Aminopeptidases from *Aspergillus niger*

Daniëlle E.J.W. van Wijk – Basten

Promotor:
Prof. dr. Ir. A.J.J. van Ooyen
Hoogleraar in de Toegepaste Microbiële Genetica
Wageningen Universiteit

Copromoter:
Dr. P.J. Schaap
Universitair docent, leerstoelgroep Microbiologie
Wageningen Universiteit

Promotiecommissie:
Prof. dr. J.A. van den Berg, Wageningen Universiteit
Prof. dr. W. J. Quax, Rijksuniversiteit Groningen
Prof. dr. H.A.B. Wösten, Universiteit Utrecht
Dr. P.J.T. Dekker, DSM foodspecialties, Delft

Aminopeptidases from *Aspergillus niger*

Daniëlle E.J.W. van Wijk – Basten

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus van
Wageningen Universiteit,
Prof. Dr. Ir. L. Speelman,
in het openbaar te verdedigen op
vrijdag 16 januari 2004
des namiddags om half twee in de Aula.

Aminopeptidases from *Aspergillus niger* / Daniëlle E.J.W. van Wijk – Basten
Thesis Wageningen University. – With ref. – With summary in Dutch
ISBN 90-5808-968-1
Subject headings: *Aspergillus* / protease / aminopeptidase / peptides

The research described in this thesis was carried out at the department Microbiology, section Fungal Genomics, formerly known as the section Molecular Genetics of Industrial Micro-organisms, Wageningen University.

Aan mijn ouders

Contents

Chapter 1

General introduction

1. Introduction

Already in the nineteenth century it was discovered that some proteins (proteases) could degrade other proteins. At that period there was however no clear understanding of the reactions involved. Progress came first from the understanding that the constituent amino acids are linked by peptide bonds, as suggested independently by Fischer (34) and by Hofmeister (51) in 1902. Convincing demonstrations that proteolytic enzymes hydrolyzed these peptide bonds were brought forward by the use of synthetic peptides and their derivatives in 1942 (13). It was noticed that there are two distinct activities, one activity catalyzed by endoproteases hydrolyzes amino acids distant from the termini of a protein, the other activity catalyzed by exopeptidases hydrolyzes amino acids at either the amino- or carboxy terminus of a protein.

Proteases from filamentous fungi are interesting to study for two main reasons. First, in the industry *Aspergilli* are being used as hosts for the production of both homologous and heterologous proteins (18, 19, 52, 76, 89). However, heterologous expression is often hampered by the proteolytic degradation of the expressed products by endogenous proteases. Second, the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa* are used as model organisms. Their genetics have been a subject of study for many decades. It is relatively easy to generate mutants and powerful genetical, biochemical and molecular biological techniques are available for analysis.

Eukaryotic microbial proteases have been reviewed by North et al, in 1982 (88), Suarez-Rendueles and Wolf have reviewed the yeast proteases (100) and more recently the molecular and biotechnological aspects of microbial proteases have been reviewed by Rao and others (90). A number of endoproteases from *Aspergilli*, especially *Aspergillus niger*, are well characterized. The aminopeptidase spectrum however, is still unclear, although some of these aminopeptidases are being used in the food industry.

Aminopeptidases have routinely been used for various purposes, such as cheese making, baking and the preparation of soy hydrolysates. Traditionally starter cultures have been used in the production of cheeses and sauces. In Camembert and Brie cheeses spores of *Penicillium camemberti* are added as starter cultures (63). Prolyl aminopeptidase activity of this fungus is important during the ripening of Camembert by decreasing the amount of bitter tasting peptides (39). The production of soy sauce starts with the grinding of soybeans after which they are mixed with water and inoculated with strains of *Aspergilli*. To increase the protein conversion and to accelerate the fermentation, proteases are added. Addition of aminopeptidases has been successful as debittering agents by hydrolyzing peptide fragments that would be bitter otherwise (42).

Aspergillus oryzae is a major source of aminopeptidase activity in commercial preparations. Aminopeptidases from this fungus are major constituents of commercial preparations such as Debitrase®DBS50 from Rhodia, Flavorpro™192 from Biocatalysts and Corolase™7093 from Röhm Tech. In these preparations one of the most common aminopeptidase activities is referred to as leucine aminopeptidase, however this actually encompasses a group of enzymes, which cleave off leucine as well as some other amino acids.

Few of these aminopeptidases are described in patents. For example patent WO96/38549 of Gist-brocades and US6.271.013 of DSM describe an aminopeptidase of *A. niger* that liberates N-terminal phenylalanine from peptides. Addition of this aminopeptidase to cheese results in accelerated ripening of the cheese without off-flavors and addition to dough liberates phenylalanine from proteins present in the dough, thereby improving flavor and aroma of the baked product.

The use of *Aspergilli* as hosts for the production of industrial proteins has resulted in detailed studies of the proteolytic spectra and construction of protease deficient strains and has improved the knowledge about protease expression and regulation in these organisms. In 1993 Taylor wrote two reviews about

aminopeptidases; one about structure and function (104) and one about action mechanism (105). A number of endoproteases are well characterized in *Aspergillus niger*, however the (intracellular) exoprotease spectrum is not.

2. Classification of aminopeptidases

Today there are two major classifications systems for proteases. The internationally recognized schemes for the classification and nomenclature of all enzymes from IUMB and MEROPS, include proteases. The update IUMB text for protease EC numbers can be found at the URL: http://www.chem.qmw.ac.uk/iubmb/enzyme/EC3/4/11/. In this system enzymes are defined by the fact that they catalyze a single reaction. This has the important implication that several different proteins are all described as the same enzyme, and a protein that catalyses more than one reaction is treated as more than one enzyme. The proteases in the EC list are divided in 13 subclasses. The aminopeptidases are in class 3.4.11. The value of the EC list for proteases resides in providing standard terminology for the various types of protease activity and especially in the assignment of a unique identification number and a recommended name to each protease. The special strength of the EC system is thus in the area of nomenclature rather than classification.

The second major classification system is described in the handbook of proteolytic enzymes (7) and at MEROPS [http://www.merops.co.uk]. It is widely used and more convenient for classification of proteases than the IUMB system. In the MEROPS system the proteases are allocated into families, which are then grouped into clans. A family of proteases is a group in which every member shows a statistically significant relationship in amino acid sequence to at least one other member of the family either throughout the whole sequence or in the part of the sequence responsible for catalytic activity. A clan is the term used to describe a group of families, the members of which have evolved from a single ancestral protein, but have diverged so far apart that we can no longer see their relationship

by comparison of the primary structures. Such indications of distant relationships come primarily from the linear order of catalytic-site residues and the tertiary structure. Occasionally other distinctive aspects of the catalytic activity such as specificity or inhibitory sensitivity are also used as indicators for classification into clans.

An alternative classification system that is commonly used is the division of proteases into endo- and exoproteases. Endoproteases are those enzymes that hydrolyze internal peptide bonds, exoproteases hydrolyze peptide bonds adjacent to a terminal α-amino group, or a terminal carboxyl group and a penultimate amino acid.

Another classification uses the number of amino acids cleaved from the amino-terminus of substrates. Enzymes that sequentially remove the amino-terminal amino acids from protein and peptide substrates are called aminopeptidases. Dipeptidylpeptidases and tripeptidylpeptidases remove di- and tripeptides from the amino-terminal end respectively.

In the field of aminopeptidases additional classification schemes are used.

1. Classification with respect to the relative efficiency with which the 20 different amino acids are removed. Aminopeptidases of broad specificity (which are capable of cleaving a range of different amino acids from the N-terminal or P1 position (according to Schechter's nomenclature (94))) as well as aminopeptidases that cleave a single type of residue at the P1 position, may be distinguished.

2. Aminopeptidases are also characterized according to their sensitivity to various protease inhibitors.

A. Metallo aminopeptidases, which are inhibited by metal chelating agents such as: EDTA, EGTA and 1,10-phenantroline.
B. Cysteine aminopeptidases, which are inhibited by Hg^{2+}, iodoacetamide, N-tethylmaleimide and *p*-chloromercuribenzoate.

C. Serine aminopeptidases, which are sensitive to phenyl methyl-sulfonyl fluoride and diisopropyl fluorophosphate.

Although aspartic endoproteases, inhibited by pepstatin are ubiquitously present, both inside and outside the cell, aspartic aminopeptidases have not been reported.

Obviously, each of these nomenclature or classification systems serves to identify the protease with respect to a topic of interest, and they are not mutually exclusive. Knowing that such a labyrinth of classifications exists, it is not surprising that in recent years several enzymes previously thought to be different were shown to be identical.

The naming of the clans in the MEROPS system starts with a letter denoting its catalytic type, M for metallo proteases, S for serine proteases, C for cysteine proteases and U for unknown catalytic types. The catalytic types of the aminopeptidases are further explained below.

2.1 Metallo aminopeptidases

Metallo aminopeptidases encompass 85% of the known aminopeptidases and are widespread among all kingdoms. According to the MEROPS classification system the metallo proteases are divided into 14 clans; MA(E), MA(M), MC to MM and clan MX. These clans are further divided into 52 families of which 9 harbor aminopeptidases. Clans and families with aminopeptidases are listed in table 1.

The M1 family contains by far the most aminopeptidases. According to MEROPS 44% of the known metallo aminopeptidases belong to the M1 family. The M1 family is composed of three phylogenetically unrelated subgroups. The first subgroup consists mainly of aminopeptidase N type aminopeptidases. The second group consists of leukotriene A4 hydrolase aminopeptidases. The metallo aminopeptidases in the remaining group are less well characterized.

Table 1: Clans and families of metallopeptidases in the MEROPS system that contain aminopeptidases

Clan	Family
MA(E)	M1 M61
MF	M17
MG	M24
MH	M18, M28, M42
MX	M29 M55

All aminopeptidases in family M1 have a HEXXH motif that is part of the zinc binding site. Although the mechanism of catalysis of metallo aminopeptidases is still poorly understood, it has been demonstrated that the metal cation forms coordinated bonds with the histidine residues from the HEXXH motif as well as with the oxygen molecule from the carbonyl goup of the peptide link targeted to the substrate (56, 92, 105). The mechanism of action of the metallo aminopeptidases is not specifically described in literature. However, the mechanism of action of the M1 family of proteases has been determined (Fig. 1). The aminopeptidases within the other families also require a metal ion for their activity, however their mechanism of action is not known, nor do they have the HEXXH motif.

Metallo protease inhibitors function through a direct interaction with the active site metal ion. Commonly used metallo protease inhibitors are therefore metal chelators such as EDTA, EGTA and 1,10 *o*-phenantroline. Inertness to serine protease inhibitors and sensitivity to these metal chelators is a first indicator for a metallo aminopeptidase, but as many proteases, including serine proteases, require calcium for activity and as EDTA is an excellent calcium chelating agent additional inhibitors are needed to unambiguously prove metallo protease activity.

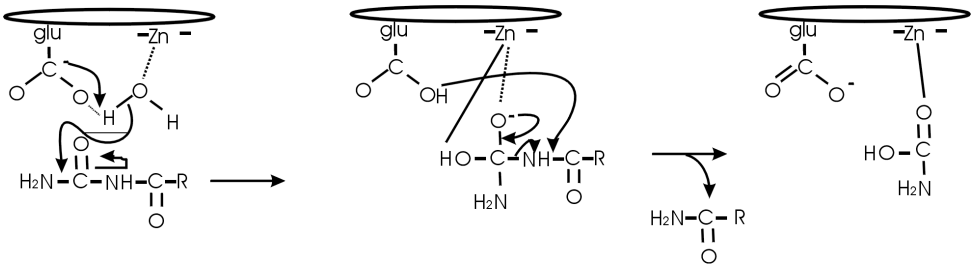

Fig. 1: The catalytic mechanism of a metallo protease of the M1 family. Water is bound to zinc and the carboxylate group of glutamic acid. After the enzyme-peptide complex is formed, the water molecule bound to the zinc is displaced by the carbonyl group of the peptide. The water molecule then attacks the carbonyl of the scissile bond of the substrate, facilitated by the glutamic acid in this motif that acts as the catalytic base. Adapted from: http://cgat.ukm.my/protease

Little is known about *Aspergillus* metallo aminopeptidases. From *A. oryzae* culture broth, 3 metallo aminopeptidases have been purified and characterized, but the corresponding genes have not been cloned (82, 84-86). A fourth metallo aminopeptidase of *A. oryzae* is cloned and characterized (15). This aminopeptidase should be added to the M28 family. From other *Aspergilli s*everal extracellular metallo aminopeptidase activities have been purified, however until now the corresponding genes have not been reported to be cloned.

2.2 Serine aminopeptidases

Serine aminopeptidases have no metal co-factor associated with their catalytic site. Catalysis requires a highly reactive serine residue. Serine aminopeptidases are less frequently found amongst aminopeptidases. Like metallo-aminopeptidases, serine aminopeptidases may also be divided into different groups, based on the organization of their catalytic site. The serine proteases are organized into the clans: SB, SC, SE, SF, SH, SK, SM, SN and SX. Families 9 and 33 are the only two families in clan SC that harbor

aminopeptidases. Furthermore family 51 of clan SN also harbors aminopeptidases.

The active site of a serine aminopeptidase consists of a serine (S), a histidine (H) and an aspartic acid (D) residue, that together form the catalytic triad (91). The catalytic mechanism of a serine protease is depicted in Fig. 2 see (64, 98) for reviews on the structure and mechanisms of serine proteases.

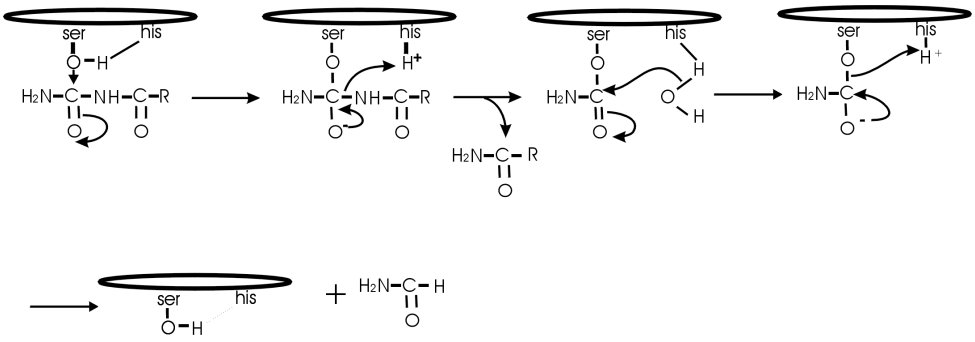

Fig. 2: Catalytic mechanism of serine proteases. Residues in the catalytic triad polarize the OH group to allow the initial nucleophilic attack on the carbonyl atom. A covalent bond is formed between the substrate carbonyl atom and the reactive serine atom. A hydrogen atom is transferred to the histidine in the active site. The intermediate breaks down to the acyl enzyme and liberates the leaving group. The leaving amino group receives a proton from the His residue of the catalytic tetrad. The acyl enzyme is than further hydrolyzed with water as the nucleophile to obtain the enzyme and the carboxylic acid product. Adapted from Http://cgat.ukm.my/protease.

Common serine protease inhibitors that act by modifying the serine active site residue are phenylmethylsulphonyl fluoride (PMSF), (44) and diisopropylfluorophosphate (DIFP). Serine protease inhibitors that act by modifying other active site residues are N-α-*p*-tosyl-lysinechloromethylketone (TLCK) (41) and L-1-tosylamide-2-phenylethylchloromethyl-ketone (TPCK) (95). In addition, several serine protease specific inhibitors of microbial origin like leupepstatin inhibit most serine proteases. Some serine aminopeptidases are

also inhibited by *p*-chloromercuribenzoate (PCMB) indicative for the involvement of a cystein residue near the active site.

To date leucine aminopeptidase IV of *A. oryzae* (82), aminopeptidase of *Penicillium camemberti* (39), prolyl aminopeptidase A (chapter 5) and aminopeptidase C (chapter 4) of *Aspergillus niger*, are the only fungal serine aminopeptidases reported. From the *A. oryzae* and *P. camemberti* aminopeptidase only the purification and characterization of the proteins have been reported while the structure of the encoding genes has not been resolved.

2.3 Cysteine proteases:

Cysteine aminopeptidases are named as such because catalysis is mediated through a highly reactive cysteine residue. The cysteine proteases are found in the clans: CA, CD, CF, CH, CJ, CK and CX. Cysteine aminopeptidases however, are restricted to family one of clan CA. The catalytic mechanism of a cysteine protease is depicted in Fig. 3; see (41, 91, 92) for reviews.

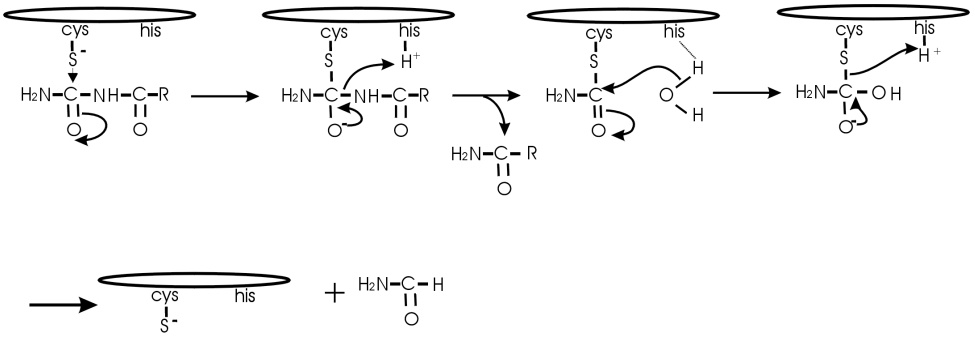

Fig. 3: Mechanism of action of cysteine proteases. Cysteine proteases contain a catalytically active cysteine (C) sulfhydryl group and a histidine (H) within the active site of the enzyme. The ion pair between C and H is the reactive nucleophile. An acyl enzyme is formed with the thiol ester of C and hydrolyzed with water as the nucleophile to obtain the enzyme and the carboxylic acid product. Adapted from http://cgat.ukm.my/protease.

Cysteine aminopeptidases are sensitive to thiol-blocking reagents like iodo- and bromoacetates and *p*-chloromercuribenzoate (73).

So far only bleomycin hydrolase (BLHI) of *S. cerevisiae* has been identified as a fungal cysteine aminopeptidase. BLH1 is a 220 kDa homotetrameric aminopeptidase with broad specificity (33).

3 Localization of aminopeptidases

An obvious role of aminopeptidases in fungi, which can utilize proteins as a nutrient source, is the complete hydrolysis of proteins into amino acids. This would involve the degradation of proteins into oligopeptides outside the cells by extracellular broad specificity proteases and further extracellular or intracellular degradation by oligopeptidases, di- tri-peptidases and finally into amino acids by aminopeptidases.

Intracellular proteolysis in addition has important roles in controlling the levels of specific proteins, the elimination of damaged or mislocalized proteins and the maintenance of amino acid pools (27, 47, 50). The major sites for proteolytic degradation are the cytoplasm for short lived proteins and the vacuole for long lived proteins (43, 70). In addition, periplasmic proteases are involved in protein degradation, while many of the membrane-bound proteases have a role in protein maturation (100).

3.1 Cytoplasmic aminopeptidases

The *in vivo* half-life of a cytoplasmic protein is related to the N-terminal amino acid, this phenomenon is described as the N-end rule (5, 109). For that reason in yeast the amino acids F,L,W,Y,R,K,I,N,Q,D and E are not often present at the N-termini of cytoplasmic proteins because they are destabilizing the protein (5, 48, 97). In the cytoplasm the proteasome is responsible for the rapid degradation of abnormal and intrinsically unstable proteins into oligopeptides (12, 26, 69, 70). The resulting oligopeptides are then broken down to amino acids by a

succession of oligopeptidases and exoproteases, both carboxy- and aminopeptidases. Aberrant or misfolded proteins destined for the secretory pathway that do not pass the quality control in the ER are subjected to retrograde transport, and then also degraded via the proteasome (110).

In the yeast cell several cytoplasmic aminopeptidases can be found, such as: APEII, AAPI, MAPI, MAPII, BLH, LTA_4 (23, 25, 40, 57, 66, 72) (Table 2). There is evidence that APEII is also partially located in the periplasmic space (36). In *Aspergillus niger* strains most aminopeptidases characterized so far (ApsA, ApsB, ApsC and PapA (8-11)) are in the cytoplasm (Table 4).

3.2 Vacuolar aminopeptidases

Vacuoles in fungi are considered to have an important function in degradation of long lived proteins (43, 70) and as a storage pool of basic amino acids (111). Several proteases in the vacuole of *S. cerevisiae* have been characterized (100). These proteases reach the vacuole by two different pathways. The majority uses the classical secretory pathway. The aminopeptidase API, however, uses the cytoplasm to vacuole targeting (Cvt) pathway. When the secretory pathway is used (Fig. 4), the proteins are synthesized in the cytoplasm, and sequestered into the lumen of the ER, where they are glycosylated and where the signal peptide is removed. The proteins transit then to the Golgi complex where further glycosylation takes place. At least yeast aminopeptidases require a sorting signal to mediate their vacuolar delivery from the Golgi apparatus. In absence of such information, these proteins are delivered to the cell surface. Upon arrival in the vacuole the propeptide is removed (20, 49, 62, 77). Aminopeptidase Y of *S. cerevisiae* is transported to the vacuole through this pathway (87, 115).

If the Cvt pathway is used, the protein does not pass through the early stages of the secretory pathway. The Cvt pathway delivers directly from the cytoplasm to the vacuole. The best described fungal aminopeptidase that uses this pathway is aminopeptidase I (API) of S. *cerevisiae.* ProAPI forms a complex in

the cytosol; a membrane is formed around the complex, separating the complex from the cytosol.

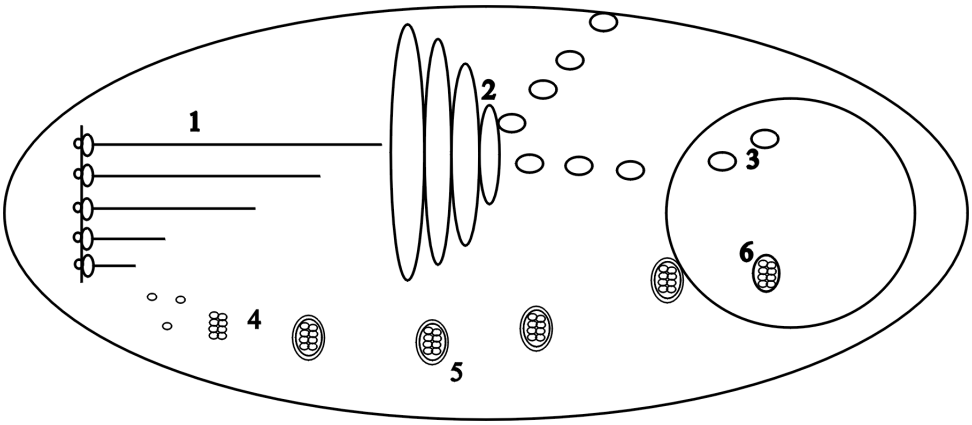

Fig. 4: Sorting of proteins to the vacuole by the secretory route (1,2,3) and the Cvt pathway (4,5,6). A: Proteins are synthesized in the cytoplasm (1), travel via the ER and Golgi apparatus (2) to the vacuole (3). B: Proteins are synthesized in the cytoplasm, form a complex (4), a membrane is formed around the complex (5) and the complex fuses to the vacuolar membrane (6).

Vacuolar delivery is achieved by fusion of the outer membrane of the vesicle with the vacuolar membrane (Fig. 4). Finally the resulting vesicles are broken down by vacuolar hydrolases to release proAPI into the vacuole lumen. The precursor form of the protein is then processed into the mature form (4, 61).

3.3 Extracellular aminopeptidases

There are two possible ways for the degradation of extracellular proteins. First the complete extracellular degradation of proteins by the subsequent action of endoproteases, oligopeptidases and aminopeptidases into amino acids, which can then be transported into the cell by amino acid transporters. An alternative way for the supply of amino acids from external sources is the degradation of proteins by endoproteases into oligopeptides, which are then transported into the cell and further degraded into di- and tripeptides and finally in amino acids by

aminopeptidases in the cytoplasm. In the *Aspergillus* family several aminopeptidase activities have been detected extracellularly (Table 4). In *S. cerevisiae* however, no extracellular aminopeptidase activity has been reported except for an aminopeptidase II activity that is located in the periplasmic space (36).

4 Regulation of aminopeptidases

Most fungal aminopeptidases are intracellular (Table 4), involved in house keeping functions and constitutively expressed. Regulation of aminopeptidases can take place at the transcriptional or post-transcriptional level, for example by on site activation of zymogens, which prevents proteolysis before arriving at the correct place. From the few extracellular aminopeptidases reported it is not known how they are regulated. There are two yeast aminopeptidases from which some features of the regulation have been described, API and APY of *S. cerevisiae*, see sections below.

4.1 Regulation at the transcriptional level

In fungi a number of important pathways are controlled by so called wide domain regulatory control. These include carbon catabolite repression, nitrogen repression and pH regulation. The wide domain regulations adapt the cell to use the easiest metabolisable C- and N-sources first. The pH regulatory system enables the cell to produce extracellular enzymes optimally adapted to the external pH of the direct environment. In the yeast *S. cerevisiae* and in the fungi *A. nidulans* and *N. crassa* and to a lesser extent in *A. niger*, wide domain regulation has been studied.

Carbon catabolite repression.

Carbon catabolite repression (CCR) results in the repression of the synthesis of a range of enzymes required for the utilization of less favored carbon

sources when more easily utilized carbon sources are present in the medium. In *A. nidulans* CCR is reviewed by Arst and Bailey (2) and Kelly (60). CCR is mediated by CreA, a negatively acting regulatory protein (2, 6). The *creA* genes of *A. nidulans* (30, 31) and *A. niger* (32) have been cloned and encode a two zinc-fingers protein of the C_2H_2 class. The consensus binding site of CreA is SYGGRG, Y being T or C and R being G or A. Yeast aminopeptidase I (*API*) is subjected to CCR (16), if glucose is present in the medium *API* mRNA is hardly detectable (37). Aminopeptidase Y (*APY*) mRNA levels increase when the cells are deprived of glucose, however the enzyme activity drops 3-fold under these conditions suggesting that there is an additional mechanism that regulates APY enzyme activity (see section below).

Nitrogen repression and pH regulation

In analogy to carbon catabolite repression a number of genes is repressed when ammonium is present in the growth medium. All enzymes and permeases in *A. nidulans* whose synthesis is subject to nitrogen metabolite repression are under the control of the positively acting regulatory gene *areA* (3, 53). The *areA* genes of *A. nidulans* (21, 65), and *A. niger* (74) have been cloned, the ortholog of *areA* in *N. crassa, nit2* has been described by Davis and Hynes (29) and Fu *et al* (38). AreA and Nit2 are both members of a multi gene family of transcription factors characterized by their zinc finger DNA binding domains and affinity for a GATA binding site (22). API of yeast is not only reported to be regulated by carbon catabolite repression but also by the nitrogen source (16, 17, 37).

4.2 Post-transcriptional level

To prevent unwanted proteolytic activities, proteases are often synthesized as zymogens. Activation of the zymogen occurs at places where the enzyme must be active. In the yeast *S. cerevisiae* the activation of the vacuolar aminopeptidases is well described. The maturation is dependent on PrA and PrB,

two vacuolar proteases. PrB is synthesized as pre-super-pro-PrB (49, 78, 81) (Fig. 5). In the Endoplasmatic Reticulum (ER) the pre-sequence is removed by signalpeptidase and autocatalytically, a part of the super-pro-PrB molecule is transformed into pro-PrB. Both super-pro-PrB and pro-PrB enter the vacuole via the Golgi apparatus. In the vacuole PrA is required for the processing of pro-PrB into active PrB. PrA is synthesized as pre-pro-PrA and in the ER the pre sequence is removed by signal peptidase. After entering the vacuole the pro-PrA is transformed into active PrA by PrB.

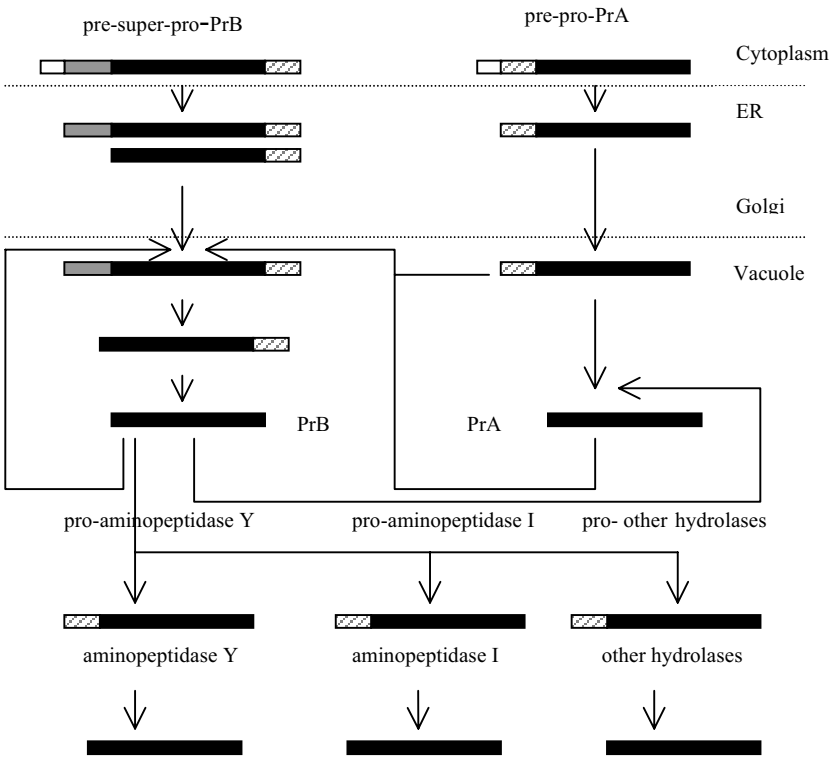

Fig. 5: Activation cascade in *S. cerevisiae* of vacuolar aminopeptidases.
pre-sequence, pro-sequence, super-sequence, mature protein.

In 1993 two genes, *pepE* and *pepC,* which are homologous to yeast *yscA* (encoding for PrA) and *yscB* (encoding for PrB) respectively, have been cloned

from *A. niger* (35, 55)) suggesting evolutionary conservation of the so called activation cascade of vacuolar proteases.

5 An inventory of fungal aminopeptidases

5.1 A list of fungal aminopeptidases

5.1.1 Yeast aminopeptidases

S. cerevisiae is a well studied organism in the scientific world and its genome is fully sequenced. I have listed the yeast aminopeptidases that are described in the literature in Table 2. I also did an extensive cross-species search in the yeast genome to find orthologous genes, putatively encoding additional aminopeptidases. These sequences are described in Table 3. Interestingly we did not find a putative prolyl aminopeptidase encoding gene in yeast so far, however, we did find putative prolidase orthologs in the genomic sequence.

Table 2: . *cerevisiae* aminopeptidases with their characteristics. Location: IC: intracellular, V: vacuolar, U: unknown

Family	Protein	Gene	Mw	pH_{opt}	Location	Reference
M01	LTA4	*LTA4*	72	7.3	IC	(66, 67, 93)
M01	LAPI	*YSCII*	100-140	7.0	IC	(36,40, 107)
M01	AAPI	*AAPI*	59		IC	(23)
M18	LAP IV	*YSCI*	570	7.5	V	(16,17,24, 28, 79, 107)
	Met-AP1	*MAP1*	42 *		IC	(25, 72)
	Met-AP2	*MAP2*	48 *		IC	(72)
M	AP-Co		100	8.5	IC	(1, 45)
M	APY	*APY*	70-75		V	(46,87, 115)
M	APCO-II	*YSCCo-II*	290	7	U	(45)
C	Ycp1	*BLH1*, *LAP3*	220	7.5	IC	(33,57,75 114)
M	yscXVI		52	7-8	U	(106)

* calculated from AA sequence

Table 3: *S. cerevisiae* putative aminopeptidases.

Accesion no of ORF	Catalytic group	Chromosome no:	Most homologous to:	Reference
NP_012129	M1	9	ApsA *Aspergillus niger*	(11)
NP_116661	M24	6	PepP *Aspergillus nidulans*	(54)
NP_011001	M24	5	AMPP *Escherichia coli*	(112)
NP_010703	M28	4	MprI *Alteromonas sp.*	(80)
NP_011981	M18	8	HUMDAP *Homo sapiens*	(113)

5.1.2 *Aspergillus* aminopeptidases

In the food industry, products of *Aspergilli* are often used to enhance flavors and to improve structure of certain food products. In the literature several aminopeptidases from *Aspergilli* have been described. For some aminopeptidases this is limited to the enzyme characteristics, while for others the cloning of the gene, has been described. All characteristics published are summarized in Table 4.

6. Outline of the thesis

There is only a limited of knowledge about aminopeptidases in *A. niger*. This thesis describes the study on the genetics and biochemistry of aminopeptidases in *A. niger*. The work was initiated in the protease group of the section Molecular Genetics of Industrial Micro-organisms, that studies the improvement of protein production in *A. niger*. The work was continued in the protease group of the section Fungal Genomics of Microbiology. Not only elimination of proteases and protease activities is important but also the characterization of the full protease spectrum is. Individual proteases have a considerable industrial importance. As stated in this chapter, proteases are important in the hydrolysis and modification of proteins and for the production of free amino acids. Aminopeptidases are especially important in the food industry for improvement of flavor and taste.

Table 4: Fungal aminopeptidases A: Metallo aminopeptidases, B: Serine aminopeptidases and C: aminopeptidases from which the catalytic mechanism is unknown. Location: IC: intracellular, EC: extracellular, U: unknown.

Organism	Protein	Gene	Mw	pH opt	Pi	Location	Reference
A: Metallo aminopeptidases							
A. niger	ApsA	*apsA*	95	7.5	5.5	EC	(11)
A. niger	ApsB	*apsB*	99	7.5	5.5	EC	(8)
A. oryzae	LapI		27	8.0		EC	(83, 84)
A. oryzae	LapII		61	8.0		EC	(83, 85)
A. oryzae	LapIII		56	8.0		EC	(83, 86)
A. oryzae	apII		57	9.5		EC	(14, 15)
A. oryzae			60	8.0		EC	(71)
A. parasiticus			32	8.0		EC	(71)
A. flavus				8.6		EC	
A. japonica			57	8.0		EC	(101-103)
A. oryzae			35			U	(58)
B: Serine aminopeptidases							
A. niger	ApsC	*apsC*	72	5.5	5.6	IC	(9)
A. niger	PapA	*papA*	50	7.5	5.1	IC	(10)
A. oryzae	LapIV		130	7.0		EC	(82)
A. oryzae	Lap1		140	8.0	4.5	EC	(68)
A. oryzae	Lap2		40	8.0	4.5	EC	(68)
A. flavus						EC	(109)
A. sojae	LapI		26	8.5		U	(83)
A. sojae	Lap II		62	8.0		U	(83)
C Unknown catalytic mechanism							
A. sojae	Lap					U	(96)
A. oryzae		leucine aminopeptidase				U	(59)
A. sojae		leucine aminopeptidase				U	(108)
A. nidulans		aminopeptidase	50		8.5	U	(99)

Although work has been done on prokaryotic aminopeptidases, especially aminopeptidases of *lactobacilli* have been studied, little is known on the aminopeptidases of fungi, except for the yeast aminopeptidases, which are extensively studied.

The aim of this thesis is to study the aminopeptidase spectrum of *A. niger*, to obtain more information on the architecture and regulation of the genes and to characterize the encoded enzymes. In chapter 2 Aminopeptidase A is described, which is a broad specificity aminopeptidase with highest specificity to synthetic substrates, which start with a lysine or arginine residue. This aminopeptidase is located in the cytoplasm were it probably functions in the last step of degradation of proteins into amino acids. In chapter 3 the ortholog of ApsA, ApsB is described. Both enzymes are metallo aminopeptidases with highest substrate specificity for lysine and arginine. In an evolutionary context, aminopeptidase A and B appear to be the result of an ancient duplication. We found that, although both enzymes have similar substrate specificities towards synthetic substrates, the specificity of the purified enzymes towards various peptides clearly differs, explaining the co-existence of both enzymes. In chapter 4 a new aminopeptidase with industrial importance has been cloned via reversed genetics. This aminopeptidase liberates aromatic amino acids (especially phenylalanine) from synthetic substrates and small peptides. Hydrolysis of proline adjacent bonds requires specific enzymes, therefore a special set of proline specific enzymes has evolved. Chapter 5 describes a proline specific aminopeptidase. Proline aminopeptidase can hydrolyze only proline and hydroxyproline from peptide substrates. Finally in chapter 6 a general discussion is given of the results presented in this thesis.

References:

1. **Achstetter, T., C. Ehmann, and D. H. Wolf.** 1982. Aminopeptidase Co, A new yeast peptidase. Biochem Biophys Res Commun **109:**341-347.
2. **Arst, H. N., and C. Bailey.** 1977. The regulation of carbon metabolism in *Aspergillus nidulans*, p. 131-146. *In* J. E. Smith (ed.), Genetics and Physiology of *Aspergillus*. Academic press, London.
3. **Arst, H. N., and D. J. Cove.** 1973. Nitrogen metabolite repression in *Aspergillus nidulans*. Mol Gen Genet **126:**111-141.
4. **Baba, M., M. Osumi, S. V. Scott, D. J. Klionsky, and Y. Ohsumi.** 1997. Two Distinct Pathways for Targeting Proteins from the Cytoplasm to the Vacuole/Lysosome. J Cell Biol **139**:1687-1695.
5. **Bachmair, A., D. Finley, and A. Varshavsky.** 1986. In vivo half-life of a protein is a function of its amino-terminal residue. Science **234:**179-186.
6. **Bailey, C., and H. N. Arst.** 1975. Carbon Catabolite Repression in *Aspergillus nidulans*. Eur J Biochem **51:**573-577.
7. **Barret, A. J., N. D. Rawlings, and J. F. Woessner.** 1998. Handbook of proteolytic enzymes. Academic Press, San Diego.
8. **Basten, D. E. J. W., S. Boeren, and P. J. Schaap.** 2004. Aminopeptidases A and B from *Aspergillus niger* have different substrate specificities. This thesis.
9. **Basten, D. E. J. W., P. J. T. Dekker, and P. J. Schaap.** 2003. Aminopeptidase C of *Aspergillus niger* is a Novel Phenylalanine Aminopeptidase. Appl Environ Microbiol **69:**1246-1250.
10. **Basten, D. E. J. W., A. P. H. A. Moers, and P. J. Schaap.** 2004. Characterization of an *Aspergillus niger* Prolyl Aminopeptidase. This thesis.
11. **Basten, D. E. J. W., J. Visser, and P. J. Schaap.** 2001. Lysine aminopeptidase of *Aspergillus niger*. Microbiology **147:**2045-2050.

12. **Baumeister, W., J. Waltz, F. Zühl, and E. Seemüller.** 1998. The proteasome: paradigm of a self-compartmentalizing protease. Cell **92:**367-380.

13. **Bergmann, M., and J. Fruton.** 1941. The specificity of proteinases. Adv Enzymol **1:**63-98.

14. **Blinkovsky, A., E. Golightly, T. Byun, and L. Kofod.** 1998. Polypeptides having aminopeptidase activity and nucleic acids encoding same. Patent application PCT WO9851804.

15. **Blinkovsky, A. M., T. Byun, K. Brown, E. J. Golightly, and A. V. Klotz.** 2000. A non-specific aminopeptidase from *Aspergillus*. Biochim Biophys Acta **1480:**171-181.

16. **Bordallo, J., R. Cueva, and P. Suarez-Rendueles.** 1995. Transcriptional regulation of the yeast vacuolar aminopeptidase yscI encoding gene (APE1) by carbon sources. FEBS Lett **364:**13-16.

17. **Bordallo, J., and P. Suarez-Rendueles.** 1995. Identification of regulatory proteins that might be involved in carbon catabolite repression of the aminopeptidase I gene of the yeast *Saccharomyces cerevisiae*. FEBS Lett **376:**120-124.

18. **Brunt, J. v.** 1986. Filamentous Fungi Join the Production Ranks. Biotechnol **4:**385-386.

19. **Brunt, J. v.** 1986. Fungi: The Perfect Hosts? Biotechnol **4:**1057-1062.

20. **Bryant, N. J., and T. H. Stevens.** 1998. Vacuole Biogenesis in *Saccharomyces cerevisiae*: Protein Transport Pathways to the Yeast Vacuole. Microbiol Mol Biol Rev **62:**230-247.

21. **Caddick, M. X., H. N. Arst, L. H. Taylor, R. I. Johnson, and A. G. Brownlee.** 1986. Cloning of the regulatory gene *areA* mediating nitrogen metabolite repression in *Aspergillus nidulans*. EMBO J **5:**1087-1090.

22. **Caddick, M. X., D. Peters, and A. Platt.** 1994. Nitrogen Regulation in Fungi. Antonie van Leeuwenhoek **65:**169-177.

23. **Caprioglio, D. R., C. Padilla, and M. Werner-Washburne.** 1993. Isolation and characterization of *AAP1*; a gene encoding an alanine / arginine aminopeptidase in yeast. J Biol Chem **268:**14310-14315.

24. **Chang, Y.-H., and J. A. Smith.** 1989. Molecular Cloning and Sequencing of Genomic DNA Encoding Aminopeptidase I from *Saccharomyces cerevisiae*. J Biol Chem **264:**6979-6983.

25. **Chang, Y.-H., U. Teichert, and J. A. Smith.** 1992. Molecular Cloning, Sequencing, Deletion, and Overexpression of a Methionine Aminopeptidase Gene from *Saccharomyces cerevisiae*. J Biol Chem **267:**8007-8011.

26. **Ciechanover, A.** 1998. The ubiquitin-proteasome pathway: on protein death and cell life. EMBO J **17:**7151-7161.

27. **Ciechanover, A., and A. L. Schwartz.** 1994. The ubiquitin-mediated proteolytic pathway: mechanisms of recognition of the proteolytic substrate and involvement in the degradation of native cellular proteins. FASEB J **8:**182-191.

28. **Cueva, R., N. Garcia-Alvarez, and P. Suarez-Rendueles.** 1989. Yeast vacuolar aminopeptidase yscI. Isolation and regulation of the APE1 (LAP4) structural gene. FEBS Lett **259:**125-129.

29. **Davis, M. A., and H. J. Hynes.** 1987. Complementation of *areA* regulatory mutants of *A. nidulans* by the heterologous regulatory gene *nit2* of *N. crassa*. Proc Natl Acad Sci USA **84:**3753-3757.

30. **Dowzer, C. E. A., and J. M. Kelly.** 1991. Analysis of the *creA* Gene, a Regulator of Carbon Catabolite Repression in *Aspergillus nidulans*. Mol Cell Biol **11:**5701-5709.

31. **Dowzer, C. E. A., and J. M. Kelly.** 1989. Cloning of *creA* from *Aspergillus nidulans*: a gene involved in carbon catabolite repression. Curr Genet **15:**457-459.

32. **Drysdale, M. R., S. E. Kolze, and J. M. Kelly.** 1993. The *Aspergillus niger* carbon catabolite repressor encoding gene, *creA*. Gene **130:**241-245.

33. **Enenkel, C., and D. H. Wolf.** 1993. *BLH1* Codes for a Yeast Thiol Aminopeptidase, the Equivalent of Mammalian Bleomycin Hydrolase. J Biol Chem **268:**7036-7043.

34. **Fischer, E.** 1902. Ueber die Hydrolyse der proteinstoffe. Chemiker-Zeitung**:**939-940.

35. **Frederick, G. D., P. Rombouts, and F. P. Buxton.** 1993. Cloning and characterisation of *pepC*, a gene encoding a serine protease from *Aspergillus niger*. Gene **125:**57-64.

36. **Frey, J., and K.-H. Röhm.** 1979. External and Internal Forms of Yeast Aminopeptidase II. Eur J Biochem **97:**169-173.

37. **Frey, J., and K.-H. Röhm.** 1978. Subcellular localization and levels of aminopeptidases and dipeptidase in *Saccharomyces cerevisiae*. Biochim Biophys Acta **527:**31-41.

38. **Fu, Y.-H., H.-J. Lee, J. L. Young, G. Jarai, and G.A.Marzluf.** 1990. Nitrogen and Sulfur Regulatory Circuits of Neurospora. Dev Biol **125:**319-335.

39. **Fuke, Y., and H. Matsuoka.** 1993. The Purification and Characterization of Prolyl Aminopeptidase from *Penicillium camemberti*. J Dairy Sci **76:**2478-2484.

40. **Garcia-Alvarez, N., R. Cueva, and P. Suarez-Rendueles.** 1991. Molecular cloning of soluble aminopeptidases from *Saccharomyces cerevisiae;* Sequence analysis of aminopeptidase yscII, a putative zinc-metallopeptidase. Eur J Biochem **202:**993-1002.

41. **Gilles, A. M., and B. Keil.** 1984. Evidence for an active-center cystein in the SH-proteinase alpha-clostripain through use of *N*-tosyl-L-lysine chloromethyl ketone. FEBS **173:**58-62.

42. **Godfrey, A.** 1998. Production of industrial enzymes and some applications in fermented foods, p. 622-657. *In* B. J. B. Wood (ed.), Microbiology of Fermented Foods, 2nd ed. Blackie Academic & Professional, Glasgow.

43. **Goldberg, A. L., T. N. Akopian, A. F. Kisselev, and D. H. Lee.** 1997. Protein degradation by the proteasome and dissection of its in vivo importance with synthetic inhibitors. Mol Biol Rep **24:**69-75.

44. **Hartley, B. S.** 1960. Proteolytic enzymes. Annu Rev Biochem **29:**45-72.

45. **Herrera-Camacho, I., R. Morales-Monterrosas, and R. Quiróz-alvarez.** 2000. Aminopeptidase yscCo-II: a new cobalt-dependent aminopeptidase from yeast - purification and biochemical characterization. Yeast **16:**219-229.

46. **Herrera-Camacho, I., and P. Suárez-Rendueles.** 1996. Regulation of the yeast vacuolar aminopeptidase Y gene (*APY1*) expression. FEMS Microbiol Lett **139:**127-132.

47. **Hershko, A., and A. Ciechanover.** 1992. The ubiquitin system for protein degradation. Annu Rev Biochem **61:**761-807.

48. **Hirel, P. H., M. J. Schmitter, P. Dessen, G. Fayat, and S. Blanquet.** 1989. Extent of N-terminal methionine excision from Escherichia coli proteins is governed by the side-chain length of the penultimate amino acid. Proc Natl Acad Sci USA **86:**8247-8251.

49. **Hirsch, H. H., H. H. Schiffer, and D. H. Wolf.** 1992. Biogenesis of the yeast vacuole (lysosome). Eur J Biochem **207:**867-876.

50. **Hochstrasser, M.** 1992. Ubiquitin and intracellular protein degradation. Curr Opin Cell Biol **4:**1024-1031.

51. **Hofmeister, F.** 1902. Uber Bau und Gruppierung der Eiweisskörper. Ergeb Physiol **1:**759-802.

52. **Hombergh van den, J. P. T.** 1996. An analysis of the proteolytic system in *Aspergillus* in order to improve protein production. Thesis, Wageningen Agricultural University, Wageningen.

53. **Hynes, M. J.** 1975. Studies on the role of the *areA* gene in the regulation of nitrogen catabolism in *Aspergillus nidulans*. Aust J Biol Sci **28:**301-313.

54. **Jalving, R., P. Bron, H. C. M. Kester, J. Visser, and P. J. Schaap.** 2002. Cloning of a prolidase gene from *Aspergillus nidulans* and characterization of its product. Mol Gen Genom **276:**218-222.

55. **Jarai, G., H. van den Hombergh, and F. P. Buxton.** 1994. Cloning and characterization of the *pepE* gene of *Aspergillus niger* encoding a new aspartic protease and regulation of *pepE* and *pepC*. Gene **145:**171-178.

56. **Jongeneel, C. V., J. Bouvier, and A. Bairoch.** 1989. A unique signature identifies a family of zinc-dependent metallo peptidases. FEBS lett **242:**211-214.

57. **Kambouris, N. G., D. J. Burke, and C. E. Creutz.** 1992. Cloning and Characterization of a Cystein Proteinase from *Saccharomyces cerevisiae*. J Biol Chem **267:**21570-21576.

58. **Kauppinen, M. S., J. Qi Si, C. Dambmann, T. Halkier, P. R. Østergaard, S. A. Patkar, and K. Hansen.** 2001. Enzymes with aminopeptidase activity. Patent US6,200,792 B1.

59. **Kauppinen, S., J. Qi Si, T. Spendler, C. Dambmann, T. Halkier, and P. R. Østergaard.** 1996. An enzyme with aminopeptidase activity. Patent application PCT WO962854.

60. **Kelly, J. M.** 1994. Carbon Catabolite Repression. Prog Ind Microbiol **29:**355-367.

61. **Kim, J., S. V. Scott, M. N. Oda, and D. J. Klionsky.** 1997. Transport of a Large Oligomeric Protein by the Cytoplasm to Vacuole Protein Targeting Pathway. J Cell Biol **137:**609-618.

62. **Klionsky, D. J., and S. D. Emr.** 1990. A new class of lysosomal/vacuolar protein sorting signals. J Biol Chem **265:**5349-5352.

63. **Kosikowski, F. V.** 1979. Cheese and Fermented Milk, 2nd ed. Edwards Brothers, MI.

64. **Kraut, J.** 1977. Serine Proteases: Structure and Mechanism of Catalysis. Ann Rev Biochem **46:**331-358.

65. **Kudla, B., M. X. Caddick, T. Langdon, N. M. Martinez-Rossi, C. F. Bennett, S. Sibley, R. W. Davies, and H. N. Arst.** 1990. The regulatory gene *areA* mediating nitrogen metabolite repression in *Aspergillus nidulans.* Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger. EMBO J **9:**1355-1364.

66. **Kull, F., E. Ohlson, and J. Z. Haeggström.** 1999. Cloning and Characterization of a Bifunctional Leukotriene A4 Hydrolase from *Saccharomyces cerevisiae*. J Biol Chem **274:**34683-34690.

67. **Kull, F., E. Ohlson, B. Lind, and J. Z. Haeggström.** 2001. *Saccharomyces cerevisiae* Leukotriene A4 Hydrolase: Formation of Leukotriene B4 and Identification of Catalytic Residues. Biochemistry **40:**12695-12703.

68. **Labbe, J.-P., and P. Rebeyrotte.** 1974. Isolation of fractions having aminopeptidase activity (EC 3.4.1.1) (arylamidase) from culture medium of *Aspergillus oryzae*. Biochimie **56:**839-844.

69. **Lee, D. H., and A. L. Goldberg.** 1998. Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol **8:**397-403.

70. **Lee, D. H., and A. L. Goldberg.** 1996. Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in *Saccharomyces cerevisiae*. J Biol Chem **271:**27280-27284.

71. **Lehmann, K. v., and H. Uhlig.** 1969. Aminopeptidases aus *Aspergilli,* I. Hoppe-Seyler's Z. Physiol. Chem. **350:**99-104.

72. **Li, X., and Y.-H. Chang.** 1995. Amino-terminal protein processing in *Saccharomyces cerevisiae* is an essential function that requires two distinct methionine aminopeptidases. Proc Natl Acad Sci USA **92:**12357-12361.

73. **Liu, W. H., G. E. Means, and R. E. Feeney.** 1971. The inhibitory properties of avian ovoinhibitors aganist proteolytic enzymes. Biochim Biophys Acta **229:**176-185.

74. **MacCabe, A. P., S. Vanhanen, M. D. Sollewign Gelpke, P. J. van de Vondervoort, H. N. Arst, Jr., and J. Visser.** 1998. Identification, cloning and sequence of the *Aspergillus niger areA* wide domain regulatory gene controlling nitrogen utilisation. Biochim Biophys Acta **1396:**163-168.

75. **Magdolen, U., G. Müller, V. Magdolen, and W. Bandlow.** 1993. A yeast gene (*BLH1*) encodes a polypeptide with high homology to vertebrate bleomycin hydrolase, a family member of thiol proteinases. Biochim Biophys Acta **1171:**299-303.

76. **Maras, M., I. Die van, Roland Contreras, and C. A. M. J. J. Hondel van den.** 1999. Filementous fungi as production organisms for glycoproteins of bio-medical interest. Glycoconj J **16:**99-107.

77. **Matile, P.** 1978. Biochemistry and function of vacuoles. Ann Rev Plan Physiol **29:**193-213.

78. **Mechler, B., H. H. Hirsch, H. Muller, and D. H. Wolf.** 1988. Biogenesis of the yeast lysosome (vacuole): biosynthesis and maturation of proteinase yscB. EMBO J **7:**1705-1710.

79. **Metz, G., and K. H. Röhm.** 1976. Yeast aminopeptidase I. Chemical composition and catalytic properties. Biochim Biophys Acta **429:**933-949.

80. **Miyamoto, K., H. Tsujibo, E. Nukui, H. Itoh, Y. Kaidzu, and Y. Inamori.** 2002. Isolation and characterization of the genes encoding two

metalloproteases (MprI and MprII) from a marine bacterium *Alteromonas* sp. strain O-7. Biosci Biotechnol Biochem **66:**416-421.

81. **Moehle, C. M., C. K. Dixon, and E. W. Jones.** 1989. Processing pathway for protease B of *Saccharomyces cerevisiae*. J Cell Biol **108:**309-325.
82. **Nakadai, T., and S. Nasuno.** 1977. Purification and properties of leucine aminopeptidase IV from *Aspergillus oryzae*. Agric Biol Chem **41:**1657-1666.
83. **Nakadai, T., S. Nasuno, and N. Iguchi.** 1972. The Action of Peptidases from *Aspergillus oryzae* in Digestion of Soybean Proteins. Agric Biol Chem **36:**261-268.
84. **Nakadai, T., S. Nasuno, and N. Iguchi.** 1973. Purification and properties of leucine aminopeptidase I from *Aspergillus oryzae*. Agric Biol Chem **37:**757-766.
85. **Nakadai, T., S. Nasuno, and N. Iguchi.** 1973. Purification and properties of leucine aminopeptidase II from *Aspergillus oryzae*. Agric Biol Chem **37:**767-774.
86. **Nakadai, T., S. Nasuno, and N. Iguchi.** 1973. Purification and properties of leucine aminopeptidase III from *Aspergillus oryzae*. Agric Biol Chem **37:**775-778.
87. **Nishizawa, M., T. Yasuhara, T. Nakai, Y. Fujiki, and A. Ohashi.** 1994. Molecular Cloning of the Aminopeptidase Y Gene of *Saccharomyces cerevisiae*. J Biol Chem **269:**13651-13655.
88. **North, M. J.** 1982. Comparative biochemistry of the proteinases of eucaryotic microorganisms. Microbiol Rev **46:**308-340.
89. **Punt, P. J., N. Biezen van, A. Conesa, A. Albers, J. Mangus, and C. A. M. J. J. Hondel van den.** 2002. Filamentous fungi as cell factories for heterologous protein production. Trends Biotechnol **20:**200-206.

90. **Rao, M. B., A. M. Tanksale, M. S. Ghatge, and V. V. Deshpande.** 1998. Molecular and Biotechnological Aspects of Microbial Proteases. Microbiol Mol Biol Rev **62:**597-635.

91. **Rawlings, N. D., and A. J. Barret.** 1993. Evolutionary families of peptidases. Biochem J **290:**205-218.

92. **Roques, B. P.** 1993. Zinc metallopeptidases: Active Site Structure and Design of Selective and Mixed Inhibitors: New Approaches in the Search for Analgesics and Anti-hypertensives. Biochem Soc Trans **21:**678-685.

93. **Rudberg, P. C., F. Tholander, M. M. G. M. Thunnissen, and J. Z. Haeggström.** 2002. Leukotriene A4 Hydrolase/Aminopeptidase. J Biol Chem **277:**1398-1404.

94. **Schechter, I., and A. Berger.** 1967. On the size of the active site in proteases. Biochem Biophys Res Commun **27:**157-162.

95. **Schoelmann, G., and E. Shaw.** 1963. Direct evidence for the presence of histidine in the active centre of chymotrypsin. Biochem **2:**252-255.

96. **Schuster, E., B. Sprössler, K. Titze, M. Gottschalk, N. Q. Khanh, S. Wolf, and H. Plainer.** 1997. Leucine aminopeptidases produced recombinantly from *Aspergillus soyae*. Patent application 97/04108.

97. **Sherman, F., J. W. Stewart, and S. Tsunasawa.** 1985. Methionine or not methionine at the beginning of a protein. Bioessays **3:**27-31.

98. **Steitz, T. A., and R. G. Shulman.** 1982. Crystallographic and NMR Studies of the Serine Proteases. Ann Rev Biophy Bioeng **11:**419-444.

99. **Stevens, L.** 1985. Regulation of proteinase activity in *Aspergillus nidulans*. Biochem Soc Trans **13:**283-285.

100. **Suarez-Rendueles, P., and D. H. Wolf.** 1988. Proteinase function in yeast: biochemical and genetic approached to a central mechanism of post-translational control in the eukaryotic cell. FEMS Microbiol Rev **54:**17-46.

101. **Sugiura, M., M. Ishikawa, and M. Sasaki.** 1978. Some Properties of Leucine Aminopeptidase from *Aspergillus japonica* as a Metalloenzyme. Chem Pharm Bull **26:**3101-3106.

102. **Sugiura, M., M. Suzuki, M. Ishikawa, and M. Sasaki.** 1976. Pharmaceutical Studies on Aminopeptidase from *Aspergillus japonica* I. Chem Pharm Bull **24:**2286-2293.

103. **Sugiura, M., M. Suzuki, M. Ishikawa, and M. Sasaki.** 1976. Purification and Properties of Leucine Aminopeptidase from *Aspergillus japonica*. II. Chem Pharm Bull **24:**2026-2031.

104. **Taylor, A.** 1993. Aminopeptidases: structure and function. FASEB J **7:**290-298.

105. **Taylor, A.** 1993. Aminopeptidases: towards a mechanism of action. TIBS **18:**167-172.

106. **Tisljar, U., and D. H. Wolf.** 1993. Purification and characterization of the cystinyl bond cleaving yeast aminopeptidase yscXVI. FEBS lett **322:**191-196.

107. **Trumbly, R. J., and G. Bradley.** 1983. Isolation and characterization of aminopeptidase mutants of *Saccharomyces cerevisiae*. J Bacteriol **156:**36-48.

108. **Umitsuki, G., and K. Abe.** 1999. Leucine aminopeptidase gene, recombinant dna, and process for producing leucine aminopeptidase. Patent application EP0967286A2.

109. **Varshavsky, A.** 1992. The N-end rule. Cell **69:**725-735.

110. **Vashist, S., W. Kim, W. J. Belden, E. D. Spear, C. Barlowe, and D. T. W. Ng.** 2001. Distinct retrieval and retention mechanisms are required for the quality control of endoplasmic reticulum protein folding. J Cell Biol **155:**355-367.

111. **Wiemken, A., and M. Durr.** 1974. Characterization of amino acid pools in the vacuolar compartment of *Saccharomyces cerevisiae*. Arch Microbiol **101:**45-57.

112. **Wilce, M. C., C. S. Bond, N. E. Dixon, H. C. Freeman, J. M. Guss, P. E. Lilley, and J. A. Wilce.** 1998. Structure and mechanism of a proline-specific aminopeptidase from *Escherichia coli*. Proc Natl Acad Sci **95:**3472-3477.

113. **Wilk, S., E. wilk, and R. P. Magnusson.** 1998. Purification, characterization, and cloning of a cytosolic aspartyl aminopeptidase. J Biol Chem **273:**15961-15970.

114. **Xu, H. E., and S. A. Johnston.** 1994. Yeast Bleomycin Hydrolase Is a DNA-binding Cysteine Protease. J Biol Chem **269:**21177-21183.

115. **Yasuhara, T., T. Nkai, and A. Ohashi.** 1994. Aminopeptidase Y, a New Aminopeptidase from *Saccharomyces cerevisiae*. J Biol Chem **269:**13644-13650.

Chapter 2

Lysine aminopeptidase of *Aspergillus niger*

Daniëlle E.J.W. Basten, Jaap Visser and Peter J. Schaap

Microbiology 2001 147:2045-2050

Summary

Conserved regions within the M1 family of metallo aminopeptidases have been used to clone a zinc aminopeptidase from the industrially used fungus *Aspergillus niger*. The derived amino acid sequence of ApsA is highly similar to two yeast zinc aminopeptidases, LAP1 and AAPI (53.3 and 50.9% overall similarity, respectively), two members of the M1 family of metallo aminopeptidases. The encoding gene was successfully overexpressed in *A. niger*, and the overexpressed product was purified and characterised. Aminopeptidase A was found to be active towards a number of amino acid *p*-nitroanilide (*p*-NA) substrates *viz* K-*p*NA, R-*p*NA, L-*p*NA, M-*p*NA, A-*p*NA and F-*p*NA. The most preferred N-terminal amino acid is lysine and not leucine, arginine or alanine, the N-terminal amino acids preferred by the yeast homologues. The K_M and K_{cat} for K-*p*NA and L-*p*NA were 0.17 mM and 0.49 μkat mg^{-1} and 0.16 mM and 0.31 μkat mg^{-1} respectively.

The pH optimum of the enzyme is between 7.5 and 8 whereas the enzyme is stable between a pH of 5 and 8. The enzyme is inhibited by the metal chelators EGTA, EDTA and 1,10-phenanthrolin. Bestatin was also able to inhibit the activity.

Introduction

Intracellular proteolytic degradation is important for the elimination of damaged proteins, for the modulation of protein levels and the maintenance of amino acid pools. The major cytoplasmic proteolytic process in eukaryotes is the ubiquitin proteasome pathway leaving the cell with short peptides, ranging from 3 to 22 residues in length (16, 25). These peptides, and peptides resulting from the breakdown of protein substrates that are imported from the external milieu via peptide transporters, are further degraded to finally amino acids by tri-, di-, carboxy- and aminopeptidases.

The majority of aminopeptidases belong to the M1 family of peptidases, they are metalloenzymes (27) which require zinc for enzymatic activity and share the zinc binding motif HEXXH (12, 15).

The evolutionary tree of the M1 family of metallo-aminopeptidases (3) shows that this family can be divided into three main groups. Two of those groups, the aminopeptidase N group and the leukotriene-A4 hydrolase group, have been fully characterised. The remaining aminopeptidases within the M1 family are grouped together, mainly because they share a high sequence similarity. However, peptidases from this group that have been biochemically characterised differ considerably in their characteristics.

Two yeast enzymes from the M1 group have been actively studied (5, 7, 9). From industrially used filamentous fungi, like *Aspergillus spp.* only aminopeptidase activities from *A. oryzae* have been described. *A. oryzae* produces at least seven aminopeptidase activities (18) from which four have been purified. (18-21) and one has been cloned (4).

Our aim is to characterise the pathways involved in protein catabolism in *Aspergillus niger*. So far 7 endopeptidases (see van den Hombergh *et al.*, (11) and references therein) 1 maturase, (13) and 2 carboxypeptidases (6, 10, 24) of *A. niger* have been cloned and characterised. To date no aminopeptidases of *A. niger* have been cloned or characterised.

Here we present the characterisation of the *aps*A gene, encoding an intracellular zinc aminopeptidase of *A. niger* and the characterisation of the purified enzyme.

Materials and methods

Strains, DNA and RNA techniques

Escherichia coli DH5α and LE392 were used as hosts for recombinant plasmids and λ DNA. *Aspergillus niger* strains N402 (*csp*A1) and NW219

(*csp*A1, *pyr*A6, *leu*A1, *nic*A1) are derived from *A. niger* N400 (CBS 120.49). Transformation and DNA digestion with restriction enzymes, ligation and agarose electrophoresis were standard procedures as described (23). Cloned fragments were sequenced using the Thermo Sequenase Fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP from Amersham Pharmacia Biotech (Uppsala, Sweden) and an ALF automated sequencer (Amersham Pharmacia Biotech). Hybridisations were done in SHB (6x SSC, 5x Denhardts' solution (23) 0.5% (w/v) sodium dodecyl sulphate (SDS) and 100 μg ml^{-1} denatured herring sperm DNA) at 65 °C, washing was performed at 65 °C using a final wash step of 1x SSC plus 0.1% (w/v) SDS. 1x SSC contains 0.15 M NaCl and 0.015 M sodium citrate. For northern analysis, strains were either grown for 17 h in minimal medium (22) supplemented with 1% (w/v) glucose and then harvested or grown for 17 h in minimal medium supplemented with 1% (w/v) fructose and then transferred to minimal medium using various other carbon or nitrogen sources as supplements. As carbon sources were used; 1% (w/v) glucose, 1% (w/v) fructose or 1% (w/v) galactose, as nitrogen sources were used 75 mM NH_4Cl or 70 mM $NaNO_3$. In addition we used 0.5% (w/v) BSA (pH 3 and pH 8), 0.5% (w/v) casein (pH 3 and pH 8), 0.5% (w/v) collagen (pH 3 and pH 8) or 0.5% (w/v) elastin (pH 3 and pH 8) as the sole carbon and nitrogen source. The cultures were grown as 50-ml cultures in 250-ml Erlenmeyer flasks in an Innova incubator shaker (New Brunswick Scientific Co., Inc. Edison N.Y., USA) at 250 rpm at 30 °C. Mycelium was collected by filtration over a nylon gauze, frozen in liquid nitrogen and then ground in a Braun II dismembrator (B. Braun Melsungen AG, Melsungen, Germany). Total RNA was isolated using Trizol Reagent (Life technologies, Rockville Md., USA). RNA concentrations were determined spectrophotometrically and equal amounts of RNA were denatured with glyoxal by standard techniques (23) and separated on a 1.6% (w/v) agarose gel. RNA blots were hybridised at 42 °C in SHB to which 10% (w/v) dextran sulphate and 50% (v/v) formamide was added. Washing was performed at 65 °C to a final

stringency of 1x SSC plus 0.1% (w/v) SDS. As loading control the blots were hybridised with the 18S rDNA gene of *A. niger.*

Cloning of apsA

An *aps*A PCR product was generated using degenerate primers based on regions conserved between the metallo aminopeptidase amino acid sequence of *S. cerevisiae*, (P37898, P32454); *Mus musculus*, (AAC52409); *Homo sapiens* (P15144); *Oryctolagus cuniculus* (S07099); *Haemonchus contortus* (CAA63897) and *Rattus norvegicus*, (AAB38021). The database accession numbers are in parenthesis.

The conserved peptide sequences GAMENWG and HELAHQW were used to design a forward primer 5'-GGIGCNATGGARAAYTGGGG-3' and a reverse primer 5'-AAICCRAACCAYTGRTGNGC-3', respectively (standard IUB-IUPAC symbols are used to indicate the nucleotide mixtures, I denotes inosine). A standard PCR was performed on genomic DNA of *A. niger* N402 using an equal amount of both primer mixtures and an annealing temperature of 50 °C. The amplified product was cloned in pGEM-T (Promega, Madison Wis., USA) and sequence analysis followed. The PCR product was used as a probe in the screening of a λ EMBL4 genomic library of *A. niger* N400 by standard methods to obtain the *aps*A gene. Three phages were isolated and from one positive phage a 1.7 kb *Eco*RI - *Bam*HI and a partially overlapping 2.7 kb *Sal*I fragment were subcloned in pUC19 and sequenced over both strands. cDNA of *aps*A was generated by RT-PCR, using the enhanced avian RT-PCR kit of Sigma (St. Louis Mo., USA), according to the suppliers' instructions.

Protein and nucleotide sequence analyses were done with the program DNAstar (Lasergene, Madison, Wis., USA). The Blast algorithm (1) was used to search the public databases. Multiple alignments were made with Clustal X (14).

Plasmid construction and overexpression of ApsA

The 1.7 kb *Eco*RI-*Bam*HI fragment and the partially overlapping 2.7 kb *Sal*I fragment were merged, resulting in pIM4102 (Fig. 1). Plasmid pIM4102 and pGW635, which contains the A. niger *pyr*A gene, were used to co-transform *A. niger* NW219 according to Kusters-van Someren *et al.*, (17). $PyrA^+$ transformants were screened for enhanced aminopeptidase activity in cell extracts. For this, ground mycelium was extracted with 100 mM Na/K phosphate buffer at pH 7.2 and clarified by centrifugation (10.000 x *g* for 15 min at 4 °C). Aminopeptidase activity in these cell extracts was determined as described below and protein concentrations were determined by the bicinchoninic acid method as described by the supplier (Sigma).

Purification of A. niger aminopeptidase A

Strain Tr7 was grown for 17 h in MM (22) supplemented with 2% (w/v) glucose, 0.2% (w/v) meat peptone, 0.1% (w/v) yeast extract, 0.1% (w/v) peptone 140 and 0.03% (w/v) yeast ribonucleic acids. The mycelium was harvested and ground. The ground mycelium was suspended in 100 mM Na/K phosphate buffer pH 7.2 and stirred for 15 min at 4 °C. Cellular debris was removed by centrifugation. Then $(NH_4)_2SO_4$ was added to 30% saturation. After centrifugation (15 min 10.000 x *g*) the supernatant was loaded onto a 20 ml Phenyl Sepharose high performance column (Pharmacia Biotech), pre-equilibrated with 50 mM triethanolamine (TEA) pH 7.0 and 1 M $(NH_4)_2SO_4$. Bound protein was eluted using a 200 ml linear gradient from 1 - 0 M $(NH_4)_2SO_4$ in 20 mM TEA pH 7.0. The fraction size was 5 ml. The active fractions were pooled, dialysed against 20 mM TEA pH 7.0 and loaded onto a 1 ml Resource Q column (Pharmacia Biotech) pre-equilibrated with 20 mM TEA pH 7.0. The bound protein was then eluted using a 15 ml linear gradient from 0 - 0.4 M NaCl in TEA pH 7.0. The fraction size was 1 ml.

Biochemical characterisation of aminopeptidase A

Aminopeptidase activity was determined as described (2). A range of amino acids coupled to *p*-nitroanilide (*p*NA) were used as substrate. Standard conditions were 2 mM substrate in 67 mM Na/K phosphate buffer at pH 7.2 and 30 °C. One unit of enzyme activity is defined as the amount of enzyme that produces 1 µMol *p*NA min^{-1}. L-*p*NA, P-*p*NA, R-*p*NA, F-*p*NA, A-*p*NA, M-*p*NA, K-*p*NA were obtained from Sigma and V-*p*NA, G-*p*NA, I-*p*NA, E-*p*NA were obtained from Bachem (Bubendorf, Switzerland). The optimal pH for enzymatic activity was determined using McIlvaine buffers at pH values ranging from 3 to 8, 200 mM Hepes plus 300 mM NaCl at pH values from 7.2 to 8.5 and 50 mM Tris/glycine buffer at pH values from 8 to 10. The pH stability of aminopeptidase A was tested by preincubation of the purified enzyme in McIlvaine buffer of different pH values ranging from 2.2 to 8 at 30 °C for 90 min followed by the standard enzyme reaction. The temperature stability of aminopeptidase A was tested by preincubation of the purified enzyme at 0, 30, 40, 50 and 60 °C for 60 min in Na/K phosphate buffer pH 7.2, followed by the standard enzyme reaction. Here the 0 °C preincubated sample was used as a reference to calculate the residual activity.

The effect of the protease inhibitors bestatin, 1,10-phenanthrolin, EDTA, EGTA, phenylmethylsulfonylfluoride (PMSF), tosyl phenylalanyl chloromethyl ketone (TPCK), tosyl lysyl chloromethyl ketone (TLCK), leupepstatin and iodoacetamide on the enzymatic activity were measured in Na/K phosphate buffer at pH 7.2. The purified enzyme was preincubated with the respective compound for 30 min at 30 °C. After the preincubation period substrate was added, followed by the standard enzyme assay. The metal ion requirement of ApsA for activity was tested by preincubating the enzyme with EDTA, EGTA or 1,10-phenanthrolin for 15 min at 37 °C or for 17 h at 4 °C.

Results and discussion

Cloning of the *A. niger* aminopeptidase A and analysis of the gene

The Amino acid sequences GAMENWG and HELAHQW which are conserved between zinc aminopeptidases of nearly all subgroups of aminopeptidases within the M1 family were used to design primers. These primers were used in a PCR reaction on genomic DNA of *A. niger* and resulted in a 130 bp product. An 1.7 kb *Eco*RI-*Bam*HI fragment and a partially overlapping 2.7 kb *Sal*I fragment which hybridised to this PCR product (Fig.1) were subcloned and sequenced over both strands. Sequence analysis showed that together these fragments harbour the complete putative aminopeptidase A gene (*aps*A) and up- and downstream sequences. RT-PCR techniques were used to generate *aps*A cDNA. The cDNA and the genomic DNA sequences were identical except for two intervening sequences in the open reading frame, one 152 bp downstream of the start codon and one 48 bp upstream of the stop codon (Fig. 1).

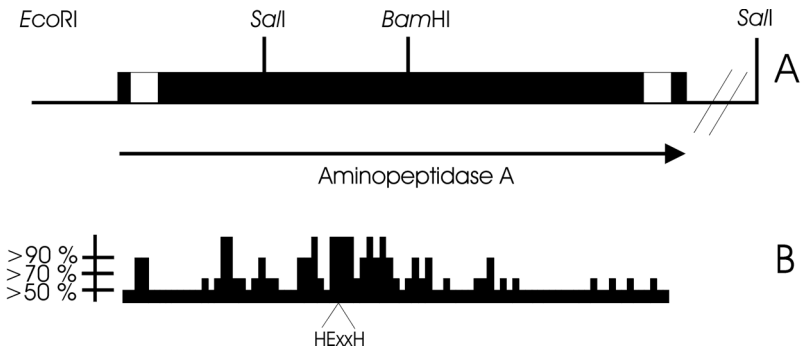

Fig. 1. Sequence characteristics of the *A. niger aps*A gene. (A) Partial restriction map of the *aps*A genomic region. The positions of the restriction enzymes used in the cloning strategy are indicated. The open reading frame is indicated with a filled box, an arrow indicates the direction of transcription. The position2 of the two introns encountered in the ORF are indicated with an open box. (B) Identity between *A. niger* ApsA and LAPI and AAP1 of yeast. The multiple alignment was created with the program Clustal X. The ApsA ORF is presented as a solid line, regions with more than 50% identity are indicated. The percentage of identity was calculated using a window size of 10. The zinc-binding motif (HEXXH) is indicated.

The encoded protein is similar to the M1 family of metallo aminopeptidases (3). ApsA has highest similarity with LAPI (encoded by *APE*2), (7) and AAPI (encoded by *AAP*I), (5), both of *S. cerevisiae* (53.3 and 50.9% overall identity, respectively, Fig. 1. The region of highest identity is found at the N-terminal part of the enzyme. The encoded protein contains the signature sequence of the M1 family of zinc peptidases (HEXXH) (12, 15) and this sequence was found in a region that is most conserved between ApsA, AAP1 and LAPI (Fig. 1).

Northern analysis of *A. niger* N402 grown on several carbon and nitrogen sources showed that the *aps*A messenger levels were independent of the carbon or nitrogen source used (results not shown). The yeast aminopeptidases *APE*2 and *AAP*I are also constitutively expressed, although yeast *AAP*I mRNA, which is present during all phases of growth, is reported to be more abundantly expressed in exponentially growing yeast cells (5).

Since ApsA does not contain a known secretion signal or a known organellar targeting signal and since *aps*A transcript levels are apparently not influenced by the carbon or nitrogen sources tested, we conclude that ApsA is like LAP1 and AAP1 located in the cytosol.

Overexpression of the aminopeptidase A encoding gene in *A. niger*

A. niger strain NW219 was transformed with plasmid pIM4102. Nine transformed strains were further analysed for the occurrence of multiple integrations of the plasmid in the genome. Southern analysis showed that transformants 7 (Tr7) and 8 (Tr8) have the highest copy numbers of the integrated plasmid (Fig. 2). Messenger levels of *aps*A of Tr7 and Tr8 were compared to the messenger level of the wild type strain by northern analysis. Scanning of the northern blots revealed that compared to the wild type strain both transformants have at least a ten-fold increased messenger level of the correct size compared to the wild type (Fig. 2).

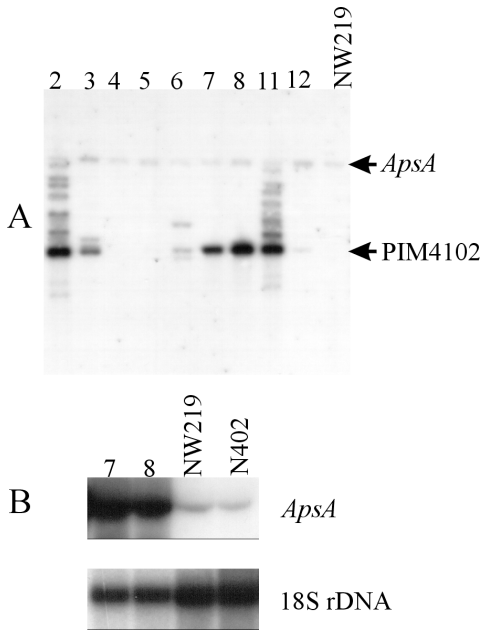

Fig. 2. Molecular characterisation of *aps*A multicopy strains. (A) Southern analysis of *aps*A transformants. *Hin*dIII digested genomic DNA of the parental strain (*A. niger* NW219) and 9 transformed strains were analysed. The two hybridising restriction fragments indicated originate from the endogenous *aps*A gene and from (multiple) integrations of pIM4102 in the genome. The other bands probably result from (partial) integrations of the plasmid at other loci. Transformed strain numbers are indicated above the lanes. (B) Northern analysis of *aps*A expression in the wild type strain (*A. niger* N402), the parental strain (*A. niger* NW219) and transformed strains Tr7 and Tr8.

Tr7 and Tr8 were analysed for increased aminopeptidase activity on four *p*NA substrates; K-*p*NA, R-*p*NA and L-*p*NA, substrates that are preferred by the two homologous yeast enzymes and F-*p*NA, a substrate which is not hydrolysed by these yeast enzymes. Compared to the wild type activity, a cell extract of Tr7 and Tr8 had a 36-fold and a 24-fold increase in activity respectively, when K-*p*NA was used as the substrate. Furthermore a 19 (Tr 7) and 12 (Tr 8) fold increase in activity towards R-*p*NA, and a 9 (Tr 7) and 6 (Tr 8) fold increase in activity towards L-*p*NA was found. A cell extract of *A. niger* hydrolyses F-*p*NA

but the transformants did not display an increased hydrolytic activity on this substrate, thus both transformed strains overexpress a specific aminopeptidase with activity towards K-, R- and L-*p*NA.

Purification and biochemical properties of the enzyme

Aminopeptidase A was purified from a cell extract of Tr7, resulting in an enzyme preparation with a specific activity of 12 U mg^{-1}. The final yield was 5% (Table 1). The low yield in the first step is probably due to the ammonium sulphate precipitation.

Table 1. Purification of Aminopeptidase A from *A. niger* Tr7. Data are means of three separate experiments in which differences did not exceed 10%.

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification (-fold)	Yield (%)
Cell extract	280	390	0.72	1	100
Phenyl Sepharose	18.6	2.5	7.4	10	6.6
Resource Q	14.6	1.2	12.2	17	5.2

One unit of enzymatic activity (U) is defined as the amount of enzyme that produces 1 µMol *p*NA min^{-1}. Activity was determined with 2 mM K-*p*NA as a substrate.

An analysis by 10% SDS-PAGE of the active fractions revealed a protein band with an apparent molecular mass of 95 kDa, identical to the calculated molecular mass of the protein inferred from the amino acid sequence. The four aminopeptidases of *Aspergillus oryzae* isolated from culture broth, differ considerably in their molecular weight from ApsA (18-21).

Fraction 11 contained the highest activity and was used for further characterisation of the enzyme (Fig 3.). The enzyme efficiently hydrolyses K-*p*NA and R-*p*NA suggesting that the enzyme prefers basic amino acids at the N-

terminal end of the substrate. The K_M and K_{cat} for K-*p*NA and L-*p*NA were 0.17 mM and 0.49 μkat mg^{-1} and 0.16 mM and 0.31 μkat mg^{-1} respectively. The enzyme also hydrolyses M-*p*NA and has some activity towards A-*p*NA and F-*p*NA (Table 2).

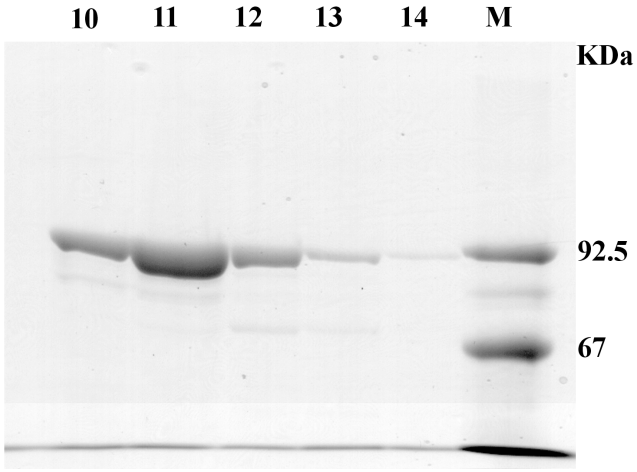

Fig. 3. Purification of aminopeptidase A from *A. niger*. Coomassie-stained SDS-polyacrylamide gel (10%) of 20 μl of the active fractions 10 to 14 eluted from the ResourceQ column. Fraction numbers are indicated above the lanes. M: Protein molecular weight marker.

Table 2: Substrate specificity of ApsA towards amino acid *p*-nitroanilide substrates. The Activity is relative to the activity towards K-*p*NA, which was 12 U mg^{-1}. The results are the mean of three independent experiments in which differences did not exceed 10%.

Substrate	**Relative activity**
K-*p*NA	1
R-*p*NA	0.6
M-*p*NA	0.4
A-*p*NA	0.1
F-*p*NA	0.1
V-*p*NA, G-*p*NA, E-*p*NA, I-*p*NA, P-*p*NA	<0.01

The yeast homologues LAPI and AAPI have highest activity towards substrates that have at their N-terminal end respectively a leucine or a lysine and an alanine or an arginine. Thus their substrate specificity differs from the *Aspergillus* enzyme. However, for AAPI Caprioglio *et al*. (5) did not report on enzyme activities on substrates other than alanine-, arginine- and leucine-napthylamide.

In the cell extracts of Tr7 and Tr8, the highest increase in activity was found towards K- and R-*p*NA, followed by L-*p*NA. However no increase in activity towards F-*p*NA was found. The purified ApsA however shows some activity towards F-*p*NA. This suggest that other, more specific F-*p*NA hydrolysing activities present in the cell extract are dominant over a relative small increase in the two transformants.

The enzyme is active between pH 5 and pH 9 (Fig. 4), the optimal pH is between 7.5 and 8.0 which is close to the pH of the *Aspergillus* cytosol which is pH 7.8 (8). This slightly differs from the optimal pH of LAPI (pH 7.5) (26). The optimal pH of AAPI has not been reported.

ApsA is stable between a pH range of 5 to 8 (Fig. 4) for 90 min with an optimal stability at pH 7. At a pH range of 2.2 to 4 the enzyme is unstable. The enzyme is furthermore stable up to a temperature of 40 °C for 1 h at pH 7.0. After 1 h at 50 °C, 60% of the activity remained. After 1 h at 60 °C, no enzyme activity was left.

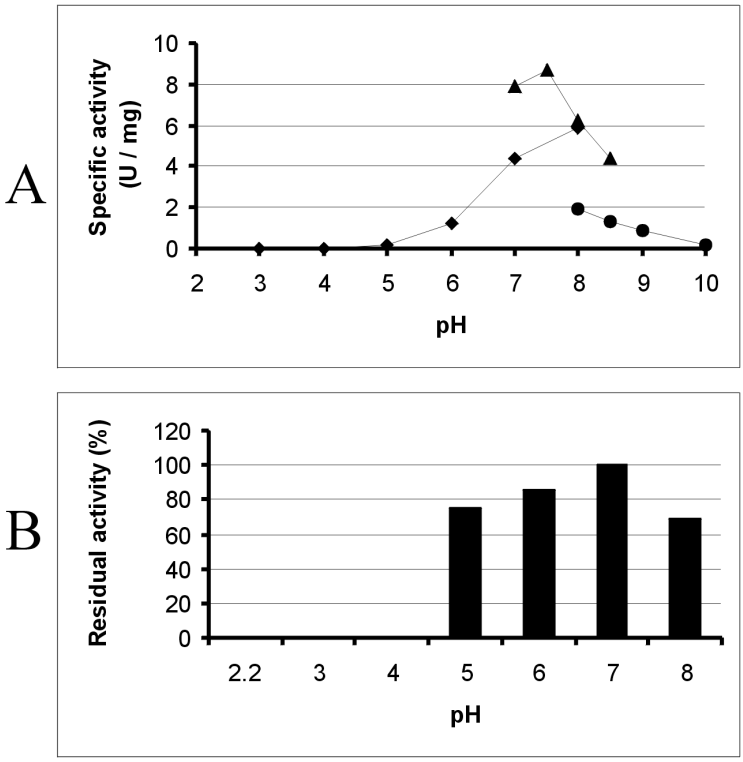

Fig. 4. Biochemical characterisation of ApsA. (A) The pH optimum of ApsA. The following buffers were used: McIlvaine **(♦)**, Hepes **(▲)** and Tris / glycine **(●)**. (B) The pH stability of ApsA. Residual activity was calculated relative to a sample kept in 20 mM TEA pH 7.0 at 30° C.

Several possible inhibitors were tested (Table 3). The three serine protease inhibitors tested had no effect on the enzyme activity. Iodoacetamide also did not have any effect on the activity of ApsA, indicating that the enzyme is not a cysteine aminopeptidase. The aminopeptidase inhibitor bestatin was able to inhibit ApsA activity. 1,10-phenanthrolin was also able to inhibit the activity of ApsA, probably by chelating the metal ion bound in the enzyme. Surprisingly, the metal chelators EDTA and EGTA were not able to reduce the activity after a pre-incubation of 30 min at 30 °C. A 17 h incubation at 4 °C with EDTA or EGTA was necessary to reduce the activity of ApsA completely. This suggests that the metal ion is bound strongly to the ApsA enzyme.

$ZnCl_2$ was also capable of reducing ApsA activity completely. This effect is probably caused by the oxidation of a cysteine residue (nine are present in ApsA), located near the active site of ApsA, since addition of 5 mM β-mercaptoethanol prevented the inhibitory effects of $ZnCl_2$.

Table 3 Effects of chemicals on the activity of the purified protease. The activity is expressed as the percentage of residual activity after 30 min incubation of the enzyme with the inhibitor. 100 % is 2.1 U mg^{-1}. Results are the mean of three experiments; differences did not exceed 10%. PMSF: phenymethylsulfonylfluoride; TPCK: tosyl phenylalanyl chloromethyl ketone; EDTA: ethylenediaminetetraacetic acid; TLCK tosyl lysyl chloromethyl ketone.

Inhibitor	Concentration (mM)	Residual activity (%)
None	0	100
PMSF	1	100
Leupepstatin	0.042	98
TPCK	0.020	95
EDTA	5	100
EGTA	5	100
1,10-*o*-phenenatrolin	5	<1
Iodoacetamide	0.040	100
Bestatin	0.002	60
TLCK	0.020	94
$ZnCl_2$	0.5	<1

In this study we cloned and characterised the first aminopeptidase gene of the M1 family of aminopeptidases from a filamentous fungus. We also determined the general biochemical characteristics of the encoded enzyme. The substrate specificity is different from that of the yeast enzymes, lysine and not leucine, arginine or alanine is preferred at the N-terminal position. This genetic and biochemical characterisation will enable further studies for the understanding of the *in vivo* roles of the aminopeptidases, in degradation of (imported) peptides.

Acknowledgements

We thank Y. Müller for technical assistance and R. Jalving for helpful discussions. This work was supported financially by DSM Food Specialities.

References

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-3402.
2. **Atlan, D., C. Gilbert, B. Blanc, and R. Portalier.** 1994. Cloning, sequencing and characterization of the *pep*IP gene encoding a proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397. Microbiology **140:**527-535.
3. **Barret, A. J., N. D. Rawlings, and J. F. Woessner.** 1998. Handbook of proteolytic enzymes. Academic Press, San Diego.
4. **Blinkovsky, A. M., T. Byun, K. Brown, E. J. Golightly, and A. V. Klotz.** 2000. A non-specific aminopeptidase from *Aspergillus*. Biochim Biophys Acta **1480:**171-181.
5. **Caprioglio, D. R., C. Padilla, and M. Werner-Washburne.** 1993. Isolation and characterization of AAP1; a gene encoding an alanine / arginine aminopeptidase in yeast. J Biol Chem **268:**14310-14315.
6. **Dal Degan, F., B. Ribadeau-Dumas, and K. Breddam.** 1992. Purification and characterization of two serine carboxypeptidases from *Aspergillus niger* and their use in C-terminal sequencing of proteins and peptide synthesis. Appl Environ Microbiol **58:**2144-2152.
7. **Garcia-Alvarez, N., R. Cueva, and P. Suarez-Rendueles.** 1991. Molecular cloning of soluble aminopeptidases from *Saccharomyces cerevisiae;* Sequence analysis of aminopeptidase yscII, a putative zinc-metallopeptidase. Eur J Biochem **202:**993-1002.
8. **Hesse, S., G. Ruijter, C. Dijkema, and J. Visser.** 2000. Measurement of intracellular (compartmental) pH by 31P NMR in *Aspergillus niger*. J. Biotech **77:**5-15.

9. **Hirsch, H. H., H. H. Schiffer, and D. H. Wolf.** 1992. Biogenesis of the yeast vacuole (lysosome). Eur J Biochem **207:**867-876.

10. **Hombergh, J. P. T. v. d., G. Jarai, F. P. Buxton, and J. Visser.** 1994. Cloning, characterization and expression of *pepF*, a gene encoding a serine carboxypeptidase from *Aspergillus*. *niger*. Gene **151:**73-79.

11. **Hombergh, J. P. T. v. d., P. J. I. van de Vondervoort, L. Fraissinet-Tachet, and J. Visser.** 1997. *Aspergillus* as a host for heterologous protein production: the problem of proteases. Trends Biotechnol **15:**256-263.

12. **Hooper, N. M.** 1994. Families of zinc metallo proteases. FEBS lett **354:**1-6.

13. **Jalving, R., P. J. I. van de Vondervoort, J. Visser, and P. J. Schaap.** 2000. Characterization of the kexin-like maturase of *Aspergillus niger*. Appl Environ Microbiol **66:**363-368.

14. **Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgens, and T. J. Gibson.** 1998. Multiple sequence alignment with Clustal X. Trends Biochem Sci **23:**403-405.

15. **Jongeneel, C. V., J. Bouvier, and A. Bairoch.** 1989. A unique signature identifies a family of zinc-dependent metallo peptidases. FEBS lett **242:**211-214.

16. **Kisselev, A. F., T. N. Akopian, K. M. Woo, and A. L. Goldberg.** 1999. The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. J Biol Chem **274:**3363-3371.

17. **Kusters van Someren, M. A., J. A. M. Harmsen, H. C. M. Kester, and J. Visser.** 1991. Structure of the *Aspergillus pelA* gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. Curr Genet **20:**293-299.

18. **Nakadai, T., and S. Nasuno.** 1977. Purification and properties of leucine aminopeptidase IV from *Aspergillus oryzae*. Agric Biol Chem **41:**1657-1666.

19. **Nakadai, T., S. Nasuno, and N. Iguchi.** 1973. Purification and properties of leucine aminopeptidase I from *Aspergillus oryzae*. Agric Biol Chem **37:**757-766.

20. **Nakadai, T., S. Nasuno, and N. Iguchi.** 1973. Purification and properties of leucine aminopeptidase II from *Aspergillus oryzae*. Agric Biol Chem **37:**767-774.

21. **Nakadai, T., S. Nasuno, and N. Iguchi.** 1973. Purification and properties of leucine aminopeptidase III from *Aspergillus oryzae*. Agric Biol Chem **37:**775-778.

22. **Pontecorvo, G., J. A. Roper, L. J. Hemmons, K. D. MacDonald, and A. W. J. Bufton.** 1953. The genetics of *Aspergillus nidulans*. Adv Genet **5:**141-238.

23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: a Laboratory Manual., 2 ed. Cold Spring Harbor, New York.

24. **Svendsen, I., and F. Dal Degan.** 1998. The amino acid sequences of carboxypeptidases I and II from *Aspergillus niger* and their stability in the presence of divalent cations. Biochim Biophys Acta **1387:**369-377.

25. **Tomkinson, B.** 1999. Tripeptidyl peptidases: enzymes that count. Trends Biochem Sci **24:**355-359.

26. **Trumbly, R. J., and G. Bradley.** 1983. Isolation and characterization of aminopeptidase mutants of *Saccharomyces cerevisiae*. J Bacteriol **156:**36-48.

27. **Van Wart, H. E.** 1996. Metallobiochemistry of aminopeptidases. Springer, Heidelberg.

Chapter three

Aminopeptidases A and B from Aspergillus niger have different substrate specificities

Daniëlle E.J.W. van Wijk - Basten, Sjef Boeren and Peter J. Schaap.

Summary

We have cloned and characterized Aminopeptidase B from *Aspergillus niger* which is involved in intracellular peptide catabolism. The inferred amino acid sequence shows 40% sequence identity to ApsA (3), but the two encoding genes differ markedly in architecture. The *apsB* open reading frame (ORF) is interrupted with nine more intervening sequences than the *apsA* ORF and none of these introns show positional conservation with the introns from the paralogous gene.

ApsB was overexpressed in *A. niger* and the protein was purified and characterized. Most of the biochemical characteristics of ApsA and ApsB are similar. Their specificity for peptide substrates however, is markedly different.
The results suggest that *apsA* and *apsB* are the result of an ancient duplication event and that both proteins have developed their own niche of intracellular peptide degradation.

Introduction

Eukaryotic cells express a large array of proteases. Similar proteases which have evolved from a common ancestor are classified into families (17) and are further grouped into clans (16). The majority of the aminopeptidases belong to the M1 family of proteases. Within the M1 family of proteases the yeast *Saccharomyces cerevisiae* appears to have at least three aminopeptidases. Arginine/Alanine aminopeptidase (AAPI) (5), Leucine aminopeptidase I (LAPI) (6) and Leukotriene A4 hydrolase (LTA4) (12, 13, 18). Although the amino acid sequences of these genes are similar, they have different substrate specificities. AAPI specifically hydrolyses artificial substrates with an aminoterminal arginine or alanine residue, LAPI preferentially hydrolyses peptides with leucine and lysine residues at their amino terminal end and LTA4 converts the fatty acid

epoxide leukotriene A4 into leukotriene B4, but can also hydrolyze artificial peptide substrates, preferentially with leucine or methionine at their amino terminal end.

The most common evolutionary mechanism giving rise to new functions is that of gene duplication, where after one of the genes may mutate without compromising the cellular processes (15). The rate of gene duplication is high, but so is the rate of gene loss. However, if gene duplicates evolve overlapping beneficial or new functions, then loss of a duplicate gene might not be easily tolerated (21). To understand the pathways involved in protein catabolism, we previously characterized aminopeptidase A of *A. niger*, an aminopeptidase which shows highest specificity towards a lysine-*p*NA substrate and thus was characterized as a lysine aminopeptidase (3). Southern analysis showed the existence of a paralog of *apsA*. For a further understanding of their role in protein catabolism we cloned the duplicate gene (*apsB*) and characterized the encoded aminopeptidase. The substrate specificity of ApsB is markedly different from ApsA, although the genes and some biochemical characteristics are similar.

Materials and methods

Strains, DNA and RNA techniques

Escherichia coli DH5α and LE392 were used as hosts for recombinant plasmids and λ DNA. *Aspergillus niger* strains N402 (*csp*A1) and NW249 (*csp*A1, *pyr*A6, *leu*A1, *nic*A1, ΔargB) are derived from *A. niger* N400 (CBS 120.49). Standard DNA and RNA manipulations were done as described in (19).

Cloning of *apsB*

A 604 bp *Hinc*II-*Bam*HI fragment of *apsA* from *A. niger* (acc. no. AJ292570) was used as a probe in a heterologous screening of the λ EMBL4 genomic library of *A. niger* N400 by standard methods to obtain the duplicate gene. 50 phages were isolated. Two *apsA* specific primers, AP-1 (5'-

AGGGATCCTCCGTTCTG-3') and AP-2 (5'- CGGTGCGCAGAAACGG), and a standard PCR using an annealing temperature of 52°C on these 50 phages, were used to counter select for phages harboring the *apsA* gene.

apsB cDNA was isolated from a cDNA library of *A. niger* N400 in λZAPII (Stratagene) by homologous hybridization using a genomic 1064 bp *XhoI* fragment as probe (Fig. 1).

Basic protein and nucleotide sequence analyses were done with the program DNAstar (Lasergene, Madison, Wis., USA). The Blast algorithm (1) was used to search the public databases. Multiple alignments were made with Clustal X (10).

Plasmid construction and overexpression or deletion of aminopeptidase B

ApsB cDNA was overexpressed in *E. coli* M15 cells using the plasmid pQE60 with a C-terminal His-tag according to the manufacture's instructions (Qiagen). For overexpression in *A. niger*, the genomic 1.2 kb *Sst*I fragment and the partially overlapping genomic 3.9 kb *Kpn*I fragment were merged, resulting in pIM 4124 (Fig.1). Plasmid pIM 4124 and pGW635, which contains the *A. niger pyr*A gene, were used to co-transform *A. niger* NW249 according to Kusters-van Someren *et al.*, (14). $PyrA^+$ transformants were screened for enhanced aminopeptidase activity in cell extracts as described (3).

An *apsB* disruption construct was made by cloning the *argB* gene of *A. niger* (acc. no. M19158) into *apsB* (Fig. 1). Linearized pIM 4126 was transformed to *A. niger* NW249. $ArgB^+$ transformants were selected and DNA was isolated from 60 transformants. A standard PCR was done on those 60 DNA samples using primers AP-3 (5-GGCCTCAAGGAGCAGGC-3') and AP-4 (5-ATATGCGGCTACTCCAC-3'). DNA samples that did not give a PCR product were digested with *Bam*HI and further screened in a Southern analysis using a 1.4 kb *Xba*I – *Bam*HI fragment as probe (Fig. 1).

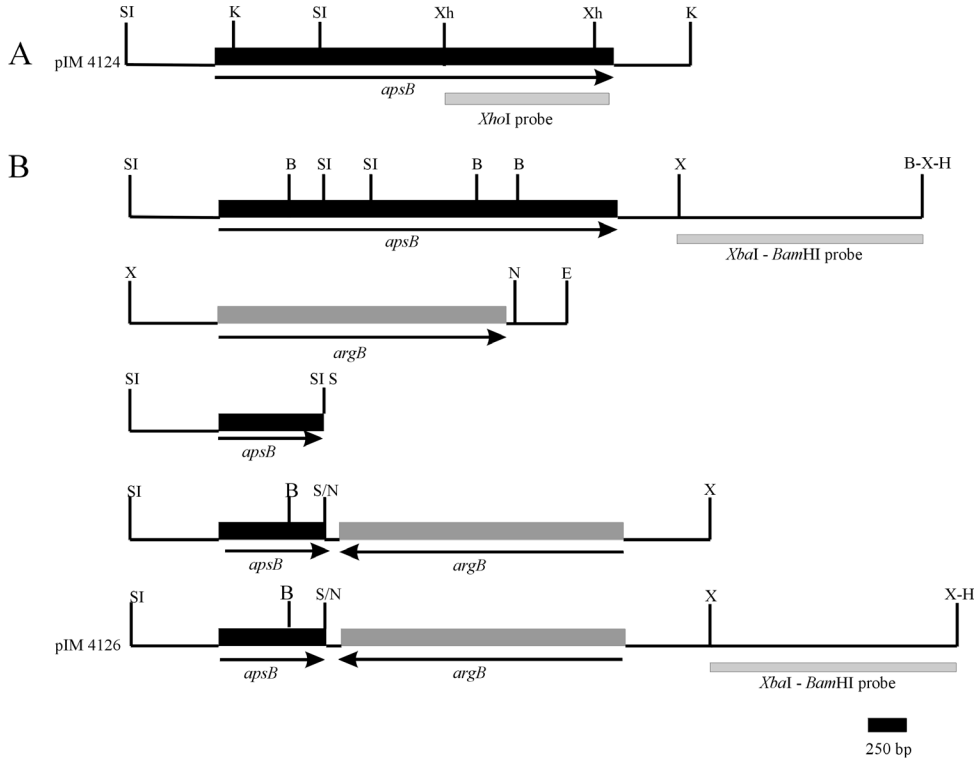

Fig. 1 A: Plasmid pIM 4124 that harbors *apsB* with up- and downstream regions. Restriction sites are indicated. SI: *Sac*I, K: *Kpn*I, Xh: *Xho*I, B: *Bam*HI, X: *Xba*I, N: *Nru*I, E: *Eco*RI, S/N: *Sma*I site ligated in *Nru*I site, X-H: *Xba*I to *Hin*dIII of pUC19, B-X-H: *Bam*HI to *Hin*dIII polylinker of pUC19.

B: Construction of the *apsB* disruption plasmid (pIM 4126) and probes used in the Southern analysis (indicated by a gray box). A *Nru*I-*Xba*I fragment of *argB* was cloned in the polylinker *Sma*I of a *Sst*I subclone of *apsB*. In the next step a *Xba*I fragment was cloned in the resulting plasmid.

Purification and biochemical characterization of *A. niger* aminopeptidase B

ApsB was purified from *E. coli* M15 cells as described by the manufacture's instructions (Qiagen). *A. niger* Tr-3 was used to purify ApsB as described (3).

Aminopeptidase activity towards artificial substrates was measured as described (3). A range of peptides was used as "natural" substrate. K-A, G-G-K, K-N, K-G, K-G-G, K-A-A, K-K, K-K-K, penta-lysine and the 15-mer K-N-N-Q-K-S-E-P-L-I-G-R-K-K-T were obtained from Bachem. Conditions for the use of the peptides were: 1 mM substrate in McIlvaine buffer at pH 8.0 and 30°C. Activity was (calculated) from the decrease in OD_{214}.

The pH optimum, pH stability, temperature stability and the effect of several protease inhibitors were determined as described (3).

LC-MS

A volume of 5 µl sample was injected on a 150*0.3 mm Prontosil 3µ C18H column (prepared in house) eluted at a flow rate of 4.6 µl min^{-1} with 5% (v/v) acetonitril in water containing 0.5 ml/l trifluoroacetic acid. A gradient from 5 to 15% (v/v) acetronitril in water containing 0.5 ml/l trifluoroacetic acid was applied in 15 min, followed by a slower increase to 40% (v/v) acetronitril in water containing 0.5 ml l^{-1} trifluoroacetic acid in 40 min (Thermoquest TSP P4000 pump, San Jose, CA, USA). Mass spectrometric analysis (Finnigan MAT 95, San Jose, Ca, USA) was performed with an electrospray atmospheric pressure source in the positive mode using a spray voltage of 4.0 kV and a capillary temperature of 180 ºC.

Results and discussion

Cloning and overexpression of the aminopeptidase B encoding gene in *A. niger*

Southern analysis using non stringent hybridization conditions was done on genomic DNA from *A. niger* using a 604 bp *Hinc*II-*Bam*HI fragment of *apsA*. This Southern analysis indicated the presence of a homologous gene (results not shown). An *A. niger* phage library was screened for this homologous gene using

standard techniques. The homologous gene was cloned and sequencing showed that the resultant plasmid pIM 4124 harbors the complete putative aminopeptidase B gene and up- and downstream sequences. The protein belongs to the M1 family of metallo aminopeptidases (2). Highest similarity was found with ApsA of *A. niger* (3), AAPI (encoded by AAP*I*, (5) and LAPI (encoded by *APE2*, (6) of *Saccharomyces cerevisiae* (all approximately 40% overall identity).

Like ApsA, AAPI and LAPI the inferred ORF of *apsB* does not contain a secretion signal or a putative organellar targeting signal and since an aminopeptidase A / B like activity was not found in the culture fluid of a shake flask culture we conclude that ApsB is like ApsA located in the cytosol.

A. niger strain NW249 was transformed with plasmid pIM 4124 (Fig. 1) to obtain an ApsB overexpressing strain and with pIM 4126 to obtain an *apsB* knock-out strain. Nine pIM 4124 transformants were analyzed for the occurrence of multiple integrations of *apsB* and 60 putative *apsB* knock-out strains were analyzed for the disruption of the *apsB* gene. Southern analysis showed that of the pIM 4124 transformants, transformant Tr-3 has the highest copy number with more then 30 copies of the integrated plasmid (results not shown) and of the pIM 4126 transformants, transformant KO-60 does have a disrupted *apsB* gene. Cell extracts of transformant Tr-3 and KO-60 were analyzed for aminopeptidase activity on K-*p*NA, R-*p*NA and L-*p*NA. These *p*NA substrates were chosen because they are preferred by ApsA and by the two homologous yeast enzymes. Compared to a wild type strain, a cell extract of Tr-3 showed a 47-fold higher activity, when K-*p*NA was used as the substrate. Tr-3 furthermore showed a 24-fold higher activity towards R-*p*NA, and only a 3-fold higher activity towards L-*p*NA. The *apsB* knock out strain showed activity levels towards K-, R-, and L-*p*NA, which were equal to the activity levels of the parental strain NW249. Thus the multi copy strain overexpresses an aminopeptidase with activity towards K- and R-*p*NA, while the aminopeptidase activities of the knock-out strain do not differ from the wild type activities when artificial substrates are being used.

Possibly ApsA, or other aminopeptidases with overlapping substrate specificity towards these substrates are able to compensate for the loss of ApsB activity.

Gene analysis

The encoded protein contains the signature sequence of the M1 family of (zinc) metallo proteases (HEXXH) (9, 11). The corresponding cDNA was isolated and sequenced. The cDNA and the genomic DNA sequences were identical except for eleven intervening sequences in the open reading frame. The intron positions between *apsA* and *apsB* are not conserved. Intron 8 has an unusual 5' splice site, GC in stead of GT (20). A GC splice site has been seen before in *A. niger pgaI* intron 2 (4). Data base searching at TIGR (www.tigr.org) revealed the *apsA* and *apsB* orthologs of *Aspergillus fumigatus* and of *Neurospora crassa* (Fig. 2). The intron positions of *apsA* are conserved between the *A. niger* gene and its homolog from *A. fumigatus* but not with the *N. crassa* homolog (Fig. 3). The same is true for the intron positions of *apsB*. Thus the two genes might be the result of an ancient duplication event. Since the two genes can also be found in *N. crassa* this duplication event must have occurred more than 670 myrs ago, before the divergence of the two *Aspergilli* and *Neurospora* (7).

The yeast *S. cerevisiae* also contains two orthologous genes, *APEII* and *AAPI*. Both *APEII* and *AAPI* are more similar to aminopeptidase A than to aminopeptidase B. Therefore the duplication event of *APEII* and *AAPI* must have been independent from the duplication of *apsA* and *apsB*. The genomes of *A. niger* and *A. fumigatus* were searched for additional paralogous sequences of aminopeptidases A and B and two were found. In yeast there are also two additional paralogs of *APEII* and *AAPI*, *LTA4* and an ORF. The half life of duplicate genes ranges from 3 myrs to 7 myrs, 90% of duplicates disappear before 50 myrs have elapsed (21). Since *apsA* and *apsB* co-exist for at least 670 myrs as functional genes, this strongly suggests that presently they have a different physiological function.

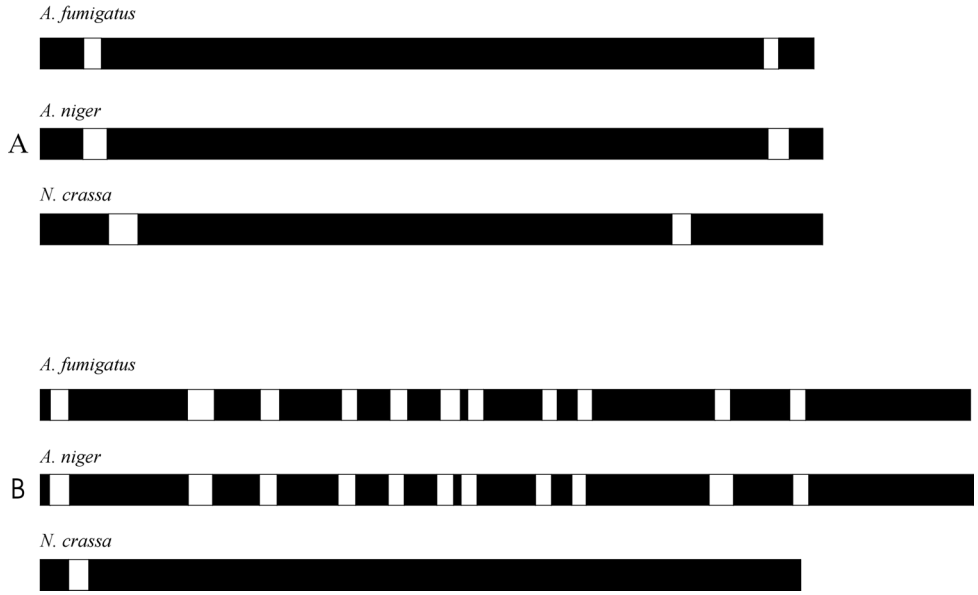

Fig. 2 Comparison of the architecture of the ORF of Aminopeptidase A (panel A) and B (panel B) from *A. niger* with similar ORFs from *A. fumigatus* and *N. crassa.* The exons are represented by a black box, the white boxes indicate introns.

Purification and biochemical properties of the enzyme

Aminopeptidase B was purified from a cell extract of Tr-3, resulting in an enzyme preparation with a specific activity of 21 U mg^{-1} (Table 1). The final yield was 35%.

Table 1 Purification of Aminopeptidase B from *A. niger* Tr-3.

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})
Cell extract	98	260	0.4
Phenyl Sepharose	130	12.6	10.3
Resource Q	35	1.7	21

The results are the mean of three independent experiments in which differences did not exceed 10%. One unit of enzymatic activity (U) is defined as the amount of enzyme that produces 1 µMol *p*NA min^{-1}. Activity was determined with 2 mM K-*p*NA as a substrate.

An analysis by 10% SDS-PAGE of the active fractions revealed a single protein band with an apparent molecular mass of 99 kDa, which is identical to the calculated molecular mass of the protein inferred from the amino acid sequence (Fig. 3).

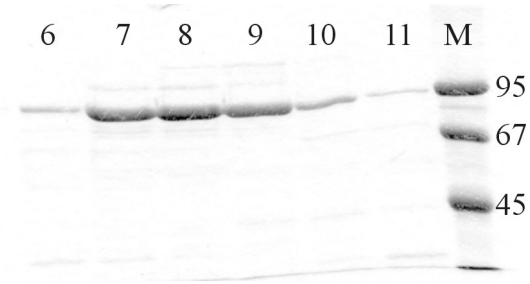

Fig. 3 Purification of aminopeptidase B from *A. niger* Tr-3. Analysis of active fractions 6 to 11 eluted from the Resource Q column were analyzed by SDS-PAGE. Fraction numbers are indicated above the lanes. M: Protein molecular weight marker.

The enzyme efficiently hydrolyses K-*p*NA and R-*p*NA suggesting that like ApsA this enzyme prefers a positively charged side group at the N-terminal end of the substrate, although ApsB hydrolyses R-*p*NA more efficiently than ApsA does (Table 2).

To exclude that this substrate specificity is caused by contaminating activities of aminopeptidase A we also expressed *apsB* in *Escherichia coli*. The purified enzyme from *E. coli* showed the same substrate specificity as the *A. niger* enzyme. The K_M and k_{cat} for K-*p*NA of ApsB were 0.42 mM and 0.57 μkat mg^{-1} and are in the same range as the K_M and k_{cat} of ApsA which are 0.17 mM and 0.49 μkat mg^{-1} respectively (Table 3) (3).

Table 2 Substrate specificity of ApsA and ApsB towards amino acid *p*-nitroanilide substrates. The Activity is relative to the activity towards K-*p*NA. *Other: V-*p*NA, G-*p*NA, E-*p*NA, I-*p*NA, P-*p*NA. The results are the mean of three independent experiments in which differences did not exceed 10%.

Substrate	Relative activity	
	ApsA	ApsB
K- *p*NA	1	1
R- *p*NA	0.6	0.8
L- *p*NA	0.5	0.09
M- *p*NA	0.4	0.04
A- *p*NA	0.1	<0.01
F- *p*NA	0.1	0.08
Other*	<0.01	<0.01

Table 3 An overview of biochemical parameters of Aminopeptidase A (ApsA) and Aminopeptidase B (ApsB).

Parameter	ApsA	ApsB
k_{cat} K-*p*NA	0.49 μKat mg^{-1}	0.57 μKat mg^{-1}
K_M K-*p*NA	0.17 mM	0.42 mM
pH optimum	pH 7.5 – 8	pH 7.5 – 8
Inhibition by $ZnCl_2$	100%	0%

The enzyme is active between pH 5 and pH 9, the optimum being between 7.5 and 8.0 which is very similar to the pH optimum of ApsA (Table 3). Like ApsA, ApsB is stable within a pH range of 5 to 8 for 90 min. The enzyme is furthermore stable up to a temperature of 40°C for 1 h at pH 7.0. After 1 h at 50°C, 60% of the activity remained. After 1 h at 60°C, 10% enzyme activity was left.

Several possible inhibitors were tested (Table 4). The aminopeptidase inhibitor bestatin was able to inhibit ApsB activity. 1,10-phenanthrolin is also able to inhibit the activity of ApsB, probably by chelating the metal ion bound in the enzyme. The metal chelators EDTA and EGTA were not able to reduce the

activity after a pre-incubation of 30 min at 30 °C. A 17 h incubation at 4 °C with EDTA or EGTA was necessary to reduce the activity of ApsB completely. This suggests that the metal ion is strongly bound to the ApsB enzyme. In contrast to ApsA, which was completely inhibited by $ZnCl_2$, ZnCl2 did not reduce ApsB activity.

Table 4 Effects of chemicals on the activity of ApsA and ApsB.

Inhibitor	Concentration (mM)	Residual activity (%) ApsA	ApsB
None	0	100	100
PMSF	1	100	101
Leupepstatin	0.04	98	96
TPCK	0.02	95	97
EDTA	5	100	85
EGTA	5	100	69
1,10-phenanthrolin	5	<1	98
Iodoacetamide	0.04	100	100
Bestatin	0.02	60	82
TLCK	0.02	94	119
$ZnCl_2$	0.5	<1	108

The activity is expressed as the percentage of residual activity after 30 min incubation of the enzyme with the inhibitor. Results are the mean of three experiments; differences did not exceed 10%. PMSF: phenymethylsulfonylfluoride; TPCK: tosyl phenylalanyl chloromethyl ketone; TLCK tosyl lysyl chloromethyl ketone.

Taken together we can conclude that ApsB has biochemical characteristics very similar to ApsA. However, ApsA has a broader substrate specificity towards *p*NA substrates.

In contrast to ApsA, ApsB hydrolyses L- F- and M-*p*NA poorly also when real peptide substrates are being used the substrate specificities of ApsA and ApsB clearly differ. Peptide substrates were incubated either with purified ApsA or purified ApsB. According to Table 2, ApsB is an aminopeptidase specific for lysine and arginine however, the enzyme is able to hydrolyze the dimer leucine arginine (LR) (Fig. 4).

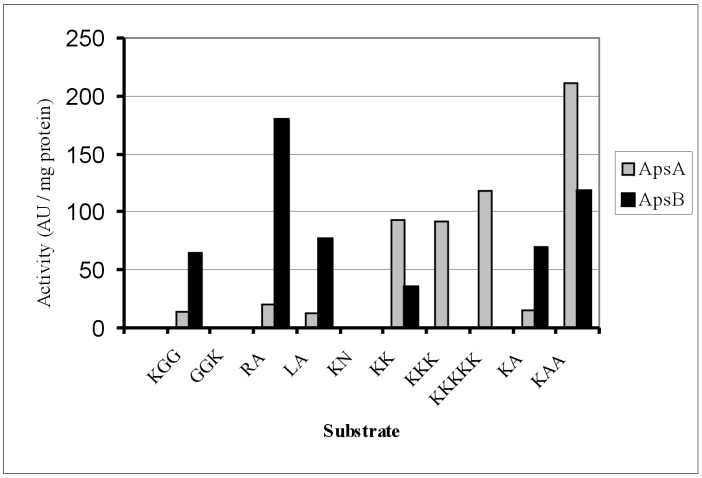

Fig. 4 A comparison of the substrate specificity of ApsA and ApsB for peptide substrates.

Furthermore, the substrate which gives the highest V_{max} does not have an N-terminal lysine but an arginine. The V_{max}, of the enzyme for the various substrates is also dependent on the peptide length. ApsB is able to hydrolyze the dimer lysine-lysine (KK) but not the trimer lysine-lysine-lysine (KKK) and the pentalysine peptide (KKKKK). ApsA on the other hand is able to hydrolyze these three peptides. Not only the substrate length appears to determine substrate specificity but also the amino acid at the penultimate position, since ApsB is not able to hydrolyze the trimer KKK, but can hydrolyze the trimers lysine-alanine-alanine (KAA) and Lysine-glycine-glycine (KGG) and ApsB is able to hydrolyze the dimer Lysine-Lysine (KK) but not the dimer lysine-aspargine (KN) (Fig. 4). Although ApsB is not able to hydrolyze the dimer KN, LC-MS analysis demonstrates hat ApsB is able to hydrolyze the 15-mer peptide KNNQKSEPLIGRKKT (KN15) into NNQKSEPLIGRKKT. ApsA, sequentially hydrolyses this peptide into SEPLIGRKKT (Fig. 5). When these results are compared to results obtained with the artificial *p*NA substrates (Table 2) it is clear that the artificial substrates only give indications about the substrate specificity towards the amino terminal amino acid, but that the specificity for real peptides can be markedly different.

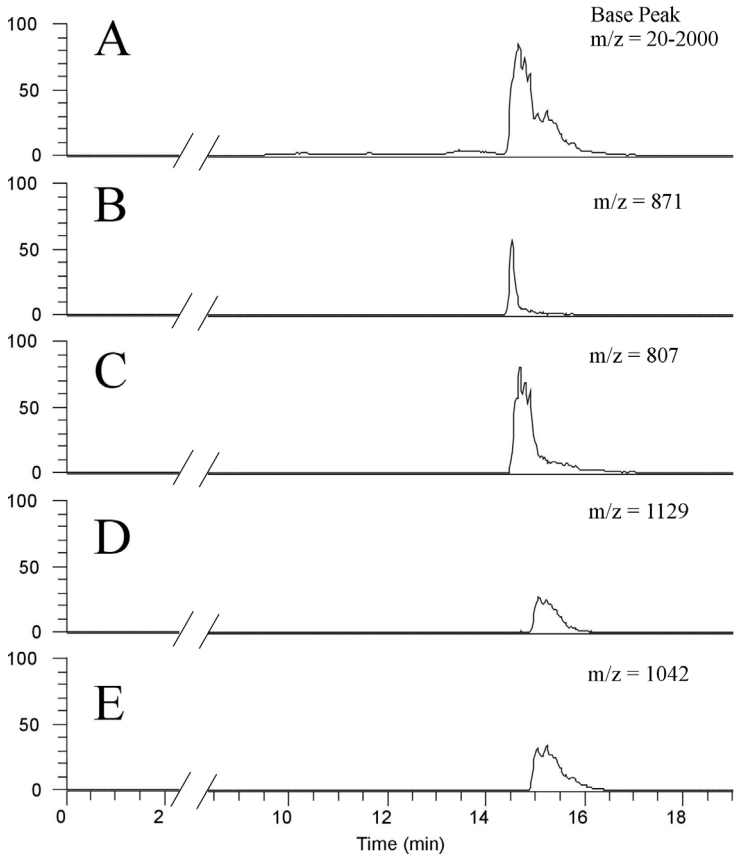

Fig. 5 Base peak (A) and single ion chromatograms for substrate (B) and three hydrolyses products. All chromatograms are shown on the same y-scale. Single ions selected are doubly charged substrate ($[KNNQKSEPLIGRKKT + 2H^+]^{2+}$, m/z=871, Fig. B) and the first hydrolyzation product ($[KNNQKSEPLIGRKKT + 2H^+]^{2+}$, m/z=807, Fig. C)). Other products shown are single charged $[SEPLIGRKKT + 2H^+]^+$, m/z=1129, Fig. D) and $[EPLIGRKKT + 2H^+]^+$, m/z=1042, Fig. E)

Thus ApsA and ApsB probably have their own role in peptide degradation. The ApsB null mutant shows that this gene is not essential under the conditions tested. However the co-existence of these genes during a long period in evolution shows that both provide the fungus an advantage in its natural habitat.

Acknowledgement

The authors thank H. Kester for assistance with the biochemical work. P. Schaap was partially supported by the Dutch Technology Foundation)STW grant WBI 4100.

References

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-3402.
2. **Barret, A. J., N. D. Rawlings, and J. F. Woessner.** 1998. Handbook of proteolytic enzymes. Academic Press, San Diego.
3. **Basten, D. E. J. W., J. Visser, and P. J. Schaap.** 2001. Lysine aminopeptidase of *Aspergillus niger*. Microbiology **147:**2045-2050.
4. **Bussink, H. J. D., K. B. Brouwer, L. H. d. Graaff, H. C. M. Kester, and J. Visser.** 1991. Identification and characterization of a second polygalacturonase gene of *Aspergillus niger*. Curr Genet **20:**301-307.
5. **Caprioglio, D. R., C. Padilla, and M. Werner-Washburne.** 1993. Isolation and characterization of AAP1; a gene encoding an alanine / arginine aminopeptidase in yeast. J Biol Chem **268:**14310-14315.
6. **Garcia-Alvarez, N., R. Cueva, and P. Suarez-Rendueles.** 1991. Molecular cloning of soluble aminopeptidases from *Saccharomyces cerevisiae;* Sequence analysis of aminopeptidase yscII, a putative zinc-metallopeptidase. Eur J Biochem **202:**993-1002.
7. **Heckman, D. S., D. M. Geiser, B. R. Eidell, R. L. Stauffer, N. L. Kardos, and S. B. Hedges.** 2001. Molecular evidence for the early colonization of land by fungi and plants. Science **293:**1129-1133.
8. **Ho, Y., A. Gruhler, A. Heilbut, G. D. Bader, L. Moore, S. L. Adams, A. Millar, P. Taylor, K. Bennett, K. Boutilier, L.Yang, C. Wolting, I. Donaldson, S. Schandorff, J. Shewnarane, M. Vo, J. Taggart, M. Goudreault, B. Musk, C. Alfarano, D. Dewar, Z. Lin, K. Michalickova, A. R. Willems, H. Sassi, P. A. Nielsen, K. J. Rasmussen, J. R. Andersen, L. E. Johansen, L. H. Hansen, H.**

Jespersen, A. Podtelejnikov, E. Nielsen, J. Crawford, V. Poulsen, B. D. Sorensen, J. Matthiesen, R. C. Hendrickson, F. Gleeson, T. Pawson, M. F. Moran, D. Durocher, M. Mann, C. W. Hogue, D. Figeys, and M. Tyers. 2002. Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. Nature **415:**180-183.

9. **Hooper, N. M.** 1994. Families of zinc metallo proteases. FEBS Lett **354:**1-6.
10. **Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgens, and T. J. Gibson.** 1998. Multiple sequence alignment with Clustal X. Trends Biochem Sci **23:**403-405.
11. **Jongeneel, C. V., J. Bouvier, and A. Bairoch.** 1989. A unique signature identifies a family of zinc-dependent metallo peptidases. FEBS lett **242:**211-214.
12. **Kull, F., E. Ohlson, and J. Z. Haeggström.** 1999. Cloning and Characterization of a Bifunctional Leukotriene A4 Hydrolase from *Saccharomyces cerevisiae*. J Biol Chem **274:**34683-34690.
13. **Kull, F., E. Ohlson, B. Lind, and J. Z. Haeggström.** 2001. *Saccharomyces cerevisiae* Leukotriene A4 Hydrolase: Formation of Leukotriene B4 and Identification of Catalytic Residues. Biochemistry **40:**12695-12703.
14. **Kusters van Someren, M. A., J. A. M. Harmsen, H. C. M. Kester, and J. Visser.** 1991. Structure of the *Aspergillus pelA* gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. Curr Genet **20:**293-299.
15. **Lynch, M., and J. S. Conery.** 2000. The Evolutionary Fate and Consequences of Duplicate Genes. Science **290:**1151-1155.
16. **Rawlings, N. D., and A. J. Barret.** 1993. Evolutionary families of peptidases. Biochem J **290:**205-218.
17. **Reeck, G. R., C. d. Haën, D. C. Teller, R. F. Doolittle, W. M. Fitch, R.**

E. Dickerson, P. Chambon, A. D. McLachlan, E. Margoliash, T. H. Jukes, and E. Zuckerkandl. 1987. 'Homology' in proteins and nucleic acids: a terminology muddle and a way out of it. Cell **50:**667.

18. **Rudberg, P. C., F. Tholander, M. M. G. M. Thunnissen, and J. Z. Haeggström.** 2002. Leukotriene A4 Hydrolase/Aminopeptidase. J Biol Chem **277:**1398-1404.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: a Laboratory Manual., 2 ed. Cold Spring Harbor, New York.
20. **Unkles, S. E.** 1992. Gene organization in industrial filamentous fungi, First ed. Blackie Academic & Professional, Glasgow.
21. **Wagner, A.** 2001. Birth and death of duplicated gene in completely sequenced eukaryotes. Trends Genet **17:**237-239.

Chapter four

Aminopeptidase C of *Aspergillus niger* is a novel phenylalanine aminopeptidase.

Daniëlle E.J.W. Basten, Peter J.T. Dekker and Peter J. Schaap

Applied and environmental microbiology 69:1246-1250

Summary

A novel enzyme with a specific phenylalanine aminopeptidase activity (ApsC) has been characterized from *Aspergillus niger* (CBS 120.49). The derived amino acid sequence is not similar to any previous characterized aminopeptidase but shares sequence similarity with some mammalian acyl-peptide hydrolases. ApsC was found to be most active towards phenylalanine β-naphthylamide (F-βNA) and phenylalanine *para*-nitroanilide (F-*p*NA), but it also displayed activity towards other amino acids with aromatic side chains coupled to βNA, while other amino acids with non-aromatic side chains coupled to either *p*NA or βNA were not or poorly hydrolyzed. ApsC was not able to hydrolyze N-acetylalanine-*p*NA, a substrate for acyl-peptide hydrolases.

Introduction

Many food products contain flavors obtained by the hydrolysis of proteins. These peptides and amino acids can taste sweet, sour and bitter. Often mixtures of endoproteases are deliberately used in conjunction with exoproteases, to improve this food flavor. Exopeptidases can reduce the amount of peptides with undesirable tastes through removal of a single or pairs of hydrophobic amino acids from the terminal ends. For example, phenylalanine containing peptides taste more than a 100-fold more bitter than free phenylalanine (13, 14). Control and termination of the hydrolytic reaction is therefore crucial for obtaining hydrolysates with the desired organoleptic properties.

Enzymes from *Aspergillus niger* have been used in food production for several decades and five different endoproteases (PepA to PepE ((26) and references therein), two carboxypeptidases (CpdI, PepF/CpdII (9, 24, 25)) and one aminopeptidase (ApsA (4)) have been cloned and characterized. Experiments have shown that a particular enzyme preparation of *A. niger* that contains a specific aminopeptidase activity can liberate phenylalanine from proteins present in dough and (semi-) hard cheeses (8) thereby improving the flavor and aroma of

these products. So far only one phenylalanine specific aminopeptidase is characterized: APF1 from the basidiomycetous fungus *Schizophyllum commune* (6). APF1 is an intracellular zinc metallo aminopeptidase that can also hydrolyze an N-terminal tyrosine.

In this report we describe a new aminopeptidase. The gene was cloned from *A. niger* and purification and characterization of the gene product shows that it encodes an aminopeptidase that specifically hydrolyzes amino terminal phenylalanine and other amino acids with an aromatic side group.

Materials and methods

Strains, DNA and RNA techniques

Escherichia coli DH5α and LE392 were used as hosts for recombinant plasmids and λ DNA respectively. *Aspergillus niger* strains N402 (*csp*A1) and NW171 (*csp*A1, *fwn*A6, *pyr*A6, *nic*A1, *pep*A::*arg*B_{nid}ΔA, *pep*B::*arg*B_{nid}ΔB, *pep*E::*arg*B_{nid}ΔE) are derived from *A. niger* N400 (CBS 120.49). DNA and RNA manipulations were done as described in (23). For Southern and northern analysis of the transformants the strains were grown in CM (MM (20) supplemented with 2% (w/v) glucose, 0.2% (w/v) meat peptone, 0.1% (w/v) yeast extract, 0.1% (w/v) peptone 140, 0.03% (w/v) yeast ribo nucleic acids and either 0.15% (w/v) KH_2PO_4 or 1.5% (w/v) KH_2PO_4). To study possible regulation of *apsC* messenger levels by the carbon and nitrogen source, strain N402 was grown on MM supplemented with either 1% glucose, 1% fructose and 0.6% NH_4Cl or 0.4% $NaNO_3$ or MM supplemented with 1% BSA, 1% elastin or 1% collagen. RNA was isolated from these cultures and subjected to northern analysis.

Purification of extracellular ApsC from *A. niger* NRRL 3112 and peptide sequencing

A. niger NRRL 3112 was grown in a medium containing 1.5% (w/v) potato flour, 2% (w/v) bactopeptone, 0.7% (w/v) yeast extract, 30 mM KH_2PO_4,

4.2 mM $MgSO_4$, 4.5 mM $CaCl_2$ and 3.7 mM $ZnCl_2$ at pH 4.8, for 120 h at 30°C. 20 ml culture broth was harvested by filtration and transferred to 10 mM Tris pH 7.5 over PD10 columns (Pharmacia, Wiesbaden, Germany). The desalted culture broth was fractionated by anion exchange chromatography on a 1 ml Mono Q column. Bound protein was eluted using a 25 ml linear gradient from 0 – 1 M NaCl in 10 mM Tris-HCl pH 7.5. The fraction size was 1 ml. The fractions containing F-AP activity were pooled and concentrated by ultrafiltration, desalted over a PD10 column and transferred to 10 mM Tris pH 7.5, concentrated 5-fold by ultrafiltration and then applied to a preparative polyacrylamide gel (15% polyacrylamide was used for separation, 5% polyacrylamide was used for stacking). Two lanes were used for a zymogram to locate F-AP activity using an overlay of 0.9 mM phenylalanine in 7.5 mM HCl. The protein band showing F-AP activity was marked and from the remaining untreated lanes, a protein band located at the same position was cut from the gel and subjected to standard (internal) amino acid sequencing procedures performed at Eurosequence (Groningen, The Netherlands) as described in (21).

Cloning of *apsC*

The N-terminal sequence of the protein and of two peptide fragments thereof, being: AEPQTAPFGTWDSPIT, VSWIQWN and WGPDGTLFFVSDR were used to design degenerate forward and reverse primers (Table 1). A standard PCR was performed on genomic DNA of *A. niger* NRRL 3112 using an equal amount of a forward and reverse primer and an annealing temperature of 54 °C. The amplified product obtained with Sap-5 and Sap-4 was cloned in pGEM-T (Promega, Madison Wis., USA) and sequence analysis followed.

Table 1 N-terminal and peptide sequences of ApsC from *A. niger* NRRL 3112 and DNA sequences of degenerate primers obtained by reverse translations of the underlined parts of these sequences. Standard IUPAC codes are used.

Peptide sequence	Forward primer	Reverse primer
VSWIQWN	Sap-1 SNTGGATHCARTGGAAY	Sap-2 RTTCCAYTGDATCCA
WGPDGTLFFVSDR	Sap-3 TGGGGNCCNGAYGGNAC	Sap-4 GTNCCRTCNGGNCCCCA
AEPQTAPFGTWDSPIT	Sap-5 GARCCICARACNGCICCNTT	

The PCR product was used as a probe in the screening of a λ EMBL4 genomic library of *A. niger* N400 by standard methods to obtain the *aps*C gene (23). Nine phages were isolated and from one positive phage a 5.5 kb *Eco*RI and a partially overlapping 1.8 kb *Xho*I fragment were subcloned in pUC19 and sequenced over both strands. cDNA of *apsC* was generated by RT-PCR, using the enhanced avian RT-PCR kit of Sigma (St. Louis Mo., USA).

Protein and nucleotide sequence analyses were done with the program DNAstar (Lasergene, Madison, Wis., USA). The inferred ApsC protein sequence was used to search the public non redundant protein and DNA databases using the blastp and tblastn algorithm respectively and a Blosum 62 substitution matrix (2). Sequences with expect values higher than 10 were ignored. Multiple alignments were made with ClustalX (15).

Plasmid construction, overexpression and purification of aminopeptidase C

The 5.5 kb *Eco*RI fragment and the partially overlapping 1.8 kb *Xho*I fragment were merged, resulting in pIM4103 (Fig. 1). Plasmid pIM4103 and pGW635 (12) which contains the *A. niger pyr*A gene, were used to co-transform *A. niger* NW171 according to (17). $PyrA^+$ transformants were screened for enhanced F-AP activity in cell extracts in 100 mM sodium/potassium phosphate buffer (PB) at pH 7.2. Strains with enhanced F-AP activity were subjected to Southern analysis and northern analysis to determine the copy number and the expression levels.

Throughout the purification procedure the fractions with F-AP activity were determined using standard conditions and F-*p*NA as substrate (see below). Multi copy strain Tr10 was grown for 17 h in 400 ml CM at 30°C. Mycelium was collected by filtration over a nylon gauze, frozen in liquid nitrogen and then ground in a Braun II dismembrator (B. Braun Melsungen A.G., Melsungen, Germany). The ground mycelium was suspended in PB pH 7.2 and stirred for 15 min at 4 °C. Cellular debris was removed by centrifugation. This cell extract of Tr10 in PB was applied to a 15.5 ml SourceQ column (Pharmacia biotech) equilibrated in 20 mM TEA, pH 7.0 and bound protein was eluted using a 124 ml linear gradient from 0 – 0.4 M NaCl in 20 mM TEA pH 7.0. The fraction size was 5 ml. The active fractions 13 and 14 were 10-fold diluted in 20 mM Bis-Tris pH 6.5 and separately loaded onto a 1 ml Resource Q column (Pharmacia Biotech) pre-equilibrated with 20 mM Bis-Tris pH 6.5. The bound protein was then eluted using a 20 ml linear gradient from 0 - 0.4 M NaCl in 20 mM Bis-Tris pH 6.5. The fraction size was 1 ml.

Biochemical characterization of aminopeptidase C

Protein concentrations were determined by the bicinchoninic acid method as described by the supplier (Sigma).

Artificial substrates: Aminopeptidase activity was determined as described by (3), using different amino acids coupled to *para*-nitroanilide (*p*NA) as substrate. Standard conditions were 1 min at 30 °C, 1 mM amino acid-*p*NA substrate in 20 mM Na-citrate pH 5.2 at a final volume of 1 ml. Aminopeptidase activity was also determined as described by (10), using amino acids coupled to β-naphthylamide (β-NA) as substrate. Standard conditions were then 1 min at 30°C, 1 mM β-NA substrate in 20 mM Na-citrate pH 5.2 at a final volume of 1 ml. One unit of enzyme activity is defined as the amount of enzyme that produces 1 µMol *p*NA or βNA min^{-1}. L-*p*NA, P-*p*NA, R-*p*NA, F-*p*NA, A-*p*NA, M-*p*NA, K-*p*NA, W-βNA, H-βNA, N-βNA, S-βNA, L-βNA and F-βNA were obtained

from Sigma and V-*p*NA, G-*p*NA, I-*p*NA, E-*p*NA, T-βNA and Y-βNA were obtained from Bachem (Bubendorf, Switzerland).

Peptide substrates: The liberated amino acids were detected by the method of (22). Standard conditions were 3 mM substrate in 20 mM Na-citrate pH 5.2. phenylalanine and leucine concentrations were calculated from a standard curve. The peptides F-G-G, L-G-G, F-G-G-F and F-L-E-E-I were obtained from Sigma (St. Louis). One unit of enzyme activity is defined as the amount of enzyme that produces 1 μMol phenylalanine or leucine min^{-1}. The optimal pH for enzymatic activity was determined using McIlvaine buffers at values ranging from pH 4 to pH 8. The pH stability of aminopeptidase C was tested by preincubation of the purified enzyme in McIlvaine buffer of different pH values ranging from 4 to 8 at 30 °C for 90 min followed by the standard enzyme reaction. The temperature stability of aminopeptidase C was tested by preincubation of the purified enzyme at 0, 30, 40, 50 and 60 °C for 60 min in 100 mM PB, pH 7.2, followed by the standard enzyme reaction. The 0 °C preincubated sample was used as a reference to calculate the residual activity.

Sequence accession number

The nucleotide sequence of the *apsC* gene from *A. niger* and the encoded amino acid sequence have been deposited in the EMBL nucleotide database under accession number AJ316576.

Results and discussion

Cloning and analysis of *A. niger apsC*

Since *A. niger* strain NRRL 3112 was used as a source for the F-AP hydrolyzing activity in a enzyme preparation described by (8), culture broth of this strain was used to purify a protein with high activity towards F-*p*NA. N-terminal sequences were determined from the N-terminal end of the mature protein and from two peptides of a tryptic digest thereof. A combination of the

forward primer Sap-5 based on the N-terminal peptide sequence AEPQTAPFGTWDSPIT and the reversed primer Sap-4 based on the peptide sequence WGPDGTLFFVSDR was used to amplify a 751 bp genomic DNA fragment of *A. niger* NRRL 3112. The third peptide sequence VSWIQWN exactly matched an internal region of the inferred amino acid sequence translated from the amplified fragment.

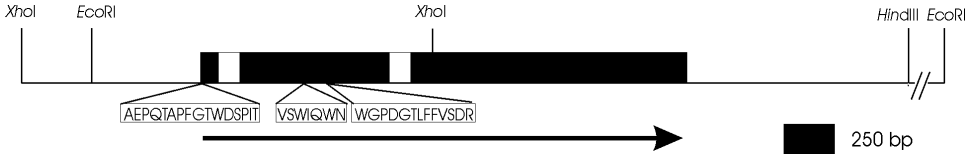

Fig. 1 Architecture of the *A. niger apsC* gene. The positions of the restriction enzymes used in the cloning strategy and construction of pIM4103 are indicated. The ORF is indicated with a filled box, an arrow indicates the direction of transcription. The positions of the two introns encountered in the ORF are indicated with an open box. The positions of the amino terminally sequenced peptides in the ORF are indicated.

A 5.5 kb *Eco*RI fragment and a partially overlapping 1.8 kb *Xho*I fragment of *A. niger* N400 which hybridized to the amplified fragment were subcloned and sequenced over both strands until the *Hin*dIII site 3' of the gene (Fig. 1). Sequence analysis showed that together these fragments harbor the complete putative aminopeptidase C gene (*apsC*) and 5'- and 3'- flanking regions. RT-PCR techniques were used to generate *apsC* cDNA, which was identical to the genomic DNA except for two intervening sequences in the open reading frame (ORF), one 111 bp and one 917 bp downstream the start codon (Fig. 1). The previously determined N-terminal sequence of the protein starts at amino acid 5 of the ORF, indicating that no cleavable amino terminal signal sequence is present in this protein. The loss of the first 4 amino acids is probably due to proteolysis. The NRRL 3112 derived N-terminal sequence differs one amino acid (a pro to ala substitution at position 7) from the N400 derived amino acid sequence which is probably due to strain differences.

Public DNA and Protein databases were searched for similar characterized aminopeptidases and none were found. Highest overall homology was found with some putative acylaminoacyl aminopeptidases also referred to as acyl-peptide hydrolases from *Synechocystis sp*: slr0825 (41% identity), *Streptomyces coelicolor* (37% identity), *Ceanorhabditis elegans* (36% identity) and *Arabidopsis thaliana* (35% identity). None of these proteins however, have been biochemically characterized. Lower scores were obtained with some genuine acyl-peptide hydrolases (ACPH) from pig, rat and human (all 15% identity), that catalyze the hydrolysis of an N-terminal acetylated peptide to release a N-acetylated amino acid. A comparison at the protein domain level shows that at the C-terminal end these mammalian ACPHs at least have a putative α/β-hydrolase fold (abhydrolase) domain, a putative S9-prolyloligopeptidase domain (S9), and a putative dienelactone hydrolase (DLH) domain (5). ApsC has a putative S9-prolyloligopeptidase domain and a putative DLH domain at the C-terminal end (Fig. 2). In the multiple alignment there are furthermore multiple insertions, of which 6 are 4 amino acids or more in the ORFs of ACPH that are not present in ApsC and the putative proteins of *S. coelicolor*, *C. elegans*., and *A. thaliana*. One of these insertions starting at coordinate 616 is part of the abhydrolase domain. In addition there is one insertion of 4 amino acids starting at coordinate 514 present in ApsC and in the putative proteins that is not present in ACPH (Fig. 2). The putative active site serine that is part of the conserved motif GXSXG (16, 18, 19) is also indicated in Fig. 2. As we will show in the next section, ApsC is not an acyl-peptide hydrolase but a novel aminopeptidase.

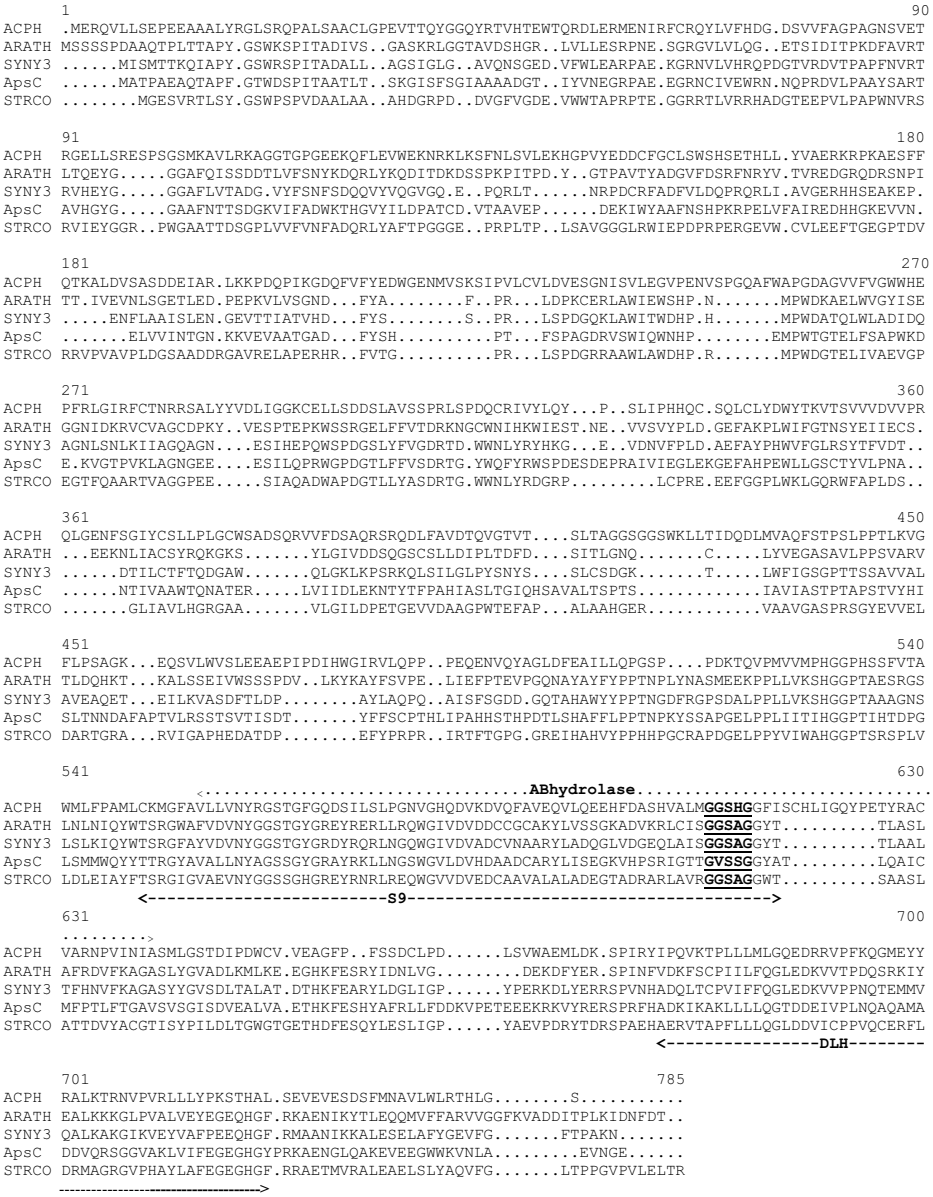

Fig. 2 Multiple alignment of *A. niger ApsC* and putative proteins from *S. coelicolor* (STRCO), *Synechocystis spp* (SYNY3), *A. thaliana* (ARATH) and ACPH from human. Only the human ACPH is shown, rat and pig ACPH are more than 90% identical to human ACPH. The conserved S9-prolyloligopeptidase (S9) and the dienelactone hydrolase (DLH) domains are indicated with a dashed line. The ACPH specific α/β-hydrolase fold (ABhydrolase) domain is indicated with a dotted line. The putative active site motif (GXSXG) is underlined and bold.

Therefore the putative proteins sequences that cluster with ApsC more likely belong to a new subfamily of aminopeptidases.

Northern analysis of *A. niger* N402 grown on several carbon and nitrogen sources showed that the *apsC* messenger levels were independent of the carbon or nitrogen source used (results not shown). *A. niger apsA* transcript levels are also independent of the C- and N- source used (4). Furthermore the yeast AapI and ApeII encoding genes also show constitutive expression.

Overexpression, purification and characterization of the enzyme.

ApsC was overexpressed in *A. niger* strain NW171 by transformation with plasmid pIM4103 (Fig. 1). Twelve transformed strains were analyzed for the occurrence of multiple integrations of the plasmid in the genome. Southern analysis showed that transformant 10 (Tr10) has the highest copy number (approximately 75 copies) of the integrated plasmid (results not shown). Scanning of northern blots revealed that Tr10 had at least a 70-fold increased messenger level of the correct size compared to the untransformed strain (results not shown).

Although ApsC was purified from culture broth of strain NRRL 3112, we were not able to detect ApsC in culture broth of strain N402. However we could find ApsC activity in cell extracts of this strain. Tr10 had a 50-fold higher activity towards F-pNA compared to the untransformed strain being 3.6 U mg^{-1} and 0.07 U mg^{-1} respectively. ApsC was purified from cell extracts of Tr10. This resulted in an enzyme preparation with a specific activity of 189 U mg^{-1} when F-*p*NA is used as the substrate (Table 2).

An analysis by 10% SDS-PAGE of the active fractions revealed a protein band with an apparent molecular mass of 72 kDa, identical to the calculated molecular mass of the protein inferred from the amino acid sequence. The fraction with the highest activity was used for further characterization of the enzyme. The N-terminal sequence of the purified protein as determined by Edman degradation exactly matched amino acids 5 to 16 of the predicted amino acid sequence.

Table 2 Purification of Aminopeptidase C from *A. niger* transformant Tr10. S.A.: specific activity

Step	**Total activity** (U)[a] *10^3	**Total protein** (mg) *10^3	**S. A.** U mg^{-1}	**Purification** (-fold)	**Yield** (%)
Cell extract	4.6	1.3	3.6	1	100
Source 30Q	1.4	0.9	15	4.2	31
Resource Q	0.42	0.0022	189	53	9

[a] One unit of enzymatic activity (U) is defined as the amount of enzyme that produces 1 µmol *p*NA min^{-1}.

Although there is no secretion signal ApsC is found both intra- and extracellularly in *A. niger* NRRL 3112. NRRL 3112 is a strain that was selected from a screen for high extracellular levels of F-AP activity. This strain probably shows more lysis than the N400 derivative strain used for overexpression under these circumstances. F-*p*NA was used as a substrate for the determination of the pH optimum, the pH stability and the temperature stability of the purified enzyme. The optimal pH is 5 which is different from pH optima reported for *A. niger* ApsA and other intracellular aminopeptidases like the yeast aminopeptidases which all have pH optima around pH 7.5 (1, 4, 7, 11).

The purified protein is stable between pH 5 and 8 (Fig. 3) for 90 min with an optimal stability at pH 8. The purified protein is furthermore stable up to a temperature of 50 °C for 60 min at pH 7.0. After 1 h at 60 °C, 5% of the activity remained. ApsC specifically hydrolyzed F-*p*NA and F-βNA substrates, but also other amino acids with aromatic side chains coupled to βNA. The activity towards amino acids with non aromatic side chains was low (at least 16 times less, Table 3).

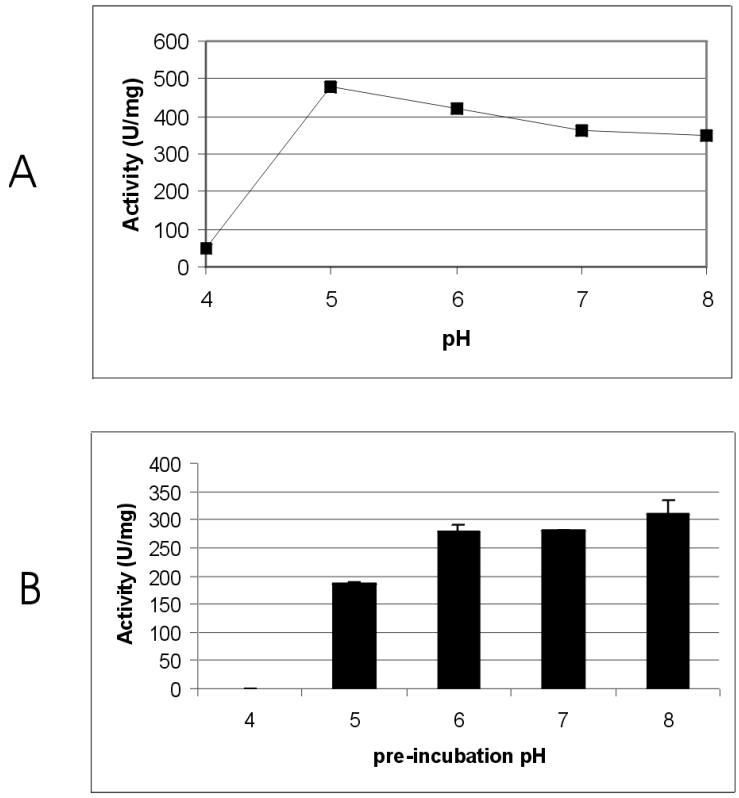

Fig. 3 Biochemical characterization of ApsC. (A) The pH optimum of ApsC using McIlvaine buffer. F-*p*NA was used as the substrate. (B) The pH stability of ApsC. McIlvaine buffer was used as pre-incubation buffer.

All acyl-peptide hydrolases tested with the substrate N-acetylalanine-*p*NA could hydrolyze this substrate. ApsC however is not able to hydrolyze this substrate, thus ApsC is not an acyl-peptide hydrolase. This apparent high specificity for amino acids with an aromatic side chain was further demonstrated by the hydrolysis rate of peptides with phenylalanine or leucine at the N-terminal position. The peptides F-G-G, F-G-G-F and F-L-E-E-I were hydrolyzed by ApsC at comparable rates (Table 4). In agreement with results obtained with the artificial substrates, ApsC is not able to hydrolyze LGG, a peptide with an amino acid with a non-aromatic side chain at the N-terminal end.

Table 3 Substrate specificity of ApsC towards (N-acetylated) amino acid *para*-nitroanilide (*p*NA) or amino acid β-naphthylamide (βNA) substrates.

100% F-*p*NA activity is 189 U mg^{-1}. NacA: N-acetyl-alanine, other *p*NA substrates tested: A-*p*NA, K-*p*NA, R-*p*NA, G-*p*NA, I-*p*NA, V-*p*NA, E-*p*NA, other βNA

Substrate	Relative Activity %	+/- SD	Substrate	Relative Activity %	+/- SD
F-βNA	100	1.1	F-*p*NA	100	0.4
Y-βNA	72	2.5	L-*p*NA	6	0.32
W-βNA	2	0.77	M-*p*NA	6	0.6
L-βNA	6	0.46	NacA-*p*NA	<1	-
Other βNA	<1	-	Other *p*NA	<1	-

In this report a novel aminopeptidase is described specific for amino acids with aromatic side chains. This aminopeptidase is most active at a slightly acidic pH therefore might be used in the controlled preparation of cheese, thereby improving flavor and aroma by reducing the content of bitter tasting phenylalanine and tyrosine containing peptides. Further research will focus on the *in vivo* role of ApsC.

References

1. **Achstetter, T., C. Ehmann, and D. H. Wolf.** 1983. Proteolysis in Eucaryotic Cells: Aminopeptidases and Dipeptidyl Aminopeptidases of Yeast revisited. Archives of biochemistry and biophysics **226:**292-305.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-3402.
3. **Atlan, D., C. Gilbert, B. Blanc, and R. Portalier.** 1994. Cloning, sequencing and characterization of the *pep*IP gene encoding a proline iminopeptidase from *Lactobacillus delbrueckii* subsp. bulgaricus CNRZ 397. Microbiology **140:**527-535.
4. **Basten, D. E. J. W., J. Visser, and P. J. Schaap.** 2001. Lysine aminopeptidase of *Aspergillus niger*. Microbiology **147:**2045-2050.
5. **Bateman, A., E. Birney, R. Durbin, S. R. Eddy, R. D. Finn, and E. L. L. Sonnhammer.** 1999. Pfam 3.1: 1313 multiple alignments and profile HMMs match the majority of proteins. Nucleic Acids Res **27:**260 - 262.
6. **Bilbrey, R. E., A. R. Penheiter, A. C. Gathman, and W. W. Lilly.** 1996. Characterization of a novel phenylalanine-specific aminopeptidase from *Schizophyllum commune*. Mycol. Res **100:**462-466.
7. **Caprioglio, D. R., C. Padilla, and M. Werner-Washburne.** 1993. Isolation and Characterization of AAP1; a gene encoding an alanine / arginine aminopeptidase in yeast. J Biol Chem **268:**14310-14315.
8. **Coenen, T. M. M., and P. Aughton.** 1998. Safety Evaluation of Amino Peptidase Enzyme Derived from *Aspergillus niger*. Food Chem Toxicol **36:**781-789.
9. **Dal Degan, F., B. Ribadeau-Dumas, and K. Breddam.** 1992. Purification and characterization of two serine carboxypeptidases from

Aspergillus niger and their use in C-terminal sequencing of proteins and peptide synthesis. Appl Environ Microbiol **58:**2144-2152.

10. **Frey, J., and K.-H. Röhm.** 1979. External and Internal Forms of Yeast Aminopeptidase II. Eur J Biochem **97:**169-173.
11. **Garcia-Alvarez, N., R. Cueva, and P. Suarez-Rendueles.** 1991. Molecular cloning of soluble aminopeptidases from *Saccharomyces cerevisiae;* Sequence analysis of aminopeptidase yscII, a putative zinc-metallopeptidase. Eur J Biochem **202:**993-1002.
12. **Goosen, T., F. van Engelenburg, F. Debets, K. Swart, K. Bos, and H. Van den Broek.** 1989. Tryptophan auxotrophic mutants in *Aspergillus niger*: inactivation of the *trp*C gene by cotransformation mutagenesis. Mol gen genet **219:**282-288.
13. **Ishibashi, N., I. Ono, K. Kato, T. Shigenaga, I. Shinoda, H. Okai, and S. Fukui.** 1988. Role of the Hydrophobic Amino Acid Residue in the Bitterness of peptides. Agric Biol Chem **52:**91-94.
14. **Ishibashi, N., K. Sadamori, O. Yamamoto, H. Kanehisa, K. Kouge, E. Kikuchi, H. Okai, and S. Fukui.** 1987. Bitterness of Phenylalanine- and Tyrosine-containing Peptides. Agric Biol Chem **51:**3309-3313.
15. **Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgens, and T. J. Gibson.** 1998. Multiple sequence alignment with Clustal X. Trends Biochem Sci **23:**403-405.
16. **Kanatani, A., T. Yoshimoto, A. Kitazono, T. Kokubo, and S. Tsuru.** 1993. Prolyl Endopeptidase from *Aeromonas hydrophila:* Cloning, Sequencing and Expression of the Enzyme Gene, and Characterization of the Expressed Enzyme. J Biochem **113:**790-796.
17. **Kusters-van-Someren, M. A., J. A. M. Harmsen, H. C. M. Kester, and J. Visser.** 1991. Structure of the *Aspergillus pelA* gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. Curr Genet **20:**293-299.

18. **Mitta, M., M. Miyagi, I. Kato, and S. Tsunasawa.** 1998. Identification of the Catalytic Triad Residues of Porcine Liver Acylamino Acid-releasing Enzyme. J Biochem **123:**924-931.

19. **Polgar, L.** 1992. Structural relationship between lipases and peptidases of the prolyl oligopeptidase family. FEBS Lett **311:**281-284.

20. **Pontecorvo, G., J. A. Roper, L. J. Hemmons, K. D. MacDonald, and A. W. J. Bufton.** 1953. The genetics of *Aspergillus nidulans*. Adv Genet **5:**141-238.

21. **Rosenfeld, J., J. Capdevielle, J. C. Guillemot, and P. Ferrara.** 1992. In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Anal Biochem **203:**173-179.

22. **Roth, M.** 1971. Fluorescence Reaction for Amino Acids. Anal Chem **43:**880-882.

23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: a Laboratory Manual., 2 ed. Cold Spring Harbor, New York.

24. **Svendsen, I., and F. Dal Degan.** 1998. The amino acid sequences of carboxypeptidases I and II from *Aspergillus niger* and their stability in the presence of divalent cations. Biochim Biophys Acta **1387:**369-377.

25. **van den Hombergh, J. P., G. Jarai, F. P. Buxton, and J. Visser.** 1994. Cloning, characterization and expression of *pepF*, a gene encoding a serine carboxypeptidase from *Aspergillus*. *niger*. Gene **151:**73-79.

26. **van den Hombergh, J. P., P. J. I. van de Vondervoort, L. Fraissinet-Tachet, and J. Visser.** 1997. *Aspergillus* as a host for heterologous protein production: the problem of proteases. Trends Biotechnol **15:**256-263.

Chapter five

Characterization of an *Aspergillus niger* prolyl aminopeptidase

Daniëlle E.J.W. van Wijk - Basten, Antoine P.H.A. Moers and Peter J. Schaap

Summary

We have cloned the prolyl aminopeptidase encoding gene from *Aspergillus niger* (*papA*). Cell extracts of strains overexpressing the gene using its own promoter showed a 3 to 5 fold increase in prolyl aminopeptidase activity but not in phenylalanine and leucine aminopeptidase activity. The overexpressed enzyme was purified and characterized. The enzyme specifically hydrolyzes N-terminal proline and hydroxyproline residues from peptides. It is the first enzyme of its kind from a eukaryotic organism which has been characterized.

Introduction

Proteases normally do not hydrolyze proline adjacent bonds. Instead a specialized group of enzymes has evolved that hydrolyzes these bonds. Their activity depends on both the isomeric state of the proline residue and the position of the proline residue in the peptide chain (7, 23). Proline aminopeptidases (Pap, prolyl iminopeptidase, EC 3.4.11.5) are serine proteases (5) that catalyze the cleavage of amino terminal proline residues from peptides and so far these enzymes have only been characterized from several bacteria. Based on their molecular mass and on their substrate specificity these enzymes can be divided into two classes. The first class consists of monomeric enzymes with a molecular mass of approximately 30 kDa. Enzymes of this class appear to have a strict specificity for amino terminal proline residues (15). Monomeric enzymes have been characterized from *Bacillus coagulans* (16), *Lactobacillus delbrueckii* (4, 9), *Neisseria gonorrhoeae* (1), *Serratia marcescens* (11, 24) and *Xanthomonas campestris* (2, 19). The second class of enzymes comprise large multimeric enzymes that are not only capable of hydrolyzing proline terminals but have also been reported to be capable of hydrolyzing N-terminal hydroxyproline residues. Enzymes from the second class have only been cloned and characterized from the prokaryotes *Aeromonas sobria* (15), *Hafnia alvei* (14) and *Propionibacterium shermanii* (18).

No prolyl aminopeptidase function appears to be present in the genome of *Saccharomyces cerevisiae* (http://mips.biochem.mpg.de). Surprisingly we found an expressed sequence tag (EST) with homology to the multimeric class of enzymes in the ascomycetous fungus *Aspergillus nidulans*. This EST was used to clone the corresponding *Aspergillus niger* gene. This paper describes the characterization of the first eukaryotic prolyl aminopeptidase from *A. niger*, a fungus from which many enzymes are used in the food industry.

Materials and Methods

Bacterial strains, DNA and RNA techniques and sequence analysis.

Escherichia coli LE392 and DH5α (Promega, Madison, Wis.) were used for phage amplification and plasmid transformation and propagation respectively. Standard DNA and RNA manipulations were carried out essentially as described (21). Protein and nucleotide sequence analyses were done using the program DNAstar (Lasergene, Madison, Wis., USA). Public databases were searched with the BLAST search tools (3). Multiple alignments were done with CLUSTAL X (12).

For Northern analysis strains were grown for 17 h in minimal medium (20) supplemented with 1% glucose as carbon source and 0.5% yeast extract in 50 ml cultures in 250 ml Erlenmeyer flasks in an incubator shaker at 250 rpm at 30^{o}C. Total RNA was isolated from mycelium samples with Trizol reagent (Life Technologies, Rockville, Md.).

Cloning and overexpression of *papA*.

A *papA* PCR-product was generated by a PCR on genomic DNA of *Aspergillus nidulans* WG096 using primers Forward, 5'-TCTTCGAGGTTCCCCTC-3' and Reverse, 5'-CTATGTATGTCGCTGCG-3'. A standard program of 30 thermal cycles was composed of 1 min at 94°C, 1 min at 48°C, and 1 min at 72°C preceded by an incubation of 4 min at 94°C and followed

by an incubation of 5 min at 72°C. The amplified fragment was cloned in vector pGEM-T (Promega, Madison, Wis.) and identified by sequencing. Genomic sequences of the *A. niger papA* gene were obtained by screening an λ EMBL4 genomic library of *A. niger* strain N400 by standard methods using the *A. nidulans* PCR-product as a probe and non-stringent hybridization conditions. From a hybridizing plaque a 3.7 kb *Eco*RI fragment was recovered and cloned in pUC19 resulting in plasmid pIM4105, containing the *papA* gene with 5' and 3' flanking regions.

Plasmids pIM4105 and pGW635 (10) which contain the *A. niger pyrA* gene encoding orotidine-5'phosphate decarboxylase were used to co-transform *A. niger* NW219 (*cspA1*, *leuA1, nicA1, pyrA6)* (17) Transformants were first selected for uridine prototrophy and subsequently screened for enhanced prolyl aminopeptidase activity in cell extracts (CE). For this, strains were grown in shake flask cultures as described for the Northern analysis. Ground mycelium was extracted with 20 mM Tris-HCl pH 7.5 and clarified by centrifugation (10.000 *g* for 15 min. at 4°C). Aminopeptidase activity in these cell extracts was assayed as described below.

Aminopeptidase enzyme assay

Artificial substrates: Aminopeptidase activity was determined as described, using different amino acids coupled to para-nitroanilide (pNA) as substrate (4). Standard conditions were 2 mM P-pNA substrate in 20 mM Tris-HCl at pH 7.5 at 30°C. Aminopeptidase activity was also determined as described using amino acids coupled to β-naphthylamide (β-NA) as substrate (8). Standard conditions were then 1 mM β-NA substrate in 20 mM Tris-HCl at pH 7.5 at 30°C. L-pNA, P-pNA, R-pNA, F-pNA, A-pNA, M-pNA and K-pNA were obtained from Sigma (St. Louis, USA). V-pNA, G-pNA, I-pNA, E-pNA, P-βNA, hydroxy-P-βNA were obtained from Bachem (Bubendorf Switzerland).

Peptide substrates. The liberated proline from peptides was determined with ninhydrin (22). Standard conditions were 1 mM substrate in 20 mM Tris-HCl pH 7.5 at 30°C. Proline concentrations were calculated from a standard curve. The peptides AP, PA, PLG(NH_2), PPGFSPFR and PLSRTLSVAAKK were obtained from Sigma (St. Louis). One unit of enzyme activity is defined as the amount of enzyme that produces 1 μMol *p*NA, βNA or proline per minute.

Protein concentrations were determined with bicinchoninic acid as described by the manufacturer (Sigma chemical Co., St Louis, USA).

Purification of *A. niger* proline aminopeptidase A.

Strain Pap-1 was pre-grown in 300 ml MM (20) supplemented with 2% (w/v) glucose, 0.2% (w/v) meat peptone, 0.1% (w/v) yeast extract, 0.1% (w/v) peptone 140 and 0.03 (w/v) yeast ribo nucleic acids. After 6.5 h pre-growth this culture was poured into 5 l MM supplemented with 2% (w/v) glucose, 0.2% (w/v) meat peptone, 0.1% (w/v) yeast extract and 0.1% (w/v) peptone 140 and growth was continued for 17 h.

The mycelium was harvested, ground and resuspended in 20 mM Tris-HCl pH 7.5 and stirred for 15 min at 4°C. Cellular debris was removed by centrifugation. Then $(NH_4)_2SO_4$ was added to 40% saturation. After centrifugation (15 min. 10.000 x *g*) $(NH_4)_2SO_4$ was added to the supernatant to a concentration of 70% saturation. After centrifugation the pellet was dissolved in 20 mM Tris-HCl pH 7.5 and dialyzed against 20 mM Tris-HCl + 1.5 M $(NH_4)_2SO_4$.

Four chromatographic steps were applied to purify the protein. The dialyzed fraction was loaded onto a 20 ml Phenyl Sepharose high performance column (Pharmacia Biotech), pre-equilibrated with 20 mM Tris-HCl + 1.5 M $(NH_4)_2SO_4$ pH 7.5 and bound protein was eluted using a 200 ml linear gradient from 1.5 – 0 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl pH 7.5. The active fractions were pooled, dialyzed against 20 mM bis-Tris pH 6.5 and loaded onto a 15.5 ml Source 30 Q column (Pharmacia Biotech), pre-equilibrated with 20 mM bis-Tris pH 6.5

and eluted using a 155 ml linear gradient from 0 – 0.5 M NaCl in 20 mM bis-Tris pH 6.5. The active fractions were pooled, 10-fold diluted in 20 mM piperazine pH 6.0 and loaded onto a 1 ml Resource Q column (Pharmacia Biotech) pre-equilibrated with 20 mM piperazine pH 6.0. Bound protein was eluted using a 50 ml linear gradient from 0 – 0.5 M NaCl in 20 mM piperazine pH 6.0. The active fractions were pooled and loaded onto a 318 ml superdex 200 column (Pharmacia Biotech) equilibrated in 20 mM Tris-HCl + 0.1 M NaCl pH 7.5.

Biochemical characterization of prolyl aminopeptidase A

The optimal pH for enzymatic activity was determined using McIlvaine buffers ranging from pH 5 to pH 8, 200 mM HEPES buffer from pH 7.2 to pH 8 and 50 mM Tris-glycine buffers from pH 8 to pH 10. P-pNA was used as the substrate.

For determination of the thermal stability, the purified enzyme was pre-incubated at 0, 30, 37, 50 and 60°C for 30 min, followed by the standard enzyme reaction. The 0°C preincubated sample was used as reference.

The pH stability of PapA was determined by preincubation of the purified enzyme in McIlvaine buffers ranging from pH 2.2 to pH 8 at 30°C, for 30 min, followed by the standard enzyme reaction.

The effect of the protease inhibitors bestatin, 1,10-phenanthroline, EDTA, EGTA, tosyl phenylalanyl chloromethyl ketone (TPCK), tosyl lysyl chloromethyl ketone (TLCK) leupeptin, iodoacetamide and 4-chloromercuribenzoic acid (PCMB) on the enzymatic activity were measured in 200 mM HEPES buffer at pH 7.5. The purified enzyme was incubated with the respective compound for 30 min at 30°C, followed by the standard enzyme assay.

Results and discussion

Cloning of the *papA* gene.

By database searching we identified two *A. nidulans* expressed sequence tags (EST) which could encode different parts of a putative fungal prolyl aminopeptidase (PapA) similar to the multimeric prokaryotic prolyl aminopeptidases. We designed two primers based on these EST sequences and amplified a 1534-bp PCR product from *A. nidulans* genomic DNA. The PCR product specified a large fragment of the open reading frame (ORF) of the putative *A. nidulans papA* gene and the 5' and 3' ends of the sequence overlapped the sequences of two selected ESTs. This indicated that the EST sequences originated from the same gene. When all available sequences were joined, more than 95% of the ORF of the putative *A. nidulans* gene could be reconstructed. Under conditions of low stringency a genomic *A. niger* 3.7 kb *Eco*RI fragment hybridized with the *A. nidulans* PCR product. The fragment was recovered from an *A. niger* genomic λ–library and cloned, yielding plasmid pIM4105. The cloned fragment contained the complete open reading frame (ORF) of the gene and approximately 1600 bp upstream and 300 bp downstream region. The ORF encodes a protein of 491 amino acids interrupted by 7 introns. With RT-PCR we could obtain overlapping cDNA fragments, which after sequencing confirmed that the predicted introns were positioned correctly. Southern analysis using conditions of low stringency indicated that the *papA* gene is a single copy gene in *A. niger* (results not shown).

Analysis of the *papA* ORF sequence.

The inferred PapA amino acid sequence was analyzed for known subcellular sorting signals and none were found suggesting that the enzyme is located in the cytoplasm. Proline aminopeptidases from the bacteria were also reported as intracellular aminopeptidases (1, 2, 4, 15, 24). In addition, we could not detect proline aminopeptidase activity in the culture fluid. In a multiple

alignment the PapA protein displayed highest identity with the multimeric prokaryotic prolyl aminopeptidases exhibiting 37% and 34% identity with the *A. sobria* and *H. alvei* prolyl aminopeptidases respectively. Only a very limited similarity was obtained with the monomeric *B. coagulans* prolyl aminopeptidase (14%). The inferred *A. niger* PapA protein sequence is 74% identical to the *A. nidulans* protein sequence translated from the reconstructed DNA sequence. There is no ortholog of *pap*A in the sequenced genome of *S. cerevisiae* (http://mips.biochem.mpg.de), but a Blast search revealed that putative orthologs exist in *Arabidopsis thaliana* and *Talaromyces emersonii*. The motif GXSXG , commonly found in regions of the reactive serine in serine proteases (Kanatani et al., 1993; Mitta et al., 1998; Polgar, 1992) was found at amino acid residues 163-167, being GQSFG. This suggests that Ser 165 is the active site serine. An identical motif has been found in the enzymes of *H. alvei* (14), *A. sobria* (15), *B. coagulans* (16) and *P. shermanii* (18). By site directed mutagenesis, the serine in the GQSFG motif of *A. sobria* and *B. coagulans* was proven to be responsible for the catalytic activity (13). This motif is also found in the monomeric class of enzymes, however the exact motif is different from the multimeric class of enzymes being in most cases GGSWG but GHSWG and GQSWG also occur.

Characterization of *papA* overexpressing strains.

We transformed *A. niger* with plasmid pIM4105, and by Northern analysis at least 5 transformants could be identified with elevated expression of the *papA* gene. Highest mRNA levels were observed in transformant Pap-1. Southern analysis of transformants Pap-1 and 2 indicated that these transformants have integrated approximately 30 (Pap-1) and 10 (Pap-2) copies of the gene in their respective genomes (results not shown). We determined the proline, phenylalanine and leucine aminopeptidase activity in cell free extracts of Pap-1 and Pap-2 and of a wild type strain with *para*-nitroanilide (*p*NA) substrates. Pap-1 and Pap-2 have a 6-fold and 4-fold increase in prolyl aminopeptidase activity

respectively. No elevated hydrolysis of the phenylalanine and leucine *p*NA substrates was measured. In concord with the elevated prolyl aminopeptidase activities, highest messenger levels and highest copy number were found in transformant Pap-1.

Purification and biochemical characterization of the *A. niger* prolyl aminopeptidase.

Five purification steps were needed to purify PapA 1.4 10^3 fold from transformant Pap-1, resulting in an enzyme preparation with a specific activity of 2.9 10^2 U mg^{-1}, and a final yield of 16% (Table 1).

A SDS-PAGE of the purified PapA (Fig. 1) showed a single band with an apparent molecular mass of 50 kDa, in agreement with the molecular mass of 50.0 kDa calculated from the ORF. The molecular mass of the native enzyme as determined by gel filtration is 340 kDa, indicating that PapA is a multimeric enzyme like the reported enzymes from *Aeromonas* and *Hafnia* which both occur as a tetramer (14, 15).

Table 1 Purification of prolyl aminopeptidase A from *A. niger* Pap1.

Data are means of three separate experiments in which differences did not exceed 10%.

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification (-fold)	Yield (%)
Cell free extract	$3.7*10^2$	$1.8*10^3$	0.21	1	100
Phenyl Sepharose	$1.4*10^2$	67	2.1	10	38
Source 30 Q	78	1.2	65	$3.1*10^2$	21
Resource Q	82	0.70	$1.2*10^2$	$5.7*10^2$	22
Superdex 200	58	0.20	$2.9*10^2$	$1.4*10^3$	16

One unit of enzymatic activity (U) is defined as the amount of enzyme that produces 1 µmol *p*NA min^{-1}. Activity was determined with 2 mM P-*p*NA as a substrate.

The substrate specificity was determined using an extensive list of *p*NA and βNA substrates and several peptide substrates (di- tri- and oligomers). PapA was not able to hydrolyze artificial substrates other than P-*p*NA and hydroxy-P-βNA.

Remarkably PapA efficiently hydrolyzes P-*p*NA but not P-βNA. Cell extracts from *A. niger* were also tested for activity towards P-βNA but no (other) activity could be detected. The hydroxy-P-βNA substrate however, can be hydrolyzed by PapA, as well as by the enzymes of *Aeromonas* and *Hafnia* (14, 15).

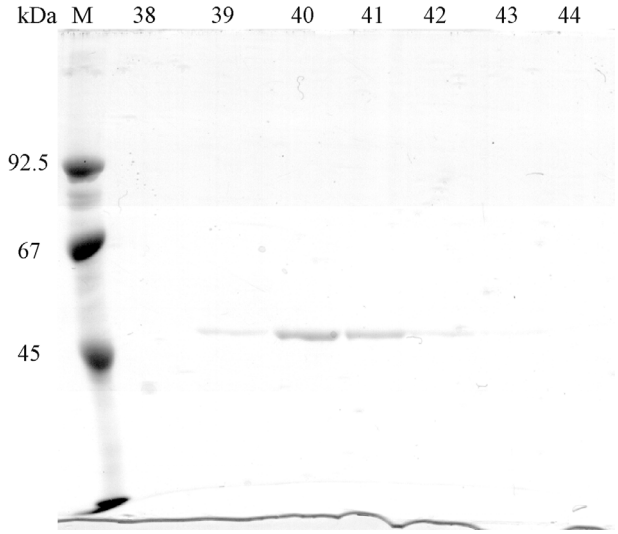

Fig. 1 Purification of prolyl aminopeptidase A from *A. niger*. Coomassie-stained SDS-polyacrylamide gel (10%) of 20 µl of the active fractions 38 to 44 eluted from the superdex 200 column. Fraction numbers are indicated above the lanes. M: Protein molecular weight marker.

The monomeric *Lactobacillus* and *Bacillus* enzymes were reported not to be active on hydroxy-P-βNA (15). The K_M of PapA for P-*p*NA is 0.037 mM, k_{cat} is 4.1 µkat mg^{-1}.

The activity towards peptide substrates was tested using peptides with increasing length and compared to the rate of hydrolysis of the *Aeromonas* and *Bacillus* enzymes (15) (Table 2). PapA is apparently able to efficiently hydrolyze both proline residues from the peptide substrate PPGFSPFR, therefore the overall hydrolysis rate for both proline residues is reported (Table 2). In contrast to the *Aeromonas* enzyme, the PLG substrate is more rapidly hydrolyzed than the longer

peptide substrates. Hydrolysis rates of dipeptides were not reported for the *Bacillus* and *Aeromonas* enzymes.

Table 2 Comparison of prolyl aminopeptidase activities of *A. niger, A. sobria* (15) and *B. coagulans* (15) on peptide substrates. n.a.: no activity detected, n.d.: not determined. PLSR(12): PLSRTLSVAAKK, PPGF(8): PPGFSPFR. *A. niger* data are the results of three independent experiments in which differences did not exceed 10%.

	Rate of hydrolysis (U mg^{-1})		
Substrate	*A. niger*	*A. sobria*	*B. coagulans*
PA	85.5	n.d.	n.d.
AP	n.a.	n.d.	n.d.
PLG(NH_2)	33.4	$5.2\ 10^{-2}$	57
PPFG(8)	55.5[1]	$4.7\ 10^{-1}$	n.a.
PLSR(12)	6.6	$3.6\ 10^{-1}$	$2.6\ 10^{-4}$

[1] Overall hydrolysis rate of both proline residues.

The pH optimum for P-*p*NA was determined. The pH optimum is broad, highest activity is found at pH 7.5, similar to a previously found intracellular aminopeptidase of *A. niger* (6) and equal to the pH optimum of the *Hafnia* enzyme (pH 7.0 – 7.5) but different from the pH optimum (pH 8.5) of the *A. sobria* enzyme (14, 15). Furthermore, the enzyme is stable from pH 5 to pH 8. Highest stability was found at pH 6 and pH 7. The enzyme is stable up to a temperature of 37°C, differing in this aspect from the *Aeromonas* and *Hafnia* enzymes which are reported to be stable up to temperatures of 57°C and 55°C, respectively (14, 15).

Several potential protease inhibitors were tested. EDTA did not influence enzyme activity, underpinning that the enzyme is not a metallo protease. The serine protease inhibitors TLCK and TPCK were able to inhibit the enzyme activity. 50% inhibition was achieved with 4 mM TLCK. However, PapA was strongly inhibited by PCMB. This inhibition is possibly caused by the modification of a cysteine residue located near the active site (11). Similar

inhibitory effects of PCMB were also reported for the *A. sobria* and *B. coagulans* prolyl aminopeptidases although these enzymes are proven to be serine proteases (13).

In this report we describe the biochemical characteristics of the first eukaryotic prolyl aminopeptidase. This aminopeptidase belongs to the multimeric class of prolyl aminopeptidases that not only hydrolyzes proline but also hydroxyproline at the amino terminal. In contrast to the bacterial enzymes, the hydrolysis rate of the fungal enzyme increases with a decrease in peptide substrate length.

Acknowledgements

The authors thank H. Kester for assistance with the biochemical work. P. Schaap was partially supported by the Dutch Technology Foundation (STW), grant WBI 4100.

References

1. **Albertson, N. H., and M. Koomey.** 1993. Molecular cloning and characterization of a proline iminopeptidase gene from *Neisseria gonorrhoeae*. Mol Microbiol **9:**1203-1211.
2. **Alonso, J., and J. L. Garcia.** 1996. Proline iminopeptidase gene from *Xanthomonas campestris* pv. *citri*. Microbiology **142:**2951-2957.
3. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-3402.
4. **Atlan, D., C. Gilbert, B. Blanc, and R. Portalier.** 1994. Cloning, sequencing and characterization of the *pep*IP gene encoding a proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397. Microbiology **140:**527-535.
5. **Barret, A. J., N. D. Rawlings, and J. F. Woessner.** 1998. Handbook of proteolytic enzymes. Academic Press, San Diego.
6. **Basten, D. E. J. W., J. Visser, and P. J. Schaap.** 2001. Lysine aminopeptidase of *Aspergillus niger*. Microbiology **147:**2045-2050.
7. **Cunningham, D. F., and B. O'Connor.** 1997. Proline specific peptidases. Biochim Biophys Acta **1343:**160-186.
8. **Frey, J., and K.-H. Röhm.** 1979. External and Internal Forms of Yeast Aminopeptidase II. Eur J Biochem **97:**169-173.
9. **Gilbert, C., D. Atlan, B. Blanc, and R. Portalier.** 1994. Proline iminopeptidase from *Lactobacillus delbrueckii* subsp *bulgaricus* CNRZ 397: purification and characterization. Microbiology **140:**537-542.
10. **Goosen, T., F. van Engelenburg, F. Debets, K. Swart, K. Bos, and H. Van den Broek.** 1989. Tryptophan auxotrophic mutants in *Aspergillus niger*: inactivation of the *trp*C gene by cotransformation mutagenesis. Mol gen genet **219:**282-288.

11. **Ito, K., T. Inoue, T. Kabshima, N. Kanada, H. Huang, X. Ma, N. Azmi, E. Azab, and T. Yoshimoto.** 2000. Substrate recognition mechanism of prolyl aminopeptidase from *Serratia marcescens.* J Biochem **128:**673-678.

12. **Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgens, and T. J. Gibson.** 1998. Multiple sequence alignment with Clustal X. Trends Biochem Sci **23:**403-405.

13. **Kitazono, A., K. Ito, and T. Yshimoto.** 1994. Prolyl aminopeptidase is not a sulfhydryl enzyme: identification of the active serine residue by site-directed mutagenesis. J Biochem **116:**943-945.

14. **Kitazono, A., A. Kitano, T. Kabashima, K.Ito, and T. Yoshimoto.** 1996. Prolyl aminopeptidase is also present in *Enterobacteriaceae*: cloning and sequencing of the *Hafnia alvei* enzyme-gene and characterization of the expressed enzyme. J Biochem **119:**468-474.

15. **Kitazono, A., A. Kitano, D. Tsuru, and T. Yoshimoto.** 1994. Isolation and characterization of the prolyl aminopeptidase gene (pap) from *Aeromonas sobria*: comparison with the *Bacillus coagulans* enzyme. J Biochem **116:**818-825.

16. **Kitazono, A., T. Yoshimoto, and D. Tsuru.** 1992. Cloning, sequencing and high expression of the proline iminopeptidase gene from *Bacillus coagulans*. J Bacteriol **174:**7919-7925.

17. **Kusters van Someren, M. A., J. A. M. Harmsen, H. C. M. Kester, and J. Visser.** 1991. Structure of the *Aspergillus pelA* gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. Curr Genet **20:**293-299.

18. **Leenhouts, K., A. Bolhuis, J. Boot, I. Deutz, M. Toonen, G. Venema, J. Kok, and A. Ledeboer.** 1998. Cloning, expression and chromosomal stabilization of the *Propionibacterium shermanii* proline iminopeptidase gene (pip) for food-grade application in *Lactococcus lactis*. Appl Environ Microbiol **64:**4736-4742.

19. **Medrano, F. J., J. Alonso, J. L. Garcia, A. Romero, W. Bode, and F. X. Gomis-Ruth.** 1998. Structure of proline iminopeptidase from *Xanthomonas campestris* pv *citri*: a prototype for the prolyl oligopeptidase family. EMBO J **17:**1-9.

20. **Pontecorvo, G., J. A. Roper, L. J. Hemmons, K. D. MacDonald, and A. W. J. Bufton.** 1953. The genetics of *Aspergillus nidulans*. Adv Genet **5:**141-238.

21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: a Laboratory Manual., 2 ed. Cold Spring Harbor, New York.

22. **Trool, W., and J. Lindsley.** 1955. A photometric method for the determination of proline. J Biol Chem **215:**655-660.

23. **Vanhoof, G., F. Goossens, I. d. Meester, D. Hendriks, and S. Scharpe.** 1995. Proline motifs in peptides and their biological processing. FASEB J **9:**736-744.

24. **Yoshimoto, T., T. Kabashima, K. Uchikawa, T. Inoue, N. Tanaka, K. Nakamura, M. Tsuru, and K. Ito.** 1999. Crystal structure of prolyl aminopeptidase from *Serratia marcescens*. J Biochem **126:**559-565.

Chapter six

General discussion

Aminopeptidases from *Aspergilli* were already described in the late sixties of the twentieth century. At the start of this research in 1997, several papers were published concerning (extracellular) aminopeptidases from different Aspergilli; mainly Aspergillus oryzae (4, 5, 23, 24, 28, 31, 35-40, 43-47, 52). In these papers, the purification and the characterization of the enzymes are described, however information about the corresponding genes is lacking. At that time there were only a limited number of fully sequenced organisms. To date there is a genomic sequence of Aspergillus niger at the Dutch company DSM (only limited available for research purposes), an annotated sequence of *A. nidulans* and a draft version of the *A. fumigatus* sequence (Table 1).

Table 1 The URLs and details of libraries used in Blast searches.# Seq: number of sequences in the database.

Organism	**Strain**	**URL**	**# Seq.**	**Release date**	**Source of DNA**
A. fumigatus	AF293	www.tigr.org	32 Mb	December 4, 2002	genomic
A. nidulans	FGSC A26	www.genome.ou.edu	6000	March 11, 1999	ÈST mixed vegetative and 24 asexual development
	FGSC A26	www-genome.wi.mit.edu	31 MB	July 8, 2003	genomic
A. flavus		www.genome.ou.edu	1400	February 22 2001	EST vegetative mycelia
A. niger	NRRL 3122	*in house*	38.8 MB		genomic

At the start of this project no aminopeptidase genes from *A. niger* (which is often used in the food industry because its products are generally regarded as safe) had been described. In this study a number of molecular techniques have been used to clone four aminopeptidase genes of *A. niger*. The architecture and regulation of these genes have been studied and the corresponding enzymes have been purified and characterized. Together, these aminopeptidases are able to hydrolyze a range of different peptides. ApsA and ApsB (chapters 2 and 3) are specific for peptides with arginine and lysine at their N-terminal end. ApsC

(chapter 4) is specific for peptides that start with an aromatic amino acid. PapA (chapter 5) specifically hydrolyses N-terminal proline and hydroxy-proline from peptides.

As all living organisms *Aspergilli* maintain a particular rate of protein turnover by continuous degradation and synthesis of proteins. Outside the cell, proteins are degraded into oligopeptides by extracellular broad specificity proteases and used as carbon and nitrogen sources. Further degradation of these oligopeptides into amino acids can be achieved by the interaction of extracellular or intracellular oligopeptidases, di-, tripeptidases and finally by carboxy- and aminopeptidases. Intracellular turnover of proteins by proteases provides a ready pool of amino acids as precursors for the synthesis of new proteins (3). In the cytoplasm, the proteasome is responsible for the rapid degradation of malfolded and intrinsically unstable proteins into oligopeptides (2, 11, 29, 30). The resulting oligopeptides are then broken down into amino acids by a succession of oligopeptidases and exopeptidases. In the archeon *Thermoplasma acidophilum* a factor called Tricorn (TRI) is found that degrades oligopeptides that result from the proteasome in a sequential manner (49). Tamura *et al.,* (48) describe that TRI is a self-compartmentalizing protease complex unrelated to the proteasome. With the Tricorn protease complex several factors seem to be associated. Some of these interacting factors have been identified as aminopeptidases. This complex acts downstream the proteasome, which leaves the cell with peptides of 6-12 amino acids. TRI then further degrades these oligopeptides into smaller peptides of 2-4 residues in size. These small peptides are than further degraded by aminopeptidases associated with TRI. Although a TRI-like complex has not been found in eukaryotes yet, the aminopeptidases (ApsA, ApsB, ApsC and PapA) described in this thesis might be associated with other proteins in a supramolecular complex.

In yeast large scale analysis of protein complexes has been done (16, 21, 27). The data sets generated in these studies are available at

http://www.mdsp.com/yeast and http://www.bind.ca. These databases have been searched for protein complexes harboring aminopeptidases. So far there is evidence that two aminopeptidases are in a complex. Methionine aminopeptidase (MAP2) has been found in a complex with RFC3 which is subunit 3 of replication factor Δ and is involved in DNA damage response and repair and DNA replication. The vacuolar aminopeptidase Y is found in a complex with PTC1, a serine-threonine protein phosphatase and SEC7, a guanine nucleotide exchange protein for ARF, involved in protein transport. Other aminopeptidases have not been found in complexes yet, but as these studies are ongoing, it is expected that more aminopeptidases will be found in complexes, taking a step forward in unraveling their physiological role.

The vacuole is responsible for the breakdown of long lived proteins (17, 18, 30). In yeast two vacuolar aminopeptidases, APY and LAPIV, are described,. We found a gene and a cDNA homologous to APY of Yeast, indicating the existence of such a function in *A. niger.* So far we have measured extracellular aminopeptidase activity only in a multi copy strain of ApsC (chapter 4). Thus the existence of genuine extracellular aminopeptidases from *A. niger* remains to be proven.

For a total hydrolysis of (small) peptides more peptidases are needed than just ApsA, ApsB, ApsC and PapA. Are there any additional aminopeptidases or does the organism deal with the remaining peptide fragments in a different way? In the yeast *Saccharomyces cerevisiae* 11 aminopeptidases have been purified and characterized (1, 6-10, 12-15, 19, 20, 22, 25, 26, 32-34, 41, 42, 50, 51, 53, 54) and from *A. niger* only four aminopeptidases have been purified and characterized (this thesis), thus it might be that there are more aminopeptidases present in *A. niger*.

In 2001, the Dutch company DSM has sequenced the complete genome of *A. niger*. The genome of *A. nidulans* has also been sequenced and annotated and there is a draft of the *Aspergillus fumigatus* genome (Table 1). We searched these

databases with the already characterized *A. niger* aminopeptidases, published aminopeptidases of other *Aspergilli* and with the yeast aminopeptidases. In addition we searched in the publical available EST libraries of *A. nidulans* and *A. flavus* (Table 1), which contain only a limited number of ESTs.

Homologs of A. niger ApsA and ApsB

A BlastP search with ApsA of *A. niger* against the annotated *A. niger* library resulted in three significant protein sequences, one being ApsA itself, one being ApsB and one additional sequence (Table 2). This additional sequence might be a leukotriene A4 hydrolase, since this sequence has highest homology with LTA4 of *Saccharomyces cerevisiae.* A BlastP search with ApsA against the annotated *A. nidulans* library resulted also in three significant protein sequences, two are orthologs of ApsA and ApsB and one which probably is a leukotriene A4 hydrolase (Table 2). A TblastN search with ApsA against the draft genome of *A. fumigatus* identified the same three proteins (Table 2).

A BlastP search with ApsB of *A. niger* resulted in four significant protein sequences. One being ApsB itself, one being ApsA, and one being probably an LTA4-like aminopeptidase (see above). In this Blast search a fourth ORF is detected, however this ORF does not contain the two characteristic motifs of the M1 family of aminopeptidases (GAMENWG and HEXXH). A further study is needed to determine if this ORF codes for an aminopeptidase.

Both the *S. cerevisiae* aminopeptidases AAPI and LAPI are most similar to ApsA and its orthologs in other *Aspergilli* (chapters 2 and 3). When a BlastP search with LTA4 of *S. cerevisiae* is done against the libraries, highest homology is found with the unknown ORF, therefore it is expected that this sequence codes for a leukotriene A4 hydrolase (LTA4). LTA4 has aminopeptidase activity.

When ApsA and ApsB were blasted against the *A. nidulans* and *A. flavus* EST data, *A. nidulans* ESTs coding for ApsB orthologs were found, indicating that ApsB is expressed in *A. nidulans*.

A more sensitive BlastP search with a HMM profile was done for the M1 family of aminopeptidases, however this did not result in any additional putative aminopeptidases.

Homologs of A. niger ApsC

Aminopeptidase C from *A. niger* seems to be a unique aminopeptidase among the *Aspergilli*. In Table 2 one sequence can be found in *A. nidulans* which has homology to ApsC from *A. niger*, however this homology is only over a small stretch of amino acids with a low expect value. ApsC hydrolyses N-terminal aromatic amino acids from peptides, thus the other *Aspergilli* must have different aminopeptidases or different ways to hydrolyze these amino acids from peptides.

Homologs of A. niger PapA

A BlastP search against the libraries with PapA revealed only PapA and its orthologs. So there is no second copy of *papA,* although it was expected that there might be an additional PapA with a narrower substrate specificity and a lower molecular mass (chapter 5). The organisms from which a prolyl aminopeptidase has been cloned seem to have either a multimeric prolylaminopeptidase with broad substrate specificity or a monomeric prolylaminopeptidase with a narrow substrate specificity. Two sequences were found in the *A. nidulans* library, however these sequences seem to originate from the same cDNA.

Homologs of S. cerevisiae BLHI

We furthermore used known *S. cerevisiae* aminopeptidases to search the genomic and EST libraries. We used bleomycin hydrolase, a cystein aminopeptidase and could find an ortholog in *A. niger* and *A. nidulans* but not (yet) in *A. fumigatus*. We also found an EST sequence in *A. nidulans*, indicating that this gene is also expressed in *A. nidulans.*

Homologs of S. cerevisiae MAP1 and MAP2

In the yeast *Saccharomyces cerevisiae*, two methionine aminopeptidases (MAP) exist. When a BlastP search is done with MAP1 and MAP2 against the libraries, six sequences can be found with homology towards these aminopeptidases in *A. nidulans* and only five and four in *A. niger* and *A. fumigatus*, respectively. However three of these *A. nidulans* sequences show only homology over a short stretch of amino acids. Only biochemical characterization of the proteins can prove if these are real aminopeptidases.

In the EST library of *A. nidulans* we find a large number of *MAP2* like sequences (21) but no *MAP1* like sequences, probably *MAP1* is only expressed at special conditions, not available in the *A. nidulans* EST library.

Homologs of the S. cerevisiae vacuolar aminopeptidases APY and LAPIV

We know that there is at least one additional aminopeptidase present in *A. niger,* an aminopeptidase with high homology to aminopeptidase Y (APY) of *S. cerevisiae*. In yeast, APY is a vacuolar aminopeptidase involved in the breakdown of peptides in the vacuole. We were able to clone a gene and the corresponding cDNA of *A. niger* similar to APY of *S. cerevisiae,* however, the sequence was only partially determined. When BlastP search with APY of *S. cerevisiae* is done against the genomic libraries then two different sequences appear in *A. fumigatus*, and three different sequences appear in *A. niger* and *A. nidulans*. In the (patent) literature additional APY like sequences of *A. oryzae* can be found. When a BlastP search is done with these sequences, additional APY-like sequences are found in *A. niger*, *A. nidulans*, and *A. fumigatus*. In total five different putative aminopeptidase genes from the M28 family are found in the *A. niger* sequence. Only biochemical characterization can prove that these enzymes are aminopeptidases. In the genomes of *A. nidulans* and *A. fumigatus* only four putative aminopeptidase are found from this family.

The most extensively investigated aminopeptidase is probably aminopeptidase I (LAPIV) of *S. cerevisiae*, a large dodecameric aminopeptidase in the vacuole. All the *Aspergilli* tested have two different sequences that might encode for a LAPIV like enzyme. There are also EST sequences of *A. nidulans,* which seem to code for LAPIV. Thus LAPIV seems to exist in the *Aspergilli* and there is also evidence for an additional copy.

With the aminopeptidases of *A. niger* cloned so far, *A. niger* is able to hydrolyze N-terminal lysine, arginine, leucine, methionine, aromatic amino acids and even proline. However, for a total hydrolysis of peptides into its amino acids, the fungus needs additional proteases.

One possibility is that the not yet cloned and characterized aminopeptidases are able to hydrolyze these N-terminal amino acids. A second possibility is that these aminopeptidases have overlapping substrate specificities, but that these enzymes are expressed under special conditions, or in specialized compartments or outside the cell. It is known that yeast APY and API are vacuolar. It might be that the additional aminopeptidases have the same substrate specificity but a different location. A third possibility is that the fungus uses additional carboxypeptidases, di-, tri peptidases and or di-, tri- peptidylpeptidases for a total hydrolysis of a protein into its amino acids.

In this thesis we elucidated (part) of the aminopeptidase spectrum of *A. niger*. Four aminopeptidases (ApsA, ApsB, ApsC and PapA) are described. The genes were cloned, the enzymes were purified, and they were biochemically characterized. The sequencing of the genomes of *A. niger* *A. nidulans* and *A. fumigatus* enlarges the knowledge about additional copies of genes, and gives information about how many additional aminopeptidase genes may be expected. However, genomic information only is not enough to get a complete picture about catabolism of peptides. Information of loss of function strains is also important to elucidate if these enzymes are essential or if other enzymes can take over the lost function. Furthermore the biochemical data are of outstanding interest. What

kinds of substrates can be hydrolyzed by the aminopeptidase, why these kinds of substrates, what are the amino acids involved in substrate binding, what are the amino acids involved in catalyses? All this information combined enables us to assess the function of a gene product. Genome information alone is of limited value.

In this study we have revealed four aminopeptidases from *A. niger*, however the whole spectrum may be far from complete yet and it remains an intriguing area of study.

Table 2 BlastP searches of aminopeptidases in the protein libraries of A. niger and A. nidulans (A. nid.). TBlastN searches of aminopeptidases in the genomic A. fumigates (A. fum.) library. Sequences with an Expect value <0.0001 are shown.

Family	***Protein***	**Organism**	**Acc. no.**	**A. niger**			***A. nid***			***A. fum***		
				Seq ID	Score (Bits)	E value	Seq ID	Score (Bits)	E value	Seq ID	Score (Bits)	E value
M1	ApsA	An	CAC38353	g03045	1677	0.0	AN1638	1473	0.0	4826	1509	0.0
				g11592	642	0.0	AN4282	662	0.0	4892	132	0.0
				g09551	69	7 e^{-12}	AN5812	83	3 e^{-16}	4856	53	1 e^{-06}
	ApsB	An	CAD10746	g11592	881	0.0	AN4281	1443	0.0	4892	414	0.0
				g03045	877	0.0	AN1638	674	0.0	4826	671	0.0
				g09551	539	1.6 e^{-18}	AN5812	100	3 e^{-21}	4856	50	1 e^{-04}
				g11146	584	1.2 e^{-05}	AN1494	55	1 e^{-07}	4416	45	1 e^{-03}
	LAPI	Sc	CAA4403	g03045	877	0.0	AN1638	943	0.0	4826	782	0.0
				g11592	881	0.0	AN4282	641	0.0	4892	89	0.0
				g09551	488	4.7 e^{-15}	AN5812	81	1 e^{-15}			
	AAPI	Sc	NP_011913	g03045	878	0.0	AN1638	907	0.0	4826	736	0.0
				g11592	881	0.0	AN4282	634	0.0	4892	107	0.0
				g09551	485	2.1 e^{-15}	AN5812	84	1 e^{-16}	4856	48	1 e^{-09}
	LTA4	Sc	CAA64237	g09551	658	5 e^{-168}	AN5812	561	1 e^{-160}	4856	327	0.0
				g11592	424	1.1 e^{-16}	AN1638	86	3 e^{-17}	4826	77	1 e^{-13}
				g03045	471	5.2 e^{-14}	AN4282	86	4 e^{-17}	4892	43	1 e^{-02}
M18	LAPIV	Sc	NP_012819	g12070	358	2.0 e^{-99}	AN8252	365	1 e^{-101}	4892	339	0.0
				g07082	265	3.0 e^{-71}	AN2966	232	2 e^{-61}	4805	165	0.0
M24	MAPI	Sc	AAA75193	g09712	367	9 e^{-127}	AN5199	444	1 e^{-125}	4963	278	0.0
				g09711	372	3.0 e^{-115}	AN5055	348	1 e^{-96}	4917	206	0.0
				g06818	323	3.0 e^{-05}	AN6641	84	8 e^{-17}			
							AN4404	50	1 e^{-06}			
							AN1582	42	4 e^{-04}			
	MAP2	Sc	AAC49142	g06818	431	4.0 e^{-123}	AN4404	434	1 e^{-122}	4917	304	0.0
				g09708	475	1.0 e^{-100}	AN0369	384	1 e^{-107}	4826	119	0.0
				g06757	330	2.8 e^{-04}				4928	64	0.0
M28	APY	Sc	AAA19559	g12069	489	8 e^{-122}	AN3918	417	1 e^{-117}	4816	341	0.0
				g07083	380	7.8 e^{-10}	AN8445	271	2 e^{-73}	4968	234	0.0
				g07080	378	1.7 e^{-09}	AN7035	61	7 e^{-10}			
	ApII	Ao		g12069	504	5.3 e^{-90}	AN8445	669	0.0	4968	479	0.0
				g08436	487	2.5 e^{-07}	AN3918	313	9 e^{-86}	4816	261	0.0
				g07080	285	9.6 e^{-07}	AN4018	45	5 e^{-05}	4516	78	1 e^{-15}
				g05298	598	2.0 e^{-04}	AN7035	44	8 e^{-05}	4932	57	1 e^{-03}
	Ap	Ao		g07080	389	3.0 e^{-106}	AN7035	382	1 e^{-106}	4882	23	0.0
				g07083	436	3.1 e^{-44}	AN3918	49	2 $^{-06}$	4816	124	1 e^{-04}
				g12069	458	8.0 e^{-08}	AN8445	45	3 e^{-05}			
							AN4018	42	3 e^{-04}			
S09	ApsC	An	CAC87723	g06359	1311	0.0	AN9372	45	5 e^{-05}			
				g12239	87	2 e^{-17}	AN1478	44	1 e^{-04}			
				g06908	42	6 e^{-04}						
S033	PapA	An	CAC40647	g03065	862	0.0	AN2092	737	0.0	4929	18	0.0
C01	BLHI	Sc	CAA48878	g07302	352	2 e^{-97}	AN6399	368	e^{-102}			

Sc: Saccharomyces cerevisiae
An: Aspergillus niger
Ao: Aspergillus oryzae

References:

1. **Achstetter, T., C. Ehmann, and D. H. Wolf.** 1982. Aminopeptidase Co, A new yeast peptidase. Biochem Biophys Res Commun **109:**341-347.
2. **Baumeister, W., J. Waltz, F. Zühl, and E. Seemüller.** 1998. The proteasome: paradigm of a self-compartmentalizing protease. Cell **92:**367-380.
3. **Bennet, J. W., and M. A. Klich.** 1992. *Aspergillus*, Biology and industrial applications. Butterworth-Heinemann, Boston.
4. **Blinkovsky, A., E. Golightly, T. Byun, and L. Kofod.** 1998. Polypeptides having aminopeptidase activity and nucleic acids encoding same. Patent application PCT WO9851804.
5. **Blinkovsky, A. M., T. Byun, K. Brown, E. J. Golightly, and A. V. Klotz.** 2000. A non-specific aminopeptidase from *Aspergillus*. Biochim Biophys Acta **1480:**171-181.
6. **Bordallo, J., R. Cueva, and P. Suarez-Rendueles.** 1995. Transcriptional regulation of the yeast vacuolar aminopeptidase yscI encoding gene (APE1) by carbon sources. FEBS Lett **364:**13-16.
7. **Bordallo, J., and P. Suarez-Rendueles.** 1995. Identification of regulatory proteins that might be involved in carbon catabolite repression of the aminopeptidase I gene of the yeast *Saccharomyces cerevisiae*. FEBS Lett **376:**120-124.
8. **Caprioglio, D. R., C. Padilla, and M. Werner-Washburne.** 1993. Isolation and characterization of AAP1; a gene encoding an alanine / arginine aminopeptidase in yeast. J Biol Chem **268:**14310-14315.
9. **Chang, Y.-H., and J. A. Smith.** 1989. Molecular Cloning and Sequencing of Genomic DNA Encoding Aminopeptidase I from *Saccharomyces cerevisiae*. J Biol Chem **264:**6979-6983.
10. **Chang, Y.-H., U. Teichert, and J. A. Smith.** 1992. Molecular Cloning, Sequencing, Deletion, and Overexpression of a Methionine

Aminopeptidase Gene from *Saccharomyces cerevisiae*. J Biol Chem **267:**8007-8011.

11. **Ciechanover, A.** 1998. The ubiquitin-proteasome pathway: on protein death and cell life. EMBO J **17:**7151-7161.
12. **Cueva, R., N. Garcia-Alvarez, and P. Suarez-Rendueles.** 1989. Yeast vacuolar aminopeptidase yscI. Isolation and regulation of the APE1 (LAP4) structural gene. FEBS Lett **259:**125-129.
13. **Enenkel, C., and D. H. Wolf.** 1993. *BLH1* Codes for a Yeast Thiol Aminopeptidase, the Equivalent of Mammalian Bleomycin Hydrolase. J Biol Chem **268:**7036-7043.
14. **Frey, J., and K.-H. Röhm.** 1978. Subcellular localization and levels of aminopeptidases and dipeptidase in *Saccharomyces cerevisiae*. Biochim Biophys Acta **527:**31-41.
15. **Garcia-Alvarez, N., R. Cueva, and P. Suarez-Rendueles.** 1991. Molecular cloning of soluble aminopeptidases from *Saccharomyces cerevisiae;* Sequence analysis of aminopeptidase yscII, a putative zinc-metallopeptidase. Eur J Biochem **202:**993-1002.
16. **Gavin, A. C., M. Bosche, R. Krause, P. Grandi, M. Marzioch, A. Bauer, J. Schultz, J. M. Rick, A. M. Michon, C. M. Cruciat, M. Remor, C. Hofert, M. Schelder, M. Brajenovic, H. Ruffner, A. Merino, K. Klein, M. Hudak, D. Dickson, T. Rudi, V. Gnau, A. Bauch, S. Bastuck, B. Huhse, C. Leutwein, M. A. Heurtier, R. R. Copley, A. Edelmann, E. Querfurth, V. Rybin, G. Drewes, M. Raida, T. Bouwmeester, P. Bork, B. Seraphin, B. Kuster, G. Neubauer, and G. Superti-Furga.** 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature **415:**141-147.
17. **Goldberg, A. L., T. N. Akopian, A. F. Kisselev, and D. H. Lee.** 1997. Protein degradation by the proteasome and dissection of its in vivo importance with synthetic inhibitors. Mol Biol Rep **24:**69-75.

18. **Goldberg, A. L., T. N. Akopian, A. F. Kisselev, D. H. Lee, and M. Rohrwild.** 1997. New insights into the mechanisms and importance of the proteasome in intracellular protein degradation. Biol Chem **378:**131-140.

19. **Herrera-Camacho, I., R. Morales-Monterrosas, and R. Quiróz-alvarez.** 2000. Aminopeptidase yscCo-II: a new cobalt-dependent aminopeptidase from yeast - purification and biochemical characterization. Yeast **16:**219-229.

20. **Herrera-Camacho, I., and P. Suárez-Rendueles.** 1996. Regulation of the yeast vacuolar aminopeptidase Y gene (*APY1*) expression. FEMS Microbiol Lett **139:**127-132.

21. **Ho, Y., A. Gruhler, A. Heilbut, G. D. Bader, L. Moore, S. L. Adams, A. Millar, P. Taylor, K. Bennett, K. Boutilier, L.Yang, C. Wolting, I. Donaldson, S. Schandorff, J. Shewnarane, M. Vo, J. Taggart, M. Goudreault, B. Musk, C. Alfarano, D. Dewar, Z. Lin, K. Michalickova, A. R. Willems, H. Sassi, P. A. Nielsen, K. J. Rasmussen, J. R. Andersen, L. E. Johansen, L. H. Hansen, H. Jespersen, A. Podtelejnikov, E. Nielsen, J. Crawford, V. Poulsen, B. D. Sorensen, J. Matthiesen, R. C. Hendrickson, F. Gleeson, T. Pawson, M. F. Moran, D. Durocher, M. Mann, C. W. Hogue, D. Figeys, and M. Tyers.** 2002. Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. Nature **415:**180-183.

22. **Kambouris, N. G., D. J. Burke, and C. E. Creutz.** 1992. Cloning and Characterization of a Cystein Proteinase from *Saccharomyces cerevisiae*. J Biol Chem **267:**21570-21576.

23. **Kauppinen, M. S., J. Qi Si, C. Dambmann, T. Halkier, P. R. Østergaard, S. A. Patkar, and K. Hansen.** 2001. Enzymes with aminopeptidase activity. Patent US6,200,792 B1.

24. **Kauppinen, S., J. Qi Si, T. Spendler, C. Dambmann, T. Halkier, and P. R. Østergaard.** 1996. An enzyme with aminopeptidase activity. Patent application PCT WO962854.

25. **Kull, F., E. Ohlson, and J. Z. Haeggström.** 1999. Cloning and Characterization of a Bifunctional Leukotriene A4 Hydrolase from *Saccharomyces cerevisiae*. J Biol Chem **274:**34683-34690.

26. **Kull, F., E. Ohlson, B. Lind, and J. Z. Haeggström.** 2001. *Saccharomyces cerevisiae* Leukotriene A4 Hydrolase: Formation of Leukotriene B4 and Identification of Catalytic Residues. Biochemistry **40:**12695-12703.

27. **Kumar, A., and M. Snyder.** 2002. Protein complexes take the bait. Nature **415:**123-124.

28. **Labbe, J.-P., and P. Rebeyrotte.** 1974. Isolation of fractions having aminopeptidase activity (EC 3.4.1.1) (arylamidase) from culture medium of *Aspergillus oryzae*. Biochimie **56:**839-844.

29. **Lee, D. H., and A. L. Goldberg.** 1998. Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol **8:**397-403.

30. **Lee, D. H., and A. L. Goldberg.** 1996. Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in Saccharomyces cerevisiae. J Biol Chem **271:**27280-27284.

31. **Lehmann, K. v., and H. Uhlig.** 1969. Aminopeptidases aus *Aspergilli,* I. Hoppe-Seyler's Z. Physiol. Chem. **350:**99-104.

32. **Li, X., and Y.-H. Chang.** 1995. Amino-terminal protein processing in *Saccharomyces cerevisiae* is an essential function that requires two distinct methionine aminopeptidases. Proc Natl Acad Sci USA **92:**12357-12361.

33. **Magdolen, U., G. Müller, V. Magdolen, and W. Bandlow.** 1993. A yeast gene (*BLH1*) encodes a polypeptide with high homology to

vertebrate bleomycin hydrolase, a family member of thiol proteinases. Biochim Biophys Acta **1171:**299-303.

34. **Metz, G., and K. H. Röhm.** 1976. Yeast aminopeptidase I. Chemical composition and catalytic properties. Biochim Biophys Acta **429:**933-949.

35. **Nakadai, T., and S. Nasuno.** 1977. Purification and properties of leucine aminopeptidase IV from *Aspergillus oryzae*. Agric Biol Chem **41:**1657-1666.

36. **Nakadai, T., S. Nasuno, and N. Iguchi.** 1972. The Action of Peptidases from *Aspergillus oryzae* in Digestion of Soybean Proteins. Agric Biol Chem **36:**261-268.

37. **Nakadai, T., S. Nasuno, and N. Iguchi.** 1972. The action of Peptidases from *Aspergillus sojae* on Soybean Proteins. Agric Biol Chem **36:**1239-1243.

38. **Nakadai, T., S. Nasuno, and N. Iguchi.** 1973. Purification and properties of leucine aminopeptidase I from *Aspergillus oryzae*. Agric Biol Chem **37:**757-766.

39. **Nakadai, T., S. Nasuno, and N. Iguchi.** 1973. Purification and properties of leucine aminopeptidase II from *Aspergillus oryzae*. Agric Biol Chem **37:**767-774.

40. **Nakadai, T., S. Nasuno, and N. Iguchi.** 1973. Purification and properties of leucine aminopeptidase III from *Aspergillus oryzae*. Agric Biol Chem **37:**775-778.

41. **Nishizawa, M., T. Yasuhara, T. Nakai, Y. Fujiki, and A. Ohashi.** 1994. Molecular Cloning of the Aminopeptidase Y Gene of *Saccharomyces cerevisiae*. J Biol Chem **269:**13651-13655.

42. **Rudberg, P. C., F. Tholander, M. M. G. M. Thunnissen, and J. Z. Haeggström.** 2002. Leukotriene A4 Hydrolase/Aminopeptidase. J Biol Chem **277:**1398-1404.

43. **Schuster, E., B. Sprössler, K. Titze, M. Gottschalk, N. Q. Khanh, S. Wolf, and H. Plainer.** 1997. Leucine aminopeptidases produced recombinantly from *Aspergillus soyae*. Patent application 97/04108.

44. **Stevens, L.** 1985. Regulation of proteinase activity in *Aspergillus nidulans*. Biochem Soc Trans **13:**283-285.

45. **Sugiura, M., M. Ishikawa, and M. Sasaki.** 1978. Some Properties of Leucine Aminopeptidase from *Aspergillus japonica* as a Metalloenzyme. Chem Pharm Bull **26:**3101-3106.

46. **Sugiura, M., M. Suzuki, M. Ishikawa, and M. Sasaki.** 1976. Pharmaceutical Studies on Aminopeptidase from *Aspergillus japonica* I. Chem Pharm Bull **24:**2286-2293.

47. **Sugiura, M., M. Suzuki, M. Ishikawa, and M. Sasaki.** 1976. Purification and Properties of Leucine Aminopeptidase from *Aspergillus japonica*. II. Chem Pharm Bull **24:**2026-2031.

48. **Tamura, N., F. Lottspeich, W. Baumeister, and T. Tamura.** 1998. The Role of Tricorn Protease and Its Aminopeptidase-Interacting Factors in Cellular Protein Degradation. Cell **95:**637-648.

49. **Tamura, T., N. Tamura, Z. Cejka, R. Hegerl, F. Lottspeich, and W. Baumeister.** 1996. Tricorn Protease - The Core of a Modular Proteolytic System. Science **274:**1385-1389.

50. **Tisljar, U., and D. H. Wolf.** 1993. Purification and characterization of the cystinyl bond cleaving yeast aminopeptidase yscXVI. FEBS lett **322:**191-196.

51. **Trumbly, R. J., and G. Bradley.** 1983. Isolation and characterization of aminopeptidase mutants of *Saccharomyces cerevisiae*. J Bacteriol **156:**36-48.

52. **Umitsuki, G., and K. Abe.** 1999. Leucine aminopeptidase gene, recombinant dna, and process for producing leucine aminopeptidase. Patent application EP0967286A2.

53. **Xu, H. E., and S. A. Johnston.** 1994. Yeast Bleomycin Hydrolase Is a DNA-binding Cysteine Protease. J Biol Chem **269:**21177-21183.

54. **Yasuhara, T., T. Nkai, and A. Ohashi.** 1994. Aminopeptidase Y, a New Aminopeptidase from *Saccharomyces cerevisiae*. J Biol Chem **269:**13644-13650.

Summary

Aspergillus niger is a filamentous fungus that can grow in many environments, on several substrates at different conditions. In the soil, *Aspergilli* recycle nutrients by the degradation of plant material. In particular, *Aspergilli* are known for their capability to spoil plant materials like grains and fruits. In the industry *Aspergilli* are often being used as hosts for the production of both homologous and heterologous (foodgrade) enzymes.

In the food industry aminopeptidases from *A. niger* are being used for the production of cheese, for baking and the preparation of soy hydrolysates. The use of *Aspergilli* as hosts for the production of industrial proteins has resulted in detailed studies on the proteolytic spectra. Although the endopeptidase spectrum is thoroughly studied, the aminopeptidase spectrum is not. In this thesis aminopeptidases of *A. niger* are analyzed. Aminopeptidases are classified according to their catalytic activity into metallo, serine and cysteine aminopeptidases.

Aminopeptidase A (ApsA) was cloned using conserved regions within the M1 family of metallo aminopeptidases. The derived amino acid sequence of this aminopeptidase is highly similar to two yeast aminopeptidases. The protein was overexpressed in *A. niger* and the purified product was characterized. ApsA prefers lysine at the N-terminal end of a peptide. The K_M and K_{cat} for the artificial substrate K-*p*NA is 0.17 mM and 0.49 μkat mg^{-1}. The pH optimum of the enzyme is between pH 7.5 and 8.

A conserved region of ApsA was used to search for a paralog. Indeed a paralog was found, aminopeptidase B (ApsB). ApsB has 40% sequence identity with ApsA, but the encoding genes differ in architecture. *apsB* is interrupted by eleven introns while the ORF of ApsA is interrupted by only one. None of these introns show positional conservation. ApsA and ApsB are encoded by similar genes but have different substrate specificities. The results suggest that *apsA* and *apsB* are the result of an ancient duplication event and that both proteins have developed their own niche of intracellular peptide degradation.

A third aminopeptidase (ApsC) of *A. niger* was cloned using degenerated primers based on sequenced fragments of purified ApsC from an industrial *A. niger* strain. This strain was selected for its high (extracellular) ApsC activity. The amino acid sequence of ApsC is not similar to any previously characterized aminopeptidase, but shares sequence similarity to mammalian acyl-peptide hydrolases, however ApsC is not able to hydrolyze N-acetylalanine-*p*NA, a substrate for acyl- peptide hydrolases. ApsC is active towards N-terminal aromatic amino acids, especially phenylalanine. The pH optimum for this aminopeptidase is between pH 5 and 5.5 which is rather uncommon for a cytoplasmic aminopeptidase.

The fourth aminopeptidase described in this thesis is PapA. PapA is capable of hydrolyzing N-terminal proline and hydroxyproline. This enzyme was described earlier in prokaryotes, however, PapA is the first enzyme of this class of enzymes described in eukaryotes.

Together these four aminopeptidases are responsible for the breakdown of a whole range of peptide fragments. However, as discussed, there are still some aminopeptidases to be found. In this world were genomic sequences become available fast the actual cloning of a gene is facilitated and less time consuming, however, the biochemical characterization of the aminopeptidases is still very important as well as the study of all the interactions of genes and enzymes in the breakdown of a (large) peptide into its amino acids.

Samenvatting

Aspergillus niger is een filamenteuze schimmel die in bijna elke omgeving kan groeien, op verschillende substraten, onder verschillende condities. Andere *Aspergilli* zijn bekend vanwege hun vermogen om granen en fruit te bederven.

In de industrie wordt A*spergillus* vaak gebruikt bij de productie van zowel homologe als heterologe "foodgrade" enzymen. In de voedselindustrie worden aminopeptidases van *A. niger* gebruikt bij de productie van kaas, bij bakken en bij de productie van soja hydrolysaten. Het gebruik van *Aspergilli* als gastheer voor de productie van industriële enzymen heeft geresulteerd in gedetailleerde onderzoeken van het proteolytische spectrum.

Alhoewel het endoprotease spectrum uitgebreid bestudeerd is, is dat niet het geval bij de aminopeptidases. In dit proefschrift wordt een deel van het aminopeptidase spectrum van *A. niger* beschreven.

Aminopeptidases worden geclassificeerd naar hun katalytische activiteit in metallo aminopeptidases, serine aminopeptidases en cysteine aminopeptidases.

Aminopeptidase A (ApsA) is gekloneerd door gebruik te maken van geconserveerde gebieden binnen de M1 familie van metallopeptidases. De verkregen aminozuur sequentie van deze aminopeptidase lijkt sterk op twee gist aminopeptidases. Het eiwit is tot overexpressie gebracht in *A. niger* en het gezuiverde product is gekarakteriseerd. ApsA heeft een voorkeur voor lysine aan de N-terminale kant van een eiwit. De K_M en de k_{Cat} voor het artificiële substraat K-*p*NA zijn 0.17 mM en 0.49 μKat mg^{-1}. Het pH optimum van het enzym ligt tussen pH 7.5 en 8.

Een geconserveerd gebied van ApsA is gebruikt om een paraloog van ApsA te vinden. Inderdaad is er een paraloog gevonden, aminopeptidase B (ApsB). De sequentie van ApsB is 40% identiek aan ApsA, maar de coderende genen zijn verschillend in architectuur. ApsB wordt onderbroken door elf intronen en ApsA door slechts twee. Geen van deze intronen is positioneel geconserveerd. ApsA en ApsB worden gecodeerd door soortgelijke genen, maar ze hebben

verschillende substraat specificiteiten. De resultaten suggereren dat *aspA* en *apsB* het gevolg zijn van een oude duplicatie waarna beide eiwitten hun eigen niche hebben ontwikkeld m.b.t. intracellulaire eiwit afbraak.

Een derde aminopeptidase van *A. niger*; ApsC, is gekloneerd door gedegenereerde primers te gebruiken die gebaseerd zijn op partiele eiwitvolgordes van gezuiverd ApsC. Dit ApsC was gezuiverd uit een industriële *A. niger* stam en geselecteerd op z'n hoge (extracellulaire) ApsC activiteit. De aminozuur sequentie van ApsC lijkt niet op enige andere eerdere gekarakteriseerde aminopeptidase, maar heeft enige overeenkomst met acyl-peptide hydrolases van zoogdieren. ApsC is echter niet in staat om N-acetylalanine *p*NA (een substraat voor acyl-peptide hydrolases) te hydrolyseren. ApsC hydrolyseert N-terminale aromatische aminozuren van eiwitten, vooral phenylalanine. Het pH optimum van dit eiwit ligt tussen pH 5 en 5.5 wat nogal ongewoon is voor een cytoplasmatisch eiwit.

De vierde aminopeptidase die in dit proefschrift wordt beschreven is PapA. PapA kan N-terminale proline en hydroxyproline hydrolyseren van eiwitten. Een soortgelijk enzym is beschreven in prokaryoten, echter PapA is het eerst beschreven enzym van deze klasse van enzymen in eukaryoten.

Samen zijn deze vier aminopeptidases in staat om verschillende eiwitten af te breken, echter zoals bediscussieerd is zijn er nog een aantal additionele aminopeptidases te verwachten. In deze wereld, waar de complete genoom sequenties van verschillende organismen steeds sneller beschikbaar komen, wordt het kloneren van een gen steeds gemakkelijker en minder tijdrovend, echter de biochemische karakterisering van aminopeptidases blijft nog steeds erg belangrijk evenals de interactie van genen en enzymen in de afbraak van een (groot) eiwit tot zijn aminozuren.

Curriculum vitae

Daniëlle Everdina Josephina Wilhelmina Basten is op 7 september 1971 in Nijmegen geboren. In 1989 behaalde ik mijn VWO diploma aan scholengemeenschap “Oost-Betuwe” te Bemmel en begon ik aan de studie Biologie aan de toenmalige Landbouw Universiteit in Wageningen. Aangezien deze studie niet was wat ik ervan verwachtte vervolgde ik in 1991 met de studie laboratoriumtechniek, richting microbiologie / biochemie aan hogeschool Larenstein te Wageningen / Velp. Na een stage en een afstudeervak bij het Leidse planten biotechnologie bedrijf MOGEN behaalde ik in 1994 mijn diploma. In dat zelfde jaar keerde ik terug naar de Landbouw Universiteit alwaar ik doorstroomde in de studie Bioprocestechnologie, de cellulaire richting met microbiologie als profiel. In maart 1997 werd deze studie afgerond na een afstudeervak bij de sectie Moleculaire Genetica van Industriële Micro-organismen (MGIM) onder leiding van Dr. J. Visser. In maart 1997 werd ik bij deze vakgroep eerst 6 maanden als toegevoegd onderzoeker aangesteld en in september werd ik bij deze zelfde vakgroep als AIO aangesteld bij het in dit proefschrift beschreven onderzoek. Sinds 1 april 2002 ben ik werkzaam als postdoc bij de leerstoelgroep plantenfysiologie van de Wageningen Universiteit in het kader van een EU project.

Publications

Papers:

Basten, D. E. J. W., P. J. T. Dekker, and P. J. Schaap. 2003. Aminopeptidase C of *Aspergillus niger* is a Novel Phenylalanine Aminopeptidase. Appl Environ Microbiol **69:**1246-1250.

Basten D. E. J. W., J. Visser and P. J. Schaap. 2001. Lysine aminopeptidase from *Aspergillus niger*. Microbiol **147**:2045-2050

De Groot, P. W. J., D. E. J. W. Basten, A. S. M. Sonnenberg, L. J. L. D. van Griensven, J. Visser, and P. J. Schaap, 1998. An endo-1,4-β-xylanase encoding gene from *Agaricus bisporus* is regulated by compost specific factors. J Mol Biol **277:**273-284

Patent:

Schuurhuizen, P. W., D. Basten, J. Visser, P. J. T. Dekker, P. J. Schaap: 2002. An isolated polypeptide with aminopeptidase activity, for preparing foodcompositions, such as bread and cheese, with enhanced flavoring. WO200216618

Nawoord

Tja… daar is dan die laatste pagina, wat heb ik hier naar uitgekeken! Er staat maar één auteur op de voorkant van dit boekje, echter, het tot stand komen van dit boekje is niet het werk van één persoon. Daarom wil ik een aantal mensen bedanken voor hun hulp bij het maken van dit boekje.

Ten eerste wil ik Prof. dr. Ir. Ab van Ooyen bedanken dat hij in een latere fase van mijn onderzoek de taak van promotor op zich wilde nemen. Alhoewel onze samenwerking met name begon toen ik al weg was bij de sectie MGIM heb ik niet alleen in wetenschappelijk opzicht maar ook in taalkundig opzicht veel van hem geleerd.

Veel dank ben ik ook schuldig aan mijn copromotor Peter Schaap. Door het wekelijkse overleg, je enthousiasme en ideeën heb ik geleerd wetenschappelijk onderzoek te bedrijven, maar ook om mijn werk af te bakenen en niet ieder idee direct uit te werken. Ook nadat ik weg was heb je nog veel tijd in mijn proefschrift gestoken, bedankt daarvoor!

Jaap Visser, Hans van den Hombergh en Bert Vroemen, zij zijn de bedenkers van dit project en onder leiding van Jaap heb ik nog ruim twee jaar mogen werken.

Ons project was een samenwerking met het toenmalige Gist-brocades uit Delft (tegenwoordig DSM foodspecialties). Onze 3 maandelijkse overleggen leverden altijd weer de nodige ideeën op. In eerste instantie werd het team geleid door Gerard Selten en Bert Vroemen, en later door Piet van Egmond en Peter Dekker. Marco Hensing, merci beaucoup voor de fermentaties die je gedaan hebt om grotere hoeveelheden ApsC te produceren. Natuurlijk wil ik ook al die anderen die vanuit DSM (in Delft en Seclin, Fr.) geholpen hebben bij het project (maar die ik niet bij name ken) bedanken voor hun hulp.

Sjef Boeren wil ik bedanken voor zijn LC-MS werk bij hoofdstuk drie. Jou kennis en praktijk ervaring van LC-MS hebben veel aan dit hoofdstuk toegevoegd.

Harrie Kools, thanks voor je hulp bij het blasten van de sequenties tegen de database van *Aspergillus niger* (in de hitte van de afgelopen zomer). Zonder jou was er lang zo veel niet te discussiëren geweest.

Op lab 1004 was het meestal vol en gezellig. Yvonne, naast al het sequence werk dat je voor me hebt gedaan heb je me heel wat keren een luisterend oor geboden, niet alleen voor frustraties en problemen op het werk, maar ook voor privé zaken. Ruud, echt wennen aan jouw werkuren heb ik nooit gekund, maar door over ons protease werk te discussiëren heb ik veel nieuwe ideeën opgedaan. Peter, ik was altijd blij als je je praktijk experimenten op het lab weer achter de rug had want dan konden we weer over tot de normale orde van de dag en hadden we opeens dubbel zoveel werkruimte.

MGIM bestond niet alleen uit mensen van lab 1004, er waren er meer. Harry, van jou heb ik veel biochemisch werk geleerd. Het omgaan met de AKTA, het bedenken van de biochemische proeven, zonder jou was het anders geweest. Peter Vo, jij was altijd bereid een stammetje voor mij uit de collectie te halen en klaar te zetten, ik hoefde alleen maar een plaatje te geven. Hetty en Ester, jullie zijn allang weg, maar jullie hebben heel wat keertjes een sequence gel voor me gedraaid, bedankt daarvoor.

In de vier jaar op lab 1004 zijn er de nodige studenten geweest. Max, Lucille en Antoine, door jullie te begeleiden heb ik veel na moeten denken waarom bepaalde experimenten gaan zoals ze gaan. Ik heb er ontzettend van genoten een deel van mijn kennis aan jullie over te dragen.

Het is onmogelijk om iedereen bij naam te noemen, maar alle anderen die op het lab werken of gewerkt hebben wil ik ook graag bedanken voor hun enthousiasme, luisterend oor, ideeën, hulp of pauzepraat.

Paps en Mams, zonder jullie zou ik nooit geworden zijn wie ik nu ben. Ik dank jullie voor de basis die jullie gelegd hebben toen ik nog een klein meisje was. Jullie hebben mij laten studeren en hebben mij daarin op verschillende manieren gesteund. Ook al begrijpen jullie weinig van wat ik deed en doe, behalve dan dat het iets met schimmels was, jullie zijn toch altijd oprecht geïnteresseerd.

Zonder een solide thuisbasis is een promotieonderzoek een stuk zwaarder dan zou hoeven. Mijn lieve Rob, jij bent mijn steun en toeverlaat, bij jou kan ik altijd terecht, al is het maar voor een luisteren oor. Je hebt mij geleerd hoe ik de dingen (soms) moet relativeren, dat niet alles zo kan gaan zoals ik wil, en dat de mensen zich vaak niet gedragen zoals ik wil. Eindelijk is er meer tijd voor ons drieën en straks voor ons vieren….. Lieve Luna, vanaf nu zijn de vrij(e)dagen echt voor ons saampjes.

Daniëlle

The research described in this thesis was financially supported by DSM foodspecialties (Delft, The Netherlands).