**B**) at three different pHs after which phosphatase expression was analyzed as described in Fig. 11.5. Track order: 1, N402, -P_i pH 8; 2, N402, -P_i pH 6; 3, N402, -P_i pH 4; 4, NW138, -P_i pH 8; 5, NW138, -P_i pH 6; 6, NW138 -P_i pH 4; 7, N402, MM pH 8; 8, N402, MM pH 6; 9, N402, MM pH 4; 10, NW138, MM pH 8; 11, NW138, MM pH 6; 12, NW138, MM pH 4.

The extracts derived from -P_i grown mycelium clearly demonstrate higher acid phosphatase activities compared to the MM derived extracts, indicative of repression by phosphate. Visualisation of all acid phosphatases present in the MM derived extracts necessitated over-staining of the -P_i-derived extracts. However all the three major acid phosphatases (PI, PII and PIII) are present in the MM (pH 4.0) derived N402 extracts and absent or severely reduced in the NW138 extracts. Apparently, PacC is involved in the regulation of all three major acid phosphatases. An additional acid phosphatase (PV) is observed in the NW138 extracts. The residual acid phosphatase activity in the *pac2C*[c] mutant is not phosphate repressible as it varies little between -P_i and MM. PV phosphatase appears also to be present in N402 extracts, although it is higher in NW138 extracts. The single alkaline phosphatase (PIV), which is an intracellular enzyme, is not repressed by phosphate and shows high expression at any ambient pH value (Fig. 11.6).

Discussion

We have constructed an *A. niger pac*C disruption strain using a single cross-over approach with an internal fragment of the *pac*C gene cloned in a plasmid containing the *arg*B selection marker. Southern analyses of *arg*B$^+$ transformants, identified by reduced acid phosphatase colony staining, showed disruption of the *pac*C locus, by virtue of site specific plasmid integration.

Northern analysis clearly showed autoregulation of the wild type *pac*C gene and de-regulation of the truncated *pac*C mRNA in the *pac*C disruption strain (NW138). NW138 is phenotypically similar to the *A. nidulans pac*C^c mutants described by Tilburn et al. (1995). This indicates that in *A. niger* the C-terminal region of PacC also negatively modulates the function of the *pac*C gene in response to ambient pH. Removing the C-terminal 323 amino acids encoding part of the *pac*C gene results in a truncated version of *pac*C which is constitutively expressed in the absence of the ambient pH signal.

It is striking that the gene truncation in *pac*2C^c occurs at a position near to that in *A. nidulans* at which a *pac*C$^{+/-}$ mutation (*pac*C$^{+/-}$ 7601) occurs (Tilburn et al., 1995). This observed *pac*C$^{+/-}$ phenotype in *A. nidulans* mutant 7601 could result from the extra 40 aa-tail, resulting from frameshifting. Apart from that particular mutant, all *A. nidulans pac*C$^{+/-}$ mutants described by Tilburn et al. (1995) result from frameshifts upstream of the putative bipartite nuclear localization signal SKKR...KRRQ (reviewed by Boulikas, 1994), which is highly conserved between $PacC_{nig}$ and $PacC_{nid}$. All the *A. nidulans pac*C^c mutants and the *A. niger pac*2C^c disruption strain encode proteins which contain (part of) the putative nuclear localization signal. Hence, possibly the two classes observed among the *A. nidulans pac*C mutants are a result of the presence or the absence of this nuclear localization signal. However, in a recent manuscript Orejas and coworkers (1995) described a novel *pac*C^{c}50 mutation, which encodes a 266 aa PacC protein lacking the KRRQ part of the putative NLS. Furthermore, they demonstrated that proteolytic truncation of the primary translation product of PacC at appoximately residue 270 renders the functional PacC protein, thus demonstrating that proteolysis at a position close to the putative NLS is essential in the regulation of gene expression by ambient pH. This *pac*C^{c}50 mutation also indicates that a role for the NLS in the distinction between *pac*C^c and *pac*C$^{+/-}$ mutations is doubtful. If the putative NLS is functional the *pac*C^{c}50 mutations shows that the KRRQ part is not an essential part of the signal or that the truncated shorter proteins can enter the nucleus without it. Furthermore, the *pac*C^{c}50 mutation indicates that also in *A. nidulans* 3'-truncations, upstream from the *pac*C$^{+/-}$7601 mutation can result in a *pac*C^c phenotype, similar as has been oberved for the *pac*2C^c mutation in *A. niger*. This could indicate that the 40-aa tail in the PacC$^{+/-}$7601 protein is involved in the loss-of function (acid mimicking) phenotype, either via preventing processing to

a functional PacC protein and/or interfering with nuclear import, binding or function of this PacC$^{+/-}$7601 protein, if it is not processed to a functional form. In this respect it is notable that the 40 aa-tail in PacC$^{+/-}$7601 contains a high degree of basic amino acids (twelve out of forty), thus rendering the C-terminal part of the protein very positively charged at physiological pH.

In cell free extracts from *A. niger* five different phosphatase activities (i.e. four acid and one alkaline) were identified. *A. niger* is known to strongly acidify its culture media and this fact could explain the high number of different acid phosphatase activities. Three of these acid phosphatases (PI, PII and PIII) are probably located in the cell wall and are regulated both by phosphate repression and pH regulation. In cell free extracts of the *pac*2C^c disruption strain an additional acid phosphatase (PV) was identified. PV shows no clear phosphate repression or pH regulation but appears to be expressed at higher levels at any ambient pH in NW138, which could be a compensatory effect for the decrease in *pac*C regulated acid phosphatases. The presence of both pH regulated and non pH-regulated acid phosphatases corresponds to the situation in *A. nidulans*, where also both pH-regulated (*pac*A) and constitutive (*pac*G) acid phosphatase genes have been identified (Caddick and Arst, 1986). The observed pH regulation is in agreement with the model presented by Tilburn et al. (1995) for acid-expressed genes. The constitutivity of *pac*C expression in NW138 leads to a lack of expression of genes normally expressed under acidic conditions, irrespective of ambient pH. The single alkaline phosphatase from *A. niger* is not phosphate regulated and is highly expressed independent of pH. Apparently this alkaline phosphatase is expressed in a similar way to the *pal*G-encoded alkaline phosphatase from *A. nidulans* (Caddick and Arst, 1986; Caddick et al., 1986a), which is also located intracellularly. Unlike in *A. nidulans*, no pH-regulated, P_i-repressible alkaline phosphatase (corresponding to the *pal*D product) was detected. The presence of only non pH-regulated alkaline phosphatases in *A. niger* could explain our inability to isolate *A. niger pal* mutants based on reduced alkaline phosphatase activity (van den Hombergh et al., unpublished results).

The characterized *pac*2C^c strain can be used as a starting point to study pH regulation of the extracellular protease genes in *A. niger*. All cloned extracellular protease genes contain one or more potential PacC target sites, as determined for the *A. nidulans* PacC transcription factor, indicating direct involvement of *pac*C in the observed wide domain pH regulation. Although the *A. niger* and *A. nidulans* PacC proteins are very similar and *pac*C^c mutations have similar mutant phenotypes, implying a strong conservation of function, functional differences with respect to regulation cannot be ruled out. Whether the observed differences in the *A. niger* and *A. nidulans*

PacC proteins - such as the absence of the QED-repeat in the C-terminal part of the *A. niger* PacC (MacCabe et al., 1996) - or the observed differences in acidification of the culture medium have any physiological relevance with respect to wide domain pH regulation is not clear. Considering the great variety of *pac*C mutants that have been isolated from *A. nidulans* and the different phenotypic effects that have been observed, it will be important to generate more pH regulatory disruptants and/or classical mutant strains covering other parts of the coding region of the *pac*C gene.

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12 Complex regulation of extracellular proteases in *Aspergillus niger*; an analysis of wide domain regulatory mutants demonstrates CREA, AREA and PACC control[1]

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Summary

Several extracellular proteases in *Aspergillus niger* are regulated by carbon catabolite repression, nitrogen metabolite repression, pH regulation and are induced by the addition of proteins to culture media. We have used a set of *are*A mutants, a set of *cre*A mutants and a *pac*2C^c disruption strain to study regulation of six protease genes at the trancriptional level. The Northern analyses demonstrate that the observed carbon, nitrogen and pH related regulatory effects on the expression of the extracellular proteases *pep*A, *pep*B and *pep*D are mediated by the *cre*A, *are*A and *pac*C wide domain regulatory genes. Although the phenotypic effects of different *are*A and *cre*A mutants on the individual proteases vary, there is clear evidence for nitrogen metabolite and carbon catabolite derepression. Surprisingly, at pH 8.0 in the *pac*2C^c disruptant the expression of acid proteases increased compared to the wild type strain. The expression of vacuolar proteases (*pep*C, *pep*E and *cpy*) is not regulated by *are*A and *pac*C but some carbon catabolite derepression has been observed in more severe *cre*A mutants. Finally, these analyses indicate that some *are*A mutations and the *pac*2C^c disruption could be used to down-regulate acid proteases, which are undesirable in homologous and heterologous protein production.

Introduction

Filamentous fungi are able to utilize a wide range of compounds as sole nitrogen and carbon sources and a large array of structural genes are dedicated to this aspect of metabolism. Efficient utilization of the various carbon and nitrogen sources necessitates tight control of the various genes involved via pathway specific and wide domain regulation. Especially for many extracellular

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enzyme systems, specific (complex) carbon and/or nitrogen sources, or rather their degradation products, are capable to induce the pathway specific set of enzymes needed to metabolize a particular substrate. In *A. nidulans* and more recently also in *A. niger*, several wide domain regulatory genes have been identified and characterized. These wide domain regulatory systems provide a means to adapt overall metabolism to use preferentially those nutrients that can be metabolised most efficiently.

In *Aspergillus* carbon catabolite repression affects the expression of genes involved in the utilization of carbon sources (Arst and Bailey, 1977; Kelly, 1994). From genetic analysis it has been shown that repression is mediated by the CREA repressor protein, encoded by the *cre*A gene, which in the presence of glucose represses the expression of genes involved in the utilization of less-favored carbon sources (Dowzer and Kelly, 1991; Drysdale et al., 1993). The CREA protein contains two C_2H_2 zinc fingers, which are very similar to those present in the C_2H_2 zinc finger family, including the *S. cerevisiae* MIG1 repressor and mammalian control genes such as Zif268, and which bind to specific CREA binding sites (consensus; 5'-G/CPyGGG/AG-3') in the promoters of target genes (Kulmburg et al., 1993). Both for *S. cerevisiae* and *A. nidulans* it has been shown that putative CREA binding sites which contain an A at position five only bind the repressor protein when a G is present at position one (Cubero and Scazzocchio, 1994; Lundin et al., 1994).

As for the utilisation of different nitrogen sources also a preferential order exists. This phenomenon is called nitrogen metabolite or 'ammonium' repression since ammonium is the preferential nitrogen source. This wide domain control mechanism leads to repression of enzymes and permeases involved in the utilization of nitrogen sources other than ammonium or L-glutamine (Arst and Scazzocchio, 1985; Wiame et al., 1985). To date, a single regulatory gene, *are*A, has been implicated in mediating nitrogen metabolite repression. Its activity is essential for the activation of a large number of the structural genes involved in the metabolism of nitrogen compounds when ammonium or L-glutamine is absent (Kudla et al., 1990; Caddick, 1994). The *are*A gene encodes a $CX_2CX_{17}CX_2C$ zinc finger protein, AREA, which shows a high degree of homology with GATA-type transcription factors (Caddick, 1994), and binds to specific binding sites (consensus; 5'-GATA-3') in the promoters of target genes (Kudla et al., 1990; Caddick, 1994).

More recently the *pac*C gene, involved in pH regulation in response to changes in ambient pH, has been cloned and characterized (Tilburn et al., 1995; MacCabe et al., 1996). Regulation of gene expression by pH ensures synthesis of permeases and extracellular enzymes, which function outside the cell permeability barrier, only at a pH value where they can function efficiently. The

*pac*C gene encodes a regulator protein, PacC, which in the model of Tilburn et al. (1995) acts as an activator of alkaline targets and as a repressor of acid target genes, upon binding to a specific binding site (consensus 5'-GCCA/GG-3') in the promoter of target genes (Tilburn et al., 1995).

A. niger is an economically important fungus because it is capable of secreting large amounts of enzymes, including pectinases, hemicellulases, lipases, oxidases, catalases and proteases into its culture fluid (Bodie et al., 1994). In order to be able to fully exploit the production potential of *A. niger*, detailed knowledge about expression of the various enzyme systems is necessary. Jarai and Buxton (1994) and van den Hombergh and coworkers (1994) have initiated regulation studies of extracellular proteases by studying expression of *pep*A, *pep*B and *pep*F gene expression in *A. niger* wild type. They showed that all three previously mentioned wide domain regulatory systems and specific induction resulting from the addition of protein to culture media are involved in the overall regulation of these extracellular proteases.

As the protease genes are indeed regulated by so many different factors, these are interesting target genes to study gene regulation in this biotechnologically important fungal species. Here we describe regulatory studies for three extracellular (*pep*A, *pep*B and *pep*D) and three intracellular (*pep*C, *pep*E and *cpy*) protease genes in *A. niger cre*A and *are*A wide domain regulatory mutants and in an alkaline mimicking wide domain regulatory *pac2*C^c disruptant. Furthermore, we describe a detailed computer analysis of the individual protease promoters.

Materials and methods

Strains, media and culture conditions. All strains were derived from *A. niger* wild type N400 (CBS 120.49); N402 has short conidiophores (*csp*A1). The areA mutants used [NW140 (*bio*A1, *csp*A1, *are*A1); NW173 (*bio*A1, *csp*A1, *are*A2); NW175 (*bio*A1, *csp*A1, *are*A4)] were selected on chlorate after UV-mutagenesis of the N573 (*bio*A1, *csp*A1) strain (MacCabe et al., unpublished results). The *cre*A mutants [NW143 (*bio*A1, *csp*A1, *are*A1, *pyr*A13, *cre*Ad2); NW145 (*bio*A1, *csp*A1, *are*A1, *pyr*A13, *cre*Ad4)] were selected in an *are*A1 background on γ-aminobutyrate (GABA) and glucose (Vanhanen et al., unpublished results); The *cre*A, *are*A1 double mutants were co-transformed with the pAREG001 plasmid, containing the *A. niger are*A gene (MacCabe, unpublished results) and the pGW635 plasmid, containing the *pyr*A selection marker (Goosen et al., 1987), to generate NW143:pAREG001 and NW145:pAREG001, respectively. The *pac*2C^c disruption strain [NW138 (*fwn*A6, *pac*2C^c-*arg*B$^+$, *csp*A1, *arg*B13, *nic*A1)] was previously described (van den Hombergh et al., 1996). The *cre*A^{d}2 recombinant [NW176 (*fwn*A1, *his*D4, *lys*A7, *nic*A1, *pab*A1)] was isolated from a cross between NW143 and N616 (*fwn*A1, *his*D4, *lys*A7, *leu*A1, *nic*A1, *pab*A1).
Minimal medium (MM) contained per liter: 6.0 g (w/v) $NaNO_3$ / 1.5 g (w/v) KH_2PO_4 / 0.5 g (w/v) KCl / 0.5 g (w/v) $MgSO_4$ / trace elements and supplements (as indicated in the figures). Complete medium (CM) contained MM supplemented with 2% (w/v) peptone / 1% (w/v) yeast extract and 0.5% (w/v) casamino acids. Supplementation of media with appropriate carbon and nitrogen sources is indicated in the figures. Glucose and glycerol were used at final concentrations of 1% (w/v) as repressing and derepressing carbon sources, respectively. Nitrogen repression was achieved by replacing $NaNO_3$ with ammonium (+)-tartrate or urea at final concentrations of 50 mM and 0.6 % (w/v), respectively. As external protein source elastin

was added at 0.5% (w/v). Unless specified otherwise, the pH of the culture media was adjusted to pH 6.0. Acidic growth conditions were achieved either through auto-acidification, as *A. niger* rapidly acidifies its medium within a few hours, or via including 0.1 M sodium citrate pH 4.0 in the transfer media. Alkaline growth conditions were achieved by including 0.1 M Tris pH 8.0 in the transfer media (and monitoring the pH of the transfer culture). Liquid cultures were inoculated with 10^6 spores per ml and grown for 20h in CM. Mycelia were filtered, washed several times with sterile distilled H_2O and transferred to MM, supplemented as described in the legends of the figures. After 6-8 h growth under the same conditions in these media, the mycelia were harvested by filtration, washed and immediately frozen in liquid nitrogen.

RNA isolation and Northern analysis. The frozen mycelia were pulverised under liquid nitrogen in a dismembrator and total RNA was isolated using TRIzol (GibcoBRL) according to the suppliers instructions. RNA concentrations were determined via OD measurements and verified by gel electrophoresis and EtBr staining. For Northern analysis 30 μg of total RNA was loaded on formaldehyde-agarose gels as decribed by Sambrook et al. (1989), transferred to Hybond-N membranes (Amersham) via capillary elution and UV-crosslinked. Hybridization was performed essentially as detailed by Sambrook et al. (1989) using 0.75 M NaCl / 0.1 % (w/v) SDS / 50 mM NaH_2PO_4 (pH 7.4) / 10 mM EDTA / 2 x Denhardt's / 10% (w/v) dextran sulphate / 50% (w/v) formamide / denatured herring sperm DNA (200 μg/ml) as hybridization buffer at 42°C. Northern blots were washed down to 0.2 x SSC [0.15 M NaCl, 0.015 M Na_3citrate pH 7.0] / 0.5 % (w/v) SDS at 65°C. The membranes were autoradiographed wet to enable stripping and reprobing of the blots. Membranes were stripped between probings with the different probes by washing several times in 1% (w/v) SDS at 100°C.

Isolation of the *A. niger* N400 *cpy* gene by PCR. Based on the *A. niger* serine carboxypeptidase Y (*cpy*) sequence presented by Yaver et al. (1995), two oligonucleotide primers were designed to PCR the complete *cpy* coding region from *A. niger* N400. For amplification of the *cpy* fragment 100 ng of genomic N400 DNA and 50 pmoles of both oligonucleotide primers were used in a standard PCR buffer (50 mM KCl, 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 0.01 % (w/v) gelatin). After 30 cycles of amplification (denaturing: 94°C, 1 min; annealing 50°C, 1 min; elongation: 72°C, 3 min) the amplified product was isolated from agarose gels and subcloned in plasmid pGEM-T (Promega). The cloned insert was partially sequenced using the dideoxy chain-termination method according to Sanger et al. (1977) to prove cloning of the *A. niger* N400 equivalent of the *cpy* gene.

Isolation of *A. niger* protease probes. Specific DNA fragments were labelled with ^{32}P using random priming (Sambrook et al., 1989). *pep*A: a 0.49 kb *Cla*I-*Hin*dIII fragment from plasmid ptz18A400, containing the complete *A. niger pep*A gene [kindly provided by Dr. F.Buxton]; *pep*B: a 0.73 kb *Bgl*II-*Sal*I fragment from plasmid pFBY175ER, containing the the *A. niger pep*B gene [kindly provided by Dr. F. Buxton]; *pep*C: a 0.56 kb *Hpa*I-*Hin*dIII fragment from plasmid pANB1 containing the *A. niger pep*C gene (Frederick et al., 1993); *pep*D: a 0.38 kb *Kpn*I-*Eco*RV fragment from plasmid PEPD12 containing the *A. niger pep*D gene (Jarai et al., 1994a); *pep*E: a 0.5 kb *Eco*RI fragment from plasmid pIM623, containing the complete *pep*E gene; *cpy*: a 2.0 kb PCR fragment containing the complete *A. niger cpy* coding region (vide infra); 28S rDNA: the 0.9 kb *Eco*RI fragment of the *Agaricus bisporus* 28S rDNA gene (Acc. No. X91812).

Computer analyses. Homology searches between a partial N400 *cpy* sequence and the complete *S. cerevisiae ysc*Y gene were performed with the CLUSTAL programme. The nucleotide sequences of the *pep*A, *pep*B, *pep*C, *pep*D, *pep*E and *pep*F promoters were analyzed using the programmes BESTFIT and FINDPATTERNS. All programmes are available in the GCG package (Genetics Computer Group).

Results and Discussion

Isolation of the *A. niger cpy* probe

In order to study the expression of three extracellular (*pep*A, *pep*B and *pep*D) and three intracellular (*pep*C, *pep*E and *cpy*) proteases in *A. niger*, we first had to generate specific probes for all these protease genes and to isolate the *A. niger* N400 *cpy* equivalent of the recently described *A. niger cpy* gene, the sequence of which was presented by Yaver et al., 1995. We used a PCR approach to amplify and isolate a DNA fragment containing the complete *cpy* coding region. The amplified PCR fragment was subcloned in pGEM-T, which was partially sequenced to verify the identity. A homology search in the combined GenBank/EMBL database indicated that the highest homology was found with the *S. cerevisiae* CPY, encoding the *ysc*Y gene, (70 % at aa level, data not shown).

Sizing of protease mRNAs and mRNA controls

Expression of the *pep*A, *pep*B, *pep*C and *pep*E genes and sizing of their mRNAs has been described previously (Jarai and Buxton, 1994, Jarai et al., 1994b. Using commercially available RNA size markers we found that the *cpy* PCR probe hybridized to a single RNA species of about 1.5 kb. The *pep*D probe hybridized also to a specific single RNA species of approximately 1.5 kb. Both results agree with the previously determined lengths of the corresponding protease genes (Jarai et al., 1994a; Yaver et al., 1995).

Previous experiments using the wild type strain indicated that the *pep*C and *pep*E genes are highly expressed under all growth conditions tested, with only very limited, growth-related variations (Jarai et al., 1994b) and thus can be used as loading controls in Northern-blot experiments. However, to exclude any regulatory effect on the *pep*E and *pep*C genes the 28S rDNA probe was used as a control to show that equal amounts of RNA were loaded on the gels. Because the size of all protease mRNAs is in the 1.3-1.8 kb range we could not use a mixture of probes. Instead, blots were stripped and probed separately with the different probes.

Nitrogen metabolite repression; *A. niger are*A mutants

Extracellular proteases

We have analyzed the expression of protease genes in relation to different nitrogen sources in Northern blot experiments using *A. niger* wild type N402 and several *are*A mutants (MacCabe et al., unpublished results) which were pre-grown on CM and transferred to media containing different nitrogen sources either in the presence or absence of glucose and/or inducing protein (Fig 12.1).

In the absence of carbon source, the nitrate containing shift cultures resulted in expression of the extracellular proteases, whereas ammonium but also urea did show repression. In the presence of inducer (elastin) ammonium still repressed expression whereas in the case of nitrate very high expression levels were obtained with elastin. The pronounced repressive effect of urea led Jarai and Buxton (1994) to postulate that besides nitrogen metabolite repression an additional indirect and as yet unknown mechanism was involved in the regulation of protease gene expression.

In N402 (Fig. 12.1A), none of the extracellular proteases was expressed in the presence of glucose except for *pep*A which showed some expression when nitrate was used as sole nitrogen source (see also Fig. 12.3, lane 3). In the carbon derepressed, non-induced situation expression of the proteases *pep*A, *pep*B and *pep*F was observed, however only in the presence of nitrate. In carbon derepressed, protein induced mycelia the highest expression levels were observed on nitrate but all extracellular proteases were expressed on urea as well. In the presence of ammonium all protease genes were still completely repressed. Apparently, urea, similar to ammonium, is also capable to very efficiently repress all extracellular proteases in the absence of inducer. Expression in the presence of inducer and urea was observed for all extracellular proteases. Although an urea-mediated repressive effect is absent for *pep*A it is still to some extent present for *pep*B and *pep*D. The results in wild type (N402) clearly demonstrate that the protease genes encoding extracellular proteases (*pep*A, *pep*B and *pep*D) are regulated by nitrogen metabolite repression. In the different *are*A mutants, expression of the extracellular protease genes is not modulated in the same way but the response depends on the combination of the protease gene and the particular *are*A mutation (Fig. 12.1B-D). The *are*A1 mutation appears to be a partial gain of function mutation, a conclusion based on the phenotypic effects observed for the expression of the *pep*A and the *pep*D genes which were higher when nitrogen sources other than ammonium were used. The ammonium repression was still present, indicating that *are*A1 is not a derepressed allele. However, for the *pep*B gene a more repressed phenotype is observed. Expression of *pep*B was also low or beyond detection on nitrogen sources other than ammonium. Only in the presence of inducer (and in the absence of carbon catabolite repressing carbon sources) *pep*B mRNA was observed in the *are*A1 strain, although significantly less than in the wild type strain. In the *are*A4 mutant (Fig. 12.1D) expression of *pep*A and *pep*B was observed on urea and on ammonium, indicative for an *are*A^{d} type of mutation. The *pep*B expression however, is still ammonium and urea repressed. The *are*A2 mutation has the severest effects on extracellular protease expression (Fig 12.1C). *pep*B expression was completely repressed, *pep*A expression was reduced and *pep*D expression was changed compared to wild type (depending on the transfer culture condition, both

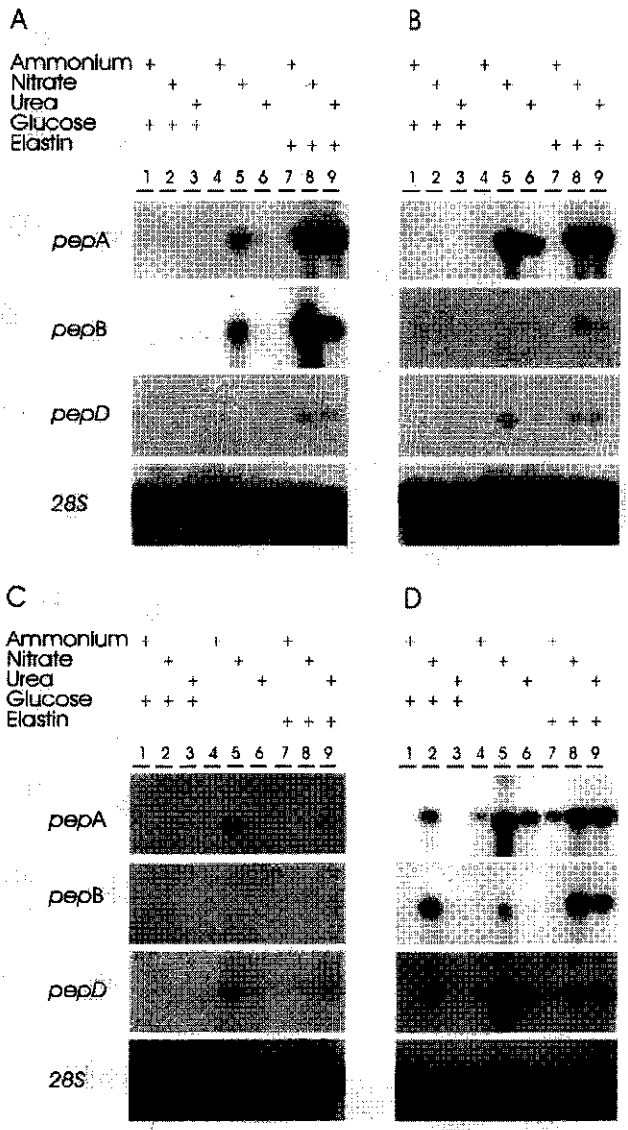

Fig. 12.1. Nitrogen metabolite repression of extracellular proteases. Cells were grown overnight in complete medium, washed and transferred into minimal medium supplemented with nitrogen and carbon sources as indicated in the figure. After 6-8 h of additional growth, mRNA was isolated as described in the experimental procedures. RNA samples were separated in agarose gels, blotted and hybridized separately with the *pep*A, *pep*B, *pep*D and 28S rDNA probes; the blots were stripped between each hybridization (for details see experimental procedures). Transfer cultures were not buffered which resulted in acidification of the cultures to a final pH between 3.5 and 4.5. Effects of nitrogen metabolite repression in wild type N402 (**A**), and *are*A mutants; (**B**) NW140 [*are*A1]; (**C**) NW173 [*are*A2]; (**D**) NW175 [*are*A4].

up and down regulation are observed). Similar differential effects of areA mutations upon the expression of individual target genes in *A. nidulans* has resulted in the identification of a third type of *are*A mutants in *A. nidulans*. Apart from the *are*A^{d} (derepressed) and *are*A^{r} (repressed or loss-of-function) mutations, *are*A mutants with an 'altered' function have been described (Caddick, 1994). In this heterogenous class of mutants the perceived wild-type hierarchy of expression is changed, resulting in derepression of some activities while others become more repressed. The heterogeneity of this mutant class was also used as formal evidence that AREA must act directly in mediating nitrogen metabolite repression in *A. nidulans* (Arst and Cove, 1973; Arst and Bailey, 1977; Arst and Scazzochio, 1985). As some of the *A. niger are*A mutations also have altered function phenotypes, AREA appears to act in *A. niger* in a very similar way. The relative high frequency of altered function phenotypes among the *A. niger are*A mutants isolated, could be a result of the complex regulation of extracellular proteases, in which a number of transcription factors are involved (see also *cre*A mutants, *pac*2C disruptant and promoter analysis).

Intracellular proteases

Three genes encoding intracellular proteases have been cloned and characterized in *A. niger*. The *pep*E, *pep*C and *cpy* (Jarai et al., 1994a; Frederick et al., 1993 and Yaver et al., 1995) encode the *A. niger* equivalents of the *S. cerevisiae PEP4*, *YSCB* and *CPY* genes (Suarez Renduelez and Wolf, 1988), respectively and thus presumably encode also vacuolar proteases in *A. niger*. In a previous study Jarai et al. (1994b) have shown that at the transcriptional level no apparent carbon catabolite repression, nitrogen metabolite repression, pH regulation or protein induction effects occurred on the expression of the *pep*C and *pep*E genes. As shown in Fig 12.2AB, at the transcriptional level no differences were observed between N402 and the *are*A4 mutant NW175 with respect to *pep*C, *pep*E and *cpy* (as is also the case for the *are*A1 and *are*A2 mutants, data not shown). Thus in *A. niger* wild type, the *cpy* gene is also constitutively expressed, like the *pep*C and *pep*E genes, and the unaltered expression of all three genes in *are*A mutants demonstrates that none of them is AREA controlled.

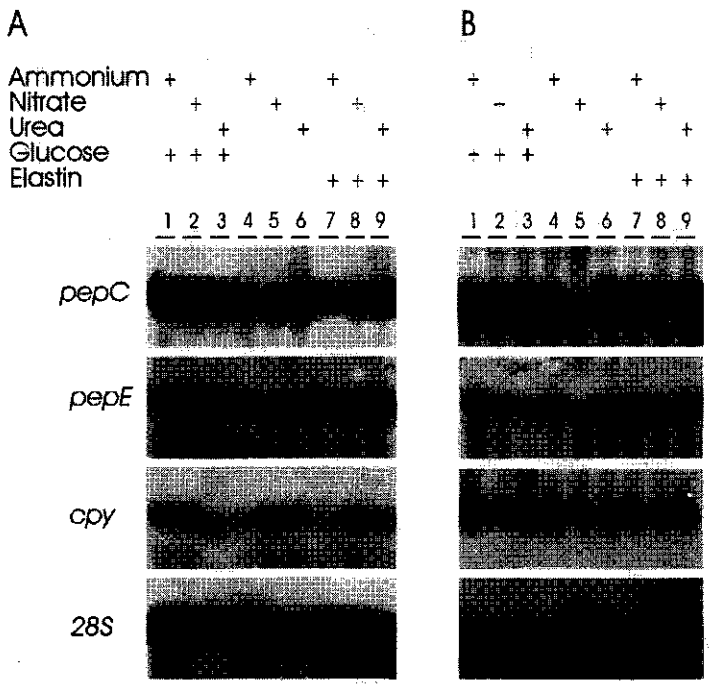

Fig. 12.2. Expression of intracellular protease; effects of nitrogen metabolite repression on the expression of *pep*C, *pep*E and *cpy*. Experiments were carried out as described in Fig. 12.1. (**A**) Expression in N402; (**B**) Expression in NW175 [*are*A4].

Carbon catabolite repression; *A. niger cre*A mutants

Extracellular proteases

Many genes involved in the utilisation of different carbon sources are regulated by the wide domain carbon catabolite repression system. Recently, van de Vondervoort (unpublished results) has isolated several *A. niger cre*A mutants, using a method described by Arst and Cove (1973). This method takes advantage of the observation that genes involved in metabolism of compounds that can supply the cell with a source of both carbon and nitrogen can be subject to both nitrogen metabolite repression and carbon catabolite repression, and that relief of either one leads to derepression. After mutagenesis in an *A. niger are*A1 genetic background and subsequent selection of mutants growing on γ-amino butyric acid (GABA) and glucose several *A. niger cre*A mutants were isolated, as carbon catabolite derepression leads to suppression of the loss of function *are*A1 mutant phenotype.

We have used several *A. niger cre*A mutants and the N402 wild type strain in transfer experiments to study the effects of repressing and de-repressing carbon sources (glucose and glycerol, respectively), in relation to nitrogen source (nitrate or ammonium) and the presence or absence of protein inducer (elastin). In wild type (Fig. 12.3A) the carbon catabolite repression effect of glucose on the expression of the extracellular proteases, both in the non-induced (lane 2, 3) and the induced (lane 6, 7) transfer cultures, resulted in decreased transcript levels. Glycerol has no effect on *pep*A mRNA levels compared to complete carbon derepressed cells. This in contrast to the response of *pep*B, in which case glycerol clearly caused repression. Furthermore, glucose was able to repress *pep*B completely even in the absence of repressing nitrogen source (lane 3) or in the presence of an inducing protein source (lane 7). In wild type, the *pep*D transcript, which was not detected in prior studies, was only observed under very specific (carbon derepressed elastin induced cultures) conditions. Apparently, the specific expression conditions required have prevented prior detection of *pep*D transcripts by Jarai et al. (1994a).

In a pilot experiment one of the isolated *cre*A, *are*A1 double mutants (NW143; *bio*A1, *csp*A1, *are*A1, *pyr*A13, *cre*A^{d}2), was compared with the parental strain (NW140; *bio*A1, *csp*A1, *are*A1) in a Northern analysis, similar to the analysis described above for the wild type (Fig. 12.3ABC). Comparing the glucose containing shift cultures (Fig. 12.3 AC, lanes 3,7), carbon derepression was observed in the *cre*A^{d}2, *are*A1 double mutant. However, as cross-control between carbon catabolite and nitrogen metabolite repression or interfering effects between carbon catabolite repression and nitrogen metabolite regulatory mutants cannot be ruled out, the NW143 strain was transformed with the *A. niger are*A gene to generate a *cre*A^{d}2:pAREG001 transformant. Additionally, the recombinant strain NW176, containing only the *cre*A^{d}2 wide domain regulatory mutation, was isolated from a cross between NW143 and N616. Both the *cre*A^{d}2 recombinant (NW176) and transformant (NW143:pAREG001) were analyzed in a set of similar Northern experiments as described for the *cre*A^{d}2, *are*A1 double mutant (Fig 12.3DE). In the *cre*A^{d}2 recombinant high expression levels were observed in glucose containing shift cultures, both for *pep*A and *pep*B. Carbon catabolite repression was not completely abolished, as shown by the higher expression levels in non-glucose containing shift cultures. The expression levels in the presence of ammonium hardly differed from cultures containing non-repressing nitrogen sources. Whether the high *cre*A^{d}2-mediated carbon catabolite derepression masks the nitrogen metabolite repression effects is unknown. In the *cre*A^{d}2 strain transformed with the *A. niger are*A gene, NW143:pAREG001 (*bio*A1, *csp*A1, *are*A1, *are*A^{+}, *cre*A^{d}2), the overall expression in relation to nitrogen source is very similar to that observed in wild type (Fig. 12.3AE). In some transfer cultures clear carbon derepression effects were observed. In the presence of ammonium and

glucose (Fig 12.3E, lane 5) expression of *pep*A, *pep*B and *pep*D is observed indicating that ammonium repression is overruled. Also, upon a transfer to glucose and nitrate containing media (Fig 12.3E, lane 3) clear derepression of *pep*B is observed. Furthermore, in a *cre*A^{d}2 genetic background the expression of *pep*D is not longer restricted to derepressed conditions in combination with induction, as is observed in wild type. Ammonium repression effects are similar to those of ammonium observed in the wild type, caused by restoration of an *are*A^{+} phenotype through the introduction of multicopy functional *are*A gene.

The results described for the *A. niger cre*A^{d}2 mutant indicated that effects of this mutation in an *are*A1 background can also be observed after restoration of a wild type *are*A phenotype in an *are*A1 background transforming this mutant with an *A. niger are*A gene containing plasmid. We have also analyzed a second *are*A-transformed *cre*A derepressed mutant strain, NW145:-pAREG001, (*bio*A1, *csp*A1, *are*A1, *are*A^{+}, *cre*A^{d}4), which also . The *cre*A^{d}2 and the *cre*A^{d}4 phenotypes were very similar (Fig. 12.3EF) but the *cre*A^{d}4 allele appeared to represent a more severely derepressed *A. niger cre*A^{d} mutation.

Comparing the effects of carbon catabolite repression and nitrogen metabolite repression on the various protease genes, it appears that the *pep*B gene is more tightly controlled than the *pep*A and *pep*D genes. In wild type glycerol or urea can completely repress *pep*B expression, in contrast to that of *pep*A and *pep*D. These differences observed for the expression of *pep*A, *pep*B and *pep*D could be directly related to the promoter structures of the individual genes. All contain several putative AREA and CREA binding sites but position and spacing of these sites is not conserved (vide intra) which probably implies that these sites vary in their binding affinity for AREA and CREA. Thus, the combination of the amount of active AREA and/or CREA present under a given set of culture conditions and the different affinities of the individual AREA and CREA binding sites, as a results of differences in gross promoter structure, are probably responsible for the complex and different phenotypic effects observed for some extracellular proteases.

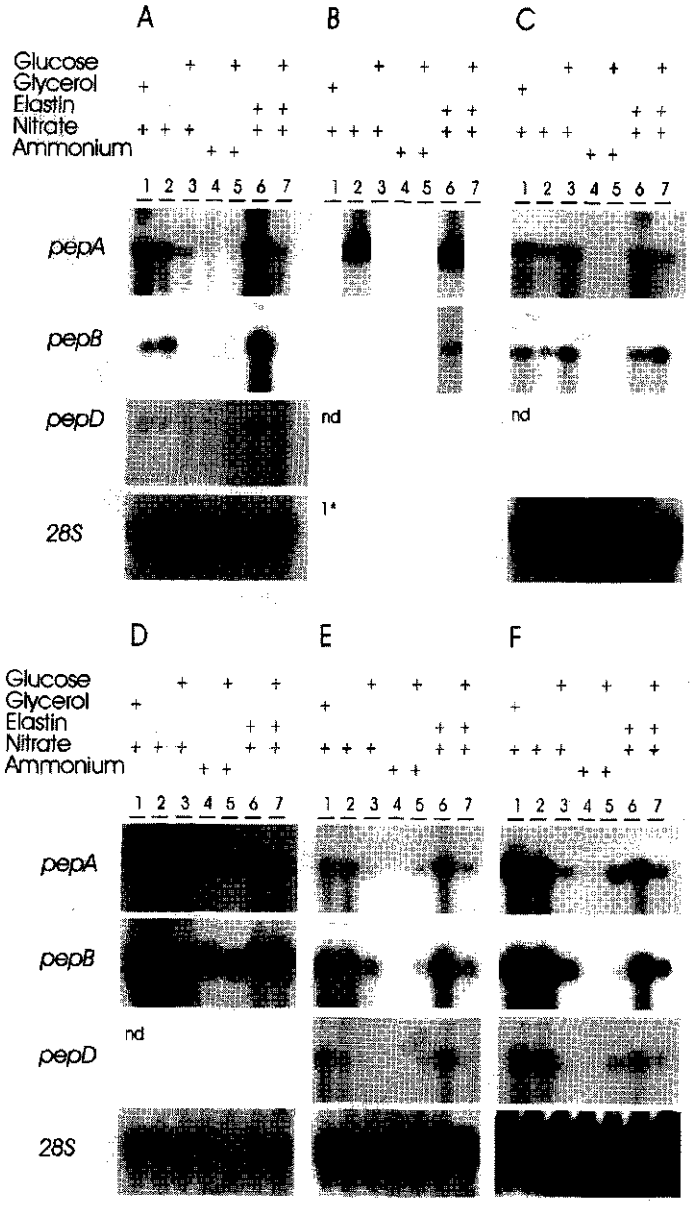

Fig. 12.3. Carbon catabolite repression of extracellular proteases. Experiments were carried out as described in Fig. 12.1. Effects of carbon catabolite repression in wild type N402 (**A**) and *creA*^{d} mutants; (**B**) NW140 [*areA*1] (only conditions for which expression was observed are shown); (**C**) NW143 [*areA*1, *creA*^{d}2]; (**D**) NW176 [*creA*^{d}2]; (**E**) NW143:pAREG001 [*areA*1, *areA*$^{+}$, *creA*^{d}2]; (**F**) NW145:pAREG001 [*areA*1, *areA*$^{+}$, *creA*^{d}4]. nd, not determined.

Intracellular proteases

We have also compared the expression of the intracellular proteases in wild type and in the two *are*A-transformed *cre*A^{d} mutants (Fig. 12.4ABC). Although in wild type no carbon catabolite repression effects were observed, it appeared that the expression levels of the intracellular protease genes (*pep*C, *pep*E and *cpy*, the latter not shown) were slightly higher in the *cre*A^{d}2 mutant and even further increased in the more severe *cre*A^{d}4 mutant. Serial dilution slot blot hybridizations confirmed this result (data not shown), indicating that carbon catabolite repression may still have an effect on these apparently constitutively expressed genes. This small effect might be an indirect effect caused by a higher need for proteolytic degradation, as in a carbon catabolite derepressed genetic background numerous CREA-controlled genes will be expressed.

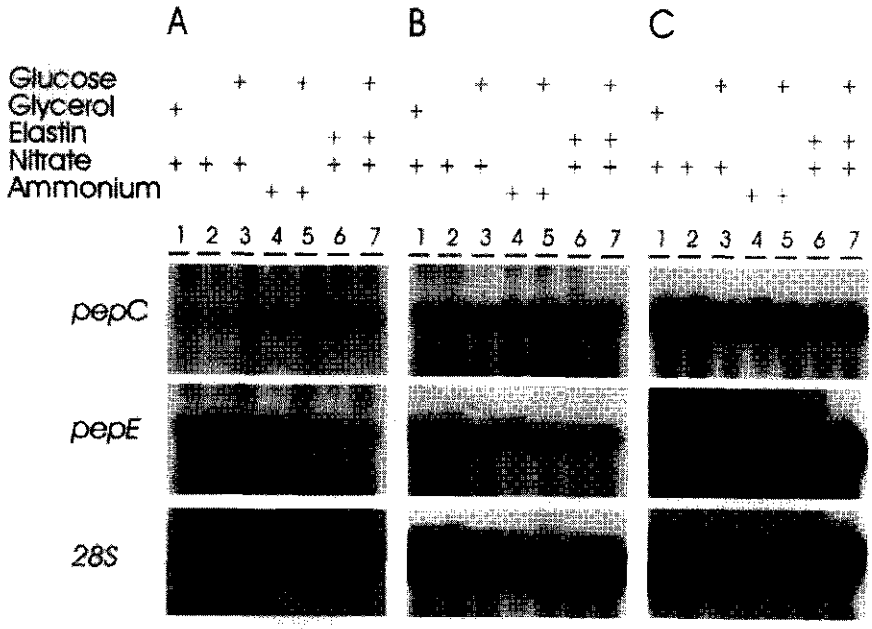

Fig. 12.4. Carbon catabolite repression of intracellular proteases. Experiments were carried out as described in Fig. 12.1. Effects of carbon catabolite repression in wild type (**A**) and *cre*A^{d} mutants; (**B**) NW143:pAREG001 [*are*A1, *are*A^{+}, *cre*A^{d}2]; (**C**) NW145:pAREG001 [*are*A1, *are*A^{+}, *cre*A^{d}4].

pH regulation; the *A. niger pac*2C^c disruption strain

The observation that *A. niger* has a number of acid proteases combined with the fact that *A. niger* acidifies its medium rapidly has initiated a detailed analysis of pH regulation in this fungus. This resulted in the recent cloning of the *A. niger pac*C gene mediating wide domain pH regulation, and its characterization (MacCabe et al., 1996). Based on a pH regulation model for gene expression in *A. nidulans* presented by Tilburn et al. (1995), the PacC protein is supposed to be only active at alkaline pH, where it acts simultaneously as an activator of alkaline target genes and as a repressor for acid target genes. The observation that *A. nidulans* alkaline mimicking (*pac*C^c) mutations express constitutively, i.e. without the need for pH signal transduction, a C-terminally truncated PacC^c protein and thus super-repress target genes normally expressed under acidic conditions, led van den Hombergh et al. (1996) to construct a *pac*2C^c alkaline mimicking disruptant in *A. niger*, designated NW138. In this disruptant the expression of pH regulated acid phosphatases is severely reduced at every ambient pH tested.

We have now analysed the *A. niger* N402 and the *A. niger* NW138 (*pac*2C^c) strains with respect to acid protease gene expression in similar transfer experiments as described for the *are*A and *cre*A mutants. To maintain either an acidic pH or an alkaline pH, the transfer cultures were buffered with 0.1 M NaCitrate (pH 4.0) or 0.1 M Tris (pH 8.0). In previous experiments we have tested other buffer systems and the addition of different salts to exclude the possibility that the observed effects were buffer or salt induced (MacCabe et al., 1996; van den Hombergh et al., 1996). The pH of the transfer cultures was monitored to ensure that the initial pH was maintained.

As shown in Fig. 12.5A, the expression of the *pep*A and *pep*B genes is clearly pH regulated as no expression is observed at pH 8.0, whereas at pH 4.0 high expression is observed in the absence of carbon source and especially in the presence of inducer. These results correspond with previous observations described by Jarai and Buxton (1994). The *pac*2C^c cultures transferred to pH 4.0 resulted in *pep*A and *pep*B expression levels which were reduced compared to the wild type situation, implicating repression by the Pac2C^c protein which is constitutively expressed and thus also present at acidic pH. However, although at an acidic ambient pH the expression of pH regulated acid proteases in a *pac*C^c strain fits with the model, the situation at alkaline ambient pH is very different. The trancript levels for both *pep*A and *pep*B are high in the *pac*2C^c strain. This effect is probably not caused by the Pal-pathway which is active at alkaline pH and which has been shown to be involved in pH signal transduction in *A. nidulans* (Tilburn et al., 1995). A similar Pal pathway is likely to be present in *A. niger* as well since recently one of the genes in this pathway, *pal*B which encodes a novel cysteine protease (Denison et al., 1995), has been cloned (van den Hombergh, unpublished results).

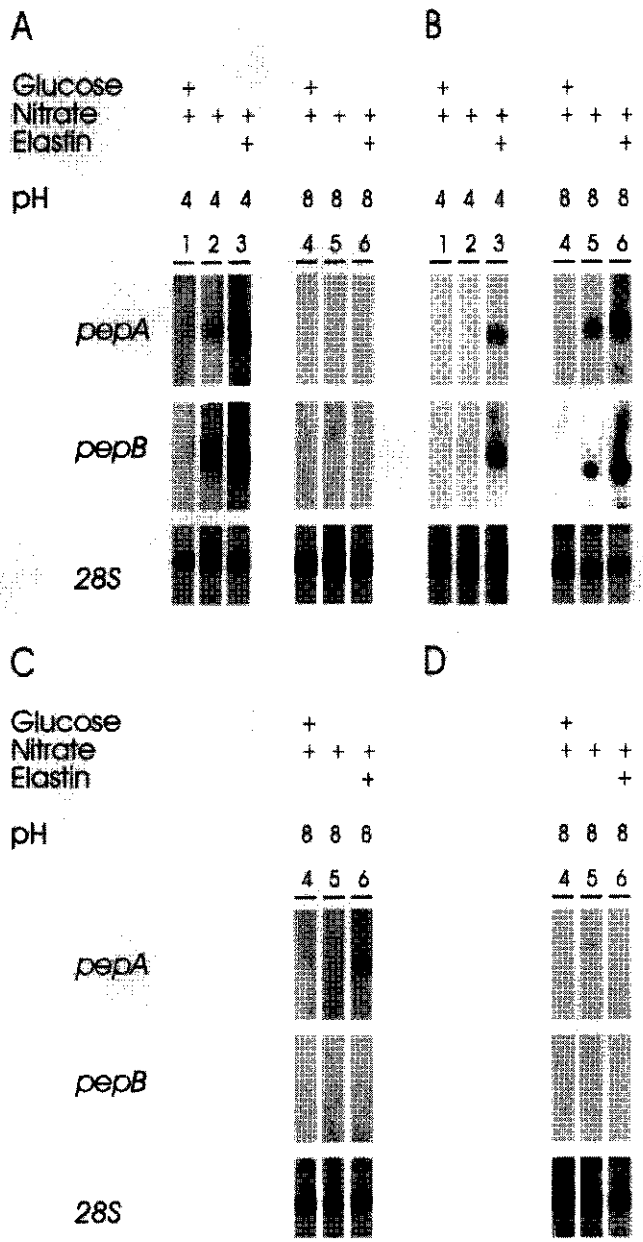

Fig. 12.5. pH regulation of extracellular proteases. Experiments were essentially carried out as described in Fig. 12.1. Effects of ambient pH in wild type (**A**) and three wide domain regulatory mutants; (**B**) NW138 [*pac*2C^c]; (**C**) NW173 [*are*A4]; (**D**) NW145:pAREG001 [*are*A1, *are*A$^+$, *cre*A^{d}4]. Different carbon and nitrogen sources, the addition of protein inducer and the controlled pH of the transfer culture are indicated in the figure.

In a paper by Orejas et al. (1995) it has been postulated that the C-terminal part of PacC contains a masking domain which has to be proteolytically removed to generate a functional PacC. The Pal-mediated pH signal transduction is hypothesized to provide a signal which interferes with or disrupts the masking function of the C-terminus and thus allows proteolytic activation of PacC. However, in a C-terminally truncated protein (encoded by *pacC*^{c} mutant genes) this masking is absent, thus resulting in constitutive expression of functional PacC. The fact that the *pac*2C^c disruptant has an alkaline mimicking phenotype was clearly demonstrated by the observation that *pacC*, being an alkaline (auto-)regulated target gene itself, is constitutively expressed in NW138 (van den Hombergh et al., 1996). The pH regulation model predicts that a functional Pal pathway would have no effect on pH regulation in a *pacC*^{c} disruptant, which is in fact observed in *A. nidulans* as *pacC* mutations are epistatic to *pal* mutations.

The very interesting observation concerning acid protease expression in *A. niger* could be specific for these acid protease genes, because down-regulation of acid phosphatase expression has been observed at both acid and alkaline pH in *A. niger* NW138 (van den Hombergh et al., 1995). Although these experiments do not completely exclude similar up-regulated transcriptional levels for acid phosphatase genes, since no acid phosphatase Northern data is available for the *A. niger* *pac*2C^c disruptant, the enzyme data seems to indicate that pH regulation of phophatase expression is similar to the situation in *A. nidulans* and thus is in agreement with the pH regulatory model. The unexpected up-regulation of acid proteases at alkaline pH, could thus be a result of the interaction of the mutant PacC protein with other transcriptional factors involved in protease regulation. However, the presence of a not yet identified factor, involved in acid pH regulation (of proteases) cannot be excluded. If an additional factor is involved, the high acid protease gene expression in *pac*2C^c and the complete repression in wild type at alkaline ambient pH could be used for mutation strategies to identify this factor. For the expression of the three intracellular proteases (*pep*C, *pep*E and *cpy*) no changes were observed in the *pac*2C^c disruptant, indicative for the absence of pH regulation (data not shown).

In Fig. 12.5C and 12.5D the effects of a transfer to alkaline pH are shown for cultures of the severest *cre*A mutation, *cre*A^{d}4 (strain NW145:pAREG001) and of the severest *are*A mutation, *are*A2 (strain NW173). In both mutants pH regulation still resulted in complete (NW173) or partial represssion (NW145:pAREG001) of both *pep*A and *pep*B at alkaline pH. Only in the *cre*A^{d}4 mutant *pep*A expression is observed but this was limited to a condition in which the inducer was present and repressing carbon and nitrogen sources absent. The expression level was low compared to pH 4.0 under inducing conditions. From these experiments it was not possible to derive a strict hierarchy in wide domain regulatory effects on extracellular protease gene

expression. Still, the pronounced effects observed at pH 8.0 both in wild type and in a *cre*A^{d}4 mutant demonstrate that pH regulation plays an important role.

Finally, it can be stated that some of the observed regulatory effects in *A. niger* wild type and several wide domain regulatory mutants indicate that modulation of protease expression through the use of wide domain regulatory mutations and/or disruptions is possible. Considering the protease-related degradation problems observed when *A. niger* is used as an expression host for the production of proteins, the present study indicates that down-regulation by modulating wide domain control could be an additional means to reduce proteolytic degradation of the proteins produced. For instance, the effects observed for the *pac*2C^{c} disruption strain combined with the instability of acid proteases above pH 6.0 indicate that extracellular proteolytic activities are reduced in a *pac*C^{c} genetic background. At low pH acid proteases are down-regulated by constitutive expression of *pac*C and at an alkaline pH any acid protease expressed is expected to be unstable. Similarly, in some of the *are*A mutants protease expression is down-regulated, which could also be exploited in protein production strains. Using a specific halo-screening medium, developed for the isolation of *A. niger prt* deficient mutants (van den Hombergh et al., 1995), we observed a strong reduction of protease activity both in the *are*A2 and in the *pac*2C^{c} disruptant (data not shown). However, before introducing some wide domain regulatory mutations in the present expression strains, the effects of the mentioned wide domain regulatory mutants/disruptants on other important parameters for protein production such as growth rate, sporulation, acidification and regulatory effects on the promoters used to drive high gene expression, have to be determined.

The effect of certain *cre*A^{d} mutations leading to increased expression of intracellular proteases, probably encoding vacuolar functions, is also very interesting. Whether in *A. niger* a higher level of mRNAs for the vacuolar proteases indeed results in a higher vacuolar protease activity, is unknown. However, in *S. cervisiae* it has been observed that inactivation of the vacuolar cascade through the disruption of the *PEP4* gene results in improved protein production. Thus, especially for expression strains which have some carbon catabolite derepressed phenotype it can become important to study gene expression of both intra- and extracellular proteases, as their levels might have increased whereas they are potentially hazardous to the desired protein products.

Promoter analysis protease genes

The experiments presented have demonstrated that at the transcriptional level several extracellular protease genes are regulated in a complex manner and at least by three wide domain regulatory genes. Combined with the fact that for the intracellular proteases no clear regulatory effects were observed, it was interesting to examine and compare the 5' non-coding sequences for all *A. niger* protease genes cloned.

Comparison of the published *pep*A-F promoter sequences showed that these were essentially non-identical although stretches with several small conserved sequences could be identified which were present in some or in all of the genes (Fig. 12.6). In *A. nidulans* the recognition sites for some of the wide domain regulatory proteins AREA, CREA and PACC have been identified (Kudla et al., 1990; Kulmburg et al., 1993; Tilburn et al., 1995). Comparison of the wide domain regulatory proteins between *A. niger* and *A. nidulans* demonstrates almost perfect conservation of the zinc finger motifs which are involved in DNA binding, suggesting that the DNA recognition sites in *A. niger* will be very similar, if not identical, to the *A. nidulans* binding sites. As the promoters of all the complex regulated extracellular proteases, contain several copies of the recognition sequences of the three wide domain regulatory proteins and as no conservation in number, positions, orientation and location towards other putative binding sites can be identified, it is impossible to identify which of these putative binding sites are functional *in vivo*. Additionally, it cannot be excluded that *cre*A, *are*A and *pac*C regulation also affects protease regulatory functions, as has been shown for other systems. In *A. nidulans* the ethanol regulon-specific *alc*R activator is CREA controlled (Felenbok and Sealy-Lewis, 1994). However, the CREA binding sites utilised *in vivo* occur as doublets which indicates cooperative binding and dimer formation. In the *pep*A, *pep*B and *pep*F promoters CREA doublets are present, but for the *pep*D gene the two CREA binding sites are more than 350 nucleotides apart. The presence of many putative wide domain regulatory binding sites in the promoter of the essentially constitutively expressed *pep*E gene, demonstrates that true identification of the functional cis-acting promoter elements requires detailed *in vitro* binding assays as well as *in vivo* footprinting experiments. Interestingly, although only a small upstream sequence is available for *pep*C, it is observed that both in *pep*C and *pep*E very large so-called CT-rich regions are observed immediately upstream of the ATG start codon (data not shown).

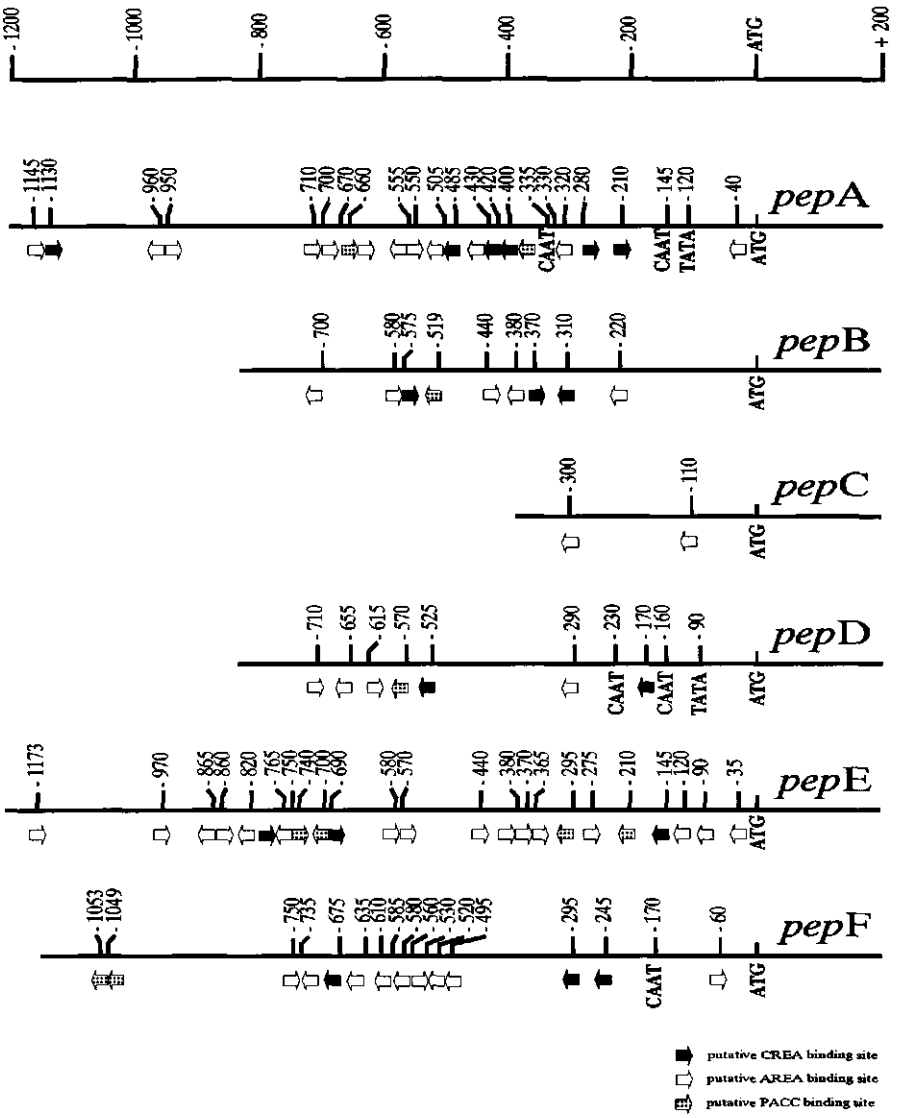

Fig. 12.6. Promoter analysis of the *A. niger* protease genes. Alignments and analyses of common sequence elements were performed based on the published 5' regions, varying from -387 (*pep*C) to -1268 (*pep*E), relative to the start codon (**ATG**) of *pep*A (kindly provided by Dr. F. Buxton), *pep*B (kindly provided by Dr. F. Buxton), *pep*C (Frederick et al., 1993), *pep*D (Jarai et al., 1994a), *pep*E (Jarai et al., 1994b), *pep*F (van den Hombergh et al., 1994). No upstream sequence data for the *A.niger cpy* gene was available. Only those sequences identical to general eukaryotic promoter elements (TATA-box and CAAT-box) are indicated. Putative CREA (G/CPyGGGG or GPyGGAG), AREA (GATA) and PACC (GCCA/GG) binding sites are indicated by distinct arrows as shown underneath the figure. Conserved sequences identified in the promoters and designated elements **A**, **B**, **C** and **D** are indicated with open boxes. For all promoter elements the relative distances to the respective translation start codons (**ATG**) are indicated.

Besides the putative recognition sites for the AREA, CREA and PACC regulatory proteins, some of the genes encoding extracellular proteases have additional regions of high homology. The 18-bp long region (5'-**TGGa/cTt/cTTCTTg/cTTCATC**-3' designated as 'element **A**' in Fig. 12.6) is present in both *pep*A and *pep*B (Jarai and Buxton, 1994), but is not present in any of the other protease genes. However, closely located to this 18-mer in *pep*A at position -94 a sequence element (5'-**AGa/cATGGAa**/t-3', designated 'element **B**' in Fig. 12.6) has been identified which is also present in the *pep*B (-219) and at a similar position in the *pep*F (-108) promoter. Furthermore, a third element (5'-**CCCGACG**-3', designated 'element **C**'in Fig. 12.6) is present in the *pep*A, *pep*B and *pep*F promoters. Comparison of the region containing element **C** in the *pep*A and *pep*F promoters revealed a large 25 bp region which contained 19 identical bp (5'-**CGGAc/gc/gC-CCGACGt/gACt/cCc/tGCCa/cGA**-3', designated 'element **D**' in Fig. 12.6.). Whether these conserved promoter elements have any functional relevance awaits further analysis.

In summary, our data show that the overall expression observed for the extracellular proteases is regulated in a very complex manner. It is the result of the interaction of several wide domain regulatory systems and other more specific regulatory systems. The complexity of overall protease regulation complicates detailed molecular analysis, especially as several factors involved in protease regulation have not yet been identified. For *A. nidulans* some reports describe effects of the phosphor and sulphur source on protease expression (Cohen, 1973ab; Stevens and Stevens, 1980; Stevens, 1985) but also very little is known about the mechanisms involved in the observed induction by extracellularly added proteins like elastin. For a better understanding of overall protease regulation these unknown regulatory factors have to be identified. The isolation of a large set of protease deficient mutants could provide the tools for cloning some very interesting additional factors involved in protease gene regulation (van den Hombergh et al., 1995). As concluded from Northern analyses, the *prt*B26 mutation results in down regulation of all extracellular proteases tested whereas in other *prt* mutants the induction mechanism in reponse to the protein inducer is affected. Finally we have no molecular data yet on the wide domain regulatory mutations in *cre*A and *are*A mutants. Sequencing of the individual mutations and making a comparison with the numerous *A. nidulans* wide domain regulatory mutants known will be very useful.

Although the experiments described for the *are*A1 mutant, the *are*A1-*cre*A^{d}2 double mutant, the transformed *are*A1-*cre*A^{d}2 double mutant and the *cre*A^{d}2 recombinant demonstrate clearly AREA and CREA mediated effects as expected, it will nevertheless be necessary to generate *are*A^{+} strains for other *cre*A^{d} alleles as well. This could be done by recombination but

considering the difficulties this should be preferentially done by transformation with a linear fragment of the *A. niger are*A gene, and selecting for a double cross-over event at the homologous *are*A locus.

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13 Reduced degradation of *Erwinia* PL3, expressed in *Aspergillus nidulans*, through modulation of pH regulation and cloning of *pep*A, an Aspergillopepsin from *Aspergillus nidulans*[1]

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Abstract

We have studied production of *Erwinia carotovora* PL3 after introducing different pH regulatory mutations in a characterized PL3 overproducing *A. nidulans* transformant. Severely reduced degradation of secreted and active PL3 was observed in *pac*C^{c}14, an alkaline mimicking pH regulatory mutation, whereas in a *pal*A and in a *pac*C$^{+/-}$ acid mimicking background the degradation was similar to the situation in wild type. In an *A. niger pac*2c^{c} alkaline mimicking genetic background we observed reduced extracellular protease activities which caused a 10-fold decrease of *in vitro* PL3 degradation and which was correlated with decreased transcription of *pep*A and *pep*B acid proteases in a *pac*2C^{c} strain. The relation between acid proteases and reduced protein degradation in *A. niger* lead us to clone the *pep*A gene from *A. nidulans*, encoding an Aspergillopepsin, and to start comparative regulatory studies of acid proteases in *A. nidulans* and *A.niger*. *A. nidulans pep*A produces a 1.4 kb mRNA and encodes probably a secreted prepro-protein which is very homologous to other aspergillopepsins.

Introduction

The development of several *Aspergillus* expression-secretion systems and their proven abilities with respect to the production of several heterologous proteins has justified the development of this eukaryotic microbial expression system. However, great intrinsic differences with respect to secretion capacity, homologous protease spectrum and glycosylation of expressed proteins are observed (Bartling et al., 1996). Proteases have been recognized as causing major problems

[1] Manuscript in prep.

especially with respect to expression of heterologous proteins. Previously, it has been shown that these intrinsic differences between *A. niger*, *A. nidulans* and *A. niger* have large effects on protein production yields. In *A. niger*, which acidifies its growth medium very rapidly, several interfering proteolytic activities have been characterized and it has been shown that especially acid proteases cause these expression problems. The extracellular acid proteinases in *A. niger* show complex regulation in which several wide domain regulatory mechanisms are involved (Jarai and Buxton, 1994; van den Hombergh et al., 1994). From transcriptional regulation studies in *A. niger* it was concluded that especially pH regulation is important in acid protease expression (Fraissinet-Tachet et al., unpublished results). *A. nidulans*, a non acidifying fungus, contains a different protease spectrum in which the major activities are neutral and alkaline activities. The major extracellular protease is a pH regulated alkaline serine protease (Katz et al., 1995; Tilburn et al., 1995) and recently two metallo proteases have been cloned as well (van den Hombergh et al., unpublished results).

The pH regulation of enzymes, including several proteases, and permeases that function beyond cell boundaries ensures that these gene products are only synthesized at pHs at which they can function efficiently. Based on detailed analyses of mutations in the *pac*C and *pal*A, -B, -C, -F, -H, and -I genes, which alter the response to ambient pH, and by cloning several genes involved (Tilburn et al., 1995; MacCabe et al., 1996; Denison et al., 1995), the pH wide domain regulatory system in *Aspergillus* has been characterized in detail, resulting in a model for pH regulation in response to ambient pH (Tilburn et al., 1995; Orejas et al., 1995). The PacC zinc finger transcriptional regulator directly regulates the expression of genes under pH control, whereas the products of the *pal* genes constitute a signal transduction pathway which, at alkaline ambient pH, triggers the conversion of PacC to a functional form as a transcriptional activator of alkaline-expressed genes and repressor of acid-expressed genes (Tilburn et al., 1995; Orejas et al., 1995). Absence of the *pal* signal at acid pH results in derepression of acid-expressed genes and lack of activation of alkaline-expressed genes. In view of the acidification-related differences in PL3 production yields in *Aspergillus* and involvement of pH regulation in protease expression, we have analyzed the effects of different pH regulatory mutations upon degradation of PL3 in *A. nidulans*.

Subsequently, the pH regulation-related differences in PL3 degradation initiated the cloning and characterization of a genomic DNA fragment containing the *A. nidulans pep*A gene, encoding an aspergillopepsin. The aspergillopepsins are a family of closely related aspartic proteases produced by certain filamentous fungi of the genus *Aspergillus*. They share extensive sequence homology with the penicillopepsins, aspartic proteinases produced by certain *Penicillium*

species (Stepanov, 1985). The aspartic proteinases (E.C. 3.4.23) seem to be confined to the eukaryotes and may be one of the younger classes of proteinases (Barrett, 1986). In fungi, aspartic proteinases are generally produced by fungi that acidify the media as many of these enzymes are unstable at pHs above neutral.

Materials and methods

Strains, library and plasmids. All *A. nidulans* strains are derived from the Glasgow strain FGSC4. The *A. nidulans* PL3 transformant (*A. nidulans* 827/3) was described by Bartling et al. (1996), whereas the *pacC*^{c}14, the *pacC*^{c}76*pacC*$^{+/-}$7601 strains and the *pal*A1 strain have been described by Tilburn et al. (1995). All *A. niger* strains were derived from wild type strain N400 (CBS 120.49). The *A. niger pac2C*^{c} strain and the *A. niger* N647:pIJ16 strains are derived from the N647 (*fwn*A6, *csp*A1, *arg*B13, *nic*A1) strain (van den Hombergh et al., 1996a). *Escherichia coli* strain DH5αF' (BRL, Life Technologies Inc., Gaithersburg, USA) was used as host for routine plasmid propagation. *E. coli* strain LE392 was used as host for phage lambda. pUC18 (Yanish-Perron et al., 1985) and pBluescript (Short et al., 1988) vectors were used for subcloning. The genomic library of *A. nidulans* WG096 was constructed by ligating partially *Sau*3AI-digested genomic DNA fragments into the lambda replacement vector Charon 4A cut with *Bam*HI.

Media. Low phosphate medium (LPM) contained per liter: 6.0 g NaNO3 / 1.5 g KH_2PO_4 / 0.5 g KCl / 0.5 g $MgSO_4$ / trace elements pH 6.0 and was supplemented with 1% (w/v) glucose as sole carbon source. For high phosphate medium the KH_2PO_4 concentration was increased 10-fold (15 g/l) compared to LPM. Liquid cultures were inoculated with 10^6 spores/ml and incubated at 30 °C on an orbital shaker at 220 rpm. Agar was added at 1.2 % for solid medium.

Genetic analysis. The procedures for genetic analysis have been described previously (Bos et al., 1988). Purified recombinants were tested for acid and alkaline phosphatase activities using a plate assay described by Dorn (1965). The analyses of PL3 expression and degradation have been described by Bartling et al. (1996).

Isolation, cloning and characterization of the *A. nidulans pep*A gene. Plaque hybridization using nylon replicas was performed according to Benton and Davis (1977). Hybridizations were performed overnight at 56°C, using hybridization buffer containing 6 x SSC / 10 x Denhardt's / 0.5% (w/v) SDS; the filters were washed with 4 x SSC / 0.5% (w/v) SDS at 56°C. Positive plaques, identified on duplicate replicas after autoradiography were recovered from the original plates and purified by rescreening at low density. For other DNA manipulations, such as plasmid DNA isolation, random priming, preparation of plaque lifts, Southern analysis and subcloning standard methods were applied (Sambrook et al., 1989). The *pep*A gene was subcloned as a 3.8-kb *Eco*RI fragment into pUC18 (Yanish-Perron et al., 1985) and both strands were completely sequenced. Sequencing of recombinant double stranded DNA was performed using the Sequenase DNA Sequencing Kit (United States Biochemical Corporation Cleveland, USA) and the ^{T7}Sequencing Kit (Pharmacia LKB), employing additional synthetic oligonucleotide primers. Nucleotide sequences were confirmed from both strands of DNA, nucleotide and amino acid sequences were analyzed using the computer programmes of Devereux *et al.* (1984). *Aspergillus* DNA was isolated according to de Graaff *et al.* (1988).

Northern analysis. *Growth conditions*: *A. nidulans* WG096 conidia were inoculated into complete YPD [1% (w/v) yeast extract / 1% (w/v) peptone / 2% (w/v) glucose] medium and grown for 16-24 h at 30°C. Mycelium was filtered, washed three times with distilled water and transferred to minimal medium containing no carbon source and supplemented with 0.5% (w/v) BSA (Boehringer, fraction V) and 0.5%

(w/v) elastin (Fluka). The transfer medium was buffered to pH 4.0 with 0.1 M NaAc or to pH 8.0 with 0.1 M Tris. Cells were then grown for an additional 5-6 h. Mycelium was harvested by filtration, freeze dried and used for RNA isolation. Total RNA was isolated using TRIzol (GibcoBRL) according to the suppliers instructions. *Blotting and hybridization*: 10 μg of total RNA was loaded on formaldehyde agarose gels (Sambrook et al., 1989), transferred to Hybond-N membranes (Amersham) after electrophoresis and UV-crosslinked. Hybridization and washing of the membranes was carried out essentially as described (Sambrook et al., 1989).

Results and discussion

In view of the effects of the *pac*2C^c disruption on the expression of two extracellular acid proteases in *A. niger* (chapter 12) and the improved protein production in strains in which acid protease expression is reduced (chapter 7 and 8) we have analyzed down-regulation of protease expression as an additional means to improve protein production. The *A. nidulans* multicopy transformant expressing the heterologous *Erwinia carotovora* pectate lyase 3 (*A. nidulans* 827/3), was generated in a *paba*A1 genetic background (Bartling et al., 1996). Furthermore, in Southern experiments on p827/3 chromosomal DNA only one single high molecular mass band was observed after digestion with restriction enzymes for which no sites are present in the plasmids p827 and pGW625 (used to generate the *A. nidulans* transformant). This indicated that the transforming DNA was probably present in a large multicopy integrate, as has been observed previously (Bussink et al., 1992). As the introduced *pl*3 DNA could be considered as one genetic locus, pH regulatory mutations could be introduced by recombination, using the *paba*A1 marker, and the putative effects on production of a hetorologous protein could be studied *in vivo*. From a cross between *A. nidulans* 827/3 (*paba*A1, *fwn*A1, *ua*Y9) and three wide domain pH regulatory mutants - i.e. two acid mimicking phenotypes, *pac*C$^{+/-}$ and *pal*A, and one alkaline mimicking phenotype, *pac*C^c - recombinants expressing PL3 and carrying the pH regulatory mutation were selected (see Table 13.1). After purification the recombinants were tested on plates for alkaline and acid phosphatase activities and for PL3 expression. Selected PL3 producing recombinants were grown on liquid LPM and HPM and expression of PL3 was monitored by SDS PAGE of cleared culture supernatant samples, Western blotting and subsequent immunochemical detection of PL3 protein (Fig. 13.1). The 827/3 transformant and the three recombinants secrete similar amounts of PL3 during growth on LPM supplemented with 5% (w/v) glucose. The two cross-reacting bands observed in all culture filtrates are probably a result of differences in glycosylation (Bartling et al. 1996).

Table 13.1. Introducing pH regulatory mutations in *A. nidulans* p827/3

Strain/ origin	Phenotype	Genotype[1]					
		I	II	III	IV	VI	VIII
p827/3 Bartling et al. 1996	Pl3 prod.	*paba*A1					*fw*A1 *ua*Y9
Tilburn et al. 1995	alk. mimic	*bi*A1				*pacC*[c]14	
Tilburn et al. 1995	acid mimic	*bi*A1	*w*A	*pal*A1			
	acid mimic				*ino*B	*pacC*[c]76 *pacC*[+/-]7601	
NW178	alk. mimic PL3 prod.					*pacC*[c]14	
NW179	acid mimic PL3 prod.	*paba*A1 *bi*A1		*pal*A1			*fw*A1 *au*Y9
NW180	acid mimic PL3 prod.	*paba*A1				*pacC*[c]76 *pacC*[+/-]7601	*fw*A1 *au*Y9

[1], linkage groups which do not contain mutations (V and VII) in any of the strains are not included

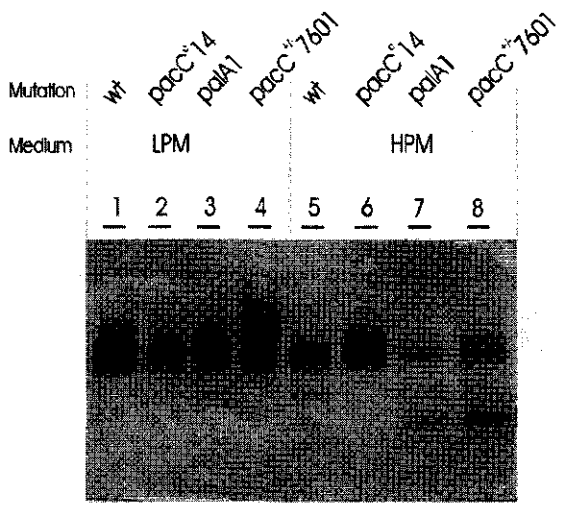


Fig. 13.1. Western analyses of PL3-producing recombinants. Strains were grown for 14 h in LPM supplemented with 5% (w/v) glucose (lanes 1-4) and in HPM supplemented with 2% (w/v) glucose (lanes 5-8). Track order: 1, p827/3; 2, [*pacC*[c]14]; 3, [*pal*A1]; 4, [*pacC*[c]76*pacC*[+/-]7601]; 5, p827/3; 6, NW178 [*pacC*[c]14]; 7, NW179 [*pal*-A1]; 8, NW180 [*pacC*[c]76*pacC*[+/-]7601-].

Judged from the absence of any small molecular weight bands on the Western blot, no degradation is observed in all four strains. After growth on HPM supplemented with 2% (w/v) glucose only for the *pacC*^{c}14 recombinant no degradation is observed. Under these conditions severe degradation is observed, both in a wild type genetic background and even some increase in degradation is observed in acid mimicking PL3 recombinants. The results presented in Fig. 13.1 show that the PL3 degradation is pH regulated and probably caused by gene products which are expressed at acidic pH, as in a *pacC*^{c}14 strain acid target genes are super-repressed by the constitutive expression of *pacC*. Additionally, in the *pacC*$^{+/-}$7601 and the *palA*1 genetic background the degradation can be repressed by high concentrations of glucose, indicating a carbon catabolite repressive effect on PL3 degradation. Two control experiments were carried out to check whether the observed differences were not caused by a loss of *pl*3 DNA or by inactivation of integrated copies during the recombination experiments. Chromosomal DNA was isolated from the recombinants and the 'parental' transformant, was digested with *Bam*HI, Southern blotted and probed with sequences encoding *pl*3. The hybridization patterns in all strains are identical (data not shown), demonstrating that no *pl*3 DNA was lost during the recombination and that the integrated *pl*3 copies were indeed linked in the 'parental' transformant. As a control for putative inactivation of functional *pl*3 expression cassettes during recombination, the p827/3 and the three recombinants were analyzed in Northern blot experiments to compare *pl*3 transcript levels. These Northern blots demonstrated similar levels of *pl*3 in all recombinants (data not shown). Thus, the observed differences in PL3 degradation clearly result from introduction of an alkaline mimicking *pacC*^{c}14 mutation.

Previously, we have generated an alkaline mimicking *pacC*2^{c} disruptant, strain NW138, in *A. niger* (van den Hombergh et al., 1996) and recently we have studied the expression of several acid proteases in this strain. In strain NW138 the expression of both the *pepA* and the *pepB* genes (encoding extracellular broad specificity acid endo proteases) is severely reduced at an acid pH (Fraissinet-Tachet et al., unpublished results). We have also tested for extracellular protease activities in this *A. niger pac*2C^{c} disruptant on a casein and gelatin containing plate medium which is used to visualize extracellular acid protease activities in *A. niger*. Judged from the reduced halo size compared to the wild type N402 strain, the extracellular acid protease activities are significantly reduced in the *pac*2C^{c} strain. The down-regulation of acid proteases in NW138 led us to test the *in vitro* degradation of the PL3 protein in serial dilutions of culture filtrates from *A. niger* N402 and *A. niger* NW138 (*pac*2C^{c}). Similar to the *in vitro* experiments described for the PELB protein (van den Hombergh et al., 1995), pure PL3 protein was incubated in serial dilutions of

wild type, N402, the *pac*2C^c strain, NW138, and as a control an *arg*B$^+$ transformant derived from strain N647 (which is also the parental strain for NW138). These *in vitro* experiments clearly demonstrate the *pac*2C^c-related reduction of PL3 degradation which is approximately 10-fold less compared to wild type.

As the reduced degradation of PL3 in *A. niger* in a pH regulatory acid mimicking *pac*C^c disruptant demonstrates the involvement of acid proteases we have tested *in vitro* whether the addition of pepstatin, a pepsin-type acid protease inhibitor, could reduce degradation of pure PL3 in cleared culture supernatants of *A. nidulans*. The results of these *in vitro* experiments indicate some effect of pepstatin, especially in the acid mimicking mutants, *pac*C$^{+/-}$ and *pal*A (Table 13.3). These observations also indicate the involvement of pepsin-type proteases in PL3 degradation in *A. nidulans* and is similar to previous observations in *A. niger*. This has then initiated the cloning of the *A. nidulans pep*A equivalent which is described below.

Table 13.2 Reduced PL3 degradation in an *A. niger pac*2C^c strain compare to two parental strains

Strain	Genotype	Residual PL3 activity[1]							
		cont	1	2	10	50	100	500	1000
N402	(*csp*A1)	**102**	0	0	5	20	43	71	**100**
N647:pIJ16	(*fwn*A6, *csp*A1, *arg*B$^+$, *arg*B13, *nic*A1)	**106**	0	0	3	26	38	80	**104**
NW138	(*fwn*A6, *csp*A1, *pac*2C^c, *arg*B$^+$ *arg*B13, *nic*A1)	**96**	0.7	1	20	74	**102**	**104**	**98**

In vitro degradation of PL3 was monitored essentially as detailed in Bartling et al. (1996). Two microgram of pure PL3 was incubated in serial dilutions of supernatants from the *A. niger* cultures. After 15 min. incubation time, the pH was adjusted to 8.5 and residual PL3 activity was measured as described by Bartling et al. (1995). [1], the PL3 activity was expressed as the percentage of residual activity compared to the control. Non affected activities are indicated in bold; cont, as a control an undiluted medium sample was boiled prior to incubation.

Table 13.3 Reduced PL3 degradation in *A. nidulans* culture supernatants

Strain	Inhibiton[1]	Residual PL3 activity[2]							
		cont	1	2	10	50	100	500	1000
wild type	-	**103**	0.5	1.2	8	51	87	**98**	**104**
wild type	pepstatin	**99**	1	4	15	88	**106**	**97**	**106**
*pal*A	-	**104**	0	0.5	2	9	28	43	**106**
*pal*A	pepstatin	**96**	0.5	2	5	29	76	**100**	**98**
pacC^{c}76*pacC*$^{+/-}$7601	-	**98**	0	0.3	3	12	21	60	**97**
pacC^{c}76*pacC*$^{+/-}$7601	pepstatin	**101**	1	3	7	20	88	**97**	**101**
pacC^{c}14	-	**106**	5	11	69	**94**	**97**	**102**	**98**
pacC^{c}14	pepstatin	**104**	6	13	43	**87**	**102**	**98**	**97**

[1], pepstatin was added to the serial dilutions prior to incubation with PL3 at 2 μM (final) concentrations; -, no inhibitor added.

[2], *in vitro* degradation of PL3 was performed and monitored as described in Table 13.3.

Cloning of pepA

Using a 1593 bp *Sal*I-*Nsi*I fragment containing the complete *pep*A gene from *A. niger* [kindly provided by Dr. F.P. Buxton, Ciba Geigy AG, Basel] we established in a series of genomic Southern analyses conditions under which restricted *A. nidulans* WG096 DNA gave specific hybridizing signals. The most stringent washing condition, allowing easily detectable and specific hybridization, was found to be 4 x SSC / 5 x Denhardt's / 0.1% (w/v) SDS at 60°C. Under the same conditions a genomic library in bacteriophage λ was screened. Three positive clones (λ1, λ2 and λ3) were identified and subsequently purified. The exact location of the hybridizing fragment within these phages was established by restriction mapping and hybridization. These hybridizations showed that the three individual phages contained sequences from the same genomic segment. From λ1 a 3.8-kb *Eco*RI fragment was subcloned into pBluescript, resulting in pIM670 which was used for further analysis.

Nucleotide sequence and copy number

The complete nucleotide sequence of the 3.8 kb *Eco*RI fragment was determined on both strands by the dideoxy-termination method using synthetic oligonucleotide primers (Sanger et al., 1977). The *pep*A nucleotide sequence is shown in Fig. 13.4 and contains an 1158 bp ORF, as well as 1282 bp upstream and 231 bp downstream non-coding sequences. The DNA and the deduced

amino acid sequence of the *pep*A gene indicated that the ORF is interrupted by several introns. Based on alignments between the nucleotide sequence of the *A. niger* and *A. nidulans pep*A genes and their encoded proteins, three putative introns were identified.

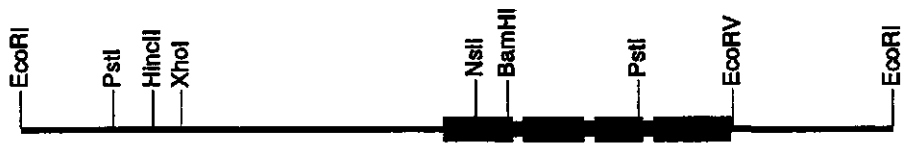
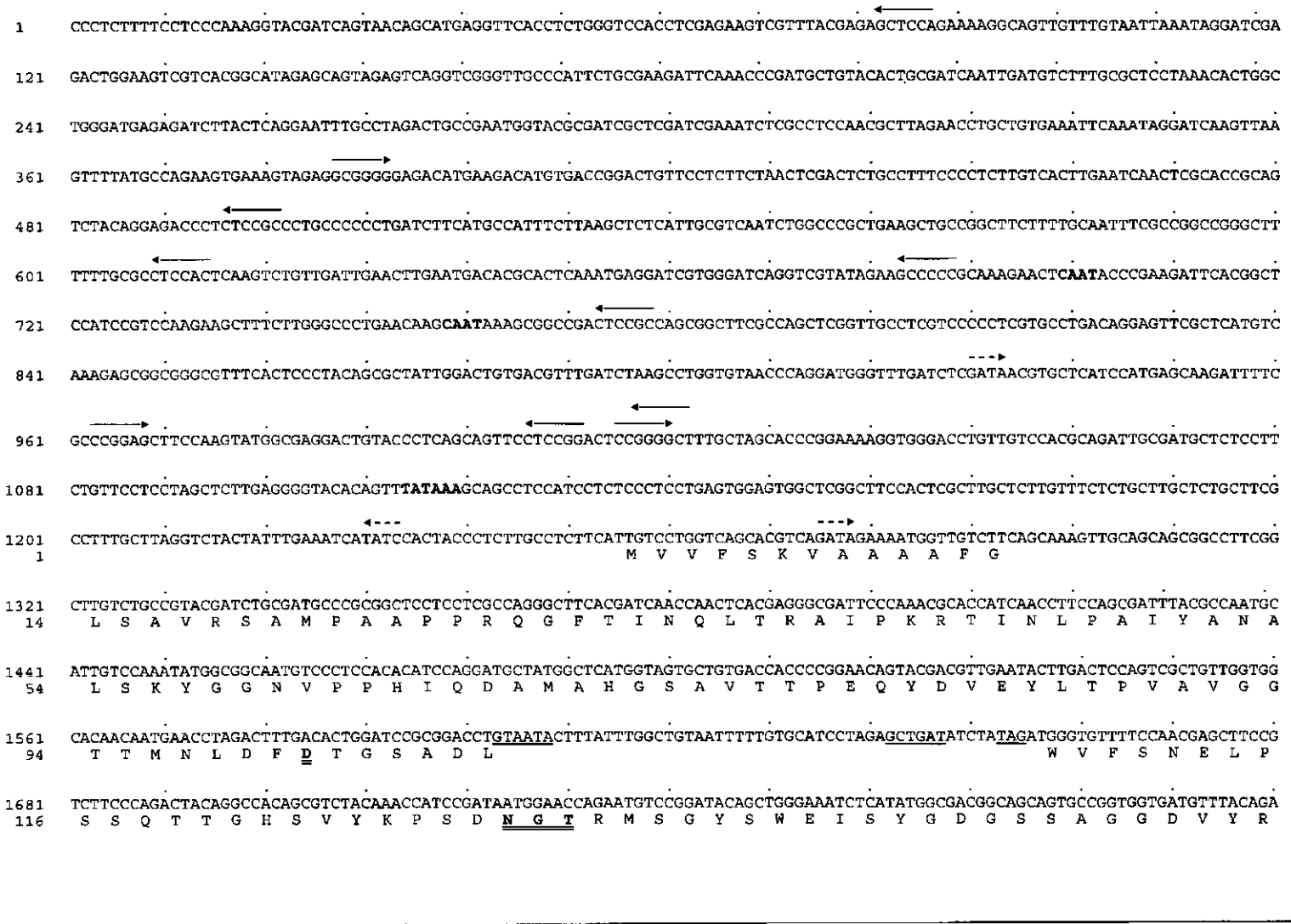


Fig. 13.3. Physical map of the *pep*A gene from *A. nidulans*. The restriction map of a 3.8-kb *Eco*RI subclone derived from lambda clone λ1 containing the *pep*A gene is shown. The coding region of the *pep*A gene is indicated with a filled bar and 5' and 3' non-coding regions are indicated with thin lines. The little gaps in the coding region indicate the positions of the three introns.

These putative introns are 53, 49 and 56 bp and contained *Aspergillus* (and other fungal) consensus sites, the 5' splice donor site (GTRNGT), the 3' splice acceptor site (YAG) and the internal element (RCTRAC), possibly needed for lariat formation during splicing. However the 5' splice site showed two and one mismatches for introns one and three, respectively. The internal element showed one mismatch in all introns. The 5' region contains several CREA and AREA binding sites which are involved in carbon catabolite repression (Kulmburg et al., 1993) and ammonium metabolite repression (Kudla et al., 1990) respectively. However, no PacC bindingsites as described by Tilburn et al. (1995) are present in the 5' region of *pep*A. Two possible CAAT-motifs are located 504 nt and 564 nt upstream of the ATG. At 149 nt upstream of the ATG a perfect copy of a TATAAA box is located. A CT-rich region, which is frequently found in fungal promoters, is located between 73 and 139 nt upstream of the ATG. The 3' region of *pep*A contains a perfect copy of a putative polyadenylation signal (AATAAA) 215 nt downstream of the stop codon.

Genomic Southern analyses were performed (both under homologous and heterologous hybridization conditions) to establish the copy number of the *pep*A gene. Total *A. nidulans* DNA was digested with *Bam*HI, *Eco*RI and *Hin*dIII, separated in agarose gels, Southern blotted and hybridized with the 0.59 kb *Bam*HI-*Pst*I *pep*A fragment (hybridization conditions; 6 x SSC / 5 x Denhardt's / 0.5% (w/v) SDS / 10 mM EDTA / 50 mM Tris pH 7.5 at 65°C; most stringent homologous wash: 0.1 x SSC / 0.5% (w/v) SDS at 65°C; most stringent heterologous wash: 4 x SSC / 0.5% (w/v) SDS at 48°C). The results of these Southern experiments indicated that the *pep*A gene was present in a single copy in the *A. nidulans* genome. No other related aspartic protease related sequences were identified (data not shown).


1 CCCTCTTTTCCTCCCAAAGGTACGATCAGTAACAGCATGAGGTTCACCTCTGGGTCCACCTCGAGAAGTCGTTTACGAGAGCTCCAGAAAAGGCAGTTGTTTGTAATTAAATAGGATCGA
121 GACTGGAAGTCGTCACGGCATAGAGCAGTAGAGTCAGGTCGGGTTGCCCATTCTGCGAAGATTCAAACCCGATGCTGTACACTGCGATCAATTGATGTCTTTGCGCTCCTAAACACTGGC
241 TGGGATGAGAGATCTTACTCAGGAATTTGCCTAGACTGCCGAATGGTACGCGATCGCTCGATCGAAATCTCGCCTCCAACGCTTAGAACCTGCTGTGAAATTCAAATAGGATCAAGTTAA
361 GTTTTATGCCAGAAGTGAAAGTAGAGGCGGGGGAGACATGAAGACATGTGACCGGACTGTTCCTCTTCTAACTCGACTCTGCCTTTCCCCTCTTGTCACTTGAATCAACTCGCACCGCAG
481 TCTACAGGAGACCCTCTCCGCCCTGCCCCCCTGATCTTCATGCCATTTCTTAAGCTCTCATTGCGTCAATCTGGCCCGCTGAAGCTGCCGGCTTCTTTTGCAATTTCGCCGGCCGGGCTT
601 TTTTGCGCCTCCACTCAAGTCTGTTGATTGAACTTGAATGACACGCACTCAAATGAGGATCGTGGGATCAGGTCGTATAGAAGCCCCCGCAAAGAACTCAATACCCGAAGATTCACGGCT
721 CCATCCGTCCAAGAAGCTTTCTTGGGCCCTGAACAAGCAATAAAGCGGCCGACTCCGCCAGCGGCTTCGCCAGCTCGGTTGCCTCGTCCCCCTCGTGCCTGACAGGAGTTCGCTCATGTC
841 AAAGAGCGGCGGGCGTTTCACTCCCTACAGCGCTATTGGACTGTGACGTTTGATCTAAGCCTGGTGTAACCCAGGATGGGTTTGATCTCGATAACGTGCTCATCCATGAGCAAGATTTTC
961 GCCCGGAGCTTCCAAGTATGGCGAGGACTGTACCCTCAGCAGTTCCTCCGGACTCCGGGGCTTTGCTAGCACCCGGAAAAGGTGGGACCTGTTGTCCACGCAGATTGCGATGCTCTCCTT
1081 CTGTTCCTCCTAGCTCTTGAGGGGTACACAGTTTATAAAGCAGCCTCCATCCTCTCCCTCCTGAGTGGAGTGGCTCGGCTTCCACTCGCTTGCTCTTGTTTCTCTGCTTGCTCTGCTTCG
1201 CCTTTGCTTAGGTCTACTATTTGAAATCATATCCACTACCCTCTTGCCTCTTCATTGTCCTGGTCAGCACGTCAGATAGAAAATGGTTGTCTTCAGCAAAGTTGCAGCAGCGGCCTTCGG
1 M V V F S K V A A A A F G
1321 CTTGTCTGCCGTACGATCTGCGATGCCCGCGGCTCCTCCTCGCCAGGGCTTCACGATCAACCAACTCACGAGGGCGATTCCCAAACGCACCATCAACCTTCCAGCGATTTACGCCAATGC
14 L S A V R S A M P A A P P R Q G F T I N Q L T R A I P K R T I N L P A I Y A N A
1441 ATTGTCCAAATATGGCGGCAATGTCCCTCCACACATCCAGGATGCTATGGCTCATGGTAGTGCTGTGACCACCCCGGAACAGTACGACGTTGAATACTTGACTCCAGTCGCTGTTGGTGG
54 L S K Y G G N V P P H I Q D A M A H G S A V T T P E Q Y D V E Y L T P V A V G G
1561 CACAACAATGAACCTAGACTTTGACACTGGATCCGCGGACCTGTAATACTTTATTTGGCTGTAATTTTTGTGCATCCTAGAGCTGATATCTATAGATGGGTGTTTTCCAACGAGCTTCCG
94 T T M N L D F D T G S A D L W V F S N E L P
1681 TCTTCCCAGACTACAGGCCACAGCGTCTACAAACCATCCGATAATGGAACCAGAATGTCCGGATACAGCTGGGAAATCTCATATGGCGACGGCAGCAGTGCCGGTGGTGATGTTTACAGA
116 S S Q T T G H S V Y K P S D N G T R M S G Y S W E I S Y G D G S S A G G D V Y R

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1801  GACACCGTCACCGTTGGTGGCGTCACTGCGCCAGGGCAGGCAGTCGAGGCCGCTTCACACATTAGTGAACAGTTCACCCGGGATCAAAACAACGATGGCTTGCTCGGTTTAGCATTCAGC
 156   D  T  V  T  V  G  G  V  T  A  P  G  Q  A  V  E  A  A  S  H  I  S  E  Q  F  T  R  D  Q  N  N  D  G  L  L  G  L  A  F  S

1921  TCCATTAACACTGGTGAGTACTTGAAACATACTTGGACCAAATTTTCCTAACTTGCCACCAGTTCAACCGAAATCCCAGACCACCTTCTTCGACAGCGTAAAGTCTCAGCTTGAATCTCC
 196   S  I  N  T                                                                V  Q  P  K  S  Q  T  T  F  F  D  S  V  K  S  Q  L  E  S  P

2041  TCTGTTTGCTGTAACACTTAAGCACCAGGCTCCTGGTTCCTACGATTTCGGCTACATCGACCAATCAAAATACACTGGCGAGCTTACCTATACAGATGTGGACAATTCTCAGGGCTTCTG
 220   L  F  A  V  T  L  K  H  Q  A  P  G  S  Y  D  F  G  Y  I  D  Q  S  K  Y  T  G  E  L  T  Y  T  D  V  D  N  S  Q  G  F  W

2161  GATGTTTTCCGCAACTGCAGGGGAGACTGACTTTGACGCCATCGCGGGTATGCCATGCCTATACCAACCCCAGCCCGACTGGCCATTTTAACCAATCTCACAGATACCGGCACAACCCTC
 260   M  F  S  A  T  A  G  E  T  D  F  D  A  I  A                                                                D  T  G  T  T  L

2281  ATTATGATCGACCAGTCTATCGCAGAGGACTATTACTCTCAGGTTCCACTCGCCTTCAACAACTTCTTCTACGGTGGTTGGACCTTCCCTTGCTCCGCCGAACTCCCGTCCTTCACTGTC
 281   I  M  I  D  Q  S  I  A  E  D  Y  Y  S  Q  V  P  L  A  F  N  N  F  F  Y  G  G  W  T  F  P  C  S  A  E  L  P  S  F  T  V

2401  ACCATCAACGGCTACGACGCCGTTGTCCCAGGCGAGCACATCAAGTACGCCCCCGTTACCGATGGCTCCTCTACCTGCTTCGGAGGTATCCAGGATAACCAGGGCCTCCCATTCTCAATT
 321   T  I  N  G  Y  D  A  V  V  P  G  E  H  I  K  Y  A  P  V  T  D  G  S  S  T  C  F  G  G  I  Q  D  N  Q  G  L  P  F  S  I

2521  CTTGGCGACGTCTTCTTGAAAAGCCAGTACGTTGTTTTCGACTCGGAGGGCCCTCAGTTGGGCTTCGCGCCCCAGGCTTAGACCTGAGATATCCGTTATTTGGGTCAGCTCAATTTCATA
 361   L  G  D  V  F  L  K  S  Q  Y  V  V  F  D  S  E  G  P  Q  L  G  F  A  P  Q  A

2641  TCTGAATTGAATACGCGTGGCTTTGATCCCTGAACATTTCCAGTTTTCTTTTCCTTCAGCATAACGGCGTGAAACGGCTAGGATTCGGCCTTCTTGATACCTTTTGTTTCTATGGTCTTC

2761  CCTATACAAGGGAAACAGCAATGCGTTTTCAGACCACTACTAATATGGGGAATAATAAATTAAGTCGTG
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Fig. 13.4. The nt and aa sequence of *pep*A from *A. nidulans*. The complete nt sequence of *pep*A from *A. nidulans* and the deduced amino acid is shown. The consensus 5' splice donor (GTRNGT), the 3' splice acceptor (YAG) and the internal element (RCTRAC) of the three introns are underlined. Possible TATAAA-like element, CAATC box and possible AATAAA-like element are in bold. Ten putative binding sites for the glucose-responsive repressor protein CREA are indicated with '◄————— '. Three putative binding sites for the ammonium-responsive repressor protein AREA are indicated with '----►'. The two putative active site residues (D_{101} and D_{275}) are in bold and double underlined.

Fig. 13.5. The aa sequence comparison of *A.nidulans* PEPA with other pepsin type acid proteases. The aa sequences were aligned using the program PILEUP, available in the GCG package. The aligned sequences are: a, *A. nidulans* PEPA; b, *A. oryzae* PEPA (aspergillopepsin I) (Ostoslavskaya et al., 1992); c, *A. satoi*; d, *Penicillium janthinellum* PENP (Penicillopepsin) (James and Sielecki, 1983); e, *A. awamori* PEPA (Berka et al., 1990); f, *Cryphonectria parasitica* EAPA (Endothiapepsin) (Razanamparany et al., 1987); g, Human CTSE (Cathepsin E) (Azuma et al., 1989); h, Human PEPA (Sogawa ett al., 1983); i, *A. niger* PEPE (Jarai et al., 1994); j, *Rhizopus niveus* RNAP (Rhizopuspepsin) (Horiuchi et al., 1988); k, *S. cerevisiae* YAP3 (aspartic proteinase 3) (Egel-Mitani et al., 1990); A dot indicates that there is no aa at that position. The active sites are double underlined in the consensus sequence and indicated with a '*' in *A. nidulans* PEPA; **Cons**: consensus sequence of all the aa sequences shown above. All sequences are shown in lower case. Aa residues identical to aa residues in *A. nidulans* PEPA are in upper case.

The PEPA polypeptide

The ORF in the *pep*A gene encodes a protein comprising 386 amino acids with a calculated molecular weight of 41.3 kDa. Sequence comparisons of the deduced amino acid sequence to other aspartic proteinases are shown in Fig 13.5. The data in Fig 13.5 shows that the aa sequences can be divided into two parts based on the similarities among them. The N-termini of the aligned aa sequences show little homology whereas there is high homology between aa 110 and the C-terminus. The PEPA protein from *A. nidulans* has not yet been purified, but several other aspartic proteases that are listed in Fig. 13.5 such as the mature mammalian pepsins and cathepsins, the yeast proteinase A and aspergillopepsin A, have been purified and subjected to N-terminal sequencing. The fact that all these proteinases are synthesized as zymogens combined with the observation that the region with the high homology corresponds to the mature part of these zymogens makes it very likely that PEPA is also synthesized as a precursor. Based on the amino acid sequence it is not possible to establish the exact N-terminus, but based on homology to *A. niger* var. *awamori* PEPA it will be located at or shortly after aa residue Met_{69}. The *A. niger* var. *awamori* prepro-protease A, which is processed after Ala_{69} (Berka et al., 1990) and the *A. nidulans* PEPA protein share a sequence of significant homology between Gly_{72} and Gly_{92}, with 16 identical aa and two conservative substitutions out of 21 aa residues, indicating that this part of the *A. nidulans* PEPA polypeptide is likely to be part of the mature enzyme. The most extensive homology was found between *A. nidulans* PEPA and other pepsins from filamentous fungi (aspergillopepsins and penicillopepsins). The regions around the active site Asp_{101} and Asp_{275} are very well conserved among all these proteases.

The structure of the very N-terminus of the PEPA polypeptide resembles that of a signal peptide (von Heijne, 1986) and signal cleavage site prediction strongly suggests that a 19 aa signal peptide is present which is cleaved off after Ser_{19} while the polypeptide is translocated to the endoplasmic reticulum. These structural features thus suggest that the PEPA protein is synthesized as a precursor which undergoes two processing steps to yield the mature enzyme consisting of approximately 318 aa residues. This mature protein has a calculated molecular mass of 34.2 kDa. The calculated isoelectric point of the mature protein is 3.64, which is close to the *A. niger* PEPA isoelectric point (3.5). *A. nidulans* PEPA contains one potential glycosylation site, Asn_{130}GlyThr (see Fig. 13.5).

***pep*A expression**

A. nidulans wild type was grown on complete medium supplemented with 1% (w/v) glucose for 20 h, mycelium was harvested, washed and transferred to minimal medium supplemented with 0.5% (w/v) BSA and 0.5% (w/v) elastin and buffered with NaAc to pH 4 or to pH 8 with Tris. After 6 h mycelium was harvested, total RNA was extracted and analyzed in a Northern blot experiment (Fig. 13.6.). The *A. nidulans pep*A probe hybridized to a single RNA species of 1.4 kb mRNA. The size of this mRNA correponds with the deduced *pep*A gene length (Fig. 13.4) and is similar to the *A. niger pep*A mRNA (Jarai and Buxton, 1994).

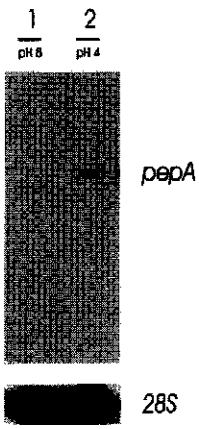

Fig. 13.6. Northern blot *A. nidulans pep*A. *A. nidulans* wild type mycelium was pre-grown on CM and transferred to carbon and nitrogen derepressed, protein induced MM which was buffered to pH 8.0 (lane 1) or to pH 4.0 (lane 2). Total RNA was isolated 6 h after transfer and analyzed in Northern hybridization experiments using a *pep*A probe and (after stripping) an *Agaricus bisporus* 28S rDNA probe (Acc. No. X91812).

Thus the *pep*A gene is transcribed at an acid pH, similar to the situation in *A. niger*. Whether this protease is pH-regulated awaits further analysis. However, the absence of PacC binding sites in the promoter of the *pep*A gene suggests at least no direct pH regulation of *pep*A expression. Experiments to study the expression of *pep*A in wide domain regulatory mutants in order to address the question whether *pep*A expression is pH regulated, and whether the reduced proteolytic degradation in *A. nidulans pacC*^{c} mutants is *pep*A related, are in progress. The cloning of the *A. nidulans* aspartyl protease will also allow comparative regulation studies between *A. niger* and *A. nidulans* acid protease expression in the future.

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14 Identification, cloning, and analysis of *pep*I and *pep*J, two metallo protease genes from *Aspergillus nidulans*; indications for an unique acid metallo protease class in filamentous fungi [1]

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Summary

We have isolated two novel metallo protease genes from *Aspergillus nidulans* after screening a genomic library with a fragment from the *A. oryzae* neutral protease II (NPII) gene as a heterologous probe. Both metallo protease genes encode prepro proteins and contain two introns in conserved positions. The PEPI and PEPJ proteins are highly homologous to the *A. oryzae* NPII metallo protease but show only very limited homology to any of the other metallo protease classes described. Detailed homology studies indicate that these PEPI and PEPJ metallo proteases are members of a new class of metallo endo-proteases, designated acid metallo proteases, due to their slight acidic pH optimum. Northern experiments demonstrated transcription of both metallo protease genes and the observed differences in expression levels in relation to pH and the carbon and nitrogen source indicate involvement of several wide domain regulatory mechanisms in transcriptional regulation.

Introduction

The metallo proteases (E.C. 3.4.24) are very ancient, being widespread in bacteria, streptomyces and fungi, as well as in higher organisms (Barret, 1986). It appears that zinc is the catalytically active metal in the natural forms of all metallo proteases, although some of them retain activity when the zinc is replaced by another metal such as cobalt. Moreover, many metallo proteases have rather unique specificities which allow them to catalyze certain peptide bond cleavages much more efficiently than other types of proteolytic enzymes (Powers and Harper, 1986). This implies that, although metallo proteases do not appear to be as abundant as serine proteases, they play key roles in numerous physiological processes.

[1] Sumitted for publication in Bio/Technology

In several *Aspergilli* metallo protease activities have been described. It has been reported that *A. oryzae* secretes two types of metallo proteases, neutral protease I (NpI) and neutral protease II (NpII) (Nakadai et al, 1973a,b). In *A. satoi* a similar situation has been described by Sekine (1972, 1973). In *A. oryzae* (Tatsumi et al., 1992) and in *Penicillium citrinum* (Matsumoto et al., 1994), NPII encoding metallo protease genes have been cloned. Very recently, Ramesh et al. (1995) described the cloning of NPII-type metallo proteases from *A. fumigatus* and *A. flavus*. Previously, a NPI-type metallo protease gene (MEP) was cloned from *A. fumigatus* (Jaton-Ogay et al., 1994).

Little is known about the biological role of metallo proteases in filamentous fungi. A direct involvement of *A. fumigatus* MEP in aspergillosis was not found as was confirmed in a MEP disruption strain (Jaton-Ogay et al., 1994) whereas a possible involvement of NPII-type metallo proteases is still unknown. In *A. oryzae* the biological role of NpII is also not clear, but it is assumed that NpII serves to utilize substrates present in the environment, since *A. oryzae* secretes large amounts (a few hundred mg/l) of NpII under normal culture conditions (Tatsumi et al., 1991). The relatively high expression observed for *A. oryzae* NpII combined with the fact that overall extracellular protease acitivity in *A. nidulans* is highest at a neutral to alkaline pH could indicate that these proteases contribute significantly to the extracellular protease activities present in *A. nidulans*.

A. nidulans is one of the *Aspergillus* species which has been developed as a host for the production of foreign protein and several heterologous human, insect, fungal and bacterial proteins have already been tested in this fungus (Davies, 1994). However, in several cases high expression yields are hampered by endogenous proteases, resulting in partial or complete degradation of products (Upshall et al., 1991; Devchand and Gwynne, 1991; Bartling et al., 1995). Some major, broad-specificity extracellular proteolytic activities are probably involved in the observed degradation of the secreted proteins, quite similar to the situation in *A. niger* and *A. oryzae*. Detailed characterization of these proteolytic activities, their expression and their contribution to the overall extracellular proteolytic spectrum will enable molecular approaches to reduce degradation in a similar way as described for other fungal expression hosts (van den Hombergh et al., 1996).

Materials and methods

Strains, library and plasmids. The *A. nidulans* strain EG096 (, *paba*A1) used as wild type is derived from the Glasgow strain FGSC4. The *A. oryzae* strain used is ATCC 20386. Minimal medium (MM) contained per litre: 6.0 g $NaNO_3$ / 1.5 g KH_2PO_4 / 0.5 g KCl / 0.5 g $MgSO_4$ / trace elements and 1% (w/v) glucose. For complete medium (CM) minimal medium was supplemented with 2% (w/v) tryptone / 1% (w/v) yeast extract / 1% (w/v) casamino acids / 0.5% (w/v) ribonucleic acids. Liquid cultures were inoculated with 10^6 spores/ml and incubated at 30 °C on an orbital shaker at 250 rpm. Agar was added at 1.2 % for solid medium. *A. nidulans* and *A. oryzae* were grown at 37°C. *Escherichia coli* strain DH5αF' (BRL, Life Technologies Inc., Gaithersburg, USA) was used as host for routine plasmid propagation. *E. coli* strain LE392 was used as host for phage λ. pUC18 (Yanish-Perron et al., 1985) and pBluescript (Short et al., 1988) vectors were used for subcloning. The genomic library of *A. nidulans* was constructed in the λ replacement vector Charon 4A.

Detection of metallo-type protease activities. *A. nidulans* was grown in liquid medium containing 0.5 g $MgSO_4$ / 1.5 g KH_2PO_4 / 0.5 g KCl / trace elements supplemented with 1% (w/v) BSA (Boehringer fraction V) and 1% (w/v) elastin (Fluka) as sole carbon and nitrogen source. After 72 h the proteolytic activities in the cleared culture supernantants were quantified as described by van den Hombergh et al. (1995). 1,10-phenanthroline (Sigma) was used to inhibit metallo-type protease activities at concentrations of 2 mM.

PCR cloning *A. oryzae* NPII. Two specific DNA primers (CGGGATCCCGCGTGAGTGAACTCGTG and CGGAATTCCGGTCACCGACTGCAAGG containing a *Bam*HI and an *Eco*RI restriction site, respectively) were used in PCR reactions to generate a 412 bp PCR fragment (annealing temperature 68°C). The purified *Bam*HI/*Eco*RI digested PCR fragment was subcloned into pUC19 and sequenced.

Isolation, cloning and characterization of the *pepI* and *pepJ* genes. Plaque hybridization using nylon replicas was performed according to Benton and Davis (1977). Hybridizations were performed overnight at 56°C and filters were washed to 4 x SSC [SSC: 0.15 M NaCl / 0.015 M Na_3-citrate pH 7.6] / 0.5% (w/v) SDS concentrations. Positive plaques, identified on duplicate replicas after autoradiography, were recovered from the original plates and purified by rescreening at low plaque density. For other DNA manipulations, such as plasmid DNA isolation, random priming, preparation of plaque lifts, Southern analysis and subcloning, standard methods were applied (Sambrook et al., 1989). *Aspergillus* DNA was isolated according to de Graaff et al. (1988). For the *pepI* gene two *Eco*RI fragments of 1.6 and 2.0 kb were subcloned in pUC18 to generate plasmids pIM667 and pIM668. For the *pepJ* gene a 6.5 kb *Hin*dIII-*Xho*I fragment was cloned in pBluescript SK^+, resulting in plasmid pIM669. Sequencing of recombinant double stranded DNA was performed using the Sequenase DNA Sequencing Kit (United States Biochemical Corporation Cleveland, USA) and the ^{T7}Sequencing Kit (Pharmacia LKB), employing additional oligonucleotide primers. Nucleotide sequences were confirmed from both strands of DNA. Nucleotide and amino acid sequences were analyzed using the computer programmes of Devereux et al. (1984).

Intron mapping. Mycelium, pregrown on complete medium, was harvested, washed and transferred to MM with 0.5% (w/v) BSA and 0.5% (w/v) elastin and grown for an additional 6 h. Total RNA was prepared from harvested mycelium using TRIzol (GibcoBRL). The splice sites of the putative introns were identified by cloning and sequencing partial cDNA copies of the *pepI* and *pepJ* transcripts, generated by reverse transcriptase PCR. First strand synthesis was performed by standard methods (Sambrook et al., 1989). The priming oligos for the *pepI* and *pepJ* introns 1 and 2 were **I2** and **I4** and **J2** and **J4**, respectively. The cDNAs were amplified by PCR using oligos **I1** + **I2** (*pepI*, intron 1), **I3** + **I4** (*pepI*, intron 2), **J1** + **J2** (*pepJ*, intron 1) and **J3** + **J4** (*pepJ*, intron 2) and cloned into pUC18. For the positions and sequences of the **I1,2,3,4** and **J1,2,3,4** primers see Fig. 14.2. Several independent clones were sequenced for each putative intron.

Southern analyses. To establish copy numbers of the *pep*I and *pep*J genes hybridizations were performed under stringent conditions using specific probes. Hybridization conditions were: 6 * SSC / 5 * Denhardt's / 0.5 % (w/v) SDS / 50 mM Tris pH 7.4 / 10 mM EDTA at 68°C; the most stringent wash was: 0.2 * SSC / 0.1% (w/v) SDS at 68°C.

Northern analysis. *Growth conditions*. *A. nidulans* WG096 conidia were inoculated into liquid CM and grown at 30°C for 20 h at 250 rpm. Mycelium was filtered, washed three times with distilled water and transferred to MM without nitrogen source and supplemented as described in the legend of Fig. 14.5. Growth at acid, neutral and alkaline pH was achieved by including 0.1 M sodium acetate buffer pH 4.0, 0.1 M sodium phosphate buffer pH 6.0 or 0.1 M Tris-Cl buffer pH 8.0 in the transfer cultures. Cells were grown for an additional 5-6 h. Mycelia were harvested by filtration and RNA was isolated (using Trizol). 10 μg of total RNA was loaded on formaldehyde agarose gels (Sambrook et al., 1989), transferred to Hybond-N membranes (Amersham) after electrophoresis and UV crosslinking. Hybridization, labelling of DNA fragments with ^{32}P using the random priming method and washing of the membranes [final wash: 0.2 x SSC / 0.5% (w/v) SDS] was carried out essentially as detailed by Sambrook et al., 1989.

Results and discussion

Cloning of *pep*I and *pep*J

In a pilot experiment we tested *A. nidulans* wild type on solid minimal medium supplemented with 1% (w/v) casein both in the presence and in the absence of EDTA. On this medium protease activity can be visualized by the formation of a clear halo, the size of which was significantly smaller in this case in the presence of EDTA. This observation indicated the presence of extracellular metallo proteases, but a possible chelating effect of EDTA on serine-type proteases could not be excluded, as several serine proteases require Ca^{2+} for activity. To measure the actual metallo protease activity, an *A. nidulans* wild type strain was grown on minimal medium supplemented with BSA and elastin. Measuring the overall proteolytic activity in the culture supernatant and its partial inhibiton by 1,10 phenanthroline (Fig. 14.1) clearly proved the presence of metallo-type activities for carbon and nitrogen derepressed, protein induced growth conditions since 1,10 phenanthroline does not chelate Ca^{2+}. These observations initiated the identification and cloning of metallo-protease encoding genes in *A. nidulans*.

Tatsumi and coworkers (1991) have described the cloning of a metallo protease from *A. oryzae* and its subsequent overexpression in *S. cerevisiae*. Comparing this fungal metallo protease with all metallo proteases in the combined GENBANK and EMBL databases revealed that only one region was conserved between the *A. oryzae* metallo protease and other metallo proteases. This region contains two histidine residues which are assumed to be involved in binding of the metal ion that is essential for catalytic action.

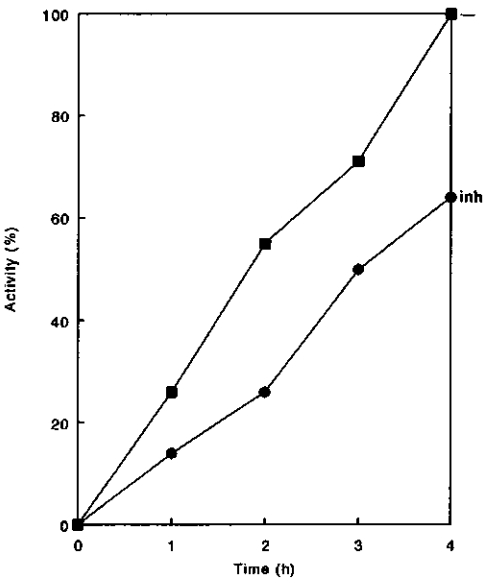


Fig. 14.1. Metallo protease activities in *A. nidulans* culture supernatants. Protease activities were measured in cleared culture supernatants and expressed as percentage of the highest activity measured (non-inhibited 4 h incubation). Symbols used: ■, activity in the absence of 1,10 phenanthroline; ●, activity in the presence of 2 mM 1,10-phenanthroline.

Thus, due to the lack of additional conserved regions within the *A. oryzae* metallo protease a direct PCR approach for cloning putative metallo protease genes of *A. nidulans* was impossible. Therefore, we decided to PCR a fragment of the *A. oryzae* metallo protease and to use this as a heterologous probe for the isolation of similar *A. nidulans* metallo protease(s). Based on the published cDNA sequence two 26-mer oligonucleotide primers (containing a *Bam*HI and an *Eco*RI restriction site, respectively) were designed to PCR a 0.41 kb fragment of the metallo protease of *A. oryzae*, encoding the mature part of the protein. At an annealing temperature of 68°C a specific PCR product was obtained which was subcloned and the resulting plasmid was sequenced to prove cloning of an *A. oryzae* NPII fragment. The cloned *A. oryzae* NPII fragment was subsequently used in a series of genomic Southern experiments using *A. oryzae* and *A.nidulans* wild type genomic DNA to establish the hybridization conditions for isolating the *A. nidulans* metallo protease sequences. Performing these experiments we observed that in all the restriction digests tested with *A. nidulans* genomic DNA and *A. oryzae* genomic DNA, used as a control, two individual hybridizing bands with differences in hybridizing intensity occurred (data not shown). These results could indicate the presence of two individual metallo protease-like genomic segments and we established conditions under which identification of both hybridizing genomic fragments was possible. Washing down to 4 x SSC and 0.1% (w/v) SDS allowed specific hybridization of both genomic segments and it was possible to distinguish between the two individual genomic loci, based on the differences in hybridization intensities. Under these

conditons the cloned *A. oryzae* NPII fragment was used to screen an *A. nidulans* genomic library in bacteriophage λ. Initially, ten positive clones (five strongly hybridizing phages, λ1-λ5, and five weakly hybridizing phages, λ6-λ10) were identified and subsequently purified. The position of the hybridizing fragment within these ten clones (λ1-λ10) was identified by restriction mapping and hybridization. The pattern obtained was compared to the hybridization patterns of the *A. nidulans* genomic DNA digests. The phages λ1-λ4 contained identical hybridizing genomic segments which corresponded to the strong hybridizing band observed in the genomic Southern analysis. The hybridizing restriction fragments from λ5-λ8 corresponded to the weaker hybridizing fragments observed in the genomic Southerns. The sizes of the hybridizing fragments in phages λ9-λ10 were not identical to either the strongly or the weakly hybridizing class of phages, probably indicative for partial clones. From λ1 a 2.4 kb hybridizing *Pst*I fragment and from λ5 a 0.7 kb hybridizing *Eco*RI fragment were initially subcloned (resulting in plasmids pIM665 and pIM666, respectively) to prove homology to the NPII encoding gene from *A. oryzae* by sequencing.

Nucleotide sequence and intron mapping

Having confirmed that both subcloned fragments contained metallo protease-like sequences, larger fragments were subcloned to complete the sequence of both genes. From the class I phages two *Eco*RI fragments (1.4 kb and 2.0 kb) and from the class II phages a 6.5 kb *Hin*dIII-*Xho*I fragment were subcloned in pUC18 resulting in plasmids pIM667, pIM668 and pIM669. The inserts from pIM667, pIM668 and part of the pIM669 insert were sequenced on both strands by the dideoxy-termination method using synthetic oligonucleotide primers (Sanger et al., 1977). Sequencing data proved that two independent metallo protease genes were cloned. Based on homologies both at the DNA and the deduced protein sequence level, the two *Eco*RI fragments appeared to contain the complete *pep*I gene. This was subsequently proven by cloning a 0.38 kb *Pst*I fragment from phage λ1 which allowed sequencing through the *Eco*RI site located in the *pep*I gene.

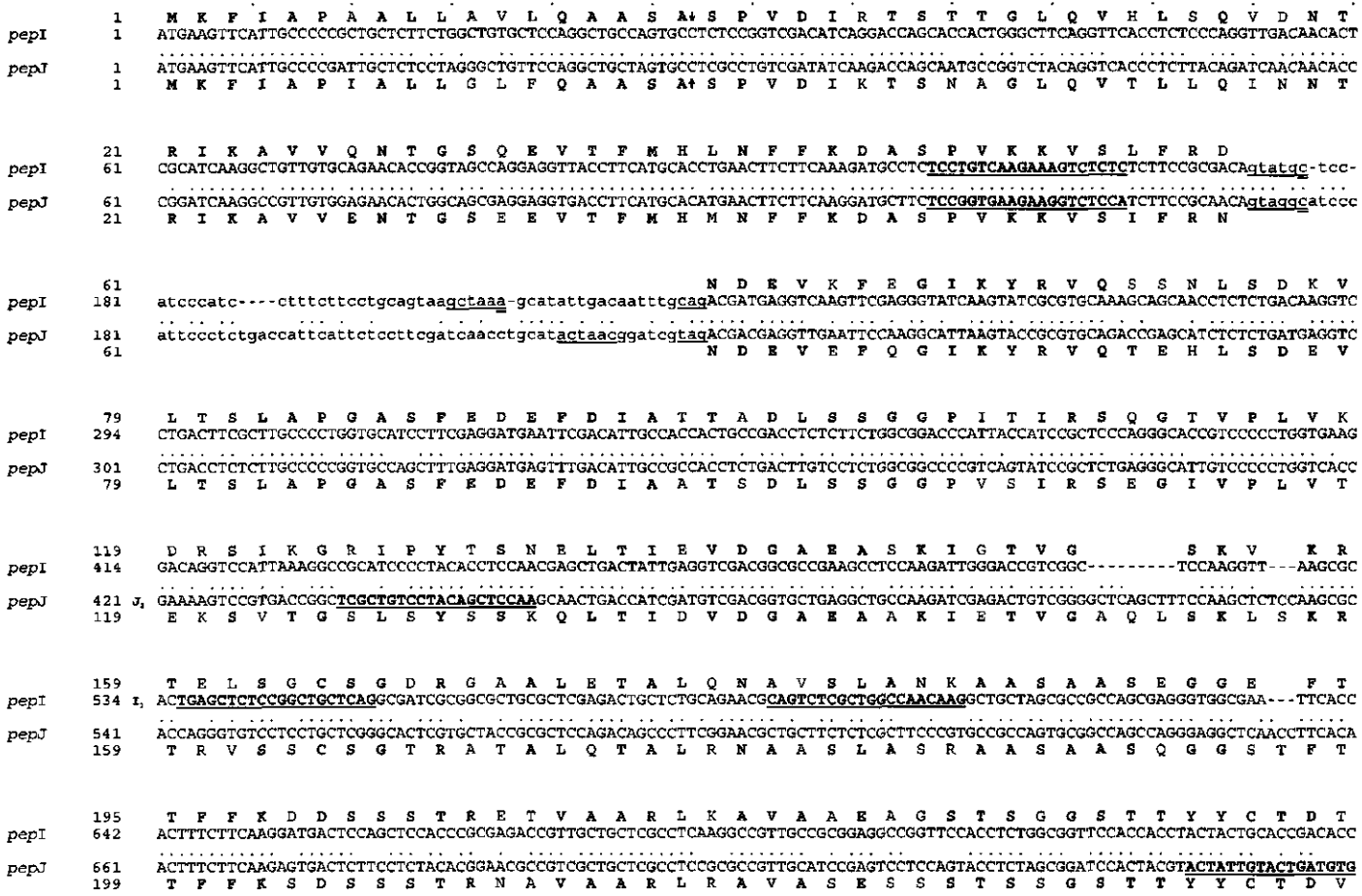
Frame shifts in the initial nucleotide sequence indicated the presence of two introns in both the *pep*I and *pep*J genes. Partial cDNAs were generated from mycelium grown under both carbon and nitrogen derepressed and protein induced conditions by reverse transcriptase PCR using specific primers for the 5' and 3' regions of the introns. Comparisons of the size of the cDNAs to the size of the fragments generated in PCR reactions on chromosomal DNA already indicated the existence of all four putative introns, which was subsequently proven after subcloning and sequencing the cDNAs.

The position of these introns are conserved between the two genes and in general the 5' and 3' border sequences as well as the lariat sequences confine to the proposed consensus sequences, except for some minor changes which are indicated in Fig. 2. Recently, Ramesh et al. (1995) have cloned metallo protease genes, related to *pep*I and *pep*J, from *A. flavus* and *A. fumigatus*. The *A. fumigatus* metallo protease contains also an intron at the position of intron 1 in *pep*I and *pep*J. However, the *A. flavus* does not contain any introns and has an additional 18 aa at the position of intron 1, indicating an unprocessed intron. Furthermore, both in the *A. flavus* and the *A. fumigatus* metallo protease gene a second intron is absent. Both *A. nidulans* metallo protease genes show 72% homology and vary with respect to their homology to the *A. oryzae* NPII encoding gene (*pep*I: 69%, *pep*J: 66%).

The PEPI and PEPJ polypeptides

The *pep*I and *pep*J genes encode proteins of 349 and 354 aa, respectively. The PEPI and PEPJ proteins are probably extracellular proteins similar to the situation in *A. oryzae* and in *P. citrinum* and thus will contain signal peptides. Signal cleavage prediction analyses identified in both *A. nidulans* metallo proteases putative signal sequences of 18 aa, which have common signal peptide characteristics (Fig. 14.3) and conform to the (-3, -1) rule (Von Heijne, 1986). Based on homologies with the *A. oryzae* NpII the mature proteins will start at a conserved threonine, Thr_{175} in PEPI and Thr_{178} in PEPJ, behind a conserved dibasic cleavage site. Whether this dibasic cleavage site implicates involvement of a KEX2 processing protease to remove the propeptides is unknown. However, KEX2-like processing events have been described in *Aspergillus* and the dibasic KR sequence is recognized and has even been used in fusion appoaches to express heterologous proteins in several *Aspergillus* species (Contreras et al., 1991). The mature PEPI and PEPJ metallo proteases thus contain 175 and 176 aa, repectively, have similar predicted molecular weights (i.e. 18.2 and 18.3 kDa) but have different predicted isoelectric points (i.e. 3.5 and 6.0). No potential N-linked glycosylation sites were found in the mature PEPI and PEPJ proteins although both the pro-peptides contain one potential N-glycosylation site.

Homologies between the primary translation products and the mature proteins of PEPI and PEPJ are high, 73% and 75%, respectively (Fig. 14.3). Fasta analyses in the combined GenBank/EMBL databases revealed significant homologies with the the *A. oryzae* NPII (Tatsumi et al., 1992), the *P. citrinum* neutral protease (Matsumoto et al., 1994) and the *A. fumigatus* and *A. flavus* MP20 metallo proteases.


pepI
pepJ

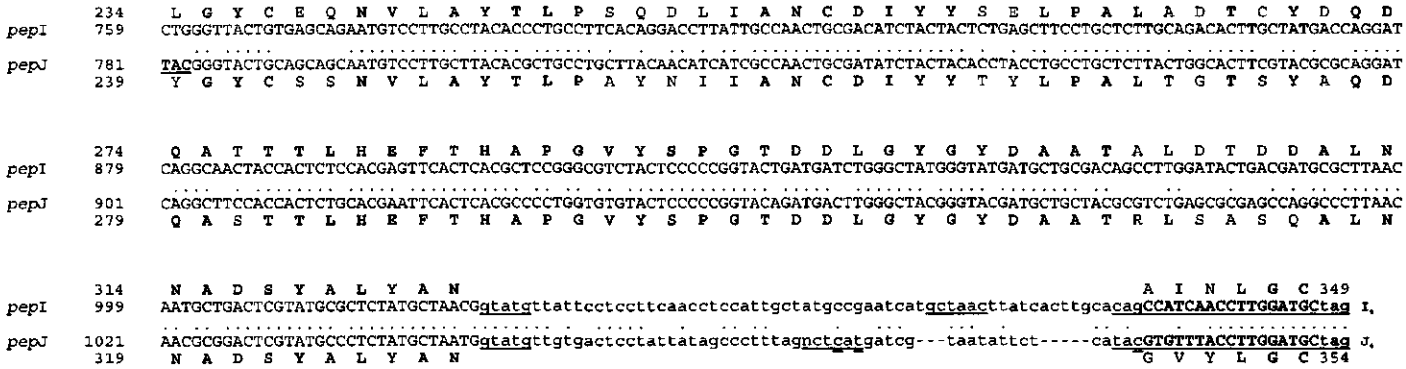


Fig. 14.2. The nucleotide sequences and deduced aa sequences of the *A. nidulans pepI* and *pepJ* genes. Exons and introns are indicated in uppercase and lowercase letters, respectively. Amino acids conserved between PEPI and PEPJ are in bold. The 8 synthetic oligoos ($\mathbf{I_1}$-$\mathbf{I_4}$ and $\mathbf{J_1}$-$\mathbf{J_4}$used to generate partial cDNAs in reverse transcriptase reactions to prove the presence of the introns are underlined and in bold. The 5' splice donor site, the 3' splice acceptor site as well as the internal element for all introns are underlined and nucleotides within these sequences which are different from the consensus sequences are double underlined. Putative signal sequences are indicated by vertical arrows (↑ or ↓).

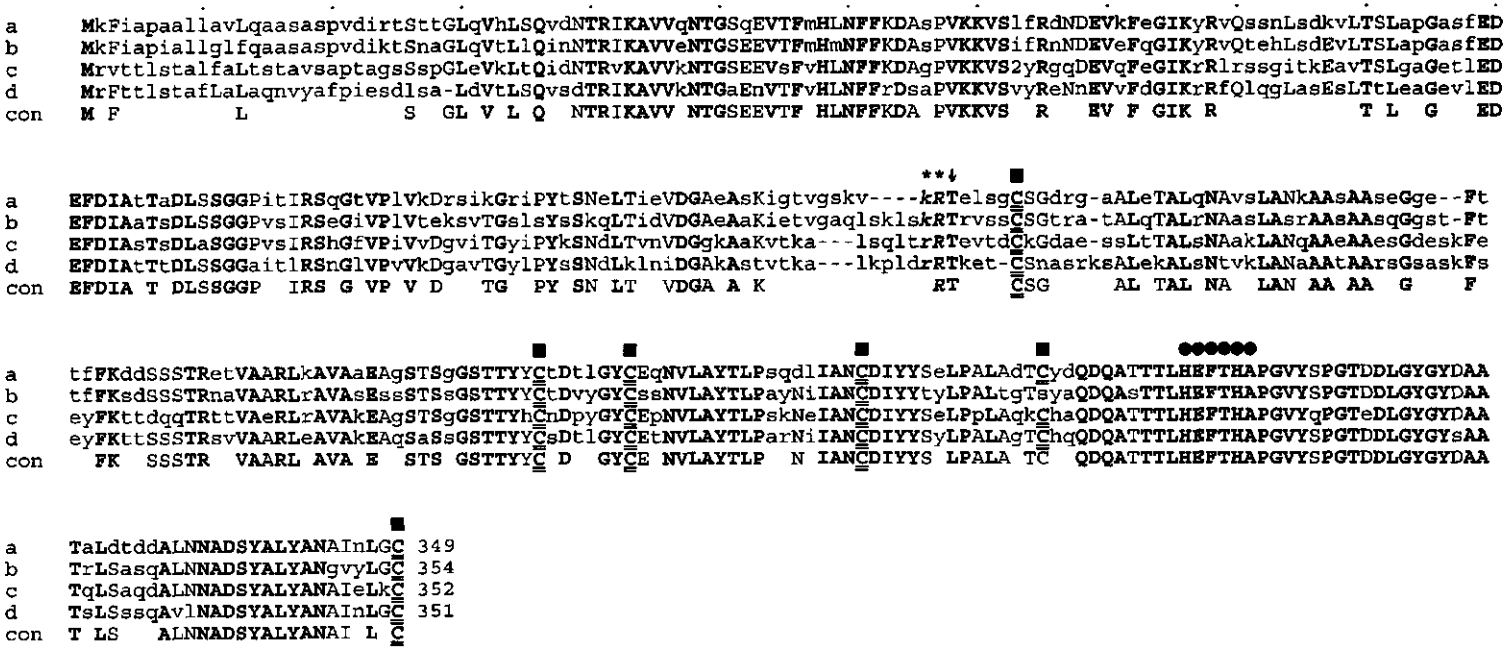


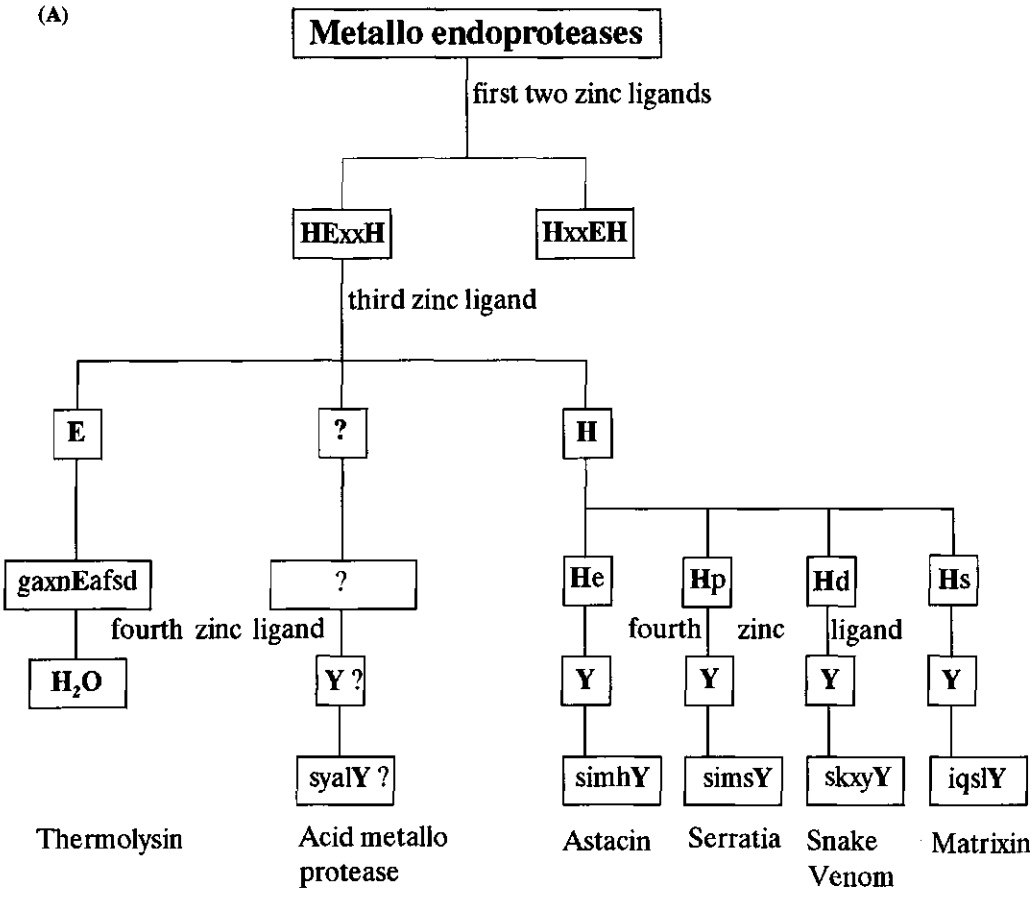
Fig. 14.3. The amino acid comparison of PEPI and PEPJ with other metallo proteases. Amino acid sequences were aligned using the program PILEUP which is available in the GCG package. Aligned sequences were as follows: a, *A. nidulans* PEPI; b, *A. nidulans* PEPJ; c, *A. oryzae* NPII (Tatsumi et al., 1991), d, *P. citrinum* metallo protease (Matsumoto et al. 1994); con, consensus sequence for which conservation is ≥ 75%; completely conserved aa are in bold; -, no aa is present at that position; **, conserved putative dibasic cleavage site; ↓, putative start of mature PEPI and PEPJ; ■, conserved cysteine residue; the HEFTHA box is indicated with ●.

All amino acid sequences of the metallo proteases characterized contain a region, **HEXXH** or HXXEH (Jiang and Bond, 1992) in which two histidine residues, presumably involved in coordinating the Zn^{2+} cation in the active center, and a glutamate involved in the bond-breaking process (Jongeneel *et al.*, 1989), are located (Fig. 14.4A). The three amino acid residues before the first histidine and after the second histidine are generally represented by hydrophobic or small and neutral amino acids in the active site of a metallo protease. The sequences found in the *A. nidulans* metallo proteases are in agreement with this. All the metallo proteases characterized sofar have been assigned to several independent classes based on the homologies in the proximity of the metal ion ligands (Jiang and Bond, 1992). The first two ligands, which in all metallo proteases studied sofar are two histidine residues, are present in either a **HEXXH** or a **HXXEH** motif (Fig. 14.4B). In the **HEXXH**-containing metallo proteases, the thermolysin-type metallo proteases, the third metal ion ligand is a glutamic acid residue which is located in a GAXNEAFSD sequence whereas the fourth ligand has been shown to be a water molecule (Kester and Matthews, 1977). For the other metallo proteases containing a **HEXXH** motif, the third ligand is also a histidine residue and the fourth ligand is a tyrosine residue. The amino acid present after the third metal ion ligand and the four amino acids present immediately before the fourth ligand did allow further differentiation into four classes, Astacin, Serratia, Matrixin and Snake Venom, respectively (Jiang and Bond, 1992). The metallo proteases from *A. nidulans*, *A. oryzae* and *P. citrinum* (listed in Fig. 4A) do not belong to any of the classes mentioned. Clearly, these metallo proteases do not have a histidine as a third ligand as, apart from the two histidine residues in the HEFTHA sequence, no additional histidine residues are present in the mature proteases. Also no GAXNE-AFSD sequence, which could indicate a thermolysin-type metallo protease, was found. The *A. fumigatus* metallo protease encoding gene (*MEP*) has also been cloned and sequenced and the deduced amino acid sequence contains in addition to the **HEYTH** zinc ligands a region resembling the third ligand region in thermolysins, ESGGMGEGWSD. This type of metallo protease has twice the size of the *A. nidulans* metallo proteases, shows no homology to the *A. nidulans* PEPI and PEPJ metallo proteases and is probably a thermolysin-type metallo protease. Recently, we have cloned the *A. niger* equivalent of the *A. fumigatus MEP*, which also contains a region with homology to the third ligand region in thermolysins (van den Hombergh et al., unpublished results). Based on these observations it appears that the metallo proteases from filamentous fungi can be divided into two separate classes. This corresponds with the characterization of two types of metallo proteases in some *Aspergillus* species. Sekine (1973) proposed, based on the slightly acidic pH optimum for the neutral proteases II from *A. oryzae* and *A. sojae*, to name this type of metallo proteases acid metallo proteases. The third and fourth metal ion ligands are unknown for

the acid metallo proteases but at approximately the same C-terminal distance of the **HEFTH** box a conserved sequence **SYALY** is found, which resembles partially the region preceeding the fourth ligand in the Astacin, Serratia, Matrixin and Snake venom classes. Whether this Tyr residue is indeed a metal ion ligand awaits site-directed mutagenesis and mechanistic studies.

The observations that in mature *A. oryzae* NPII 6 Cys residues are present and that in the *A. satoi* NPII no free sulfhydryl has been observed (Sekine, 1972b), led Tatsumi et al. (1991) to propose that perhaps three disulphide bridges are formed in this relatively small molecule (19 kDa), explaining its high thermal stability. The PEPI and the *P. citrinum* NPII also contain 6 cysteine residues and their positioning is well-conserved, which could indicate the importance of these postulated disulphide bridges. However, in mature PEPJ only five cysteine residues are conserved and no other cysteine residues have been identified, which would predict a less thermostable form.

(A)



(B)

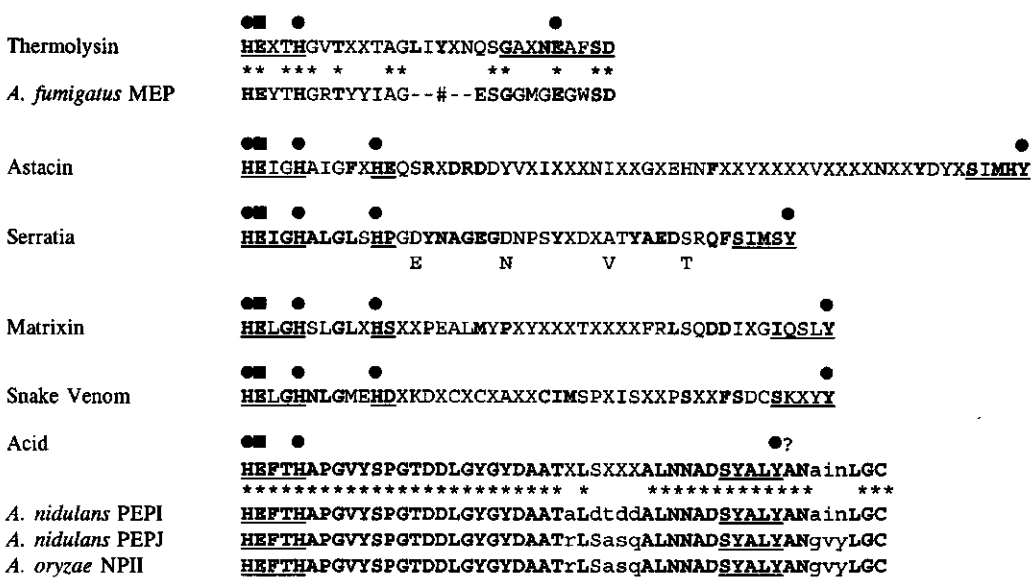


Fig. 14.4 Classes of metallo proteases based on their zinc binding site (**A**) The residues in bold are zinc-ligands (modified from Jiang and Bond, 1992). (**B**) Comparison of consensus sequences for regions in the close proximity of the zinc binding site in HEXXH-containing metallo proteases which were used to identify individual classes (thermolysin, astacin, serratia, matrixin, snake venom and acid) within the metallo protease family (Jongeneel et al., 1989; Jiang and Bond, 1992). The zinc binding sites of the *A. fumigatus*, the *A. nidulans* and the *A. oryzae* metallo proteases were aligned with the consensus sites to identify homologies. Sequences used to generate Fig. 14.4A are underlined. Strongly conserved aa are in bold; --#-- indicates that the spacing between the putative third zinc ligand and the HEXXH motif is larger in *A. fumigatus* MEP; ●, putative zinc ligand; ■, glutamic acid probably involved in the bond breaking process.

The strong conservation of a dibasic cleavage site could indicate KEX2 involvement in maturation of prepro acid metalloproteases. Upon overexpression of the *A. oryzae* NPII in *S. cerevisiae*, actual processing was dependent on the presence of Zn^{2+} in the medium and pro-NPII was processed in *vitro* to mature NPII. These observations show no direct involvement of KEX2 and suggest that the pro-NPII removes the propeptide autocatalytically. The mature NPII generated *in vitro* however, was one amino acid shorter (Glu_2) than the native NPII (Thr_1). A possible explanation for this could be a conformational change, possibly induced by the removal of Ca^{2+} during the purification (*A. sojae* NPII possesses one molecule of Ca^{2+}; Sekine, 1973b) in the presence of EDTA during the normal purification procedure, leading to a shift in the normal cleavage site. On the other hand, the described *in vitro* result may be an artefact, as processing in yeast could be the result of a yeast protease.

Genomic Southern analyses

In a series of genomic Southern experiments we have investigated the copy number of the *pep*I and *pep*J genes. Genomic DNA digested with *Bam*HI, *Eco*RI, *Hind*III and *Sal*I was Southern blotted and then probed with specific *pep*I and *pep*J probes using stringent hybridization conditions. These experiments showed that both the *pep*I and *pep*J genes were present in the genome as single copy genes (data not shown).

Regulation of *pep*I and *pep*J expression

Little is known about regulation of proteases in *A. nidulans*. Cohen (1972, 1973a,b) and Stevens and Stevens (1980) described the presence of neutral proteases in culture supernatants and mycelial extracts. The extracellular proteases were shown to respond to carbon, nitrogen and sulphur starvation, indicative for wide domain regulation (Cohen, 1973a,b). Two proteases, one intracellular and one extracellular, have been purified from *A. nidulans* and shown to be serine-type proteases (Ansari and Stevens, 1983). Comparison of the *A. nidulans* protease data by Stevens (1985) indicates that at least five proteases have been identified, i.e. protease I (also referred to as ß), proteinase II (also referred to as γ), protease III (also referred to as ϵ), protease α and an aminopeptidase. Protease I is an intracellular protease, insensitive to nitrogen derepression, whereas protease II is the principal extracellular serine protease activity when *A. nidulans* is grown under nitrogen derepression conditions. Recently, a serine-type protease (*prt*A), probably encoding protease II, has been cloned (Katz et al., 1995). For the other two extracellular proteases (III and α) little is known. However, both proteases are insensitive to the serine protease inhibitor phenylmethylsulphonyl fluoride (PMSF) and both proteases are repressed by ammonium and derepressed upon nitrogen starvation (Stevens, 1985; Cohen, 1973a).

We have initiated expression studies of the *pep*I and *pep*J genes in relation to the use of different carbon, nitrogen and pH conditions in transfer cultures via Northern analysis. *A. nidulans* mycelium, pre-grown on complete medium was transferred to minimal medium containing different carbon and/or nitrogen sources. After an additional 5 h growth period in the transfer media, the cultures were harvested, mycelia were washed, freeze dried and used for RNA extraction. Northern blots were prepared and probed with specific *pep*I and *pep*J probes and, after stripping, with a fragment of the *Agaricus bisporus* 28S ribosomal rDNA gene to prove loading of identical RNA concentrations.

Priliminary results indicated that both glucose and ammonium have repressive effects on the expression of both *pep*I and *pep*J, similar to the situation for the extracellular proteases in *A. niger* (data not shown). Interestingly we also have observed differences in expression in relation to

the pH of the transfer culture (Fig. 14.5). Transfer to an alkaline pH results in high expression of *pep*I whereas no expression is observed for the *pep*J gene. The opposite is observed for the acid transfer culture, i.e. high expression of *pep*J and low expression of *pep*I. These observations indicate that the two metallo proteases are expressed at a different ambient pH.

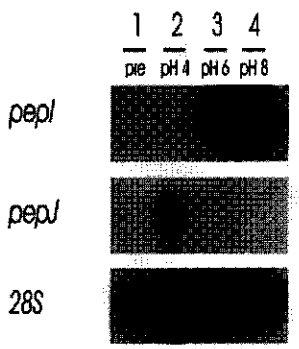


Fig. 14.5. Northern analysis of *pep*I and *pep*J expression in relation to ambient pH. *A. nidulans* mycelium, pre-grown on complete medium was transferred to medium containing 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 0.5 % (w/v) BSA and 0.5 % (w/v) elastin per litre. Addition of 0.1 M actetate, phosphate and Tris buffers ensured a constant pH at 4.0, 6.0 and 8.0, respectively. Track order: 1, immediately prior to transfer; 2, acetate-buffered medium pH 4.0; 3, phosphate-buffered medium pH 6.0; 4, Tris-buffered medium pH 8.0; pre, prior to transfer.

Definite proof for the involvement of wide domain regulatory systems in metallo protease gene expression in *A. nidulans* awaits similar expression studies as described for *A. niger*, using characterized wide domain regulatory *cre*A, *are*A and *pac*C mutants in *A. nidulans*

The data presented demonstrates that two novel acid metallo protease gene have been cloned from *A. nidulans* and detailed homology analyses indicate that two different classes of metallo proteases are present in filamentous fungi. Whether both the *A. nidulans* metallo proteases encode functional proteins and, if so, to what extent they contribute to the described extracellular metallo protease activities awaits further analysis. In several *Aspergillus* species both neutral protease I and neutral protease II activities have been described. Since in *A. fumigatus* both a thermolysin-type and an acid-type metallo protease have been cloned, it is well possible that a thermolysin-type metallo protease is also present in *A. nidulans*.

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15 General discussion: towards further improving protein production

Aspergilli have proved to be potentially very useful microorganisms for the expression and study of a variety of eukaryotic gene product, both for basic research and for industrial and pharmaceutical applications. The validity of producing recombinant proteins from filamentous fungi has already been established as production processes for chymosin (Genencor) and a detergent lipase (Novo) have been approved recently. The *Aspergillus* expression-secretion system was considered attractive to develop because of its exceptionally high protein secretion capacity. However, other factors have stimulated the exploration of this system as well. These microorganisms have a long history of industrial use, mainly to produce enzymes. Several *Aspergillus* species have Generally Regarded As Safe (GRAS) status. Furthermore, in many cases correct processing and folding of products, efficient disulphide bridge formation and less overglycosylation occur. Finally it has been shown that certain proteins could not be expressed properly in *E. coli* or *S. cerevisiae*, thus opening the need for additional expression systems. However, the increasing number of reports describing partial or complete degradation of heterologous proteins produced in *Aspergillus* indicates major proteolytic expression problems and has resulted in the molecular analysis of the proteolytic system described in this thesis.

The proteolytic system in *Aspergillus* is very versatile, as demonstrated by the number and complex regulation of the proteolytic functions identified. The observed complexity of proteolytic degradation and the large differences in wild type protease spectra complicated the construction of protease deficient strains as significant reduction required inactivation of several proteolytic activities. The differences in homologous protease spectra, acidification of cultures and glycosylation during the expression of PL3 in *A. niger*, *A. awamori* and *A. nidulans* demonstrated that the *Aspergillus* expression host can have a large influence on functionality and yields of the produced protein. This implicates that selection of expression strains based on the effects of their homologous proteases on the protein of interest will be critical to select succesful expression strategies. The correlation between the *in vitro* degradation tests for the *A. niger* pectin lyase B presented in chapter 8 and the *in vivo* data (chapter 9), demonstrated that *in vitro* testing of different expression hosts can be very useful, but also that effects of acidification on product stability have to be considered, as *A. niger* acidifies its environment very rapidly in contrast to *A. nidulans* or *A. oryzae*.

Two *Aspergillus* species which have been developed as expression secretion hosts and which have very different homologous protease spectra, *A.niger* and *A. nidulans*, were chosen to study their proteolytic system. The well developed genetics in *A. nidulans* and more recently also in *A. niger* enabled a combined molecular and classical genetical approach to address the protease problem. The

production strains momentarily used in industry are mainly the result of intensive screening of strain collections often followed by repeated mutation and selection programmes to further improve the strain of choice. Although this approach has been shown to be very valuable it does not allow strain improvement by recombination as this requires the development of the genetics of the particular host strain. Furthermore, repeated mutagenesis could finally lead to an accumulation of background damage which could interfere with growth and/or production yields of the production strain. The present study, in which three strategies to reduce proteolytic degradation - i.e. (I) selective gene disruption of interfering protease activities, (II) classical mutagenesis and (III) down regulation of protease activities - were employed, demonstrates how the combination of both molecular and classsical genetic techniques in a genetically well developed strain allows controlled construction of improved production strains without the accumulation of background damage.

Gene disruption

The construction of several *A. niger* protease disruptants and the controlled construction of multiple disruptants demonstrated the involvement of several acid proteases in the degradation of proteolytically susceptible proteins. However, although severely reduced, even in the Δ*pep*A, Δ*pep*B, Δ*pep*E (triple) disruptant the degradation was not absent, indicating the need for further strain improvement. Detailed knowledge about the regulation of proteases, zymogen activation and cascade activation allows simultaneous modulation of several proteases, either via transcriptional regulation (see modulation of protease expression) or via inactivation of proteolytic cascades (Δ*pep*E). The different extracellular protease spectrum present in *A. nidulans* required the cloning of major neutral and alkaline protease activities. Cloning of the alkaline subtilisin encoding *prt*A gene (Katz et al., 1995), the apergillopepsin encoding *pep*A gene and the *pep*I and *pep*J metallo protease genes allows a similar targetted gene disruption approach to construct *A. nidulans* strains in which the major protease activities have been inactivated, as has been described here for *A. niger*. In analogy with *S. cerevisiae* it will also be important to study the effects of several intracellular proteases which are potentially harmful to the proteins produced, either within the cell or extracellularly upon mislocalisation or cell lysis.

Classical mutagenesis

After classical UV mutagenesis a large set of protease deficient *A. niger* (*prt*) mutants has been isolated and characterized. In some of the seven *prt* complementation groups the degradation of an 'aspecific' substrate as BSA was reduced to 2% of that of wild type. Both *in vitro* and *in vivo* degradation of a model protein, PELB, was reduced, especially in the *prt*A, *prt*B, *prt*D, *prt*F and *prt*G

mutants. Surprisingly, the transcription levels of extracellular proteases in several *prt* mutants were not severely affected. However, extracellular protease activities and immunochemical analyses with anti-PEPA antibodies showed clearly reduction of extracellular protease activities, indicating that some of the *prt* mutations affect protease expression post-transcriptionally. Cloning of the individual *prt* genes will be necessary in order to understand the impact of the *prt* mutations and this could provide additional means to modulate protease expression (see below).

Similar to the situation in the disruptants (Chapter 7), proteolytic degradation is not absent in the *prt* mutants (even not in the strongest *prt*C7, *prt*A33 double mutant). For further reduction of proteolytic degradation the residual activities have to be characterized. Using specific inhibitors and substrates in sensitive protease assays the class(es) of proteases that are still (partially) expressed in a particular *prt* background should be determined. Additionally, degradation products of tester proteins can be sequenced to determine specificities of the residual protease activities. It might be possible to increase the expression of residual proteases by introducing specific carbon catabolite de-repressed (*cre*A^d) mutations or other wide domain regulatory mutations in a *prt* or disruption background. Partial characterization of the residual protease spectrum in some *A. niger prt* mutants has already resulted in the cloning of the metallo protease *pep*H, and, considering the effects of metallo protease inhibition upon the residual protein degradation in cleared culture supernatants of *prt*F mutants, disruption of *pep*H in a *prt*F background is expected to further improve the stability of proteins which are susceptable to proteolytic degradation.

Comparing the improved *in vitro* and *in vivo* stability of the PELB protein it is obvious that the *in vitro* 500-1000 fold reduced PELB degradation in *prt*F28 cleared culture supernatant is not seen *in vivo* to the same extent when PELB is produced in a *prt*F mutant. Apparently, other factors are limiting the potential improvement observed *in vitro*. Having reduced the high level extracellular degradation of produced proteins, new mutagenesis and selection programmes in protease mutants or disruptants could enable the isolation of mutations affecting other key factors involved in protein production.

Besides the direct use in expression studies as a starting point for further strain improvement in *Aspergillus*, these strains also can be used to screen for specific and/or low-level enzymatic activities. As the major proteolytic activities have been mutated and/or disrupted (both intracellularly and extracellularly) the residual protease spectrum can be screened for new/specific protease activities. In addition, combining multiple *prt* mutations and disruptions with *cre*A mutations would enable the identification of specific (derepressed) enzymatic activities without the degradation problems, as observed in wild type.

Down regulation of proteases

Protease regulation studies in *A. niger* have shown that expression of especially the extracellular proteases is regulated in a complex manner. Three wide domain regulatory mechanisms (carbon catabolite repression, nitrogen metabolite repression and pH regulation) have been shown to be involved in the regulation of *pep*A, *pep*B, *pep*D and *pep*F. Expression of these genes is induced by externally added protein. Furthermore, some of the isolated *prt* mutations affect several protease activities, indicating the involvement of additional unidentified factors in protease expression. These observations make the extracellular proteases interesting target genes to study complex gene regulation in *A. niger*. The *are*A and *cre*A wide domain regulatory mutations have to be sequenced in order to determine their impact on the functionality of the regulatory proteins. Cloning of *prt* genes, especially those involved in protein induction, can further contribute to a better understanding of the regulation of the proteolytic system. Very little is known about induction by externally added protein and about the subsequent uptake of the protein degradation products. Questions concerning how and in which form degraded proteins are imported and whether (and if so which) single amino acids are involved in induction in *A. niger* still remain. Initial plate experiments with some of the *prt* mutants indicated that some mutants are not able to grow without a supplement of several amino acids. How these mutations affect the various amino acid pools is unknown and has to be determined. Additionally, these amino acid requirements could also provide a direct cloning approach for the involved *prt* gene.

Interestingly, it has been shown that in *cre*A and *are*A mutants at an ambient pH of 4.0 derepression can be observed for the extracellular *A. niger* proteases. However, at pH 8.0 no de-repression is observed, indicative for an overruling pH regulation. Combined with the observed down-regulated expression of acid proteases in a *A. niger pac*2C^c strain this initiated studies on the effects of proteolytic degradation in pH regulatory mutants. The analysis of pH regulatory recombinants, isolated from a cross between an *E. carotovora* PL3 producing *A. nidulans* transformant and individual *pac* and *pal* mutant strains, showed reduced degradation of the recombinant protein. The subsequent construction of an *A. niger pac*2C^c disruption strain, which also resulted in reduced PL3 degradation *in vitro*, demonstrated the possibilities of down-regulation of protease expression. However, although the degradation of the PL3 produced is reduced, it is not completely absent. Proteases which are not regulated by *pac*C could be involved but also alkaline *pac*C-regulated proteases which are probably constitutively expressed in a *pac*C^c background, similar to the constitutive expression of *A. nidulans prt*A, could contribute. Therefore, the effects on the non-acid expressed spectrum in a *pac*C^c strain will have to be analyzed as well. Especially constitutive expression of alkaline genes could result in the need for specific alkaline expressed protease gene disruptions in a *pac*C^c background to optimize expression.

The reduced degradation in *A. nidulans pac*C^{c} mutants could indicate the involvement of acid expressed proteases in a wild type or *pac*C$^{+/-}$ background. This has initiated the cloning of the aspergillopepsin A encoding gene, *pep*A, from *A. nidulans*. Preliminary results indicate that the *pep*A gene is transcribed at pH 4.0 and that a 43 kD protein cross-reacting with the polyclonal *A. niger* anti-PEPA antibodies is observed in *A. nidulans* cleared culture supernatants. Whether *A. nidulans pep*A is indeed pH regulated and whether the differences in PL3 degradation in pH regulatory mutants are indeed *pep*A related has to be investigated. Remarkably, no PacC consensus binding sites are identified in the *A. nidulans pep*A promoter, in contrast to the *A. niger pep*A gene. The cloning of the *A. niger* and the *A. nidulans pep*A genes and the isolation and characterization of different regulatory mutations in both *Aspergilli* will allow future comparative studies on acid protease gene regulation.

The observation that some intracellular proteases appear to have higher transcriptional expression levels in *A. niger cre*A is also important. Industrial production strains usually have a history of repeated mutation selection procedures and probably some of these strains will contain wide domain regulatory mutations. If, and if so to what extent, a *cre*A mutation increases proteolytic activities in an expression host and whether this influences expression yields is unknown, but should certainly be taken into consideration.

Optimizing expression strategies in protease deficient strains

The controlled strain improvement will eventually result in (a set of) expression strains suited for homologous and heterologous protein production. As a number of different modifications will be combined in these strains, they will have to be tested in fermentations to establish conditions which are optimal for protein production. Obtaining optimal production yields probably requires adjusting expression strategies to these conditions. For the construction of efficient expression-secretion cassettes many different systems are available at the moment and a number of them can be applied in several *Aspergillus* species. Apart from the strong constitutive promoters, which are ideal for continuous protein production, also a set of inducible promoters is available, enabling controlled induction of the production process. Unfortunately, some of these inducible promoters can only be induced under derepressed conditions, which is often less favourable, as several proteases will also be derepressed. Hybrid promoters which show no repression and which are still inducible have been described and can be of help in such cases. Additionally, promoters which are highly expressed under the chosen fermentation conditions could be cloned. Apart from the choice of the promoter and terminator, the protein can be expressed intracellularly or extracellularly and in the latter case either with or without fusion approach. In many cases the extracellular production is preferred as this

enables easy product recovery, especially as the expression approach ensures specific expression of the product of interest preferably combined with repression of other (homologous) enzyme systems. In the expression cassette used to express the *E. carotovora* PL3 protein, the secretion was ensured by including either the pectin lyase or the polygalacturonase secretion signal and the *pki* promoter enabled high expression on glucose and carbon catabolite repression of many homologous extracellular enzyme systems. Constructs in which the gene of interest is fused to a highly expressed homologous gene can further improve expression as is shown for the interleukin 6 expression (Contreras et al., 1991). Apparently, fusion to the *A. niger* glucoamylase gene ensures high co-expression of interleukin 6 and furthermore protects the interleukin 6 also from degradation within the secretory pathway. Including a KEX2 dibasic cleavage site results in production and secretion of functionally interleukin 6.

It is of course apparent that besides decreasing proteolytic degradation in expression strains many other factors can be important during protein production in *Aspergillus*. Although the exceptional protein secretion capacity in especially *A. niger* has been known for many years, no molecular basis for this observation has been reported yet which made Peberdy (1994) refer to it as 'a highly productive black box'. Efficient future modulation of protein secretion requires detailed knowledge about the secretion process in filamentous fungi. Especially, if in future specific posttranslational modification mechanisms which reside in the secretion pathway have to be modified to ensure 'product authenticity' (as will be discussed below).

Many different factors can contribute to design a succesful expression strategy for *A. niger*. As in *Aspergilli* foreign DNA is readily integrated into the genome upon transformation the copy number and methods for increasing copy number were among the first techniques which really improved expression. Little is known about transcription initiation, RNA processing, RNA stability and translation efficiency in *Aspergillus*, but these are factors which should be looked into in more detail to understand and improve protein production in *Aspergilli*. Apart from the site of integration and copy number of introduced DNA, the transcription and translation and the secretion of proteins should be studied. Are the proteins of interest corretly folded, which proteolytic enzymes present in the secretion pathway are potentially hazardous for the expressed protein and what are the effects of glycosylation? Some observations seem to indicate that glycosylation can be beneficial for production yields (either by reducing proteolytic degradation through shielding or by stimulating overall secretion of the product), whereas glycosylation, and especially over-glycosylation, also can modify the enzyme structure and activity.

The most important technological hurdles to overcome before filamentous fungal systems can be universally applied for the production of recombinant proteins still involve increasing expression levels of certain proteins and adapting fungal systems to the production of certain proteins which need specific post-translational modifications for activity. In most cases the expression yields of non-fungal (heterologous) proteins from fungal hosts has been significantly lower than those obtained for the expression of (homologous) fungal products. Using high efficiency promoters it has been demonstrated that the barriers to obtain high heterologous gene expression yields occur post-transcriptionally. However, a point has to be made concerning the genetic structure of the transformant in relation to expression. Stable transformants can be obtained when foreign DNA is integrated into the genome. Large variation in site of integration and production levels between transformants is often found, suggesting that the site of integration is important to achieve high expression levels. Identification of fungal attachment elements, similar to those described in a mammalian system (Stief et al., 1989), that mediate increased expression levels in a position-independent manner, could be very useful. Development of targetting systems, designed to specifically integrate at those 'high expression' loci, preferably in high copy number, could both further increase expression and circumvent the large differences between individual transformants as seen in the expression systems thusfar used. From a process validation point of view a well-defined targetting system will also be very useful, as it is crucial that the genetic structure of a recombinant production strain is established and subsequently validated as unaltered during the production process. This is relatively simple for plasmid-based expression systems, but very difficult for some filamentous fungi in which DNA can integrate at multiple sites.

In addition to the concerns about product yield, the problem of 'product authenticity' will become more important when more therapeutical proteins are being expressed in filamentous fungi. Many non-fungal proteins, especially those which are secreted, are posttranslationally modified. One of the most important modifications is the addition of carbohydrates (glycosylation). Carbohydrate addition may be required for full activity in certain proteins. The presence of appropriate types of carbohydrates may also be required for other purposes. Especially, for pharmaceutical proteins where aberrantly glycosylated proteins can be immunogenic or may be cleared very quickly from the blood. Filamentous fungi typically attach high mannose carbohydrates in N-linked structures and sometimes tend to hyperglycosylate foreign proteins as is the case with certain strains of *Saccharomyces*. The *A. nidulans*-produced tPA seems to be glycosylated to a similar degree as the native mammalian protein (Upshall et al., 1987), unlike the recombinant product from *Saccharomyces* (Lemontt et al., 1985; MacKay, 1987). The endogenous *T. reesei* reesei CBHI contains N-linked glycans which are quantitatively similar to mammalian glycans of the $(Man)_5(GlcNAc)_2$ and $(Man)_9(GLcNAc)_2$ type

(Salovuori et al., 1987). There is no evidence that fungi produce the complex, sialic acid containing, glycoproteins which are required for both stability and non-immunogenicity in serum. Given this fact, one of the future goals for expression of therapeutical proteins in filamentous fungi should be the analysis of 'product authenticity' of secreted recombinant proteins. At present the product range for which filamentous systems are useful is limited to non-glycosylated injectable therapeutic proteins, and glycosylated proteins for topical therapeutic, diagnostic and industrial use only which applies also for *E. coli* and *S. cerevisiae*, because of the potential occurrence of minor differences in glycosylation between proteins produced in filamentous fungi and mammalian cells. In future it might be possible to modify the glycosylation system of the host and to add the residue(s) and structure(s) for seroprotection of recombinant proteins. One possible way will be the cloning and expression of appropriate glycosyl transferase enzymes in the selected production strains.

Finally it can be stated that the filamentous fungi are beginning to be recognized as a useful and promising group of hosts for the expression of proteins from both fungal and other sources, as some products which have been derived from recombinant fungal processes have recently received government regulatory approval. These organisms have been accepted alongside *E. coli* and *S. cerevisiae* as viable recombinant production systems for food and detergent use at least. Genetic engineering approaches have allowed to exploit (at least partially) the astonishing protein secretion capacity of *Aspergillus* species. It is obvious that still a lot needs to be learned about the molecular biology and physiology of gene expression and secretion. Nevertheless, the development of *Aspergillus* species into competitive production strains and their application has proven the rational behind developing filamentous fungal expression systems.

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Summary

The filamentous fungus *Aspergillus niger* is capable of producing and secreting large amounts of homologous proteins (20-40 g/l; Montenecourt and Eveleigh, 1985; Nevalainen et al., 1991). Because of this very efficient secretion large effort is being made to use *Aspergilli* as hosts for expression of many heterologous products. Especially *A. niger* is an interesting option because of the very high secretion capacity and because it has GRAS (Generally Regarded As Safe) status, which enables food-grade applications. Unfortunately, in spite of the very high expression and secretion capacity of *A. niger*, it has been shown both in homologous and heterologous expression studies that overexpression of gene products is often hampered by homologous proteases. The *Aspergillus* expression strains produce several proteases which cause partial or complete degradation of target products. In order to study these protease-related problems and to construct *Aspergillus* strains in which proteolytic degradation is decreased or absent, the protease project was started. The results of these elaborate studies to improve protein production in relation to proteolytic degradation are presented in this thesis.

Aspergillus strains express many different proteolytic activities as discussed in the introduction (chapter I). Therefore, the protease spectra of *A. nidulans* and *A. niger* have been characterized (chapter III). The very different protease spectra, the differences in acidification of the growth medium and glycosylation of the proteins produced, caused large differences in expression yields, stability and activities of the expressed products. This clearly proved the importance of the choice of expression strain for heterologous expression. Many different proteolytic activities, comprising acid, semi-alkaline and alkaline proteases and serine carboxpeptidases, have been isolated from *A. niger*. Some of these activities were cloned (*pep*A; Berka et al., 1990; *pep*B, Inoue et al., 1991 and *pep*C, Frederick et al., 1993) at the beginning of the protease project in which five additional *A. niger* protease genes were cloned, *pep*D, *pep*E, *pep*F, *pep*H and *pal*B (van den Hombergh et al., unpublished results). In chapter IV the cloning of a pepstatin-repressible intracellular acid protease, *pep*E, is described. The PEPE protein is probably located in the vacuole and involved in the cascade activation of two additional vacuolar proteolytic enzymes (PEPC and CPY, carboxypeptidase Y). After cloning and characterization of *pep*F, an extracellular serine carboxypeptidase it was shown that this extracellular exo-protease was regulated in a very complex manner (chapter V). Both carbon catabolite repression and nitrogen metabolite repression are involved in the regulation of *pep*F at the level of mRNA amounts. Furthermore, pathway specific induction by protein and regulation by external pH have been described. Additionally, an extracellular metallo protease, *pep*H, was cloned from *A. niger*. This endo-protease was cloned after the observation that in the residual activities of some isolated protease (*prt*) mutants, metallo protease activities were still present (see also chapter

VIII). Cloning of the responsible gene(s) and subsequent disruption enables specific strain improvement of these protease mutants. Gene disruption of the three cloned acid proteases (two extracellular (*pep*A and *pep*B) and one intracellular (*pep*E), resulted in a significant reduction of degradation of 'a-specific' proteins (chapter VII). Also, for specific tester-proteins such as PELB a similar reduced *in vitro* degradation was observed. Furthermore, it was shown that disruption of *pep*E inactivated the cascade activation of Pro-PEPC and Pro-CPY, resulting in a strongly reduced vacuolar proteolytic activity. Besides generating specific gene disruptions, a large number of protease (*prt*) deficient mutants were isolated (chapter VIII). Classical genetic analyses of the *prt* mutants identified at least seven complementation groups (*prt*A-G). Residual activities in some of these *prt* mutants varied from 2-80 % compared to wild type and *in vitro* degradation of tester proteins was reduced 500-1000 fold in some of these mutants. Construction of multiple *prt* deficient mutants, either by recombination of characterized mutations or by additional mutagenesis in existing *prt* mutants, resulted in further reduction of degradation. For six *prt* complementation groups transformable strains were constructed and subsequently expression of a *pkipel*B expression-secretion cassette was studied (chapter IX). These experiments proved that *in vivo* degradation of PELB was also significantly reduced and overall expression yields were improved. The highest expression was observed in the *prt*F28 mutant and the overall expression was improved by a factor of 13 compared to expression in wild type. The regulation of several proteases in *A. niger* is very complex, as already mentioned in chapters I, IV, V and VI. The observed pH regulation of several extracellular proteases combined with the fact that *A. niger* acidifies its medium very rapidly, initiated analysis of pH regulation which finally resulted in the isolation of the wide domain pH regulatory gene, *pac*C, in *A. niger* (chapter X). The *pac*C gene encodes a zinc-finger regulator protein which binds to specific sequences (5'-GCCA/GG-3') in the promoters of target genes. A disruption construct, designed in a way that upon integration at the homologous *pac*C locus a C-terminally truncated PacC protein is produced, resulted in constitutive expression of *pac*C (chapter XI). From expression studies with this *pac*2C^c disruptant it was concluded that the *pac*C gene is auto-regulated and that the C-terminal part of PacC (deleted in the *pac*2C^c strain) has a negative regulatory effect. Expression of several phosphatases identified at least three acid phosphatases (PI, PII and PIII) which are regulated both by phosphate and by pH. The observed pH regulatory effects were shown to be *pac*C mediated, as expression of PI, PII and PIII decreased severely in the *pac*2C^c disruptant. The recent isolation of both *cre*A and *are*A mutants in *A. niger* combined with the construction of a *pac*2C^c disruption strain enabled a detailed analysis of protease expression at the level of mRNA content (chapter XII). Northern analyses using the wide domain regulatory mutants and disruptant proved that the observed regulatory effects for extracellular proteases (carbon catabolite repression, nitrogen metabolite repression and pH regulation) were indeed

mediated by the *cre*A, *are*A and *pac*C genes, respectively. Apart from identification of severe *cre*A and *are*A alleles and the expected derepression phenomena for carbon catabolite repression and nitrogen metabolite repession of extracellular protease, also elevated mRNA levels for the vacuolar proteases were observed. Possibly, the derepressed strains need higher degradation capacities in order to remove the increased levels of damaged or unwanted protein. Furthermore, in the *pac*2C^c disruption strain the expression of the extracellular acid proteases is increased upon transfer to alkaline pH. As the current model for pH regulation predicts a strong reduced expression for acid target genes in *pac*C^c strains, this observation could imply that for acid pH regulation not all the components involved have been identified.

A. nidulans has a very different protease spectrum compared to *A. niger* (chapter III). However, although the pH optimum for degradation of 'a-specific' proteins is much higher than for *A. niger*, *A. nidulans* can also express an acid protease gene, *pep*A, which is probably regulated in a similar complex way as described for the *A. niger* extracellular proteases (chapter XIII). Cloning and characterization of two novel metallo protease genes, *pep*I and *pep*J, from *A. nidulans* is described in chapter XIV. These two metallo proteases appear (together with the *A. oryzae* neutral protease II, the *A. fumigatus* and *A. flavus* mp20 and the *P. citrinum* neutral metallo protease) to belong to a novel class of metallo proteases, as no significant homologies with any of the known metallo protease families can be detected. As the metallo protease genes are expressed in *A. nidulans* and metallo proteases in general have neutral pH optima, these metallo proteases could well be involved in the overall degradation of expressed heterologous proteins, which is also observed in *A. nidulans*. Disruption of these metallo proteases together with the major extracellular serine protease could be a starting point for construction of *A. nidulans* expression strains with reduced proteolytic activities.

Samenvatting

Aspergillus niger, een filamenteuze schimmel, is in staat bepaalde eiwitten in zeer hoger concentraties uit te scheiden (20 - 40 g/l; Montenecourt and Eveleigh, 1985; Nevalainen et al., 1991). Door deze efficiente secretie bestaat er binnen de biotechnologie grote belangstelling om *Aspergilli* als gastheer voor expressie van tal van soortvreemde (heterologe) eiwitten te gebruiken. Met name *A. niger* is hierbij interessant omdat deze schimmel in de voedselindustrie wordt toegepast en zgn GRAS (Generally Regarded As Safe) status bezit. Echter uit studies naar zowel homologe als heterologe expressie in *A. niger* zijn belangrijke complicaties gebleken. Door de geschikte *Aspergillus* stammen worden ook verschillende proteases aangemaakt die tot verminderde en soms zelfs tot niet detecteerbare niveau's van het gewenste product leiden.

Om deze problemen nader te bestuderen en om uiteindelijk *Aspergillus* stammen te construeren die geschikter zijn voor homologe en heterologe expressie is ruim vier jaar geleden het protease project gestart, hetgeen geleid heeft tot dit proefschrift. Het doel van het in dit proefschrift beschreven onderzoek was het verbeteren van eiwitproduktie door eleminatie van storende protease activiteiten. Zoals beschreven in de inleiding (hoofdstuk I), worden door de afzonderlijke *Aspergillus* stammen, vele maar ook vaak verschillende proteases aangemaakt. Initieel zijn daarom de protease spectra van *A. niger* en *A. nidulans* in kaart gebracht (hoofdstuk III). De zeer verschillende protease spectra, de verschillen in verzuringsgraad van het medium en glycosylering zorgen voor een grote variatie in de opbrengst, stabiliteit en activiteit van de tot expressie gebrachte eiwitten, hetgeen impliceert dat de keuze van de gastheer zeer belangrijk is bij heterologe expressie in *Aspergillus*.

Uit *A. niger* is de afgelopen jaren een breed spectrum aan proteolytische activiteiten geïsoleerd. Dit spectrum omvat o.a. zure, semi-alkalische en alkalische serine proteases en serine carboxypeptidases. Sommige van deze activiteiten waren reeds gekloneerd tijdens het begin van dit onderzoek (*pep*A; Berka et al., 1990; *pep*B, Inoue et al., 1991 en *pep*C; Frederick et al., 1993) en tijdens het onderzoek zijn nog vijf additionele protease genen in *A. niger* gekloneerd, *pep*D, *pep*E, *pep*F, *pep*H en *pal*B (van den Hombergh et al., ongepubliceerde resultaten). In hoofdstuk IV wordt de klonering van een pepstatine-geremd intracellulair aspartyl protease, *pep*E, beschreven. Het genproduct is waarschijnlijk in de vacuole gelocaliseerd en betrokken bij de cascade activiatie van twee andere vacuolaire enzymen (PEPC en CPY). Uit de klonering, karakterisering en de expressie van *pep*F, een extracellulair serine carboxypeptidase uit *A. niger* blijkt dat dit protease op transcriptieniveau zeer complex gereguleerd is (hoofdstuk V). Zowel koolstof kataboliet repressie, stikstof metaboliet repressie en pH regulatie zijn aangetoond voor dit protease gen. Naast deze drie wide domain regulatie mechanismen blijkt ook specifieke inductie door eiwit te worden waargenomen. Ook een metallo protease gen, *pep*H, is gekloneerd uit *A. niger* (hoofdstuk VI). Dit extracellulaire

protease werd gekloneerd nadat in de restactiviteiten van sommige van de geïsoleerde protease mutanten (zie hoofdstuk VIII) metallo protease activiteiten werden waargenomen. Klonering van het verantwoordeijke metallo protease gen en disruptie van deze activiteit maakt verdere stamverbetering van de protease mutanten mogelijk. Gendisruptie van drie gekloneerde zure proteases, twee extracellulaire proteases (*pep*A en *pep*B) en een intracellulair protease (*pep*E), in *A. niger* resulteerde in een aanzienlijke verlaging van de afbraak van 'a-specifieke' eiwitten (hoofdstuk VII). Ook bij specifieke test-eiwitten werden zowel *in vitro* als *in vivo* een verminderde afbraak van gesecreteerd functioneel product waargenomen. Verder werd aangetoond dat door disruptie van het *pep*E gen een cascade activatie van PEPC en CPY onmogelijk werd, resulterend in een sterk verlaagde vacuolaire protease activiteit. Naast het genereren van specifieke gen-disrupties zijn ook een groot aantal protease (*prt*) mutaties geisoleerd (hoofdstuk VIII). Deze mutanten konden na klassiek genetische studies onderverdeeld worden in tenminste zeven complementatie groepen (*prt*A-G). Residuele activiteiten in sommige van deze *prt* mutanten waren sterk verlaagd, variërend van 2-80 %, in vergelijking met de wild type stam en de *in vitro* afbraak van tester eiwitten verminderde hierdoor 500-1000 voudig. Constructie van dubbel *prt* mutanten, zowel door middel van recombinatie als herhaalde mutagenese, heeft deze *in vitro* afbraak nog verder verlaagd. Voor zes verschillende complementatiegroepen zijn vervolgens transformeerbare expressiestammen geconstrueerd waarna expressie van een *pkipel*B expressie cassette in deze stammen is bestudeerd (hoofdstuk IX). Hieruit bleek dat ook de *in vivo* afbraak van PELB sterk verminderd was. De *prt*F28 stam bleek voor *pkipel*B expressie het meest geschikt en de uiteindelijke expressie verbetering voor dit specifieke systeem bleek ongeveer een factor 13 te zijn (hetgeen bewerkstelligd wordt door één enkele *prt* mutatie, waarvoor nog steeds 20% restactiviteit wordt waargenomen). Zoals vermeld in hoofdstukken I, IV, V en VI blijkt de regulatie van verschillende proteases zeer complex te zijn. De waargenomen pH regulatie van enkele extracellulaire proteases, gecombineerd met het feit dat vooral voor *A. niger* sterke verzuring van het cultuurmedium is waargenomen, heeft geleid tot nadere bestudering van pH regulatie, resulterend in de klonering van het wide domain pH regulatoire gen, *pac*C (hoofdstuk X). Het *pac*C gen codeert voor een 'Zinc-finger' eiwit dat is staat is om te binden aan specifieke sequenties (5'-GCCA/GG-3') in de promoter van target genen. Een disruptie construct, zodanig geconstrueerd dat na integratie op de homologe *pac*C locus een C-terminaal getrunceerd eiwit ontstaat, resulteert in constitutieve expressie van *pac*C (hoofdstuk XI). Uit expressie studies in deze *pac*2C^c disruptie stam blijkt dat *pac*C auto-regulatie vertoont en dat het C-terminale deel van het PacC eiwit hierbij betrokken is. Vervolgens is de expressie van verschillende fosfatases bestudeerd waarbij bleek dat in *A. niger* tenminste drie zure fosfatases (PI, PII en PIII) zowel door fosfaat als door de pH gereguleerd worden en dat deze waargenomen pH regulatie *pac*C afhankelijk is. De beschikbaarheid van zowel *cre*A als

*are*A mutanten en het genereren van een *pac*2C^c mutatie maakten een uitvoerige protease expressie studie in *A. niger* mogelijk (hoofdstuk XII). Northern analyses van de genoemde mutanten toonden aan dat de waargenomen regulatoire effecten (koolstof katabolietrepressie, stikstof metaboliet repressie en pH regulatie) inderdaad door respectievelijk CREA, AREA en PacC werden gemediëerd. Naast identificatie van sterke *cre*A en *are*A allelen en de verwachte derepressie fenomenen mbt de expressie van extracellulaire proteases werden ook zeer opmerkelijke resultaten mbt expressie van intracellulaire proteases en mbt de pH regulatie verkregen. De vacuolaire proteases vertoonden hogere mRNA niveaus in *cre*A mutanten, mogelijk veroorzaakt door indirecte effecten zoals verhoogde behoefte aan degradatie capaciteit. Verder werden verhoogde transcriptie niveaus van verschillende zure extracellulaire proteases waargenomen in de *pac*2C^c stam, na transfer naar pH 8.0. Aangezien in het pH regulatie model voor een *pac*C^c mutant sterk verlaagde expressie van zure target genen wordt voorspeld kan deze waarneming betekenen dat (nog) niet alle componenten van het pH regulatie systeem (met name betrokken bij de zure pH regulatie) zijn geïdentificeerd.

A. nidulans bezit een sterk afwijkend protease spectrum in vergelijking met *A. niger*. Alhoewel het pH optimum voor afbraak van 'a-specifieke' eiwitten veel hoger ligt dan bij *A. niger*, bezit ook *A. nidulans* een zuur protease, *pep*A, dat waarschijnlijk op een vergelijkbare wijze als het *A. niger pep*A gen wordt gereguleerd (hoofdstuk XIII). Klonering en karakterisatie van twee nieuwe metallo protease activiteiten uit *A. nidulans* wordt tenslotte beschreven in hoofdstuk XIV. Deze metallo proteases blijken samen met andere neutrale metallo proteases uit *A. oryzae*, *A. flavus*, *A. fumigatus* en *P. citrinum* een nieuwe metallo protease familie te vormen en zij kunnen vanwege hun neutrale pH optimum betrokken zijn bij de waargenomen afbraak van tot expressie gebrachte eiwitten in *A. nidulans*.

Curriculum Vitae

Johannes Petrus Theodorus Wilhelmus van den Hombergh werd geboren op 14 oktober 1967 te Venlo. In 1986 behaalde hij het diploma gymnasium ß aan het St. Thomascollega aldaar en begon vervolgens in september in Wageningen aan een studie Moleculaire Wetenschappen. Deze studie werd in 1991 afgerond met hoofdvakken in de Moleculaire Genetica en de Proceskunde. Verder bracht hij een 6-maandse stage periode door bij de vakgroep Biochemistry & Genetics van de University of Newcastle upon Tyne in Newcastle upon Tyne, Engeland. Het in dit proefschrift beschreven onderzoek werd uitgevoerd bij de sectie Moleculaire Genetica van Industriële Microorganismen in de periode september 1991 - september 1995. Gedurende dit onderzoek was hij een van de docenten van de sectie MGIM die in samenwerking met de Carlsberg Laboratories de EMBO cursus 'Heterologous protein production in yeast and filamentous fungi' (gehouden te Kopenhagen, augustus 1993) organiseerde. Deze gezamelijke organisatie heeft geresulteerd in een samenwerking waarbij heterologe expressie van prokaryote genen in verschillende *Aspergilli* wordt bestudeerd. Verder heeft hij in 1993 gedurende vier maanden bij Ciba Geigy binnen het Dept. of Biotechnology (Basel, Zwitserland) het PEPF serinecarboxypeptidase gekarakteriseerd. Per 1 september 1995 is hij als toegevoegd onderzoeker aangesteld bij sectie MGIM van de Landbouwuniversiteit.

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