A Molecular Analysis of L-Arabinan Degradation in *Aspergillus niger* and *Aspergillus nidulans*

Promotor: dr. ir. A.J.J. van Ooyen
hoogleraar in de Genetische Technieken
in de Levensmiddelentechologie

Co-promotor: dr. ir. J. Visser
universitair hoofddocent sectie Moleculaire Genetica
van Industriële Micro-organismen

Michel J. A. Flipphi

A Molecular Analysis of L-Arabinan Degradation in *Aspergillus niger* and *Aspergillus nidulans*

Proefschrift
ter verkrijging van de graad van doctor
in de Landbouw- en Milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op dinsdag 2 mei 1995
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

The research described in this thesis was financially supported by the Dutch Ministries of Economic Affairs and of Agriculture and Fisheries, through a grant from the Programme Committee of Agricultural Biotechnology (PcLB), and by the European Communitee in the framework of BRIDGE, and was performed at the section Molecular Genetics of Industrial Microorganisms at the Wageningen Agricultural University.

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Flipphi, Michel J.A.

A molecular analysis of L-arabinan degradation
in *Aspergillus niger* and *Aspergillus nidulans* /
Michel J.A. Flipphi – [S.l. : s.n.]
Thesis Landbouw Universiteit Wageningen. – With ref.
– With summary in Dutch.
ISBN 90-5485-392-1
Subject headings: *Aspergillus niger* /
α-L-arabinofuranosidase / gene regulation.

Printing of this thesis was supported by Gist-brocades.

Stellingen

1. Hydrolyse van artificiële aryl-glycoside derivaten mag niet als bewijs van de substraat specificiteit van glycosyl hydrolases op natuurlijke substraten beschouwd worden, doch slechts als indicatie hiervoor.

   Schram AW, Tager JM (1981) TIBS **6**:328-330

2. *Aspergillus niger* α-L-arabinofuranosidases A en B katalyseren nucleofiele substitutie van de alcoholfunctie in de glycoside binding met behoud van de α-configuratie van het anomere C_1 atoom in de L-arabinofuranose (hemi)acetaal.

   Sinnott ML (1990) Chem Rev **90**:1171-1202
   Fielding AH, Sinnott ML, Kelly MA, Widdows D (1981) J Chem Soc Perkin Trans I: 1013-1014

3. Om vast te kunnen stellen of L-arabinose dan wel L-arabitol een directe "inducer" van arabinase gen-expressie in *Aspergillus nidulans* is, is een systeem nodig waarin interconversie van deze twee stoffen onmogelijk is.

4. De kataboliet repressie zoals die zich volgens Arst en Bailey in aanwezigheid van bepaalde koolstofbronnen manifesteert in de stofwisseling van *Aspergillus nidulans*, is niet universeel van toepassing op alle *Aspergillus* enzym-systemen die ontvankelijk zijn voor koolstof kataboliet repressie.

   Arst HN Jr, Bailey CR (1977) In: Smith JE, Pateman JA (eds) Genetics and Physiology of *Aspergillus*. Academic Press, London. pp.131-146
   Dit Proefschrift

5. Oxido-reductieve verwerking van D-galactose via galactitol en D-tagatose is niet de alternatieve katabole route in *Aspergillus nidulans* bij groei in alkalisch medium op D-galactose als enige koolstofbron, een conditie waarbij de "Leloir route" geblokkeerd is.

   Roberts CF (1970) Biochim Biophys Acta **201**:267-283
   Lindberg M (1967) Nature **213**:395-396

6. Het D-galactose katabolisme is een potentieel doelwit voor het medium pH-afhankelijke regulatie systeem in *Aspergillus nidulans*.

   Roberts CF (1970) Biochim Biophys Acta **201**:267-283
   Caddick MX, Brownlee AG, Arst HN Jr (1986) Mol Gen Genet **203**:246-353

7. De nucleotide sequentie van het *Klebsiella pneumoniae* pullulanase gen zoals gepresenteerd door Janse *et al.*, is niet correct.

Janse BJH, Steyn AJC, Pretorius IS (1993) Curr Genet **24**:400-407
Katsuragi N, Takizawa N, Marooka Y (1987) J Bacteriol **169**:2301-2306
Kornacker MG, Pugsley AP (1989) Mol Microbiol **4**:73-85

8. Het feit dat de door Kubicek *et al.* gepresenteerde resultaten, betrekking hebbende op de inhibitie van eiwit-glycosylering in *Trichoderma reesei* door 2-deoxy-D-glucose, voor meerdere interpretaties vatbaar zijn, contrasteert met de stelligheid waarmee één van deze interpretaties in de titel van de publikatie wordt benadrukt.

Kubicek CP, Panda T, Schreferl-Kunar G, Gruber F, Messner R (1987) Can J Microbiol **33**:698-703

9. Het succes van de nucleotide sequentie bepaling bij de karakterisering van genen en gen-produkten heeft geleid tot een devaluatie van de waardering voor de prestaties van diegenen die dit interessante werk mogen doen.

10. De militaire dienstplicht is een door de grondwet gelegitimeerde vorm van dwangarbeid.

11. Een wettelijk verbod van ondemocratische partijen is een machtsmiddel waarmee de geloofwaardigheid, de principes en de kracht van het democratisch bestel door haar eigen instituten wordt ondermijnd.

Stellingen behorende bij het proefschrift

A Molecular Analysis of L-Arabinan Degradation in *Aspergillus niger* and *Aspergillus nidulans*

Wageningen, 2 Mei 1995
M.J.A. Flipphi

aan mijn ouders

List of abbreviations

ABF	α-L-arabinofuranosidase (EC 3.2.1.55)
ABN	endo-1,5-α-L-arabinase (EC 3.2.1.99)
AP-1	activating protein 1 (mammalian)
AP-2	activating protein 2 (mammalian)
ATF	activating transcription factor (mammalian)
cAMP	cyclic AMP, adenosine-3'5'-monophosphate
CBH	cellobiohydrolase (EC 3.2.1.91)
CNBr	cyanogen bromide
cre	catabolic repression (*Aspergillus nidulans*)
CRE	cAMP-responsive element (mammalian)
EGL	endo-1,4-β-D-glucanase (EC 3.2.1.4)
αGAL	α-D-galactosidase (raffinase) (EC 3.2.1.22)
βGAL	β-D-galactosidase (lactonase) (EC 3.2.1.23)
βGLU	β-D-glucosidase (EC 3.2.1.21)
PAGE	polyacrylamide gel electrophoresis
pfu	plaque-forming unit
pI	iso-electric point
PKC	protein kinase C (mammalian / *Saccharomyces cerevisiae*)
PNP-A	*para*-nitrophenyl-α-L-arabinofuranoside
PNP-X	*para*-nitrophenyl-β-D-xylopyranoside
SDS	sodium dodecyl sulphate
U	Unit (of enzyme activity)
βXYL	β-D-xylosidase (EC 3.2.1.37)
XYN	endo-1,4-β-D-xylanase (EC 3.2.1.8)

Contents

Chapter 1

Introduction
Occurrence, properties and applications of L-arabinan-degrading enzymes

Introduction

The cell wall provides rigidness and protects the plant from various external influences. It consists of polysaccharides, glycosylated protein (glycoproteins and proteoglycans), and polyphenols (lignin). Plant cell wall polysaccharides are subdivided in cellulose, hemicellulose and pectin (acid polysaccharides) (*cf.* Stephen 1983). The last two decades featured an increased scientific and commercial interest in plant biomass as an alternative source for energy and chemicals. Reviews monitoring developments in the research on plant polysaccharide structure and means for the mobilization and utilization of plant biomass and agro-industrial by-products are regularly updated and extended (*e.g.* Coughlan 1989; Lewis and Paice 1989; Schneider 1989; Leatham and Himmel 1991; Ajay Singh *et al.* 1992; Visser *et al.* 1992; Suominen and Reinikainen 1993).

Cellulose, the most abundant polysaccharide fraction in the plant cell wall, is present as fibrils. These cellulose fibrils are believed to be embedded in a matrix of hemicelluloses, proteoglycans and pectic substances, which determines the physical characteristics of the cell wall. For efficient utilization of plant biomass the cell wall matrix has to be disrupted. Tentative models of the dicotyl primary cell wall suggest that the neutral sugar side chains of pectin would be eminently involved in covalent crosslinking of the main cell wall components (Keegstra *et al.* 1973; Monro *et al.* 1976; Chambat *et al.* 1984; Dey and Brinson 1984). Microbial enzyme systems, naturally "designed" for efficient mobilization and utilization of plant polysaccharides, could provide powerful tools in efficient and clean conversion of plant biomass as an alternative for or in addition to conventional chemical and mechanical methods. This thesis is devoted to one such system involved in disruption of the cell wall matrix structure, the L-arabinan-degrading complex of the saprophytic hyphal fungi *Aspergillus niger* and *Aspergillus nidulans*.

The structure of L-arabinan and L-arabinofuranose-containing heteroglycans

The pentose sugar L-arabinose, mainly in its furanosyl configuration, is present various hemicellulosic and pectic plant polysaccharides. In heteroglycans, like arabinoxylan, arabinogalactan and hydroxyproline-rich proteoglycans (extensin), L-arabinofuranosyl residues are present in or as side chains (*e.g.* Clarke *et al.*

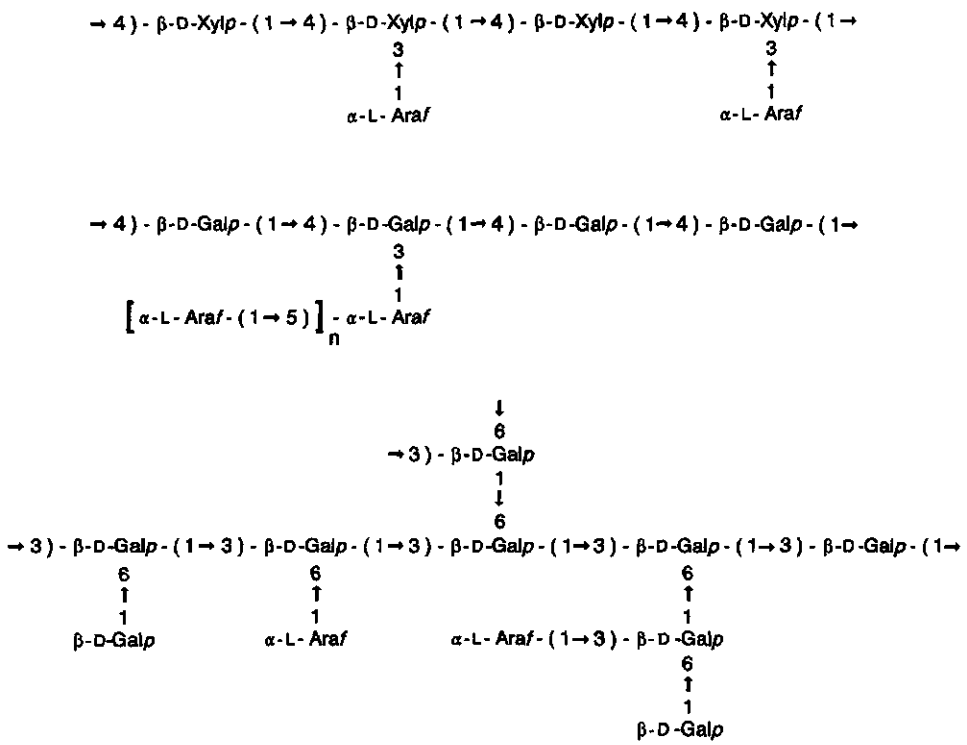


Fig.1. Generalized structures of graminaceous arabinoxylan (L-arabino-D-xylan) **(top)**, pectic arabinogalactan (type-I L-arabino-D-galactan) **(middle)** and hemicellulosic arabinogalactan (type-II L-arabino-D-galactan) **(bottom)**, showing the most common linkages of terminal α-L-arabinofuranose groups to the main frame of the respective polysaccharides. (*cf.* Stephen 1983).

1979; Wilkie 1979; Stephen 1983; Fincher *et al.* 1984; McNeil *et al.* 1984; Dey and Brinson 1984; Selvendran 1985; Saulnier *et al.* 1992; Pellerin *et al.* 1993; see also Visser *et al.* 1992). Simplified structures of arabinoxylan, type-I (pectic) arabinogalactan and type-II (hemicellulosic) arabinogalactan are given in Fig.1. In arabinoxylan and arabinogalactan the L-arabinofuranosyl residues are mainly α-linked, whereas in proteoglycans they are predominantly β-linked.

An L-arabinofuranose homoglycan is found to be an integral component of, or closely associated with, pectin. L-Arabinofuran, better known as L-arabinan, is almost completely made up of α-glycosidically-linked L-arabinofuranose residues. In old literature this polymer is referred to as L-araban. Due to the intimate

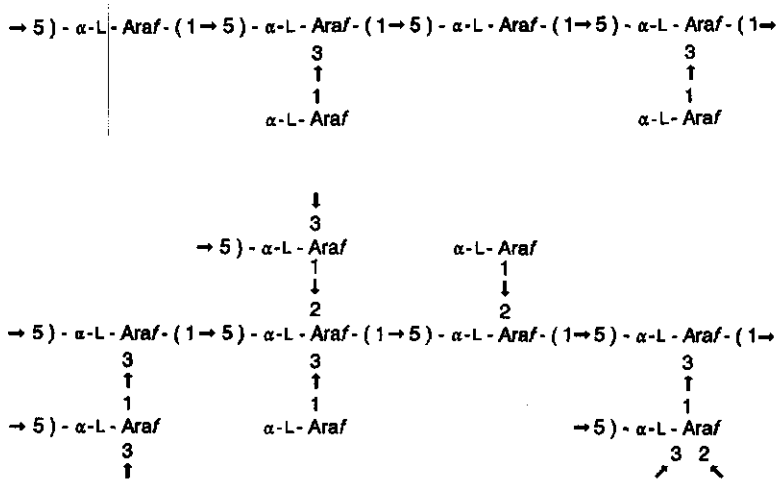


Fig.2. Structures of L-arabinan. The ordered, repetitive structure of the peanut polymer as proposed by Hirst and Jones (1947) **(top)** versus a generalized highly-branched structure showing all linkages occurring in sugar beet L-arabinan (*cf.* Beldman *et al.* 1993) **(bottom).**

relation to pectin, it is historically considered to be a pectic polysaccharide. The natural polymer is highly soluble and due to the furanose configuration of L-arabinose, acid-labile (Green 1966; BeMiller 1967; Sakai *et al.* 1993). For research and diagnostic purposes, L-arabinan can simply be obtained in a highly pure form from sugar beet pulp, an abundant and cheap waste residue (Hirst and Jones 1948; Tagawa and Kaji 1988a; Sakai *et al.* 1993).

L-Arabinan structure is addressed in various reviews (*cf.* Stephen 1983; Dey and Brinson 1984; Selvendran 1985; Voragen *et al.* 1987). L-Arabinans from various sources all conform to the general architecture first described for the peanut polymer (Hirst and Jones 1947). A backbone consisting of (1→5)-*α*-linked L-arabinofuranoses is substituted with side chains of (1→5)-, (1→3)- and (1→2)-linked units attached to either C2 or C3 of main-chain residues. Simplified representations of possible L-arabinan structure are depicted in Fig.2. Recently, the three-dimensional structure of linear (1→5)-*α*-L-arabinan has been addressed (Cros *et al* 1994). L-Arabinans differ in the occurrence, extent and complexity of the side chains, resulting in different physical properties. Essentially linear (1→5)-*α*-L-arabinan arises in fruit juices due to external pectinase activity, applied to optimize yield during pressing (Churms *et al.* 1983; Whitaker 1984; Babsky and Schobinger 1985; Schmitt 1985). This material features a low solubility. The existence of an unbranched L-arabinan "in planta" was recently reported (Zawadzki-Baggio *et al.* 1992). Most L-arabinans described to date are, however,

highly branched. In sugar beet L-arabinan the side chains are predominantly attached at C3 or at both C2 and C3 of main-chain residues, and feature a highly branched structure themselves (Guillon and Thibault 1989; Beldman *et al.* 1993). L-Arabinopyranose residues can be found at non-reducing termini of L-arabinans (*e.g.* Tanaka *et al.* 1981; Fry 1983; Swamy and Salimath 1991; Beldman *et al.* 1993). In alkali-extracted sugar beet pectin, the L-arabinopyranose represents 1.5 % of the L-arabinose contents (Guillon and Thibault 1989). L-Arabinans also contain variable amounts of D-galactopyranose (*e.g.* Hough and Powell 1961; Tanaka *et al.* 1981; Tagawa and Kaji 1988; Beldman *et al.* 1993; Sakai *et al.* 1993; Tharananthan *et al.* 1994).

Functional aspects of L-arabinan in the plant cell wall

Several hemicellulosic and pectic polysaccharides contain small amounts of hydroxycinnamic acid derivatives, predominantly ferulic (3-[4-hydroxy-3-methoxyphenyl]-2-propenoic) acid and *p*-coumaric (3-[4-hydroxyphenyl]-2-propenoic) acid. Oligosaccharides containing such phenolic acids were isolated after enzymic degradation of hemicellulosic and pectic cell wall components (*e.g.* Fry 1982; Smith and Hartley 1983; Kato and Nevins 1985; Mueller-Harvey *et al.* 1986; Nishitani and Nevins 1988). The presence of ferulic acid in pectin-associated L-arabinan has been demonstrated (Fry 1983; Rombouts and Thibault 1986a, 1986b; Guillon and Thibault 1989, 1990; Guillon *et al.* 1989). Dependent on the method used to solubilize pectin from sugar beet pulp, 0.6 to 2.5 % of the neutral sugar residues present carry feruloyl groups. Notably, ferulic acid is only found in pectins from *Centrospermae* and not in *e.g.* potato, apple and citrus pectins (Rombouts and Thibault 1986a; references therein). In arabinoxylan, the ferulic acid is esterified to the fifth carbon atom (C5) of terminal non-reducing L-arabinofuranose residues (Colquhoun *et al.* 1994). In sugar beet pectin, non-terminal D-galactose and L-arabinofuranose residues constituting the backbone of (1→4)-*β*-D-galactan and (1→5)-*α*-L-arabinan, respectively, carry the feruloyl substituents (Ralet *et al.* 1994a; Colquhoun *et al.* 1994). Here, ferulic acid is esterified to the second carbon atom (C2) of L-arabinofuranose. Cinnamic acid to glycosyl ester linkages are alkali-labile. The cinnamoyl groups can form both homo- and heterodimers by oxidative coupling with either peroxidase in the presence of hydrogenperoxide or ammonium persulphate, and by light-induced cyclomerization (*e.g.* Geissman and Neukom 1971, 1973; Thibault and Rombouts 1986; Hartley and Ford 1989; Guillon and Thibault 1990; Hartley *et al.* 1990; Morrison *et al.* 1992). Such cinnamic acid dimers have been isolated from cell wall material (Hartley and Jones 1976, 1977; Markwalder and Neukom 1976). Accumulating evidence indicates that hemicelluloses, pectins and lignin are covalently crosslinked through phenolic bridges, involving both ester and ether linkages (*e.g.* Fry 1986; Conchie *et al.* 1988; Fry and Miller 1989; Watanabe *et al.* 1989; Iiyama *et al.* 1990; Ishii *et al.* 1990; Jeffries 1990; Nishitani and

Nevins 1990; Minor 1991; Kato and Nevins 1992). This implies an important contribution of such crosslinks to the cell wall matrix structure of both monocotyls and dicotyls. The occurrence and significance of phenolic acid derivatives in plant cell walls was recently reviewed by Christov and Prior (1993) and Iiyama *et al.* (1994).

Ehrlich and Schubert (1928) indicated an intimate association of L-arabinan with D-galacturonan in naturally occurring, insoluble protopectin of various plants, which could be disturbed in hot lime water. Currently, pectin is known to consist of alternating regions of linear homogalacturonan and highly substituted rhamnogalacturonan (*e.g.* Voragen and Pilnik 1989; Guillon *et al* 1989; Lerouge *et al.* 1993a; Sakai *et al.* 1993). The latter structure carries extensive branches of neutral sugars. The D-galactopyranose present in some isolated L-arabinans are the remainders of the β-D-galactan stretches linking L-arabinan to L-rhamnose within the rhamnogalacturonan regions in pectin (Jarvis *et al.* 1981; Lerouge *et al.* 1993a; Sakai *et al.* 1993), but in others it is found as non-reducing terminal residues (Guillon *et al.* 1989). The neutral sugar branches carry the feruloyl groups involved in covalent crosslinking of cell wall components, as described above. In sugar beet pectin, 50 % of the feruloyl groups present are carried by the L-arabinan component (Ralet *et al.* 1994a). The best evidence for the involvement of L-arabinan in cell wall matrix crosslinking in some dicotyls is the observation that an L-arabinan-degrading activity from *Bacillus subtilis* is able to solubilize sugar beet protopectin (Sakai and Sakamoto 1990; Sakai *et al.* 1993). Extraction of sugar beet fibres with hot dilute acid releases L-arabinose and eliminates L-arabinan, thereby solubilizing part of the resident pectin and disorganizing the tissue structure (Bertin *et al.* 1988). Moreover, the same *B. subtilis* enzyme is able to disintegrate potato tissue (Yoshihara and Kaji 1984) although ferulic acid is absent in pectin from potato (*cf.* Rombouts and Thibault 1986a). Besides in pectin, short linear L-arabinan-like side chains are reported to occur in type-I arabinogalactan, type-II arabinogalactan and in monocotyl arabinoxylan (*e.g.* Jarvis *et al.* 1981; Carre *et al.* 1985; Nishitani and Nevins 1989; van de Vis *et al.* 1991; Will and Dietrich 1992).

Enzymes involved in degradation and modification of L-arabinan and structural cell wall polysaccharides containing L-arabinofuranose

Enzymes can cleave glycosidic linkages in polysaccharides either by hydrolysis or transelimination. In L-arabinan degradation only hydrolases are known. Subdivisions are based on differences in mode of action. Exo-type hydrolases (glycosidases) liberate mono- or oligosaccharide fragments from non-reducing termini, whereas the activity decreases with increasing degree of polymerization of the substrate. Endo-type hydrolases (glycanases) randomly hydrolyze glycosidic bonds while the activity decreases with decreasing degree of polymerization. Furthermore, glycohydrolase action results in either retention or

inversion of the anomeric configuration of C1 (α or β) of the generated reducing sugar unit (*cf.* Sinnott 1990). The reaction mechanism of retaining hydrolases involves the formation of an intermediate covalent glycosyl-enzyme complex enabling transfer of that glycosyl group to acceptor compounds other than H_2O, like *e.g.* other sugars. Enzymes for which the transfer activity prevails over the hydrolytic activity are called glycosyl transferases. Hydrolases and transferases can be grouped in families based on similarities in their amino acid sequence structure (Henrissat 1991; Henrissat and Bairoch 1993). To date 47 families are described, harbouring the vast majority of the 500 or so glycosyl hydrolases of which the primary structure is known. The families indicate structural and evolutionary relationship, and enable assignment of residues potentially involved in catalysis or substrate binding.

L-Arabinan-degrading glycosyl hydrolases are generally known as arabinases or arabinanases. Sometimes they are referred to as arabanases or, incorrectly, as arabinosidases. α-L-Arabinofuranosidase (ABF), an exo-type hydrolase and endo-α-L-arabinase (ABN), an endo-type enzyme, are involved in L-arabinan degradation. Arabinases are produced by a wide variety of plants and micro-organisms – the latter include phytopathogenic, saprophytic and ruminal species. Microbial ABFs caught the attention of phytopathologists in the late sixties and the start of the seventies, after Fuchs *et al.* (1965) reported constitutive production of this activity by phytopathogenic fungi. Interest ceased after it became clear that ABFs were not able to macerate plant material and were rejected as a potent virulence factor (*cf.* Bateman 1976), despite the fact that ABF production by the brown-rot fungus *Monilinia fructigena* was shown to correlate with pathogenicity (Laborda *et al.* 1974; Howell 1975). Two independent impulses, *viz.* an increased interest in the application of microbial xylanolysis, and problems related to the formation of insoluble linear L-arabinan during the preparation of fruit juices, have recently boosted research on arabinases (*e.g.* Whitaker 1984; Biely 1985; Voragen and Pilnik 1989; Visser *et al.* 1992; Coughlan and Hazlewood 1993a, 1993b). L-Arabinan-degrading enzymes have been addressed in several reviews (*cf.* Dekker and Richards 1976; Dey and Del Campillo 1984; Whitaker 1984; Matheson and McCleary 1985; Ward and Moo-Young 1989). Kaji, the main contributor in the field, specifically focused on these enzymes (Kaji 1984, Tagawa and Kaji 1988b).

α-L-Arabinofuranosidases

Glycosidases releasing monomeric L-arabinose from α-linked L-arabinofuranose-containing polysaccharides or fragments thereof, are referred to as α-L-arabino-furanosidases (ABF). These enzymes are classified as α-L-arabinofuranoside arabinofuranohydrolases (EC 3.2.1.55). ABF is generally acknowledged as an important ancillary activity in the degradation of arabinoxylan (*cf.* Biely 1985). ABF activity was first noted by Ehrlich and Schubert (1928) upon incubation of sugar beet L-arabinan with Takadiastase, a classical α-amylase preparation from

the mold *Aspergillus oryzae*. ABFs are isolated from a wide range of microorganisms and from plants (listed in Table 1). In plants, this activity is thought to be involved in modification of the cell wall matrix, affecting the physical characteristics of the plant cell wall (*e.g.* Yamaki and Kakiuchi 1979; Tanimoto 1985; Tong and Gross 1988; Nishitani and Nevins 1990). Most microbial ABFs are reported to be extracellular but intracellular (cell-associated) enzymes are also known (*e.g.* Laborda *et al.* 1973; Hespell and O'Bryan 1992; Manin *et al.* 1994). Isozymes obtained from a single source and exhibiting distinct physico-chemical properties are not uncommon – the brown-rot fungus *Monilinia fructigena* produces three different ABFs. Both in *M. fructigena* and *A. niger*, the isozymes are immunochemically unrelated (Hislop *et al.* 1974; van der Veen *et al.* 1991).

Generally, ABF activity can easily be assayed by measuring the hydrolysis of synthetic aryl-*α*-L-arabinofuranosides – commonly used are the phenyl, *para*-nitrophenyl (PNP-A), and 4-methylumbelliferyl derivatives (Börjeson *et al.* 1963; Fielding and Hough 1965; Kelly *et al.* 1988). Alternatively, release of L-arabinose from oligomeric or polymeric substrates can be determined upon oxidation with NAD^+-dependent D-galactose dehydrogenase (EC 3.1.1.48) from *Pseudomonas fluorescens*, spectrophotometrically measuring the formation of NADH (Melrose and Sturgeon 1983). This assay is specific for L-arabinose when D-galactose and D-fucose, the two other substrates of the dehydrogenase are not liberated. Kaji (1984) distinguished two kinds of ABFs based on differences in substrate affinity. Some ABFs act only on synthetic and small oligomeric substrates, while others can use polymers as substrates as well. Only *A. niger* ABF A and the octameric ABF from *Streptomyces purpurascens* IFO 3389 (see Table 1) can be clearly assigned to the former class. Usually microbial ABFs are highly specific with respect to the furanose configuration of L-arabinose whereas both L-arabinose and L-arabino-(1,4)-*γ*-lactone are powerful competitive inhibitors (*e.g.* Tagawa 1970a; Schwabe *et al.* 1978; Fielding *et al.* 1981; Tanaka *et al.* 1981). Although ABF was introduced as a typical exo-type hydrolase activity involved in L-arabinan degradation, some species feature distinct characteristics. The ABFs from the cellulolytic fungus *Geotrichum candidum* for instance, hydrolyze PNP-A and liberate L-arabinose from arabinogalactan and arabinoglucuronoxylan, but do not act on L-arabinan (Golubev *et al.* 1993). Extracellular ABF from *Streptomyces diastatochromogenes* acts preferably on high molecular weight L-arabinan and thus does not behave as a typical glycosidase (Higashi 1983; Higashi *et al.* 1983). The ABF from radish seed (Hata *et al.* 1992) is also exceptional. Sugar beet L-arabinan is a relatively bad substrate. The enzyme releases D-xylose from synthetic aryl-xylopyranosides, xylobiose, xylotriose and 4-*O*-methyl-glucuronoxylan, and therefore could be considered a *β*-D-xylosidase (*β*XYL) (EC 3.2.1.37) as well. Acting on oat spelt xylan both L-arabinose and D-xylose are liberated. Mixed-substrate kinetics with PNP-derivatives suggests that a single catalytic site is responsible for both activities. Dualism in the hydrolysis of aryl-derivatives of

Table 1. Source and some physico-chemical characteristics of α-L-arabinofuranosidases (ABFs)

Source	MW [kDa] (SDS-PAGE) [a]	MW [kDa] (GPC) [b]	pI	Main substrate(s)	Reference(s)
bacterial					
***Bacillus* sp.**				PNP-A / L-arabinan	Yasuda *et al.* (1983)
Strain No. 430 (sugar beet pulp)				type-II arabinogalactan	Yasuda and Tokuzato (1988)
ABF I / ABF II	ND [c]	ND	ND		
Bacillus stearothermophilus	52.5 / 57.5	114.8	ND	PNP-A	Bezalel *et al.* (1993)
Strain L1 (L-arabinose)					
Bacillus subtilis					
Strain F-11 (sugar beet pulp)	65	ND	5.3	PNP-A / L-arabinan	Weinstein and Albersheim (1979)
Strain 3-6 (arabinoxylan)	61	ND	ND	PNP-A / L-arabinan	Kaneko *et al.* (1994)
Novo Ban L-120	57	ND	ND	PNP-A / arabinoxylan	Nishitani and Nevins (1989)
Butyrivibrio fibrisolvens	31	240	6.0	PNP-A / L-arabinan	Hespell and O'Bryan (1992)
Strain GS113 (arabinoxylan)				arabinoxylan	
				arabinofuranoxylobiose	
Clostridium acetobutylicum	94	90	8.15	PNP-A	Lee and Forsberg (1987)
Strain ATCC 824 (arabinoxylan)				L-arabinan	
Clostridium stercorarium	45 / 55	180	ND	PNP-A	Bronnenmeier *et al.* (1990)
Strain NCIB 11745 (xylan)					
Pseudomonas fluorescens	58	ND	ND	arabinoxylan	Kellett *et al.* (1990)
subsp. *cellulosa*.					
(recombinant *E. coli*)					
Ruminococcus albus	75	305-310	3.8	PNP-A	Greve *et al.* (1984)
Strain 8 (alfalfa cell walls)				cell wall pectin / hemicellulose	
Streptomyces diastaticus				PNP-A / (1,5)-L-arabinan	Tajana *et al.* (1992)
Strain ET/BW200 (wheat bran)				arabinoxylan	
ABF C1	38	ND	8.8		
ABF C2	60	ND	8.3		
Streptomyces diastatochromogenes				phenyl-α-L-arabinofuranoside	Higashi *et al.* (1981)
Strain No.065 (L-arabinan)				L-arabinan	Higashi *et al.* (1983)
EC-AFase (EF-2)	ND	74	8.4		Higashi (1983)
IC-AFase	ND	ND	ND		
Streptomyces lividans 66	69	380	4.6	PNP-A	Manin *et al.* (1994)
(recombinant *S. lividans*)				arabinoxylanosides	
(oat spelt xylan)					

Streptomyces massasporeus Strain IFO 3841 (L-arabinan)	ND	54	ND	PNP-A L-arabinan	Kaji *et al.* (1982)
Streptomyces purpurascens Strain IFO 3389 (L-arabinan)	62	495	3.9	PNP-A di- / tri-α-L-arabinofuranosides	Komae *et al.* (1982)
***Streptomyces* sp.** Strain No. 17-1 (L-arabinan)	92	ND	4.4	L-arabinan PNP-A	Kaji *et al.* (1981)
Thermoanaerobacter ethanolicus Strain ATCC 31550 (D-xylose)	85	165	4.6	aryl-β-D-xylosides / PNP-A PNP-α-L-arabinopyranoside (1→4)-β-D-xylosides arabinoxylan	Shao and Wiegel (1992)
Thermomonospora fusca Strain BD21 (arabinoxylan)	46	92	ND	PNP-A	Bachmann and McCarthy (1991)
fungal					
Aspergillus aculeatus Pectinex Ultra SP	37	ND	ND	PNP-A / L-arabinan	Beldman *et al.* (1993)
ABFs B1 / B2					
Aspergillus awamori Strain CMI 142717 (arabinoxylan)					
ABF	66	ND	ND	PNP-A / arabinoxylanosides	Wood *et al.* (1992)
AXH	32	ND	ND	arabinoxylan arabinoxylanosides	Kormelink *et al.* (1991) Kormelink *et al.* (1993)
Aspergillus nidulans					
Strain FGSC 187 (L-arabitol)	65	ND	3.3	PNP-A / L-arabinan	Ramón *et al.* (1993)
Strain CECT 2544 (L-arabinan)	36	ND	± 4.3	PNP-A / L-arabinan	Fernández-Espinar *et al.* (1994)
Aspergillus niger [d] Strain CBS 120.49 (sugar beet pulp)					van der Veen *et al.* (1991)
ABF A	83	ND	3.3	PNP-A	
ABF B	67	ND	3.5	PNP-A / L-arabinan	
Cladosporium fulvum Strain: not specified				L-arabinan	Higashi *et al.* (1986)
(L-arabinan) ara-ABF	ND	ND	ND	phenyl-α-L-arabinofuranoside	
(soluble starch) 3 sla-ABFs	ND	ND	ND		
Colletotrichum lindemuthianum γ Strain ATCC 56987 (pectate)	ND	131 / 139.5	3.4 / 3.6	PNP-A	Wijesundera *et al.* (1989)
Corticium rolfsii Strain K2 (wheat bran)	ND	ND	ND	phenyl-α-L-arabinofuranoside L-arabinan	Kaji and Yoshihara (1969) Kaji and Yoshihara (1970)

Table 1. Source and some physico-chemical characteristics of α-L-arabinofuranosidases (ABFs). continued (2)

Source	MW (kDa) (SDS-PAGE) [a]	MW (kDa) (GPC) [b]	pI	Main substrate(s)	Reference(s)
fungal					
Dichomitus squalens (Karst) Reid Strain CBS 432.34 (arabinoxylan)	ND	60	5.1	PNP-A / L-arabinan arabinoxylan	Brillouet *et al.* (1985)
Fusarium roseum "Avenaceum" (various polymeric C-sources)					Mullen and Bateman (1975)
ABF 1 (pH 5.0)	ND	67	ND	L-arabinan	
ABF 2 (pH 8.8)	ND	56	ND	L-arabinan	
***Fusarium* sp.** Strain I 50 (rye-bran)	ND	ND	ND	PNP-A	Butschak *et al.* (1989)
Geotrichum candidum Strain 3C (wheat bran/pressed sugar beet)				PNP-A / arabinogalactan arabinoglucuronoxylan	Golubev *et al.* (1993)
ABF 1	80	ND	4.0		
ABF 2	69	ND	3.85		
Glomerella miyabeana var. Arx Strain A 72 (D-glucose)	ND	ND	ND	PNP-A	Butschak *et al.* (1989)
Monilinia fructigena Alderh. & Ruhl. Strain ATCC 26106 (pectate)				PNP-A / L-arabinan 2-naphthyl-α-L-arabinofuranoside	Laborda *et al.* (1973) Laborda *et al.* (1974) Kelly *et al.* (1987)
ABF I	ND	125 - 220	3.0		
ABF II	ND	350	4.5		
ABF III	34 - 35	40	6.5		
Mycosphaerella pinodes Strain A 9 (rye-bran)	ND	ND	ND	PNP-A	Butschak *et al.* (1989)
Penicillium capsulatum [e]				PNP-A / L-arabinan arabinoxylan	Coughlan *et al.* (1993)
Ara I	64.5	ND	4.15		
Ara II	62.7	ND	4.55		
Phanerochaete chrysosporium [e]	55	ND	7.34	arabinoxylan	Coughlan *et al.* (1993)
Phytophthora palmivora Butl. Strain CMI 63552 (pectin / maltose)	ND	63.1	ND	PNP-A	Akinrefon (1968)
Rhodotorula flava IFO 0407 (L-arabinan)	ND	ND	ND	PNP-A / L-arabinan arabinoxylan	Uesaka *et al.* (1978)
Sclerotinia sclerotiorum de Bary isolate 214 (L-arabinose)	62	ND	7.5	PNP-A / L-arabinan type-II arabinogalactan	Baker *et al.* (1979)

Talaromyces emersonii [e]	105	210	3.5	arabinoxylan	Coughlan *et al.* (1993)
Trichoderma lignorum	50	50	ND	arabinoxylan	Schmidt *et al.* (1984)
Strain: not specified (arabinoxylan)					
Trichoderma reesei	53	30	7.5	PNP-A / L-arabinan	Poutanen (1988)
Strain VTT-D-79125				arabinoxylan	
(D-glucose / distiller's spent grain)					
plant					
Carrot (*Daucus carota* L. cv. Kintoki)				PNP-A / L-arabinan	
(cell culture)				pectin from carrot cell walls	
ABF-I	94	110	4.7		Konno *et al.* (1987)
ABF-II	80	84	5.6		Konno *et al.* (1994)
Lupin (*Lupinus luteus* cv. Weiko III)				PNP-A / type-I arabinogalactan	Matheson and Saini (1977)
(cotyledon of seedlings)				pectin from lupin cell walls	
ABF I	ND	70	ND		
ABF II	ND	110	ND		
Radish (*Raphanus sativus* L.)	64	63	4.7	PNP-A / PNP-X	Hata *et al.* (1992)
(var. *hortensis* cv. Aokubi)				α-L-arabinofuranosyl heteroglycans	
(seed)				β-(1→4)-D-xylosides	
Scopolia japonica	ND	62	8.0	PNP-A	Tanaka and Uchida (1978)
(callus material)				L-arabinan	Tanaka *et al.* (1981)
				α-L-arabinofuranobioses	Uchida and Tanaka (1988)
Soybean	87	ND	ND	PNP-A	Hatanaka *et al.* (1991)
(*Glycine max* L. cv. Akishirome)				L-arabinan	
(cotyledon of seedlings)					
Spinach (*Spinacia oleracea* L.)					
(leaf mesophyl tissue)					
ABFI	ND	118	4.2	PNP-A / PNP-X	Hirano *et al.* (1994)
ABFII	ND	68	4.2	PNP-A / L-arabinan	
				type-II arabinogalactan	

[a] Apparent molecular weight determined with SDS-PAGE.
[b] Apparent molecular weight determined with gel permeation chromatography.
[c] Not determined.
[d] Several reports document the isolation of *A. niger* ABF from various sources; a full survey is given in Table 4. The data of van der Veen *et al.* (1991) are enlisted.
[e] Unpublished; data adapted from Coughlan *et al.* (1993).

L-arabinofuranose and D-xylopyranose is quite commonly found for βXYLs, including the enzyme from *A. niger* (Rodionova *et al.* 1983). The physiological implications remain obscure and only in a few cases this phenomenon was further investigated. For the *Trichoderma reesei* and *Thermoanaerobacter ethanolicus* βXYLs, the affinity for the arabinofuranoside is in fact much lower than for the xylopyranoside (Poutanen and Puls 1988; Shao and Wiegel 1992). The specific activity for the arabinofuranoside is, however, much higher for *T. ethanolicus* βXYL. This enzyme hydrolyzes (1→4)-linked D-xylose oligomers, but (1→5)-linked L-arabinofuranoses are not degraded. Like radish seed ABF, it releases both L-arabinose and D-xylose from wheat arabinoxylan. Based on these results, this βXYL could be considered an ABF with quite specific substituent requirements.

Recently two distinct ABFs were described which could not hydrolyze aryl-arabinofuranosides or L-arabinan. These glycosidases, from *Pseudomonas fluorescens* (Kellett *et al.* 1990) and from *Aspergillus awamori* (Kormelink *et al.* 1991), specifically release L-arabinose from arabinoxylan. The *A. awamori* enzyme is resistant to inhibition by L-arabino-(1,4)-γ-lactone. This specialized ABF is referred to as 1,4-β-D-arabinoxylan arabinofuranohydrolase (AXH). Cooper *et al.* (1988) found a constitutive L-arabinan-degrading glycosidase activity inert towards PNP-A, in *Rhizoctonia cerealis*-infected wheat seedlings. Since monocotyls do not have much pectin in their cell wall, the authors suggested that this activity could be involved in debranching of arabinoxylan. Such an enzyme has been identified as a vital component of a fungal hydrolytic system responsible for the release of oligomeric arabinoxylan fragments from cell walls, which are lethal for maize cells (Kauffmann *et al.* 1990). It is not unlikely that such enzymes are the frontrunners of more specialized ABFs yet to be discovered.

Synthetic aryl-glycosides allow accurate determination of kinetic properties of most ABFs, although it must be emphasized that those parameters probably do not reflect the kinetics of the enzyme when acting towards natural substrates. In this respect all ABFs are ill-characterized as suitable, well-defined homo- and hetero-oligoglycans are not generally available. Information on possible linkage preferences, substituent requirements and reaction mechanisms is scarce. The ABF from *Scopolia japonica* callus tissue has a slight preference for the (1→3) linkage compared to the (1→5) linkage, comparing the kinetics of hydrolysis of the two isomeric α-linked L-arabinofuranobioses (Tanaka *et al.* 1981). 1,5-α-linked L-Arabinofuranosyl oligomers, only recently commercially available, partially fulfil the need for adequate substrates to investigate the "natural" kinetic properties of ABFs (*cf.* McCleary 1991). The extracellular ABFs from *Monilinia fructigena* (ABF I / ABF III) both are retaining hydrolases, implying potential transferase activity (Marshall *et al.* 1981; Fielding *et al.* 1981). The reaction mechanism of ABF III has been further studied by Sinnott and co-workers, using various aryl-arabinofuranosides, pH-dependent kinetics and 1,4-dideoxy-1,4-imino-alditol inhibitors, acting similarly to the β-D-glucosidase inhibitor (1-deoxy)

Table 2. Cloned α-L-arabinofuranosidase-encoding genes

Source organism	Gene code / clone	sequence determination	protein MW [kDa] [a]	Main substrate(s)	Reference(s)
Aspergillus niger [b] Strain CBS 120.49	*abfA*	yes	83 [c] / 65.4 [d]	PNP-A (1,5)-α-L-arabinofuranosides	Flipphi *et al.* (1993c) & (1994) Chapters 2 & 5
	abfB	yes	67 [c] / 50.7 [d]	PNP-A / L-arabinan (1,5)-α-L-arabinofuranosides	Flipphi *et al.* (1993a) Chapter 4
Bacillus stearothermophilus	pMG1	no	–	(1→4)-β-D-xylosides	Oh *et al.* (1992)
Bacteriodes ovatus Strain V975	pOX30	yes [f]	38 [c]	PNP-X / PNP-A (1→4)-β-D-xylosides	Whitehead and Hespell (1990) T.R. Whitehead (unpublished)
Butyrivibrio fibrisolvens Strain GS113	*xylB*	yes	60 [c]	PNP-X / PNP-A (1→4)-β-D-xylosides	Sewell *et al.* (1989) Utt *et al.* (1991)
Cellumonas biazotea Strain DSM 20112	pAF1	no	-	aryl-α-L-arabinofuranosides	Liebl *et al.* (1992)
Clostridium stercorarium	*bxlB*	no	-	PNP-X / PNP-A / xylobiose	Schwarz *et al.* (1991)
Strain NCIB 11745	*arfA*	no	-	PNP-A / PNP-X	
	arfB	no	-	PNP-A	
Strain F-9	*xylA*	yes	53 [c] / 53.34 [d] 220 [e]	PNP-X / PNP-A	Sakka *et al.* (1993)
Pseudomonas fluorescens subsp. *cellulosa*	*xynC*	yes	59 [d]	oat spelt arabinoxylan	Kellett *et al.* (1990)
Ruminococcus flavefaciens Isolate 17	L9	no	-	xylan / lichenan [g]	Flint *et al.* (1989)
Streptomyces lividans Strain 66	*abf*A	yes	69 [c] / 72.49 [d] 380 [e]	PNP-A arabinofuranoxylosides	Manin *et al.* (1994)

[a] Molecular weight of the gene product.
[b] See also Table 4.
[c] Apparent molecular weight determined with SDS-PAGE.
[d] Molecular weight deduced from nucleic acid sequence.
[e] Apparent molecular weight determined with gel permeation chromatography.
[f] DNA sequence (EMNEW:BO04957) and features available under accession number U04957.
[g] Non-cellulosic homoglucan: β-(1→3,1→4)-D-glucan.

nojirimycin (Kelly *et al.* 1987; Selwood and Sinnott 1989; Axamawaty *et al.* 1990). The single catalytic proton-donating amino acid residue has a pKa-value of 5.9 in the absence, and of 6.7 in the presence of substrate.

To date several bacterial ABF-encoding genes have been isolated (Table 2), next to the *A. niger* genes coding for ABF A and ABF B (Flipphi *et al.* 1993a, 1993c, 1994; Chapters 2, 4 and 5 of this thesis). For most of the bacterial genes, the gene product has been characterized as an aryl-α-L-arabinofuranoside-hydrolyzing activity usually co-inciding with a main aryl-β-D-xylosidase activity. The *Pseudomonas fluorescens xynC* gene was co-incidently cloned as a second open reading frame directly adjacent to the xylanase-encoding *xynB* (Kellett *et al.* 1990). Its gene product is an ABF which liberates L-arabinose from oat spelt arabinoxylan and is inactive against PNP-A, as already mentioned. L-Arabinan degradation has not been considered as means to further identify or characterize most of these bacterial genes. Only the intracellular *Streptomyces lividans abf*A gene product features a limited activity towards L-arabinan as well as towards polymeric arabinoxylans, whereas oligomeric arabinofuranoxylosides are the preferred substrates (Manin *et al.* 1994).

Arabinose-releasing endo-1,4-β-D-xylanases

endo-1,4-β-D-Xylanase (XYN) (EC 3.2.1.8) randomly hydrolyzes the (1→4)-β-glycosidic bonds in the xylan backbone (*cf.* Fig.1). Generally, microbes produce multiple XYNs differing in their mode of action and exhibiting various requirements with respect to side-chain substitution (*e.g.* Coughlan *et al.* 1993). In the seventies, some XYNs were isolated which could liberate L-arabinose from arabinoxylan in addition to the XYN activity, leading to the historical subdivision in debranching and non-debranching XYNs (*cf.* Dekker and Richards 1976; Dekker 1985). For a long time, L-arabinose-releasing XYNs were only known from fungal sources including *A. niger*, as surveyed by Dekker (1985). Recently such enzymes were found in xylanolytic bacteria as well (Grabski and Jeffries 1991; Matte and Forsberg 1992; Wang *et al.* 1993). Specific inhibition of the arabinose-liberating activity of XYN I of *Ceratocystis paradoxa* by xylotriose indicated that two distinct catalytic domains are involved (Dekker and Richards 1975). Therefore L-arabinose-liberating XYNs could be regarded as ABFs with restricted substrate specificity. *Fibrobacter succinogenes* endoxylanase 1 was found to release up to 60 % of the L-arabinose substituting two different arabinoxylans, but was hardly active on sugar beet L-arabinan and PNP-A (Matte and Forsberg 1992). Like endoxylanase 1 from *Trichoderma koningii* (Wood and McCrae 1986), the *F. succinogenes* enzyme features its ABF activity prior to xylanolysis. It has been suggested that the observed activity is due to contaminant ABF (Coughlan *et al.* 1993) – in the case of *F. succinogenes* this then would involve an enzyme which is essentially inert towards L-arabinan and PNP-A. However, a *Bacillus polymyxa* xylanase gene (*xynD*) encodes two heterologous proteins which both exhibit xylanase and aryl-α-L-arabinofuranoside-

hydrolyzing activities when expressed in the non-xylanolytic host *E. coli* (Gosalbes *et al.* 1991). This provides evidence that arabinose-releasing XYNs do exist.

endo-1,5-α-L-Arabinases

Glycanases hydrolyzing the α-glycosidic linkages in L-arabinans in a random fashion are referred to as endo-1,5-α-L-arabinases (ABN). These enzymes are classified as 1,5-α-L-arabinan 1,5-α-L-arabinohydrolase (EC 3.2.1.99). The existence of this hydrolase was first described for *Clostridium felsineum* var. *sikokianum* by Kaji *et al.* (1963b). To date, a limited number of microbial ABNs have been purified and characterized (Table 3). Except for the one from *A. niger* (Flipphi *et al.* 1993b; Chapter 3 of this thesis) ABN-encoding genes have not been described in literature. All ABNs are extracellular and the fungal enzymes are glycosylated. There are no indications for multiplicity of (eukaryotic) ABN activity, which is quite commonly found for fungal glycan-hydrolyzing systems (see for instance: Wong *et al.* 1988; Araujo and Ward 1989; Kester and Visser 1990; Wood 1992). However, all microbes which produce ABN (Table 3) also synthesize ABF activity (*cf.* Table 1; Beldman *et al.* 1993; see also Karimi and Ward 1989). The *B. subtilis* ABN from Sakai and Sakamoto (1990) was isolated and purified for its protopectin-solubilizing properties, releasing high molecular weight pectin (Sakai *et al.* 1993). Weinstein and Albersheim (1979) described an ABN from a different *B. subtilis* strain that could release 20 % of the L-arabinose contents from cultured sycamore cells. *B. subtilis* ABN from a third isolate is reported to disintegrate potato tissue (Yoshihara and Kaji 1984). These results strongly suggest that *B. subtilis* ABN, possibly in concert with ABF activity, is able to macerate plant tissue. Notably, the *B. subtilis* enzyme is not inhibited by L-arabino-(1,4)-γ-lactone (Yoshihara and Kaji 1983). Linear L-arabinan, prepared by enzymic debranching of purified sugar beet L-arabinan (Tagawa and Kaji 1988a; Sakai *et al.* 1993), is most commonly used for kinetic characterization. Subsequently, ABN activity has to be quantified with the highly aspecific Nelson/Somogyi reducing sugar assay (Somogyi 1952). Recently, dyed carboxymethylated linear L-arabinans were introduced. These substrates are highly specific for ABN and enable direct measurement of ABN activity in crude enzyme preparations (*cf.* McCleary 1990; Dunkel and Amadò 1994a).

Distinct exo-type arabinases

An L-arabinan degrading enzyme from the phytopathogenic bacterium *Erwinia carotovora* IAM 1024 features quite distinct properties compared to the activities described above. It acts on branched sugar beet L-arabinan releasing mainly arabinotriose, and does not hydrolyze arabinofuranose-containing heteroglycans, linear L-arabinan or PNP-glycosides (Kaji and Shimokawa 1984). Recently, a similar activity was found in a commercial enzyme preparation of *Aspergillus aculeatus*, though the major product is arabinobiose (Lahaye and Thibault 1990).

Table 3. Source and some physico-chemical characteristics of endo-1,5-α-L-arabinases (ABNs)

Source	MW [kDa] (SDS-PAGE) [a]	MW [kDa] (GPC) [b]	pI	Main substrate(s)	Reference(s)
bacterial					
Bacillus subtilis Strain F-11 (sugar beet pulp)	32	36	9.3	(1→5)-L-arabinan L-arabinan sycamore cell walls	Kaji and Saheki (1975) Weinstein and Albersheim (1979)
Bacillus subtilis Strain IFO 3022 (sugar beet pulp)	33	ND [c]	7.9 / 9.7	(1→5)-L-arabinan L-arabinan	Yoshihara and Kaji (1984) Kaji (1984)
Bacillus subtilis Strain IFO 3134 (dextran / soybean flour) [Protopectinase PPase-C]	30	27	9.0	(1→5)-L-arabinan sugar beet protopectin	Sakai and Sakamoto (1990) Sakai *et al.* (1993) Sakamoto *et al.* (1993)
fungal					
Aspergillus aculeatus Pectinex Ultra SP	45	ND	ND	(1→5)-L-arabinan L-arabinan	Beldman *et al.* (1993)
Aspergillus nidulans Strain FGSC 187 (L-arabitol)	40	ND	3.25	L-arabinan	Ramón *et al.* (1993)
Aspergillus niger [d] Strain CBS 120.49 (sugar beet pulp)	43	ND	3.00	L-arabinan	van der Veen *et al.* (1991)

[a] Apparent molecular weight determined with SDS-PAGE.
[b] Apparent molecular weight determined with gel permeation chromatography.
[c] Not determined.
[d] Several reports document the isolation of *A. niger* ABN from various sources; a full survey is given in Table 4. The data of van der Veen *et al.* (1991) are enlisted.

The apparent molecular weight of this enzyme is 67 kDa. In 1975, Sturdy and Cole reported the isolation of an "endo-type" arabinase from dry rot lesions caused by the fungus *Fusarium caeruleum* when cultured on potato tubers. It liberates arabinose as well as di- and tetra-arabinofuranosides from commercial sugar beet L-arabinan and from potato tubers. In contrast to *B. subtilis* ABN (Yoshihara and Kaji 1984), it fails to macerate tubers. From the data provided it is unclear whether this enzyme is a true ABN, as it was not tested for activity towards PNP-A and linear L-arabinan. Its reported apparent molecular weight (62 kDa) suggests that this enzyme is more likely to be an exo-type arabinase rather than an ABN (*cf.* Mullen and Bateman 1975; Tables 1 & 3).

Ancillary enzymes involved in L-arabinan degradation

Considering the structure of the polymer and the specificity of most arabinases for the furanose configuration of L-arabinose, complete degradation would require additional enzyme activities releasing the D-galactopyranose, L-arabinopyranose and feruloyl constituents from L-arabinan (as mentioned earlier in this Chapter). The relevance of such accessory activities in L-arabinan degradation was demonstrated by Tanaka *et al.* (1981). They isolated branched L-arabinan fragments enriched in D-galactose, from partially acid-hydrolyzed sugar beet L-arabinan. Up to five times more L-arabinose could be released from this substrate by *Scopolia japonica* ABF in the mutual presence of α-D-galactosidase (αGAL) (EC 3.2.1.22) and β-D-galactosidase (βGAL) (EC 3.2.1.23). It is not unlikely that these two glycosidases also facilitate the release of L-arabinopyranose, as some αGALs hydrolyze β-L-arabinopyranosides while some βGALs degrade α-L-arabinopyranosides (*cf.* Dey and Pridham 1972; Dey and Del Campillo 1984). α-L-Arabinosidase is present as a separate catalytic unit in a multimeric βGAL from apple (Dick *et al.* 1990). Both galactosidases belong to the extracellular glycosidase repertoire of *A. niger* (*e.g.* Bahl and Agrawal 1969; Adya and Elbein 1977; Widmar and Lueba 1979; Kumar *et al.* 1992). However, their potential L-arabinopyranosidase activity was never investigated. Specialized glycosidases could also be involved (Riou *et al.* 1991). The characterization of a plant β-L-arabinosidase (EC 3.2.1.88) lacking αGAL activity has been documented for example (Dey 1983). Ferulic acid esterase is considered a prime ancillary activity in the degradation of arabinoxylans (*cf.* Christov and Prior 1993). Recently, its involvement in pectinolysis has been addressed experimentally (Ralet *et al.* 1994b). Interestingly, *A. niger* produces at least three isozymes featuring distinct substrate specificities (Faulds and Williamson 1993, 1994; Ralet *et al.* 1994b).

Table 4. Source and some physico-chemical characteristics of *Aspergillus niger* arabinases

Source	MW [kDa] (SDS-PAGE) [a]	MW [kDa] (GPC) [b]	pI	Main substrate(s)	Reference(s)
α-L-arabinofuranosidase A (ABF A) [c]					
Pectinase 29	83	ND [d]	3.3	PNP-A (1,5)-α-L-arabinofuranosides arabinofuranoxylosides	Voragen *et al.* (1987) Rombouts *et al.* (1988) van der Veen *et al.* (1991) Kormelink *et al.* (1993)
Strain CBS 120.49 (sugar beet pulp)	83	ND	3.3	PNP-A	van der Veen *et al.* (1991)
Arabanase 8001/8002 [e]	80	ND	ND	PNP-A type-II arabinogalactan-protein	Saulnier *et al.* (1992)
α-L-arabinofuranosidase B (ABF B) [f]					
Strain K1 (sugar beet pulp)	ND	53	3.6	PNP-A / L-arabinan arabinoxylan type-II arabinogalactan	Kaji *et al.* (1967) Kaji and Tagawa (1970) Tagawa and Kaji (1988b)
Pectinol R-10	ND	ND	3.3	PNP-A / L-arabinan arabinoxylan type-II arabinogalactan	Neukom *et al.* (1967) Gremli and Neukom (1968) Neukom and Markwalder (1975) Waibel *et al.* (1980)
Pectinol 59-L	ND	ND	ND	arabinoxylan	Andrewartha *et al.* (1979)
Ultrazym 20	ND	ND	ND	PNP-A	Waibel *et al.* (1980)
Pectinase 29	67	ND	3.5	PNP-A / L-arabinan (1,5)-α-L-arabinofuranosides arabinofuranoxylosides type-II arabinogalactan	Voragen *et al.* (1987) Rombouts *et al.* (1988) van der Veen *et al.* (1991) Kormelink *et al.* (1993)

Hemicellulase REG 2	ND	61	< 3.7	PNP-A monoterpyl-diglycosides [g]	Gunata *et al.* (1990)
Pectinex AR	ND	52.9	3.6	PNP-A / L-arabinan	Schöpplein and Dietrich (1991)
Strain CBS 120.49 (sugar beet pulp)	67	ND	3.5	PNP-A / L-arabinan	van der Veen *et al.* (1991)
Strain 5-16 (arabinoxylan)	67	ND	3.5	PNP-A / L-arabinan A_1X_2 [h]	Kaneko *et al.* (1993)
endo-1,5-α-L-arabinase (ABN A) [i]					
Pectinase 29	43	ND	3.0	(1→5)-L-arabinan / L-arabinan penta-(1,5)-α-L-arabinofuranoside type-I arabinogalactan	Voragen *et al.* (1987) Rombouts *et al.* (1988) van der Veen *et al.* (1991) van de Vis *et al.* (1991)
Pectinex AR	ND	34.5	3.0	(1→5)-L-arabinan / L-arabinan type-I arabinogalactan	Schöpplein and Dietrich (1991)
Strain CBS 120.49 (sugar beet pulp)	43	ND	3.0	L-arabinan	van der Veen *et al.* (1991)
Pectinex AR KPG 027 [j]	42.5	41	2.9	(1→5)-L-arabinan / L-arabinan	Dunkel and Amadò (1994b)

[a] Apparent molecular weight determined with SDS-PAGE.
[b] Apparent molecular weight determined with gel permeation chromatography.
[c] Encoded by *abfA* gene (Flipphi *et al.* 1993c, 1994; Chapters 2 & 5). Molecular weight deduced from DNA sequence: 65.4 kDa; deduced pI: 3.7.
[d] Not determined.
[e] This enzyme is only briefly characterized. Given the molecular weight and substrate specificities, it is considered here to be ABF A rather than ABF B. However, it might be a distinct *A. niger* ABF with a restricted specificity towards type-II arabinogalactan-protein.
[f] Encoded by *abfB* gene (Flipphi *et al.* 1993a; Chapter 4). Molecular weight deduced from DNA sequence: 50.7 kDa; deduced pI: 3.8.
[g] [*O*-α-L-arabinofuranosyl-(1→6)]-β-D-glucopyranosides of various terpenols (Gunata *et al.* (1990).
[h] [*O*-α-L-arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose.
[i] Encoded by *abnA* gene (Flipphi *et al.* 1993b; Chapter 3). Molecular weight deduced from DNA sequence: 32.5 kDa; deduced pI: 3.5.
[j] Molecular weight determined with "matrix-assisted laser desorption ionization mass spectroscopy": 33.9 kDa (Dunkel and Amadò 1994b).

The L-arabinan-degrading system of *Aspergillus niger*

Amongst molds *A. niger* is a prominent producer of ABF and ABN activity (*e.g.* Tagawa 1970c; Butchak *et al.* 1976; Johnston *et al.* 1989; Karimi and Ward 1989; Tuohy *et al.* 1989). Some details on purified *A. niger* arabinases are compiled in Table 4. The interest in *A. niger* arabinases, particulary in ABF B, is related to application of these enzymes. ABN A, ABF A and ABF B, like most *A. niger* extracellular glycosidases, are optimally active in the acidic pH-range. Beyond doubt, *A. niger* ABF B is the most widely studied arabinase – more than a dozen different groups purified, characterized or applied this glycosidase. Kaji *et al.* (1963a) were the first to describe ABF B in the medium of a wheat bran-grown culture. Its main characteristics were documented back in 1970 (Kaji and Tagawa 1970; Tagawa 1970a, 1970b; as reviewed by Tagawa and Kaji 1988b). Waibel *et al.* (1980) reported the co-existence of two PNP-A-hydrolyzing enzymes in *A. niger*: ABF B can be specifically separated from the other enzyme by affinity chromatography on crosslinked L-arabinan as ABF B binds to this matrix. More recently, Voragen *et al.* (1987) isolated and characterized ABF A and ABN A for the first time. All three extracellular arabinases are glycosylated (Voragen *et al.* 1987).

ABF A and B differ in physico-chemical properties and substrate requirements (see Table 4). Both are highly specific for the furanose configuration of L-arabinose as deduced from their activity against a number of *para*-nitrophenyl glycosides under standard reaction conditions for PNP-A hydrolysis (unpublished observations). Only the structurally related β-linked D-galactofuranoside proved to be hydrolyzed to some extent by both enzymes (1.5 to 5 % compared to PNP-A), while upon extended reaction times weak hydrolysis of PNP-X (less than 0.2 %) could be observed. Under the latter conditions some hydrolysis of the α-linked D-galactopyranoside was found in the presence of ABF A. Arabinofuranosyl-transfer occurs with both ABFs using PNP-A as the donor and methanol as the acceptor (unpublished data), which strongly suggests that ABF A and ABF B act with retention of the anomeric configuration (*cf.* Sinnott 1990). The catalytic proton-donating amino acid in ABF B has a pKa-value of 5.65 as determined with pH-dependent kinetics using sugar beet L-arabinan as the substrate (Tagawa 1970a). ABF A and ABF B seem to have similar linkage preferences amongst α-linked L-arabinofuranobioses considering the evolvement of the formation of the four possible dimers and their steady state levels upon ABF-catalyzed condensation in saturated L-arabinose solution (unpublished results). ABF B is reported to act on polymers containing terminal non-reducing α-linked L-arabinofuranoses and debranching such polymers is considered one of its functions. ABF A, however, only acts on small oligomers containing only α-linked L-arabinofuranosyl groups (Voragen *et al.* 1987). This historical functional distinction between the two ABFs seems to be somewhat oversimplified, since: 1) The percentages of L-arabinose reported to be liberated by ABF B from arabinoxylan and type-II

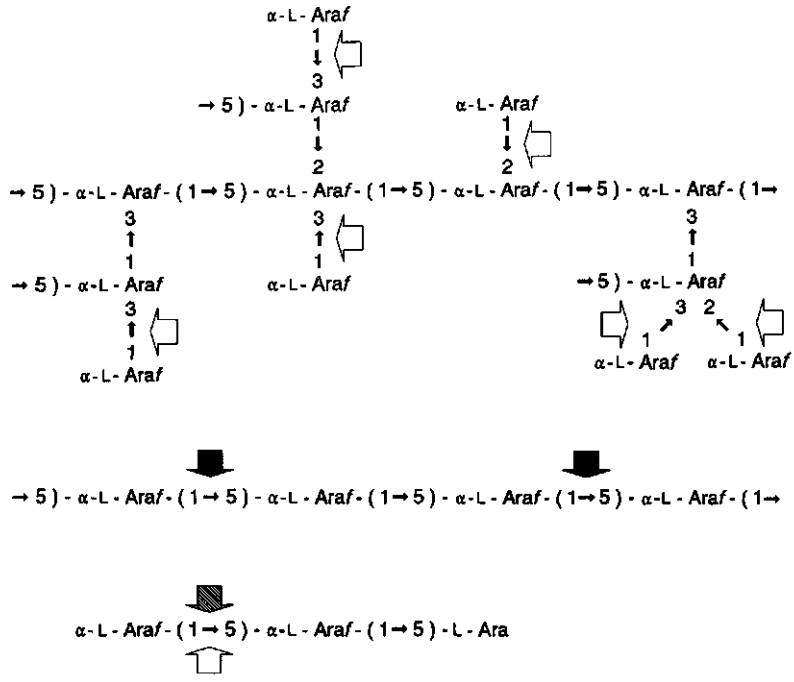


Fig.3. Simplified scheme showing the action of the three *A. niger* arabinases in the degradation of branched L-arabinan as deduced from their synergistic interactions (*cf.* Voragen *et al.* 1987). ABF B (open arrows) acts in debranching the polymer **(top)** while ABN A (black arrows) degrades the backbone in debranched regions **(middle)**. Both ABF A (hatched arrow) and ABF B depolymerize short oligomeric structures resulting from ABN A action to the endproduct of degradation, L-arabinose **(bottom)**. **[ara]** denotes the reducing residue of the latter structure, subject to continuous interconversion of all four hemiacetal configurations.

arabinogalactan show enormous variation. Although its synthesis was induced on arabinoxylan, Kaneko *et al.* (1993) found no activity towards the polymeric substrate; 2) Specialized debranching enzymes are described in related fungi (Sturdy and Cole 1975; Lahaye and Thibault 1990; Kormelink *et al.* 1991). The specific activity of *A. awamori* AXH on arabinoxylan is more than twenty times that of *A. niger* ABF B (Kormelink *et al.* 1991). Moreover, besides AXH, *A. awamori* produces an ABF B-like enzyme which is hardly active on polymeric arabinoxylan (Wood *et al.* 1992); 3) Oligomers are far better substrates for ABF B than polymers, as the activity on linear (1→5)-*α*-L-arabinofuranosides declines with increasing length (Rombouts *et al.* 1988). Upon sustained purification of a

commercial ABF B preparation, activity towards linear L-arabinan is lost (Lerouge *et al.* 1993b); 4) Both ABFs act on arabinofuranoxylosyl oligomers originating from wheat arabinoxylan, though ABF A and ABF B feature quite different substituent requirements (Kormelink *et al.* 1993); 5): *Aspergillus nidulans* does not produce an equivalent for ABF A (Ramón *et al.* 1993); 6): The kinetic properties of the ABFs for PNP-A hydrolysis are essentially identical, both at pH 3.5 and pH 5.0 (Rombouts *et al.* 1988; van der Veen *et al.* 1991). Unfortunately, kinetic data for defined α-L-arabinofuranosides and arabinofuranoxylosides are not available to date.

Linear (1→5)-α-L-arabinan is the preferred substrate for fungal ABN and reduced activity is found on branched L-arabinan. The activity declines with decreasing chain-length and increasing degree of substitution (Rombouts *et al.* 1988; McCleary 1991; Lerouge *et al.* 1993b; Beldman *et al.* 1993). *A. niger* ABN A liberates primarily arabinobiose and arabinotriose from linear L-arabinan, and L-arabinose and arabinobiose upon prolonged incubation (Rombouts *et al.* 1988). The enzyme contains either a pentameric or a hexameric substrate-binding site (McCleary 1991). Neither L-arabinose nor its γ-lactone inhibit degradation of linear L-arabinan (Dunkel and Amadò 1994b). PNP-A- and arabinoxylan-degrading activity are essentially absent, but type-I arabinogalactan is shown to be a substrate for ABN A (Schöpplein and Dietrich 1991; van de Vis *et al.* 1991).

Efficient degradation of L-arabinan requires cooperative action of the three *A. niger* arabinases as mutual action of ABN A with either ABF leads to enhanced formation of reducing sugar equivalents, suggesting synergism in L-arabinan degradation (Voragen *et al.* 1987; Rombouts *et al.* 1988; see also: Guillon and Thibault 1990; Beldman *et al.* 1993). The mode of action of the *A. niger* arabinases which has been deduced from these observations is schematically drawn in Fig.3. Similar forms of cooperation are documented for ABN and endo-(1,4)-D-galactanase in the degradation of type-I arabinogalactan (van de Vis *et al.* 1991). Degradation of arabinoxylan, type-II arabinogalactan and pectin by XYN, exo-(1,3)-β-galactanase, βGAL and endo-polygalacturonase, respectively, is enhanced by ABF activity (Greve *et al.* 1984; Lee and Forsberg 1987; Poutanen 1988; Kauffmann *et al.* 1990; Hirano *et al.* 1994; van de Vis 1994).

Current use and potential applications of arabinases

The availability of pure and defined arabinases can be considered a useful tool in further studies on optimalization of enzymic conversion of plant biomass, particularly because the L-arabinan component is involved in covalent crosslinking of structural cell wall components. Action of *A. niger* ABN A on sugar beet pectin eliminates a portion of its ferulic acid contents (Guillon *et al.* 1989; Guillon and Thibault 1990), which is the substituent directly involved in covalent crosslinking in sugar beet. The protopectin-solubilizing activity described for *B. subtilis* ABN (Sakai *et al.* 1993) is an excellent example of how L-arabinan degradation can

affect the cell-wall matrix structure. Moreover, *B. subtilis* ABN disintegrates tissue from potato (Yoshihara and Kaji 1984) eventhough potato pectin does not carry feruloyl groups. Disruption of the cell wall matrix increases the accessibility of the individual cell wall components for enzymic degradation. The debranching activity of ABFs might be an important accessory function in endolytic degradation of pectin and hemicellulose, by acting towards the L-arabinofuranosyl-containing components. Such a function would explain the forementioned synergistic interactions. Furthermore, gradual enzymic disruption of the matrix structure circumvents feedback inhibition and catabolite repression of the various glycan-degrading systems by released monomeric sugars during biomass conversion. This is an important concept in the development of continuous systems for down-stream processing of agro-industrial by-products to chemical feedstock or biogas (*e.g.* Kusakabe *et al.* 1975; Kjaergaard 1984; Coughlan *et al.* 1986; Considine *et al.* 1986, 1988; Buchholz *et al.* 1987; Khan *et al.* 1988). Moreover, enzymic pretreatment of the pentosan fraction in live stock forage improves the digestibility and the nutritional value for non-herbivores (*e.g.* Groot Wassink *et al.* 1989; Charlick *et al.* 1991; Evans *et al.* 1992; van Heuvel *et al.* 1992).

Currently both ABF and ABN are widely applied as highly specific tools to study polysaccharide structure and crosslinking, especially with respect to pectins and arabinoxylans (*e.g.* Saulnier *et al.* 1988; Guillon *et al.* 1989; Nishitani and Nevins 1989; Renard *et al.* 1991; Schöpplein *et al.* 1991; Saulnier *et al.* 1992; Lerouge *et al.* 1993a; Pellerin *et al.* 1993). Commercial application of ABF is mentioned in bio-bleaching of paper (Zamost and Elm-Durrschmidt 1991; Bezalel *et al.* 1993), improvement of the gelation properties of sugar beet pectin (Guillon and Thibault 1987, 1990; Matthew *et al.* 1990, 1991) and the enhancement of flavour by the degradation of natural glycosyl precursors of odoriferous compounds in wine-must (Gunata *et al.* 1990, 1991; van Heuvel *et al.* 1992). Enzymic modification of the L-arabinan residue, obtained from sugar beet upon commercial sugar production, leads to debranched L-arabinan. In concentrated form this behaves like a paste and can be utilized in food industry, for instance as a fat substitute (McCleary *et al.* 1990). A curious application of ABF is the targeting of chemostatics by *in situ* pH-dependent hydrolysis of inert arabinofuranosyl-derivatives as an alternative concept in cancer therapy (*cf.* Butschak *et al.* 1989b, 1989c). Fungal ABN could be applied to complement pectic enzyme preparations devoid of this activity, circumventing the formation of insoluble colloids during the preparation of fruit juices (Voragen *et al.* 1982; Voragen and Pilnik 1989; McCleary 1990; van Heuvel *et al.* 1992). The forementioned protopectin-solubilizing activity of *B. subtilis* ABN could be useful to release high molecular weight pectin from agro-industrial by-products and in preparing plant protoplasts, as an alternative for the chemical and mechanical processes currently in use (Sakai *et al.* 1993).

Induction of fungal arabinases

Surveying the literature on ABFs (*cf.* Table 1), the enzymes are mostly detected in cultures containing complex polysaccharides: L-arabinan, pectin, pectate, arabinoxylan, sugar beet pulp, wheat bran or even crude cell wall fractions. L-Arabinose, the end-product of L-arabinan degradation, L-arabitol, D-galactose and D-galacturonic acid are documented to mediate expression of fungal ABF activity (*e.g.* Laborda *et al.* 1974; Cooper and Wood 1975; Baker *et al.* 1979; Ramón *et al.* 1993; Kristufek *et al.* 1994). However, Cooper and Wood (1973 & 1975) elegantly proved that L-arabinose suppresses expression of ABF by feed-back inhibition when cultures of *Verticillium albo-atrum* and *Fusarium oxysporum* f.sp. *lycopersici* are supplied with more carbon source than needed to maintain metabolism, reflected by the levels of L-arabinose residing in the culture medium. Restricted supply of L-arabinose resulted in ten- to twenty-fold higher expression levels in *F. oxysporum* and even up to thirtyfive-fold higher levels in *V. albo-atrum.* Some phytopathogenic fungi feature constitutive secretion of ABF (*e.g.* Fuchs *et al.* 1965; Cole and Wood 1970; Higashi *et al.* 1986; Butschak *et al.* 1989a). ABF expression requires induction in the soft-rot fungus *Trichoderma reesei* but is not subject to carbon catabolite repression (Poutanen 1988; Kristufek *et al.* 1994). In most saprophytic fungi expression requires induction and is subject to carbon catabolite repression. As shown in Table 4, *A. niger* arabinases are mainly obtained from commercial enzyme preparations. Kaji, Tagawa and co-workers purified their ABF B from culture medium. Induction studies showed that ABF B was produced in the presence of either L-arabinan, L-arabinose or L-arabitol, the polymer being the most powerful inducer (Tagawa and Terui 1968). Both D-glucose and L-arabinose conferred catabolite repression of ABF B formation mediated by L-arabinan. Recently, Kaneko *et al.* (1993) isolated ABF B as a mycelium-associated enzyme upon growth on arabinoxylan.

In 1988, research on the genetics of L-arabinan degradation by *A. niger* was initiated in our department. Within the course of these studies, we purified three arabinases from an *A. niger* sugar beet pulp culture and determined some of their physico-chemical and kinetic properties (van der Veen *et al.* 1991; Table 4). In addition, specific antisera were raised which enabled us to screen the induction characteristics of various carbon sources. Crude residues as well as polysaccharides containing L-arabinan proved to be powerful inducers of the L-arabinan-degrading complex, sugar beet pulp being the most effective one. Other L-arabinofuranose-containing polysaccharides mediated the formation of only ABF B. These results conform the generally accepted concept that microbial polysaccharide-degrading systems are induced by low-molecular-weight substances derived from their substrates (*cf.* Reese 1977). When added abundantly, L-arabinose only induced formation of ABF B. L-Arabitol, an intermediate of *A. niger* L-arabinose catabolism (Witteveen *et al.* 1989), was found to mediate expression of all three arabinases, though less efficiently than sugar beet pulp (van der Veen

et al. 1993). Arabinase expression was repressed upon co-cultivation with D-glucose. Upon depletion of D-glucose as sole carbon source, PNP-A-hydrolyzing activity was produced at low levels. With respect to ABF B, our induction results confirm those of Tagawa and Terui (1968).

The putative status of L-arabitol as a low-molecular-weight inducer is further strengthened by the induction characteristics of an *A. niger* mutant, which is unable to grow on both L-arabinose and D-xylose (Witteveen *et al.* 1989). Upon mycelial transfer to L-arabitol, it produced up to three times more PNP-A-hydrolyzing activity than the wild-type strain (van der Veen *et al.* 1993). In contrast to the wild-type, transfer to D-xylose or xylitol did also lead to expression of arabinases in the mutant together with intracellular accumulation of L-arabitol.

Aim and outline of the thesis

In this introductionary Chapter I have surveyed the current knowledge on L-arabinan as an important plant cell wall component, as well as on the enzyme system involved in degradation and modification of this component. Despite their obvious importance in the degradation of plant biomass, arabinases did not receive much attention in the past. This is essentially due to the delicate nature of L-arabinan and its ferulic acid substituents as well as the lack of simple and defined substrates to study the biochemistry of arabinases. Genetic research could lead to novel insights in microbial strategies to degrade plant cell walls and the regulatory mechanisms involved. Moreover, such an approach would provide means to overproduce the individual enzyme components, enabling further characterization and application.

The availability of highly purified enzymes, specific antisera, the low-molecular-weight inducer L-arabitol as well as an *A. niger* mutant strain unable to grow on L-arabinose and L-arabitol, provided the means for the molecular cloning and primary characterization of the structural genes coding for the enzymes of the *A. niger* L-arabinan-degrading system. Chapters 2, 3 and 4 describe the cloning of the structural genes encoding ABF A, ABN A and ABF B, respectively. In each case, the identity of the cloned material was established upon the introduction of multiple gene copies in both *A. niger* and the heterologous host *A. nidulans*. The transformant strains thus generated overproduced the respective arabinases when grown on sugar beet pulp as the carbon source. The DNA sequences of the genes were determined and the primary structures of the encoded proteins were analyzed in Chapters 3 (ABN A), 4 (ABF B) and 5 (ABF A). The enzymes of the L-arabinan-degrading system lack structural sequence similarity with each other. The mechanisms affecting expression of the three genes were studied in Chapter 5, by analyzing the effects of multiple copies of either ABF-encoding gene on the synthesis of the other ABF as well as of ABN A upon induction with sugar beet pulp and L-arabitol. The regulatory mechanisms governing the expression of

arabinases were further addressed in a study of an *A. nidulans* mutant unable to grow on L-arabinose. In Chapter 6, this mutant is characterized biochemically and the effects of the mutation on the regulation of the L-arabinose catabolic pathway as well as of the L-arabinan-degrading complex were studied. In Chapter 7, the results obtained are discussed in relation to further biochemical characterization of *A. niger* arabinases as well as to the mechanisms involved in their expression. Preliminary and unpublished results are presented to support these considerations.

Acknowledgement. I am grateful towards Dr. G. Beldman and Prof. Dr. A.G.J. Voragen (Section of Food Chemistry and Microbiology, Dept. of Food Technology, Wageningen Agricultural University, The Netherlands) for their willingness to provide me with material prior to publication.

References

Adya SW, Elbein AD (1977) Glycoprotein enzymes secreted by *Aspergillus niger*: purification and properties of α-galactosidase. J Bacteriol **129**:850-856

Ajay Singh, Kumar PKR, Schügerl K (1992) Bioconversion of cellulosic materials to ethanol by filamentous fungi. Adv Biochem Eng Biotechnol **45**:30-55

Akinrefon OA (1968) Studies on the α-L-arabinofuranosidase of *Phytophthora palmivora* (Butl.) Butl. New Phytol **67**:543-556

Andrewartha KA, Phillips DR, Stone BA (1979) Solution properties of wheat-flour arabinoxylans and enzymically modified arabinoxylans. Carbohydr Res **77**: 191-204

Araujo A, Ward OP (1990) Purification and some properties of the mannanases from *Thielavia terrestris*. J Industr Microbiol **6**:269-274

Axamawaty MTH, Fleet GWJ, Hannah KA, Namgoong SK, Sinnott ML (1990) Inhibition of the α-L-arabinofuranosidase III of *Monilinia fructigena* by 1,4-dideoxy-1,4-imino-L-threitol and 1,4-dideoxy-1,4-imino-L-arabinitol. Biochem J **266**:245-249

Babsky NE, Schobinger U (1986) An arabinan in pear juice concentrate. Alimenta **5**:125-127

Bachmann SL, McCarthy AJ (1991) Purification and cooperative activity of enzymes constituting the xylan-degrading system of *Thermomonospora fusca*. Appl Environ Microbiol **57**:2121-2130.

Bahl OP, Agrawal KML (1969) Glycosidases of *Aspergillus niger*. I. Purification and characterization of α- and β-galactosidases and β-N-acetylglucosaminidase. J Biol Chem **244**: 2970-2978

Baker CJ, Whalen CH, Korman RZ, Bateman DF (1979) α-L-Arabinofuranosidase from *Sclerotinia sclerotiorum*: purification, characterization, and effects on plant cell walls and tissue. Phytopathology **69**:789-793

Bateman DF (1976) Plant cell wall hydrolysis by pathogens. In: Friend J, Threlfall DR (eds) Biochemical aspects of host-pathogen relationships. Academic Press, London. pp.79-103

Beldman G, Searle-van Leeuwen MJF, Ruiter GA de, Siliha HA, Voragen AGJ (1993) Degradation of arabinans by arabinanases from *Aspergillus aculeatus* and *Aspergillus niger*. Carbohydr Polym **20**:159-168

BeMiller JN (1967) Acid-catalyzed hydrolysis of glycosides. Adv Carbohydr Chem **22**:25-108

Bertin C, Rouau X, Thibault J-F (1988) Structure and properties of sugar beet fibers. J Sci Food Agric **44**:15-29

Bezalel L, Shoham Y, Rosenberg E (1993) Characterization and delignification activity of a thermostable α-L-arabinofuranosidase from *Bacillus stearothermophilus*. Appl Microbiol Biotechnol **40**:57-62

Biely P (1985) Microbial xylanolytic systems. Trends Biotechnol **3**:286-290

Börjeson H, Jerkeman P, Lindberg B (1963) Phenylglycofuranosides. Synthesis of phenyl-β-D-xylofuranoside and phenyl-α-L-arabinofuranoside. Acta Chem Scand **17**:1705-1708

Brillouet J-M, Moulin J-C (1985) Production, purification, and properties of an α-L-arabinofuranosidase from *Dichomitus squalens*. Carbohydr Res **144**:113-126

Bronnenmeier K, Ebenbichler C, Staudenbauer WL (1990) Separation of the cellulolytic and xylanolytic enzymes of *Clostridium stercorarium*. J Chromatogr **521**:301-310

Buchholz K, Stoppok E, Emmerich R, Bartz U (1987) Zur Anaeroben fermentation von rübenschnitzeln und rübenabfall – reaktionstechnik und scale up. Zuckerind **112**:605-611

Butschak G, Förster W, Graffi A (1976) Spaltung des α-L-arabinofuranosids, β-D-glucopyranosids und β-cellobiosids von 4-nitrophenol durch enzyme verscheidener pilze – ein beitrag in verbindung mit versuchen zur steigerung der selektivität der tumortherapie. Z Alg Mikrobiol **16**:507-519

Butschak G, Küster A, Schulze B, Graffi A (1989a) Temperature dependent pH-instability of some α-L-arabinofuranosidases. Arch Geschwulstforsch **59**:165-170

Butschak G, Schulze B, Küster A, Niederhausen T, Niemeyer U, Graffi A (1989b) Pharmakokinetische untersuchungenn der aktivität einer pH-labilen α-L-arabinofuranosidase nach i.v.-injektion in tumortragende mäuse. Arch Geschwulstforsch **59**:325-331

Butschak G, Schulze B, Küster A, Niederhausen T, Niemeyer U, Graffi A (1989c) Versuche zur beeinflussung der verteilung der aktivität verschiedener α-L-arabinofuranosidasen nach i.v. applikation in tumortragende mäuse. Arch Geschwulstforsch **59**:407-413.

Carre B, Brillouet J-M, Thibault J-F (1985) Characterization of polysaccharides from white lupin (*Lupinus albus* L.) J Agric Food Chem **33**:285-292

Chambat G, Barnoud F, Joseleau J-P (1984) Structure of the primary cell walls of suspension cultured *Rosa glauca* cells. I. Polysaccharides associated with cellulose. Plant Physiol **74**:687-693

Charlick R, Hissa K, Hissa P, Virkki M (1991) Treatment of forage with enzymes and lactic acid bacteria for silage. PCT Int. Appl. WO 91 15,966. 31-Okt-1991. 87 pp. [Chem Abstracts **116**:104852j]

Christov LP, Prior BA (1993) Esterases of xylan-degrading microorganisms: production, properties, and significance. Enzyme Microb Technol **15**:460-475

Churms SC, Merrifield EH, Stephen AM, Walwijn DR, Polson A, Merwe K van de, Spies HCS, Costa N (1983) An L-arabinan from apple-juice concentrates. Carbohydr Res **113**:339-344

Clarke AE, Anderson RL, Stone BA (1979) Form and function of arabinogalactans and arabinogalactan-proteins. Phytochemistry **18**:521-540

Cole ALJ, Wood RKS (1970) Production of hemicellulases by *Penicillium digitatum*. Phytochemistry **9**:695-699

Colquhoun IJ, Ralet M-C, Thibault J-F, Faulds CB, Williamson G (1994) Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy. [Feruloylated oligosaccharides from cell-wall polysaccharides. II.] Carbohydr Res **263**:243-256

Conchie J, Hay AJ, Lomax JA (1988) Soluble lignin-carbohydrate complexes from sheep rumen fluid: their composition and structural features. Carbohydr Res **177**:127-151

Considine PJ, Mehra RK, Hackett TJ, O'Rorke A, Comerford FR, Couglan MP (1986) Upgrading the value of agricultural residues. Ann NY Acad Sci **469** [Biochem Eng IV]: 304-311

Considine PJ, O'Rorke A, Hackett TJ, Couglan MP (1988) Hydrolysis of beet pulp polysaccharides by actracts of solid-state cultures of *Pencicillium capsulatum*. Biotechnol Bioeng **31**:433-438

Cooper RM, Wood RKS (1973) Induction of synthesis of extracellular cell-wall degrading enzymes in vascular wilt fungi. Nature **246**:309-311

Cooper RM, Wood RKS (1975) Regulation of synthesis of cell wall degrading enzymes by *Verticilium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici*. Physiol Plant Pathol **5**: 135-156

Cooper RM, Longman D, Campbell A, Henry M, Lees PE (1988) Enzymic adaptation of cereal pathogens to the monocotyledonous primary wall. Physiol Mol Plant Pathol **32**:33-47

Coughlan MP (1989) Enzyme systems for lignocellulose degradation. [Proceedings of a workshop on Production, Characterization and Application of Cellulose-, Hemicellulose- and Lignin-degrading Enzyme Systems. Galway, Ireland, april 12-14 1989.] Elsevier Applied Science, London.

Coughlan MP, Hazlewood GP (1993b) Hemicellulose and hemicellulases. Portland Press research Monograph IV. Portland Press, London & Chapel Hill.

Coughlan MP, Considine PJ, O'Rorke A, Moloney AP, Mehra RK, Hackett TJ, Puls J (1986) Energy from biomass: saccharification of beet pulp using fungal enzymes. Ann NY Acad Sci **469** [Biochem Eng IV]: 294-303

Coughlan MP, Tuohy MG, Filho EXF, Puls J, Claeyssens M, Vršanská M, Hughes MM (1993) Enzymological aspects of microbial hemicellulases with emphasis on fungal systems. In: Coughlan MP, Hazlewood GP (eds) Hemicellulose and hemicellulases. Portland Press research Monograph IV. Portland Press, London & Chapel Hill. pp.53-84

Cros S, Imberty A, Bouchemal N, Hervé du Penhoat C, Perez S (1994) Modeling of arabinofuranose and arabinan. II. NMR and conformational analysis of arabinobiose and arabinan. Biopolym **34**:1433-1447

Dekker RFH (1985) Biodegradation of the hemicelluloses. In: Higuchi T (ed) Biosynthesis and biodegradation of wood components. Academic Press, Orlando. pp.505-533

Dekker RFH, Richards GN (1975) Purification, properties, and mode of action of hemicellulase I produced by *Ceratocytis paradoxa*. [Studies on hemicellulases. II.] Carbohydr Res **39**: 97-114

Dekker RFH, Richards GN (1976) Hemicellulases: their occurrence, purification, properties, and mode of action. Adv Carbohydr Chem Biochem **32**:277-352

Dey PM (1983) Further characterization of β-L-arabinosidase from *Cajanus indicus*. Biochim Biophys Acta **746**:8-13

Dey PM, Brinson K (1984) Plant cell-walls. Adv Carbohydr Chem Biochem **42**:265-382

Dey PM, Del Campillo E (1984) Biochemistry of the multiple form of glycosidases in plants. Adv Enzymol & Rel Areas Mol Biol **56**:141-249

Dey PM, Pridham JB (1972) Biochemistry of α-galactosidases. Adv Enzymol & Rel Areas Mol Biol **36**:91-130

Dick AJ, Opoku-Gyamfua A, DeMarco AC (1990) Glycosidases of apple fruit: a multi-functional β-galactosidase. Physiol Plant **80**:250-256

Dunkel MPH, Amadó R (1994a) A simple, rapid, and semiquantitative assay for the determination of *endo*-(1→5)-α-L-arabinanase (EC 3.2.1.99) activity in column chromatography eluate fractions. Carbohydr Res **260**:131-135

Dunkel MPH, Amadó R (1994b) Purification and physico-chemical properties of an *endo*-(1→5)-α-L-arabinanase (EC 3.2.1.99) isolated from an *Aspergillus niger* pectinase preparation. Carbohydr Polym **24**:247-263

Ehrlich F, Schubert F (1928) Über tetra-araban und seine beziehung zur tetragalakturonsäure, dem hauptkomplex der pektinstoffe. Biochem Z **203**:343-350

Evans CT, Mann SP, Charley RC, Parfitt D (1992) A carbohydrolase and microorganism-containing mixture for treating silage to improve nutritional quality. PCT Int. Appl. WO 92 10,945. 09-Jul-1992. 17 pp. [Chem Abstracts **117**:232770p]

Faulds CB, Williamson G (1993) Ferulic acid esterase from *Aspergillus niger*: purification and partial characterization of two forms from a commercial source of pectinase. Biotechnol Appl Biochem **117**:349-359

Faulds CB, Williamson G (1994) Purification and characterization of a ferulic acid esterase (FAE-III) from *Aspergillus niger*: specificity for the phenolic moiety and binding to microcristalline cellulose. Microbiology **140**:779-787

Fernández-Espinar MT, Peña JL, Piñaga F, Vallés S (1994) α-L-Arabinofuranosidase production by *Aspergillus nidulans*. FEMS Microbiol Lett **115**:107-112

Fielding AH, Hough L (1965) The synthesis of *p*-nitrophenyl α-L-arabinofuranoside. Carbohydr Res **1**:327-329

Fielding AH, Sinnott ML, Kelly MA, Widdows D (1981) Product stereochemistry and some inhibitors of the α-L-arabinofuranosidases of *Monilinia fructigena*. J Chem Soc Perkin Trans I:1013-1014

Fincher GB, Stone BA, Clarke AE (1984) Arabinogalactan-proteins: structure, biosynthesis, and function. Ann Rev Plant Physiol **34**:47-70

Flint HJ, McPherson CA, Bisset J (1989) Molecular cloning of genes from *Ruminococcus flavefaciens* encoding xylanase and β-(1-3,1-4)-glucanase activities. Appl Environ Microbiol **55**:1230-1233

Flipphi MJA, Heuvel M van, Veen P van der, Visser J, Graaff LH de (1993a) Cloning and characterization of the *abf*B gene coding for the major α-L-arabinofuranosidase (ABF B) of *Aspergillus niger*. Curr Genet **24**:525-532

Flipphi MJA, Panneman H, Veen P van der, Visser J, Graaff LH de (1993b) Molecular cloning, expression and structure of the *endo* 1,5-α-L-arabinase gene of *Aspergillus niger*. Appl Microbiol Biotechnol **40**:318-326

Flipphi MJA, Visser J, Veen P van der, Graaff LH de (1993c) Cloning of the *Aspergillus niger* gene encoding α-L-arabinofuranosidase A. Appl Microbiol Biotechnol **39**:335-340

Flipphi MJA, Visser J, Veen P van der, Graaff LH de (1994) Arabinase gene expression in *Aspergillus niger*: indications for coordinated regulation. Microbiology **140**:2673-2682

Fry SC (1982) Phenolic components of the primary cell wall. Feruloylated dissaccharides of D-galactose and L-arabinose from spinach polysaccharide. Biochem J **203**:493-504

Fry SC (1983) Feruloylated pectins from the primary cell wall: their structure and possible functions. Planta **157**:111-123

Fry SC (1986) Cross-linking of matrix polymers in the growing cell walls of angiosperms. Ann Rev Plant Physiol **37**:156-186

Fry SC, Miller JG (1989) Towards a working model of the growing plant cell wall: phenolic crosslinking reactions in the primary cell walls of dicotyledons. In: Lewis NG, Paice MG (eds). Plant cell wall polymers. Biogenesis and biodegradation. ACS Symp Ser **399**. American Chemical Society, Washington DC. pp.33-46

Fuchs A, Jobsen JA, Wouts WM (1965) Arabanases in phytopathogenic fungi. Nature **206**: 714-715

Geissman T, Neukom H (1971) Vernetzung von phenolcarbonsäure-estern von polysacchariden durch oxydative phenolische kupplung. Helv Chim Acta **54**:1108-1112

Geissman T, Neukom H (1973) On the composition of the water-soluble pentosans and their oxidative gelation. Lebensm Wis Technol **6**:59-62

Golubev AM, Kilimnik AY, Rodionova NA, Kaprel'yants LV, Neustroev KN (1993) Isolation and properties of arabinofuranosidase from *Geotrichum candidum* 3C. Biochemistry Russia **58**: 124-128

Gosalbes MJ, Pérez-González JA, González R, Navarro A (1991) Two β-glycanase genes are clustered in *Bacillus polymyxa*: molecular cloning, expression and sequence analysis of genes encoding a xylanase and an endo-β-(1,3)-(1,4)-glucanase. J Bacteriol **173**: 7705-7710

Grabski AC, Jeffries TW (1991) Production, purification, and characterization of β-(1,4)-endoxylanase of *Streptomyces roseiscleoticum*. Appl Environ Microbiol **57**:987-992

Green JW (1966) The glycofuranosides. Adv Carbohydr Chem **21**:95-142

Gremli H, Neukom H (1968) The effect of a purified α-L-arabinofuranosidase on some arabinose-containing polysaccharides. Carbohydr Res **8**:110-112

Greve LC, Labavitch JM, Hungate RE (1984) α-L-Arabinofuranosidase from *Ruminococcus albus* 8: purification and possible role in the hydrolysis of alfalfa cell wall. Appl Environ Microbiol **47**:1135-1140

Groot Wassink JWD, Campbell GL, Classen HL (1989) Fractionation of crude pentosanase (arabinoxylanase) for improvement of the nutritional value of rye diets for broiler chickens. J Sci Food Agric **46**:289-300

Guillon F, Thibault J-F (1987) Structural study of the side chains of beet pectins. CR Seances Soc Biol Ses Fil **181**:707-717. [Chem Abstracts **110**:39271d]

Guillon F, Thibault J-F (1989) Methylation analysis and mild acid hydrolysis of the "hairy" fragments of sugar-beet pectins. [Structural investigation of the neutral sugar side-chains of sugar-beet pectins. I.] Carbohydr Res **190**:85-96

Guillon F, Thibault J-F (1990) Oxidate cross-linking of chemically and enzymatically modified sugar-beet pectin. Carbohydr Polym **12**:353-374

Guillon F, Thibault J-F, Rombouts FM, Voragen AGJ, Pilnik W (1989) Enzymic hydrolysis of the "hairy" fragments of sugar-beet pectins. [Structural investigation of the neutral sugar side-chains of sugar-beet pectins. II.] Carbohydr Res **190**:97-108

Gunata Z, Brillouet J-M, Voirin S, Baumes R, Cordonnier R (1990) Purification and some properties of an α-L-arabinofuranosidase from *Aspergillus niger*. Action on grape monoterpenyl arabinofuranosylglucosides. J Agric Food Chem **38**:772-776

Gunata Z, Bitteur S, Baumes R, Brillouet J-M, Tapiero C, Bayonove C, Cordonnier R (1991) Enzymic preparation of odoriferous compounds from glycosidic precursors. Eur. Pat. Appl. WO 91 09,537. 11-Jul-1991. 59 pp. [Chem Abstracts **116**:127347m]

Hartley RD, Ford CW (1989) Phenolic constituents of plant cell walls and wall biodegradability. In: Lewis NG, Paice MG (eds) Plant cell wall polymers. Biogenesis and biodegradation. ACS symposium series **399**. American Chemical Society, Washington DC. pp.137-145

Hartley RD, Jones EC (1976) Diferulic acid as a component of cell walls of *Lolium multiflorum*. [Lignin-carbohydrate linkages in plant cell walls. 4.] Phytochemistry **15**:1157-1160

Hartley RD, Jones EC (1977) Phenolic components and digestability of cell walls of grass and legume species. [Lignin-carbohydrate linkages in plant cell walls. 5.] Phytochemistry **16**: 1531-1534

Hartley RD, Morrison WH III, Himmelsbach DS, Borneman WS (1990) Cross-linking of cell wall phenolic arabinoxylans in graminaceous plants. Phytochemistry **29**:3705-3709

Hata K, Tanaka M, Tsumuraya Y, Hashimoto Y (1992) α-L-Arabinofuranosidase from Radish (*Raphanus sativus* L.) seeds. Plant Physiol **100**:388-396

Hatanaka H, Imaoka H, Tajima S, Kasai T (1991) Purification and properties of an α-L-arabinofuranosidase from cotyledons of soybean seedlings. Agric Biol Chem **55**:2599-2605

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J **280**:309-316

Henrissat B, Bairoch A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J **293**:781-788

Hespell RB, O'Bryan PJ (1992) Purification and characterization of an α-L-arabinofuranosidase from *Butyrivibrio fibrisolvens* GS113. Appl Environ Microbiol **58**:1082-1088

Heuvel M van, Bakhuis JG, Coutel Y, Harder A, Graaff LH de, Flipphi MJA, Veen P van der, Visser J, Andreoli PM (1992) Cloning and expression of gene encoding fungal arabinan-degrading enzymes. Eur Pat Appl EP 506,190. 30-Sep-1992. 75 pp. [Chem Abstracts **118**:97139t]

Higashi K (1983) Studies on α-L-arabinofuranosidase produced by *Streptomyces diastatochromogenes* 065. Bull Agric Fac Tamagawa Univ **23**:69-118.

Higashi K, Kusakabe I, Yasui T (1981) Studies on α-L-arabinofuranosidase from *Streptomyces* sp. No. 065. Purification and properties. Bull Agric Fac Tamagawa Univ **21**:46-58.

Higashi K, Kusakabe I, Yasui T, Ishiyama T, Okimoto Y (1983) Arabinan-degrading enzymes from *Streptomyces diastatochromogenes* 065. Agric Biol Chem **47**:2903-2905

Higashi K, Ishiyama T, Okimoto Y (1986) Plant cell wall degrading enzymes from phytopathogenic fungi. Bull Agric Fac Tamagawa Univ **26**:64-74.

Hirano Y, Tsumuraya Y, Hashimoto Y (1994) Characterization of spinach leaf α-L-arabinofuranosidases and β-galactosidases and their synergistic action on an endogenous arabinogalactan-protein. Physiol Plant **92**:286-296

Hirst EL, Jones JKN (1947) Pectic substances. VI. The structure of the araban from *Arachis hypogea*. J Chem Soc:1221-1225

Hirst EL, Jones JKN (1948) Pectic substances. VIII. The araban component of sugar-beet pectin. J Chem Soc:2311-2313

Hislop EC, Shellis C, Fielding AH, Bourne FJ, Chidlow JW (1974) Antisera produced to purified extracellular pectolytic enzymes from *Sclerotinia fructigena*. J Gen Microbiol **83**: 135-143

Hough L, Powell DB (1960) Methylation and peroxidate oxidation studies of the alkali-stable polysaccharide of sugar-beet pectin. J Chem Soc:16-22

Howell HE (1975) Correlation of virulence with secretion *in vitro* of three wall-degrading enzymes in isolates of *Sclerotinia fructigena* obtained after mutagen treatment. J Gen Microbiol **90**:32-40

Iiyama K, Lam TBT, Stone BA (1990) Phenolic acid bridges between polysaccharides and lignin in wheat internodes. Phytochemistry **29**:733-737

Iiyama K, Lam TBT, Stone BA (1994) Covalent cross-links in the cell wall. Plant Physiol **104**: 315-320

Ishii T, Hirroi T, Thomas JR (1990) Feruloylated xyloglucan and *p*-coumaroyl arabinoxylan oligosaccharides from bamboo shoot cell-walls. Phytochemistry **29**:1999-2003

Jarvis MC, Threlfall DR, Friend J (1981) Potato cell wall polysaccharides: degradation with enzymes from *Phytophthora infectans*. J Exp Bot **32**:1309-1319

Jeffries TW (1990) Biodegradation of lignin-carbohydrate complexes. Biodegradation **1**: 163-176

Johnson KG, Silva MC, MacKenzie CR, Schneider H, Fontana JD (1989) Microbial degradation of hemicellulosic materials. Appl Biochem Biotechnol **20/21**:245-258

Kaji A (1984) L-Arabinosidases. Adv Carbohydr Chem Biochem **42**:383-394

Kaji A, Saheki T (1975) Endo-arabinanase from *Bacillus subtilis* F-11. Biochim Biophys Acta **410**: 354-360

Kaji A, Shimokawa K (1984) New *exo*-type arabinase from *Erwinia carotovora* IAM 1024. Agric Biol Chem **48**:67-72

Kaji A, Tagawa K (1970) Purification, chrystallization and amino acid composition of α-L-arabinofuranosidase from *Aspergillus niger*. Biochim Biophys Acta **207**:456-464

Kaji A, Yoshihara O (1969) Production and properties of α-L-arabinofuranosidase from *Corticium rolfsii*. Appl Microbiol **17**:910-913

Kaji A, Yoshihara O (1970) Production and properties of α-L-arabinofuranosidase from various strains of *Corticium rolfsii*. Agric Biol Chem **34**:1249-1253

Kaji A, Taki H, Shimazaki A, Shinkai T (1963a) Studies on the enzymes acting on arabans. II. Action and seperation of arabanase produced by Aspergilli. Tech Bull Fac Agric Kagawa Univ **15**:34-39

Kaji A, Anabuki Y, Taki H, Oyama Y, Okada T (1963b) Studies on the enzymes acting on arabans. III. Action and seperation of arabanase produced by *Clostridium felsineum* var. *sikokianum*. Tech Bull Fac Agric Kagawa Univ **15**:40-44

Kaji A, Tagawa K, Matsubara K (1967) Studies on the enzymes acting on araban. VIII. Purification and properties of arabanase produced by *Aspergillus niger*. Agric Biol Chem **31**:1023-1028

Kaji A, Sato M, Tsutsui Y (1981) An α-L-arabinofuranosidase produced by wild-type *Streptomyces* sp. No. 17-1. Agric Biol Chem **45**:925-931

Kaji A, Sato M, Yoshihara O, Adachi A (1982) α-L-Arabinofuranosidase from *Streptomyces massasporeus* IFO 3841. Tech Bull Fac Agr Kagawa Univ **34**:79-85

Kaneko S, Shimasaki T, Kusakabe I (1993) Purification and properties of intracellular α-L-arabinofuranosidase from *Aspergillus niger* 5-16. Biosci Biotech Biochem **57**:1161-1165

Karimi S, Ward OP (1989) Comparative study of some microbial arabinan-degrading enzymes. J Industr Microbiol **4**:173-180

Kato Y, Nevins DJ (1985) Isolation and identification of *O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose as a component of *Zea* shoot cell-walls. Carbohydr Res **137**:139-150

Kato Y, Nevins DJ (1992) Structural characterizayion of an arabinoxylan-rhamnogalacturonan complex from cell walls of *Zea* shoots. [Enzymatic dissociation of *Zea* shoot cell-wall polysaccharides. VI.] Carbohydr Res **227**:315-329

Kauffmann S, Doares SH, Albersheim P, Darvill A (1990) Involvement of pathogen-secreted enzymes and plant cell wall derived xylan fragments in plant cell death in the rice blast system. 5th Int Symp Mol Genet. Plant-microbe Interact. Interlaken. Abstr SO106. p.32. [*As referred to in*: Ryan CA, Farmer EE (1991) Oligosaccharide signals in plants: a current assessment. Ann Rev Plant Physiol Mol Biol **42**:651-674]

Keegstra K, Talmadge KW, Bauer WD, Albersheim P (1973) The structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. Plant Physiol **51**:188-196

Kellett LE, Poole DM, Ferreira LMA, Durrant AJ, Hazlewood GP, Gilbert HJ (1990) Xylanase B and an arabinofuranosidase from *Pseudomonas fluorescens* subsp. *cellulosa* contain identical cellulose-binding domains and are encoded by adjacent genes. Biochem J **272**:369-376

Kelly MA, Sinnott ML, Herrchen M (1987) Purification and mechanistic properties of an extracellular α-L-arabinofuranosidase from *Monilinia fructigena*. Biochem J **245**:843-849

Kelly MA, Sinnott ML, Widdows D (1988) Preparation of some aryl α-L-arabinofuranosides as substrates for arabinofuranosidase. Carbohydr Res **181**:262-266

Kester HCM, Visser J (1990) Purification and characterization of polygalacturonases produced by the hyphal fungus *Aspergillus niger*. Biotech Appl Biochem **12**:150-160

Khan AW, Lamb KA, Schneider H (1988) Recovery of fermentable sugars from the brewer's spent grains by the use of fungal enzymes. Process Biochem **23**:172-75

Kjaergaard L (1984) Examples of biotechnological utilization of beet pulp and bagasse. Sugar Tech Rev **10**:183-237

Komae K, Kaji A, Sato M (1982) An α-L-arabinofuranosidase from *Streptomyces purpurascens* IF03389. Agric Biol Chem **46**:1899-1905

Konno H, Yamasaki Y, Katoh K (1987) Purification of an α-L-arabinofuranosidase from carrot cell cultures and its involvement in arabinose-rich polymer degradation. Physiol Plant **69**:405-412

Konno H, Tanaka R, Katoh K (1994) An extracellular α-L-arabinofuranosidase secreted from cell suspension cultures of carrot. Physiol Plant **91**:454-460

Kormelink FJM, Searle-van Leeuwen MJF, Wood TM, Voragen AGJ (1991) Purification and characterization of a (1,4)-β-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori*. Appl Microbiol Biotechnol **35**:753-758

Kormelink FJM, Gruppen H, Voragen AGJ (1993) Mode of action of (1→4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) and α-L-arabinofuranosidases on alkali-extractable wheat-flour arabinoxylan. Carbohydr Res **249**:345-353

Kristufek D, Hodits R, Kubicek CP (1994) Coinduction of α-L-arabinofuranosidase and α-D-galactosidase formation in *Trichoderma reesei* RUT C-30. FEMS Microbiol Lett **115**: 259-264

Kumar V, Ramakrihsnan S, Teeri TT, Knowles JKC, Hartley BS (1992) *Saccharomyces cerevisae* cells secreting an *Aspergillus niger* β-galactosidase grow on whey permeate. BIO/TECHNOLOGY **10**:82-85

Kusakabe I, Yasui T, Kobayashi T (1975) Enzymes degrading arabinose-containing polysaccharides. I. Properties of araban-degrading enzymes produced by microorganisms and enzymatic preparation of arabinose from sugar beet pulp. Nippon Nogei Kagaku Kaishi **49**:295-305. [Chem Abstracts **83**:189627n]

Laborda F, Fielding AH, Byrde RJW (1973) Extra- and intra-cellular α-L-arabinofuranosidase of *Sclerotinia fructigena*. J Gen Microbiol **79**:321-329

Laborda F, Archer SA, Fielding AH, Byrde RJW (1974) Studies of the α-L-arabinofuranosidase complex from *Sclerotinia fructigena* in relation to brown rot of apple. J Gen Microbiol **81**:151-163

Lahaye M, Thibault J-F (1990) Purification of arabinanases and galactanases from *Aspergillus niger*. Poster displayed at the 3rd International Workshop on Plant Polysaccharides, Structure and Function. Le Croisic, France, September 19-21 1990. [As referred to in: Beldman G, Schols HA, Searle-van Leeuwen MJF, Voragen AGJ (1995) Arabinans and arabinan degrading enzymes. Adv Macromol Carbohydr Res, in press]

Leatham GF, Himmel ME (1991) Enzymes in biomass conversion. ACS Symp Ser **460**. American Chemical Society, Washington DC.

Lee SF, Forsberg CW (1987) Purification and characterization of an α-L-arabinofuranosidase from *Clostridium acetobutylicum* ATCC 824. Can J Microbiol **33**:1011-1016

Lerouge P, O'Neill MA, Darvill AG, Albersheim P (1993a) Structural characterization of endo-glycanase-generated oligoglycosyl side chains of rhamnogalacturonan I. Carbohydr Res **243**:359-371

Lerouge P, O'Neill MA, Darvill AG, Albersheim P (1993b) The purification of commercially available endo-α-L-arabinanase and α-L-arabinosidase for use in the structural analysis of pectic polysaccharides. Carbohydr Res **243**:373-378

Lewis NG, Paice MG (1989) Plant cell wall polymers. Biogenesis and biodegradation. ACS Symp Ser **399**. American Chemical Society, Washington DC.

Liebl W, Bohmann A, Zubek E, Schleifel KH (1992) Isolation of genes for xylanase, β-xylosidase, and arabinofuranosidase of *Cellumonas biazotea* DSM 20112. In: Visser J, Beldman G, Kusters-van Someren MA, Voragen AGJ (eds) Xylans and xylanases. Progress in Biotechnology. Vol.7. Elsevier, Amsterdam. pp.475-478

Manin C, Shareek F, Morosoli R, Kluepfel D (1994) Purification and characterization of an α-L-arabinofuranosidase from *Streptomyces lividans* 66 and DNA sequence of the gene (*abf*A). Biochem J **302**:443-449

Markwalder HU, Neukom H (1976) Diferulic acid as a possible crosslink in hemicelluloses from wheat germ. Phytochemistry **15**:836-837

Marshall PJ, Sinnott ML, Smith PJ, Widdows D (1981) Active-site-directed irreversible inhibition of glycosidases by the corresponding glycosylmethyl-(*p*-nitrophenyl)triazenes. J Chem Soc Perkin Trans I:366-376

Matheson NK, McCleary BV (1985) Enzymes metabolizing polysaccharides and their application to the analysis of structure and function of glycans. In: Aspinall GO (ed) The polysaccharides. Vol.3. Academic Press, NY. pp.1-105

Matheson NK, Saini HS (1977) α-L-Arabinofuranosidases and β-D-galactosidases in germinating-lupin cotyledons. Carbohydr Res **57**:103-116

Matte A, Forsberg CW (1992) Purification, characterization, and mode of action of endoxylanases 1 and 2 from *Fibrobacter succinogenes* S85. Appl Environ Microbiol **58**: 157-168

Matthew JA, Howson SJ, Keenan MHJ, Belton PS (1990) Improvement of the gelation properties of sugarbeet pectin following treatment with an enzyme preparation derived from *Aspergillus niger* – comparison with a chemical modification. Carbohydr Polym **12**: 295-306

Matthew JA, Wyatt GM, Archer DB, Morgan MRA (1991) Purification of a sugarbeet pectin modifying enzyme system from *Aspergillus niger* by antibody affinity column chromatography. Carbohydr Polym **16**:381-390

McCleary BV (1990) Novel and selective substrates for the assay of endo-arabinanase. In: Phillips GO, Williams PA, Wedlock DJ (eds) Gums and Stabilisers for the Food Industry. Vol.5. IRL Press, Oxford. pp.291-298

McCleary BV (1991) Comparison of endolytic hydrolases that depolymerize 1,4-β-D-mannan, 1,5-α-L-arabinan and 1,4-β-D-galactan. In: Leatham GF, Himmel ME (eds) Enzymes in biomass conversion. ACS Symp Ser **460**. American Chemical Society, Washington DC. pp.437-449

McCleary BV, Cooper JM, Williams EL (1990) Debranched araban and its use as a fat substitute. PCT Int. Appl. WO 90 06,343. 14-Jun-1990. 46 pp. [Chem Abstracts **113**:150887k]

McNeil M, Darvill AG, Fry SC, Albersheim P (1984) Structure and function of the primary cell walls of plants. Ann Rev Biochem **53**:625-663

Melrose J, Sturgeon RJ (1983) An enzymic assay of L-arabinose, using β-D-galactose dehydrogenase: its application in the assay of α-L-arabinofuranosidase. Carbohydr Res **118**:247-253

Minor JL (1991) Localisation of lignin-bonded pectic polysaccharides. J Wood Chem Technol **11**:159-169

Monro JA, Penny D, Bailey RW (1976) The organisation and growth of primary cell walls of lupin hypocotyl. Phytochemistry **15**:1193-1198

Morrison WH III, Hartley RD, Himmelsbach DS (1992) Synthesis of substituted truxillic acids from *p*-coumaric and ferulic acid: stimulation of photodimerization in plant cell walls. J Agric Food Chem **40**:766-771

Mueller-Harvey I, Hartley RD, Harris PJ, Curzon EH (1986) Linkage of *p*-coumaroyl and feruloyl groups to cell wall polysaccharides of barley straw. Carbohydr Res **148**:71-85

Mullen JM, Bateman DF (1975) Polysaccharide degrading enzymes produced by *Fusarium roseum* "Avenaceum" in culture and during pathogenesis. Physiol Plant Pathol **6**: 233-246

Neukom H, Markwalder H (1975) Isolation and characterization of an arabinogalactan from wheat flour. Carbohydr Res **39**:387-389

Neukom H, Providoli L, Gremli H, Hui PA (1967) Recent investigations on wheat flour pentosans. Cereal Chem **44**:238-244

Nishitani K, Nevins DJ (1988) Enzymic analysis of feruloylated arabinoxylans (feraxan) derived from *Zea mays* cell walls. I. Purification of novel enzymes capable of dissociating feraxan fragments from *Zea mays* coleoptile cell wall. Plant Physiol **87**:883-890

Nishitani K, Nevins DJ (1989) Enzymic analysis of feruloylated arabinoxylans (feraxan) derived from *Zea mays* cell walls. II. Fractionation and partial characterization of feraxan fragments dissociated by a *Bacillus subtilis* enzyme (feraxanase). Plant Physiol **91**: 242-248

Nishitani K, Nevins DJ (1990) Enzymic analysis of feruloylated arabinoxylans (feraxan) derived from *Zea mays* cell walls. III. Structural changes in the feraxan during coleoptile elongation. Plant Physiol **93**:396-402

Oh SW, Park SS, Park YI, Choi YJ (1992) Molecular cloning and expression of *Bacillus stearothermophilus* β-D-xylosidase gene in *E. coli*. Sanop Misaengmul Hakhoechi **20**: 136-142 [Chem Abstracts **119**:44204a]

Pellerin P, Waters E, Brillouet J-M (1993) Characterization of two arabinogalactan-proteins from red wine. Carbohydr Polym **22**:187-192

Poutanen K (1988) An α-L-arabinofuranosidase of *Trichoderma reesei*. J Biotechnol **7**:271-282

Poutanen K, Puls J (1988) Characteristics of *Trichoderma reesei* β-xylosidase and its use in the hydrolysis of solubilized xylans. Appl Microbiol Biotechnol **28**:425-432

Ralet M-C, Thibault J-F, Faulds CB, Williamson G (1994a) Isolation and purification of feruloylated oligosaccharides from cell walls of sugar-beet pulp. [Feruloylated oligosaccharides from cell-wall polysaccharides. I.] Carbohydr Res **263**: 227-241

Ralet M-C, Faulds CB, Williamson G, Thibault J-F (1994b) Degradation of feruloylated oligosaccharides from sugar-beet pulp and wheat bran by ferulic acid esterases from *Aspergillus niger*. [Feruloylated oligosaccharides from cell-wall polysaccharides. III.] Carbohydr Res **263**:257-269

Ramón D, Veen P van der, Visser J (1993) Arabinan degrading enzymes from *Aspergillus nidulans*: induction and purification. FEMS Microbiol Lett **113**:15-22

Reese ET (1977) Degradation of polymeric carbohydrates by microbial enzymes. In: Loewus FA, Runeckles VC (eds) The structure, biosynthesis, and degradation of wood. [Proceedings of the sixteenth annual meeting of the phytochemical society of North America, Vancouver, British Colombia, Canada, august 1976.] Recent Adv Phytochem **11**. Plenum Press, NY. pp.311-367

Renard CMGC, Searle-van Leeuwen MJF, Voragen AGJ, Thibault J-F, Pilnik W (1991) Studies on apple protopectin. II. Apple cell wall degradation by pure polysaccharidases and their combinations. Carbohydr Polym **14**:295-314

Riou C, Freyssinet G, Fevre M (1991) Production of cell wall-degrading enzymes by the phytopathogenic fungus *Sclerotinia sclerotiorum*. Appl Eviron Microbiol **57**:1478-1484

Rodionova NA, Tavobilov IM, Bezborodov AM (1983) β-Xylosidase from *Aspergillus niger* 15: purification and properties. J Appl Biochem **5**:300-312

Rombouts FM, Thibault J-F (1986a) Feruloylated pectic substances from sugar-beet pulp. Carbohydr Res **154**:177-187

Rombouts FM, Thibault J-F (1986b) Enzymic and chemical degradation and the fine structure of pectins from sugar-beet pulp. Carbohydr Res **154**:189-203

Rombouts FM, Voragen AGJ, Searle-van Leeuwen MF, Geraerds CCJM, Schols HA, Pilnik W (1988) The arabinanases of *Aspergillus niger* – purification and characterisation of two α-L-arabinofuranosidases and an *endo*-1,5-α-L-arabinanase. Carbohydr Polym **9**:25-47

Sakai T, Sakamoto T (1990) Purification and some properties of a protopectin-solubilizing enzyme that has potent activity on sugar beet protopectin. Agric Biol Chem **54**:879-889

Sakai T, Sakamoto T, Hallaert J, Vandamme EJ (1993) Pectin, pectinase, and protopectinase: production, properties, and applications. Adv Appl Microbiol **39**:213-294

Sakamoto T, Yoshinaga J, Shogaki T, Sakai T (1993) Studies on protopectinase-C mode of action – analysis of the chemical structure of the specific substrate in sugar beet protopectin and characterization of the enzyme activity. Biosci Biotech Biochem **57**: 1832-1837

Sakka K, Yoshikawa K, Kojima Y, Karita S-I, Ohmiya K, Shimada K (1993) Nucleotide sequence of the *Clostridium stercorarium xyl*A gene encoding a bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities, and properties of the translated product. Biosci Biotech Biochem **57**:268-272

Saulnier L, Brillouet J-M, Joseleau J-P (1988) Structural studies of pectic substances from the pulp of grape berries. Carbohydr Res **182**:63-78

Saulnier L, Brillouet J-M, Moutounet M, Hervé du Penhoat C, Michon V (1992) New investigations of the structure of grape arabino-galactan-protein. Carbohydr Res **224**: 219-235

Schneider H (1989) Conversion of pentoses to ethanol by yeast and fungi. CRC Crit Rev Biotechnol **9**:1-40

Schmidt J, John M, Wandrey C, Sahm H (1984) Abbau von hemicellulose durch ein leistungfähiges xylanase-system. Dechema-Monograph **95**:223-229

Schmitt R (1985) Turbidity in apple juices – arabanes? Confructa Studien **29**:22-26

Schöpplein E, Dietrich H (1991) Charakterisierung polysaccharidspaltender begleitaktivitäten in einem technischen pektinasepräparat. Dtsch Lebensm-Rundsch **87**:212-219

Schöpplein E, Dietrich H, Wucherpfennig K (1991) Isolierung und characterisierung von kolloidal gelösten polysacchariden in himbeersäften. Z Lebensm Unters Forsch **193**:1-8

Schwabe K, Grossmann A, Fehrmann B, Tschiersch B (1978) Inhibition of α-L-arabinofuranosidase (*Aspergillus niger*) and non-inhibition of α-L-arabinopyranosidase (almond emulsin and barley) by L-arabinono-1,4-lactone. Carbohydr Res **67**:541-544

Schwarz WH, Adelsberger H, Jauris S, Hertel C, Funk B, Staudenbauer WL (1990) Xylan degradation by the thermophile *Clostridium stercorarium*: cloning and expression of xylanase, β-D-xylosidase, and α-L-arabinofuranosidase genes in *Escherichia coli*. Biochem Biophys Res Commun **170**:368-374

Selvendran RR (1985) Developments in the chemistry and biochemistry of pectic and hemicellulosic polymers. In: Roberts K, Johnston AWB, Lloyd CW, Shaw P, Woolhouse HW (eds) The cell surface in plant growth and development. J Cell Sci Suppl **2**. The Company of Biologists Ltd., Cambridge. pp.51-88

Selwood T, Sinnott ML (1988) One-proton catalysis by the α-L-arabinofuranosidase III of *Monilinia fructigena*. Biochem J **254**:899-901

Sewell GW, Utt EA, Hespell RB, MacKenzie KF, Ingram LO (1989) Identification of the *Butyrivibrio fibrisolvens* xylosidase gene (*xylB*) coding region and its expression in *Escherichia coli*. Appl Environ Microbiol **55**:306-311

Shao W, Wiegel J (1992) Purification and characterization of a thermostable β-xylosidase from *Thermoanaerobacter ethanolicus*. J Bacteriol **174**:5848-5853

Sinnott ML (1990) Catalytic mechanisms of enzymic glycosyl transfer. Chem Rev **90**: 1171-1202

Smith MM, Hartley RD (1983) Occurence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. Carbohydr Res **118**:65-80

Somogyi (1952) Notes on sugar determination. J Biol Chem **195**:19-23

Stephen AM (1983) Other Plant Polysaccharides. In: Aspinall GO (ed) The Polysaccharides. Vol.2. Academic Press, NY. pp.97-193

Sturdy ML, Cole ALJ (1975) A cell wall-degrading α-L-1,3-arabinofuranosidase produced by the potato dry rot pathogen *Fusarium caeruleum* (Lib.) Sacc. Ann Bot **39**:331-335

Suominen P, Reinikainen T (1993) *Trichoderma reesei* cellulases and other hydrolases. Enzyme structures, biochemistry, genetics and applications. [Proceedings of the Tricel93 Symposium, june 2-5, 1993, Espoo, Finland]. Foundation for Biotechnical and Industrial Fermentation Research. Vol.8. Foundation for Biotechnical and Industrial Fermentation Research, Helsinki.

Swamy NR, Salimath PV (1991) Arabinans from *Cajanus cajan* cotyledon. Phytochemistry **30**: 263-265

Tagawa K (1970a) Kinetic studies of α-L-arabinofuranosidase from *Aspergillus niger* (I) General kinetic properties of the enzyme. J Ferment Technol **48**:730-739

Tagawa K (1970b) Kinetic studies of α-L-arabinofuranosidase from *Aspergillus niger* (II) Stability of the enzyme. J Ferment Technol **48**:740-746

Tagawa K (1970c) Selection of fungal strains which produce the enzymes acting on arabinan and some properties of the enzymes. Tech Bull Fac Agric Kagawa Univ **21**:186-195

Tagawa K, Kaji A (1988a) Preparation of L-arabinan and 1,5-L-arabinan. Methods Enzymol **160**: 542-545

Tagawa K, Kaji A (1988b) α-L-Arabinofuranosidase from *Aspergillus niger*. Methods Enzymol **160**:707-712

Tagawa K, Terui G (1968) Studies on the enzymes acting on araban. IX. Culture conditions and process of arabanase production by *Aspergillus niger*. J Ferment Technol **46**: 693-700

Tajana E, Fiechter A, Zimmermann W (1992) Purification and characterization of two α-L-arabinofuranosidases from *Streptomyces diastaticus*. Appl Environ Microbiol **58**: 1447-1450

Tanaka M, Uchida T (1978) Purification and properties of α-L-arabinofuranosidase from plant *Scopolia japonica* calluses. Biochim Biophys Acta **522**:531-540

Tanaka M, Abe A, Uchida T (1981) Substrate specificity of α-L-arabinofuranosidase from plant *Scopolia japonica* calluses and a suggestion with reference to the structure of beet araban. Biochim Biophys Acta **658**:377-386

Tanimoto E (1985) Axial distribution of glycosidases in relation to cellular growth and ageing in *Pisum sativum* root. J Exp Bot **36**:1267-1274

Thibault J-F, Rombouts FM (1986) Effects of some oxidising agents, especially ammonium persulfate, on sugar-beet pectins. Carbohydr Res **154**:205-215

Tong CBS, Gross KC (1988) Glycosyl-linkage composition of tomato fruit cell wall hemicellulosic fractions during ripening. Physiol Plant **74**:365-370

Tuohy MG, Buckley RJ, Griffin TO, Connelly IC, Shanley NA, Filho EXF, Hughes MM, Grogan P, Coughlan MP (1989) Enzyme production by solid-state cultures of aerobic fungi on lignocellulosic substrates. In: Coughlan MP (ed) Enzyme systems for lignocellulose degradation. Elsevier Applied Science, London. pp.293-312

Uchida T, Tanaka M (1988) α-L-Arabinofuranosidase from *Scopolia japonica*. Methods Enzymol **160**:712-719

Uesaka E, Sato M, Raiju M, Kaji A (1978) α-L-Arabinofuranosidase from *Rhodotorula flava*. J Bacteriol **133**:1073-1077

Utt EA, Eddy CK, Keshav KF, Ingram LO (1991) Sequencing and expression of the *Butyrivibrio fibrisolvens xylB* gene encoding a novel bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities. Appl Environ Microbiol **57**:1227-1234

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1991) Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. Arch Microbiol **157**:23-28

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1993) Induction of extracellular arabinases on monomeric substrates in *Aspergillus niger*. Arch Microbiol **159**:66-71

Vis JW van de (1994) Characterization and mode of action of enzymes degrading galactan structures of arabinogalactans. Ph.D. Thesis. Wageningen Agricultural University, The Netherlands. 161 pp.

Vis JW van de, Searle-van Leeuwen MF, Siliha HA, Kormelink FJM, Voragen AGJ (1991) Purification and characterization of *endo*-1,4-β-D-galactanases from *Aspergillus niger* and *Aspergillus aculeatus*: use in combination with arabinanases from *Aspergillus niger* in enzymatic conversion of potato arabinogalactan. Carbohydr Polym **16**: 167-187

Visser J, Beldman G, Kusters-van Someren MA, Voragen AGJ (1992) Xylans and xylanases. [Proceedings of an international symposium. Wageningen, The Netherlands, December 8-11, 1991.] Progress in Biotechnology. Vol.7. Elsevier, Amsterdam.

Voragen AGJ, Pilnik W (1989) Pectin-degrading enzymes in fruit and vegetable processing. In: Whitaker JR, Sonnet PE (eds) Biocatalysis in agricultural biotechnology. ACS Symp Ser **389**. American Chemical Society, Washington DC. pp.93-115

Voragen AGJ, Geerst F, Pilnik W (1982) Hemicellulase in enzymic fruit processing. In: Dupuy P (ed) *Utilisation des Enzymes en Technologie Alimentaire*. [Symposium International Versailles 1982.] Technique et Documentation Lavoisier, Paris. pp.497-502

Voragen AGJ, Rombouts FM, Searle-van Leeuwen MF, Schols HA, Pilnik W (1987) The degradation of arabinans by endo-arabinanase and arabinofuranosidases purified from *Aspergillus niger*. Food Hydrocoll **1**:423-437

Waibel R, Amadò R, Neukom H (1980) Purification of an α-L-arabinofuranosidase from *Aspergillus niger* by substrate affinity chromoatography. J Chromatogr **197**:86-91

Wang P, Mason JC, Broda P (1993) Xylanases from *Streptomyces cyaneus*: their production, purification characterization. J Gen Microbiol **139**:1987-1993

Ward OP, Moo-Young M (1989) Enzymatic degradation of cell wall and related plant polysaccharides. CRC Crit Rev Biotechnol **8**:237-274

Watanabe T, Ohnishi J, Yamasaki Y, Kaizu S, Koshijma (1989) Binding-site analysis of the ether linkages between lignin and hemicelluloses in lignin-carbohydrate complexes by DDQ-oxidation. Agric Biol Chem **53**:2233-2252

Weinstein L, Albersheim P (1979) Structure of plant cell walls. IX. Purification and partial characterization of a wall-degrading endo-arabanase and an arabinosidase from *Bacilllus subtilis*. Plant Physiol **63**:425-432

Whitaker JR (1984) Pectic substances, pectic enzymes and haze formation in fruit juices. Enzyme Microb Technol **6**: 341-349

Whitehead TR, Hespell RB (1990) The genes for three xylan-degrading activities from *Bacteroides ovatus* are clustered in a 3.8-kilobase region. J Bacteriol **172**:2408-2412

Widmar F, Leuba J-L (1979) β-Galactosidase from *Aspergillus niger*. Separation and characterization of three multiple forms. Eur J Biochem **100**:559-567

Wijesundera RLC, Bailey JA, Byrde RJW, Fielding AH (1989) Cell wall degrading enzymes of *Colletotrichum lindemuthianum*: their role in the development of bean anthracnose. Physiol Mol Plant Pathol **34**:403-413

Wilkie KCB (1979) The hemicelluloses of grasses and cereals. Adv Carbohydr Chem Biochem **36**: 215-264

Will F, Dietrich H (1992) Isolation, purification and characterization of neutral polysaccharides from extracted apple juices. Carbohydr Polym **18**:109-117.

Witteveen CFB, Busink R, Vondervoort P van de, Dijkema C, Swart K, Visser J (1989) L-Arabinose and D-xylose catabolism in *Aspergillus niger*. J Gen Microbiol **135**: 2163-2171

Wong KKY, Tan LUL, Saddler JN (1988) Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. Microbiol Rev **52**:305-317

Wood TM (1992) Fungal cellulases. Biochem Soc Trans **20**:46-53

Wood TM, McCrae SI (1986) Studies of two low-molecular-weight endo-(1→4)-β-D-xylanases constitutively synthesised by the cellulolytic fungus *Trichoderma koningii*. Carbohydr Res **148**:321-330

Wood TM, Castanares A, Smith DC, McCrae SI, Brown JA (1992) Xylan-degrading enzyme systems of the fungi *Aspergillus awamori* and *Penicillium pinophilum*: studies on the isolation of over-producing mutant strains and aspects of the enzymology. In: Visser J, Beldman G, Kusters-van Someren MA, Voragen AGJ (eds) Xylans and xylanases. Progress in Biotechnology. Vol.7. Elsevier, Amsterdam. pp.187-202

Yamaki S, Kakiuchi N (1979) Changes in hemicellulose-degrading enzymes during development and ripening of Japanese pear fruit. Plant Cell Physiol **20**:301-309

Yasuda M, Tokuzato M (1988) Studies on arabinan degrading enzymes produced by microorganisms. IV. Purification and properties of α-L-arabinofuranosidase II from *Bacillus* sp No. 430. Sci Bull Coll Agric Univ Ryukyus **35**:53-62

Yasuda M, Tokuzato M, Sesoko M (1983) Studies on arabinan degrading enzymes produced by microorganisms. III. Purification and properties of α-L-arabinofuranosidase I from *Bacillus* sp. Sci Bull Coll Agric Univ Ryukyus **30**:201-210

Yoshihara O, Kaji A (1983) An endo-1,5-α-L-arabinase, which can disintegrate potato tissue. Agric Biol Chem **47**:1935-1940

Zamost BL, Elm-Durrschmidt D (1991) Thermostable hemicellulases from *Bacillus stearothermophilus* for pulp bleaching. PCT Int. Appl. WO 91 18,976. 12-Dec-1991. 65 pp. [Chem Abstracts **116**:123933c]

Zawadzki-Baggio SF, Sierakowski M-R, Corrêa JBC, Reicher F (1992) A linear (1→5)-linked α-L-arabinofuranan from the seeds of guapuruvu (*Schizolobium parahybum*). Carbohydr Res **233**: 265-269

Chapter 2

Cloning of the *Aspergillus niger* gene encoding α-L-arabinofuranosidase A [1]

Using L-arabitol as an inducer, simple induction conditions were established which result in high-level expression of α-L-arabinofuranosidase A (ABF A) by an *Aspergillus niger* D-xylulose kinase mutant strain. These conditions were adapted to construct a cDNA expression library from which an ABF A cDNA clone was isolated using specific antiserum. The corresponding gene encoding ABF A (*abf*A) was isolated from a genomic library and cloned into a high-copy number plasmid vector. By co-transformation of uridine auxotrophic mutants lacking orotidine-5'-phosphate decarboxylase activity, the *abf*A gene was introduced both in *A. niger* and *A. nidulans*, using the *A. niger pyr*A gene as the selection marker. The identity of the *abf*A gene was confirmed by overexpression of the gene product in *A. niger* and *A. nidulans* transformants, upon growth using sugar beet pulp as the carbon source.

Introduction

Degradation of the plant cell wall compound L-arabinan by the hyphal fungus *Aspergillus niger* is a topic of interest as the enzymes produced can be applied in agro-industrial processes, *e.g.* in fruit juice technology and in the valorization of agricultural waste (Voragen *et al.* 1982, 1987; Churms *et al.* 1983). The fungus is known to produce the three different types of L-arabinan-degrading activities described by Kaji (1984); an *endo*-1,5-α-L-arabinase (EC 3.2.1.99) and two distinct α-L-arabinofuranosidases (EC 3.2.1.55). These three enzymes have been purified from a technological pectinase preparation (Voragen *et al.* 1987; Rombouts *et al.* 1988) as well as from medium of an *A. niger* culture, using sugar beet pulp as the carbon source, leading to their further characterization (van der Veen *et al.* 1991). The *A. niger* L-arabinan-degrading system is the most complex one currently described – *endo*-1,5-α-L-arabinase proves to have strong synergistic interactions with both ABFs (Voragen *et al.* 1987).

---

[1] published in: Appl Microbiol Biotechnol **39**:335-340 (1993)

A. niger α-L-arabinofuranosidase A (ABF A) (83 kDa) is active on small 1,5-linked α-L-arabinofuranosides as *para*-nitrophenyl α-L-arabinofuranoside (PNP-A) and unbranched 1,5-α-L-arabinofuranosyl oligosaccharides. Unlike α-L-arabinofuranosidase B (ABF B) (67 kDa), it does not act on polysaccharides as linear 1,5-α-L-arabinan, branched sugar beet arabinan, arabinoxylan and arabinogalactan. Apparently the enzyme is not able to hydrolyze 1,3-α- and 1,2-α-linkages. An ABF produced by the actinomycete *Streptomyces purpurascens* IFO 3389 shows a similar substrate specificity (Komae *et al.* 1982). There are indications that such an enzyme could also be produced by *Bacillus subtilis* F-11 (Weinstein and Albersheim 1979).

In a previous paper, data are presented on the induction of arabinase expression in wild-type *A. niger* by different carbon sources (van der Veen *et al.* 1991). Polysaccharides with a high arabinose content, present in raw materials as apple pulp and sugar beet pulp (Rombouts *et al.* 1988), proved to be powerful inducers of all three arabinases. L-Arabinose, the product of ABF activity, was found to induce only ABF B. Growth on L-arabinose leads to intracellular accumulation of L-arabitol and xylitol, which are intermediates in L-arabinose catabolism (Witteveen *et al.* 1989). Growth on L-arabitol results, however, in secretion of all three arabinases (van der Veen *et al.* 1993). These observations are consistent with the results obtained by Tagawa and Terui (1968), studying ABF induction of an *A. niger* strain by L-arabinose and L-arabitol upon mycelial transfer.

The antiserum, obtained against ABF A purified from sugar beet pulp culture medium (van der Veen *et al.* 1991), has been used to probe a cDNA expression library. This enabled isolation and cloning the *abf*A gene encoding ABF A. The identity of this gene is established by overexpression in both *A. niger* and *A. nidulans*.

Materials and methods

Fungal strains, bacterial strains, phages and plasmids. The *A. niger* strains used all descend from wild-type strain N400 (CBS 120.49); N402 (*cspA1*), N593 (*cspA1, pyrA6*) (Goosen *et al.* 1987) and N572 (*cspA1, nicA1, xkiA1*) (Witteveen *et al.* 1989) have been described previously. *A. nidulans* WG096 (*pabaA1, yA2*) is a standard strain originating from the Glasgow collection (FGSC 187) (Uitzetter 1982). *A. nidulans* strain G191 (*pabaA1, pyrG89, fwA1, uAY9*) was described by Ballance and Turner (1985). *Escherichia coli* strains PLK-F', BB4 and JM101 were supplied by Stratagene; PLK-F' was used for phage amplification, BB4 for immunochemical screening, phagemid excision and lambda DNA isolation. JM101 was used for propagation of plasmids. Phage R408 (Stratagene) was used as a helper phage for phagemid excision. Plasmid vector pGEM-7Zf(+) was obtained from Promega. Plasmid pGW613 contains the *A. niger* orothidine-5'-phosphate decarboxylase (*pyr*A) gene (Goosen *et al.* 1987).

Aspergillus cultivation: media and conditions. To obtain conidiospores, *Aspergillus* strains were grown on 1.5 % agar-solidified complete medium (Pontecorvo *et al.* 1953) with 1.5 % sucrose as a carbon source. Minimal medium used for submerged culturing consisted of 1.5 g/l ofK H_2PO_4, 4 g/l of NH_4Cl, 0.5 g/l of $MgSO_4$, 0.5 g/l of KCl, 0.1 g/l of yeast extract, 0.04 ml/l of trace element solution according to Vishniac and Santer (1957) and 1 % (w/v) of the carbon source indicated; the initial pH was set at 6.0 with NaOH. For expression studies of the transformed strains however, 15 g/l of KH_2PO_4 was added (Archer *et al.* 1990). Culture media for auxotrophic strains were supplemented with 2 mg/l of *para*-aminobenzoate, 10 mM uridine or 1 mg/l of nicotinamide. Medium was inoculated with 10^6 spores/ml and incubated at 30°C using a New Brunswick orbital shaker (250 rpm). L-Arabitol was obtained from Sigma. Sugar beet pulp, a gift from CSM (Breda, The Netherlands), was ground in a Waring blendor prior to use.

Isolation of RNA and poly A^+ RNA *A. niger* N572 was cultivated for 24 h using D-glucose as the carbon source; then the mycelium was harvested by filtration and washed with sterile saline. The mycelium was subsequently transferred to fresh medium containing 1 % (w/v) L-arabitol. After 16 h of induction mycelium was collected by filtration and thoroughly washed with sterile saline; the mycelial pellet was squeezed between papersheets to reduce redundant liquid, before freezing in liquid nitrogen. Frozen mycelium was powdered using a micro-dismembrator (Braun). Total RNA was isolated from mycelial powder in accordance with the guanidine monothiocyanate / LiCl protocol of Cathala *et al.* (1983), except that sodium dodecyl sulfate (SDS) was omitted from the solubilization buffer. Poly A^+ RNA was isolated from 1 mg of total RNA by oligo(dT)-cellulose chromatography (Aviv and Leder 1972; Sambrook *et al.* 1989) with the following modifications: 10 mM *N*-(2-hydroxymethyl)-piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), pH 7.6, was used as a buffer and SDS was omitted from all solutions, the loading buffer was supplemented with 9 % (v/v) dimethyl-sulfoxide.

Construction of an L-arabitol cDNA expression library. cDNA was synthesized from 5 μg poly A^+ RNA using the ZAP–cDNA synthesis kit (Stratagene). After ligation of the cDNA into UniZAP–XR vector arms (Stratagene), the phage DNA was packaged using Packagene extracts (Promega) and the resulting library was subsequently titrated using *E. coli* PLK-F'. The primary library was amplified using PLK-F', titrated and stored at 4°C.

Immunochemical screening. Screening of the cDNA expression library was basically performed according the method of Young and Davis (1983). In short, 5000 plaque-forming units (pfu) of the amplified stock were plated with BB4 cells in 0.6 % agarose per 94 mm plate. Plates were incubated for 5 h at 37°C. Nitrocellulose filters, previously soaked in 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and subsequently air-dried, were carefully layered upon the growing cells. Plates were incubated for 6 h at 37°C, and then cooled to 4°C. The position of the filters on the plates was marked prior to removal. Filters were incubated twice for 15 min in 0.5 M NaCl/ 0.05 % Tween20 (BioRad)/20 mM TRIS/HCl, pH 7.5, with gentle shaking and cell debris was removed by gentle scrubbing. Positive plaques were identified by probing the filters with anti-ABF A antiserum and subsequently visualising antigen-antibody binding using an alkaline phosphatase assay, according the procedure described for Western blots in BioRad's immun–blot assay kit. Positive phages were recovered from the original plate and purified by rescreening at low density. Phagemids were excised from positive phages by co-infection with helper phage R408 and rescued by plating with BB4 on ampicillin-containing plates, as described in the Instruction manual for Stratagene's ZAP–cDNA synthesis kit. Excision results in cDNA unidirectionally cloned in pBluescript SK^-.

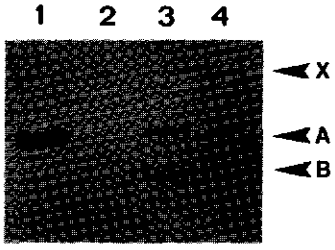


Fig.1. Expression of ABF A by *A. niger* N572 upon 16 h of L-arabitol induction. **Lane** 1: 1 μg purified ABF A; **2**: culture medium L-arabitol transfer; **3**: 20-times concentrated culture medium L-arabitol transfer; **4**: 20-times concentrated culture medium D-glucose transfer. The positions of ABF A (83 kDa) as well as of ABF B (65 kDa) and the cross-reactive protein of high-molecular weight (> 100 kDa) are indicated with arrows marked **[A]**, **[B]** and **[X]**, respectively.

Isolation and cloning of the *abf*A gene. Plaque hybridization using nitrocellulose replicas was performed according to Benton and Davis (1977); 1 x 10^4 pfu were plated with BB4 cells in 0.6 % agarose per 94 mm plate. Hybridizations were performed overnight at 68°C, using standard hybridization buffer containing 6 x SSC (Sambrook *et al.* 1989). The filters were washed twice for 30 min in 2 x SSC/0.1 % SDS and subsequently washed twice for 30 min in 0.2 x SSC/0.1 % SDS, all at 68°C. Positive plaques, identified on duplicate replicas after autoradiography were recovered from the original plates and purified by rescreening at low density. For other DNA manipulations standard methods were applied (Sambrook *et al.* 1989).

Transformation of *A. niger* and *A. nidulans*. Both *A. niger* and *A. nidulans* uridine auxotrophic strains, N593 and G191 respectively, were transformed to prototrophy with a mixture of pGW613 and pIM900, carrying the *A. niger abf*A gene, using the co-transformation protocol described by Kusters-van Someren *et al.* (1991). In each experiment 3 μg pGW613 DNA and 50 μg of the co-transforming plasmid was used.

Analysis of culture medium protein. Culture samples were centrifuged (3 krpm, 15 min) to pellet debris. ABF activity was assayed using PNP-A as described previously (van der Veen *et al.* 1991). Culture medium samples were concentrated as follows: the media were extensively dialyzed against 2 mM sodium phospate buffer, pH 6.0, at 4°C and subsequently lyophilized. Dry material was resolved in 1/20 of the original volume in sterile bidistilled water. Secreted protein was analyzed by electrophoresis in 10 % polyacrylamide gels containing 0.1 % SDS according to Laemmli (1970), and subsequent staining using Coomassie brilliant blue R250 (Weber and Osborn 1969). Purified *A. niger* ABF A (van der Veen *et al.* 1991) was used as a marker. For specific detection of ABF A, Western blots of SDS-PAGE-separated medium protein were probed with anti-ABF A antiserum, and antigen-antibody binding was visualised using the alkaline phosphatase assay as described above.

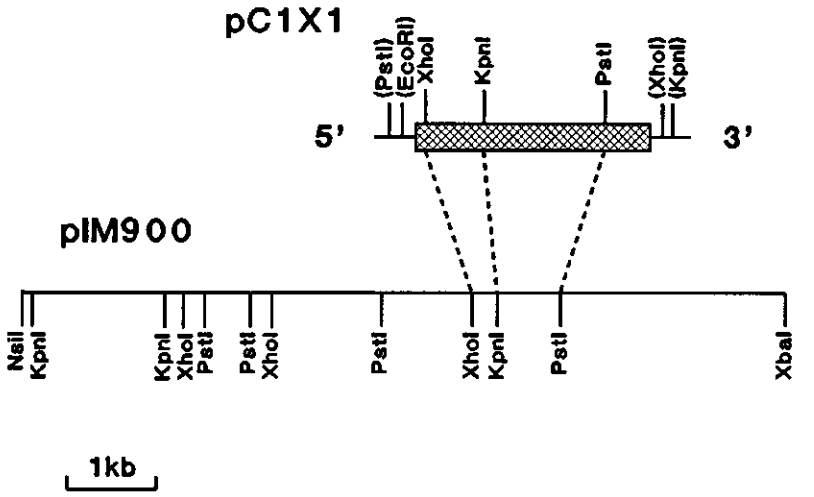


Fig.2. Restriction maps of cDNA clone pC1X1 **(top)** and genomic clone pIM900 **(bottom).** The *Kpn*I, *Pst*I and *Xho*I sites are given. The map of pC1X1 is presented enlarged; the cDNA insert is represented by a box. The cDNA is unidirectionally cloned in the *Eco*RI and *Xho*I sites of pBluescript SK$^-$ and its orientation is indicated. Restriction sites situated in the polylinker of the vector part of pC1X1 are denoted between brackets and not drawn on scale.

Results

L-Arabitol induced expression of ABF A by *A. niger* D-xylulose kinase mutant N572

The mutant *A. niger* strain N572, lacking D-xylulose kinase activity, is not able to grow on L-arabinose and is shown to accumulate L-arabitol and xylitol to higher levels than the wild-type N402 upon transfer to medium containing only L-arabinose (Witteveen *et al.* 1989). Expression of arabinases by N572 upon transfer to minimal medium containing L-arabitol was screened, applying an induction period of 16 h as used by Witteveen *et al.* (1989). Similar to wild-type strain N402, all three arabinases were secreted in the culture medium. However, for the mutant higher levels of expression were found (van der Veen *et al.* 1993). Fig.1 (lane 3) shows the level of ABF A expression in N572 upon L-arabitol transfer using immunochemical detection after SDS-PAGE and western blotting. The antiserum has apparently also affinity for ABF B (lane 3), abundantly present upon L-arabitol induction as monitored using anti-ABF B antiserum (not shown), and for an unknown protein moiety produced upon transfer to medium containing D-glucose (lane 4). Whether this cross-reactivity is due to structural similarities amongst the proteins involved or results from minor impurities within the material used for immunization, is not known – neither ABF A nor the protein of high molecular weight are recognized by anti-ABF B antiserum.

Isolation of an ABF A cDNA clone

The induction conditions described above were chosen to construct a cDNA expression library from strain N572. PolyA$^+$ RNA was isolated from 16 h L-arabitol-induced mycelium and converted into cDNA. Ligation of 200 ng cDNA in 1 μg vector arms and subsequent packaging of one fifth of the ligation mixture, resulted in a primary library consisting of 2.5 x 10^5 independent recombinant phages. In two independent experiments 5 x 10^3 and 5 x 10^4 pfu of the amplified library were immunochemically screened for expression of ABF A cDNA; two and nine positives were found respectively. Upon purification and excision the resulting plasmids were isolated and cDNA insert lengths were determined by digestion with *Eco*RI and *Xho*I and subsequent agarose electrophoresis. The clone containing the largest insert found (1.3 kb), called pC1X1, was subjected to more detailed restriction analysis (Fig. 2); *Xho*I, *Kpn*I and *Pst*I sites were found within the cDNA.

Isolation of the *abf*A gene

An *A. niger* N400 genomic library (Harmsen *et al.* 1990) was screened to isolate the gene encoding ABF A, using a nicktranslated 1.0 kb *Pst*I fragment of cDNA clone pC1X1 (Fig. 2) as a probe: screening of 6 * 10^4 pfu (representing 20 times the *A. niger* genome) resulted in the isolation of 18 positive clones. The inserts of four of these phages were partially characterized by restriction analysis and subsequent Southern blotting. Based on the position of the cDNA in the restriction patterns of the phages, a hybridizing 8.5 kb *Nsi*I/*Xba*I fragment was selected to be subcloned in pGEM-7Zf(+) resulting in pIM900. The position of the *abf*A gene in pIM900, as deduced from the position of the cDNA, is such that the clone contains several kb of both upstream and downstream sequences, and therefore the gene present on pIM900 is likely to be functional (Fig. 2).

Expression of the *abf*A gene by *A. nidulans* and *A. niger* transformed strains

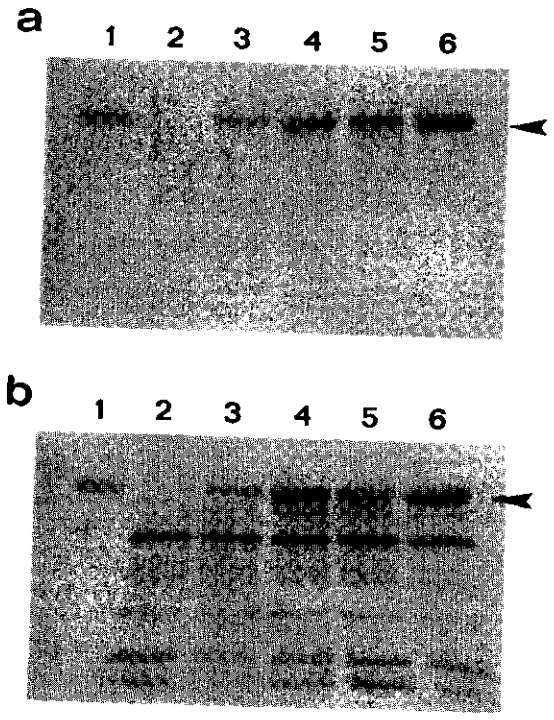
In order to establish identity and functionality of the gene isolated, uridine auxotrophic mutants of both *A. niger* and *A. nidulans* lacking orotidine-5'-phosphate decarboxylase activity were transformed with pGW613, containing the *A. niger pyr*A gene, and pIM900 as co-transforming plasmid. In general, an increase in gene copy number leads to an increase in expression level of its gene product, provided that *trans*-acting factors are not limiting (Gwynne and Devchand 1992). The ratio between both plasmids was chosen such that high percentages of co-transformation and multiple copy integration were likely, as has been decribed for a number of other co-transformations using *pyr*A complementation of uridine-auxotrophic *Aspergillus* recipients (*e.g.* Kusters-van Someren *et al.* 1991; de Graaff *et al.* 1992). Transformants were selected for uridine prototrophy. From both transformations four transformants were randomly chosen and analyzed for overexpression of the *abf*A gene. The transformants were grown on minimal medium using sugar beet pulp as the carbon source, and

medium samples were taken at time intervals of 12 h. Samples were analyzed by SDS-PAGE and subsequent Coomassie brilliant blue staining, and the PNP-A-hydrolyzing activity was determined, using culture samples of *A. nidulans* WG096 and *A. niger* N402 as controls.

As shown in Fig.3a, all four *A. nidulans* transformants tested secreted different amounts of a protein with the same apparent molecular weight as purified *A. niger* ABF A. Even after 36 h of growth (Fig.3b) *A. nidulans* wild-type strain WG096 did not produce this protein, although several other proteins appear in the culture medium. Expression of the *A. niger abf*A gene in the *A. nidulans* transformants was confirmed immunochemically (not shown). On a western blot the protein band which specifically appears in the culture media of the transformants is recognized by the anti-ABF A antiserum. Wild-type strain WG096 does not produce a protein which is recognized by the antiserum, even after 36 h of growth. The differences in the expression level of the heterologous gene product amongst the transformants correlate well with the PNP-A-hydrolyzing activities measured in the culture media taken at 24 h of growth (Table 1). The PNP-A-hydrolyzing activity produced by WG096, presumably is due to endogenous ABF B, as immunochemically monitored on western blot using anti-ABF B antiserum (not shown). Analysis of the culture media after 36 h of growth (Fig.3b) shows that in the additional growth period a considerable level of endogenous protein was produced, while the quantities of ABF A are comparible to those present after 24 h of growth.

After 24 and 36 h of growth, the *A. niger* transformants tested produced different amounts of two proteins which appear as sharp bands, with slightly different apparent molecular weights (Fig.4a, b). The apparent molecular weights of both proteins correspond with that of purified ABF A, which appears as a fuzzy band. Immunochemical visualization of ABF A shows that both moieties are recognized by the antiserum (Fig.4c); the wild-type level of ABF A as produced by N402, is hardly visible under the conditions applied. Table 2 shows that the PNP-A-hydrolyzing activity in the culture medium has considerably increased in the transformants as compared to the wild-type activity. Since both ABF A and B are measured in the PNP-A assay, the given activity is determined by both enzymes. Introduction of the *abf*A gene present on pIM900 in *A. niger* as well as in the heterologous host *A. nidulans* leads to overexpression of ABF A by the transformants tested upon growth on sugar beet pulp.

A. nidulans



A. niger

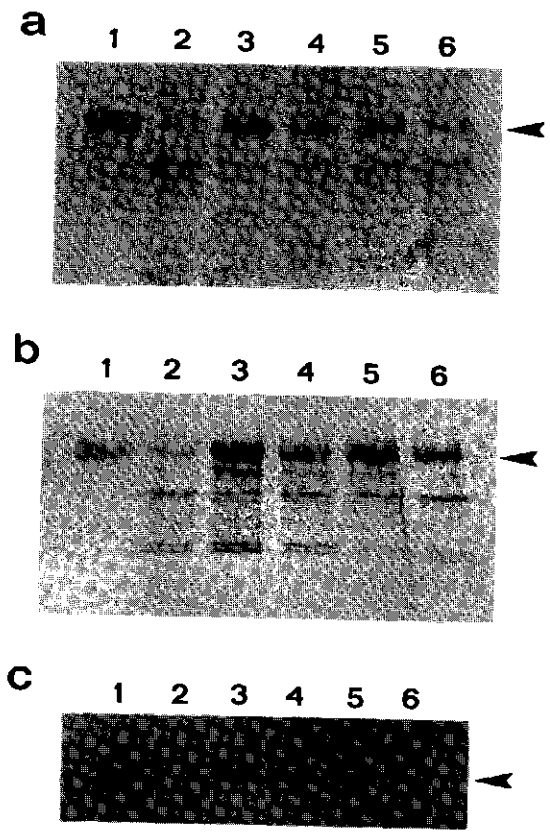


Fig.3 (left). SDS-PAGE of culture medium protein of *A. nidulans afb*A transformants and wild-type WG096 grown on sugar beet pulp. Gels are stained using Coomassie brilliant blue R250. **a**: culture media 24 h after inoculation; **b**: culture media 36 h after inoculation. **Lane 1**: 1 μg purified ABF A; **2**: WG096; **3 – 6**: *abf*A transformants G191 :: pIM900-1 / -2 / -3 / -4. The position of ABF A (83 kDa) is marked with an arrow.
Fig.4 (right) SDS-PAGE of culture medium protein of *A. niger afb*A transformants and wild-type N402 grown on sugar beet pulp. **a**: culture media 24 h after inoculation, stained using Coomassie brilliant blue R250; **b**: culture media 36 h after inoculation, stained using Coomassie brilliant blue R250; **c**: culture media 24 h after inoculation, immunochemical analysis. **Lane 1**: 1 μg purified ABF A; **2**: N402; **3 – 6**: *abf*A transformants N593::pIM900-1 / -2 / -3 / -4. The position of ABF A (83 kDa) is marked with an arrow.

Strain	Activity
WG096	0.16
G191::pIM900-1	1.34
G191::pIM900-2	2.64
G191::pIM900-3	2.13
G191::pIM900-4	2.63

Strain	Activity
N402	0.35
N593::pIM900-1	2.48
N593::pIM900-2	1.25
N593::pIM900-3	1.99
N593::pIM900-4	1.79

Table 1 (left). PNP-A-hydrolyzing activity in culture medium of *A. nidulans* wild-type and *abf*A transformants after 24 h of growth on sugar beet pulp as the carbon source. Activity is given in Units/ml culture medium, and determined as described in Materials and Methods.
Table 2 (right). PNP-A-hydrolyzing activity in culture medium of *A. niger* wild-type and *abf*A transformants after 24 h of growth on sugar beet pulp as the carbon source. Activity is given in Units/ml culture medium, and determined as described in Materials and Methods.

Discussion

From the results described it can be concluded that the *A. niger* gene encoding α-L-arabinofuranosidase A (ABF A) has been cloned. The plasmid pIM900 contains all sequences essential for gene expression and proper regulation both in *A. niger* and in *A. nidulans*. Overexpression by the heterologous host provides elegant and definite proof of the identity of the *abf*A gene, since this fungus does not produce an endogenous equivalent of the *A. niger* enzyme. Although expressed in an organism, which obviously lacks a corresponding ABF A function, the *abf*A gene seems to retain its regulatory properties. It is properly induced on sugar beet pulp and repressed on D-glucose, which is responsible for carbon catabolite repression of the arabinase complex in *A. niger* (Tagawa and Terui 1968; van der Veen *et al.* 1993). As in *A. niger* (*cf.* Fig.1; lane 4), none of the *A. nidulans* transformants secrete detectable levels of the gene product when grown on medium containing D-glucose as sole carbon source. Heterologous expression of the *abf*A gene differs remarkably from that of the *A. niger* pectin lyase-encoding *pel*A gene; *A. nidulans pel*A transformants grown on D-glucose secrete considerable amounts of heterologous gene product (Kusters-van Someren *et al.* 1991), suggesting an impaired regulation of *pel*A in this host.

Comparison of *abf*A expression in *A. nidulans* and *A. niger* reveals some remarkable differences. Firstly, heterologous expression leads to considerable levels of secreted of ABF A after 24 h of growth on sugar beet pulp, a stage of growth in which there is hardly any background of endogenous extracellular protein. Since the enzyme is produced in considerable amounts by such *A. nidulans* transformants, heterologous expression is useful with respect to production and purification of large quantities of the protein for further research purposes. However, inferring the GRAS status of the host, only *A. niger abf*A transformants are suitable for applications in food technology. A second feature of interest is the occurrence of enzyme microheterogeneity upon secretion by transformed strains. Like in the case of purified *A. niger* ABF A, the heterologous protein produced by *A. nidulans* transformants appears as a diffuse, fuzzy band after SDS-PAGE, while *A. niger* transformants apparently produce two moieties condensed in sharp bands with slightly different apparent molecular weights. Variation in glycosylation patterns or other post-translational modifications mediated by the two hosts can be resonsible for the phenomena observed. Also, it has been reported that fungal glycoprotein can spontaneously be modified during purification and storage (Niku-Paavola *et al.* 1985).

Recently, the molecular cloning of some genes from prokaryotic origin encoding PNP-A-hydrolyzing activity were reported (Schwarz *et al.* 1990; Utt *et al.* 1991). The *A. niger abf*A gene is to our knowledge, the first eukaryotic gene encoding an ABF to be cloned. Cloning of this gene is the first step towards a further molecular analysis of the regulation of L-arabinan degradation by *A. niger*.

References

Archer DB, Roberts IN, MacKenzie DA (1990) Heterologous protein secretion from *Aspergillus niger* in phosphate-buffered batch culture. Appl Microbiol Biotechnol **34**:313-315

Aviv H, Leder P (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci USA **69**:1408-1412

Ballance DJ, Turner G (1985) Development of a high-frequency transforming vector for *Aspergillus nidulans*. Gene **36**:321-331

Benton WD, Davis RW (1977) Screening lambda gt recombinant clones by hybridization to single plaques *in situ*. Science **196**:180-182

Cathala G, Savouret J-F, Mendez B, West BL, Karin M, Martial JA, Baxter JD (1983) A method for isolation of intact, translationally active ribonucleic acid. DNA **2**:329-335

Churms SC, Merrifield EH, Stephen AM, Walwijn DR, Polson A, Merwe K van de, Spies HCS, Costa N (1983) An L-arabinan from apple-juice concentrates. Carbohydr Res **113**:339-344

Goosen T, Bloemheuvel G, Gysler C, Bie DA de, Broek HWJ van den, Swart K (1987) Transformation of *Aspergillus niger* using the homologous orotidine-5'-phosphate-decarboxylase gene. Curr Genet **11**:499-503

Graaff L de, Broeck H van den, Visser J (1992) Isolation and characterization of the *Aspergillus niger* pyruvate kinase gene. Curr Genet **22**:21-27

Gwynne DI, Devchand M (1992) Expression of foreign proteins in the genus *Aspergillus*. In: Bennett JW, Klich MA (eds) *Aspergillus*: Biology and industrial applications. Biotechnology series. Vol.23. Butterworth-Heinemann, Boston. pp.203-214

Harmsen JAM, Kusters-van Someren MA, Visser J (1990) Cloning and expression of a second *Aspergillus niger* pectin lyase gene (*pel*A): indications of a pectin lyase gene family in *Aspergillus niger*. Curr Genet **18**:161-166

Kaji A (1984) L-Arabinosidases. Adv Carbohydr Chem Biochem **42**:383-394

Komae K, Kaji A, Sato M (1982) An α-L-arabinofuranosidase from *Streptomyces purpurascens* IF03389. Agric Biol Chem **46**:1899-1905

Kusters-van Someren MA, Harmsen JAM, Kester HCM, Visser J (1991) Structure of the *Aspergillus niger pel*A gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. Curr Genet **20**: 293-299

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685

Niku-Paavola M-L, Lappalainen A, Enari T-M, Nummi, M (1985) A new appraisal of the endoglucanases of the fungus *Trichoderma reesei*. Biochem J **231**:75-81

Pontecorvo G, Roper JA, Hemmons LJ, MacDonald KD, Bufton AWJ (1953) The genetics of *Aspergillus nidulans*. Adv Genet **5**:141-238

Rombouts FM, Voragen AGJ, Searle-van Leeuwen MF, Geraerds CCJM, Schols HA, Pilnik W (1988) The arabinanases of *Aspergillus niger* – purification and characterisation of two α-L-arabinofuranosidases and an *endo*-1,5-α-L-arabinanase. Carbohydr Polym **9**:25-47

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.

Schwarz WH, Adelsberger H, Jauris S, Hertel C, Funk B, Staudenbauer WL (1990) Xylan degradation by the thermophile *Clostridium stercorarium*: cloning and expression of xylanase, β-D-xylosidase, and α-L-arabinofuranosidase genes in *Escherichia coli*. Biochem Biophys Res Commun **170**:368-374

Tagawa K, Terui G (1968) Studies on the enzymes acting on araban. IX. Culture conditions and process of arabanase production by *Aspergillus niger*. J Ferment Technol **46**:693-700

Uitzetter JHAA (1982) Studies on carbon metabolism in wild-type and mutants of *Aspergillus nidulans*. PhD Thesis Wageningen Agricultural University, Wageningen, The Netherlands.

Utt EA, Eddy CK, Keshav KF, Ingram LO (1991) Sequencing and expression of the *Butyrivibrio fibrisolvens xyl*B gene encoding a novel bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities. Appl Environ Microbiol **57**:1227-1234

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1991) Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. Arch Microbiol **157**:23-28

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1993) Induction of extracellular arabinases on monomeric substrates in *Aspergillus niger*. Arch Microbiol **159**:66-71

Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev **21**:195-213

Voragen AGJ, Geerst F, Pilnik W (1982) Hemicellulase in enzymic fruit processing. In: Dupuy P (ed) Use of enzymes in food technology. Technique et Documentation Lavoisier, Paris. pp.497-502

Voragen AGJ, Rombouts FM, Searle-van Leeuwen MF, Schols HA, Pilnik W (1987) The degradation of arabinans by endo-arabinanase and arabinofuranosidases purified from *Aspergillus niger*. Food Hydrocoll **1**:423-437

Weber K, Osborn M (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem **244**:4406-4412

Weinstein L, Albersheim P (1979) Structure of plant cell walls. IX. Purification and partial characterization of a wall-degrading endo-arabanase and an arabinosidase from *Bacilllus subtilis*. Plant Physiol **63**:425-432

Witteveen CFB, Busink R, Vondervoort P van de, Dijkema C, Swart K, Visser J (1989) L-Arabinose and D-xylose catabolism in *Aspergillus niger*. J Gen Microbiol **135**:2163-2171

Young RA, Davis RW (1983) Yeast RNA polymerase II genes: isolation with antibody probes. Science **222**:778-782

Chapter 3

Molecular cloning, expression and structure of the endo-1,5-α-L-arabinase gene of *Aspergillus niger* [1]

Secretion of endo-1,5-α-L-arabinase A (ABN A) by an *Aspergillus niger* D-xylulose kinase mutant upon mycelium transfer to medium containing L-arabitol was immunochemically followed with time to monitor its induction profile. A cDNA expression library was made from polyA$^+$ RNA isolated from the induced mycelium. This library was immunochemically screened and one ABN A specific clone emerged. The corresponding *abn*A gene was isolated from an *A. niger* genomic library. Upon Southern blot analysis, a 3.1 kb *Hin*dIII fragment was identified and subcloned to result in plasmid pIM950. By means of co-transformation using the *A. niger pyr*A gene as a selection marker, the gene was introduced in both *A. niger* and *A. nidulans* uridine auxotrophic mutants. Prototrophic *A. niger* and *A. nidulans* transformants overproduced *A. niger* ABN A upon growth in medium containing sugar beet pulp as the sole carbon source, thereby establishing the identity and functionality of the cloned gene. The DNA sequence of the complete *Hin*dIII fragment was determined and the structure of the *abn*A gene as well as of its deduced gene product were analyzed. *abn*A contains three introns within its structural region and codes for a protein of 321 amino acids. Signal peptide processing results in a mature protein of 302 amino acids with a deduced molecular weight of 32.5 kDa. *A. niger abn*A is the first gene encoding an ABN to be isolated and characterized.

Introduction

Branched L-arabinan is a common polysaccharide component of the cell wall of higher plants, in which it is associated with pectic substrates. The polymer from sugar beet consists of a backbone of mainly 1,5-α-coupled L-arabinofuranose residues, which is substituted with 1,2-α- and 1,3-α-linked side chains of L-arabinofuranose residues (Hirst and Jones 1948; Tanaka *et al.* 1981). Two main enzymatic activities are involved in degradation of L-arabinan, namely α-L-arabinofuranosidase (ABF) (EC 3.2.1.55) and endo-1,5-α-L-arabinase (ABN)

[1] published in: Appl Microbiol Biotechnol 40:318-326 (1993)
The nucleotide sequence data reported in this Chapter are accessable from the EMBL and GenBank Nucleotide Sequence Databases under accession numbers X74757 and L23430.

(EC 3.2.1.99) (Kaji 1984; Whitaker 1984). Various phytopathogenic and saprophytic microorganisms are known to produce these activities (*e.g.* Karimi and Ward 1989). From the hyphal fungus *Aspergillus niger* two distinct ABFs (ABF A & B) and one ABN (ABN A) are purified and characterized (Voragen *et al.* 1987; Rombouts *et al.* 1988; van der Veen *et al.* 1991). ABN A is glycosylated and has an apparent molecular weight of 43 kDa as determined upon SDS-PAGE. It hydrolyzes the 1,5-α-glycosidic bonds in L-arabinan in a random fashion, eventually resulting in arabinotriose, arabinobiose and substituted oligomers. Linear 1,5-α-L-arabinan is shown to be the most preferable substrate, the enzyme activity is progressively reduced as 1,5-α-linked chains become shorter or more highly substituted (Voragen *et al.* 1987; McCleary 1991). The *A. niger* ABN A acts synergistically with both ABFs in degrading L-arabinan (Voragen *et al.* 1987). The properties of the fungal enzyme resemble those of ABNs isolated from various *Bacillus subtilis* strains, although the prokaryotic enzymes are reported to produce mono- and dimers as end products (Kaji and Saheki 1975; Weinstein and Albersheim 1979; Sakai and Sakamoto 1990; Yoshihara and Kaji 1983).

Previous studies (van der Veen *et al.* 1991) indicate that ABN A expression is induced upon growth on crude materials, such as sugar beet pulp, that contain L-arabinan as well as on polysaccharides such as arabinogalactan, which contain sequences of 1,5-α-linked L-arabinofuranose residues. Next to polymers, L-arabitol, an intermediate of L-arabinose catabolism, induces ABN A expression (van der Veen *et al.* 1993). *A. niger* mutant N572, which lacks D-xylulose kinase activity (Witteveen *et al.* 1989), has been shown to produce higher levels of all arabinases than *A. niger* wild-type upon induction with L-arabitol after mycelial transfer (van der Veen *et al.* 1993). This mutant was previously used to isolate the *abf*A gene encoding the minor ABF from *A. niger* (ABF A) by means of immunochemical screening of a cDNA expression library (Chapter 2; Flipphi *et al.* 1993b). A similar approach was followed to isolate the *abn*A gene encoding ABN A, which is reported in this Chapter. The identity of the gene was confirmed by overexpression of ABN A from both *A. niger* and *A. nidulans* transformants, in which the cloned gene was introduced through co-transformation. The structure of the *abn*A gene is presented.

Materials and methods

Fungal strains, bacterial strains, phages and plasmids. The *A. niger* strains used in this study all descend from wild-type strain N400 (CBS 120.49): N402 (*cspA1*); N593 (*cspA1*, *pyrA6*); N572 (*cspA1*, *nicA1*, *xkiA1*), and were described previously (Goosen *et al.* 1987; Witteveen *et al.* 1989). *A. nidulans* WG096 (*pabaA1*, *yA2*) is a standard strain originating from the Glasgow collection (FGSC 187). *A. nidulans* G191 (*pabaA1*, *pyrG89*, *fwA1*, *uAY9*) (Ballance and Turner 1985) was kindly provided by Dr. D.J. Ballance. *Escherichia coli* strains PLK-F', BB4 and JM101 were supplied by Stratagene. Phage R408 (Stratagene) was used as a helper phage for phagemid excision. pEMBL (Dente and Cortese 1987), pBluescript (Stratagene) and pGEM plasmids (Promega) were used in DNA subcloning. Plasmid pGW635 contains the *A. niger pyr*A gene (Goosen *et al.* 1989).

Aspergillus cultivation. Media used for conidiation and submerged growth were composed essentially as previously in Chapter 2 (Flipphi *et al.* 1993b), although 6 g/l $NaNO_3$ was added as a nitrogen source instead of NH_4Cl. For growth of auxotrophic strains the medium was supplemented with either 2 mg/l *para*-aminobenzoate, 10 mM uridine or 1 mg/l nicotinamide, respectively. Liquid medium was inoculated with 10^6 spores/ml and incubated either at 30°C for *A. niger* strains or at 37°C for *A. nidulans* strains using an orbital shaker (250 rpm). L-arabitol was obtained from Sigma. Sugar beet pulp, a gift from CSM (Breda, The Netherlands), was ground in a Waring blendor prior to use.

Construction and screening of an L-arabitol-induced cDNA expression library. *A. niger* N572 was cultivated for 24 h using sucrose as a carbon source. The mycelium was harvested by filtration and thoroughly washed with sterile saline. The mycelium was devided into eight portions of equal weight, seven of which were transferred to minimal medium containing 1 % L-arabitol. The eighth portion was transferred to mimimal medium containing 1 % D-glucose to serve as a control. During a time course of seven hours, a culture was removed each hour from the incubator and the mycelium was separated from the medium by filtration. A 10 ml sample of culture medium was collected to be used for an immunochemical screening of ABN A secretion. PolyA$^+$ RNA was isolated from the mycelium obtained, as described in Chapter 2 (Flipphi *et al.* 1993b). cDNA was synthesized from 5 μg polyA$^+$ RNA using Stratagene's ZAP−cDNA synthesis kit. After ligation of the cDNA into Uni−ZAP XR vector-arms (Stratagene), the phage DNA was packaged using Packagene extracts (Promega) and the resulting library was subsequently titrated using *E. coli* PLK-F'. This primary library was amplified in *E. coli* PLK-F', titrated and stored at 4°C.

The amplified cDNA library was immunochemically screened for ABN A expression and positive phages were purified as described in Chapter 2 (Flipphi *et al.* 1993b), using anti-ABN A antiserum as a probe. Phagemids were excised from positive phages by co-infection with helper phage R408 and rescued by plating with *E. coli* BB4 on ampicillin-containing plates, resulting in unidirectionally cloned cDNA in pBluescript SK$^-$.

Isolation and cloning of the *abnA* gene. An *A. niger* N400 genomic library in λEMBL4 (Harmsen *et al.* 1990) was screened using a 550 bp *Kpn*I fragment from cDNA clone pC1N1 as the probe. Screening by plaque filter hybridization using nitrocellulose replicas was performed as decribed previously (Chapter 2; Flipphi *et al.* 1993b). The filters were washed at 68°C, using 0.2 x SSC/0.1 % SDS as the final washbuffer. For all other DNA manipulations standard methods were applied (Sambrook *et al.* 1989).

***Aspergillus* transformation and isolation of *Aspergillus* genomic DNA.** *Aspergillus* transformation was carried out according to the protocol of Kusters-van Someren *et al.* (1991) using *A. niger* and *A. nidulans* uridine auxotrophic mutants N593 and G191 as recipients. pIM950 was added in a 20-fold excess over pGW635 to obtain high co-transformation frequencies. Genomic DNA from *A. niger* and *A. nidulans* transformed and wild-type strains as used in Southern blot analyses, was isolated as described by de Graaff *et al.* (1988); the hybridization and washing conditions used were identical to those described for plaque filter hybridization.

Analysis of secreted protein. Medium samples were filtrated though filterpaper and centrifuged (3000 rpm, 15 min) to pellet debris. ABN activity was assayed using ArabinaZyme tablets (Megazyme, Warriewood (Sydney), Australia) and calculated according to the manufacturer's instructions − one unit (U) is the amount of enzyme required to release 1 μmole of L-arabinose reducing-sugar equivalents per minute. Samples were concentrated as follows: the media were extensively dialyzed against 1 mM sodium phospate buffer, pH 6.0, at 4°C and subsequently lyophilised. The dry material was resolved in 1/50 of the original volume in sterile bidistilled water. Protein samples were separated by electrophoresis in 10 %

polyacrylamide gels containing 0.1 % SDS according to Laemmli (1970), and either electroblotted onto nitrocellulose or stained with Coomassie brilliant blue R250. The blots were probed with anti-ABN A antiserum (Chapter 2; Flipphi *et al.* 1993b). Purified ABN A having an apparent molecular weight of 43 kDa (van der Veen *et al.* 1991) was used as a molecular weight marker.

Amino acid sequence determination and cyanogen bromide cleavage. Approximately 1 – 2 nmoles of purified ABN A was directly used for amino acid sequencing of the N-terminus of the enzyme, utilizing a gas phase sequencer equipped with a PTH analyzer as decribed by Amons (1987). Purified protein was cleaved with cyanogen bromide (Pierce) according to Steers *et al.* (1965), using a 200-fold excess cyanogen bromide at a protein concentration of 1 mg/ml. The peptides were separated by electrophoresis in a 15 % polyacrylamide gel containing 0.1 % SDS (Laemmli 1970) and electroblotted onto Immobilon–P polyvinylidene difluoride membrane (Millipore) (Matsudaira 1987). The piece of membrane containing a peptide with an apparent molecular weight of 27 kDa was recovered and used in sequence analysis as described by Amons (1987).

Isolation of a full length cDNA clone. The amplified cDNA library was screened as described for the genomic library using a 300 bp *Sph*I/*Bam*HI fragment from the genomic clone pIM950 as the probe. Plasmids were recovered from positive phages by excision as described above.

DNA sequence determination, sequence analysis and primer extension mapping. DNA fragments were cloned both in plasmid vectors (pEMBL; pBluescript; pGEM) and in M13 mp18/19. DNA sequences were determined with the dideoxynucleotide chain termination method (Sanger *et al.* 1977) using the ^{T7}Sequencing Kit from Pharmacia LKB according to the suppliers instructions. Single-stranded M13 DNA as well as alkali-denatured plasmid DNA were used as template, and both commercially available oligonucleotides and gene-specific oligonucleotides were employed as primers. Computer analysis was done using the PC/GENE program (IntelliGenetics).

Primer extension mapping to deduce the transcription initiation sites was performed according to Calzone *et al.* (1987) using polyA$^+$ RNA isolated from *A. niger abn*A transformant strain N593::pIM950-14 as described above. The sequence of the 17-mer oligonucleotide which served as primer is complementary to nucleotides 95 – 111 (see Fig.5).

Results

Isolation of an ABN A cDNA clone

Combining the characteristics of *A. niger* D-xylulose kinase mutant N572 with respect to arabinase expression and the specific induction capacity of L-arabitol, previously enabled cloning of the *abf*A gene encoding ABF A after immunochemically identifying cDNA clones from a cDNA expression library (Chapter 2; Flipphi *et al.* 1993b). Analogously it was attempted to isolate ABN A cDNA clones from the same cDNA library of 16 h L-arabitol-induced mycelium. However, such clones were not found upon screening of 1.5 x 10^5 plaque-forming units (pfu), which corresponds to more than half of the primary library. The medium of the culture which was used to generate the library contained however considerable amounts of ABN A (not shown).

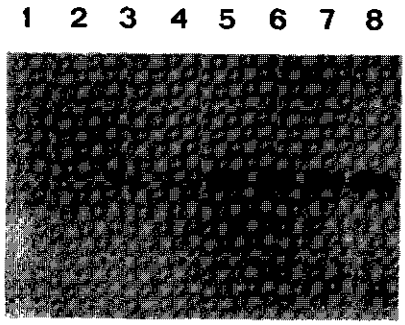


Fig.1. Immunochemical study of a time course following induction of ABN A upon mycelial transfer to medium containing 1 % L-arabitol. Medium samples were concentrated 50 times prior to SDS-PAGE. **Lane 1**: medium from the D-glucose transfer collected 4 h after transfer; **2–8**: medium from the L-arabitol transfer collected each hour, up to 7 h after transfer.

Since isolating ABN A cDNA clones did not succeed, one could assume that such clones might be under-represented in this library due to rapid decline of the mRNA level after the initial induction. Therefore secretion of ABN A by N572 cultivated in minimal medium containing L-arabitol was followed immunochemically during the first 7 h after transfer. As shown in Fig.1, extracellular ABN A could be detected 2 h after transfer, and within the next 2 h the ABN A level was quicky elevated to its maximum. This level is roughly maintained throughout the last 3 h of the time course. Fig.1 also shows accumulation of immunoreactive low molecular weight moieties during the experiment, presumably resulting from proteolytic breakdown of ABN A.

Using conditions described above, a new cDNA library was constructed from *A. niger* N572 mycelium harvested at, respectively, 1, 2, 3, 4, 5 and 6 h after transfer. Half of the RNA isolated from the mycelium samples was pooled, the polyA$^+$ RNA fraction was isolated and used to synthesize cDNA. Ligation of 100 ng cDNA in 1 μg vector arms and packaging of one fifth of the ligation mixture resulted in a library consisting of 3×10^4 independent recombinant phages. After amplification of the library, 5×10^4 *pfu were immunochemically* screened for expression of ABN A cDNA. One positive plaque was found from which upon purification and excision the plasmid pC1N1 emerged. The cDNA insert of pC1N1 was found to be approximately 700 bp in length as determined after digestion with *Eco*RI and *Xho*I. Further restriction analysis revealed the presence of a *Sal*I and a *Kpn*I site within the cDNA; digesting pC1N1 with *Kpn*I resulted in a fragment of approximately 550 bp containing the 3' end of the cDNA.

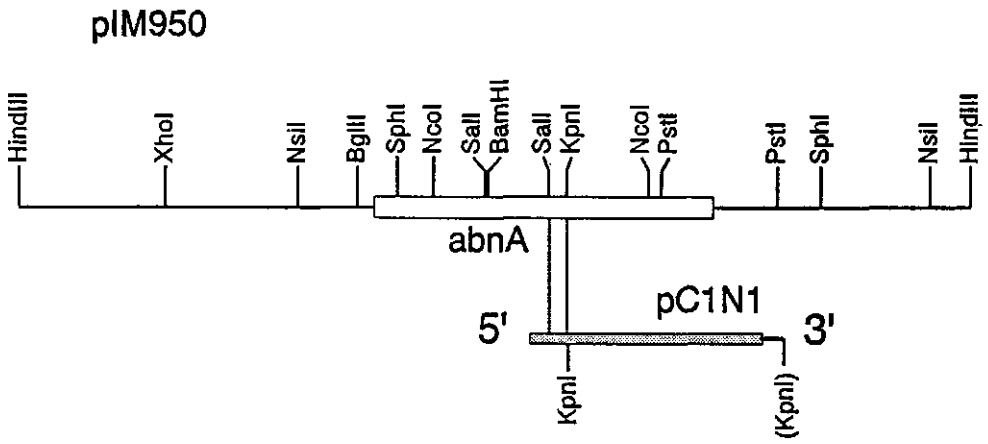


Fig.2. Physical map of the insert from the genomic clone pIM950. The position and orientation of the cDNA insert from pC1N1 are also indicated. There is no *Pst*I site in pC1N1 – the 5' *Pst*I site in pIM950 is situated within the third intron of the gene, as established by sequencing (*cf.* Fig.5).

Isolation and expression of the abnA gene

The 550 bp *Kpn*I fragment from cDNA clone pC1N1 was used to probe an *A. niger* N400 genomic library. Screening of 6 x 10^4 pfu resulted in six positive λ-clones. The inserts of these phages were characterized by restriction analysis using a limited number of restriction enzymes. Upon Southern blotting and hybridization using the *Kpn*I cDNA fragment as the probe, a hybridizing 3.1 kb *Hin*dIII fragment was found in five of the λ-clones. This fragment was isolated and subcloned in vector pEMBL19 resulting in plasmid pIM950. A detailed restriction map of the *Hin*dIII insert was made, which is shown in Fig.2.

In order to prove the functionality of the cloned *abn*A gene present on pIM950, it was introduced in uridine auxotrophic mutants of both *A. niger* and *A. nidulans* by co-transformation using the *A. niger pyr*A gene as primary selection marker. Six *A. niger* and six *A. nidulans* prototrophic transformants were chosen to be analyzed for their expression of ABN A. Southern blot analysis of *Eco*RI-cut genomic *Aspergillus* DNA using the 550 bp *Kpn*I cDNA fragment as the probe, indicated that all transformants except *A. niger* N593::pIM950-10 contain additional copies of the *abn*A gene. The transformants as well as the *A. niger* and *A. nidulans* wild-type strains were grown submerged on medium containing sugar beet pulp as the carbon source. Medium samples were taken at 24 h, 36 h and 48 h after inoculation. Expression was demonstrated upon SDS-PAGE of

culture fluid, immunochemically upon electroblotting as well as by direct Coomassie staining. The levels of secreted enzyme were quantified by measuring ABN activity in the culture media.

As shown in Fig.3a, after 24 h of growth several of the selected *A. niger* transformants overproduced a single immunoreactive extracellular protein as compared to the wild-type level produced by *A. niger* N402 (lane 2). Fig.3b provides a control for the western blot, showing that this protein was overproduced by these transformants whereas other extracellular proteins were produced at comparible levels by all strains analyzed. The overproduced protein has the same apparent molecular weight as purified ABN A of *A. niger* (lane 1). The amount of protein produced by individual transformants shows a correlation with the level of ABN activity present in the culture media (Fig.3c).

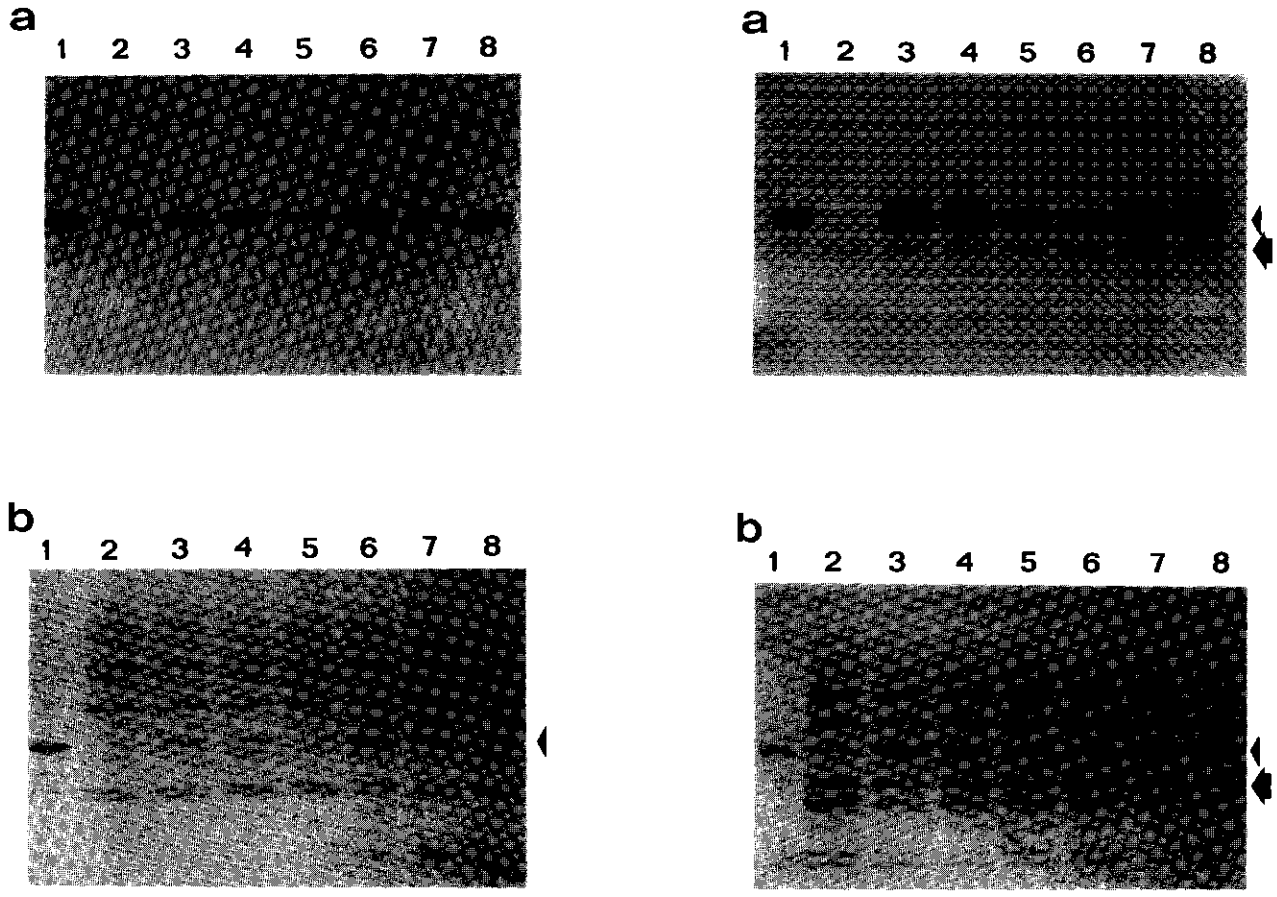
After 24 h of growth, all *A. nidulans* transformants secreted two main proteins which were recognized by anti-ABN A antiserum (Fig.4a). A protein with a lower apparent molecular weight than *A. niger* ABN A (lane 1) was produced at comparable levels by all transformants as well as by the wild-type. Obviously this band represents endogenous ABN from *A. nidulans*, as the wild-type produces such an activity upon growth on sugar beet pulp (Fig.4c). The protein with the highest apparent molecular weight was produced in various, but vast amounts by the individual transformants, but was not found in *A. nidulans* wild-type strain WG096. Fig.4b shows that this protein was overproduced by all transformants whereas all other proteins were produced at comparable levels. The protein has the same apparent molecular weight as purified *A. niger* ABN A (lane 1). The differences in overexpression of ABN A are also refected by the extracellular ABN activity produced by the various transformants as compared to wild-type *A. nidulans* (Fig.4c).

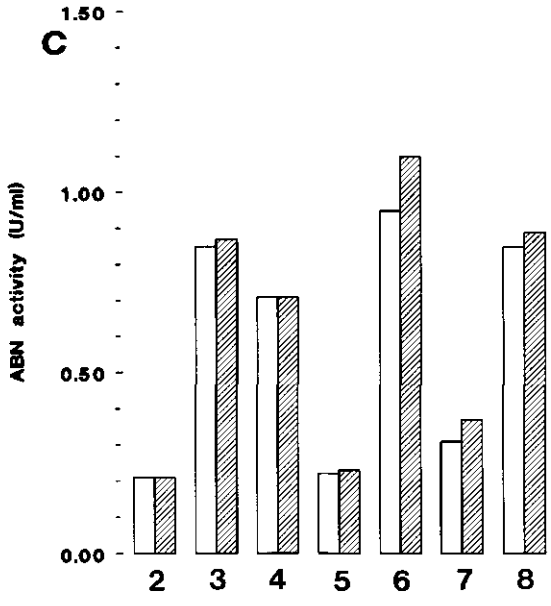
Determination of amino acid sequences

The ABN A enzyme was purified from medium filtrates of sugar beet culture of *A. niger* N400. The apparent molecular weight of the purified enzyme, as determined by SDS-PAGE using a 10% gel, was 43 kDa. The isoelectric point was determined to be 3.0, fully in accordance with van der Veen *et al.* (1991). Amino acid sequences were determined from the N-terminus of the intact protein, as well as of the N-terminal end from a 27 kDa peptide obtained after treatment of ABN A with cyanogen bromide. The N-terminal amino acid sequence of the whole protein was determined to be [Y A D P G A X S G V X T T] (13 residues). The amino acids at position 7 and 11 could not be assigned and are therefore denoted "X". For the internal cyanogen bromide fragment the N-terminal amino acid sequence [E Y G S W T D H G S T G I A S] (15 residues) was found.

A. niger

A. nidulans





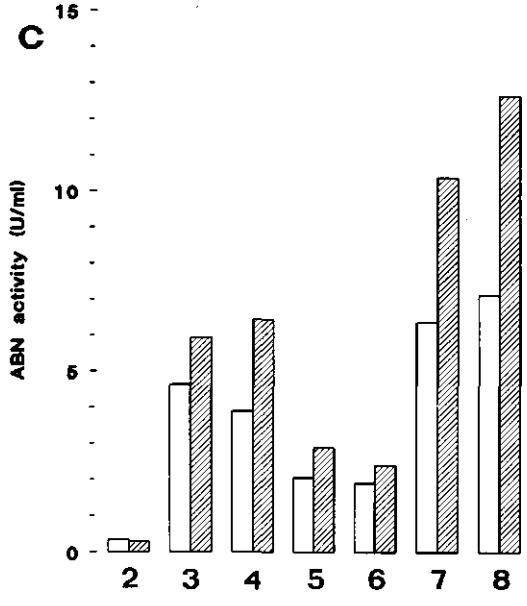


Fig.3 (left). Analysis of ABN A expression by *A. niger abn*A transformants and *A. niger* wild-type N402 upon 24 h of growth on minimal medium containing sugar beet pulp as the carbon source. From each strain 15 μl of a culture medium sample was loaded on the gel. The position of ABN A (43 kDa) is marked (◂). **a**: ABN A-specific immunochemical detection upon SDS-PAGE and western blotting; **b**: Coomassie brilliant blue R250-stained gel upon SDS-PAGE; **c**: Bar diagram representing ABN activities (U/ml medium) determined in the culture filtrates at 24 hrs (open bars) and 36 hrs (hatched bars) after inoculation. **Lane 1**: 1 μg purified ABN A; **2**: N402; **3**: N593::pIM950-8; **4**: -9; **5**: -10; **6**: -11; **7**: -13; **8**: -14.

Fig.4 (right). Analysis of ABN A expression by *A. nidulans abn*A transformants and *A. nidulans* wild-type WG096 upon 24 h of growth on minimal medium containing sugar beet pulp as carbon source. From each strain 15 μl of a culture medium sample was loaded on the gel. The position of the heterologous ABN A is indicated (◂); the position of the endogenous ABN is marked with an arrow. **a**: ABN A-specific immunochemical detection upon SDS-PAGE and western blotting; **b**: Coomassie brilliant blue R250-stained gel upon SDS-PAGE; **c**: Bar diagram representing ABN activities (U/ml medium) determined in the culture filtrates at 24 hrs (open bars) and 36 hrs (hatched bars) after inoculation. **Lane 1**: 1 μg purified ABN A; **2**: WG096; **3**: G191::pIM950-7; **4**: -11; **5**: -12; **6**: -16; **7**: -17; **8**: -20.

Primary structure of the *A. niger abn*A gene and deduced protein structure

The nucleotide sequence of the *Hin*dIII insert of pIM950 was determined over both strands and is shown in Fig.5. 3151 bp in total were determined; 1177 bp of the 5' non-coding area, 1126 bp of structural sequences and 848 bp of the 3' non-coding area.

The position of the coding region of the *A. niger abn*A gene has been determined using the terminal sequences of the insert of cDNA clone pC2N1. This presumable "full length" cDNA clone was isolated from the cDNA library using the 300 bp *Sph*I/*Bam*HI fragment from pIM950 (Fig.2) as the probe. This fragment enabled screening for clones with larger inserts than pC1N1. Upon screening of 1×10^4 pfu of the amplified library, 36 positive plaques were found. Three of these were purified and excised. The terminal sequences of the cDNA inserts of the resulting plasmids were determined and were found to be identical. By matching these terminal cDNA sequences and the available amino acid sequence data on the genomic sequence, the structural region of *abn*A was determined. Upon analysis of the deduced amino acid sequence of the 5'-DNA sequence of pC2N1, the complete N-terminal sequence of ABN A could be identified (not shown). The coding region was found to start with the ATG codon 16 bases downstream from the 5'-end of the pC2N1 cDNA insert (corresponding with position 1 in Fig.5), and was shown to end with the TAA codon 162 bases upstream from the 3'-end of the cDNA (corresponding with position 1124 in Fig.5). The structural region is interrupted by three introns, which were established by sequencing the corresponding parts of cDNA clone pC2N1: intron A is found from position 268 to 317, intron B from position 795 to 853 and intron C from position 940 to 990. Primer extension experiments indicated major transcription initiation sites at positions -33 and -29 upstream of the proposed start codon, and weaker sites at -38 and -32.

Upon removal of the introns, an open reading frame of 963 bp is formed that encodes a protein of 321 amino acid residues (Fig.5). The N-terminal amino acid sequence as determined for the purified extracellular ABN A is preceded by a sequence of 19 mainly hydrophobic residues, which presumably serves as signal peptide. The cleavage site between residues -1 and $+1$ is conform the "-3 -1 rule" proposed by von Heijne (1986). The unassignable amino acids at positions 7 and 11 of the determined N-terminal sequence both turn out to be cysteine, which could not be detected in gas-phase sequencing since the ABN A protein was not S-pyridylethylated prior to sequence analysis. The amino acid sequence of the internal cyanogen bromide fragment was found from position 106 to 120 in the amino acid sequence (Fig. 5). Removal of the proposed signal sequence leaves a mature protein of 302 amino acid residues, which has a deduced molecular weight of 32.5 kDa and a theoretical isoelectric point of 3.5.

Discussion

In this Chapter the molecular cloning of the *A. niger* gene encoding *endo*-1,5-α-L-arabinase A (ABN A) is described. Transformant *Aspergillus* strains have been constructed in which additional copies of the *abn*A gene were introduced through co-transformation. The results show that the gene present on pIM950 is functional in both *A. niger* and *A. nidulans*. Comparison of enzyme activities (Fig.3c & 4c) shows that introduction of the *A. niger abn*A gene in *A. nidulans* leads to much higher expression levels of ABN A upon induction, than found for *A. niger abn*A transformants. Moreover, heterologous expression proceeds between 24 and 36 h after inoculation whereas in the homologous system the expression seems to be suppressed after 24 h of growth. Southern blot analyses of genomic DNA of *abn*A transformants (not shown) did not reveal large differences in the approximate number of extra copies of *abn*A found in the highest producing *A. niger* transformants and in the highest producing *A. nidulans* transformants. Upon additional screening of *A. niger* transformants recovered from a second, independent transformation experiment did not result in the isolation of transformants that produced significantly higher levels of ABN activity than produced by the transformants described above (results not shown). All these observations indicate possible differences in regulation between the two hosts, although the described phenomena could also be explained by differences in proteolytic activity produced by these two organisms, as suggested by Kusters-van Someren *et al.* (1992).

Heterologous expression by *A. nidulans* transformants has previously been used for identification of the *A. niger pel*B and *abf*A genes (Kusters-van Someren *et al.* 1992; Chapter 2; Flipphi *et al.* 1993b). In case of the *abn*A gene, growth of the *A. nidulans* strains on sugar beet pulp results in secretion of an immuno-reactive endogenous protein, which correlates with ABN activity of *A. nidulans* wild-type (Fig.4a, c). The apparent molecular weight of the *A. nidulans* protein clearly differs from that of the *A. niger* enzyme. These results strongly indicate that *A. nidulans* produces an equivalent ABN. Southern analysis using stringent hybridization and washing conditions could not reveal the presence of a related gene in *A. nidulans*.

The deduced amino acid sequence of ABN A has no significant similarities with the sequences of two exo-type α-L-arabinosidases of prokaryotic origin – a β-D-xylosidase from *Butyrivibrio fibrisolvens* exhibiting a strong *para*-nitrophenyl α-L-arabinofuranoside-hydrolyzing activity (Utt *et al.* 1991) and an ABF from *Pseudomonas fluorescens* subsp. *cellulosa* acting only on oat spelt xylan (Kellett *et al.* 1990). Comparison with the EMBL and GenBank databases did not result in identification of significant primary sequence similarities. The deduced properties of ABN A – its molecular weight and its isoelectric point – differ from those determined for the purified protein from *A. niger* wild-type. A similar discrepancy

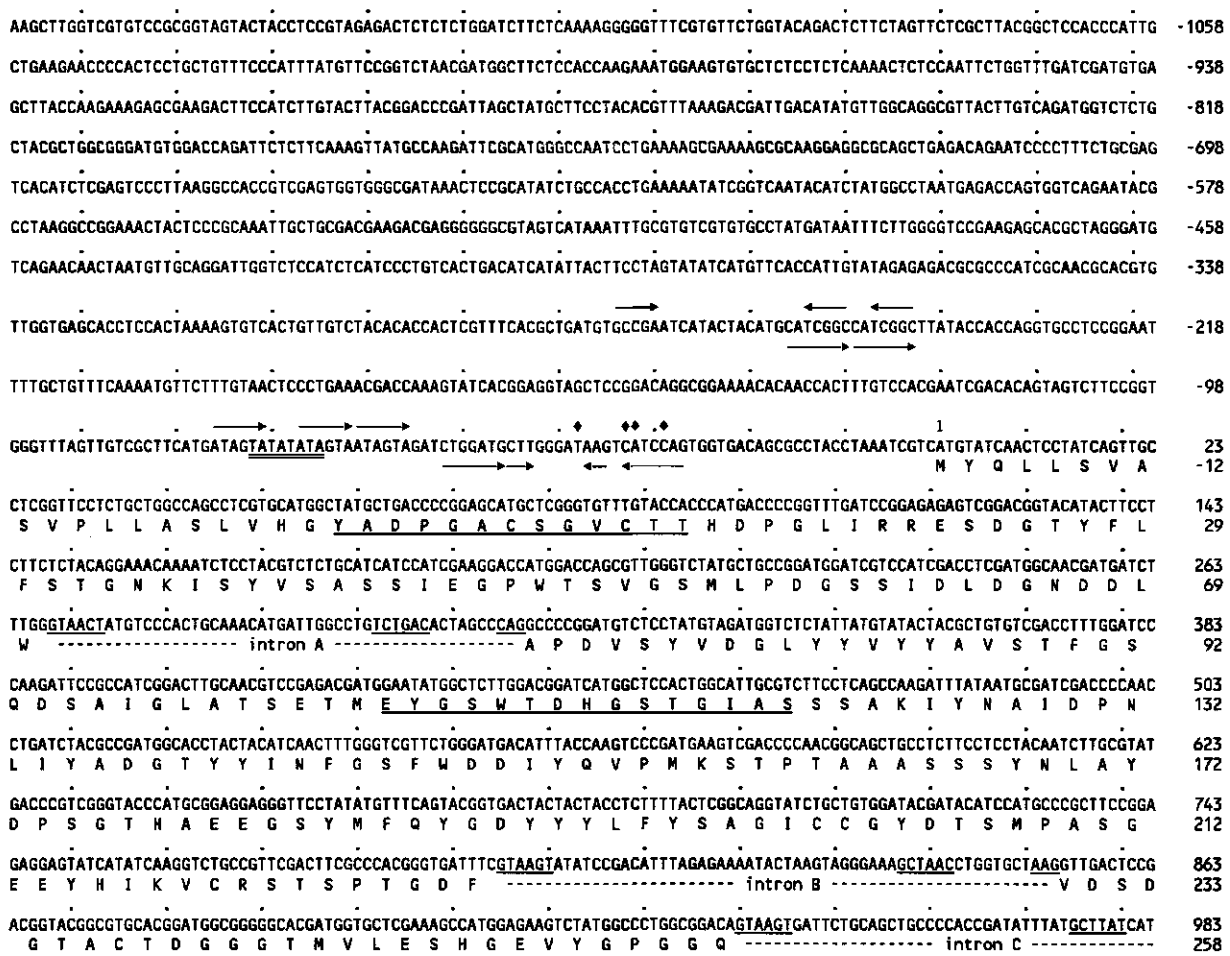

AAGCTTGGTCGTGTCCGCGGTAGTACTACCTCCGTAGAGACTCTCTCTGGATCTTCTCAAAAGGGGGTTTCGTGTTCTGGTACAGACTCTTCTAGTTCTCGCTTACGGCTCCACCCATTG -1058
CTGAAGAACCCCACTCCTGCTGTTTCCCATTTATGTTCCGGTCTAACGATGGCTTCTCCACCAAGAAATGGAAGTGTGCTCTCCTCTCAAAACTCTCCAATTCTGGTTTGATCGATGTGA -938
GCTTACCAAGAAAGAGCGAAGACTTCCATCTTGTACTTACGGACCCGATTAGCTATGCTTCCTACACGTTTAAAGACGATTGACATATGTTGGCAGGCGTTACTTGTCAGATGGTCTCTG -818
CTACGCTGGCGGGATGTGGACCAGATTCTCTTCAAAGTTATGCCAAGATTCGCATGGGCCAATCCTGAAAAGCGAAAAGCGCAAGGAGGCGCAGCTGAGACAGAATCCCCTTTCTGCGAG -698
TCACATCTCGAGTCCCTTAAGGCCACCGTCGAGTGGTGGGCGATAAACTCCGCATATCTGCCACCTGAAAAATATCGGTCAATACATCTATGGCCTAATGAGACCAGTGGTCAGAATACG -578
CCTAAGGCCGGAAACTACTCCCGCAAATTGCTGCGACGAAGACGAGGGGGGCGTAGTCATAAATTTGCGTGTCGTGTGCCTATGATAATTTCTTGGGGTCCGAAGAGCACGCTAGGGATG -458
TCAGAACAACTAATGTTGCAGGATTGGTCTCCATCTCATCCCTGTCACTGACATCATATTACTTCCTAGTATATCATGTTCACCATTGTATAGAGAGACGCGCCCATCGCAACGCACGTG -338
TTGGTGAGCACCTCCACTAAAAGTGTCACTGTTGTCTACACACCACTCGTTTCACGCTGATGTGCCGAATCATACTACATGCATCGGCCATCGGCTTATACCACCAGGTGCCTCCGGAAT -218
TTTGCTGTTTCAAAATGTTCTTTGTAACTCCCTGAAACGACCAAAGTATCACGGAGGTAGCTCCGGACAGGCGGAAAACACAACCACTTTGTCCACGAATCGACACAGTAGTCTTCCGGT -98
GGGTTTAGTTGTCGCTTCATGATAGTATATATAGTAATAGTAGATCTGGATGCTTGGGATAAGTCATCCAGTGGTGACAGCGCCTACCTAAATCGTCATGTATCAACTCCTATCAGTTGC 23
M Y Q L L S V A -12
CTCGGTTCCTCTGCTGGCCAGCCTCGTGCATGGCTATGCTGACCCCGGAGCATGCTCGGGTGTTTGTACCACCCATGACCCCGGTTTGATCCGGAGAGAGTCGGACGGTACATACTTCCT 143
S V P L L A S L V H G Y A D P G A C S G V C T T H D P G L I R R E S D G T Y F L 29
CTTCTCTACAGGAAACAAAATCTCCTACGTCTCTGCATCATCCATCGAAGGACCATGGACCAGCGTTGGGTCTATGCTGCCGGATGGATCGTCCATCGACCTCGATGGCAACGATGATCT 263
F S T G N K I S Y V S A S S I E G P W T S V G S M L P D G S S I D L D G N D D L 69
TTGGGTAACTATGTCCCACTGCAAACATGATTGGCCTGTCTGACACTAGCCCAGGCCCCGGATGTCTCCTATGTAGATGGTCTCTATTATGTATACTACGCTGTGTCGACCTTTGGATCC 383
W ---------- intron A ---------- A P D V S Y V D G L Y Y V Y Y A V S T F G S 92
CAAGATTCCGCCATCGGACTTGCAACGTCCGAGACGATGGAATATGGCTCTTGGACGGATCATGGCTCCACTGGCATTGCGTCTTCCTCAGCCAAGATTTATAATGCGATCGACCCCAAC 503
Q D S A I G L A T S E T M E Y G S W T D H G S T G I A S S S A K I Y N A I D P N 132
CTGATCTACGCCGATGGCACCTACTACATCAACTTTGGGTCGTTCTGGGATGACATTTACCAAGTCCCGATGAAGTCGACCCCAACGGCAGCTGCCTCTTCCTCCTACAATCTTGCGTAT 623
L I Y A D G T Y Y I N F G S F W D D I Y Q V P M K S T P T A A A S S S Y N L A Y 172
GACCCGTCGGGTACCCATGCGGAGGAGGGTTCCTATATGTTTCAGTACGGTGACTACTACTACCTCTTTTACTCGGCAGGTATCTGCTGTGGATACGATACATCCATGCCCGCTTCCGGA 743
D P S G T H A E E G S Y M F Q Y G D Y Y Y L F Y S A G I C C G Y D T S M P A S G 212
GAGGAGTATCATATCAAGGTCTGCCGTTCGACTTCGCCCACGGGTGATTTCGTAAGTATATCCGACATTTAGAGAAAATACTAAGTAGGGAAAGCTAACCTGGTGCTAAGGTTGACTCCG 863
E E Y H I K V C R S T S P T G D F ---------- intron B ---------- V D S D 233
ACGGTACGGCGTGCACGGATGGCGGGGGCACGATGGTGCTCGAAAGCCATGGAGAAGTCTATGGCCCTGGCGGACAGTAAGTGATTCTGCAGCTGCCCCACCGATATTTATGCTTATCAT 983
G T A C T D G G G T M V L E S H G E V Y G P G G Q ---------- intron C ---------- 258

Fig.5. The DNA sequence of the *Hin*dIII fragment from pIM950 containing the *A. niger abn*A gene and the deduced amino acid sequence of the gene product ABN A. The DNA sequence is numbered relative to the first base of the deduced startcodon, the amino acid sequence is numbered relative to the N-terminal tyrosine residu of mature ABN A. The consecutive introns are numbered (**A**), (**B**) and (**C**); sequences matching the fungal splice consensus sequences within these introns are underlined. Transcription initiation sites are marked (♦). The 3′ end of the cDNA covered sequence is marked (▾). Repeats in the upstream non-coding sequence are indicated by arrows. The putative TATA-box in the upstream non-coding area and the putative transcription termination/polyadenylation motifs in the downstream non-coding area, are double underlined. Determined amino acid sequences are underlined. The only putative *N*-glycosylation site (amino acid position 276) is marked (▲).

between deduced and apparent molecular weight has been found for several other extracellular carbohydrolases from *A. niger*, *e.g.* pectin lyases A and B (Kusters-van Someren *et al.* 1991, 1992). Since ABN A is known to be glycosylated (Voragen *et al.* 1987) and only one putative *N*-glycosylation site is found in the deduced sequence (Fig.5), *O*-linked glycosylation might account for the difference of approximately 10 kDa found in the case of ABN A.

In the upstream non-coding region of the *abn*A gene, a TATA-like element (TATATATA) is found at position −72 to −65, approximately 40 bp in front of the major transcription initiation sites (Fig.5). The TATA-box is one of the elements involved in transcription initiation, as established in higher eukaryotes (Nussinov 1990). A CAAT-box, another such element, could not be identified. Also *abn*A lacks a CT-rich region in front of any of the transcription initiation sites; this motif has been described to play a role in transcription of several highly expressed fungal genes (Gurr *et al.* 1987). The upstream region does, however, contain direct and indirect repeats as tentative candidates for a possible *abn*A-specific regulatory promoter element, as indicated in Fig.5. Interestingly, both major transcription initiation sites map within the most proximal half of the dyad symmetry found between the putative TATA-box and the proposed translational start.

The DNA sequence directly preceding the start codon (T C G T C – A T G) is identical to what has been reported for the *A. niger trp*C gene (Kos *et al.* 1988). This region is thought to effect initiation of translation and the sequence resembles the consensus defined for higher eukaryots by Kozak (1984) (C C Pu C C – A T G), but not the yeast consensus (N A/Py A A/T A – A T G) proposed by Cigan and Donahue (1987).

Downstream of the proposed stop codon, the mRNA contains a non-coding area of 162 bases as indicated by the 3′ sequence of cDNA clone pC2N1 (Fig.5). This region does not contain specific signals for polyadenylation as indicated by Gurr *et al.* (1987). Downstream of the 3′-terminal base of cDNA clone pC2N1 however, a T-rich region is present that could be involved in transcription termination, as described for mammalian genes (Proudfoot 1991). More distal (approximately 320 bp downstream) a AATAAA (AATAAC) and a CCTGTTCC motif (CGTGTTAC) (McLauchlan *et al.* 1985) were present (Fig.5).

The *abn*A gene contains three introns of which the sizes, determined as 50 bp, 59 bp and 51 bp respectively, are common amongst introns of fungal genes (Rambosek and Leach 1987). In each intron, divergences from the fungal splice consensus sequences according to Rambosek and Leach (1987), *viz.* GTPuNGT for the 5′ donor site; PuCTPuAC for the lariat; PyAG for the 3′ acceptor site, are found. The 5′ border sequence of intron A (GTAACT) contains an odd C at the fifth position – the third intron of the *Aspergillus oryzae amd*S gene (GTAGCT) (Gomi *et al.* 1991) is the only other intron known thus far harbouring C at this position. Intron B is the first *A. niger* intron described containing the rarely found

A at the first position of the 3' sequence (Gurr *et al.* 1987; Unkles 1992). The putative lariat of intron C (GCTTAT) resembles the one proposed for the only intron found in the *A. niger pyr*G gene (ACTTAT) (Wilson *et al.* 1988; Unkles 1992).

The *A. niger abn*A gene is the first ABN-encoding gene to be cloned and sequenced. Previously the structural genes coding for the two ABFs from *A. niger* L-arabinan-degrading complex were cloned (Chapters 2 & 4; Flipphi *et al.* 1993a, b). The availability of these three genes offers the possibility of studying both induction and repression at the RNA level and could lead towards a better understanding of the regulation of L-arabinan degradation by *A. niger*.

References

Amons R (1987) Vapor-phase modification of sulfhydryl groups in proteins. FEBS Lett **212**: 68-72

Ballance DJ, Turner G (1985) Development of a high-frequency transforming vector for *Aspergillus nidulans*. Gene **36**:321-331

Calzone FJ, Britten RJ, Davidson EH (1987) Mapping of gene transcripts by nuclease protection assays and cDNA primer extension. Methods Enzymol **152**: 611-632

Cigan AM, Donahue TF (1987) Sequence and structural features associated with translational initiator regions in yeast – a review. Gene **59**:1-18

Dente L, Cortese R (1987) pEMBL: a new family of single-stranded plasmids for sequencing DNA. Methods Enzymol **155**:111-119

Flipphi MJA, Heuvel M van, Veen P van der, Visser J, Graaff LH de (1993a) Cloning and characterization of the *abf*B gene coding for the major *a*-L-arabinofuranosidase (ABF B) of *Aspergillus niger*. Curr Genet **24**:525-532

Flipphi MJA, Visser J, Veen P van der, Graaff LH de (1993b) Cloning of the *Aspergillus niger* gene encoding *a*-L-arabinofuranosidase A. Appl Microbiol Biotechnol **39**:335-340

Gomi K, Kitamoto K, Kumagai C (1991) Cloning and molecular characterization of the acetamidase-encoding gene (*amd*S) from *Aspergillus oryzae*. Gene **108**:91-98

Goosen T, Bloemheuvel G, Gysler C, Bie DA de, Broek HWJ van den, Swart K (1987) Transformation of *Aspergillus niger* using the homologous orotidine-5'-phosphate-decarboxylase gene. Curr Genet **11**:499-503

Goosen T, Engelenburg F van, Debets F, Swart K, Bos K, Broek H van den (1989) Tryptophan auxotrophic mutants in *Aspergillus niger*: Inactivation of the *trp*C gene by cotransformation mutagenesis. Mol Gen Genet **219**:282-288

Graaff L de, Broek H van den, Visser J (1988) Isolation and transformation of the pyruvate kinase gene of *Aspergillus nidulans*. Curr Genet **13**:315-321

Gurr SJ, Unkles SE, Kinghorn JR (1987) The structure and organization of nuclear genes of filamentous fungi. In: Kinghorn JR (ed) Gene structure in eukaryotic microbes. SGM Special Publication. Vol.23. IRL Press, Oxford. pp.93-139

Harmsen JAM, Kusters-van Someren MA, Visser J (1990) Cloning and expression of a second *Aspergillus niger* pectin lyase gene (*pel*A): indications of a pectin lyase gene family in *Aspergillus niger*. Curr Genet **18**:161-166

Heijne G von (1986) A *new method for predicting signal sequence cleavage sites*. Nucleic Acids Res **14**:4683-4691

Hirst EL, Jones JKN (1948) Pectic substances. VIII. The araban component of sugar-beet pectin. J Chem Soc:2311-2313

Kaji A (1984) L-Arabinosidases. Adv Carbohydr Chem Biochem **42**:383-394

Kaji A, Saheki T (1975) Endo-arabinanase from *Bacillus subtilis* F-11. Biochim Biophys Acta **410**: 354-360

Karimi S, Ward OP (1989) Comparative study of some microbial arabinan-degrading enzymes. J Industr Microbiol **4**:173-180

Kellett LE, Poole DM, Ferreira LMA, Durrant AJ, Hazlewood GP, Gilbert HJ (1990) Xylanase B and an arabinofuranosidase from *Pseudomonas fluorescens* subsp. *cellulosa* contain identical cellulose-binding domains and are encoded by adjacent genes. Biochem J **272**: 369-376

Kos T, Kuyvenhoven A, Hessing HGM, Pouwels PH, Hondel CAMJJ van den (1988) Nucleotide sequence of the *Aspergillus niger trp*C gene: structural relationship with analogous genes of other organisms. Curr Genet **13**:137-144

Kozak M (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation. J Biol Chem **266**:19867-19870

Kusters-van Someren MA, Harmsen JAM, Kester HCM, Visser J (1991) Structure of the *Aspergillus niger pel*A gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. Curr Genet **20**:293-299

Kusters-van Someren M, Flipphi M, Graaff L de, Broeck H van den, Kester H, Hinnen A, Visser J (1992) Characterization of the *Aspergillus niger pel*B gene: structure and regulation of expression. Mol Gen Genet **234**:113-120

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685

Matsudaira P (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem **262**:10035-10038

McCleary BV (1991) Comparison of endolytic hydrolases that depolymerize 1,4-β-D-mannan, 1,5-α-L-arabinan, and 1,4-β-D-galactan. In: Leatham GF, Himmel ME (eds) Enzymes in biomass conversion. ACS Symp Ser **460**. American Chemical Society, Washington DC. pp.437-449

McLauchlan J, Gaffney D, Whitton JL, Clements JB (1985) The consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for efficient formation of mRNA 3′ termini. Nucleic Acids Res **13**:1347-1368

Nussinov R (1990) Sequence signals in eukaryotic upstream regions. CRC Crit Rev Biochem Mol Biol **25**:185-224

Proudfoot N (1991) Poly(A) signals. Cell **64**:671-674

Rambosek J, Leach J (1987) Recombinant DNA in filamentous fungi: progress and prospects. CRC Crit Rev Biotechnol **6**:357-393

Rombouts FM, Voragen AGJ, Searle-van Leeuwen MF, Geraerds CCJM, Schols HA, Pilnik W (1988) The arabinanases of *Aspergillus niger* – purification and characterisation of two α-L-arabinofuranosidases and an *endo*-1,5-α-L-arabinanase. Carbohydr Polym **9**:25-47

Sakai T, Sakamoto T (1990) Purification and some properties of a protopectin-solubilizing enzyme that has potent activity on sugar beet protopectin. Agric Biol Chem **54**:879-889

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74**:5463-5467

Steers E Jr, Craven GR, Anfinsen CB, Bethune JL (1965) Evidence for nonidentical chains in the β-galactosidase of *Escherichia coli* K12. J Biol Chem **240**:2478-2484

Tanaka M, Abe A, Uchida T (1981) Substrate specificity of α-L-arabinofuranosidase from plant *Scopolia japonica* calluses and a suggestion with reference to the structure of beet araban. Biochim Biophys Acta **658**:377-386

Unkles SE (1992) Gene organization in industrial filamentous fungi. In: Kinghorn JR, Turner G (eds) Applied molecular genetics of filamentous fungi. 1st edn. Blackie Academic & Professional, London. pp.28-53

Utt EA, Eddy CK, Keshav KF, Ingram LO (1991) Sequencing and expression of the *Butyrivibrio fibrisolvens xylB* gene encoding a novel bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities. Appl Environ Microbiol **57**:1227-1234

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1991) Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. Arch Microbiol **157**:23-28

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1993) Induction of extracellular arabinases on monomeric substrates in *Aspergillus niger*. Arch Microbiol **159**:66-71

Voragen AGJ, Rombouts FM, Searle-van Leeuwen MF, Schols HA, Pilnik W (1987) The degradation of arabinans by endo-arabinanase and arabinofuranosidases purified from *Aspergillus niger*. Food Hydrocoll 1:423-437

Weinstein L, Albersheim P (1979) Structure of plant cell walls. IX. Purification and partial characterization of a wall-degrading endo-arabanase and an arabinosidase from *Bacilllus subtilis*. Plant Physiol **63**:425-432

Whitaker JR (1984) Pectic substances, pectic enzymes and haze formation in fruit juices. Enzyme Microb Technol **6**:341-349

Wilson LJ, Carmona CL, Ward M (1988) Sequence of the *Aspergillus niger pyrG* gene. Nucleic Acids Res **16**:2339

Witteveen CFB, Busink R, Vondervoort P van de, Dijkema C, Swart K, Visser J (1989) L-Arabinose and D-xylose catabolism in *Aspergillus niger*. J Gen Microbiol **135**:2163-2171

Yoshihara O, Kaji A (1983) An endo-1,5-α-L-arabinase, which can disintegrate potato tissue. Agric Biol Chem **47**:1935-1940

Chapter 4

Cloning and characterization of the *abf*B gene coding for the major α-L-arabinofuranosidase (ABF B) of *Aspergillus niger* [1]

Based on amino acid sequence data from *Aspergillus niger* α-L-arabinofuranosidase B (ABF B) and cyanogen bromide fragments derived thereof, deoxyoligonucleotide mixtures were designed to be employed as primers in polymerase chain reaction (PCR) on *A. niger* genomic DNA. This resulted in amplification of three related PCR products. The *abf*B gene encoding ABF B was isolated from a genomic library using such an amplification product as a probe. A 5.1 kb *Bam*HI fragment was subcloned to result in plasmid pIM991. Upon introduction by co-transformation into both *A. niger* and *A. nidulans* uridine auxotrophic strains, pIM991 was shown to contain the functional gene as prototrophic transformants overproduced ABF B upon growth on the inducing carbon source sugar beet pulp. A plate assay was developed enabling quick selection of ABF B-overproducing transformants. The sequence of a 4122 bp long *Bam*HI / *Sst*I fragment was determined. The *abf*B gene does not contain introns and codes for a protein of 499 amino acids. The mature ABF B, 481 amino acids in lenght, has a deduced molecular weight of 50.7 kDa. *A. niger abf*B is the first eukaryotic gene encoding an ABF to be characterized.

Introduction

α-L-Arabinofuranosidase (ABF) (EC 3.2.1.55) is an exo-type hydrolase which liberates L-arabinose from oligo- or polysaccharides containing α-linked L-arabinofuranosyl residues at non-reducing ends. This activity is known to be produced, extracellularly in most cases, by plants, bacteria and fungi as reviewed by Dekker and Richards (1976), Kaji (1984) and Whitaker (1984). Degradation of branched L-arabinan (from sugar beet) by the saprophytic hyphal fungus *Aspergillus niger* involves two distinct ABFs, ABF A (83 kDa) and ABF B (67 kDa), and an endo-1,5-α-L-arabinase (ABN A) (Voragen *et al.* 1987; Rombouts *et al.* 1988; van der Veen *et al.* 1991). Though both active on the synthetic substrate *para*-nitrophenyl α-L-arabinofuranoside (PNP-A), the ABFs differ in

[1] a shortened version of this Chapter is published in: Curr Genet **24**:525-532 (1993). The nucleotide sequence data reported in this Chapter are accessable from the EMBL and GenBank Nucleotide Sequence Databases under accession numbers X74777 and L23502.

specificity towards natural substrates. ABF A only acts on small linear 1,5-α-linked L-arabinofuranosyl oligosaccharides while ABF B is less specific. It is able to hydrolyze 1,5-, 1,3- and 1,2-α-linkages not only in L-arabinofuranosyl oligosaccharides, but also in polysaccharides containing terminal non-reducing L-arabinofuranoses in side chains, like L-arabinan, arabinogalactan and arabinoxylan.

A. niger ABF B was first described by Kaji *et al.* (1963) when reporting on the production of extracellular ABF activity upon growth on wheat bran as the carbon source. In several papers Kaji, Tagawa and coworkers described the induction and purification of this enzyme, finally resulting in its general kinetic and physicochemical characterization (*cf.* Kaji 1984; Tagawa and Kaji 1988). Several other researchers have purified ABF B and investigated its properties or used it in various applications (Neukom *et al.* 1967; Gremli and Neukom 1968; Neukom and Markwalder 1975; Andrewartha *et al.* 1979; Gunata *et al.* 1990; Will and Dietrich 1992; Beldman *et al.* 1993; Lerouge *et al.* 1993a, b). Only recently, Voragen *et al.* (1987) reported the isolation of a second ABF isozyme from *A. niger*, ABF A.

Cloning of the structural arabinase genes would enable study of L-arabinan degradation by *A. niger* at the molecular level. Recently the genes coding for ABF A and ABN A were cloned (Chapters 2 & 3; Flipphi *et al.* 1993a, b). In this Chapter, the cloning and expression of the third gene involved, the *abf*B gene encoding ABF B, is described and its derived structure is presented.

Materials and methods

Fungal and bacterial strains, and plasmids used. *A. niger* N593 (*cspA1, pyrA6*) and *A. nidulans* G191 (*pabaA1, pyrG89, fwA1, uAY9*) were previously described (Goosen *et al.* 1987; Ballance and Turner 1985); *A. nidulans* G191 was kindly provided by Dr. D.J. Ballance. *A. niger* N402 (*cspA1*) originates from the wild-type strain N400 (CBS 120.49) and is a morphological mutant with short conidiophores. *A. nidulans* WG096 (*pabaA1, yA2*) is a standard strain originating from the Glasgow collection (FGSC 187). Media and growth conditions used in the cultivation of *Aspergillus* strains were essentially as described in Chapter 2 (Flipphi *et al.* 1993b), using 6 g/l $NaNO_3$ instead of NH_4Cl as the nitrogen source. *A. niger* was grown at 30°C and *A. nidulans* at 37°C. *Escherichia coli* strains JM101 and DH5α were supplied by Stratagene. Plasmid vectors pEMBL (Dente and Cortese 1987) and pGEM (Promega) were used in DNA subcloning. Plasmid pGW635 contains the *A. niger pyr*A gene encoding orotidine-5'-phosphate decarboxylase (Goosen *et al.* 1989).

Materials for growth and enzyme assays. Fungal growth restrictor dichloran (2,6-dichloro-4-nitro-aniline) was obtained from Merck. Grünband apple pectin DE 61.2 was obtained from ObiPectin (Bischofszell, Switzerland). L-Arabitol and the ABF-substrate compounds PNP-A and 4-methylumbelliferyl α-L-arabinofuranoside (4MU-A) were supplied by Sigma. Sugar beet pulp, a gift from CSM (Breda, The Netherlands), was ground in a Waring blender prior to use.

Cyanogen bromide cleavage and amino acid sequence determination. ABF B was cleaved with cyanogen bromide (CNBr) according to the protocol of Steers *et al.* (1965) as decribed in Chapter 3 (Flipphi *et al* 1993a). The generated peptides were separated with SDS-PAGE using a 15 % polyacrylamide gel containing 0.1 % SDS (Laemmli 1970), together with appropriate molecular weight markers, and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore) (Matsudaira 1987). Upon staining with Coomassie brilliant blue R250, the pieces of membrane containing ABF B-derived peptides were recovered. Amino acid sequences were determined using a gas phase sequenator equipped with a PTH analyzer as described by Amons (1987), and shown in Table 1 (top half).

Design of deoxyoligonucleotide mixtures. Deoxyoligonucleotide mixture [a] contains all individual oligonucleotides which in all degenerations code for peptide [A]; mixtures [b] and [c] contain all individual oligonucleotides which complement in all degenerations the sequences that code for peptides [B] and [C], respectively. To reduce the number of components within these mixtures, deoxyinosine was introduced at those positions which correspond with or complement the third base of four-fold degenerated codons (Ohtsuka *et al.* 1985). Both mixtures [b] and [c] had to be synthesized in two different reactions due to the presence of a six-fold degenerated codon (leucine and serine, respectively). The sequences of the synthesized mixtures are shown in Table 1 (bottom half).

Polymerase chain reaction (PCR). The concentration of deoxyoligonucleotide solutions was deduced from their absorbance at 260 nm. Mixtures [b1] and [b2], as well as [c1] and [c2], were mixed to result in equal concentrations for each single component within the final mixtures [b] and [c]. A PCR sample contained 100 pmoles of each of the deoxyoligonucleotide mixtures which serve as primers, buffer solution (10 mM TRIS/HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 100 μg/ml gelatine), dNTPs (Boehringer Mannheim) to a final concentration of 200 μM, 2.5 U *Taq* DNA polymerase (Gibco-BRL) and template DNA in a final volume of 100 μl. Amplifications were done using the ATAQ Controller from Pharmacia LKB, executing 25 amplification cycles following the sequence: 1 min 94°C; 1 min 48°C; 2 min 72°C. Prior to amplification the samples were incubated for 3 min at 94°C, and the reaction was terminated after a final 5 min incubation at 72°C following amplification. The reaction mixtures were subsequently analyzed by electrophoresis in a 2 % agarose gel containing 25 μg/l ethidium bromide together with appropriate molecular length markers. As template for amplification either 1 μg *A. niger* N402 high-molecular weight genomic DNA or PCR-amplified material was used. *Aspergillus* genomic DNA was isolated as described by de Graaff *et al.* (1988).

Screening of an *A. niger* genomic library. An *A. niger* N400 genomic library in λEMBL4 (Harmsen *et al.* 1990) was screened by plaque filter hybridization using nitrocellulose replicas, performed as described previously (Chapter 2; Flipphi *et al.* 1993b). The filters were washed at 68°C, using 0.2 x SSC/0.1 % SDS as the final washing buffer. Positive plaques, identified on duplicate replicas after autoradiography, were recovered from the original plates and purified by rescreening at low plaque density.

General DNA manipulations. General DNA manipulations were performed essentially as described by Sambrook *et al.* (1989).

Aspergillus transformation. Transformation was carried out according to the protocol of Kusters-van Someren *et al.* (1991) using *A. niger* and *A. nidulans* uridine auxotrophic mutants N593 and G191 as recipients. pIM991 was added in a 20-fold excess over pGW635 to obtain high co-transformation frequencies. Six transformants resulting from the *A. niger* transformation and six from the *A. nidulans* transformation were randomly selected and purified, to be analyzed for expression of the *abf*B gene introduced.

Isolation of ABF B-overproducing *A. niger abf*B transformants. The screening plates were prepared as follows: medium consisting of 9 g/l KH_2PO_4, 2 g/l yeast extract, 2 g/l casein amino acids, 8 g/l NH_4Cl, 1 g/l KCl, 1 g/l $MgSO_4$, 0.08 ml/l of trace element solution according to Vishniac and Santer (1957) and 26 g/l agar, and a solution of 1 % (w/v) apple pectin were sterilised separately. The initial pH of both components was set at 5.0. Medium and carbon source solution were mixed in equal volumes and fungal growth restrictor dichloran was added to a final concentration of 20 μg/ml (King *et al.* 1979; Lamsa and Bloebaum 1990). For screening plates the fluorogenic ABF substrate 4MU-A was added to a final concentration of 1mM before pouring; master plates did not contain 4MU-A. Sixty additional *A. niger* transformants as well as the six previously selected transformants were transferred in duplicate to screening plates and master plates; *A. niger* N402 was included in ten-fold on each plate to serve as a wild-type control. Plates were incubated at 30°C. The screening plates were regularly checked for 4MU-A hydrolysis simply by monitoring the formation of fluorescent halos around fungal colonies while the plates were subjected to UV transillumination. The master plates were grown for 4 days, and mycelium of four selected ABF B overproducers was transferred to minimal medium plates without uridine. Upon sporulation, the transformants were purified over minimal medium plates without uridine.

Analysis of *Aspergillus abf*B transformants. ABF activity was assayed by PNP-A hydrolysis and calculated accordingly, as described by van der Veen *et al.* (1991). Activities are given in units (U) – the amount of enzyme releasing 1 μmol *para*-nitrophenol per minute. The 12 randomly-selected transformants were also studied by SDS-PAGE of medium samples using 10 % polyacrylamide gels containing 0.1 % SDS according to Laemmli (1970); purified ABF B and Protein test mixture 4 (Serva) were used as markers. The gels were either stained directly using Coomassie brilliant blue R250, or the separated proteins were electroblotted onto nitrocellulose. Western blots were probed with anti-ABF B antiserum (van der Veen *et al.* 1991) and ABF B was visualised with an alkaline phosphatase assay, using the Immun-Blot GAM-AP assay kit from Bio-Rad and following the supplier's instructions.

DNA sequencing and sequence analysis. DNA fragments were cloned both in plasmid vectors (pEMBL, pGEM) and in M13 mp18/19. DNA sequences were determined with the dideoxy-nucleotide chain-termination method (Sanger *et al.* 1977) using the ^{T7}Sequencing Kit from Pharmacia LKB according to the supplier's instructions. Single-stranded M13 DNA as well as alkali-denatured plasmid DNA were used as template, and general sequencing primers as well as gene-specific oligonucleotides were employed as primers. Computer analysis was done using the PC/GENE program (IntelliGenetics) and the sequence analysis software package of the Genetics Computer Group.

Determination of the transcription initiation sites. L-Arabitol-induced mycelium of *A. niger abf*B transformant N593::pIM991-112 was generated by performing a medium-transfer experiment, essentially as described in Chapter 2 (Flipphi *et al.* 1993b). Six hours after transfer the mycelium was harvested. PolyA$^+$ RNA was isolated as previously described (Chapter 2; Flipphi *et al.* 1993b). Approximately 5 μg polyA$^+$ RNA was used in primer-extension mapping of the transcription initiation sites of the *abf*B gene, according the method described by Calzone *et al.* (1987). The 18-mer oligonucleotide which served as primer (5' [TGCTGTGCG CGGCTACGC] 3') is complementary to nucleotides 92 – 109 of the determined *abf*B sequence (see Fig.4). The positions of transcription initiation sites were deduced from an autoradiographed polyacrylamide sequencing gel enabling determination of the length of primer-extension reaction products, using dideoxy-terminated sequencing reaction products as markers.

Table 1. Amino acid sequences determined from *A. niger* ABF B and the nucleotide sequences from the synthesized deoxyoligonucleotide mixtures.

	Amino acid sequence [a]	**residue position** [b]	**Origin**
[1]	(D)P X D I Y E A G D T P	1 – 12	ABF B
[2]	G P X D I Y E A G D T P X V A A	1 – 16	CNBr 29 kDa
[3]	E N N L F S(G)A D E(G)Y N S	203 – 216	CNBr 20 kDa
[4]	S K E G A I I L G I G G D N S N G A Q G	267 – 286	CNBr 15 kDa
[5]	T S G Y P S D D V E N(S)V	294 – 306	CNBr 23.5 kDa

Deoxyoligonucleotide mixtures [cd]

[a]	5′ G A Y A T H T A Y G A R G C I G G I G A Y A C I C C 3′	(26 mer; 48 components)
[b1]	5′ C C I C C D A T I C C I A G D A T D A T I G C I C C Y T C Y T T 3′	(32 mer; 108 components)
[b2]	5′ C C I C C D A T I C C Y A A D A T D A T I G C I C C Y T C Y T T 3′	(32 mer; 216 components)
[c1]	5′ T T Y T C I A C R T C R T C I G A I G G R T A I C C 3′	(26 mer; 16 components)
[c2]	5′ T T Y T C I A C R T C R T C R C T I G G R T A I C C 3′	(26 mer; 32 components)

[a] Residues which could not be assigned are denoted "**X**", ambiguous residues are between brackets.
[b] The positions of the determined sequences refer to their respective positions found in the deduced ABF B amino acid sequence (*cf.* Fig.4).
[c] D = A, G or T; H = A,C or T; I = deoxyinosine; R = A or G; Y = C or T; according to the IUPAC-IUB code.
[d] Underlined bases mark the differences between mixtures [b1] and [b2], and mixtures [c1] and [c2], respectively.

Results

Isolation of a probe specific for the ABF B-encoding gene

In order to generate a probe enabling cloning of the encoding gene, the N-terminal amino acid sequence of the previously purified ABF B protein (van der Veen *et al.* 1991) was determined, as shown in Table 1 (top half). Within this sequence the first residue was ambiguous while the third was not assignable. To obtain more amino acid sequence data, ABF B was subjected to cyanogen bromide (CNBr) cleavage and the N-termini of several of the resulting peptides were sequenced (Table 1, top half). The N-terminus of a 29 kDa peptide turned out to be identical to the N-terminus from intact ABF B, and from this the N-terminal residue could be assigned as glycine. However, two unassignable residues remain at positions 3 and 13.

Based on the amino acid sequence data, three sets of deoxyoligonucleotide mixtures were designed by reversed translation of the peptide sequences [A]: [DIYEAGDTP], derived from the ABF B N-terminal sequence; [B]: [KEGAIILGIGG], derived from the internal 15 kDa peptide sequence; and [C]: [GYPSDDVEN], derived from the internal 23.5 kDa peptide sequence (Table 1, bottom half). The individual mixtures were first directly used as hybridization probes (*e.g.* Ohtsuka *et al.* 1985), but this strategy did not result in cloning of the ABF B-encoding gene.

Because mixture [a] was designed as a coding sequence, and mixtures [b] and [c] as complementary to the coding sequence, the deoxyoligonucleotide mixtures could also be utilized as primers in polymerase chain reaction (PCR). When PCR was performed using *A. niger* genomic DNA as a template and mixtures [a] and [b] as primers, using an annealing temperature of 48°C, a discrete product of approximately 820 bp was found upon analysis of the reaction sample (data not shown). Under the same conditions with mixtures [a] and [c] as primers, PCR resulted in the amplification of two discrete products approximately 310 and 900 bp in length (data not shown). Control experiments using either single mixture as primer did not result in amplification of DNA. Further experiments showed that both the 820 bp fragment and the 310 bp fragment could be amplified using the 900 bp PCR product as the template, using the distinguished pairs of oligonucleotide mixtures as primers (data not shown). These results strongly suggest that all three DNA fragments amplified are specific for the same genomic locus in *A. niger* N402 and that either of these PCR products could be used as a probe to screen for the ABF B-encoding gene.

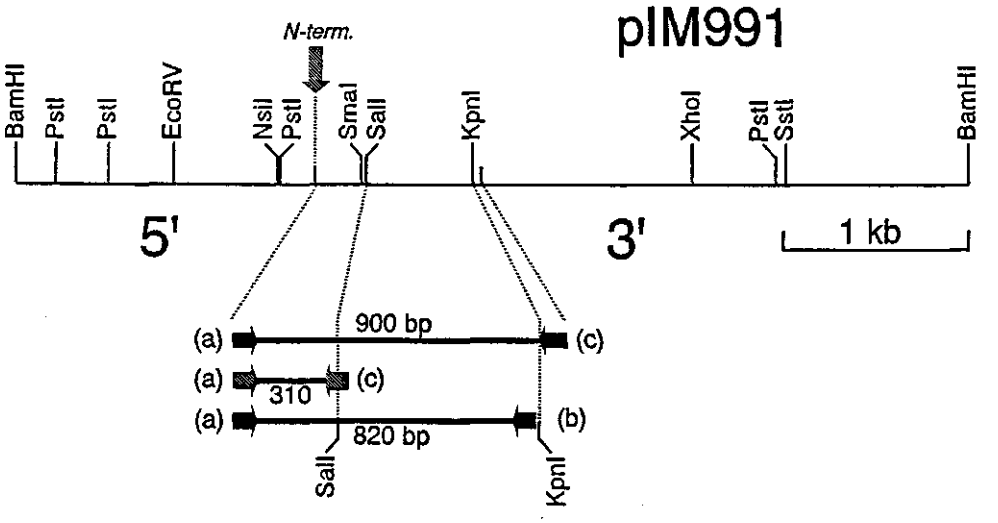


Fig.1. Restriction map of 5.1 kb *Bam*HI insert from pIM991. The approximate position corresponding to the N-terminus of ABF B, as well as the orientation of the *abf*B gene are indicated. The PCR amplification products are displayed underneath the restriction map; the determined lengths are indicated. The arrows symbolizing the deoxyoligonucleotide mixtures used as PCR primers (Table 1) are not to scale.

Cloning of the *abf*B gene

The 820 bp PCR product was used to probe an *A. niger* N400 genomic library. Screening of 1 x 10^5 plaque-forming units (pfu) resulted in 28 positive λ-clones. Five of these phages were purified by two cycles of rescreening at low density and subsequently phage DNA was isolated. The phages were characterized by restriction analysis using a limited number of restriction enzymes. Upon Southern blotting and hybridization using the 820 bp PCR product as a probe, a hybridizing 5.1 kb *Bam*HI fragment was found in all five λ-clones. This fragment was isolated from λ5 and cloned in vector pEMBL19 resulting in plasmid pIM991. A detailed restriction map of the *Bam*HI insert was made which is shown in Fig. 1. The orientation of the cloned *abf*B gene could be determined after restriction analysis of the 820 and 900 bp PCR amplification products (data not shown). Both were cut by *Sal*I, resulting in a fragment of approximately 280 bp and a distinctive fragment of respectively 540 bp and 620 bp in length. *Kpn*I-digestion of the 900 bp PCR product resulted in a fragment approximately 840 bp in length. *Pst*I did not cut the PCR amplification products. From these data the orientation of the gene was deduced and the approximate position of the sequence encoding the N-terminus of ABF B was mapped as drawn in Fig.1.

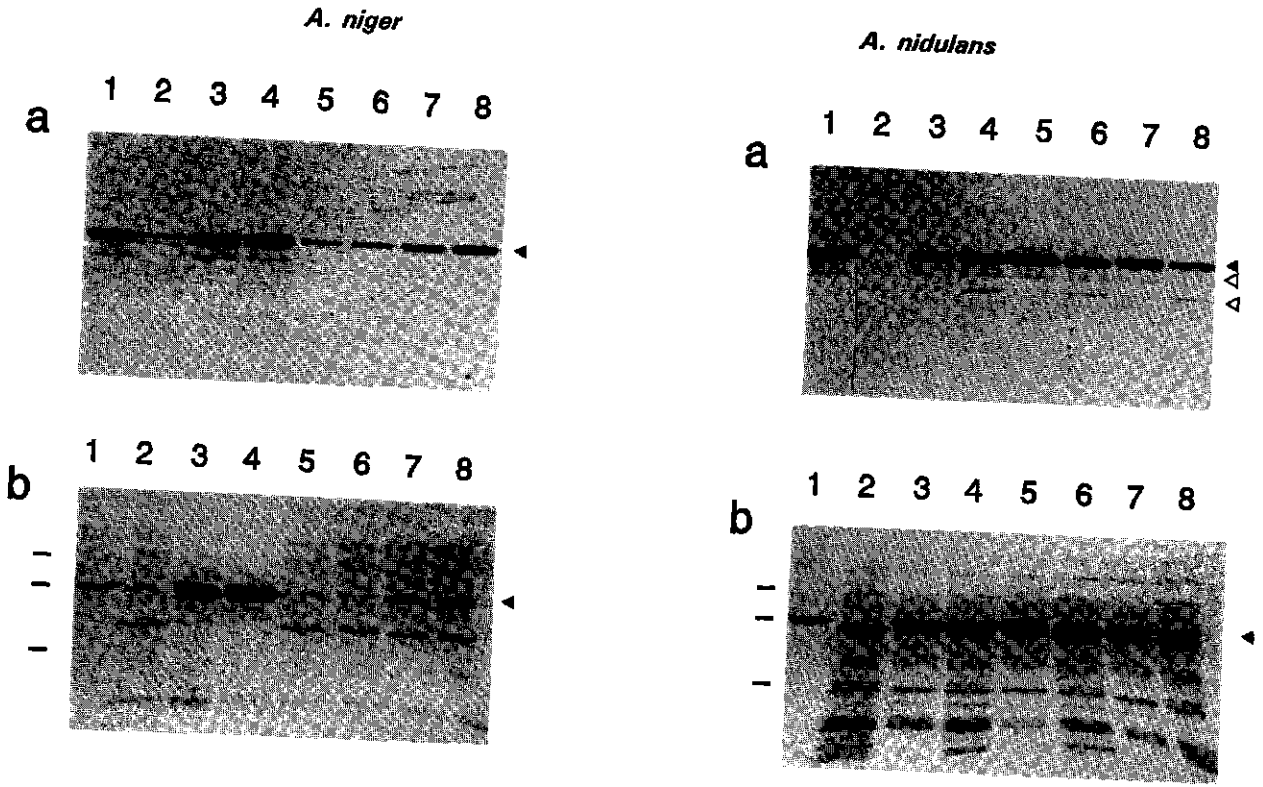


Fig.2 (left). Analysis of ABF B expression by *A. niger abf*B transformants and *A. niger* wild-type N402 upon 36 h of growth on minimal medium containing sugar beet pulp as the carbon source. **a**: Immunochemical detection upon SDS-PAGE and western blotting; 15 μl of medium was loaded per lane; **b**: Coomassie brilliant blue R250-stained gel upon SDS-PAGE; 3.25 μl of medium was loaded per lane. The position of ABF B is marked (◂) in both panels. Indicated molecular weight markers (left): ovalbumin, 45 kDa; bovine serum albumin, 67 kDa; phosphorylase B, 92.5 kDa. **Lane 1**: 1 μg purified ABF B; **2**: culture medium N402; **3–8**: culture medium *abf*B transformants N593::pIM991-4 / -10 / -11 / -13 / -17 / -18, respectively.

Fig.3 (right) Analysis of ABF B expression by *A. nidulans abf*B transformants and *A. nidulans* wild-type WG096 upon 36 h of growth on minimal medium containing sugar beet pulp as the carbon source. **a**: Immunochemical detection upon SDS-PAGE and western blotting; 15 μl of medium was loaded per lane; **b**: Coomassie brilliant blue R250-stained gel upon SDS-PAGE; 3.25 μl of medium was loaded per lane. The position of ABF B is indicated (◂); the positions of the endogenous ABFs are marked (◁). Indicated molecular weight markers (left): ovalbumin, 45 kDa; bovine serum albumin, 67 kDa; phosphorylase B, 92.5 kDa. **Lane 1**: 1 μg purified ABF B; **2**: culture medium WG096; **3–8**: culture medium *abf*B transformants G191::pIM991-2 / -4 / -6 / -7 / -10 / -12, respectively.

Strain	Activity after 24 h	Activity after 36 h	Strain	Activity after 24 h	Activity after 36 h
N402	0.33	0.34	WG096	0.07	0.06
N593::pIM991-4	4.79	5.78	G191::pIM991-2	1.53	2.49
N593::pIM991-10	4.45	6.05	G191::pIM991-4	3.13	4.59
N593::pIM991-11	0.34	0.35	G191::pIM991-6	2.28	3.46
N593::pIM991-13	0.32	0.34	G191::pIM991-7	2.68	3.00
N593::pIM991-17	0.54	0.55	G191::pIM991-10	1.66	2.55
N593::pIM991-18	1.26	1.25	G191::pIM991-12	0.84	0.92
N402 [a]	0.43	0.49			
N593::pIM991-4 [a]	4.86	5.95			
N593::pIM991-101	4.24	5.14			
N593::pIM991-112	4.84	5.58			
N593::pIM991-121	3.09	3.38			
N593::pIM991-128	3.43	4.01			

Table 2 (left). PNP-A-hydrolyzing activity in culture medium of *A. niger* wild-type and *abf*B transformants after 24 h and 36 h of growth on sugar beet pulp as the carbon source. Activity is given in Units/ml culture medium, and determined as described in Materials and Methods incubating for 10 min, using either 10 μl undiluted filtrated culture fluid, or 10 μl of a 5 times or 20 times dilution in McIlvaine buffer pH 3.5.

[a] The data given for randomly picked transformants and 4MU-A selected transformants originate from independent experiments; N402 and N593::pIM991-4 are used as references for the 4MU-A selected transformants.

Table 3 (right). PNP-A-hydrolyzing activity in culture medium of *A. nidulans* wild-type and *abf*B transformants after 24 h and 36 h of growth on sugar beet pulp as the carbon source. Activity is given in Units/ml culture medium, and determined as described in Materials and Methods incubating for 10 min, using either 10 μl undiluted filtrated culture fluid, or 10 μl of a 5 times or 20 times dilution in McIlvaine buffer pH 3.5.

Expression of the *abf*B gene in *A. niger* and *A. nidulans*

To probe the functionality of the cloned gene, pIM991 was introduced in uridine auxotrophic mutants of both *A. niger* and *A. nidulans* by co-transformation with pGW635 using the *A. niger pyr*A gene as the primary selection marker. Six *A. niger* and six *A. nidulans* prototrophic transformants were randomly chosen to be analyzed for expression of ABF B. Additionally, sixty other *A. niger* transformants were screened on plates for ABF overexpression utilizing the ABF B-inducing capacity of apple pectin (van der Veen *et al.* 1991) and the ABF-substrate compound 4MU-A. Hydrolysis of 4MU-glycosides results in the release of the fluorophore 4-methylumbelliferone allowing direct selection of overproducing transformants (see Sewell *et al.* 1989; Schwarz *et al.* 1990). Upon UV transillumination, distinctive fluorescent halos formed around the fungal colonies were easily detectable at 18 h after inoculation (results not shown). The four transformants showing the most massive halos were isolated and purified to be analyzed for production of PNP-A-hydrolyzing activity.

Southern blot analysis of *Xho*I-cut genomic *Aspergillus* DNA using the 820 bp PCR product as the probe, indicated that all these transformants except *A. niger* N593::pIM991-11 and -13 contain additional copies of the *abf*B gene (data not shown). Expression was studied upon submerged growth of *abf*B transformants and *Aspergillus* wild-type strains (*A. niger* N402 and *A. nidulans* WG096) using sugar beet pulp as the sole carbon source and $NaNO_3$ as the nitrogen source, essentially as described in Chapter 2 (Flipphi *et al.* 1993b). Samples of the growth media were taken at 24 h, 36 h and 48 h after inoculation.

As shown in Fig.2a, after 36 h of growth four of the randomly selected *A. niger* transformants (N593::pIM991-4; -10; -17; -18) produced higher amounts of the main immunoreactive extracellular protein as compared to *A. niger* N402, whereas the two transformants without additional copies (nos. -11 & -13) produced this protein in the same amount as N402 (lane 2). This protein has the same apparent molecular weight as purified *A. niger* ABF B (lane 1). Fig.2b provides a Coomassie-stained control for the western blot, showing that the transformants producing the highest amounts of the immunoreactive protein (nos. -4 & -10) differ from the other strains in the overall pattern of extracellular protein produced. The amount of immunoreactive protein produced by individual transformants correlates with the PNP-A-hydrolyzing activity found in the culture media (Table 2; top half). The four transformants selected by 4MU-A plate screening, all secreted over six times more PNP-A-hydrolyzing activity than wild-type strain N402 (Table 2; bottom half).

After 36 h of growth, all *A. nidulans* transformants analyzed secreted one main protein which was recognized by anti-ABF B antiserum (Fig.3a) and has the same apparent molecular weight as purified *A. niger* ABF B (lane 1). Fig.3b shows the Coomassie-stained control for the western blot. The immunoreactive protein was produced in various, but vast amounts by the individual transformants, but was not found in *A. nidulans* wild-type strain WG096 (lane 2).

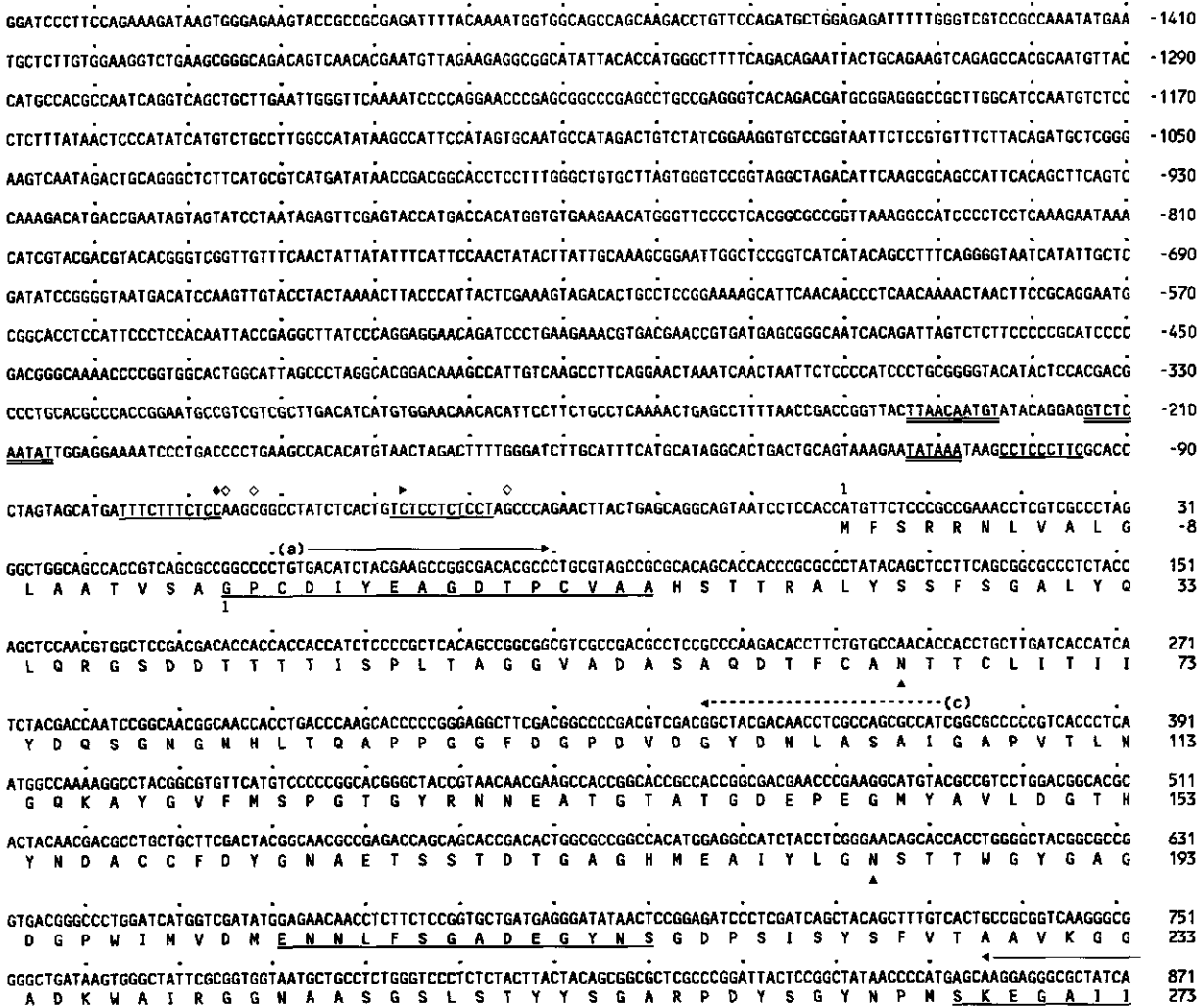
The differences in overexpression of the heterologous ABF B are also reflected by the PNP-A-hydrolyzing activity produced by the various transformants as compared to wild-type WG096 (Table 3). Two less-abundant immunoreactive proteins with a lower apparent molecular weight than *A. niger* ABF B were produced by the transformants as well as by wild-type *A. nidulans* (Fig.3a). These proteins could represent endogenous ABFs from *A. nidulans* although wild-type WG096 produced only residual ABF activity under the growth conditions employed (Table 3).

Primary structure of the *abf*B gene and its deduced protein

The nucleotide sequence of the 4122 bp *Bam*HI / *Sst*I fragment of pIM991 (Fig.1) was determined over both strands and is shown in Fig.4. In addition, three cDNA clones were isolated from a cDNA expression library previously used to isolate cDNA clones specific for the ABN A-encoding *abn*A gene (Chapter 3; Flipphi *et al.* 1993a), using the 5'-specific 280 bp *Sal*I fragment of the 820 bp PCR amplification product as the probe. Upon terminal sequence analysis of the cDNA inserts, these clones were found to be identical (data not shown).

The determined sequence contains a large open reading frame of 1500 bp ranging from the start codon at position 1 to a TAA stop codon 1498 bp downstream, which codes for a protein of 499 amino acids. The *abf*B gene does not contain introns, as was confirmed by sequence analysis of one of the isolated cDNAs. Within the deduced amino acid sequence of the primary gene product all four determined amino acid sequences (Table 1) were present, as displayed in Fig.4. The determined N-terminal residue of ABF B is preceded by a sequence of 18, mainly hydrophobic residues. Signal sequence processing between residues −1 and +1 implicates the presence of small, neutral residues at position −3 and −1, as predicted by von Heijne (1986). Removal of the proposed signal sequence leaves a mature protein of 481 amino acid residues, which has a deduced molecular weight of 50.7 kDa and a theoretical isoelectric point of 3.8.

Upon primer-extension mapping (results not shown) using L-arabitol-induced polyA$^+$ RNA from *A. niger abf*B transformant N593::pIM991-112 as template, one major transcription initiation site at position −67 upstream of the startcodon and three additional weak sites at −66, −63 and −36 could be identified, as shown in Fig.4. The ultimate 3' base of the cDNAs was situated at position 1632, implying that the *abf*B transcript contains a 132 bp-long 3' non-coding sequence. The structural sequence predicts PCR amplification products of 824 bp and 902 bp for PCR using the distinctive pairs of deoxyoligonucleotide mixtures ([a] and [b], respectively [a] and [c]) as primers, confirming the results reported above. A second sequence is present to which deoxyoligonucleotide mixture [c] could hybridize, leading to an illegitimate product of 308 bp in PCR amplification with mixtures [a] and [c] corresponding with the 310 bp product actually found.



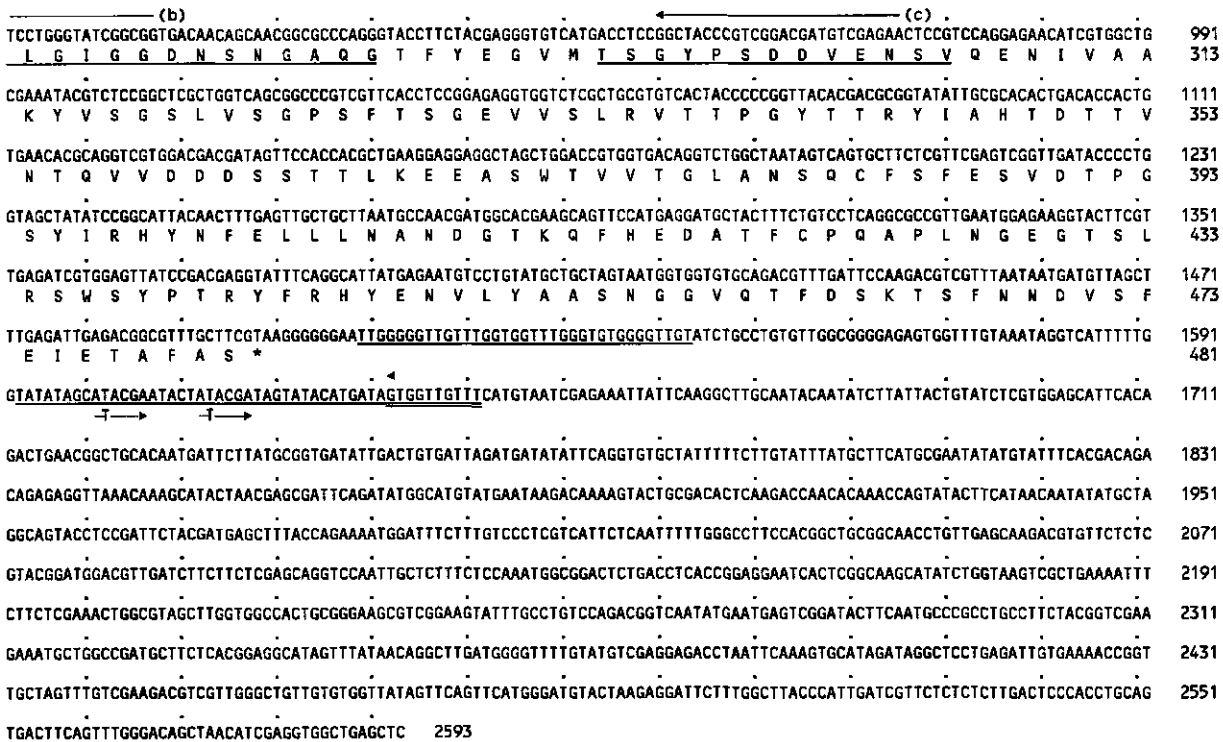


Fig.4. The nucleotide sequence of the *A. niger abf*B gene and the deduced amino acid sequence of the gene product ABF B. The nucleotide sequence is numbered relative to the first base of the deduced startcodon, the amino acid sequence is numbered relative to the N-terminal glycine residue of mature ABF B. The major transcription initiation site is marked (♦), the weak sites (◇). The 5′ ultimate base of the cDNA (position −47) is marked (▸), the 3′ ultimate base is marked (◂). Putative promoter elements in the upstream non-coding sequence thought to effect transcription initiation are underlined (CT-stretches) or double underlined (CAAT / TATA boxes). The sequences homologous to the deoxyoligonucleotide mixtures are indicated by arrows above the sequence; the sequence leading to the illegitimate amplification product by a dashed arrow. Elements found in the 3′ non-coding sequence are underlined or double underlined; the direct repeat is indicated by arrows underneath the sequence and marked **T**. Determined amino acid sequences are underlined. The two putative *N*-glycosylation sites (residues 65 and 184) are indicated (▲).

Discussion

After cloning the structural genes for ABF A and ABN A (Chapters 2 & 3; Flipphi *et al.* 1993a, b,) cloning the *A. niger abf*B gene encoding the third enzyme involved in L-arabinan degradation, ABF B, has been accomplished. The gene, resident on pIM991, is functional in both *A. niger* and the heterologous host *A. nidulans*. High-level overproducing *A. niger* transformants have been selected utilizing a simple plate screening based on hydrolysis of the fluorogenic ABF substrate 4MU-A. In contrast to what was found previously in case of the *A. niger abn*A gene, where the higher levels of expression were found in *A. nidulans* transformants (Chapter 3; Flipphi *et al.* 1993a), for *abf*B there are no significant differences in expression levels found between high-level producing *A. niger* and *A. nidulans* transformants (Tables 2 & 3). Fig.2b shows that the high-level overproducing *A. niger abf*B transformants do not produce the normal spectrum of extracellular protein found in the wild-type N402, which might be indicative for a possible malfunction in protein synthesis or in secretion.

Analysis of the 5' non-coding area of the determined *abf*B sequence reveals the presence of sequence elements resembling functional general promoter elements as they are described for fungal genes (Gurr *et al.* 1987; Unkles 1992) – a TATAAA element, two CAAT boxes and three CT-stretches (Fig.4). The central CT-stretch is located directly in front of the major transcription initiation site and the third in front the most proximal site found. The start codon is preceded by [CCACC], the preferred sequence found directly upstream functional startcodons in higher eukaryotes (Kozak 1991). The structure of the 3' non-coding sequence of the *abf*B transcript is quite remarkable as it consists of a large GT-rich region of 93 bp (84 % GT), including a 35 bp long GT-stretch, and an adjacent AT-rich region (72.5 % AT) of 39 bp. The latter region contains a direct repeat [ATACGA], respectively 31 bp and 20 bp upstream of the putative polyadenylation site. Directly downstream a 9 bp-long GT-stretch is found which could be involved in the termination of transcription (Proudfoot 1991).

A. niger ABF B is the first ABF active on branched L-arabinan and α-L-arabinofuranosyl oligomers for which the derived amino acid sequence is published. It does not show significant sequence similarities with *A. niger* ABN A nor with the published sequences of two prokaryotic arabinosidase-like enzymes – a β-D-xylosidase from *Butyrivibrio fibrisolvens* exhibiting strong PNP-A-hydrolyzing side-activity (Utt *et al.* 1991) and an ABF from *Pseudomonas fluorescens* subsp. *cellulosa* acting only on oat spelt xylan (Kellett *et al.* 1990). Comparison with protein sequences from the EMBL/GenBank databases did not result in the identification of common amino acid patterns within the ABF B primary structure.

Table 4. Comparison of the deduced amino acid composition of ABF B and the composition of *A. niger* K1 ABF as determined by Kaji and Tagawa (1970).

Amino acid	Deduced [a]	Determined [b]
N & D	63	60
Q & E	35	32
S	49	37
G	58	67
H	8	6
R	11	9
T	49	49
A	45	54
P	20	5
Y	29	27
V	27	23
M	7	5
C	8	17
I	17	14
L	23	28
F	19	19
W	5	22
K	8	7
Total	481	481

[a] Deduced from the *abf*B DNA sequence (Fig.4).

[b] Composition according to Kaji and Tagawa (1970) calculated on a molecular weight of 64 kDa, accounting for 79.23 % (w/w) of the ABF B apparent molecular weight. Quantities given are expressed to the nearest integer.

The deduced molecular weight of ABF B (50.7 kDa) is similar to the apparent molecular weight of 53 kDa reported by Kaji and Tagawa (1970) using gel filtration, but differs from those described more recently (60 kDa / 61 kDa / 67 kDa), where the molecular weight was determined either by gel filtration techniques or by SDS-PAGE (Voragen *et al.* 1987; Gunata *et al.* 1990; van der Veen *et al.* 1991). ABF B is known to be glycosylated – the amino acid content of the enzyme from *A. niger* strain K1 is reported to account for 79.23 % of the total molecular weight (Kaji and Tagawa 1970). If the percentage of non-amino acid substances are taken into account, one could then deduce a total molecular weight of 64 kDa for *A. niger* ABF B based on the sequence data. Determination of the primary sequence allows comparison with the amino acid composition determined by Kaji and Tagawa (1970), after recalculation to a molecular weight of 64 kDa accounting for 79.23 % of this molecular weight (Table 4). The major differences, in proline, tryptophan and cysteine contents, may be attributed to

errors in their determination. Furthermore, the prominent differences in serine and alanine contents might result from the procedure employed by Kaji and Tagawa. Upon deglycosylation under alkaline conditions and subsequent reduction during acid hydrolysis of the protein, those serine residues which carry *O*-linked sugars are converted into alanines (*cf.* Neustroev *et al.* 1993). This suggests that 9 to 12 serine residues within ABF B are glycosylated. The threonine contents, however, are identical, hence only serines are *O*-glycosylated. Notably, ABF B is not *N*-glycosylated (Flipphi *et al.* 1994; Chapter 5). The amino acid-contents comparison strongly suggests that the cloned *abf*B gene from *A. niger* CBS 120.49 codes for an enzyme corresponding to the ABF from *A. niger* K1 (Kaji and Tagawa 1970).

References

Amons R (1987) Vapor-phase modification of sulfhydryl groups in proteins. FEBS Lett **212**: 68-72

Andrewartha KA, Phillips DR, Stone BA (1979) Solution properties of wheat-flour arabinoxylans and enzymically modified arabinoxylans. Carbohydr Res **77**:191-204

Ballance DJ, Turner G (1985) Development of a high-frequency transforming vector for *Aspergillus nidulans*. Gene **36**:321-331

Beldman G, Searle-van Leeuwen MJF, Ruiter GA de, Siliha HA, Voragen AGJ (1993) Degradation of arabinans by arabinanases from *Aspergillus aculeatus* and *Aspergillus niger*. Carbohydr Polym **20**:159-168

Calzone FJ, Britten RJ, Davidson EH (1987) Mapping of gene transcripts by nuclease protection assays and cDNA primer extension. Methods Enzymol **152**:611-632

Dekker RFH, Richards GN (1976) Hemicellulases: their occurrence, purification, properties, and mode of action. Adv Carbohydr Chem Biochem **32**:277-352

Dente L, Cortese R (1987) pEMBL: a new family of single-stranded plasmids for sequencing DNA. Methods Enzymol **155**:111-119

Flipphi MJA, Panneman H, Veen P van der, Visser J, Graaff LH de (1993a) Molecular cloning, expression and structure of the endo-1,5-α-L-arabinase gene of *Aspergillus niger*. Appl Microbiol Biotechnol **40**:318-326

Flipphi MJA, Visser J, Veen P van der, Graaff LH de (1993b) Cloning of the *Aspergillus niger* gene encoding α-L-arabinofuranosidase A. Appl Microbiol Biotechnol **39**:335-340

Flipphi MJA, Visser J, Veen P van der, Graaff LH de (1994) Arabinase gene expression in *Aspergillus niger*: indications for coordinated regulation. Microbiology **140**:2673-2682

Goosen T, Bloemheuvel G, Gysler C, Bie DA de, Broek HWJ van den, Swart K (1987) Transformation of *Aspergillus niger* using the homologous orotidine-5'-phosphate-decarboxylase gene. Curr Genet **11**:499-503

Goosen T, Engelenburg F van, Debets F, Swart K, Bos K, Broek H van den (1989) Tryptophan auxotrophic mutants in *Aspergillus niger*: inactivation of the *trp*C gene by cotransformation mutagenesis. Mol Gen Genet **219**:282-288

Graaff L de, Broek H van den, Visser J (1988) Isolation and transformation of the pyruvate kinase gene of *Aspergillus nidulans*. Curr Genet **13**:315-321

Gremli H, Neukom H (1968) The effect of a purified α-L-arabinofuranosidase on some arabinose-containing polysaccharides. Carbohydr Res **8**:110-112

Gunata Z, Brillouet J-M, Voirin S, Baumes R, Cordonnier R (1990) Purification and some properties of an α-L-arabinofuranosidase from *Aspergillus niger*. Action on grape monoterpenyl arabinofuranosylglucosides. J Agric Food Chem **38**:772-776

Gurr SJ, Unkles SE, Kinghorn JR (1987) The structure and organization of nuclear genes of filamentous fungi. In: Kinghorn JR (ed) Gene structure in eukaryotic microbes. SGM Special Publication. Vol.23. IRL Press, Oxford. pp.93-139

Harmsen JAM, Kusters-van Someren MA, Visser J (1990) Cloning and expression of a second *Aspergillus niger* pectin lyase gene (*pel*A): indications of a pectin lyase gene family in *Aspergillus niger*. Curr Genet **18**:161-166

Heijne G von (1986) A new method for predicting signal sequence cleavage sites. Nucleic Acids Res **14**:4683-4691

Kaji A (1984) L-Arabinosidases. Adv Carbohydr Chem Biochem **42**:383-394

Kaji A, Tagawa K (1970) Purification, chrystallization and amino acid composition of α-L-arabinofuranosidase from *Aspergillus niger*. Biochim Biophys **Acta** 207:456-464

Kaji A, Taki H, Shimazaki A, Shinkai T (1963) Studies on the enzymes acting on arabans. II. Action and seperation of arabanase produced by Aspergilli. Tech Bull Fac Agric Kagawa Univ **15**:34-39

Kellett LE, Poole DM, Ferreira LMA, Durrant AJ, Hazlewood GP, Gilbert HJ (1990) Xylanase B and an arabinofuranosidase from *Pseudomonas fluorescens* subsp. *cellulosa* contain identical cellulose-binding domains and are encoded by adjacent genes. Biochem J **272**: 369-376

King AD Jr, Hocking AD, Pitt JI (1979) Dichloran-rose bengal medium for enumeration and isolation of molds from foods. Appl Environ Microbiol **37**:959-964

Kozak M (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation. J Biol Chem **266**:19867-19870

Kusters-van Someren MA, Harmsen JAM, Kester HCM, Visser J (1991) Structure of the *Aspergillus niger pel*A gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. Curr Genet **20**: 293-299

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685

Lamsa M, Bloebaum P (1990) Mutation and screening to increase chymosin yield in a genetically-engineered strain of *Aspergillus awamori*. J Industr Microbiol **5**:229-238

Lerouge P, O'Neill MA, Darvill AG, Albersheim P (1993a) Structural characterization of endo-glycanase-generated oligoglycosyl side chains of rhamnogalacturonan I. Carbohydr Res **243**:359-371

Lerouge P, O'Neill MA, Darvill AG, Albersheim P (1993b) The purification of commercially available endo-α-L-arabinanase and α-L-arabinosidase for use in the structural analysis of pectic polysaccharides. Carbohydr Res **243**:373-378

Matsudaira P (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem **262**:10035-10038

Neukom H, Markwalder H (1975) Isolation and characterization of an arabinogalactan from wheat flour. Carbohydr Res **39**:387-389

Neukom H, Providoli L, Gremli H, Hui PA (1967) Recent investigations on wheat flour pentosans. Cereal Chem **44**:238-244

Neustroev KN, Golubev AM, Ibatullin FM, Firsov LM (1993) Effect of inhibitors of glycosylation on glycosidase secretion in *Aspergillus awamori* var. X100/D27. Biochemistry Russia **58**:370-374

Ohtsuka E, Matsuki S, Ikehara M, Takahashi Y, Matsubara K (1985) An alternative approach to deoxyoligonucleotides as hybridization probes by insertion of deoxyinosine at ambiguous codon positions. J Biol Chem **260**:2605-2608

Proudfoot N (1991) Poly(A) signals. Cell **64**:671-674

Rombouts FM, Voragen AGJ, Searle-van Leeuwen MF, Geraerds CCJM, Schols HA, Pilnik W (1988) The arabinanases of *Aspergillus niger* – purification and characterisation of two α-L-arabinofuranosidases and an *endo*-1,5-α-L-arabinanase. Carbohydr Polym **9**:25-47

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74**:5463-5467

Schwarz WH, Adelsberger H, Jauris S, Hertel C, Funk B, Staudenbauer WL (1990) Xylan degradation by the thermophile *Clostridium stercorarium*: cloning and expression of xylanase, β-D-xylosidase, and α-L-arabinofuranosidase genes in *Escherichia coli*. Biochem Biophys Res Commun **170**:368-374

Sewell GW, Utt EA, Hespell RB, MacKenzie KF, Ingram LO (1989) Identification of the *Butyrivibrio fibrisolvens* xylosidase gene (*xylB*) coding region and its expression in *Escherichia coli*. Appl Environ Microbiol **55**:306-311

Steers E Jr, Craven GR, Anfinsen CB, Bethune JL (1965) Evidence for nonidentical chains in the β-galactosidase of *Escherichia coli* K12. J Biol Chem **240**:2478-2484

Tagawa K, Kaji A (1988) α-L-Arabinofuranosidase from *Aspergillus niger*. Methods Enzymol **160**:707-712

Unkles SE (1992) Gene organization in industrial filamentous fungi. In: Kinghorn JR, Turner G (eds) Applied molecular genetics of filamentous fungi. 1st edn. Blackie Academic & Professional, London. pp.28-53

Utt EA, Eddy CK, Keshav KF, Ingram LO (1991) Sequencing and expression of the *Butyrivibrio fibrisolvens xylB* gene encoding a novel bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities. Appl Environ Microbiol **57**:1227-1234

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1991) Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. Arch Microbiol **157**:23-28

Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev **21**:195-213

Voragen AGJ, Rombouts FM, Searle-van Leeuwen MF, Schols HA, Pilnik W (1987) The degradation of arabinans by endo-arabinanase and arabinofuranosidases purified from *Aspergillus niger*. Food Hydrocoll **1**:423-437

Whitaker JR (1984) Pectic substances, pectic enzymes and haze formation in fruit juices. Enzyme Microb Technol **6**:341-349

Will F, Dietrich H (1992) Isolation, purification and characterization of neutral polysaccharides from extracted apple juices. Carbohydr Polym **18**:109-117

Chapter 5

Arabinase gene expression in *Aspergillus niger* : indications for coordinated regulation [1]

Aspergillus niger **secretes three glycosylated glycosyl hydrolases which are involved in degradation of the plant cell wall polysaccharide L-arabinan: α-L-arabinofuranosidases (ABF) A and B, and endo-1,5-α-L-arabinase (ABN) A. The nucleotide sequence of the previously cloned gene encoding ABF A (*abf*A) from *A. niger* was determined. The coding region contains seven introns. Mature ABF A comprises 603 amino acids with a molecular weight of 65.4 kDa as deduced from the nucleotide sequence. The secreted enzyme is *N*-glycosylated. The primary structures of the three *A. niger* arabinases characterized lack similarity. Regulation of arabinase expression upon induction by sugar beet pulp and by L-arabitol was studied as a function of time. This was done in wild-type *A. niger* as well as in transformants carrying multiple copies of either one of the ABF-encoding genes. Each arabinase gene responded differently upon a mycelial transfer to L-arabitol-containing medium. Extra copies of *abf*A or *abf*B led to a decreased expression level of ABN A, though the repression elicited by *abf*B is stronger and more persistent than that effected by *abf*A. Multiple copies of both *abf* genes influence expression of the other ABF similary, but to a far less pronounced degree than they affect ABN A synthesis. Four putative promoter elements, shared by all three arabinase genes, could be involved in coordination of L-arabinan degradation by *A. niger*.**

Introduction

Enzymes degrading L-arabinan, a branched polymer largely made up of (1→5)-, (1→2)- and (1→3)-α-linked L-arabinofuranosyl residues, are commonly applied in structural studies of pectic and hemicellulosic plant cell wall heteropolysaccharides (*e.g.* Nishitani and Nevins 1989; Sakai and Sakamoto 1990; van de Vis *et al.* 1991; Will and Dietrich 1992; Lerouge *et al.* 1993). The results of such studies indicate that the L-arabinan component is involved in covalent cross-linking of cell wall polysaccharides. This has led to renewed interest in

[1] published in: Microbiology **140**:2673-2682 (1994)
The nucleotide sequence data reported in this Chapter are accessable from the GenBank Nucleotide Sequence Database under accession number L29005.

arabinases, with respect to their potential use in agro-industrial processes such as fruit juice technology, valorization of agricultural waste and bioconversion of plant biomass (*cf.* Whitaker 1984; Voragen *et al.* 1987).

The hyphal fungus *Aspergillus niger* secretes an endo-1,5-α-L-arabinase (ABN A) (EC 3.2.1.99) and two distinct α-L-arabinofuranosidases (ABF A and B) (EC 3.2.1.55) which are considered the main L-arabinan-degrading activities (Voragen *et al.* 1987; Rombouts *et al.* 1988; van der Veen *et al.* 1991). These glycosylated enzymes act synergistically in degrading branched L-arabinan: the preferred substrate for ABN A is linear (debranched) (1→5)-α-L-arabinan, the backbone of the polymer, while the ABFs are typical glycosidases increasingly active against oligosaccharides of a lower degree of polymerization (Rombouts *et al.* 1988; McCleary 1991; Beldman *et al.* 1993; Lerouge *et al.* 1993). Both ABFs might also be involved in arabinoxylan degradation as debranching hydrolases, removing α-linked L-arabinofuranosyl residues from wheat flour arabinoxylan fragments (Kormelink *et al.* 1993). Previously the production of the *A. niger* arabinases was studied using enzyme-specific antisera (van der Veen *et al.* 1991, 1993). All three were found to be secreted when grown on plant cell wall fractions containing L-arabinan, *e.g.* sugar beet pulp. L-Arabitol, an intermediate of *A. niger* L-arabinose catabolism (Witteveen *et al.* 1989), also mediates arabinase expression. Recently the three arabinase-encoding genes from *A. niger* were cloned and the structure of two of these genes, *abn*A encoding ABN A and *abf*B encoding ABF B, was analyzed (Chapters 2 to 4; Flipphi *et al.* 1993a, b, c).

In this Chapter, arabinase expression induced by sugar beet pulp or L-arabitol has been studied as a function of time, both in wild-type *A. niger* and transformed *A. niger* strains containing multiple copies of either the *abf*A or the *abf*B gene. The nucleotide sequence of the *abf*A gene encoding ABF A and its structure are presented. Upon comparison of the three *A. niger* arabinase genes, common sequence elements were identified which might be involved in regulation of arabinase gene expression.

Materials and methods

***Escherichia coli* strains and plasmids.** *E. coli* DH5α was used to generate and propagate plasmids; *E. coli* JM101 was used to generate recombinant M13 mp18/19 phages for sequencing. Plasmid vectors pEMBL (Dente and Cortese 1987) and pGEM (Promega) were used in DNA subcloning. Plasmids pIM900, containing the *A. niger abf*A gene, and pC1X1, an *abf*A cDNA clone, were described previously (Chapter 2; Flipphi *et al.* 1993c). Plasmids pIM950 and pIM991 contain the *A. niger abn*A and *abf*B genes, respectively (Chapters 3 & 4; Flipphi *et al.* 1993a, b). Plasmid pSF5, kindly provided by Dr. J.R. Kinghorn (Plant Molecular Genetics Unit, University of St Andrews, Scotland, UK), carries the *A. nidulans* gene encoding γ-actin described by Fidel *et al.* (1988). General DNA manipulations were performed according to Sambrook *et al.* (1989).

***Aspergillus* strains and growth conditions.** *A. niger* N402 (*cspA1*) originates from wild-type strain N400 (CBS 120.49) and is a morphological mutant with short conidiophores. Generation of *A. niger abf*A and *abf*B transformants was described previously (Chapter 2 & 4; Flipphi *et al.* 1993a, c). Growth conditions and media used in cultivation were as described in Chapter 2 (Flipphi *et al.* 1993c), using 6 g/l $NaNO_3$ instead of NH_4Cl as the nitrogen source and 1 % (w/v) of the carbon source indicated. Transfer experiments were performed according to Chapter 3 (Flipphi *et al.* 1993b). L-Arabitol was obtained from Sigma. Sugar beet pulp, a gift from CSM (Breda, The Netherlands), was ground in a Waring blendor prior to use.

Sequence determination and analysis. DNA fragments were cloned both in plasmid vectors (pEMBL, pGEM) and in M13 mp18/19. DNA sequences were determined by the dideoxynucleotide chain-termination method (Sanger *et al.* 1977) using the ^{T7}Sequencing Kit from Pharmacia LKB according to the supplier's instructions. Single-stranded M13 DNA as well as alkali-denatured plasmid DNA were used as template; both general sequencing primers and gene-specific oligonucleotides were employed as primers. All nucleotide sequences were determined on both strands. Computer analysis was done using the PC/GENE program (IntelliGenetics) and the sequence analysis software package of the Genetics Computer Group. Cyanogen bromide (CNBr) cleavage of ABF A and amino acid sequence determination were performed as previously described (Chapter 3; Flipphi *et al.* 1993b). The transcription initiation site was deduced upon primer-extension mapping according to the method of Calzone *et al.* (1987) as described in Chapter 4 (Flipphi *et al.* 1993a). PolyA^+ RNA, isolated from *A. niger abf*A transformant N593::pIM900-1 after 1 h of L-arabitol induction as described previously (Chapter 2; Flipphi *et al.* 1993c), was used as template. The 17-mer oligonucleotide which served as primer [TGTTGCCGGCTGTCTGG] is complementary to nucleotides −16 to +1 of the determined *abf*A sequence, as shown in Fig.2.

Enzymic deglycosylation. *N*-Linked carbohydrate moieties were removed from purified *A. niger* ABF A (van der Veen *et al.* 1991) with peptide-N^4-(*N*-acetyl-β-glucosamidyl) asparagine amidase (EC 3.5.1.52), which cleaves off all three types of *N*-linked carbohydrate oligomers (Hirani *et al.* 1987; Tarentino and Plummer 1987). *N*-Glycanase from Genzyme was used according the manufacturer's instructions. Protein was analyzed by SDS-PAGE using 10 % (w/v) polyacrylamide gels containing 0.1 % (w/v) SDS (Laemmli, 1970). Protein test mixture 4 (Serva) provided molecular weight markers. The gels were stained with Coomassie brilliant blue R250.

Determination of arabinase activity in culture medium. Medium samples were filtered over filterpaper and centrifuged (3000 r.p.m., 15 min) to pellet debris. ABF was assayed by measuring hydrolysis of *para*-nitrophenyl α-L-arabinofuranoside (PNP-A) (Sigma) as described previously (van der Veen *et al.* 1991). ABN activity was determined using ArabinaZyme tablets (Megazyme, Warriewood (Sydney), Australia) according to the manufacturer's instructions.

Isolation of RNA and genomic DNA. Mycelium was harvested from liquid cultures, washed with saline and powdered as previously described (Chapter 2; Flipphi *et al.* 1993c). *A. niger* genomic DNA was isolated according to the method of de Graaff *et al.* (1988). Total RNA was isolated from the mycelial powder by guanidinium thiocyanate extraction (Chirgwin *et al.* 1979) and subsequent centrifugation over a 5.7 M CsCl cushion overnight at 32.000 r.p.m. (Glišin *et al.* 1974). The quality of the RNA isolated was tested by native electrophoresis in 1 % (w/v) agarose in the presence of 25 μg/l ethidium bromide. The RNA concentration was calculated from the absorbance at 260 nm.

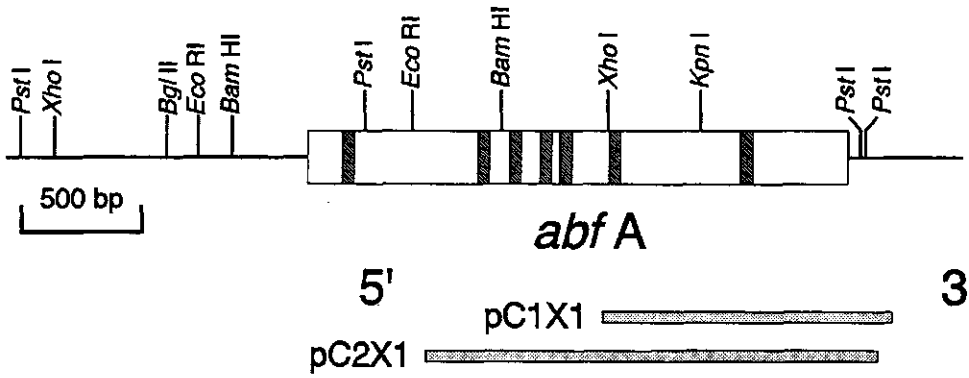


Fig.1. Restriction map of the 3958 bp long fragment of pIM900 from which the DNA sequence is determined. The sites for *Pst*I, *Xho*I and *Kpn*I (*cf.* Chapter 2; Flipphi *et al.* 1993c) as well as for *Bgl*II, *Eco*RI and *Bam*HI are indicated. The coding region of the *abf*A gene is represented by a box, the shaded areas mark the introns. Underneath, the orientation and extent of the cDNA inserts from pC1X1 and pC2X1 are indicated. Upon sequence analysis, pC1X1 turned out to be a hybrid clone containing two fused cDNAs (two polyA stretches) of which only the 5' one (approximately 1.0 kb in length) is *abf*A-specific.

Slot blot analysis of RNA. Total RNA was denatured with glyoxal and dimethylsulfoxide according to McMaster and Carmichael (1977). Denatured RNA samples were diluted in 10 mM sodium phosphate buffer, pH 7.0. Either 5 or 1 μg of RNA was applied to each slot of a Hybri-Slot manifold (Gibco BRL), and was transferred to Hybond-N membrane (Amersham) using 10 mM sodium phosphate buffer, pH 7.0, as transfer buffer. The RNA was fixed to the nylon support by baking at 80°C. Prior to hybridization glyoxal was removed as indicated by Sambrook *et al.* (1989). The slot blots were probed with ^{32}P-labelled DNA fragments specific for either one of the RNAs of interest using standard prehybridization and hybridization solutions containing 50 % (v/v) formamide (Merck) and 6 x SSC at 42°C, essentially according to Sambrook *et al.* (1989) – the hybridization solution, however, additionally contained 10 % (w/v) dextran sulphate (Pharmacia LKB) and 0.1 % (w/v) sodium pyrophosphate (Merck). Excess of probe was removed by two sequential 20-min washing steps at 68°C in 2 x SSC/0.1 % (w/v) SDS/0.1 % (w/v) sodium pyrophosphate. The *A. nidulans* γ-actin gene was used as a probe to quantify the amount of constitutively expressed γ-actin mRNA, which served as a reference. Hybridization with the heterologous actin probe was carried out at 37.1°C and washing was done at 60°C, using the same buffers as for the homologous probes.

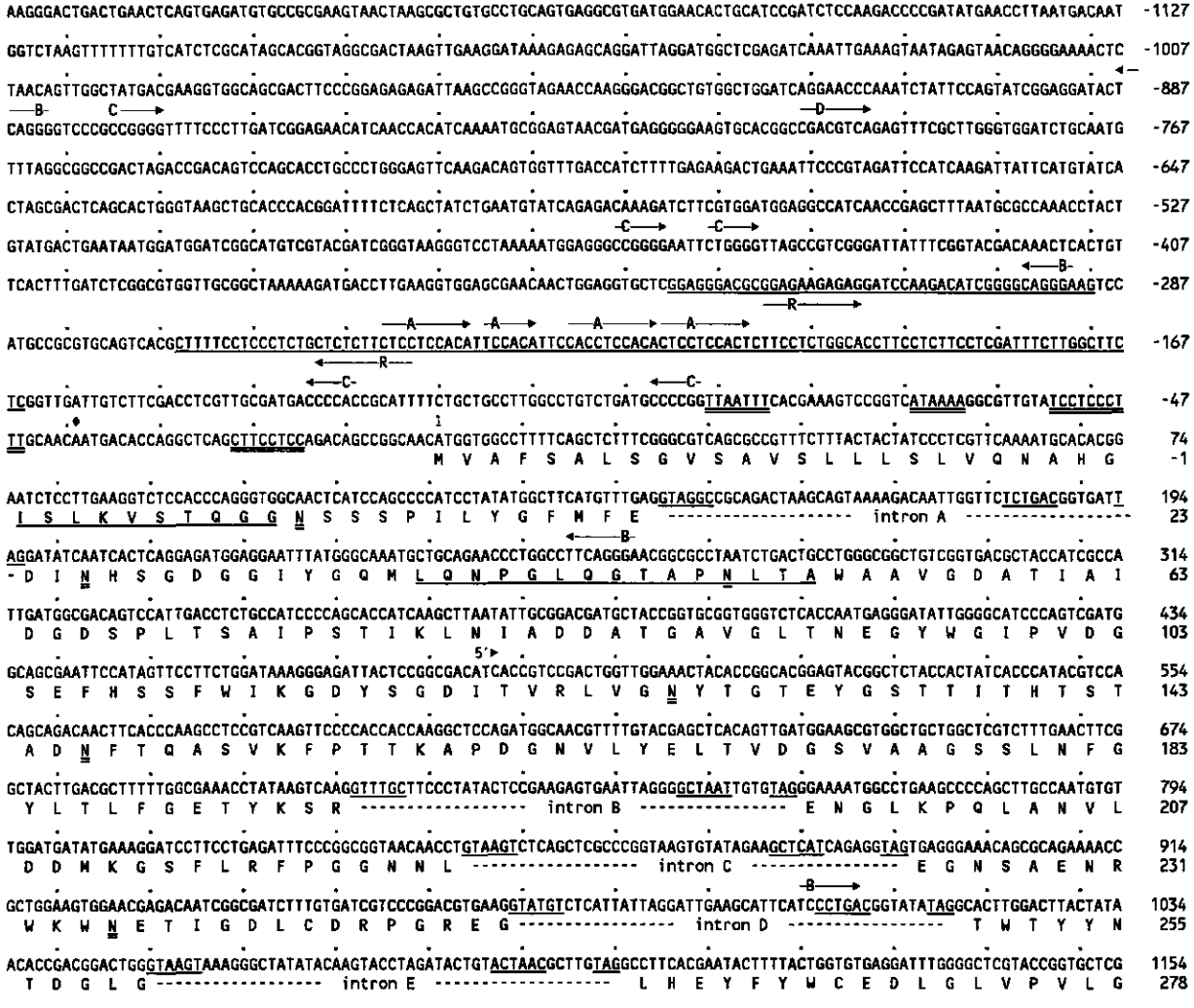
Results

Primary structure of the *abf*A gene

In Chapter 2, the isolation of the *A. niger abf*A gene was reported and its identity was proven by overexpression of ABF A by *A. niger* and *A. nidulans* transformants in which additional copies of the *abf*A gene were introduced by co-transformation (see also Flipphi *et al.* 1993c). Fig. 1 shows the part of plasmid pIM900, which carries the cloned *abf*A gene, from which the nucleotide sequence was determined. In addition, the N-terminal amino acid sequences of purified ABF A and of a peptide derived from ABF A upon CNBr treatment, were determined. The sequence [X X L K V X T Q G G] (10 residues) was found for the N-terminus of ABF A, while for the N-terminus of the CNBr-generated peptide the sequuence [L Q N P G L Q G T A P X L T A] (15 residues) was determined. Residues that could not be assigned are indicated as "X".

The amino acid sequence data enabled deduction of the position of the coding region of the *abf*A gene within the determined DNA sequence, starting at position 1 and ending with a TAG stop codon situated at position 2237 (Fig.2). The structural region contains seven introns of which the two most 3'-positioned (introns F and G) were established by sequencing pC1X1, the cDNA clone used to isolated the *abf*A gene (Chapter 2; Flipphi *et al.* 1993c). Sequence analysis of a second cDNA clone (pC2X1 – approximately 1.5 kb cDNA insert), isolated from the same cDNA library from which pC1X1 originates, established introns B, C, D and E (results not shown). Intron A remains unestablished but its border sequences and the putative lariat formation site conform to the generally established consensus sequences proposed for introns of fungal genes (*cf.* Rambosek and Leach 1987; Gurr *et al.* 1987; Unkles 1992). Moreover, it contains stop codons in all frames and removal of this 52 bp sequence brings the two determined amino acid sequences within the same reading frame (Fig.2).

The two isolated cDNAs differ with respect to the length of the 3' non-coding sequence of the *abf*A transcript. The ultimate 3' *abf*A-specific base of pC2X1 was found to correspond to the C at position 2371 (a non-coding sequence of 132 bp) and that of pC1X1 to the T at position 2419 (a non-coding sequence of 180 bp). This strongly suggests the presence of (at least) two sites for polyadenylation. Upon primer-extension mapping using L-arabitol-induced polyA$^+$ RNA from *A. niger abf*A transformant N593::pIM900-1 as template (results not shown), a single site for transcription initiation was identified at position −39 relative to the start codon.


AAGGGACTGACTGAACTCAGTGAGATGTGCCGCGAAGTAACTAAGCGCTGTGCCTGCAGTGAGGCGTGATGGAACACTGCATCCGATCTCCAAGACCCCGATATGAACCTTAATGACAAT -1127
GGTCTAAGTTTTTTTGTCATCTCGCATAGCACGGTAGGCGACTAAGTTGAAGGATAAAGAGAGCAGGATTAGGATGGCTCGAGATCAAATTGAAAGTAATAGAGTAACAGGGGAAAACTC -1007
TAACAGTTGGCTATGACGAAGGTGGCAGCGACTTCCCGGAGAGAGATTAAGCCGGGTAGAACCAAGGGACGGCTGTGGCTGGATCAGGAACCCAAATCTATTCCAGTATCGGAGGATACT -887
CAGGGGTCCCGCCGGGGTTTTCCCTTGATCGGAGAACATCAACCACATCAAAATGCGGAGTAACGATGAGGGGGAAGTGCACGGCCGACGTCAGAGTTTCGCTTGGGTGGATCTGCAATG -767
TTTAGGCGGCCGACTAGACCGACAGTCCAGCACCTGCCCTGGGAGTTCAAGACAGTGGTTTGACCATCTTTTGAGAAGACTGAAATTCCCGTAGATTCCATCAAGATTATTCATGTATCA -647
CTAGCGACTCAGCACTGGGTAAGCTGCACCCACGGATTTTCTCAGCTATCTGAATGTATCAGAGACAAAGATCTTCGTGGATGGAGGCCATCAACCGAGCTTTAATGCGCCAAACCTACT -527
GTATGACTGAATAATGGATGGATCGGCATGTCGTACGATCGGGTAAGGGTCCTAAAAATGGAGGGCCGGGGAATTCTGGGGTTAGCCGTCGGGATTATTTCGGTACGACAAACTCACTGT -407
TCACTTTGATCTCGGCGTGGTTGCGGCTAAAAAGATGACCTTGAAGGTGGAGCGAACAACTGGAGGTGCTCGGAGGGACGCGGAGAAGAGAGGATCCAAGACATCGGGGCAGGGAAGTCC -287
ATGCCGCGTGCAGTCACGCTTTTCCTCCCTCTGCTCTCTTCTCCTCCACATTCCACATTCCACCTCCACACTCCTCCACTCTTCCTCTGGCACCTTCCTCTTCCTCGATTTCTTGGCTTC -167
TCGGTTGATTGTCTTCGACCTCGTTGCGATGACCCCACCGCATTTTCTGCTGCCTTGGCCTGTCTGATGCCCCCGGTTAATTTCACGAAAGTCCGGTCATAAAAGGCGTTGTATCCTCCCT -47
TTGCAACAATGACACCAGGCTCAGCTTCCTCCAGACAGCCGGCAACATGGTGGCCTTTTCAGCTCTTTCGGGCGTCAGCGCCGTTTCTTTACTACTATCCCTCGTTCAAAATGCACACGG 74
M V A F S A L S G V S A V S L L L S L V Q N A H G -1
AATCTCCTTGAAGGTCTCCACCCAGGGTGGCAACTCATCCAGCCCCATCCTATATGGCTTCATGTTTGAGGTAGGCCGCAGACTAAGCAGTAAAAGACAATTGGTTCTCTGACGGTGATT 194
I S L K V S T Q G G N S S S P I L Y G F M F E ---------- intron A ---------- 23
AGGATATCAATCACTCAGGAGATGGAGGAATTTATGGGCAAATGCTGCAGAACCCTGGCCTTCAGGGAACGGCGCCTAATCTGACTGCCTGGGCGGCTGTCGGTGACGCTACCATCGCCA 314
- D I N H S G D G G I Y G Q M L Q N P G L Q G T A P N L T A W A A V G D A T I A I 63
TTGATGGCGACAGTCCATTGACCTCTGCCATCCCCAGCACCATCAAGCTTAATATTGCGGACGATGCTACCGGTGCGGTGGGTCTCACCAATGAGGGATATTGGGGCATCCCAGTCGATG 434
D G D S P L T S A I P S T I K L N I A D D A T G A V G L T N E G Y W G I P V D G 103
GCAGCGAATTCCATAGTTCCTTCTGGATAAAGGGAGATTACTCCGGCGACATCACCGTCCGACTGGTTGGAAACTACACCGGCACGGAGTACGGCTCTACCACTATCACCCATACGTCCA 554
S E F H S S F W I K G D Y S G D I T V R L V G N Y T G T E Y G S T T I T H T S T 143
CAGCAGACAACTTCACCCAAGCCTCCGTCAAGTTCCCCACCACCAAGGCTCCAGATGGCAACGTTTTGTACGAGCTCACAGTTGATGGAAGCGTGGCTGCTGGCTCGTCTTTGAACTTCG 674
A D N F T Q A S V K F P T T K A P D G N V L Y E L T V D G S V A A G S S L N F G 183
GCTACTTGACGCTTTTTGGCGAAACCTATAAGTCAAGGTTTGCTTCCCTATACTCCGAAGAGTGAATTAGGGGCTAATTGTGTAGGGAAAATGGCCTGAAGCCCCAGCTTGCCAATGTGT 794
Y L T L F G E T Y K S R ---------- intron B ---------- E N G L K P Q L A N V L 207
TGGATGATATGAAAGGATCCTTCCTGAGATTTCCCGGCGGTAACAACCTGTAAGTCTCAGCTCGCCCGGTAAGTGTATAGAAGCTCATCAGAGGTAGTGAGGGAAACAGCGCAGAAAACC 914
D D M K G S F L R F P G G N N L ---------- intron C ---------- E G N S A E N R 231
GCTGGAAGTGGAACGAGACAATCGGCGATCTTTGTGATCGTCCCGGACGTGAAGGTATGTCTCATTATTAGGATTGAAGCATTCATCCCTGACGGTATATAGGCACTTGGACTTACTATA 1034
W K W N E T I G D L C D R P G R E G ---------- intron D ---------- T W T Y Y N 255
ACACCGACGGACTGGGTAAGTAAAGGGCTATATACAAGTACCTAGATACTGTACTAACGCTTGTAGGCCTTCACGAATACTTTTACTGGTGTGAGGATTTGGGGCTCGTACCGGTGCTCG 1154
T D G L G ---------- intron E ---------- L H E Y F Y W C E D L G L V P V L G 278

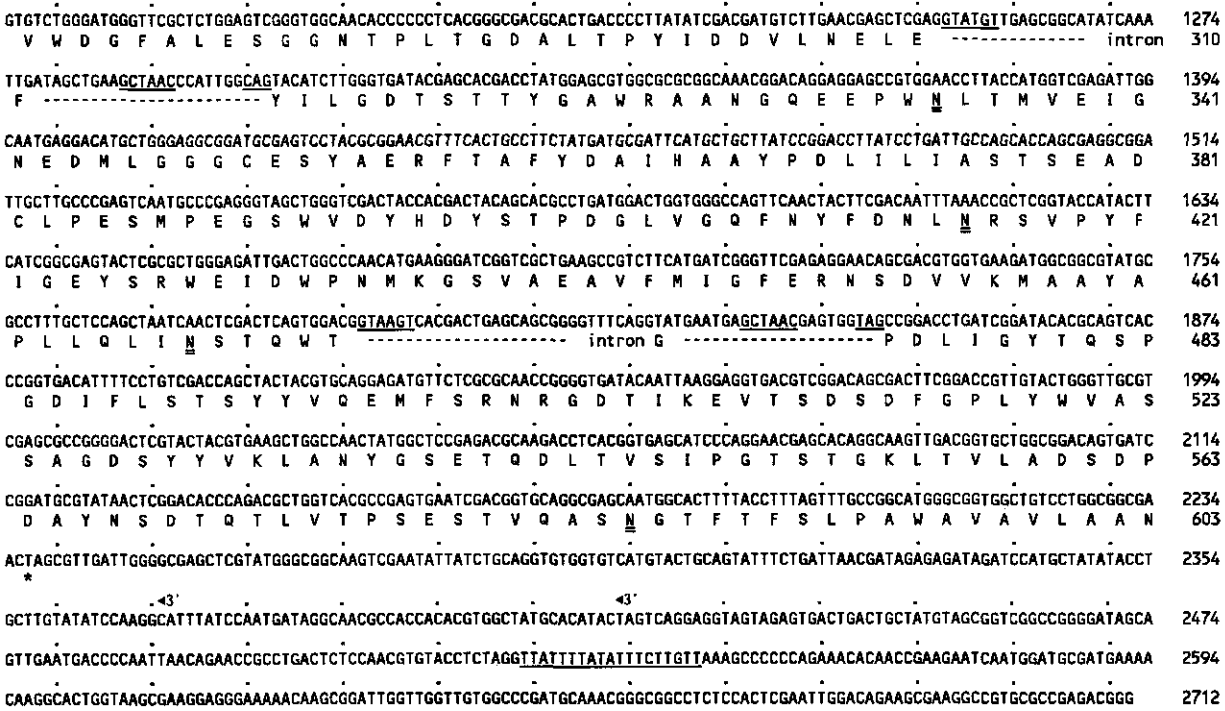


Fig.2. The nucleotide sequence of the *A. niger abf*A gene and the deduced amino acid sequence of the gene product ABF A. The nucleotide sequence is numbered relative to the first base of the deduced startcodon, the amino acid sequence is numbered relative to the N-terminal isoleucine residue of mature ABF A. The transcription initiation site is marked (♦). The 5′ ultimate base of the cDNA insert from pC2X1 (position 486) is marked (5′▸); the 3′ ultimate bases of the cDNA inserts from pC2X1 and pC1X1 are marked (◂3′). Putative promoter elements in the upstream non-coding sequence thought to effect transcription initiation are double underlined (TATA boxes / CT-stretches). The CT-rich and GA-rich upstream sequences are marked by underlining. Various putative sequence elements are indicated by arrows either above or underneath the sequence, and marked with uppercase letters. A T-rich sequence found in the 3′ non-coding sequence is underlined. Determined amino acid sequences are underlined. Putative *N*-glycosylation sites are indicated (N).

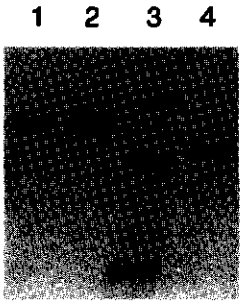


Fig.3. SDS-PAGE analysis of deglycosylation of ABF A. Deglycosylation of 2 μg ABF A was performed as described in Methods. Loading buffer was applied to stop the reaction and half the volume of each sample (20 μl) was directly applied on a 10 % polyacrylamide gel. **Lane 1,** untreated ABF A (1 μg); **2,** ABF A incubated in the absence of *N*-Glycanase; **3,** Molecular weight markers (ovalbumin, 45 kDa; bovine serum albumin, 67 kDa; phosphorylase B, 92.5 kDa); **4,** ABF A incubated in the presence of *N*-Glycanase. The figure shows representative results for one out of two independent experiments.

Deduced primary structure of ABF A

Removal of the seven intron sequences from the coding region of *abf*A leads to an open reading frame of 1884 bp coding for a protein of 628 amino acids (Fig.2). Within the deduced sequence of the primary gene product the determined N-terminal sequence of mature extracellular ABF A is preceded by a 25 residue-long sequence which could serve as a signalpeptide for secretion. Small neutral amino acid residues are present at positions −3 (alanine) and −1 (glycine) relative to the proposed signal processing site, as predicted using the method described by von Heijne (1986). Mature ABF A consists of 603 amino acids with a deduced molecular weight of 65.4 kDa and a theoretical isoelectric point of 3.7. These characteristics differ from those previously reported for the purified ABF A protein, where an apparent molecular weight of 83 kDa and an isoelectric point of 3.3 were found (van der Veen *et al.* 1991). This discrepancy is likely to be due to carbohydrate moieties as ABF A is a glycoprotein (Voragen *et al.* 1987). The amino acid sequence reveals the presence of ten putative sites for *N*-linked glycosylation (Fig.2). Removal of *N*-linked oligosaccharides from purified ABF A with *N*-Glycanase resulted in a reduction of the apparent molecular weight of over 15 %, as determined upon SDS-PAGE (Fig.3). The apparent molecular weight of the deglycosylated protein (68 kDa) is only slightly higher than the deduced molecular weight (65.4 kDa). Time course study of the deglycosylation (results not shown) suggested at least three *N*-linked oligosaccharides are present. One of these is highly resistent to enzymic hydrolysis, which leads to the minor band just above the main band present in lane 4 of Fig.3.

Table 1. Conserved amino acid sequence elements found in ABF A and two Klebsiella pullulanases by primary structure alignment.

Protein [a]	Residue no. [b]	Amino acid sequence [c]	Sequence element [d]
A. niger ABF A	209	**D** M **K** **G** S **F** L **R** F	
K. aerogenes PUL	368	**D** L **K** **G** A **F** Y **R** Y	Element e
K. pneumoniae PUL	358	**D** L **K** **G** A **F** Y **R** Y	
A. niger ABF A	270	**D** **L** **G** **L** V **P** **V** L G **V**	
K. aerogenes PUL	611	**D** **L** **G** M N V I M D **V**	
	491	I E **L** **L** **P** **V** F D L	
K. pneumoniae PUL	601	**D** **L** **G** M N V I M D **V**	
	481	V E **L** **L** **P** **V** F D L	
A. niger ABF A	302	I D D **V** **L** **N** E **L**	
K. aerogenes PUL	532	V E E **V** **L** **N** Q **L**	
K. pneumoniae PUL	531	V E E **V** **L** **N** Q **L**	
A. niger ABF A	386	S M **P** **E** **G** **S** W **V** D Y **H** D **Y**	
K. aerogenes PUL	582	T V **P** **E** **G** **S** Y	
	689	D G F R F D L M G **Y**	Element g
	846	Y V S K H D	Element j
K. pneumoniae PUL	572	T V **P** **E** **G** **S** Y	
	679	D G F R F D L M G **Y**	
	836	Y V S K H D	
A. niger ABF A	427	**R** W **E** **I** D W P - **N** **L** **K** **G** S	
K. aerogenes PUL	732	**R** F **E** **I** A S Q I **N** **L** **K** **G** T	
K. pneumoniae PUL	722	**R** F **E** **I** A S Q I **N** **L** **K** **G** T	
A. niger ABF A	475	**D** **L** I **G** **Y**	
K. aerogenes PUL	694	**D** **L** M **G** **Y**	
K. pneumoniae PUL	684	**D** **L** M **G** **Y**	
A. niger ABF A	589	**T** F S **L** **P** **A** **W** A **V** **A** **V** **L**	
K. aerogenes PUL	1072	S V T **L** **P** **A** **W** S **V** **A** **V** **L**	
K. pneumoniae PUL	1062	**T** V T **L** **P** **A** **W** S **V** **A** **V** **L**	

[a] *Klebsiella aerogenes* pullulanase (PUL A) (Katsuragi *et al.* 1987). *Klebsiella pneumoniae* pullulanase (PUL A) (Kornacker and Pugsley 1990).

[b] The residue numbers apply to the N-terminal residue of the aligned sequences in the respective proteins.

[c] Identical amino acids are in bold face. Residues conserved amongst glycosyl hydrolases from family 13 are underlined.

[d] Sequence elements described by Melasniemi *et al.* (1990).

Comparison of ABF A with other glycosyl hydrolases

The deduced amino acid sequence of ABF A does not show homology with the two other *A. niger* L-arabinan-degrading enzymes, ABF B and ABN A. ABF A and ABN A have one sequence [E Y G S T/W T I/D T/– H T/G S T] in common, in ABF A present at position [132 – 143] and in ABN A at position [106 – 116] (Chapter 3; Flipphi *et al.* 1993b). No similarities were found upon comparison with three prokaryotic enzymes having ABF-like activities, *i.e.* two β-D-xylosidases exhibiting PNP-A-hydrolyzing activity and an α-L-arabinofurano-hydrolase only acting on oat spelt xylan (Kellett *et al.* 1990; Utt *et al.* 1991; Sakka *et al.* 1993). Screening the major protein data libraries for similarity, however, resulted in the identification of several short, highly similar amino acid sequences (listed in Table 1) shared by ABF A and two strongly related *Klebsiella* pullulanases (α-dextrin endo-1,6-α-D-glucosidase; EC 3.2.1.41) (Katsuragi *et al.* 1987; Kornacker and Pugsley 1989). Pullulanases belong to glycosyl hydrolase family 13, which contains only α-amylase-like hydrolases and transferases (Henrissat 1991). Two of the sequences recognized overlap with homologous regions commonly found within α-amylases (Melasniemi *et al.* 1990; Table 1). However, hydrophobic cluster analysis (*cf.* Gaboriaud *et al.* 1987) could not verify the similarities indicated by primary structure alignment.

Study of arabinase expression in wild-type and transformant *A. niger* strains

As the three *A. niger* arabinases are involved in degradation of the same polysaccharide and their synthesis is simultanously induced upon growth on polymeric substrates and L-arabitol (van der Veen *et al.* 1991, 1993), regulation of expression of the corresponding genes could well be coordinated. Arabinase expression has been studied in *A. niger* wild-type N402 and in transformant strains containing additional copies of either the *abf*A or the *abf*B gene. Several *abf*A and *abf*B transformants covering the available range from low to high copy numbers, as deduced from Southern blot analyses (results not shown), were selected.

The selected strains were grown submerged in liquid medium containing sugar beet pulp as the carbon source and 0.1 % (w/v) yeast extract to initiate efficient germination of the inoculum. Medium samples were taken at 24, 36 and 48 h after inoculation, in which both the ABN activity and the PNP-A-hydrolyzing activity were measured (Table 2). This approach enables to monitor the effect of either *abf* gene on the expression of the ABN A-encoding gene (*abn*A) with time. The activity assay for ABN is highly specific, while the PNP-A-hydrolyzing activity represents the combined activities of ABF A and ABF B. In general, the levels of ABN activity produced by all *abf* transformants are progressively lower with increasing PNP-A-hydrolyzing activity. However, the reduction of the ABN A expression levels is rather correlated with the *abf* gene dosage than with increasing ABF activity. Transformant N593::pIM991-17 features a reduction

Table 2. PNP-A-hydrolyzing activities and ABN activities produced by *A. niger* wild-type and *abf*-transformed strains upon growth on sugar beet pulp

Timepoint	Strain	relative ABN activity [a]	relative PNP-A activity [b]
24 h	N402	1.00	1.00
	N593::pIM900-4	0.80	1.85
	N593::pIM900-11	0.67	3.00
	N593::pIM900-1	0.53	6.83
	N593::pIM991-18	0.41	4.25
	N593::pIM991-135	0.28	5.15
	N593::pIM991-17	0.29	2.37
	N593::pIM991-121	0.34	7.61
	N593::pIM991-112	0.18	12.44
36 h	N402	1.00	1.00
	N593::pIM900-4	0.87	1.87
	N593::pIM900-11	0.61	3.03
	N593::pIM900-1	0.51	6.43
	N593::pIM991-18	0.42	3.59
	N593::pIM991-135	0.30	4.97
	N593::pIM991-17	0.33	2.14
	N593::pIM991-121	0.35	6.82
	N593::pIM991-112	0.21	11.17
48 h	N402	1.00	1.00
	N593::pIM900-4	0.92	1.88
	N593::pIM900-11	0.69	2.99
	N593::pIM900-1	0.58	6.31
	N593::pIM991-18	0.45	3.50
	N593::pIM991-135	0.33	4.65
	N593::pIM991-17	0.34	2.06
	N593::pIM991-121	0.36	6.35
	N593::pIM991-112	0.22	10.51

Transformant strains are ordered with increasing copy numbers as indicated by Southern blot analyses (not shown). Representative values from one out of two independent experiments are given.

[a] ABN activity was determined in duplicate, as described in Methods assaying 50 μl of culture medium for 10 min. Activities are given relative to the wild-type level at each consecutive timepoint. The wild-type level produced by N402 is 0.241 U/ml (± 0.004) at 24 h, 0.246 U/ml (± 0.008) at 36 h, and 0.238 U/ml (± 0.008) at 48 h.

[b] PNP-A-hydrolyzing activity was determined in quadruplicate, as described in Methods assaying 10 μl of culture medium for 10 min. Activities are given relative to the wild-type level at each consecutive timepoint. The wild-type level produced by N402 is 0.241 U/ml (± 0.015) at 24 h, 0.301 U/ml (± 0.010) at 36 h, and 0.342 U/ml (± 0.013) at 48 h.

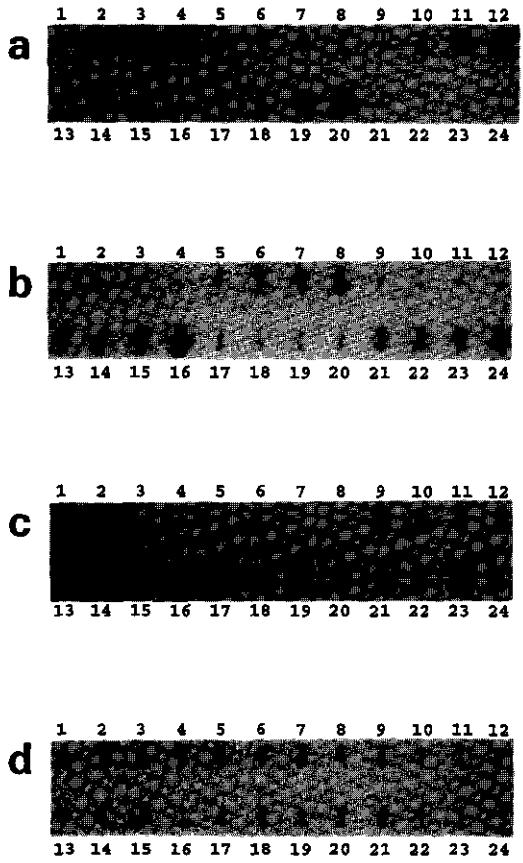


Fig.4. Slot blot analysis of L-arabitol-induced arabinase expression by *A. niger* wild-type and *abf*-transformed strains. Transformant strains are ordered with increasing copy numbers as indicated by Southern blot analyses (not shown). Each slot is loaded with 1 μg of total RNA. **a**: Blot probed with a 2.0 kb *Pst*I fragment from pIM900, containing most of the coding area of the *abf*A gene (*cf.* Fig.1); **b**: Blot probed with a 2.8 kb *Pst*I fragment from pIM991, containing the complete coding area of the *abf*B gene (Chapter 4; Flipphi *et al.* 1993a); **c**: Blot probed with the whole 3.1 kb *Hin*dIII insert from pIM950, carrying the *abn*A gene (Chapter 3; Flipphi *et al.* 1993b); **d**: Blot probed with a 830 bp *Nco*I/*Kpn*I fragment from pSK5, containing mainly coding sequences from the heterologous *A. nidulans* γ-actin gene (Fidel *et al.* 1988). **Slots 1 to 8**: N402; N593::pIM900-4; -11; -1; N593::pIM991-18; -135; -121; -112, mycelia isolated 1 h after transfer; **slots 9 to 16**; *ibid*, isolated 3 h after transfer; **slots 17 to 24**; *ibid*, isolated 5 h after transfer.

These blots show representative results from one out of two independent experiments and should not be used to compare the expression levels of the three arabinase genes, as the specific activity of the probes used was not determined.

intermediate between strains -135 and -121 while these three strains harbor similar amounts of additional *abf*B copies (data not shown). N593::pIM991-17 is the most moderate overproducer of PNP-A-hydrolyzing activity (*cf.* Table 2), which could be due to abundant presence of non-functional *abf*B copies. This strongly suggests that extra *abf* copies titrate a common transcriptional activator. In *abf*A transformants the repression never exceeds 50 %, while the ABN A expression level is reduced to 20 to 40 % in the *abf*B multiple copy strains. This indicates that distinct factors are titrated by the respective ABF-encoding genes. Notably, the wild-type ABN level remains constant while in all transformants except N593::pIM900-11, ABN expression increases with time.

To study the effect of additional *abf* gene copies on the expression of the individual ABFs, arabinase expression was investigated more directly at the level of transcription. The experiment was set up essentially as previously described (Chapter 3; Flipphi *et al.* 1993b; van der Veen *et al.* 1993), performing mycelial transfer of sucrose-grown biomass to minimal medium containing the simple and defined substrate L-arabitol as sole carbon source. In such an experiment expression of all three arabinases is accomplished, and their secretion reaches steady state levels within six hours after transfer, without the lag phase characteristic for induction on sugar beet pulp upon mycelial transfer (unpublished results). Mycelium was recovered at 1, 3 and 5 h after transfer. Biomass isolated 2 h after transfer to minimal medium containing D-glucose, was used as a control for proper induction. Slot blot analysis of total RNA (10 μg) revealed the presence of small amounts of *abf*A and *abf*B mRNA in D-glucose-transferred mycelium of all strains (data not shown). This suggests low constitutive expression of both ABF-encoding genes on D-glucose.

Slot blot analysis of the time course of induction (Fig.4) cleary shows that L-arabitol induces arabinase expression. The mRNA levels of the various transformant strains correlate with the overexpression of extracellular PNP-A-hydrolyzing activity (Table 2). The three genes studied respond differently on transfer to medium containing L-arabitol. For *abf*A, the expression levels were high 1 h after transfer and decreased with time. In constrast, *abn*A was hardly expressed after 1 h but the mRNA level sharply rose between 1 and 3 h after transfer. The *abf*B gene was clearly expressed after 1 h of induction and its expression level slowly increased with time. In all transformants, the presence of multiple copies of either *abf* gene led to a decrease of the expression level of *abn*A correlating with increasing gene dosage. This effect was most evident after 1 h but for the *abf*B transformants was also clear at both other timepoints. These data verify the activity measurements from the sugar beet pulp cultures, although repression of ABN A expression is far more persistent on sugar beet pulp. The slot blots (Fig.4) suggest that extra *abf*B copies affect ABF A expression, although there is no clear relation between the reduction of the expression level and the *abf*B gene dosage. In case of ABF B in *abf*A transformants, effects

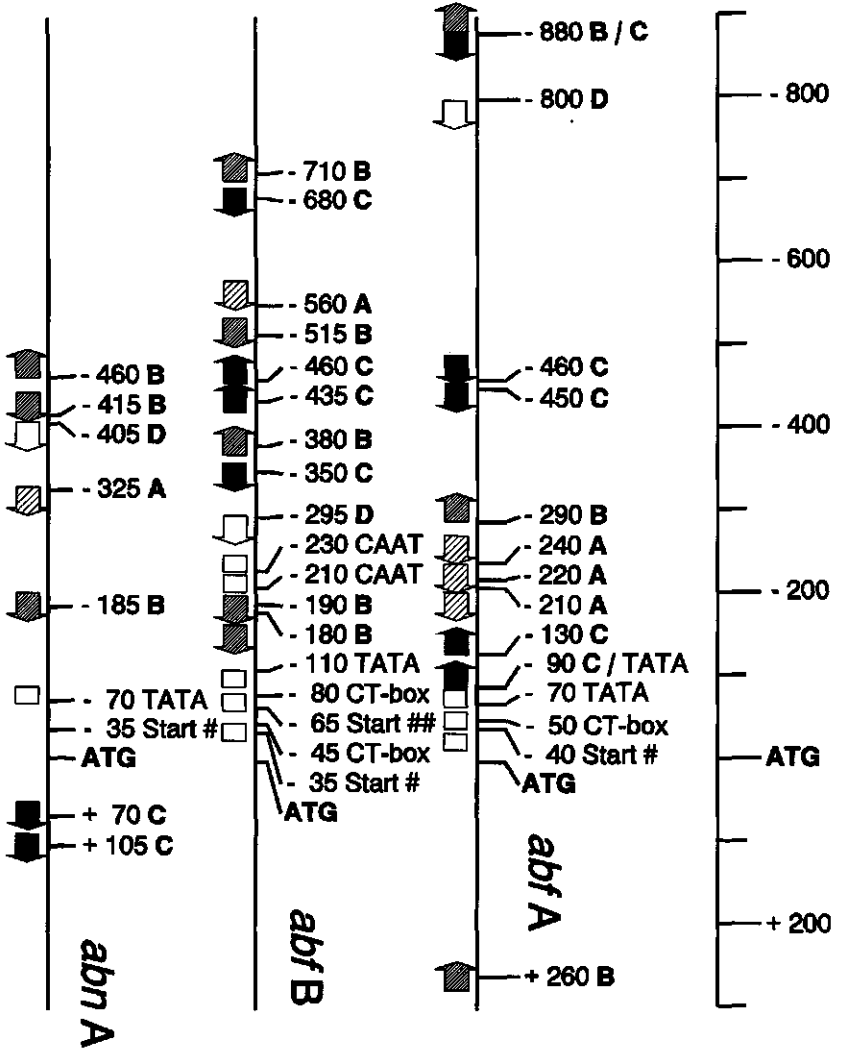

- 460 B
- 415 B
- 405 D
- 325 A
- 185 B
- 70 TATA
- 35 Start #
ATG
+ 70 C
+ 105 C
abn A
- 710 B
- 680 C
- 560 A
- 515 B
- 460 C
- 435 C
- 380 B
- 350 C
- 295 D
- 230 CAAT
- 210 CAAT
- 190 B
- 180 B
- 110 TATA
- 80 CT-box
- 65 Start ##
- 45 CT-box
- 35 Start #
ATG
abf B
- 880 B / C
- 800 D
- 460 C
- 450 C
- 290 B
- 240 A
- 220 A
- 210 A
- 130 C
- 90 C / TATA
- 70 TATA
- 50 CT-box
- 40 Start #
ATG
abf A
+ 260 B
- 800
- 600
- 400
- 200
ATG
+ 200

Fig.5. Schematic overview showing the localization and orientation of common putative sequence elements in the 5′ regions (− 900 to + 300 relative to the translation start codon (**ATG**) of the *A. niger* arabinase genes (*abf*A, *abf*B and *abn*A). The four different elements (**A, B, C, D**) are represented by distinct arrows and the approximate distances from the respective translation start codon (**ATG**) are indicated, as for the major transcription initiation sites of the respective genes. Sequences silimar to functional general eukaryotic promoter elements (CAAT-box, TATA-box and CT-stretch) are represented by open boxes. Only those putative CREA-sites (**C**) are given which are found in conjunction with either another CREA-site or with any other of the indicated elements.

similar to those found for ABN A were evident though far less pronounced. Expression of *abf*B was reduced in strains N593::pIM900-11 and -1, mainly at 1 h after transfer.

Discussion

Previously two of the genes involved in L-arabinan degradation by *A. niger*, *abf*B and *abn*A, were isolated and characterized. In this Chapter, the structure of a third gene involved, *abf*A, and its gene product ABF A are presented. The deduced amino acid sequences from the three *A. niger* arabinases do not exhibit sequence similarity, although all act on (1→5)-*α*-glycosidic linkages between L-arabinofuranosyl residues. Primary structure alignments indicate that none of the arabinases is related to any of the prokaryotic glycosyl hydrolases known to exhibit PNP-A-hydrolyzing or L-arabinose-releasing activities. It seems likely that the arabinases represent a novel class of glycosyl hydrolase families, reflecting a specific architecture fitting the furanosyl configuration of L-arabinose. Biochemically, a major difference between ABF A and the other two arabinases is that ABF A acts only on small oligomers – its activity was found to decrease progressively with increasing length of (1→5)-*α*-linked oligomers (Rombouts *et al.* 1988). This coincides with the fact that ABF A is *N*-glycosylated whereas ABF B and ABN A are not – *N*-Glycanase does not affect the apparent molecular weights of ABF B and ABN A (results not shown), indicating that these glycoproteins are *O*-glycosylated. Morosoli *et al.* (1992) suggested that *N*-glycosylation affects the substrate specificity as well as the kinetic properties of a xylanase from the yeast *Cryptococcus albidus*. Unglycosylated xylanase, produced intracellularly by transformed *E. coli*, exhibited reduced substrate affinity whereas the substrate specificity was shifted from oligomers towards polymers. However, *N*-linked glycosyl moieties in fungal extracellular enzymes are generally considered to be involved in protection against host-specific proteases and in heat stability, rather than in activity (*cf.* Tanner and Lehle 1987). One of the putative sites for *N*-glycosylation (Fig.2) is located at position 12 of the determined N-terminal sequence of the internal CNBr-generated peptide. Attachment of a glycosyl group to the asparagine on that position might be the reason that this residue could not be assigned upon Edman degradation of the CNBr-derived peptide (*cf.* Meldgaard and Svendsen 1994). Enzymic deglycosylation not only reduces the apparent molecular weight, it also changes the appearance of ABF A upon SDS-PAGE from a broad diffuse band to a condensed sharp band (Fig.3), indicating that the fuzzy appearance of purified (glycosylated) ABF A could well be a result of modifications of *N*-linked sugars.

Analysis of the 5′ non-coding sequence of the *abf*A gene (Fig.2) shows that the transcription initiation site (39 bp upstream of the start codon) is located 6 bases downstream of a 10 bp-long CT-stretch, an element involved in initiation of transcription of several highly expressed fungal genes (*cf.* Gurr *et al.* 1987; Unkles 1992). Further upstream two AT-stretches ([TTAATTT] (−91 to −85 relative to the start codon) and [ATAAAA] (−69 to −64) are present, located such that these sequences could operate as functional TATA-elements. Upstream of the most distal TATA-like element, *abf*A is highly structured. A CT-rich area of 104 bp (84.6 % CT) is preceded by a 46 bp GA-rich area (82.6 % GA) as reflected by the presence of a perfect 11 bp-long dyad symmetry (denoted R in Fig.2). The CT-rich area includes a complex 40 bp region devoid of G's exhibiting extensive internal homology, *i.e.* there are five perfect 5 bp repeats [T C C A C] present. In Fig.2 the internal homology is indicated by three 10 bp direct repeats of the sequence [C T/A C C T C C A C A/T] (element A) together with a truncated 6 bp copy [T C C A C A]. The most distal copy of this element partly overlaps the most proximal half of the dyad symmetry element R. Recent studies indicated that all three arabinase genes are subjected to carbon catabolite repression by D-glucose (van der Veen *et al.* 1993). *abf*A contains several sequence elements (marked C in Fig.2) perfectly matching the consensus sequence of the *cis*-acting element found in the *A. nidulans alc*A and *alc*R genes [G/C C/T G G G G], involved in D-glucose-mediated carbon catabolite repression of the ethanol utilization regulon by the DNA-binding repressor protein CREA (Kulmburg *et al.* 1993). Several copies of this element are also present in *abn*A and *abf*B (Fig.5).

The results indicate that regulation of arabinase synthesis exhibits coordinated features, which could be mediated through common *cis*-acting sequence elements. Comparing the sequences of the arabinase genes, several of such elements were found (Fig.5; see Fig.2 for *abf*A). Element B [T C C C T G A A] is present as a single copy in each gene, though several truncated and complementary versions are present in all genes. The core of this element [C C C T G A] is found in all pectinolytic *A. niger* genes of which sequences are available (Bussink *et al.* 1992). Element B bears similarity to a cAMP-responsive element (UAS_{PDS}) in a yeast heat shock protein HSP70-encoding gene [T T/A A G G G A T], reported to mediate repression in the presence of fermentable carbon sources (Boorstein and Craig 1990). A single copy of element D [T/C G A C A/G T C A] is present in each gene and is highly similar to a cAMP-responsive element [T G A C G T C A] (CRE/ATF) found in mammalian genes, conferring both activating and repressing effects (*e.g.* Roesler *et al.* 1988). Element A [C T/A C C T C C A C A/T], the repeated element in the upstream region of *abf*A as discussed above, is found as a single copy in *abn*A and as a truncated copy [C A C C T C C A T T] in *abf*B. No functional analogues could be found for this element but it is also present in the upstream non-coding region of a number of totally unrelated fungal genes, *e.g.* amongst others, *Ascobolus immersus met2* (homoserine *O*-trans-

acetylase) (Goyon *et al.* 1988), *Neurospora crassa grg-1* (glucose repressible gene) (McNally and Free 1988), and *Aspergillus awamori phy* (phytase) (Mullaney *et al.* 1991) (data not shown). It seems likely that all the common sequence elements represent more general regulatory features. Whether these elements are actually involved in regulation of arabinase expression is under investigation.

Expression of arabinases in *A. niger* is inducible both by complex substrates containing L-arabinan and by L-arabitol. The two expression experiments described here were conducted under quite different experimental and physiological conditions. The different timescale and detection method chosen are a consequence of the totally distinct nature of the two compounds employed as inducing agents. Nevertheless, clear indications were found that additional copies of either *abf* genes influence the expression of ABN A on sugar beet pulp and on L-arabitol similarly. In both experiments the influence of *abf*B is stronger than of *abf*A. However, even in the case of the highest *abf*B gene dosage (strain N593::pIM991-112) ABN A expression still reaches 20 % of the wild-type level on sugar beet pulp (Table 2). The effect of extra *abf*B copies is also more persistent than that of extra *abf*A copies.

Extra copies of either *abf* gene seem to titrate distinct regulatory factors which could contribute to coordination of arabinase gene expression, especially with reference to ABN A. Another feature concerning ABN A expression was noticed previously (Chapter 3; Flipphi *et al.* 1993b). In *A. niger* transformants carrying multiple copies of the *abn*A gene grown on sugar beet pulp, overexpression of ABN A did not exceed a limit of five times the wild-type level. The PNP-A-hydrolyzing activity was not affected in these transformants (unpublished results). This limitation does not occur in heterologous *Aspergillus nidulans* multiple copy transformants (Chapter 3) nor in case of the two *abf* genes (see Chapters 2 & 4; Flipphi *et al.* 1993a, c). Similar behaviour has been described in other fungal systems, *e.g.* cellobiohydrolase II (*cbh2* gene) of *Trichoderma reesei* and alcohol dehydrogenase I (*alcA* gene) of *A. nidulans* (Kubicek-Pranz *et al.*, 1991; Gwynne *et al.*, 1989). In the latter case this was due to limited expression of ALCR, the pathway-specific activator of the ethanol utilization regulon. In conclusion, the data presented suggest that at least three different activators, one titrated by multiple *abf*A copies, one titrated by multiple *abf*B copies and an *abn*A-specific one, are involved in ABN A expression. Moreover, arabinase expression seems to be co-regulated to some extent. The different behaviour of the three genes in wild-type and multiple copy transformants implies however that expression is basically governed by gene-specific factors, while coordinated control is restricted merely to modulation of the expression level.

Acknowledgement. I would like to express my gratitude towards Dr. Bernard Henrissat (Centre de Recherches sur les Macromolécules Végétales, CNRS, Grenoble, France) for his help and interest concerning hydrophobic cluster analysis.

References

Beldman G, Searle-van Leeuwen MJF, Ruiter GA de, Siliha HA, Voragen AGJ (1993) Degradation of arabinans by arabinanases from *Aspergillus aculeatus* and *Aspergillus niger*. Carbohydr Polym **20**:159-168

Boorstein WR, Craig EA (1990) Regulation of a yeast HSP70 gene by a cAMP responsive transcriptional control element. EMBO J **9**:2543-2553

Bussink HJD, Buxton FP, Fraaye BA, Graaff LH de, Visser J (1992) The polygalacturonases of *Aspergillus niger* are encoded by a family of diverged genes. Eur J Biochem **208**:83-90

Calzone FJ, Britten RJ, Davidson EH (1987) Mapping of gene transcripts by nuclease protection assays and cDNA primer extension. Methods Enzymol **152**:611-632

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294-5299

Dente L, Cortese R (1987) pEMBL: a new family of single-stranded plasmids for sequencing DNA. Methods Enzymol **155**:111-119

Fidel S, Doonan JH, Morris NR (1988) *Aspergillus nidulans* contains a single actin gene which has unique intron locations and encodes a γ-actin. Gene **70**:283-293

Flipphi MJA, Heuvel M van, Veen P van der, Visser J, Graaff LH de (1993 a) Cloning and characterization of the *abf*B gene coding for the major α-L-arabinofuranosidase (ABF B) of *Aspergillus niger*. Curr Genet **24**:525-532

Flipphi MJA, Panneman P, Veen P van der, Visser J, Graaff LH de (1993 b) Molecular cloning, expression and structure of the endo-1,5-α-L-arabinase gene of *Aspergillus niger*. Appl Microbiol Biotechnol **40**:318-326

Flipphi MJA, Visser J, Veen P van der, Graaff LH de (1993 c) Cloning of the *Aspergillus niger* gene encoding α-L-arabinofuranosidase A. Appl Microbiol Biotechnol **39**:335-340

Gaboriaud C, Bissery V, Benchetrit T, Mornon JP (1987) Hydrophobic cluster analysis: an efficient new way to compare and analyse amino acid sequences. FEBS Lett **224**:149-155

Glišin V, Crkvenjakov R, Byus C (1974) Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry **13**:2633-2637

Goyon C, Faugeron G, Rossignol J-L (1988) Molecular cloning and characterization of the *met2* gene from *Ascobolus immersus*. Gene **63**:297-308

Graaff L de, Broek H van den, Visser J (1988) Isolation and expression of the *Aspergillus nidulans* pyruvate kinase gene. Curr Genet **13**:315-321

Gurr SJ, Unkles SE, Kinghorn JR (1987) The structure and organization of nuclear genes of filamentous fungi. In: Kinghorn JR (ed) Gene structure in eukaryotic microbes. SGM Special Publication. Vol. 23. IRL Press, Oxford. pp.93-139

Gwynne DI, Buxton FP, Williams SA, Sills AM, Johnstone JA, Buch JK, Guo Z-M, Drake D, Westphal M, Davies RW (1989) Development of an expression system in *Aspergillus nidulans*. Biochem Soc Trans **17**:338-340

Heijne G von (1986) A new method for predicting signal sequence cleavage sites. Nucleic Acids Res **14**:4683-4691

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J **280**:309-316

Hirani S, Bernasconi RJ, Rasmussen JR (1987) Use of peptide-*N*-glycosidase F to release asparagine-linked oligosaccharides for structural analysis. Anal Biochem **162**:485-492

Katsuragi N, Takizawa N, Murooka Y (1987) Entire nucleotide sequence of the pullulanase gene of *Klebsiella aerogenes* W70. J Bacteriol **160**:2301-2306

Kellett LE, Poole DM, Ferreira LMA, Durrant AJ, Hazlewood GP, Gilbert HJ (1990) Xylanase B and an arabinofuranosidase from *Pseudomonas fluorescens* subsp. *cellulosa* contain identical cellulose-binding domains and are encoded by adjacent genes. Biochem J **272**: 369-376

Kormelink FJM, Gruppen H, Voragen AGJ (1993) Mode of action of (1→4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) and α-L-arabinofuranosidases on alkali-extractable wheat-flour arabinoxylan. Carbohydr Res **249**:345-353

Kornacker MG, Pugsley AP (1989) Molecular characterization of *pulA* and its product, pullulanase, a secreted enzyme of *Klebsiella pneumoniae* UNF5023. Mol Microbiol **4**:73-85

Kubicek-Pranz EM, Gruber F, Kubicek CP (1991) Transformation of *Trichoderma reesei* with the cellobiohydrolase II gene as a means for obtaining strains with increased cellulase production and specific activity. J Biotechnol **20**:83-94

Kulmburg P, Mathieu M, Dowzer C, Kelly J, Felenbok B (1993) Specific binding sites in the *alcR* and *alcA* promoters of the ethanol regulon for the CREA repressor mediating carbon catabolite repression in *Aspergillus nidulans*. Mol Microbiol **7**:847-857

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685

Lerouge P, O'Neill MA, Darvill AG, Albersheim P (1993) Structural characterization of endo-glycanase-generated oligoglycosyl side chains of rhamnogalacturonan I. Carbohydr Res **243**:359-371

McCleary BV (1991) Comparison of endolytic hydrolases that depolymerize 1,4-β-D-mannan, 1,5-α-L-arabinan and 1,4-β-D-galactan. In: Leatham GF, Himmel ME (eds) Enzymes in biomass conversion. ACS Symp Ser **460**. American Chemical Society, Washington DC. pp.437-449.

McMaster GK, Carmichael GG (1977) Analysis of single- and double stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc Natl Acad Sci USA **74**:4835-4838

McNally MT, Free SJ (1988) Isolation and characterization of a *Neurospora* glucose-repressible gene. Curr Genet **14**:545-551

Melasniemi H, Paloheimo M, Hemiö L (1990) Nucleotide sequence of the α-amylase-pullulanase gene from *Clostridium thermohydrosulfuricum*. J Gen Microbiol **136**:447-454

Meldgaard M, Svendsen I (1994) Different effects of *N*-glycosylation on the thermostability of highly homologous bacterial (1,3-1,4)-β-glucanases secreted from yeast. Microbiology **140**: 159-166

Morosoli R, Durand S, Moreau A (1992) Cloning and expression in *Escherichia coli* of a xylanase-encoding gene from the yeast *Cryptococcus albidus*. Gene **117**:145-150

Mullaney EJ, Gibson DM, Ullah AH (1991) Positive identification of a lambda gt11 clone containing a region of fungal phytase gene by immunoprobe and sequence verification. Appl Microbiol Biotechnol **35**:611-614

Nishitani K, Nevins DJ (1989) Enzymic analysis of feruloylated arabinoxylans (feraxan) derived from *Zea mays* cell walls. II. Fractionation and partial characterization of feraxan fragments dissociated by a *Bacillus subtilis* enzyme (feraxanase). Plant Physiol **91**:242-248

Rambosek J, Leach J (1987) Recombinant DNA in filamentous fungi: progress and prospects. CRC Crit Rev Biotechnol **6**:357-393

Roesler WJ, Vanderbark GR, Hanson RW (1988) Cyclic AMP and the induction of eukaryotic gene transcription. J Biol Chem **263**:9063-9066

Rombouts FM, Voragen AGJ, Searle-van Leeuwen MF, Geraerds CCJM, Schols HA, Pilnik W (1988) The arabinanases of *Aspergillus niger* – purification and characterisation of two α-L-arabinofuranosidases and an *endo*-1,5-α-L-arabinanase. Carbohydr Polym **9**:25-47

Sakai T, Sakamoto T (1990) Purification and some properties of a protopectin-solubilizing enzyme that has potent activity on sugar beet protopectin. Agric Biol Chem **54**:879-889

Sakka K, Yoshikawa K, Kojima Y, Karita S-i, Ohmiya K, Shimada K (1993) Nucleotide sequence of the *Clostridium stercorarium xylA* gene encoding a bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities, and properties of the translated product. Biosci Biotech Biochem **57**:268-272

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74**:5463-5467

Tanner W, Lehle L (1987) Protein glycosylation in yeast. Biochim Biophys Acta **906**:81-99

Tarentino AL, Plummer TH Jr (1987) Peptide-N-(*N*-acetyl-β-glucosamidyl) asparagine amidase and endo-β-N-acetylglucosaminidase from *Flavobacterium meningosepticum*. Methods Enzymol **138**:770-778

Unkles SE (1992) Gene organization in industrial filamentous fungi. In: Kinghorn JR, Turner G (eds) Applied molecular genetics of filamentous fungi. 1st edn. Blackie Academic & Professional, London. pp.28-53

Utt EA, Eddy CK, Keshav KF, Ingram LO (1991) Sequencing and expression of the *Butyrivibrio fibrisolvens xyl*B gene encoding a novel bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities. Appl Environ Microbiol **57**:1227-1234

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1991) Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. Arch Microbiol **157**:23-28

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1993) Induction of extracellular arabinases on monomeric substrates in *Aspergillus niger*. Arch Microbiol **159**:66-71

Vis JW van de, Searle-van Leeuwen MF, Siliha HA, Kormelink FJM, Voragen AGJ (1991) Purification and characterization of *endo*-1,4-β-D-galactanases from *Aspergillus niger* and *Aspergillus aculeatus*: use in combination with arabinanases from *Aspergillus niger* in enzymatic conversion of potato arabinogalactan. Carbohydr Polym **16**:167-187

Voragen AGJ, Rombouts FM, Searle-van Leeuwen MF, Schols HA, Pilnik W (1987) The degradation of arabinans by endo-arabinanase and arabinofuranosidases purified from *Aspergillus niger*. Food Hydrocoll **1**:423-437

Whitaker JR (1984) Pectic substances, pectic enzymes and haze formation in fruit juices. Enzyme Microb Technol **6**:425-432

Will F, Dietrich H (1992) Isolation, purification and characterization of neutral polysaccharides from extracted apple juices. Carbohydr Polym **18**:109-117

Witteveen CFB, Busink R, Vondervoort P van de, Dijkema C, Swart K, Visser J (1989) L-Arabinose and D-xylose catabolism in *Aspergillus niger*. J Gen Microbiol **135**:2163-2171

Chapter 6

Characterization of an *Aspergillus nidulans* L-arabitol dehydrogenase mutant [1]

The degradation pathway for L-arabinose, which consists of a sequence of alternating reduction and oxidation reactions prior to ultimate phosphorylation was studied in *Aspergillus nidulans* wild-type as well as in an L-arabinose non-utilizing mutant. The inability of the mutant to use L-arabinose was caused by the absence of NAD^+-dependent L-arabitol dehydrogenase activity. The effect of the mutation on polyol accumulation patterns was studied upon growth on various carbon sources. The presence of L-arabinose resulted in intracellular accumulation of arabitol in this mutant. Moreover, the mutant secreted arabitol under these conditions and, in contrast to the wild-type, featured enhanced expression of enzymes involved in L-arabinose catabolism as well as of extracellular glycosyl hydrolases involved in degradation of the plant cell wall polysaccharide L-arabinan.

Introduction

Catabolism of D-xylose in fungi has been thoroughly studied during the last decade due to increased interest in direct conversion of plant biomass to ethanol (see recent reviews: Schneider 1989; Ajay Shing *et al.* 1992; Mishra and Ajay Singh 1993). L-Arabinose utilization received far less attention, despite the eminent presence of L-arabinofuranose in hemicellulosic and pectic plant polysaccharides (Stephen 1983). Previously L-arabinose catabolism has been studied in *Penicillium chrysogenum* and *Aspergillus niger* (Chiang and Knight 1961; Witteveen *et al.* 1989). In both organisms degradation involves a series of reduction and oxidation reactions (Fig. 1). L-Arabinose is subsequently converted into L-arabitol, L-xylulose, xylitol and D-xylulose. The latter two metabolites are common intermediates of L-arabinose and D-xylose catabolism. The reduction steps are catalyzed by NADPH-dependent reductases and the oxidation steps by NAD^+-dependent dehydrogenases. The equilibrium in the route is towards the formation of the pentitols, hence their accumulation, and catabolism is driven by irreversible phosphorylation of D-xylulose to D-xylulose 5-phosphate which is further metabolized via the non-oxidative pentose phosphate pathway. In *A. niger*

[1] a shortened version of this Chapter is published in: FEMS Microbiol Lett **123**:83-90 (1994)

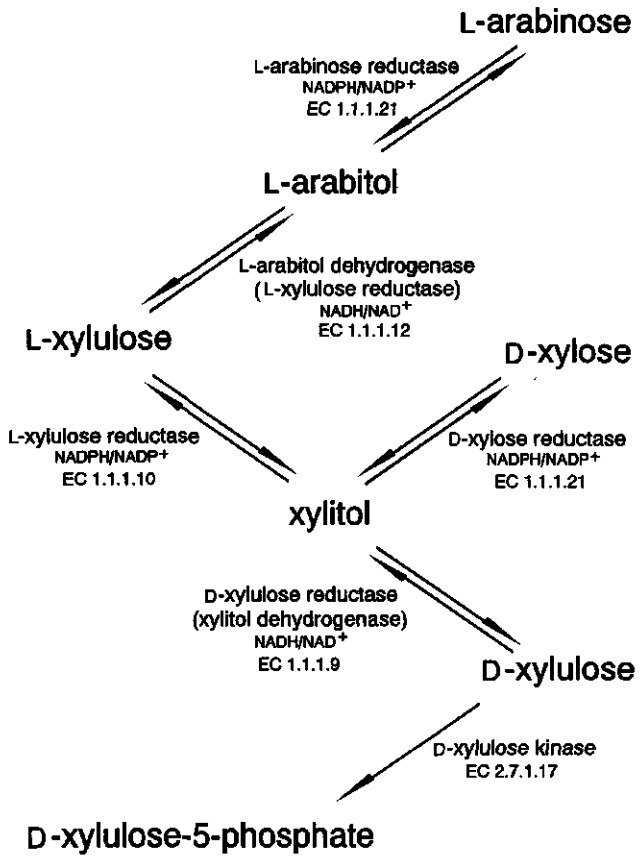


Fig.1. Pentose catabolism in *Aspergillus niger*

a D-xylulose kinase negative mutant (*xkiA1*) unable to utilize D-xylose and L-arabinose, has been isolated (Witteveen *et al.* 1989). *Aspergillus nidulans* strain G094, originally described as a mutant in D-galactose utilization (Roberts 1963, 1970), was reinvestigated by Clutterbuck (1981) and found to be an L-arabinose non-utilizing mutant (*araA1*). This Chapter describes the nature of the biochemical defect of this mutant and how this affects the physiology of the strain. Furthermore, in view of the accumulation of L-arabitol, the impact of the mutation for the expression of the L-arabinan-degrading enzymes has been investigated.

The ability of L-arabitol to mediate expression of α-L-arabinofuranosidase (ABF) (EC 3.2.1.55) and endo-1,5-α-L-arabinase (ABN) (EC 3.2.1.99), the main activities in the extracellular degradation of the plant cell wall polysaccharide L-arabinan (Kaji 1984; Voragen *et al.* 1987), has been documented in both *A. niger* and *A. nidulans* (Tagawa and Terui 1968; van der Veen *et al.* 1993; Ramón *et al.* 1993). The relevance of L-arabitol as an inducer of arabinases is further indicated by the induction characteristics of formentioned *A. niger* D-xylulose kinase mutant. Upon transfer to L-arabitol, this strain features enhanced expression of three different arabinases, *viz.* ABF A, ABF B and ABN A (van der Veen *et al.* 1993). In *A .nidulans* one of the ABF activities (ABF A) lacks (Chapter 2; Flipphi *et al.* 1993; Ramón *et al.* 1993). The other two are expressed on L-arabitol and L-arabinose (Ramón *et al.* 1993). The present Chapter provides further evidence towards a role for L-arabitol in fungal arabinase induction.

Materials and methods

Strains. *A. nidulans* WG096 (*pabaA1, yA2*) (FGSC 187) was used as a wild-type with respect to L-arabinose catabolism. *A. nidulans* G094 (*biA1, wA3, araA1*) is an L-arabinose non-utilizing strain (Clutterbuck 1981), as is *A. nidulans* G844 (*yA2, wA2, cnxH4, araA1*). All strains descend from the Glasgow strain FGSC 4.

Media and growth conditions. Minimal medium was composed essentially as described by Witteveen *et al.* (1989), using either $NaNO_3$ (6 g/l) or NH_4Cl (4 g/l) as the nitrogen source. The pH of the medium was set at 6.0. For plates, medium was solidified with 1.5 % (w/v) agar. Carbon sources and supplements were sterilised separately as concentrated stock solutions. Carbon sources were added to a final concentration of 100 mM and all media were supplemented with 4 μg/l biotin and 2 mg/l *para*-aminobenzoic acid. *A. nidulans* was grown at 37°C. Liquid cultures contained $NaNO_3$ as the nitrogen source and were inoculated with 10^6 spores/ml. Fungal biomass for enzyme assays and polyol extractions was generated during 16 h of submerged growth in conical flasks using a rotary shaker at 250 rpm. For transfer experiments biomass was generated on D-glucose as described above. Mycelium was harvested by filtration and washed with saline. This was transferred to fresh minimal medium with L-arabinose as sole carbon source and incubated for another 8 h.

Chemicals. L-Arabinose, D-arabitol, L-arabitol, D-xylose, xylitol, D-xylulose and L-xylulose and *para*-nitrophenyl α-L-arabinofuranoside (PNP-A) were obtained from Sigma, D-galactose from BDH, and galactitol (dulcitol) from Fluka. ArabinaZyme tablets were obtained from Megazyme (Warriewood (Sydney), Australia). Auxiliary enzymes used in D-xylulose kinase assays, pyruvate kinase (EC 2.7.1.40) and L-lactate dehydrogenase (EC 1.1.1.27), were supplied by Boehringer Mannheim.

Enzyme assays. Enzyme activities of the L-arabinose degradation pathway were determined in cell-free extracts as described by Witteveen *et al.* (1989). Assays were performed at 37°C. D-Xylulose kinase activity was corrected for D-xylulose reductase and ATP-ase activities. The protein concentration of cell-free extracts were determined with the Bicinchoninic acid protein assay kit (Sigma) according to the supplier's instructions and using bovine serum albumin as a standard. For determination of extracellular arabinases culture medium was dialyzed against 1 mM sodium phosphate buffer, pH 6.0, and subsequently lyophilized. The dry material was redissolved in such a volume of bidistilled water, that all samples were normalized to equal biomass. ABF was assayed by measuring hydrolysis of PNP-A (van der Veen *et al.* 1991) at

Table 1. Growth of *A. nidulans* wild-type strain WG096 and of L-arabinose non-utilizing strain G094 on different carbon sources.[a]

Carbon source	WG096			G094		
	no addition	+ L-arabinose (100 mM)	+ L-arabitol (100 mM)	no addition	+ L-arabinose (100 mM)	+ L-arabitol (100 mM)
D-glucose	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
D-fructose	+ + +	+ + +	+ + +	+ + +	−	+
D-galactose	+ +	+ + +	+ + +	+	*	*
D-xylose	+ + +	+ + +	+ + +	+ + +	+	+ +
L-arabinose	+ + +	nd [b]	nd	−	nd	nd
galactitol	+ +	+ + +	+ + +	−	−	−
L-arabitol	+ +	+ + +	nd	−	−	nd
D-arabitol	+ +	+ + +	+ +	+ +	+ + +	+
xylitol	+	+ + +	+ +	+	+	+
glycerol	+ +	+ + +	+ + +	+ +	−	+
L-glutamate	+ +	+ + +	+ + +	+ +	+	+

[a] Growth tests were done in duplicate except for galactitol and D-arabitol.
Score: [+ + +], good growth; [+ +], moderate growth; [+], poor growth; [*], very poor growth; [−], no growth.
Essentially identical results were obtained using either NH_4Cl or $NaNO_3$ as the nitrogen source.
[b] Not done.

37°C, using 25 µl of (concentrated) sample and an incubation time of 1 h. ABN activity was determined using ArabinaZyme tablets following the manufacturer's instructions, using 50 µl of (concentrated) sample and an incubation time of 1 h.

Determination of polyols in mycelium and culture medium. Harvesting and extraction of the mycelial samples was done as described by Witteveen *et al.* (1994). Medium samples were diluted 50 times with bidistilled water and insoluble material was removed by centrifugation, prior to polyol analysis. Polyol contents were determined on a HPLC system (Dionex) equipped with a CarboPac MA-1 column using isocratic elution with 0.48 M NaOH and pulsed electrochemical detection. HPLC can not differentiate between D- and L-configurations of polyols.

Results and Discussion

Growth tests with the L-arabinose non-utilizing A. nidulans mutant G094

A. nidulans strain G094 was isolated as a mutant less able to utilize D-galactose featuring an abberant morphology (Roberts 1963, 1970). Clutterbuck (1981) found that this strain was unable to grow on L-arabinose but could not recover the original phenotype on D-galactose. This mutant (*araA1*) was reinvestigated to establish whether it provided a useful tool to investigate L-arabinose catabolism and arabinase induction in *A. nidulans*.

The results of the growth tests (Table 1) confirmed the major conclusions from Clutterbuck (1981). Except for L-arabinose, L-arabitol and galactitol, all carbon sources tested supported growth of G094, including D-galactose. Compared to WG096, the mutant grew slower on all growth-supporting substrates. The presence of either L-arabinose or L-arabitol inhibited growth of G094 to various extent, except in case of D-glucose and D-arabitol. Inhibition by L-arabinose was found to be stronger in most cases. Interestingly, L-arabitol suppressed growth on D-arabitol while L-arabinose seemed to enhance growth. The growth characteristics were identical regardless whether ammonium or nitrate salts were used as the nitrogen source (data not shown). *A. nidulans* G844, which carries the *araA1* mutation in a distinct genetic background, features identical growth characteristics.

The biochemical defect of the *araA1* mutation

As a first step to elucidate the nature of the mutation present in strain G094, the polyol levels in wild-type and mutant mycelium which was pregrown on D-glucose and transferred to L-arabinose medium, were determined (Table 2). Both arabitol and xylitol accumulated in wild-type mycelium. The same was previously observed in L-arabinose-grown *A. niger* (Witteveen *et al.* 1989, 1994), suggesting that the same pathway is used in *A. nidulans*. In the mutant the intracellular arabitol level was about four times higher than in the wild-type, whereas the xylitol level was negligable. Erythritol and mannitol levels were much lower in the mutant and became reduced to 10 % of the wild-type levels, presumably because these pools were remetabolized. As shown in Table 1, G094

Table 2. Levels of intracellular polyols in *A. nidulans* wild-type strain WG096 and the L-arabinose non-utilizing strain G094 grown on different carbon sources.[a]

Strain	Carbon source	glycerol	erythritol	xylitol	arabitol	mannitol
WG096	L-arabinose [transfer][b]	23	19	26	184	335
G094	L-arabinose [transfer][b]	20	2	1	825	38
WG096	D-glucose	9	82	0	7	385
WG096	L-arabinose	15	26	41	207	417
WG096	L-arabinose/D-glucose	37	115	3	37	727
G094	L-arabinose/D-glucose	17	6	0	563	155
WG096	D-xylose	7	39	62	20	404
G094	D-xylose	19	32	116	17	623
WG096	L-arabinose/glycerol	355	22	19	153	483
G094	L-arabinose/glycerol	254	1	3	482	1

[a] Growth was for 16 h at 37°C. Polyol concentrations are expressed in μmoles/mg mycelial dry weight. The data given are the average of two independent experiments except for L-arabinose and D-xylose which were done in triplicate.

[b] Mycelium was grown on D-glucose (100 mM) for 16 h and then transferred to fresh minimal medium with L-arabinose (100 mM) for another 8 h.

Table 3. Levels of enzymes involved in L-arabinose and D-xylose catabolism upon growth on different carbon sources of *A. nidulans* wild-type strain WG096 and L-arabinose non-utilizing strain G094.[a]

Strain	Carbon source	NADPH-dependent L-arabinose reductase	NADPH-dependent D-xylose reductase	NAD^+-dependent L-arabitol dehydrogenase	NADH-dependent L-xylulose reductase	NADPH-dependent L-xylulose reductase	NAD^+-dependent xylitol dehydrogenase	NADH-dependent D-xylulose reductase	D-xylulose kinase
WG096	L-arabinose [transfer][b]	365	201	121	143	107	399	120	411
G094	L-arabinose [transfer][b]	794	514	12	0	408	899	383	450
WG096	D-glucose	0	0	10	0	0	23	27	28
WG096	L-arabinose	730	336	367	289	271	360	228	719
WG096	L-arabinose/D-glucose	13	0	32	0	52	32	41	123
G094	L-arabinose/D-glucose	424	444	11	0	126	238	300	564
WG096	D-xylose	412	2350	33	0	33	438	327	478
G094	D-xylose	406	1860	0	0	0	199	176	336
WG096	L-arabinose/glycerol	442	ND[c]	183	103	103	228	ND	ND
G094	L-arabinose/glycerol	1411	1287	0	0	482	413	ND	ND

[a] Growth was for 16 h at 37°C. Enzyme activities given are the average of two independent experiments and are expressed in μmoles of substrate converted per min per mg of protein.

[b] Mycelium was grown on D-glucose (100 mM) for 16 h and then transferred to fresh minimal medium with L-arabinose (100 mM) for another 8 h.

[c] Not determined.

did not grow on L-arabinose or L-arabitol, whereas D-xylose and xylitol supported growth. This fact and the polyol accumulation pattern indicated that one of the two enzymatic steps between L-arabitol and xylitol (*cf.* Fig.1) is blocked. Enzyme activity measurements in mycelial extracts of L-arabinose-grown wild-type *A. nidulans* (Table 3) confirmed the presumed pathway. Mutant strain G094 clearly lacked NAD^+-dependent L-arabitol dehydrogenase activity after transfer to L-arabinose (Table 3). The observation that the mutant could not grow on galactitol (Table 1) suggested that the same enzyme catalyzes the conversion of galactitol in D-tagatose (EC 1.1.1.16).

Enzyme induction in L-arabinose catabolism and effects of the *araA1* mutation

Once the biochemical defect of the *araA1* mutation was established, the induction of the enzymes involved in the L-arabinose degradation pathway was investigated in more detail. Various carbon sources and mixed carbon sources were tested for their effects (Table 3). From the data, the following was concluded. (1) On D-glucose no or very low activities were measured both in wild-type and mutant, whereas pentitols were low or even absent (*cf.* Table 2). (2) All the enzyme activities of the L-arabinose catabolic pathway were induced in the wild-type and the mutant strain upon transfer to L-arabinose-containing medium except for NAD^+-dependent L-arabitol dehydrogenase, which was absent in the mutant. This is also indicated by the absence of NADH-dependent L-xylulose reductase activity catalyzing the reverse reaction (*cf.* Fig.1). In the mutant there was also induction of those enzymes catalyzing the conversions after the L-arabitol dehydrogenase step. This indicated a common inducer (either L-arabinose or L-arabitol) for the entire catabolic route. Moreover, the induction pattern suggested that the *araA1* mutation concerned the structural gene for NAD^+-dependent L-arabitol dehydrogenase rather than a regulatory gene. (3) On D-xylose, NAD^+-dependent L-arabitol dehydrogenase and NADPH-dependent L-xylulose reductase were absent. These enzymes were found to be specific for the L-arabinose pathway suggesting that the catabolic routes for D-xylose and L-arabinose are induced independently. This was further emphasized by the differences in the levels of L-arabinose and D-xylose reductase activities found upon growth on L-arabinose and D-xylose, respectively. Similar observations were previously made for *A. niger* and *Aureobasidium pullulans* (Witteveen *et al.* 1989; Machová 1992). The enzymes converting the intermediates common to both pathways were induced on both pentoses. (4) In mutant and wild-type mycelia grown on D-xylose, the levels of D-xylose reductase and D-xylulose kinase were comparible, but the NAD^+-dependent xylitol dehydrogenase activity was only half of the wild-type level. This reduced activity correlated with a twofold increase in intracellular xylitol (Table 2). This might be the explanation for the somewhat poorer growth of the mutant on D-xylose. Obviously, xylitol did not induce its own conversion. The relation of this lower xylitol dehydrogenase activity upon growth on D-xylose with the mutation in L-arabitol dehydrogenase is

not clear. (5) The enzyme activities in the wild-type were repressed in mycelia grown on a mixture of L-arabinose and D-glucose. This indicated that L-arabinose catabolism is subject to carbon catabolite repression. On a mixed carbon source of glycerol and L-arabinose the pathway was induced in the wild-type, although at a lower level than on L-arabinose only. In the *araA1* mutant, only a weak repression was observed on a mixture of L-arabinose and D-glucose. Apparently catabolic repression by D-glucose was overruled in the mutant, most likely as a result of the gradual accumulation of L-arabitol (see below) due to some L-arabinose uptake which then triggered a further cascade in the induction of the pathway. On solid medium the mutant strain G094 did not grow on a mixture of L-arabinose and glycerol (Table 1). However, as submerged culture there was growth although reduced. The mutant overexpressed NADPH-dependent L-arabinose reductase, NADPH-dependent L-xylulose reductase and NAD^+-dependent xylitol dehydrogenase activity under these conditions. The effect on D-xylulose kinase was not determined but is expected to be identical, considering the proposed coordinated regulation of the pathway.

Polyol accumulation

Polyol levels have been determined in the same mycelia that were used for enzyme determinations (Table 2) and also in the media of the cultures (Table 4). In the wild-type grown on D-glucose, mannitol and erythritol prevailed whereas no or only low levels of pentitols were observed. When wild-type *A. nidulans* was grown on L-arabinose, a polyol accumulation pattern was observed comparible to that found upon mycelial transfer to L-arabinose-containing medium. Mainly mannitol and arabitol were present. The erythritol level was threefold reduced as compared to growth on D-glucose whereas some xylitol appeared. The high level of arabitol present in mutant mycelium upon transfer to L-arabinose was already mentioned above. As expected, xylitol lacked in the mutant due to the metabolic block. Likewise, erythritol and mannitol levels were low compared to the wild-type since these polyols were metabolized in the mutant after transfer. The pentitol levels were strongly reduced in the wild-type when L-arabinose and D-glucose were provided simultaneously, which is in agreement with the enzyme activity measurements (*cf.* Table 3). The less effective repression of the enzymes of the L-arabinose catabolic pathway in the *araA1* mutant resulted in arabitol accumulation, concomitant with an approximately 80 % reduction in the size of the mannitol pool. When L-arabinose and glycerol were provided simultaneously the major polyols in the wild-type were mannitol, glycerol and arabitol, compared to arabitol and glycerol in the mutant. Remarkable is the absence of mannitol in the mutant although the strain is perfectly able to accumulate mannitol in the absence of L-arabinose (see *e.g.* data on D-xylose in Table 2). When in a previous study glycerol was used as the only carbon source high levels of mannitol were found in wild-type *A. nidulans* (Dijkema *et al.* 1985). The lack of mannitol in the mutant might be related to increased mannitol conversion to meet the energy

Tabel 4. Concentration of extracellular polyols in culture media of *A. nidulans* wild-type strain WG096 and L-arabinose non-utilizing strain G094 upon growth on or transfer to different carbon sources.[a]

Strain	Carbon source	glycerol	erythritol	arabitol	mannitol
WG096	L-arabinose [transfer][b]	0	0	0	0
G094	L-arabinose [transfer][b]	0	0	3.9	0
WG096	D-glucose	0.15	0.16	0	0.14
WG096	L-arabinose	0	0	0	0
WG096	D-glucose/L-arabinose	0.19	0.04	0	0.22
G094	D-glucose/L-arabinose	0.03	0	0.22	0
WG096	D-xylose	0	0	0	0
G094	D-xylose	0	0	0	0
WG096	L-arabinose/glycerol	> 50 [c]	0	0	0
G094	L-arabinose/glycerol	> 50 [c]	0	6.4	0

[a] Growth was for 16 h at 37 °C. Polyol concentrations are expressed in mM. The data given are the average of two independent experiments except for L-arabinose and D-xylose which were done in triplicate.

[b] Mycelium was grown on D-glucose (100 mM) for 16 h and then transferred to fresh minimal medium with L-arabinose (100 mM) for another 8 h.

[c] Glycerol is used as a carbon source for growth, present at 100 mM at the start of the culture.

Table 5. Induction of L-arabinan-degrading enzymes in *A. nidulans* wild-type strain WG096 and of L-arabinose non-utilizing strain G094 on mixed carbon sources.

Carbon source	Strain	Biomass yield [a]	Extracellular arabitol concentration [b]	ABF activity [c]	ABN activity [d]
L-arabinose	WG096	2.7	0.00	0.29	3.45
L-glutamate/L-arabinose	WG096	3.3	0.00	0.05	1.87
	G094	1.1	2.90	4.85	16.72
glycerol/L-arabinose	WG096	2.5	0.00	0.01	0.00
	G094	1.6	2.66	4.08	26.75
D-galactose/L-arabinose	WG096	3.1	0.00	0.10	1.97
	G094	1.3	1.11	1.05	8.77
D-fructose/L-arabinose	WG096	3.4	0.00	0.00	0.00
	G094	2.1	2.00	0.88	4.85
D-xylose/L-arabinose	WG096	3.2	0.00	0.09	2.13
	G094	3.0	0.50	0.66	3.97
D-glucose/L-arabinose	WG096	3.6	0.00	0.01	0.00
	G094	3.2	0.23	0.14	1.76

All data are the average of two independent experiments.

[a] Biomass generated upon 24 h of cultivation expressed in mg mycelial dry weight/ml culture medium.

[b] Extracellular arabitol concentration expressed in μmoles/mg mycelial dry weight.

[c] Enzyme activity was determined as described in Materials and Methods and is expressed in mUnits/mg mycelial dry weight. One Unit is the amount of enzyme required to release 1 μmol of *para*-nitrophenol per min.

[d] Enzyme activity was determined as described in Materials and Methods and is expressed in mUnits/mg mycelial dry weight. One Unit is the amount of enzyme required to release 1 μmol of L-arabinose reducing-sugar equivalents per min.

requirement and the requirement for NADPH in the mutant since in the presence of L-arabinose glycerol utilization is impaired (*cf.* Table 5). L-Arabitol is not likely to be toxic since the intracellular L-arabitol concentration in the mutant is even higher on a mixture of D-glucose and L-arabinose, which results in normal growth, than on glycerol and L-arabinose (*cf.* Table 2).

In some cultures secretion of polyols into the medium was observed (Table 4). This was *e.g.* the case for wild-type *A. nidulans* grown for 16 h on D-glucose and on a mixture of D-glucose and L-arabinose. Erythritol, glycerol and mannitol were found in a range between 50 – 200 μM. However, in the *araA1* mutant high levels of extracellular arabitol (up to 6.4 mM) appeared after mycelial transfer to L-arabinose and upon growth on a mixture of glycerol and L-arabinose. Of the total amount of arabitol in these cultures (intra- and extra-cellular) more than 95 % was found to be present in the medium. The fungus apparently has mechanisms to secrete excess of polyols if required (Witteveen 1993).

Effect of the araA1 mutation on the expression of L-arabinan-degrading enzymes

Since the L-arabitol dehydrogenase-deficient *A. nidulans* mutant G094 accumulated arabitol in the presence of L-arabinose, the effects of mixed carbon sources consisting of carbon catabolite repressing or non-repressing substrates and L-arabinose on the expression levels of the *A. nidulans* arabinases were tested. The results are presented in Table 5. In submerged cultures of mutant strain G094 biomass formation from most carbon sources was restricted in the presence of L-arabinose, but in neither case completely inhibited as was the case on solid media (*cf.* Table 1). The inability to maintain a proper redox balance is probably causing growth problems when the mutant is grown on L-glutamate, D-fructose, D-galactose or glycerol in the presence of L-arabinose. After 24 h of growth the cultures were harvested and the medium was analyzed for polyol contents and for arabinase activities present. Wild-type *A. nidulans* produced considerable levels of arabinase in the presence of L-glutamate, D-galactose and D-xylose, whereas D-glucose, D-fructose and glycerol repressed L-arabinose-mediated expression. The mutant produced arabitol and arabinases under all conditions tested while in identically grown cultures of wild-type strain WG096, no arabitol was observed in the medium. Notably, the substrates on which the mutant secreted high amounts of arabitol simultaneously exhibited high arabinase expression and featured reduced growth. In contrast to what has been observed in the wild-type, glycerol proved to be one of the best co-substrates with respect to arabinase expression in mutant strain G094. When glycerol or L-glutamate were used as the co-substrate, the arabinase expression levels were up to 20 times higher than found in the wild-type in the sole presence of L-arabinose. Furthermore, similar to what has been observed for the enzymes of the L-arabinose catabolic pathway, the mutant synthesized considerable amounts of arabinase on a mixed carbon source of D-glucose and L-arabinose.

The data presented confirm that L-arabitol plays an eminent role in the induction of the arabinase enzyme spectrum in *A. nidulans*. Carbon catabolite repression, however, is an important factor in determining the final levels of these enzymes in wild-type *A. nidulans*.

Acknowledgement. I would like to thank Dr. A.J. Clutterbuck (Institute of Genetics, Glasgow University, Glasgow, Scotland) for his kind gift of the *A. nidulans* strains G094 and G844.

References

Ajay Singh, Kumar PKR, Schügerl K (1992) Bioconversion of cellulosic materials to ethanol by filamentous fungi. Adv Biochem Eng/Biotechnol **45**:30-55

Chiang C, Knight SG (1961) L-Arabinose metabolism by cell-free extracts of *Penicillium chrysogenum*. Biochim Biophys Acta **46**:271-278

Clutterbuck AJ (1981) An arabinose non-utilizing mutant *araA1*. Aspergillus Newsletter **15**:21

Dijkema C, Kester HCM, Visser J (1985) ^{13}C NMR studies of carbon metabolism in the hyphal fungus *Aspergillus nidulans*. Proc Natl Acad Sci USA **82**:14-18

Flipphi MJA, Visser J, Veen P van der, Graaff LH de (1993) Cloning of the *Aspergillus niger* gene encoding *α*-L-arabinofuranosidase A. Appl Microbiol Biotechnol **39**:335-340

Kaji A (1984) L-Arabinosidases. Adv Carbohydr Chem Biochem **42**:383-394

Machová E (1992) Induction of aldose reductase and polyol dehydrogenase activities in *Aureobasidium pullulans* by D-xylose, L-arabinose and D-galactose. Appl Microbiol Biotechnol **37**:374-377

Mishra P, Ajay Singh (1993) Microbial pentose utilization. Adv Appl Microbiol **39**:91-152

Ramón D, Veen P van der, Visser J (1993) Arabinan degrading enzymes from *Aspergillus nidulans*: Induction and purification. FEMS Microbiol Lett **113**:15-22

Roberts CF (1963) The genetic analysis of carbohydrate utilization in *Aspergillus nidulans*. J Gen Microbiol **31**:45-58

Roberts CF (1970) Enzyme lesions in galactose non-utilizing mutants of *Aspergillus nidulans*. Biochim Biophys Acta **201**:267-283

Schneider H (1989) Conversion of pentoses to ethanol by yeast and fungi. CRC Crit Rev Biotechnol **9**:1-40

Stephen AM (1983) Other Plant Polysaccharides. In: Aspinall GO (ed) The Polysaccharides. Vol.2. Academic Press, New York NY. pp.97-193

Tagawa K, Terui G (1968) Studies on the enzymes acting on araban. IX. Culture conditions and process of arabanase production by *Aspergillus niger*. J Ferment Technol **46**: 693-700

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1991) Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. Arch Microbiol **157**:23-28

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1993) Induction of extracellular arabinases on monomeric substrates in *Aspergillus niger*. Arch Microbiol **159**:66-71

Voragen AGJ, Rombouts FM, Searle-van Leeuwen MF, Schols HA, Pilnik W (1987) The degradation of arabinans by endo-arabinanase and arabinofuranosidases purified from *Aspergillus niger*. Food Hydrocoll **1**:423-437

Witteveen CFB (1993) Gluconate formation and polyol metabolism in *Aspergillus niger*. Ph.D. thesis. Wageningen Agricultural University, Wageningen, The Netherlands. 135 pp.

Witteveen CFB, Busink R, Vondervoort P van de, Dijkema C, Swart K, Visser J (1989) L-Arabinose and D-xylose catabolism in *Aspergillus niger*. J Gen Microbiol **135**:2163-2171

Witteveen CFB, Weber F, Busink R, Visser J (1994) Purification and characterization of two xylitol dehydrogenases from *Aspergillus niger*. Microbiology **140**:1679-1685

Chapter 7

General discussion

Synopsis of preceding Chapters

In Chapters 2 to 5 of this thesis, the cloning and characterization of the structural genes coding for three extracellular glycosyl hydrolases constituting the *Aspergillus niger* L-arabinan-degrading complex were described. ABF A, ABF B and ABN A are genetically unrelated. ABF A is *N*-glycosylated whereas ABF B and ABN A are not – these two enzymes are only *O*-glycosylated. Arabinase-overproducing strains were generated by introducing multiple copies of the arabinase-encoding genes both in *A. niger* and *Aspergillus nidulans* uridine auxotrophic strains through co-transformation. Transformed strains were isolated by primary selection for uridine prototrophy. Subsequent overproduction of the enzyme encoded by the gene introduced in such transformants, was demonstrated both immunochemically and by assaying enzyme activity. ABN A overproduction in *A. niger abn*A transformants was found to be limited to approximately five times the wild-type level. This is in sharp contrast to what has been found in the heterologous host *A. nidulans*.

In Chapters 5 and 6, the regulation of L-arabinan degradation was addressed. Additional copies of either ABF-encoding gene in *A. niger* were shown to reduce, but not to silence expression of the wild-type ABN A-encoding gene upon induction with either sugar beet pulp or L-arabitol. The reduction of the expression level of *abn*A correlated with the *abf* gene dosage. The repression effected in the presence of extra *abf*B gene copies was more stringent and more persistent than that elicited by additional *abf*A copies. Similar effects were observed for the expression of the other *abf* gene in multiple copy *abf*A- and *abf*B-transformants. It was proposed that the *abf* genes titrate two distinct gene activators both involved in coordination of arabinase gene expression. Four distinct sequence motifs were found in common in the promoter regions of the three genes which might be involved in coordinated regulation. However, the three genes were shown to respond differently upon a mycelial transfer to L-arabitol-containing medium, indicating that induction operates primarily gene-specific. The presumed role of L-arabitol in the induction of fungal arabinases was further emphasized by the induction characteristics of an *A. nidulans* mutant strain unable to grow on the end-product of L-arabinan degradation, L-arabinose. This mutant was shown to lack NAD^+-dependent L-arabitol dehydrogenase activity resulting in L-arabitol accumulation, both intracellularly and in the culture

medium, whenever L-arabinose was present. Upon submerged growth on various carbon sources in the presence of L-arabinose, the mutant featured enhanced expression of the enzymes involved in extracellular L-arabinan degradation, and of those of intracellular L-arabinose catabolism. The co-substrates on which the mutant secreted large amounts of arabitol simultaneously exhibited high arabinase expression and featured reduced growth. L-Arabitol secretion and enzyme production were also observed on a mixed carbon source of D-glucose and L-arabinose, resulting in normal growth. Hence, in the presence of L-arabinose, the carbon catabolite repression normally conferred by D-glucose is overruled in the mutant.

In this Chapter, a remote sequence similarity amongst ABN A and some bacterial xylanolytic glycosyl hydrolases is presented. Furthermore, some of the results described in this thesis are discussed in relation to the enzymology and function of the *A. niger* arabinases, and to the regulatory mechanisms involved in the expression of the three arabinase genes.

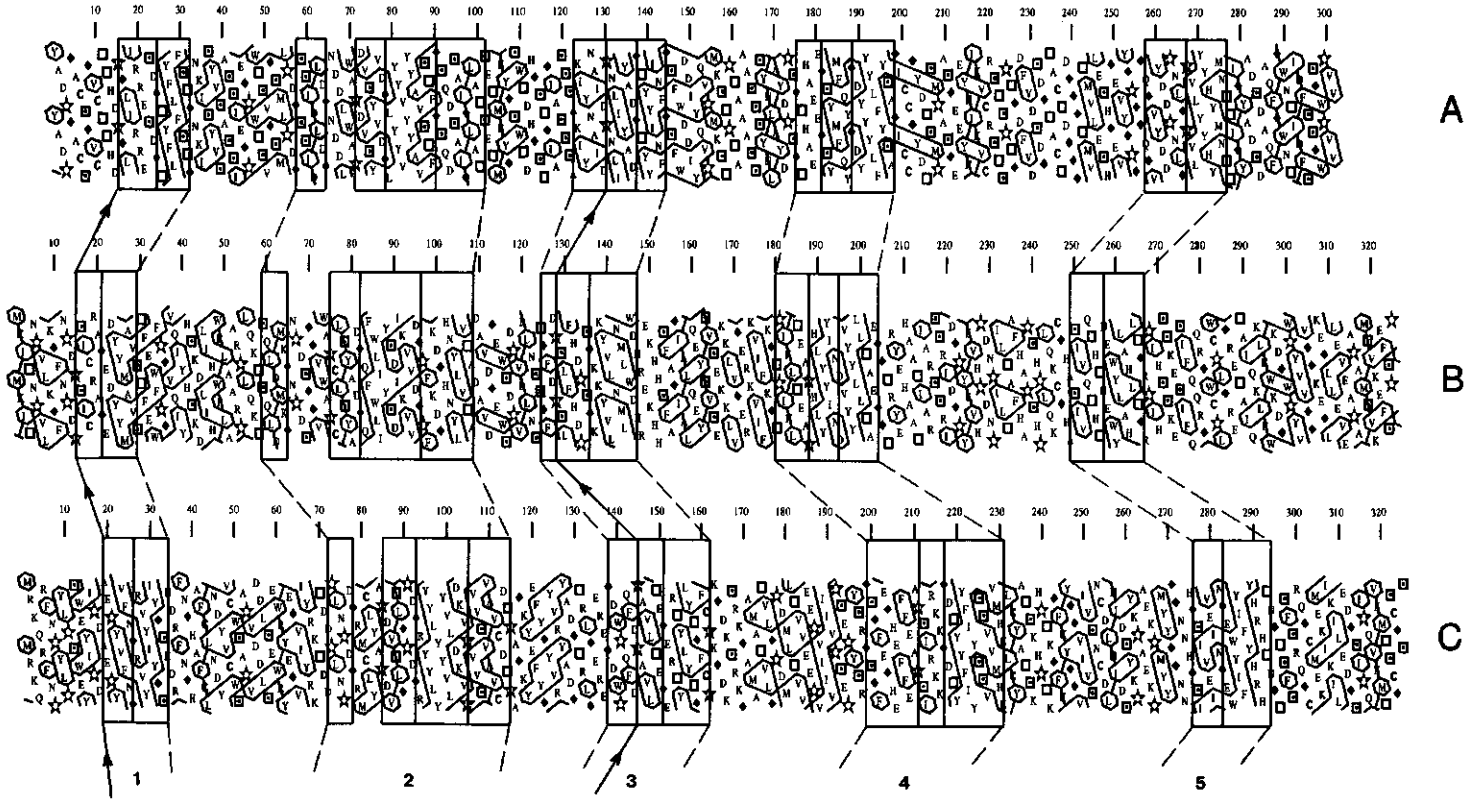
Glycosyl hydrolase family 43 might reflect an L-arabinofuranosyl-binding domain

Although acting on the same substrate, no sequence similarity could be detected amongst the three *A. niger* arabinases upon analysis using three distinct comparison modules available from the GCG analysis software package, version 6.0. (*cf.* Devereux *et al.* 1984; see Chapters 3, 4 & 5). Sequence similarity could indicate which amino acids are involved in substrate binding or hydrolysis (*cf.* Henrissat 1991). Moreover, similarities were not found comparing either *A. niger* arabinase with any other glycosyl hydrolase from which sequences are available, assuming that a similarity is only relevant when it is detected by both BLASTP (Altschul *et al.* 1990) and FASTA (Pearson and Lipman 1988). However, the analyses with ABN A yielded some interesting scores.

Protein library screening with the primary sequence of ABN A using BLASTP indicated similarities in common with the endo-1,4-β-D-xylanase XYN D from *Bacillus polymyxa* (Gosalbes *et al.* 1991). This XYN could not be assigned to either glycosyl hydrolase family 10 or 11, but is able to hydrolyze aryl-α-L-arabinofuranosides suggesting it is an L-arabinose-releasing XYN. FASTA analysis scored similarity with β-D-xylosidase XYL A from *Clostridium stercorarium* which also hydrolyzes PNP-A (Sakka *et al.* 1993). These two bacterial hydrolases, both involved in xylanolysis, scored similarity to each other in both FASTA and BLASTP library screenings. These results encouraged me to have a closer look at all available βXYL and XYN sequences by primary structure alignment using the GCG PILEUP module. No substantial similarities were found amongst XYNs using *B. polymyxa* XYN D, except with *Clostridium thermocellum* XYN Z (Grépinet *et al.* 1988) as was already reported by Gosalbes *et al.* (1991). Amongst the five βXYLs, I could clearly distinguish two groups; one containing XYN B from

"*Caldocellum saccharolyticum*" (Lüthi *et al.* 1990) and XYN B from *Thermoanaerobacterium saccharolyticum* (Lee and Zeikus 1993), the other XYN B from *Bacillus pumilus* (Xu *et al.* 1991) and XYL B from *Butyrivibrio fibrisolvens* (Utt *et al.* 1991). This was comfirmed by Henrissat and Bairoch (1993): glycosyl hydrolase families 41 and 43 comprise the same enzymes, respectively. Triple-sequence PILEUP alignments indicated that certain hydrophobic stretches present in both *B. pumilus* XYN B and *B. fibrisolvens* XYL B are also present in *C. stercorarium* XYL A, *B. polymyxa* XYN D and *A. niger* ABN A. This could be confirmed by hydrophobic cluster analysis of the primary structures of these five enzymes according to Gaboriaud *et al.* (1987) (see Fig.1). The five clusters have more or less similar spacing and occur in the same consecutive order in each of the enzymes. They map within the first 330 amino acid residues in all sequences. Notably, the βXYLs and XYN D are much larger than ABN A; the homology common to *C. thermocellum* XYN Z and *B. polymyxa* XYN D maps beyond this region. The observations indicate that *C. stercorarium* XYL A, *B. polymyxa* XYN D and *A. niger* ABN A might be assigned, at least provisionally, to glycosyl hydrolase family 43. A βXYL/ABF-encoding gene from *Bacteriodes ovatus*, available from the EMBL data library (T.R. Whitehead, unpublished; *cf.* Chapter 1), encodes a protein which is likely to belong to family 43 as well, as suggested by BLASTP and FASTA analyses.

Classification of glycosyl hydrolases could provide indications for common characteristics like substrate binding and the reaction mechanism (*cf.* Henrissat 1991). The cell-associated *B. pumilus* βXYL is the only enzyme out of family 43 which has been thoroughly studied biochemically. Its major physico-chemical characteristics were described by Claeyssens *et al.* (1975). The enzyme hydrolyzes only β-(1→4)-D-xylopyranosides following Michealis-Menten kinetics and lacks glycosyl-transferase activity (Kersters-Hilderson *et al.* 1978; van Doorslaer *et al.* 1985). Hydrolysis works with inversion of the anomeric configuration at carbon atom C1 of the product D-xylose (Kersters-Hilderson *et al.* 1976). The pH-dependence of its inactivation by diethyl pyrocarbonate and sulfhydryl-oxidizing compounds indicates that a histidine (pKa 7.0) and a protonated cysteine (pKa 8.0) are involved in catalysis (Saman *et al.* 1978; Kersters-Hilderson *et al.* 1984). The similarities conserved in family 43 might indicate that all βXYLs as well as XYN D hydrolyze β-(1→4)-linkages between D-xylopyranoses with inversion of configuration. Notably, almost all known XYNs are retaining hydrolases and assigned to glycosyl hydrolase families 10 or 11 (Gebler *et al.* 1992), but an *A. niger* XYN is documented to act conform an inverting mechanism (Frederick *et al.* 1981). Unfortunately, ABN A is not active against arabinoxylan (Voragen *et al.* 1987; Rombouts *et al.* 1988), thus unlikely to hydrolyze β-(1→4)-linkages between D-xylopyranoses. Whether the catalytic site for hydrolysis of these linkages is located within the clusters outlined in Fig.1, is doubtful since neither cysteine nor histidine residues are conserved, also when *A. niger* ABN A is excluded from the comparison.


A
B
C
1
2
3
4
5

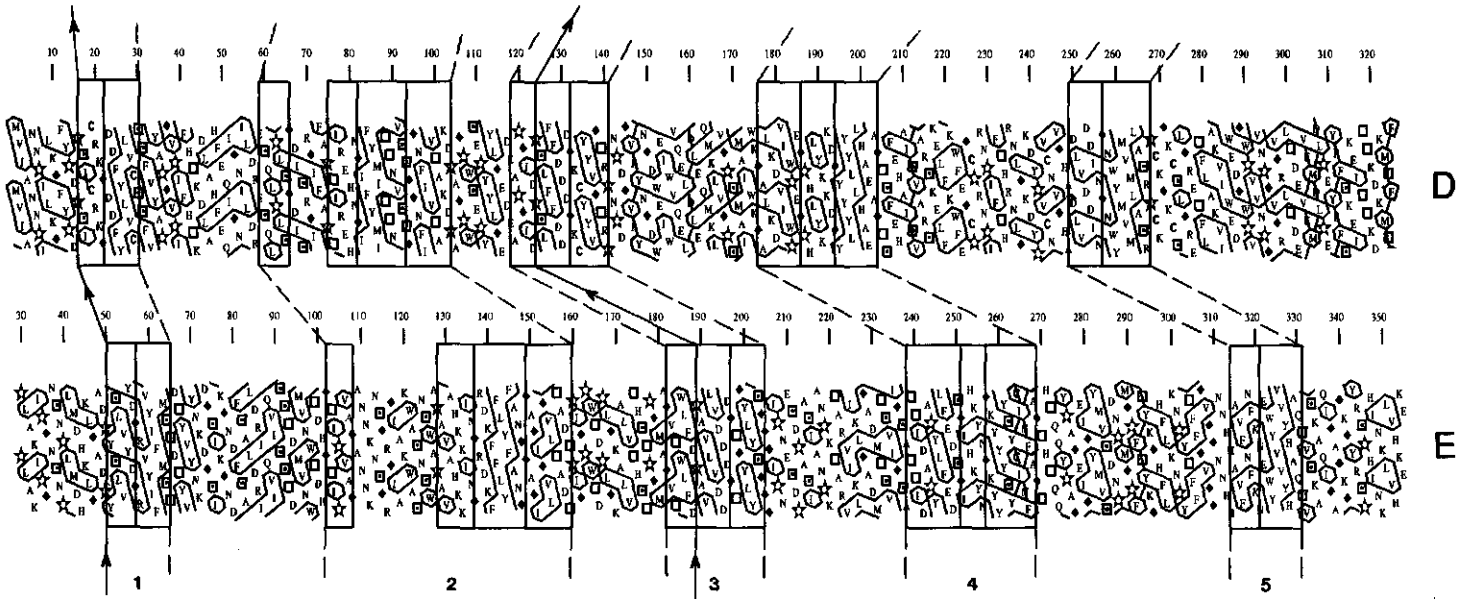


Fig.1. Hydrophobic cluster analysis (HCA) plots of *A. niger* ABN A (**A**), *B. pumilis* βXYL (**B**), *C. stercorarium* βXYL (**C**), *B. fibrisolvens* βXYL (**D**) and *B. polymyxa* XYN D (**E**). Amino acids are represented by their standard one-letter code, except the following which were represented by symbols: proline (⋆), glycine (◆), serine (□), threonine (⊡). The clusters which are considered to be conserved in terms of shape and position, are numbered 1 to 5 and connected by dashed lines. Two arrowed lines connect the two topologically conserved aspartic acid residues [D], present at the left border of cluster 1 and within cluster 3. *B. pumilis* βXYL and *B. fibrisolvens* βXYL were previously assigned to glycosyl hydrolase family 43 (Henrissat and Bairoch 1993).

HCA was conducted following published guidelines (Gaboriaud et al. 1987; Lemesle-Varloot et al. 1990). The amino acids isoleucine [I], leucine [L], methionine [M], phenylalanine [F], tryptophan [W], tyrosine [Y] and valine [V], are considered hydrophobic. In the plots, the amino acid sequence is written on a duplicated helical net and clusters formed by contiguous hydrophobic residues are drawn. HCA plots with automatic hydrophobic cluster contouring were generated using the plot program from Doriane S.A. (France) operating from a Macintosh computer.

Whenever family 43 can be firmly established to contain three quite distinct types of glycosyl hydrolase activities, *viz.* ABNs, βXYLs and L-arabinose-releasing XYNs, the similarity is likely to specify an L-arabinofuranose-binding domain as a common element. aryl-α-L-Arabinofuranosides are not hydrolyzed by *B. pumilus* βXYL, but act as potent competitive inhibitors of PNP-X hydrolysis mutually exclusive to D-xylopyranose-like inhibitors (Marshall and Sinnott 1983). L-Arabino-γ-lactone acts similarly. The other βXYLs of family 43 and XYN D are reported to be able hydrolyze aryl-α-L-arabinofuranosides but kinetic data are not available. PNP-A is not hydrolyzed by *A. niger* ABN A (van der Veen *et al.* 1991), however, in contrast to *B. pumilus* βXYL this enzyme is not inhibited by the γ-lactone (Dunkel and Amadò 1994). Unfortunately, release of L-arabinose from arabinofuranoxylosides or arabinoxylan by the βXYLs and XYN D has never been investigated, neither their ability to hydrolyze α-(1→5)-L-arabinofuranosides or L-arabinan. Such experiments are needed to probe the physiological significance of the observed PNP-A hydrolysis. Notably, ABF from radish (Hata *et al.* 1992) and βXYL from *Thermoanaerobacter ethanolicus* (Shao and Wiegel 1992) are shown to be able to release both D-xylose and L-arabinose from arabinoxylan, whereas a XYN from *Fibrobacter succinogenes* is demonstrated to liberate L-arabinose from arabinoxylan (Matte and Forsberg 1992). Two aspartic acid residues conserved in the first and the third cluster (Fig.1), could be involved in hydrolysis or binding of L-arabinofuranose residues. Such could be established upon site-directed mutagenesis of the corresponding codons within some of the encoding genes. To this end, it is important to realize that extracellular *A. niger* ABN A is active in the acidic pH-range (optimum at pH 4.6) while intracellular *B. pumilus* βXYL has its pH-optimum at pH 7.4 and *C. stercorarium* βXYL at pH 7.0 (both for PNP-X hydrolysis). At these latter pH-values aspartic acid might not be adequately protonated. Hence, hydrolysis of an α-glycosidic bond at the reducing side of an L-arabinofuranose residue by the relevant members of family 43, might well be catalyzed by a catalytic centre distinct from the one responsible for the βXYL or XYN activity. The L-arabinose-releasing activity of most bacterial members of family 43 is likely to be exo-type, which contradicts with the random mode of action of *A. niger* ABN A. The homologous domain in ABN A could therefore be specific for a single (1→3)-linked terminal residue attached to the (1→5)-linked L-arabinan backbone, and situated at such a distance that it does not interfere with endo-catalytic activity. This would enable ABN A to act on moderately, single-residue branched substrate, regardless whether the enzyme is able to remove such a substituent. In this respect ABN A may act similarly to debranching XYNs, like *B. polymyxa* XYN D, or XYNs with preference for the branched areas in the substrate, like forementioned inverting XYN from *A. niger* (Frederick *et al.* 1981).

Bacillus subtilis ABN (PPase-C) produces L-arabinose from sugar beet L-arabinan in higher amounts than bi- and trioses during the first phase of hydrolysis (Sakai *et al.* 1993; Sakamoto *et al.* 1993), indicating an additional debranching activity. Sequence data from PPase-C (*cf.* Sakai *et al.* 1993) might

verify the assignment of *A. niger* ABN A to family 43 provided that these enzymes are related. However, the amino acid composition and the isoelectric point of the *B. subtilis* protein indicate that it differs substantially from ABN A. Primary sequence alignment does resolve similarity. [T M I (K) E G S S] is found in PPase-C from position 14 to 21, with an ambiguous residue at the fourth position, whereas [S M L P D G S S] is present in ABN A at position 53 to 60. This similarity is located directly N-terminal of the second cluster in Fig.1. Hence, the N-terminal amino acid sequence of *B. subtilis* ABN does not verify any of the clusters from the provisional glycosyl hydrolase family 43 but this could also indicate that *B. subtilis* PPase-C is not related to *A. niger* ABN A.

ABF multiplicity in *A. niger*

A. niger produces at least two, genetically distinct ABFs both involved in L-arabinan degradation. The model proposed by Voragen *et al.* (1987) (*cf.* Chapter 1), is deduced from the observed enhanced release of reducing sugar units by ABN A *in presence of* either ABF. However, *the two ABFs do not act* cooperatively. Why does *A. niger* produce ABF A while *A. nidulans* lacks an ABF A equivalent ? In *A. niger*, ABF B is responsible for 80 to 85 % of the extracellular PNP-A-hydrolyzing activity produced when grown on sugar beet pulp (unpublished observations). Moreover, ABF B seems to perform identically to ABF A on small α-L-arabinofuranosides, in addition to a polymer-debranching activity. The co-occurrence of two ABFs in *A. niger* remains enigmatic considering the various contrasting observations I surveyed in Chapter 1. Especially, the physiological relevance of the ABF B debranching activity and of the involvement of ABF A in xylanolysis needs to be elucidated. Kinetic studies with oligomeric substrates might reveal possible differences in substituent requirements of the two *A. niger* ABFs. The performance of the ABFs on branched α-L-arabinofuranosides (single or double substituted at either terminal and non-terminal residues) could be comparable to their distinct action on arabinofuranoxylosides (Kormelink *et al.* 1993). Such studies require both defined substrates and highly purified enzymes – cloning of the structural genes provides the means to produce these enzymes in homologous high-level expression systems under non-inducing conditions. The profits of producing both ABFs could also emerge from studies with recombinant *A. niger* strains, in which either of the ABF-encoding genes is disrupted or deleted.

The ABFs from *Monilinia fructigena*

The brown-rot fungus *Monilinia fructigena* provides the only other fungal system investigated in sufficient depth to discuss the occurrence and functionality of ABF multiplicity. Physico-chemical properties as well as kinetic behaviour of the three isozymes are quite distinct (Laborda *et al.* 1973, 1974; Hislop *et al.* 1974a; *cf.* Chapter 1, Table 1), strongly suggesting that they are

genetically unrelated. This is further supported by the lack of cross-affinity of antiserum raised against ABFI (Hislop *et al.* 1974b). ABFI (220 kDa, pI 3.0), representing 13 % of the PNP-A-hydrolyzing activity in the medium upon submerged growth on pectate, has a pH-optimum between pH 3 and 4, and seems to be primarily cell-wall associated. ABFII (350 kDa, pI 4.5) is exclusively intracellular and optimally active around pH 6.0. ABFIII (40 kDa, pI 6.5), the major extracellular activity, exhibits an optimum pH of 5.0. In contrast to the *A. niger* ABFs, all three isozymes are active against L-arabinan. Two observations suggest functional differences between the two extracellular ABFs. ABFIII shows reduced activity towards PNP-A and is more labile under acidic conditions than ABFI, *viz.* in apple juice. On the other hand, mutant *M. fructigena* strains which feature strongly reduced growth on apples and reduced extra-cellular ABF activity upon submerged growth on pectate, lack ABFIII (Laborda *et al.* 1974; Howell 1975). ABFIII is therefore suspected to act preferentially at the start of the degradation process, to enhance the susceptibility of cell wall polysaccharides for macerating endolytic hydrolases. Simultaneously L-arabinose is released early in the infection process. ABFI might function primarily in the ultimate degradation of polysaccharide material after cell maceration, in an acidified environment. The *M. fructigena* system enables me to propose distinct functions for *A. niger* ABF A and ABF B in L-arabinan degradation and in plant tissue maceration.

ABF A might be essentially cell-wall associated

In Chapter 5, some sequence similarity was found comparing *A. niger* ABF A and the pullulanases of two related *Klebsiella* species. The homology was only indicated by FASTA analysis and could not be established by hydrophobic cluster analysis. Pullulanase (*α*-dextrin endo-1,6-*α*-glucosidase; EC 3.2.1.41) is only known in prokaryotes and belongs to glycosyl hydrolase family 13 consisting of *α*-amylase-like enzymes (*cf.* Henrissat 1991). The enzyme hydrolyses pullulan, a fungal extracellular polysaccharide from the basidiomycete *Aerobasidium pullulans* composed of (1→4)- and (1→6)-*α*-linked D-glucopyranoses, essentially (1→6)-*α*-linked maltotriose units (*cf.* Gorin and Barreta-Bergter 1983). The *A. niger* cell wall may contain similar *α*-glucan. It is unlikely that ABF A is involved in fungal cell wall degradation, since *A. niger* is known to produce pullulan-degrading enzymes (isopullulanase; EC 3.2.1.57) with physico-chemical properties distinct from those of ABF A (Sakano *et al.* 1972, 1990). The noted regions of similarity might, however, outline a domain in ABF A which binds a pullulan-like polysaccharide or cell wall structure, to function as a cell wall anchor. Hence, one could anticipate that ABF A is a cell-wall associated activity equivalent to *M. fructigena* ABFI. Upon induction with L-arabitol, over 25 % of the total PNP-A-hydrolyzing activity produced by *A. niger* in submerged culture was found wall associated (van der Veen *et al.* 1993), although it has not been further specified whether ABF A is predominantly present in the cell wall. A possible cell wall localization of ABF A would rationalize its inability to act against

polymeric substrates. Fixation of glycosidase activity in the fungal cell wall might have functional implications, as this could result in local accumulation of the monomeric end-product of L-arabinan degradation promoting efficient utilization and uptake of the carbon source.

Cell wall-associated glycosidases with a multimeric structure are not uncommon in fungi, whereas monomeric extracellular isozymes have also been described in the same organisms (*e.g.* Civas *et al.* 1984a, 1984b; Matsuo *et al.* 1987). Usually, such isozymes have different genetic backgrounds and substrate specificity, and are differentially induced. Native ABFI from *M. fructigena* has a high molecular weight and is found to be uniformly distributed along the hyphae of pectate-grown mycelium, suggesting that this particular isozyme is essentially cell-wall associated (Hislop *et al.* 1974b). Unfortunately, a possible (native) quaternary structure of *A. niger* ABF A has never been addressed experimentally. Recently, an essentially cell-wall associated α-D-galactosidase (αGAL) from the phytopathogenic mold *Cephalosporium acremonium* was shown to be able to agglutinate (precipitate) starch granules as well as dextran-type α-glucans (Zaprometova *et al.* 1990). The lectin activity features an alkaline pH-optimum contrasting with the acidic pH-optimum for the hydrolytic activity, and is partially inhibited by D-glucose and methyl-α-D-glucopyranoside. If ABF A is indeed anchored in the fungal cell wall, it could be released upon medium acidification which occurs when *A. niger* is grown submerged. Extracellular ABF A might alternatively be the result of cell wall turn-over, the carbon source or the cultivation regime. *Trichoderma reesei* extracellular β-D-glucosidase (βGLU) is shown to be immunochemically related to the cell-associated isozymes (Hofer *et al.* 1989), but is only found upon submerged growth on lactose or cellulose and represents only a minor part of the total βGLU activity under these conditions (Strauss and Kubicek 1990). Also, extracellular ABF A may feature kinetic properties which differ from those of wall-associated enzyme. Interestingly, both D-glucose and D-mannose are reported to inhibit the catalytic activity of cell-wall associated αGALs from *Aspergillus tamarii* in an uncompetitive fashion (Civas *et al.* 1984a).

ABF B might function in substrate sensing

There are indications that the major extracellular isozyme, ABF B, could perform similarly to ABFIII from *M. fructigena*. Transfer of *A. niger* mycelium to medium devoid of a carbon source leads to prominent derepression of, specifically, ABF B synthesis (unpublished observations). Under starvation conditions, the transcription level of the single wild-type *abf*B gene equals that found in high-copy transformants upon L-arabitol induction, as revealed by RNA analyses. Similar results are described by Uchida *et al.* (1992). It is unlikely that ABF B is involved in fungal cell wall autolysis as it is abundantly produced under certain non-starvation conditions. The high expression levels found might suggest that ABF B is produced to sense the presence of polymeric substrates for growth

when the fungus suffers starvation, and possibly to make plant cell wall components subsequently more accessible for endolytic enzymes. Such a function could explain the observed debranching activity against virtually all plant polysaccharides containing α-linked L-arabinofuranosyl groups. L-Arabinose would thus be liberated at the start of cell wall degradation, which could induce certain lytic enzyme systems (see later). Interestingly, *A. niger* also produces PNP-A-hydrolyzing activity upon mycelial transfer to medium containing D-galacturonic acid, the main end-product of pectin degradation (unpublished results). This suggests that an ABF is produced in response to breakdown of polygalacturonic acid, indirectly signalling the presence of associated L-arabinan.

In line with what has been discussed, it would be interesting to probe the extent to which the *A. niger abf*B gene is able to complement the forementioned *M. fructigena* mutants. The presence of conidial-bound enzymes is considered to be essential for the initial release of low-molecular-weight inducers from polymeric substrates in order to induce corresponding glycan-degrading enzyme systems (Reese 1977). In line with a substrate-sensing function, one would expect ABF B to be present on *A. niger* conidiospores (*cf.* Podila *et al.* 1989; Kristufek *et al.* 1994). The observed expression of *abf*B after mycelial transfer to D-glucose (Chapter 5) provides indications to this end. Furthermore, elimination of the *abf*B gene by reversed genetics could provide valuable clues about the proposed role of ABF B in *A. niger*.

Additional activities in L-arabinan degradation

In line with the proposed functional distinction between ABF A and ABF B and considering the different characteristics of the various fungal ABF activities (*cf.* Chapter 1), it is unlikely that ABF multiplicity in *A. niger* is limited to ABF A and B only. Additional ABFs could have a broad substrate range or feature restricted specificity towards arabinoxylan, arabinogalactan or L-arabinan, or fragments thereof. Some of them could be inert towards aryl-α-L-arabinofuranosides (*cf.* Kormelink *et al.* 1991). An exo-type arabinase corresponding to the one described in the related fungus *Aspergillus aculeatus* (Lahaye and Thibault 1990), could be an important debranching activity in the degradation of L-arabinan. It is worthwhile to notice that the apparent molecular weight of *A. aculeatus* exo-arabinase, determined upon SDS-PAGE, is the same as that of ABF B (67 kDa). A strictly intracellular isozyme, equivalent to *M. fructigena* ABFII or *Streptomyces lividans* ABFA (Manin *et al.* 1994), might also be postulated as a component of the L-arabinan-degrading system of *A. niger*. Such an activity would be required to hydrolyze oligomeric arabinofuranosides in case these would be taken up by the fungus. PNP-A-hydrolyzing activity has not been detected in disrupted *A. niger* protoplasts (van der Veen *et al.* 1993) but such an isozyme is unlikely to be active under the assay conditions applied (pH 3.5) and could be inert towards synthetic substrates.

Induction of the L-arabinan-degrading system

The experiments described in Chapter 5 show that induction of arabinases in *A. niger* is a complex process as the expression of the three genes studied, seems interdependent. The system is regulated at the level of transcription. Induction of ABN A involves at least three distinct activating regulatory factors: one titrable by multiple *abf*A copies, one titrable by multiple *abf*B copies and a gene-specific one. The latter one limits ABN A overexpression in *A. niger abn*A multiple-copy transformants but does not affect expression of the ABFs. Such limitation was not observed in recombinant *A. nidulans* strains harbouring multiple *abn*A copies. A similar co-regulation mechanism might be operative in *A. nidulans* in which the heterologous *abf*A gene can be expressed upon growth on sugar beet pulp, although *A. nidulans* lacks an *abf*A gene equivalent (Chapter 2). Although multiple copies of either *abf* gene reduce ABN A expression in *A. niger* significantly, titration of the individual factors does not result in total silencing. It would be interesting to study ABN A synthesis in the presence of multiple copies of both *abf* genes. Further indications could be obtained by studying recombinant strains harbouring multiple copies of upstream non-coding sequences from either gene. Such strains might feature total silencing of the respective genes (a deficient phenotype) if sufficient copies are present, whereas the other arabinases could still be expressed to a certain extent. Such an approach could allow identification of the *cis*-acting sequence elements involved in coordination of L-arabinan degradation, along with gene-specific elements. Indications for possible ABN multiplicity could emerge when *abn*A promoter sequences are analyzed. Whether the observed coordination is system-specific or part of some wide domain regulatory circuit remains to be elucidated. Induction studies in strains from which complete *abf* genes are deleted (*cf.* Suominen *et al.* 1993), could provide indications to this end.

Polymeric substrate (sugar beet pulp) is the supreme inducing compound in both *A. niger* and *A. nidulans* (van der Veen *et al.* 1991, 1993; Ramón *et al.* 1993). The actual inducer of the system, however, needs to be small enough to be taken up by the fungus (Reese 1977; Kubicek *et al.* 1993a). Both L-arabinose, the end-product of extracellular L-arabinan degradation, and L-arabitol, an intermediate of fungal L-arabinose catabolism, can fulfil the role of the direct inducer. This is supported by the observation that both *A. niger abf* genes have a low basal expression upon mycelial transfer to medium containing the non-inducing and repressing carbon source D-glucose (Chapter 5). Action of the extracellular *A. niger* ABFs yields L-arabinose outside the cell. The results from Chapter 6 indicate that uptake of L-arabinose occurs even in the presence of D-glucose. Moreover, the production of ABF B upon starvation, which suggests a substrate-sensing function (as mentioned earlier in this Chapter), also points in this direction. L-Arabinose is described to be an inducer of various fungal glycosyl hydrolases involved in pectinolysis and xylanolysis, *i.e. Fusarium oxysporum* and

Vertillium albo-atrum βGAL (Cooper and Wood 1975), *Aureobasidium pullulans* XYN (Leathers *et al.* 1986) and *T. reesei* αGAL (Zeilinger *et al.* 1993). However, oligomeric fragments arising from endolytic or exolytic action on L-arabinan or other substrate polysaccharides, might have extra inducing capacities in addition to that from the L-arabinose present. One has to note that other substances, not related to L-arabinose, are documented to mediate fungal ABF expression. I mention here D-galacturonic acid (Cooper and Wood 1975), D-galactose (Laborda *et al.* 1974) and 1-*O*-methyl β-D-xyloside (Uchida *et al.* 1992). In the latter cases, expression was accompanied by strongly reduced growth and therefore I prefer to consider those as derepression effects. D-Galacturonic acid was shown to induce substantial PNP-A-hydrolyzing activity in *A. niger* as well (unpublished results).

In *A. nidulans*, both ABF and ABN synthesis are induced on L-arabinose but L-arabitol is more effective (Ramón *et al.* 1993). In *A. niger*, only ABF B production is observed upon growth on L-arabinose (van der Veen *et al.* 1993). The inducing competence of the pentose sugar is masked by its capacity to confer carbon catabolite repression in *A. niger* and *A. nidulans*. Cooper and Wood (1975) elegantly proved that L-arabinose represses the synthesis of PNP-A-hydrolyzing activity in *V. albo-atrum* and *F. oxysporum* when the supply exceeds the needs of cellular metabolism. In *A. niger*, addition of L-arabinose to L-arabinan-grown mycelium leads to reduced ABF expression (Tagawa and Terui 1968). On the other hand, L-arabinose is superior over L-arabitol in inducing PNP-A-hydrolyzing activity in *T. reesei*, which system is not subject to carbon catabolite repression (Kristufek *et al.* 1994). Given the differential kinetics of pH-dependent cellular uptake of the sugar and the polyol (*cf.* Kristufek *et al.* 1994), uptake efficiency could also be involved. Gradual breakdown of polymeric L-arabinan provides a continuous release of L-arabinose with minimal repression effects, hence its superior inducing capacity in *A. niger* and *A. nidulans*. One might be able to determine the optimal L-arabinose concentration in fermenter experiments. To this end, it would also be interesting to probe the extracellular concentration of free L-arabinose and the intracellular amounts of L-arabitol upon growth on sugar beet pulp or L-arabinan.

The *in vivo* interconversion of L-arabinose and L-arabitol (Witteveen *et al.* 1989; Chapter 6) makes it impossible to discriminate between the inducing capabilities of these two compounds. Medium transfer studies using ill-convertible derivatives like 1-*O*-methyl α-L-arabinofuranoside, 1-*O*-methyl α-L-arabinopyranoside and 1-methoxy-L-arabitol, in combination with a non-repressive carbon source like L-glutamate (*cf.* Chapter 6), might generate clues to this end. The inducing capacity of L-arabino-γ-lactone could also be investigated. Decisive evidence can only be obtained using a mutant strain which is unable to interconvert L-arabinose and L-arabitol. Such a strain could be selected for if the (NADPH-dependent) L-arabinose and D-xylose reductases are the only enzymes capable to metabolize L-arabinose, using the *A. nidulans* L-arabitol dehydrogenase

mutant described in Chapter 6 as the parental strain and provided that these enzymes are not co-induced in this mutant.

The phenomena observed in Chapter 5 indicate titration of mutual activating transcription factors. However, competition amongst gene-specific activators for a common low-molecular-weight inducer could also explain the observations. The effects were found to be similar in both experiments, although more persistent in the sugar beet pulp experiment. This could be due to a continuous, restricted availability of such an inducer in case of growth on sugar beet pulp. Such a competition could also explain the induction profiles of the three *A. niger* arabinase genes emerging from the time course experiment using L-arabitol to induce expression. The decreasing amounts of *abf*A messenger indicate that either the structural gene or its activator would be most susceptible to repression when fungal metabolism becomes adapted to the new, abundantly present carbon source. The low *abn*A mRNA levels one hour after the mycelial transfer would imply that the *abn*A activator has the lowest affinity for the inducer. Alternatively, *de novo* protein synthesis may be required for transcriptional activation of *abn*A whereas the onset of ABF production is faster due to a constitutive presence of inactive activator complexes. Such inactive activator complexes are known to occur, for instance in the *Saccharomyces cerevisiae* galactose utilization (*GAL*) regulon (*cf.* Johnston and Carlson 1992). The basal mRNA levels observed for both *abf* genes upon mycelial transfer to D-glucose, a non-induced and repressing growth condition, and the concomitant absence of *abn*A mRNA, support the suggestion for ABF expression. If induction of *abn*A depends on inducer-mediated, *de novo* synthesis of its private transcriptional activator, extra copies of the structural *abn*A gene are not expected to affect ABF synthesis, which indeed seems to be the case (*cf.* Chapter 5). To this end, it is interesting to mention the presence of the 11-bp long sequence element [5′ CTGCCTTGGCC 3′] within the upstream non-coding regions of the *abf* genes, but not in *abn*A.

Carbon catabolite repression of arabinase expression

In fungi, carbon catabolite repression occurs when so called "preferable" carbon sources, of which D-glucose is considered the most eminent one, are sufficiently present to fulfil the needs of cellular metabolism. If such is the case, the expression of facultative enzyme systems involved in mobilization and catabolism of other, "less preferable" carbon sources is suppressed, even in the presence of inducers of such systems. This suppression is effected by direct repression of the transcription of one or more genes comprising a responsive system. In *A. niger* and *A. nidulans* this feature involves the transcriptional repressor CREA. Acting at several levels, the mechanism leads to both rapid transient and long-term repression of structural gene expression. Induced expression of the *A. niger* arabinase genes is subject to tight repression in the

presence of D-glucose (van der Veen *et al.* 1993). Induction studies in carbon catabolite repression (*cre*) mutants of *A. nidulans* in the presence of both L-arabitol and D-glucose (van der Veen *et al.* 1994) show that the repression of *A. nidulans* arabinase expression by D-glucose involves CREA. In the most severe mutant (*creA*^{d}-30) up to 15 times higher levels of arabinase activity were found than in the wild-type, in the presence of both D-glucose and L-arabitol. Interestingly, similar differences were found when only L-arabinose or L-arabitol were supplied. This suggests that end-product (feedback) inhibition of arabinase expression could be mediated by CREA as well.

CREA is an essential, DNA-binding protein which belongs to the C_2H_2 class of zinc-finger proteins (Dowzer and Kelly 1991). The mode of action of CREA has been extensively studied in the ethanol utilization regulon of *A. nidulans* (Hintz and Lagosky 1993; Kulmburg *et al.* 1993; Mathieu and Felenbok 1994). It binds to defined bidirectionally functional promoter elements [G/C C/T G G G G] present in the genes encoding alcohol dehydrogenase I (*alcA*) and the auto-regulated regulon-specific activator (*alcR*). CREA effectively competes with activator binding and affects transcription of the target genes directly. Repression of both the structural and the regulator genes magnifies the effect on the expression of *alcA*. The CREA-binding sites utilized *in vivo* occur as doublets which indicates cooperative binding and dimer formation. Equivalent DNA-binding repressor proteins have been described in *S. cerevisiae* (MIG1 or CAT4) (Nehlin and Ronne 1990; Griggs and Johnston 1991; Nehlin *et al.* 1991; Schüller and Entian 1991) and in *A. niger* (CREA) (Drysdale *et al.* 1993).

Our studies on catabolite repression of the L-arabinan-degrading system and L-arabinose catabolism in *creA* mutants (van der Veen *et al.* 1994), show a typical non-hierarchical pattern of phenotypic variation amongst different mutant alleles as described for other target genes (*cf.* Arst and Bailey 1977), *i.e.* there is no general allele hierarchy with respect to relieved repression of individual target genes. This indicates that CREA directly affects transcription of the *A. nidulans* arabinase genes. All *A. niger* arabinase genes contain various copies of the *A. nidulans* consensus CREA-binding sequence [G/C C/T G G G G], postulated by Kulmburg *et al.* (1993) (Chapter 5, Fig.5). Some of these occur as doublets and others in close conjunction with distinct putative promoter elements. The number of putative CREA-responsive elements is not necessarily limited to those presented in Chapter 5. Both *S. cerevisiae* MIG1 and *A. nidulans* CREA bind to sequences in which an A is present at the fifth position of the consensus, provided a G is present at the first position (Cubero and Scazzocchio 1994; Lundin *et al.* 1994). [G C G G A G] is half of the CREA-binding site which functions *in vivo* as responsive element in the *A. nidulans* proline utilization cluster. Elimination of a putative CREA site from the cellobiohydrolase I-encoding gene from the soft-rot fungus *T. reesei*, enabled expression in the presence of D-glucose (Penttilä *et al.* 1993). This suggests that CREA might be involved in D-glucose-mediated repression of the *A. niger* L-arabinan-degrading system as well.

Identification of the functional CREA-responsive *cis*-acting promoter elements as well as the extent of the direct influence of CREA on the transcription of the structural arabinase genes, requires *in vitro* binding assays, site-directed mutagenesis of putative sites, and studies in appropriate *creA*-mutant backgrounds. The arabinase genes do not contain sequences similar to two *cis*-acting elements described to confer D-glucose repression on two other fungal systems, *viz.* the *Neurospora crassa grg-1* gene (McNally and Free 1988; Wang *et al.* 1994) and the *Aspergillus oryzae* Taka-amylase gene, which was studied in *A. nidulans* as heterologous host (Nagata *et al.* 1993).

Besides direct repression of gene expression, an "inducer exclusion" mechanism could give rise to carbon catabolite repression (*cf.* Biely 1982; Ullmann 1985). In this case expression should not be repressed when D-glucose is added to induced biomass, which indicates that D-glucose mainly interferes in the uptake of inducing compounds. Unfortunately, the effects of inducer exclusion have not been investigated for the *A. niger* and *A. nidulans* arabinase systems. Inducer exclusion can be effected by various means. D-Glucose directly inhibits the action of the permeases responsible for uptake of the inducing compounds of the *Cryptococcus albidus* xylan-hydrolyzing system (Krátký and Biely 1980) and of the *T. reesei* cellulase complex (Kubicek *et al.* 1993b). Furthermore, in the *S. cerevisiae* D-galactose utilization (*GAL*) regulon the CREA-equivalent MIG1 was found to repress the expression of the permease involved in D-galactose uptake, and thus to be responsible for the D-glucose repression of this system at three levels (Johnston *et al.* 1994).

The results with the L-arabitol dehydrogenase mutant (Chapter 6) indicate that repression of arabinase expression in *A. nidulans* is not governed solely by direct repression of transcription of the structural genes by CREA. The mutation allows substantial expression of arabinases, half the amounts produced by the wild-type on L-arabinose only, and accumulation of arabitol on a mixed carbon source of D-glucose and L-arabinose. The effect of inducer exclusion is circumvented in the mutant due to the metabolic block which indicates that this mechanism is prominently involved in repression in wild-type *A. nidulans*, in the presence of D-glucose and L-arabinose. In the wild-type, the pentose catabolic route acts at a basal level on D-glucose / L-arabinose to convert intracellular inducer entering the cell by basal uptake, since a small amount of xylitol was found in the mycelium (Chapter 6, Table 2). Also twice as much arabitol was present compared to mutant mycelium grown on D-xylose, which in this case can only be D-arabitol, and five times more than observed in the wild-type grown on D-glucose. In the presence of glycerol and L-arabinose, arabinases were absent in the wild-type while in the mutant, expression levels were eight to sixteen times higher (for ABN and ABF, respectively) than found in the wild-type on L-arabinose. A partial explanation for these observations could be that the mutation severely affects the competence of L-arabinose to act as a carbon catabolite repressor. When D-xylose is the co-substrate, especially the ABN level is derepressed in the wild-

type to more than half the amount found in the mutant and in the wild-type on L-arabinose only. This could be a kinetic effect as L-arabinose and D-xylose seem to be more or less equally good substrates for L-arabinose reductase but not for D-xylose reductase (Chapter 6, Table 3), slowing down L-arabinose conversion in the wild-type. If such is the case this might indicate that the L-arabinose (end-product) repression of arabinase expression is deduced from the rate of its conversion into L-arabitol and not from its intracellular concentration. Such would be conform the suggestion of Sierkstra *et al.* (1993) that the flux through glycolysis signals D-glucose repression in bakers yeast. Note that L-arabinose-induced arabinase expression in the *A. niger* wild-type is repressed in the presence of glycerol and allowed in the presence of D-xylose. This contrasts with the results of previous investigations on the carbon catabolite repression of various intracellular enzyme systems (*cf.* Arst and Bailey 1977; Arst 1984).

Another factor involved in repression might be related to the growth rate of the fungus. The mutation seems to have only minor implications in the presence of D-glucose and D-xylose as co-substrates, whereas biomass generation on the other mixed carbon sources is slowed down compared to the wild-type. Reduced mutant biomass correlates with higher amounts of extracellular arabitol and elevated arabinase expression. On the other hand, addition of either D-glucose or glycerol to cellulose-grown *A. nidulans* leads to higher growth rates and a cessation of cellulase synthesis (Bagga *et al.* 1989). A relationship between derepression and growth rate was suggested to explain non-induced expression of β-D-glucosidase (βGLU) by the rumen bacteria *Prevotella* (*Bacteroides*) *ruminicola* in D-glucose-limiting continuous cultures (Strobel and Russell 1987).

Cyclic AMP as a modulator of arabinase gene expression

Upon comparison of the three *A. niger* arabinase gene sequences, two common sequence elements were found which are highly homologous to cyclic AMP (cAMP)-responsive elements known from other organisms (Chapter 5). Here I want to discuss how cAMP could be involved in the regulation of arabinase expression in *Aspergilli*. cAMP could well be involved in many of the features related to induction and repression which were discussed earlier in this Chapter.

The experiments with *A. nidulans* wild-type and the L-arabitol dehydrogenase mutant on mixed carbon sources (Chapter 6) show that each co-substrate tested influences the L-arabinose-induced arabinase expression and growth to different extent, discernible in each of these two strains. cAMP has been suggested to mediate similar responses in some microbial cellulase systems (Linden and Shiang 1991; Šesták and Farkaš 1993a & b). Furthermore, cAMP is shown to be essential for the utilization of, amongst others, glycerol, L-arabinose and D-mannitol as substrates for growth in *N. crassa*, whereas catabolism of *e.g.* D-xylose and D-fructose is stimulated by cAMP (Terenzi *et al.* 1979). In fungi, cAMP is believed to function as a second messenger in the control of a wide

variety of features in response to both internal and external stimuli, amongst others, the utilization of exogenous and endogenous carbon sources, protein synthesis and degradation, and cell differentiation (Pall 1981). The cellular cAMP level is directly modulated via its synthesis by adenylate cyclase, its breakdown by phosphodiesterase and by excretion. cAMP exerts its effects through phosphorylation of responsive transcription factors by cAMP-dependent protein kinases (*cf.* Roessler *et al.* 1988; Gibbs and Marshall 1989; Hunter and Karin 1992). Subtle changes in the intracellular cAMP level have an enormous effect on the activity of cAMP-dependent protein kinases (*cf.* Thevelein 1991, 1992). cAMP has been shown to be involved in carbon catabolite repression in bakers yeast and other fungi, but cAMP-independent mechanisms (*e.g.* MIG1) also exist (*cf.* Wills 1990; Saier 1991; Gancedo 1992; Trumbly 1992; Costigan and Snyder 1994).

cAMP elicits discernible and even opposite effects with respect to fungal extracellular enzyme systems. Addition of exogenous cAMP led to partial repression of starch-induced synthesis of *A. awamori* glucoamylase (Bhella and Altosaar 1988). In contrast, supply of cAMP intensifies induced expression of other systems which are repressed in the presence of D-glucose, like XYN from *C. albidus* and lignin-degrading peroxidases from *Phanerochaete chrysosporium* (Morosoli *et al.* 1989; Boominathan and Reddy 1992). Synthesis of invertase in *N. crassa* is initiated by externally supplied cAMP even in absence of inducing compounds (Terenzi *et al.* 1992). Sophorose-induced endo-(1,4)-D-glucanase (EGL) production in *T. reesei* resting cells was found to be enhanced by exogenous supply of cAMP at low (μM) concentrations, and suppressed at higher (mM) concentrations (Šesták and Farkaš 1993a, b). Addition of monomeric sugars resulted in elevated intracellular cAMP levels but only "non-repressive" compounds stimulated the induced EGL expression. Catabolite repression was thus found to be dominant over the stimulative effect of cAMP in this particular system. However, in *Aspergillus sidowii* D-glucose repression of the xylan-degrading system could be partially relieved by external supply of dibutyl-cAMP, an ill-convertible analogue, in a concentration-dependent fashion (Ghosh and Nanda 1994).

Sequence element B [TCCCTGAA] as found in the *A. niger* arabinase genes (Chapter 5; *cf.* Fig.2), is highly homologous to a cAMP-responsive element (UAS_{PDS}) in the *S. cerevisiae* heat shock protein HSP70-encoding *SSA3* gene (Boorstein and Craig 1990). UAS_{PDS} confers repression to *SSA3* during exponential, fermentative growth and drives expression in the stationary growth phase. In *S. cerevisiae*, cAMP seems to be involved in signalling the availability of fermentable carbon sources, triggering the metabolic transition. The stationary growth phase, entered upon depletion of supplied D-glucose, features slow metabolism, derepression of enzyme systems subject to carbon catabolite repression and a decreased intracellular cAMP level (Gibbs and Marshall 1989;

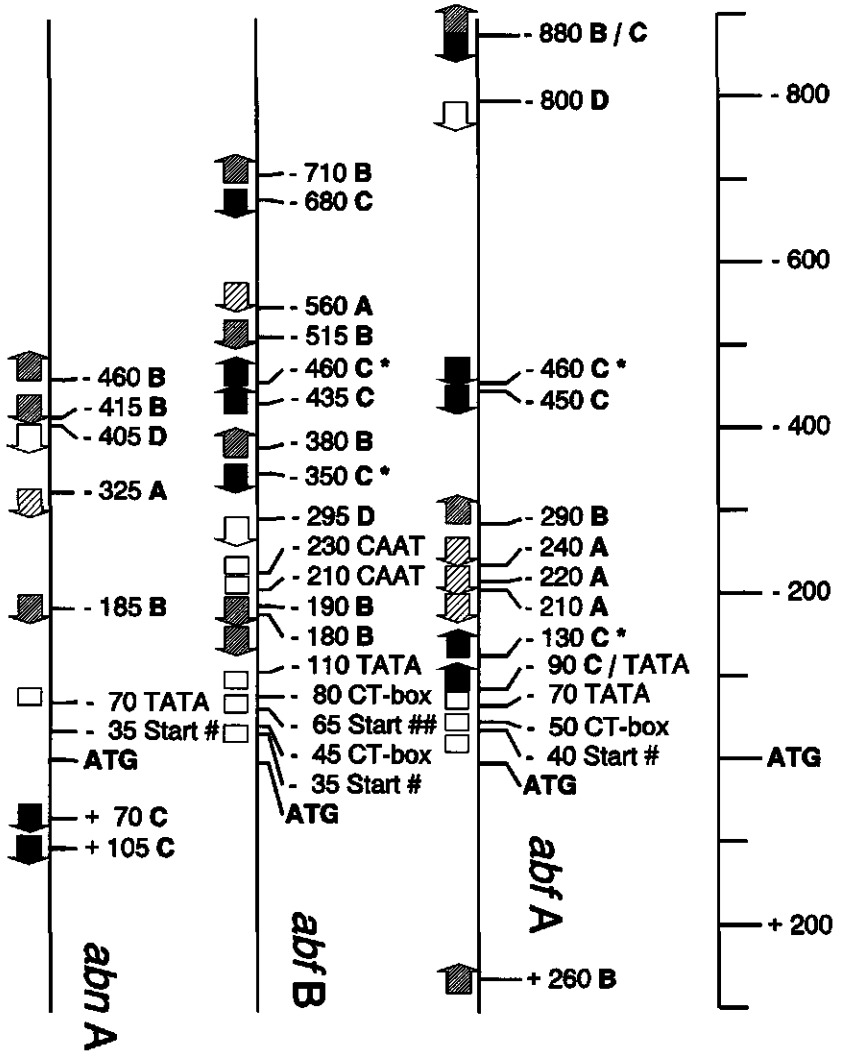

- 460 B
- 415 B
- 405 D
- 325 A
- 185 B
- 70 TATA
- 35 Start #
ATG
+ 70 C
+ 105 C
abn A
- 710 B
- 680 C
- 560 A
- 515 B
- 460 C *
- 435 C
- 380 B
- 350 C *
- 295 D
- 230 CAAT
- 210 CAAT
- 190 B
- 180 B
- 110 TATA
- 80 CT-box
- 65 Start ##
- 45 CT-box
- 35 Start #
ATG
abf B
- 880 B / C
- 800 D
- 460 C *
- 450 C
- 290 B
- 240 A
- 220 A
- 210 A
- 130 C *
- 90 C / TATA
- 70 TATA
- 50 CT-box
- 40 Start #
ATG
abf A
+ 260 B
- 800
- 600
- 400
- 200
ATG
+ 200

Fig.2. Schematic overview showing the localization and orientation of common putative sequence elements in the 5' regions (– 900 to + 300 relative to the translation start codon (**ATG**) of the *A. niger* arabinase genes (*abf*A, *abf*B and *abn*A), as decribed in Chapter 5 (*cf.* Chapter 5, Fig.5). The four different elements (**A, B, C, D**) are represented by distinct arrows and the approximate distances from the respective translation start codon (**ATG**) are indicated, as for the major transcription initiation sites of the respective genes. Sequences silimar to functional general eukaryotic promoter elements (CAAT-box, TATA-box and CT-stretch) are represented by open boxes. Only those putative CREA-sites (**C**) are indicated which are found in conjunction with either another CREA-site or with any other of the indicated elements. The CREA-sites found in conjunction with putative AP2-binding sites in *abf*A and *abf*B, are marked (**C***).

Thevelein 1991, 1992). However, whereas in *S. cerevisiae* the intracellular cAMP level decays upon carbon source depletion, the opposite response occurs in several higher fungi (Farkaš *et al.* 1987; Kämper *et al.* 1994; Zonneveld 1976). In *A. nidulans* and *A. niger*, a direct relation between cAMP metabolism and CREA, the DNA-binding protein mediating D-glucose repression, has been suggested based on sequence homology with the *S. cerevisiae RGR1* gene product, which is distinct from the MIG1-equivalent DNA-binding domain (Davis *et al.* 1993). RGR1 is involved in carbon catabolite repression as *rgr1* mutants feature derepressive phenotypes for D-glucose-repressible functions together with high internal cAMP levels (Sakai *et al.* 1990). In *A. niger*, element B might function as an effector of secondary metabolism, which is initiated upon depletion of the supplied carbon source. All three *A. niger* arabinase genes are found to be derepressed under carbon source starvation conditions (unpublished results). Promoter analysis of a *Fusarium solani* (f. sp. *pisi*) cutinase (cutin esterase) gene identified a region harbouring a silencer element (Kämper *et al.* 1994). This area contains the sequence [TGCCTGAA], homologous to element B, while 11 bp upstream a second copy [TTCCTGAA] is present. The silencer functions bidirectionally and in a distance-independent fashion. These observations are interesting since: (1) *abf*B contains two tandem-orientated copies of element B adjacent to a putative CAAT box; (2) the complete copy in *abf*A is located in the coding region of the gene; and (3) the element is also found in D-glucose-repressible *A. niger* genes encoding (other) extracellular pectinolytic enzymes (*cf.* Chapter 5).

Element D [T/C G A C A/G T C A] (*cf.* Chapter 5; Fig.2) is highly homologous to the palindromic ATF/CRE element [TGACGTCA] functional in vertebrate systems (*cf.* Roessler *et al.* 1988). Moreover, it is one of two *cis*-acting elements required to mediate D-glucose repression on the *N. crassa grg-1* gene (Wang *et al.* 1994). Although involvement of cAMP was not addressed in this study, the element proved to be functional a filamentous fungus. In mammals, the ATF/CRE element seems to be a focal point through which positive and negative regulation is conferred and it is subject to both cAMP and calcium signal transduction (*cf.* Hunter and Karin 1992; Lalli and Sassone-Corsi 1994). The sequence element may bind at least ten different transcription factors from the basic region/leucine zipper (bZIP) type, all substrates for both cAMP-dependent and -independent protein kinases (*cf.* Lalli *et al.* 1993). Some of these are activators and others repressors. ATF/CRE-binding transcription factors can form heterodimers which are likely to confer a wide variety of effects. The gene encoding the yeast repressor analogue, *ACR1* (*SKO1*), was isolated with selection systems using the palindromic vertebrate ATF/CRE-element (Nehlin *et al.* 1992; Vincent and Struhl 1992). However, a functional ACR1-binding site [GTACGTCA] in the *S. cerevisiae SUC2* (invertase) gene which differs at two positions from the mammalian consensus, was found to be involved in carbon catabolite repression independent from MIG1 (Nehlin *et al.* 1992). Furthermore, the ATF/CRE-site in

the *N. crassa grg-1* gene is required to suppress transcription in the presence of D-glucose, but seems to be occupied by distinct DNA-binding proteins in the presence and absence of D-glucose, respectively (Wang *et al.* 1994). ATF/CRE-binding proteins can also dimerize with effectors from the "Activating Protein 1" (AP-1) class, bZIP proteins recognizing the cognate sequence [TGACTCA] responding to cAMP and protein kinase C (PKC) signal transduction (*cf.* Lee *et al.* 1987; Hunter and Karin 1992; Lalli *et al.* 1993). Representatives from the AP1-class of transcriptional activators are known in fungi (Harshman *et al.* 1988; Tamaru *et al.* 1994).

Screening of a promoter element library using the GCG TFDSEARCH program indicated the presence of sequences homologous to a distinct mammalian cAMP-responsive element, "Activating Protein 2" (AP-2) [C C C C/A N C/G C/G C/G] (Williams and Tjian 1991b; Faisst and Meyer 1992), in the upstream non-coding regions of the *A. niger abf*A and *abf*B genes. In *abf*B, two of such elements, [CCCATCCC] (−360 / −353) and [CGCATCCC] (−458 / −451) (*cf.* Chapter 4, Fig.4), could be found. Notably, the latter site overlaps with the distal half of a CREA-doublet in *abf*B, while the perfect match is close to a single putative CREA-site (Fig.2). The *abf*A gene also contains two AP-2-like elements, *viz.* [CCCCGG CC] (− 456/ −463) and [CCCCACCG] (−134 / −127) (*cf.* Chapter 5, Fig.2), each of which overlaps the distal half of a CREA-doublet (Fig.2). AP-2(A) is a helix-span-helix/basic region-type transcriptional activator responsive to cAMP and PKC signal transduction (*cf.* Imagawa *et al.* 1987; Williams *et al.* 1988; Williams and Tjian 1991a). A non-DNA-binding, antagonizing factor (AP-2B) is also known (Buettner *et al.* 1993). AP-2-like transcription factors have been suggested to be involved in the regulation of fungal PKC- and cAMP-responsive genes. In spores of the phytopathogenic fungus *F. solani* (f. sp. *pisi*), induction of the virulence factor cutinase and subsequent germination requires phosphorylation of a nuclear factor (Podila *et al.* 1989; Bajar *et al.* 1991). Cutin monomers, liberated by conidial-bound cutinase, trigger this phosphorylation reaction resulting in *de novo* synthesis of cutinase within minutes. The responsive promoter area of the cutinase gene harbouring the binding site for the phosphorylated transcription factor contains (Soliday *et al.* 1989; Bajar *et al.* 1991) a putative AP-2/CREA site [GCGGGGAGGGG] (−327 / −317). This element was found to act as a modulator. It amplifies the response to both specific induction by cutin monomers and catabolite repression by D-glucose although it confers neither induction nor repression by itself (Kämper *et al.* 1994). The element antagonizes the action of a silencer element, containing a sequence highly homologous to element B as mentioned before. It was suggested that these two elements act in concert in modulating the cutinase expression level (Kämper *et al.* 1994). Furthermore, germination of condiospores of *Colletrotrichum trifolii*, a related phytopathogen, and subsequent formation of a specialized penetration structure (apressorium) were found to be stimulated by phorbol esters (Dickman *et al.* 1993). The occurrence and functionality of PKC in

filamentous fungi was thereby established. From *S. cerevisiae* several PKC isozymes have been isolated (*e.g.* Ogita *et al.* 1990; Simon *et al.* 1991). Recently, PKC signal transduction was shown be involved in cAMP-independent nutrient sensing in *S. cerevisiae* (Costigan and Snyder 1994).

The action of fungal AP-2-like transcription factors was suggested in several other systems. They may be involved in the onset of secondary metabolism in the white-rot fungus *Phanerochaete chrysosporium* in relation to the production of lignin-degrading enzymes (Dhawale 1993). Some of these enzymes are strongly derepressed in nitrogen- and carbon-limited cultures (Gold and Alic 1993), conditions which generally lead to an increase in the internal cAMP level in higher fungi. As mentioned earlier in this Chapter, *A. niger* ABF B is prominently produced upon carbon starvation. A putative AP-2 target site in the *N. crassa* nitrogen regulatory gene *nit2*, subject to nitrogen and D-glucose repression, overlaps a putative CREA-binding site (Dhawale 1993). Such an AP-2/CREA element is also present in the promoter area of the *N. crassa* hydrophobin-encoding *bli7/eas* gene, which is shown to contain the *cis*-acting elements responsive to both nitrogen and D-glucose starvation (Eberle and Russo 1992; Kaldenhoff and Russo 1993).

The presented putative cAMP-responsive elements might allow cAMP to mediate discernible effects on arabinase expression simultaneously through multiple signal transduction pathways. For the fourth element common to all three *A. niger* arabinase genes, element A [C T/A C C T C C A C A/T] (*cf.* Chapter 5; Fig.2), I could not find functional analogues. However, FASTA analysis of the major nucleic acid data libraries revealed that identical sequences are present in the upstream non-coding regions of several unrelated fungal genes, *viz. N. crassa grg-1* (McNally and Free 1988), *A. niger* phytase (*myo*-inositol hexaphosphate 3-phosphohydrolase) (*phy*) (Mullaney *et al.* 1991), *Ascobolus immersus* homoserine *O*-transacetylase (*met2*) (Goyon *et al.* 1988), *Trichoderma harzianum* alkaline protease (*prb1*) (Geremia *et al.* 1993), *Candida albicans* chitin synthase (*chs1*) (Au-Young and Robbins 1990), *Physarum polycephalum* Eα-tubulin (Walden *et al.* 1989) and *S. cerevisiae* NAD^+-dependent cytosolic glycerol-3-phosphate dehydrogenase (*GPD1*) (Sleep *et al.* 1991; Albertyn *et al.* 1994). An interesting score is the *Yarrowia lipolytica ryl1* gene (accession n° L06969; B. Pertuiset *et al.* unpublished) coding for a functional equivalent of *S. cerevisiae* SEC4, a regulatory protein required for cellular secretion (Salminen and Novick 1987). The complement of element A is present in the upstream regions of other fungal genes (not shown). Interesting in view of the results from Chapter 6, the *S. cerevisiae GPD1* gene product is the key enzyme for glycerol synthesis in response to osmotic stress, mediated by an osmosensing signal-transduction pathway (Brewster *et al.* 1993; Albertyn *et al.* 1994). The functionality of all putative promoter elements found shared amongst the *A. niger* arabinase genes, needs to be confirmed by *in vitro* nuclear-protein binding assays and promoter analysis. In line with what has been discussed, it is highly unlikely that

transcription factors binding to any of these elements, are system-specific.

Epilogue

In this final Chapter I have discussed aspects of arabinase enzymology and regulatory mechanisms involved in their expression in *A. niger* and *A. nidulans*. Although I drew on information from other fungal extracellular enzyme systems, one should note that the generalizations do not have to apply on each fungal L-arabinan degradation system. Even different strains from the same organism, isolated from distinct environments, are likely to have evolved towards divergent degradation strategies and coherent regulatory mechanisms. Strain CBS 120.49, the wild-type *A. niger* strain from our laboratory, features an efficient pectinolytic (L-arabinan-degrading) system whereas its xylanolytic capacity is rather poor (Conrad 1981). Certainly, the L-arabinan-degrading systems of *A. niger* and *A. nidulans* feature similarities, but also major differences. I refer to the lack of an ABF A equivalent in *A. nidulans*, the ABF expression levels, *abn*A expression in multiple copy transformant strains and the induction characteristics on L-arabinose. Moreover, in the brown-rot fungus *M. fructigena* and in the soft-rot fungus *T. reesei*, the system could be adapted to defence strategies of the host plant, leading to constitutive or non-repressible expression. Despite these latter considerations, the *A. niger* L-arabinan-degrading system may evolve into a valuable model system for studying homologous and heterologous gene expression in this important industrial host organism as it is inducible on the well-defined carbon source L-arabitol. Furthermore, the results of the study described in this thesis could contribute to the establishment of arabinases as important tools in efficient microbial and enzymic conversion of plant biomass.

Acknowledgement. I want to thank Dr. Bernard Henrissat (Centre de Recherches sur les Macromolécules Végétales, CNRS, Grenoble, France) for his help and interest concerning hydrophobic cluster analysis.

References

Albertyn J, Hohmann S, Thevelein JM, Prior BA (1994) *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol Cell Biol 14:4135-4144

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403-410

Arst HN Jr (1984) Regulation of gene expression in *Aspergillus nidulans*. Microbiological Sciences 1:137-140

Arst HN Jr, Bailey CR (1977) The regulation of carbon matabolism in *Aspergillus nidulans*. In: Smith JE, Pateman JA (eds) Genetics and physiology of *Aspergillus nidulans*. Academic Press, NY. pp. 131-146

Au-Young J, Robbins PW (1990) Isolation of a chitin synthase gene (*CHS1*) from *Candida albicans* by expression in *Saccharomyces cerevisiae*. Mol Microbiol 4:197-207

Bagga PS, Sandhu DK, Sharma S (1989) Catabolite repression of cellulase production in *Aspergillus nidulans*. Process Biochem **24**:41-45

Bajar A, Podila GK, Kolattukudy PE (1991) Identification of a fungal cutinase promoter that is inducible by a plant signal via a phosphorylated trans-acting factor. Proc Natl Acad Sci USA **88**:8208-8212

Bhella RS, Altosaar I (1988) Role of cAMP in the mediation of glucose catabolite repression of glucoamylase synthesis in *Aspergillus awamori*. Curr Genet **14**:247-252

Biely P (1982) Catabolic repression of β-xylanase synthesis in the yeast *Cryptococcus albidus*. Biologia (Bratislava) **37**:799-807

Boominathan K, Reddy CA (1992) cAMP-mediated differential regulation of lignin peroxidase and manganese-dependent peroxidase production in the white-rot basidiomycete *Phanerochaete chrysosporium*. Proc Natl Acad Sci USA **89**:5586-5590

Boorstein WR, Craig EA (1990) Regulation of a yeast HSP70 gene by a cAMP responsive transcriptional control element. EMBO J **9**:2543-2553

Brewster JL, de Valoir T, Dwyer ND, Winter E, Gustin MC (1993) An osmosensing signal transduction pathway in yeast. Science **259**:1760-1763

Buettner R, Kannan P, Imhof A, Bauer R, Kim SO, Glockshuber R, Dyke MW van, Tainsky MA (1993) An alternative spliced mRNA from the AP-2 gene encodes a negative regulator of transcriptional activation by AP-2. Mol Cell Biol **13**:4174-4185

Civas A, Eberhard R, Le Dizet P, Petek F (1984a) Glycosidases induced in *Aspergillus tamarii*. Mycelial α-D-galactosidases. Biochem J **219**:849-855

Civas A, Eberhard R, Le Dizet P, Petek F (1984b) Glycosidases induced in *Aspergillus tamarii*. Secreted α-D-galactosidase and β-D-mannanase. Biochem J **219**:857-863

Claeyssens M, Saman E, Kersters-Hilderson H, Bruyne CK de (1975) β-D-Xylosidase from *Bacillus pumilus*. Molecular properties and oligomeric structure. Biochim Biophys Acta **405**: 475-481

Conrad D (1981) Enzymatic hydrolysis of xylans. I. A high xylanase and ß-xylosidase producing strain of *Aspergillus niger*. Biotechnol Lett **3**:345-350

Cooper RM, Wood RKS (1975) Regulation of synthesis of cell wall degrading enzymes by *Verticilium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici*. Physiol Plant Pathol **5**: 135-156

Costigan C, Snyder M (1994) SLK1, a yeast homolog of MAP kinase activators, has a RAS/cAMP-independent role in nutrient sensing. Mol Gen Genet **243**:286-296

Cubero B, Scazzocchio C (1994) Two different, adjacent and divergent zinc finger binding sites are necessary for CREA-mediated carbon catabolite repression in the proline gene cluster of *Aspergillus nidulans*. EMBO J **13**:407-415

Davis MA, Kelly JM, Hynes MJ (1993) Fungal catabolic gene regulation: molecular genetic analysis of the *amdS* gene of *Aspergillus nidulans*. Genetica **90**:133-145

Devereux J, Haerberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res **12**:1431-1442

Dhawale SS (1993) Is an activator protein-2-like transcription factor involved in regulating gene expression during nitrogen limitation in fungi ? Appl Environ Microbiol **59**:2335-2338

Dickman MB, Warwar V, Nietfeldt J (1993) Protein kinase C activity in *Colletotrichum trifolii*. Fungal Genetics Newslett **40A** [Supplement. Abstracts from the 17th Fungal Genetics Conference. Pacific Grove Ca., USA, march 23-28 1993]:34

Doorslaer E van, Kersters-Hilderson H, Bruyne CK de (1985) Hydrolysis of β-D-xylo-oligosaccharides by β-D-xylosidase from *Bacillus pumilus*. Carbohydr Res **140**:342-346

Dowzer CEA, Kelly JM (1991) Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. Mol Cell Biol **11**:5701-5709

Drysdale MR, Kolze SE, Kelly JM (1993) The *Aspergillus niger* carbon catabolite repressor encoding gene, *creA*. Gene **130**:241-245

Dunkel MPH, Amadò R (1994) Purification and physico-chemical properties of an *endo*-1,5-α-L-arabinanase (EC 3.2.1.99) isolated from an *Aspergillus niger* pectinase preparation. Carbohydr Polym **24**:247-263

Eberle J, Russo VEA (1992) *Neurospora crassa* blue-light-inducible gene *bli-7* encodes a short hydrophobic protein. DNA Sequence - J DNA Seq Mapping **3**:131-141

Faisst S, Meyer S (1992) Compilation of vertebrate-encoded transcription factors. Nucleic Acids Res **20**:3-26

Farkaš V, Šesták S, Grešík M, Kolarova M, Labudová I, Bauer Š (1987) Induction of cellulases in *Trichoderma reesei* grown on lactose. Acta Biotechnol **7**:425-429

Frederick MM, Frederick JR, Fratzke AR, Reilly PJ (1981) Purification and characterization of a xylobiose- and xylose-producing endo-xylanase from *Aspergillus niger*. Carbohydr Res **97**: 87-103

Gaboriaud C, Bissery V, Benchetrit T, Mornon JP (1987) Hydrophobic cluster analysis: an efficient new way to compare and analyse amino acid sequences. FEBS Lett **224**:149-155

Gancedo JM (1992) Carbon catabolite repression in yeast. Eur J Biochem **206**:297-313

Gebler J, Gilkes NR, Claeyssens M, Wilson DB, Béguin P, Wakarchuk WW, Kilburn DG, Miller RC Jr, Warren RAJ, Withers SG (1992) Stereoselective hydrolysis catalyzed by related β-1,4-glucanases and β-1,4-xylanases. J Biol Chem **267**:12559-12561

Geremia RA, Goldman GH, Jacobs D, Ardiles W, Vila SB, Montagu M van, Herrera-Estrella A (1993) Molecular characterization of the proteinase-encoding gene, *pbr1*, related to mycoparasitism by *Trichoderma hazianum*. Mol Microbiol **8**:603-613

Ghosh M, Nanda G (1994) Physiological studires on xylose induction and glucose repression of xylanolytic enzymes in *Aspergillus sydowii* MG49. FEMS Microbiol Lett **117**:151-156

Gibbs JB, Marshall MS (1989) The *ras* oncogene - an important regulatory element in lower eukaryotic organisms. Microbiol Rev **53**:171-185

Gold MH, Alic M (1993) Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol Rev **57**:605-622

Gorin PAJ, Barreto-Bergter E (1983) The chemistry of polysaccharides of fungi and lichens. In: Aspinall GO (ed) The Polysaccharides. Vol.2. Academic Press, NY. pp.365-409

Gosalbes MJ, Pérez-González JA, González R, Navarro A (1991) Two β-glycanase genes are clustered in *Bacillus polymyxa*: Molecular cloning, expression and sequence analysis of genes encoding a xylanase and an endo-β-(1,3)-(1,4)-glucanase. J Bacteriol **173**: 7705-7710

Goyon C, Faugeron G, Rossignol J-L (1988) Molecular cloning and characterization of the *met2* gene from *Ascobolus immersus*. Gene **63**:297-308

Grépinet O, Chebrou M-C, Béguin P (1988) Nucleotide sequence and deletion analysis of the xylanase gene (*xynZ*) of *Clostridium thermocellum*. J Bacteriol **170**:4582-4588

Griggs DW, Johnston M (1991) Regulated expression of the *GAL4* acticator gene in yeast provides a sensitive genetic swith for glucose repression. Proc Natl Acad Sci USA **88**: 8597-8601

Harshman KD, Moye-Rowley WS, Parker CS (1988) Transcriptional activation by the SV40 AP-1 recognition element in yeast is mediated by a factor similar to AP-1 that is distinct from GCN4. Cell **53**:321-330

Hata K, Tanaka M, Tsumuraya Y, Hashimoto Y (1992) α-L-Arabinofuranosidase from Radish (*Raphanus sativus* L.) seeds. Plant Physiol **100**:388-396

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J **280**:309-316

Henrissat B, Bairoch A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J **293**:781-788

Hintz WE, Lagosky PA (1993) A glucose-derepressed promoter for expression of heterologous products in the filamentous fungus *Aspergillus nidulans*. BIO/TECHNOLOGY **11**:815-818

Hislop EC, Barnaby VM, Shellis C, Laborda F (1974a) Localization of α-L-arabinofuranosidase and acid phosphatase in mycelium of *Sclerotinia fructigena*. J Gen Microbiol **81**:79-99

Hislop EC, Shellis C, Fielding AH, Bourne FJ, Chidlow JW (1974b) Antisera produced to purified extracellular pectolytic enzymes from *Sclerotinia fructigena*. J Gen Microbiol **83**: 135-143

Hofer F, Weissinger E, Mischak H, Messner R, Meixner-Monori B, Blaas D, Visser J, Kubicek CP (1989) A monoclonal antibody against the alkaline extracellular β-glucosidase from *Trichoderma reesei*: reactivity with other *Trichoderma* β-glucosidases. Biochim Biophys Acta **922**:298-306

Howell HE (1975) Correlation of virulence with secretion *in vitro* of three wall-degrading enzymes in isolates of *Sclerotinia fructigena* obtained after mutagen treatment. J Gen Microbiol **90**:32-40

Hunter T, Karin M (1992) The regulation of transcription by phosphorylation. Cell **70**:375-387

Imagawa M, Chiu R, Karin M (1987) Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell **51**:251-260

Johnston M, Carlson M (1992) Regulation of carbon and phosphate utilization. In: Jones EW, Pringle JR, Broach JR (eds) The molecular and cellular biology of the yeast *Saccharomyces cerevisiae*. Vol.2. Gene expression. Cold Spring Harbor Press, Cold Spring Harbor NY. pp.193-281

Johnston M, Flick JS, Pexton T (1994) Multiple mechanisms provide rapid and stringent glucose repression of *GAL* gene expression in *Saccharomyces cerevisiae*. Mol Cell Biol **14**: 3834-3841

Kaldenhoff R, Russo VEA (1993) Promoter analysis of the *bli-7/eas* gene. Curr Genet **24**: 394-399

Kämper JT, Kämper U, Rogers LM, Kolattukudy PE (1994) Identification of regulatory elements in the cutinase promoter from *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*). J Biol Chem **269**:9195-9204

Kersters-Hilderson H, Claeyssens M, Doorslaer E van, Bruyne CK de (1976) Determination of the anomeric configuration of D-xylose with D-xylose isomerases. Carbohydr Res **47**: 269-273

Kersters-Hilderson H, Doorslaer E van, Bruyne CK de (1978) β-D-Xylosidase from *Bacillus pumilus* PRL B12: hydrolysis of aryl β-D-xylopyranosides. Carbohydr Res **65**:219-227

Kersters-Hilderson H, Doorslaer E van, Lippens M, Bruyne CK de (1984) The pH dependence and group modification of β-D-xylosidase from *Bacillus pumilus*: evidence for sulfhydryl and histidyl groups. Arch Biochem Biophys **234**:61-72

Kormelink FJM, Searle-van Leeuwen MJF, Wood TM, Voragen AGJ (1991) Purification and characterization of a (1,4)-β-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori*. Appl Microbiol Biotechnol **35**:753-758

Kormelink FJM, Gruppen H, Voragen AGJ (1993) Mode of action of (1→4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) and α-L-arabinofuranosidases on alkali-extractable wheat-flour arabinoxylan. Carbohydr Res **249**:345-353

Krátký Z, Biely P (1980) Inducible β-xyloside permease as a constituent of the xylan-dgrading enzyme system of the yeast *Cryptococcus albidus*. Eur J Biochem **112**:367-373

Kristufek D, Hodits R, Kubicek CP (1994) Coinduction of α-L-arabinofuranosidase and α-D-galactosidase formation in *Trichoderma reesei* RUT C-30. FEMS Microbiol Lett **115**: 259-264

Kubicek CP, Messner R, Gruber F, Mach RL, Kubicek-Pranz EM (1993a) The *Trichoderma* cellulase regulatory puzzle: from the interior life of a secretory fungus. Enzyme Microb Technol **15**:90-99

Kubicek CP, Messner R, Gruber F, Mandels M, Kubicek-Pranz EM (1993b) Triggering of cellulase biosynthesis by cellulose in *Trichoderma reesei*. Involvement of a constitutive, sophorose-inducible, glucose-inhibited β-diglucoside permease. J Biol Chem **268**: 19364-19368

Kulmburg P, Mathieu M, Dowzer C, Kelly J, Felenbok B (1993) Specific binding sites in the *alc*R and *alc*A promoters of the ethanol regulon for the CREA repressor mediating carbon catabolite repression in *Aspergillus nidulans*. Mol Microbiol **7**:847-857

Laborda F, Fielding AH, Byrde RJW (1973) Extra- and intra-cellular α-L-arabinofuranosidase of *Sclerotinia fructigena*. J Gen Microbiol **79**:321-329

Laborda F, Archer SA, Fielding AH, Byrde RJW (1974) Studies of the α-L-arabinofuranosidase complex from *Sclerotinia fructigena* in relation to brown rot of apple. J Gen Microbiol **81**: 151-163

Lahaye M, Thibault J-F (1990) Purification of arabinanases and galactanases from *Aspergillus niger*. Poster displayed at the 3rd Int Workshop on Plant Polysaccharides, Structure and Function. Le Croisic, France, September 19-21 1990. [As referred to in: Beldman G, Schols HA, Searle-van Leeuwen MJF, Voragen AGJ (1995) Arabinans and arabinan degrading enzymes. Adv Macromol Carbohydr Res, in press]

Lalli E, Sassone-Corsi P (1994) Signal transduction and gene regulation: the nuclear response to cAMP. J Biol Chem **269**:17359-17362

Lalli E, Lee JS, Masquiller D, Schlotter F, Foulkes NS, Molina CA, Sassone-Corsi P (1993) Nuclear response to cyclic AMP: central role of transcription factor CREM (cyclic-AMP-responsive-element modulator). Biochem Soc Trans **21**:912-917

Leathers TD, Detroy RW, Bothast RJ (1986) Induction and glucose repression of xylanase from a color variant strain of *Aerobasidium pullulans*. Biotechnol Lett **8**:867-872

Lee W, Mitchell AP, Tjian R (1987) Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell **49**:741-752

Lee Y-E, Zeikus JG (1993) Genetic organization, sequence and biochemical characterization of recombinant β-xylosidase from *Thermoanaerobacterium saccharolyticum* strain B6A-RI. J Gen Microbiol **139**:1235-1243

Lemesle-Varloot L, Henrissat B, Gaboriaud C, Bissery V, Morgat A, Mornon JP (1990) Hydrophobic cluster analysis: procedures to derive structural and functional information from 2-D-representation of protein sequences. Biochimie **72**:555-574

Linden JC, Shiang M (1991) Bacterial cellulases. Regulation of synthesis. In: Leatham GF, Himmel ME (eds) Enzymes in biomass conversion. ACS Symp Ser **460**. American Chemical Society, Washington DC. pp.331-348

Lundin M, Nehlin JO, Ronne H (1994) Importance of a flanking AT-rich region in target site recognition by the GC box-binding zinc finger protein MIG1. Mol Cell Biol 14:1979-1985

Lüthi E, Love DR, McAnulty J, Wallace C, Caugley PA, Saul D, Bergquist PL (1990) Cloning, sequence analysis, and expression of genes encoding xylan-degrading enzymes from the thermophile "*Caldocellum saccharolyticum*". Appl Environ Microbiol **56**:1017-1024

Manin C, Shareek F, Morosoli R, Kluepfel D (1994) Purification and characterization of an α-L-arabinofuranosidase from *Streptomyces lividans* 66 and DNA sequence of the gene (*abf*A). Biochem J **302**:443-449

Marshall PJ, Sinnott ML (1983) Competative inhibition of the inverting β-xylosidase of *Bacillus pumilus* 12 by monosaccharide derivatives of different structural and conformational types. A possible natural substrate. Biochem J **215**:67-74

Mathieu M, Felenbok B (1994) The *Aspergillus nidulans* CREA protein mediates glucose repression of the ethanol regulon at various levels through competition with the ALCR-specific transactivator. EMBO J **13**:4022-4027

Matsuo M, Fujie A, Maung Win, Yasui T (1987) Four types of β-xylosidases from *Penicillium wortmanni* IFO 7237. Agric Biol Chem **51**:2367-2379

Matte A, Forsberg CW (1992) Purification, characterization, and mode of action of endoxylanases 1 and 2 from *Fibrobacter succinogenes* S85. Appl Environ Microbiol **58**: 157-168

McNally MT, Free SJ (1988) Isolation and characterization of a *Neurospora* glucose-repressible gene. Curr Genet **14**:545-551

Morosoli R, Durand S, Boucher F (1989) Stimulation of xylanase synthesis in *Crytococcus albidus* by cyclic AMP. FEMS Microbiol Lett **57**:57-60

Mullaney EJ, Gibson DM, Ullah AH (1991) Positive identification of a lambda gt11 clone containing a region of fungal phytase gene by immunoprobe and sequence verification. Appl Microbiol Biotechnol **35**:611-614

Nagata O, Takashima T, Tanaka M, Tsukagoshi N (1993) *Aspergillus nidulans* nuclear proteins bind to a CCAAT element and the adjacent upstream sequence in the promoter region of the starch-inducible Taka-amylase A gene. Mol Gen Genet **237**:251-260

Nehlin JO, Ronne H (1990) Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. EMBO J **9**:2891-2898

Nehlin JO, Carlberg M, Ronne H (1991) Control of yeast *GAL* genes by MIG1 repressor: a transcriptional cascade in the glucose response. EMBO J **10**:3373-3377

Nehlin JO, Carlberg M, Ronne H (1992) Yeast *SKO1* gene encodes bZIP protein that binds to the CRE motif and acts as a repressor of transcription. Nucleic Acids Res **20**:5271-5278

Ogita K, Miyamoto S-i, Koide H, Iwai T, Oka M, Ando K, Kishimoto A, Ikeda K, Fukami Y, Nishizuka Y (1990) Protein kinase C in *Saccharomyces cerevisiae*: comparison with the mammalian enzyme. Proc Natl Acad Sci USA **87**:5011-5015

Pall ML (1981) Adenosine 3',5'-phosphate in fungi. Microbiol Rev **45**:462-480

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence analysis. Proc Natl Acad Sci USA **85**:2444-2448

Penttilä M, Saloheimo A, Ilmén M, Onnela M-L (1993) Regulation of expression of *Trichoderma reesei* cellulases at mRNA and promoter level. In: Suominen P, Reinikainen T (eds) *Trichoderma reesei* cellulases and other hydrolases. Enzyme structures, biochemistry, genetics and applications. Foundation for Biotechnical and Industrial Fermentation Research. Vol.8. Foundation for Biotechnical and Industrial Fermentation Research, Helsinki. pp. 189-197

Podila GK, Dickman MB, Rodgers LM, Kolattukudy, PE (1989) Regulation of expression of fungal genes by plant signals. In: Nevalainen H, Penttilä M (eds) Proceedings of the EMBO-Alko Workshop on Molecular Biology of Filamentous Fungi, Helsinki 1989. Foundation for Biotechnical and Industrial Fermentation Research. Vol.6. Foundation for Biotechnical and Industrial Fermentation Research, Helsinki. pp. 217-226

Ramón D, Veen P van der, Visser J (1993) Arabinan degrading enzymes from *Aspergillus nidulans*: induction and purification. FEMS Microbiol Lett **113**:15-22

Reese ET (1977) Degradation of polymeric carbohydrates by microbial enzymes. In: Loewus FA, Runeckles VC (eds) Recent Adv Phytochem **11**. The structure, biosynthesis, and degradation of wood. Proceedings of the sixteenth annual meeting of the phytochemical society of North America, Vancouver, British Colombia, Canada, august 1976. Plenum Press, NY: pp 311-367

Roesler WJ, Vandenbark GR, Hanson RW (1988) Cyclic AMP and the induction of eukaryotic gene transcription. J Biol Chem **263**:9063-9066

Rombouts FM, Voragen AGJ, Searle-van Leeuwen MF, Geraerds CCJM, Schols HA, Pilnik W (1988) The arabinanases of *Aspergillus niger* - purification and characterisation of two α-L-arabinofuranosidases and an *endo*-1,5-α-L-arabinanase. Carbohydr Polym **9**:25-47

Saier MH Jr (1991) A multiplicity of potential carbon catabolite repression mechanisms in prokaryotic and eukaryotic microorganisms. New Biologist **3**:1137-1147

Sakai A, Shimizu Y, Kondou S, Chibazakura T, Hishinuma F (1990) Structure and molecular analysis of *RGR1*, a gene required for glucose repression of *Saccharomyces cerevisiae*. Mol Cell Biol **10**:4130-4138

Sakai T, Sakamoto T, Hallaert J, Vandamme EJ (1993) Pectin, pectinase, and protopectinase: production, properties, and applications. Adv Appl Microbiol **39**:213-294

Sakamoto T, Yoshinaga J, Shogaki T, Sakai T (1993) Studies on protopectinase-C mode of action - analysis of the chemical structure of the specific substrate in sugar beet protopectin and characterization of the enzyme activity. Biosci Biotech Biochem **57**: 1832-1837

Sakano Y, Higushi M, Kobayashi T (1972) Pullulan 4-glucanohydrolase from *Aspergillus niger*. Arch Biochem Biophys **153**:180-187

Sakano Y, Taguchi A, Hisamatsu R, Kobayashi S, Fujimoto D, Kobayashi T (1990) Comparison of cell-bound and extracellular isopullulanase from *Aspergillus niger*. Denpun Kagaku **37**:39-41. [Chem Abstracts **113**:111319g]

Sakka K, Yoshikawa K, Kojima Y, Karita S-I, Ohmiya K, Shimada K (1993) Nucleotide sequence of the *Clostridium stercorarium xylA* gene encoding a bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities, and properties of the translated product. Biosci Biotech Biochem **57**:268-272

Salminen A, Novick PJ (1987) A *ras*-like protein is required for a post-golgi event in yeast secretion. Cell **49**:527-538

Saman E, Claeyssens M, Bruyne CK de (1978) Study of the sulfhydryl groups of β-D-xylosidase from *Bacillus pumilus*. Eur J Biochem **85**:301-307

Schüller H-J, Entian K-D (1991) Extragenic suppressors of yeast glucose derepression mutants leading to constitutive synthesis of several glucose-repressible enzymes. J Bacteriol **173**: 2045-2052

Šesták S, Farkaš V (1993a) Metabolic regulation of endoglucanase synthesis in *Trichoderma reesei*: participation of cyclic AMP and glucose-6-phosphate. Can J Microbiol **39**:342-347

Šesták S, Farkaš V (1993b) Involvement of cyclic AMP in the regulation of cellulase synthesis in *Trichoderma reesei*. In: Suominen P, Reinikainen T (eds) *Trichoderma reesei* cellulases and other hydrolases. Enzyme structures, biochemistry, genetics and applications. Foundation for Biotechnical and Industrial Fermentation Research. Vol.8. Foundation for Biotechnical and Industrial Fermentation Research, Helsinki. pp. 217-225

Shao W, Wiegel J (1992) Purification and characterization of a thermostable β-xylosidase from *Thermoanaerobacter ethanolicus*. J Bacteriol **174**:5848-5853

Sierkstra LN, Silljé HHW, Verbakel JMA, Verrips CT (1993) The glucose-6-phosphate isomerase reaction is essential for normal glucose repression in *S. cerevisiae*. Eur J Biochem **214**:121-127

Simon AJ, Milner Y, Saville SP, Dvir A, Mochly-Rosen D, Orr E (1991) The identification and purification of a mammalian-like protein kinase C in the yeast *Saccharomyces cerevisiae*. Proc R Soc Lond, B **243**:165-171

Sleep D, Ogden JE, Roberts NA, Goodey AR (1991) Cloning and characterization of the *Saccharomyces cerevisiae* glycerol-3-phosphate dehydrogenase (*GUT2*) promoter. Gene **101**:89-96

Soliday CL, Dickman MB, Kolattukudy PE (1989) Structure of the cutinase gene and detection of promoter activity in the 5'-flanking region by fungal transformation. J Bacteriol **171**: 1942-1951

Strauss J, Kubicek CP (1990) β-Glucosidase and cellulase formation by a *Trichoderma reesei* mutant defective in constitutive β-glucosidase formation. J Gen Microbiol **136**:1321-1326

Strobel HJ, Russell JB (1987) Regulation of β-glucosidase in *Bacteroides ruminicola* by a different mechanism: growth rate-dependent derepression. Appl Environ Microbiol **53**: 2505-2510

Suominen PL, Mäntylä A, Karkunen T, Hakola S, Nevalainen KMH (1993) High frequency one-step gene replacement in *Trichoderma reesei*. II. Effects of deletions of individual cellulase genes. Mol Gen Genet **241**:523-530

Tagawa K, Terui G (1968) Studies on the enzymes acting on araban. Part IX. Culture conditions and process of arabanase production by *Aspergillus niger*. J Ferment Technol **46**:693-700

Tamaru H, Nishida T, Harashima T, Inoue H (1994) Transcriptional activation of a cyclohexidine-inducible gene encoding laccase is mediated by *cpc-1*, the cross-pathway control gene, in *Neurospora crassa*. Mol Gen Genet **243**:548-554

Terenzi H, Terenzi HF, Jorge JA (1992) Effect of cyclic AMP on invertase activity in *Neurospora crassa*. J Gen Microbiol **138**:2433-2439

Terenzi HF, Jorge JA, Roselino JE, Migliorini RH (1979) Adenylate cyclase deficient *cr-1* (Crisp) mutant of *Neurospora cassa*: cyclic AMP-dependent nutritional deficienties. Arch Microbiol **123**:251-258

Thevelein JM (1991) Fermentable sugars and intracellular acidification as specific activators of the *RAS*-adenylate cyclase signalling pathway in yeast: the relationship to nutrient-induced cell cycle control. Mol Microbiol **5**:1301-1307

Thevelein JM (1992) The *RAS*-adenylate cyclase pathway and cell cycle control in *Saccharomyces cerevisiae*. Anthonie van Leeuwenhoek **62**:109-130

Trumbly RJ (1992) Glucose repression in the yeast *Saccharomyces cerevisiae*. Mol Microbiol **6**:15-21

Uchida H, Kusakabe I, Kawabata Y, Ono T, Murakami K (1992) Production of xylose from xylan with intracellular enzyme system of *Aspergillus niger* 5-16. J Ferment Bioeng **74**: 153-158

Ullmann A (1985) Catabolite repression 1985. Biochimie **67**:29-34

Utt EA, Eddy CK, Keshav KF, Ingram LO (1991) Sequencing and expression of the *Butyrivibrio fibrisolvens xyl*B gene encoding a novel bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities. Appl Environ Microbiol **57**:1227-1234

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1991) Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. Arch Microbiol **157**:23-28

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1993) Induction of extracellular arabinases on monomeric substrates in *Aspergillus niger*. Arch Microbiol **159**:66-71

Veen P van der, Arst HN Jr, Flipphi MJA, Visser J (1994) Extracellular arabinases in *Aspergillus nidulans*: the effect of different *cre* mutations on enzyme levels. Arch Microbiol **162**:433-440

Vincent AC, Struhl K (1992) ACR1, a yeast ATF/CREB repressor. Mol Cell Biol **12**:5394-5405

Voragen AGJ, Rombouts FM, Searle-van Leeuwen MF, Schols HA, Pilnik W (1987) The degradation of arabinans by endo-arabinanase and arabinofuranosidases purified from Aspergillus niger. Food Hydrocoll **1**:423-437

Walden PD, Monteiro MJ, Gull K, Cox RA (1989) Structure and expression of an α-tubilin gene of *Physarum polycephalum*. Eur J Biochem **181**:583-592

Wang Z, Deak M, Free SJ (1994) A *cis*-acting region required for the regulated expression of *grg-1*, a Neurospora glucose-repressible gene. Two regulatory sites (CRE and NRS) are required to repress *grg-1* expression. J Mol Biol **237**:65-74

Williams T, Tjian R (1991a) Characterization of a dimerization motif in AP-2 and its function in heterologous DNA-binding proteins. Science **251**:1067-1071

Williams T, Tjian R (1991b) Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. Genes Dev **5**:670-682

Williams T, Admon A, Lüscher B, Tjian R (1988) Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements. Genes Dev **2**: 1557-1569

Wills C (1990) Regulation of sugar and ethanol metabolism in *Saccharomyces cerevisiae*. CRC Crit Rev Biochem Mol Biol **25**:245-280

Witteveen CFB, Busink R, Vondervoort P van de, Dijkema C, Swart K, Visser J (1989) L-Arabinose and D-xylose catabolism in *Aspergillus niger*. J Gen Microbiol **135**:2163-2171

Xu W-Z, Shima Y, Negoro S, Urabe I (1991) Sequence and properties of β-xylosidase from *Bacillus pumilus* IPO. Contradiction of the previous nucleotide sequences. Eur J Biochem **202**:1197-1203

Zaprometova OM, Ulezlo IV, Lakhtin VM (1990) Structure and properties of a *Cephalosporium acremonium* α-galactosidase. Glycoconjugate J **7**:287-300

Zeilinger S, Kristufek D, Arisan-Atac I, Hodits R, Kubicek CP (1993) Conditions of formation, purification, and characterization of an α-galactosidase of *Trichoderma reesei* RUT C-30. Appl Environ Microbiol **59**:1347-1353

Zonneveld BJM (1976) The effect of glucose and manganese on adenosine-3',5'-monophosphate levels during growth and differentiation of *Aspergillus nidulans*. Arch Microbiol **108**:41-44

Summary

This thesis describes a molecular study of the genetics of L-arabinan degradation in *Aspergillus niger* and *Aspergillus nidulans*. These saprophytic hyphal fungi produce an extracellular hydrolytic enzyme system to depolymerize the plant cell wall polysaccharide L-arabinan. **Chapter 1** surveys the occurrence, properties and applications of L-arabinanolytic enzymes (arabinases). The *A. niger* system, which constitutes an endolytic endo-1,5-α-L-arabinase (ABN A) and two distinct α-L-arabinofuranosidases (ABF A and ABF B), has been a frequent subject of investigation in the past and represents the best characterized L-arabinanolytic system to date. These three enzymes are all glycosylated. Current knowledge on the induction of fungal arabinase expression is summarized in this Chapter. Furthermore, the structure of the polysaccharide substrate and its function in the plant cell wall matrix are introduced.

In Chapters 2 to 5, the cloning and characterization of the structural genes coding for the three glycosyl hydrolases from the *A. niger* L-arabinan-degrading complex are described. *A. niger abf*A and *abf*B are the first eukaryotic ABF-encoding genes to be isolated and sequenced, *abn*A is the first ABN-encoding gene published. **Chapter 2** reports on the isolation of the *abf*A gene encoding ABF A, the minor extracellular ABF. This gene could be cloned by utilizing ABF A-specific cDNA as the probe. This cDNA was immunochemically identified from a cDNA library generated from L-arabitol-induced mycelium of an *A. niger* D-xylulose kinase mutant. This mutant is unable to grow on L-arabitol and features enhanced expression of all three arabinases when transferred to medium containing this pentitol as sole carbon source. In **Chapter 3**, the cloning of the ABN A-encoding gene (*abn*A) is described. This gene was isolated following the same strategy as with *abf*A, although a second cDNA library had to be generated first. The induction process was immunochemically monitored in order to establish the proper induction conditions for the new library. The *abn*A gene and the gene product were characterized by DNA sequence analyses of the cloned genomic DNA and the cDNA. The N-terminal amino acid sequences of ABN A and a CNBr-derived peptide were determined. Several transcription initiation sites and one polyadenylation site could be identified. The structural region codes for a protein of 321 amino acids and is interrupted by three introns. Extracellular ABN A consists of 302 amino acid residues with a deduced molecular weight of 32.5 kDa and a theoretical pI of 3.5. For the protein, an apparent pI of 3.0 and an apparent molecular weight of 43 kDa, determined upon SDS-PAGE, were previously reported. **Chapter 4** documents the isolation and characterization of the *abf*B gene, coding for the major extracellular ABF. The determination of N-

terminal amino acid sequences from ABF B and CNBr-generated peptides allowed the design of deoxyoligonucleotide mixtures which enabled the cloning of *abf*B. When utilized as primers in a polymerase chain reaction (PCR), ABF B-specific amplification products emerged, one of which was used to probe the gene. The *abf*B gene and the gene product were characterized by DNA sequence analyses of the cloned genomic DNA and of ABF B-specific cDNA isolated from the library described in Chapter 3. Several transcription initiation sites and one polyadenylation site could be identified. The structural region is a single open reading frame and codes for a protein of 499 amino acids. The mature enzyme consists of 481 amino acid residues with a deduced molecular weight of 50.7 kDa and a theoretical pI of 3.8. An apparent pI of 3.5 and an apparent molecular weight of 67 kDa, determined upon SDS-PAGE, were previously reported. The *abf*B gene product was suggested to be identical to the ABF purified and characterized by Kaji and Tagawa (Biochim Biophys Acta **207**: 456-464 (1970)). Considering the non-amino acid content of the latter protein, a molecular weight of 64 kDa could be deduced for ABF B. In **Chapter 5**, the *abf*A gene and its gene product were characterized by DNA sequence analyses of the genomic DNA and of the cDNA for which the isolation was described in Chapter 2. The N-terminal amino acid sequences of ABF A and a CNBr-derived peptide were determined. One transcription initiation site and two polyadenylation sites could be identified. The structural region is interrupted by seven introns and codes for a protein of 628 amino acids. Mature ABF A consists of 603 amino acid residues with a deduced molecular weight of 65.4 kDa and a theoretical pI of 3.7. For this ABF, an apparent pI of 3.3 and an apparent molecular weight of 83 kDa, determined upon SDS-PAGE, were previously documented.

Although the three enzymes are all active against (1→5)-*α*-glycosidic bonds between L-arabinofuranosides, ABF A, ABF B and ABN A are genetically unrelated. ABF A was found to be *N*-glycosylated whereas ABF B and ABN A were not – these enzymes are only *O*-glycosylated. For each gene, arabinase-overproducing strains were generated by introducing multiple gene copies in *A. niger* or in *A. nidulans* uridine auxotrophic strains through co-transformation. Transformants were isolated upon primary selection for uridine prototrophy. Subsequent overproduction of the genes introduced was demonstrated in these recombinant strains upon growth on sugar beet pulp, both immunochemically and by assaying enzyme activity. *abf*A was shown to be expressed in the heterologous host *A. nidulans*, despite the absence of an *abf*A gene equivalent in this organism. High-copy number *A. niger abf*B transformants featured impaired secretion of other extracellular proteins upon growth on sugar beet pulp. ABN A overproduction was found to be limited to approximately five times the wild-type level in *A. niger abn*A transformants, but not in *A. nidulans* transformants. Such a limitation was not observed in case of the ABFs.

In Chapters 5 and 6, the regulation of L-arabinan degradation is addressed. The structural genes seem to be regulated mainly at the transcriptional level. Additional copies of either ABF-encoding gene in *A. niger* were shown to result in a reduction, but not in total silencing of the expression of the wild-type ABN A-encoding gene upon induction with either sugar beet pulp or L-arabitol (**Chapter 5**). The reduction of the expression level of *abn*A correlated with the *abf* gene dosage. The repression effected by extra *abf*B gene copies was more stringent and more persistent than that elicited by additional *abf*A copies. Although observed with both inducers, these phenomena were more outspoken and more persistent on sugar beet pulp. Similar, but more moderate effects were observed towards the expression of the other *abf* gene in multiple copy *abf*A- and *abf*B-transformants. It was proposed that the *abf* genes titrate two distinct gene activators both involved in coordination of arabinase gene expression. However, the three genes were shown to respond differently upon a mycelial transfer to L-arabitol-containing medium, indicating that gene-specific factors are also involved. Four distinct sequence motifs were found in common in the promoter regions of the three genes. One of these elements is identical to the *A. nidulans* CREA-motif, which has been shown to mediate carbon catabolite repression on several *A. nidulans* enzyme systems. Arabinase expression in *A. niger* is known to be repressed in the presence of D-glucose. Two other motifs are highly homologous to cAMP-responsive elements described in other organisms. For the fourth motif no functional analogues could be found, but the element was found to be present in several other fungal genes which are not involved in L-arabinan degradation at all. It is therefore likely that none of these common elements confer system-specific regulation.

The presumed involvement of L-arabitol in the induction process of fungal arabinases was further emphasized by the induction characteristics of an *A. nidulans* mutant unable to grow on the end-product of L-arabinan degradation, L-arabinose, nor on L-arabitol (**Chapter 6**). L-Arabitol is an intermediate of L-arabinose catabolism in *Aspergilli*. This mutant was shown to lack NAD^+-dependent L-arabitol dehydrogenase activity resulting in L-arabitol accumulation, both intracellularly and in the culture medium, whenever L-arabinose is present. Upon submerged growth on various carbon sources in the presence of L-arabinose, the mutant featured enhanced expression of the enzymes involved in extracellular L-arabinan degradation, and of those of the intracellular L-arabinose catabolism. The co-substrates on which the mutant secreted large amounts of arabitol simultaneously exhibited high arabinase expression and featured reduced growth. L-Arabitol secretion and enzyme production were also observed on a mixed carbon source of D-glucose and L-arabinose, resulting in normal growth. Hence, in the presence of L-arabinose, the carbon catabolite repression conferred by D-glucose in the wild-type, is overruled in the mutant.

In **Chapter 7**, ABN A is shown to have remote sequence similarity with four bacterial xylanolytic glycosyl hydrolases (three β-D-xylosidases and an endo-1,4-β-D-xylanase), three of which feature activity against *para*-nitrophenyl-α-L-arabinofuranoside. This synthetic compound is commonly utilized to assay potential ABF activity, whereas it is known to be an inhibitor of the fourth enzyme. The homology became evident only after multiple-sequence alignments and hydrophobic cluster analysis. It was proposed that these enzymes share a binding site for a terminal non-reducing α-linked L-arabinofuranosyl residue and that they all belong to glycosyl hydrolase family 43. Implications from these suggestions were discussed. The ABFs could not be assigned to an established glycosyl hydrolase family.

Based on the L-arabinolytic system of the brown-rot fungus *Monilinia fructigena*, the sequence similarity found amongst ABF A and bacterial pullulan-degrading enzymes, and ABF expression levels under carbon starvation conditions and on D-glucose as the carbon source, distinct functions in L-arabinan and plant cell-wall degradation were proposed for ABF A and ABF B. ABF A would be essentially cell-wall associated and act to degrade L-arabinan fragments generated by ABN A. ABF B activity would be important for the primary release of small amounts of L-arabinose which initiate induction of various endolytic systems to degrade plant cell walls, and thus function in substrate sensing. In line with these considerations, the involvement of other, not yet identified glycosyl hydrolases in L-arabinan degradation by *A. niger* was suggested.

Induction and repression of arabinase gene expression are further discussed in **Chapter 7**. The results of the studies in *A. niger* (Chapter 5) and *A. nidulans* (Chapter 6) were interpreted in a mutual context. The identity of the low-molecular-weight compound directly responsible for induction of arabinase gene expression, was addressed. Both L-arabinose and L-arabitol are likely candidates to fulfil such a role. However, it was not possible to weigh the actual inductive capacities of L-arabinose and L-arabitol due to their *in vivo* convertibility and the carbon catabolite repression elicited by the pentose. Competition for such a compound provides an alternative explanation for the phenomena observed in Chapter 5. The involvement of the transcriptional repressor CREA in arabinase gene expression is not limited to the direct repression of structural and regulatory genes of the L-arabinan-degrading system. It also plays a role in inducer exclusion and end-product repression, two processes shown to be eminently involved in the regulation of L-arabinan degradation in wild-type *A. nidulans*. Fungal growth rate was suggested to be related to derepression of the L-arabinan-degrading system. The possible involvement of cAMP in arabinase gene expression, as suggested by the presence of potential *cis*-acting cAMP-responsive elements in the structural genes, was considered. Various ways by which cAMP might modulate arabinase synthesis were surveyed.

Samenvatting

Dit proefschrift beschrijft een moleculaire studie naar de genetica van L-arabinan afbraak door *Aspergillus niger* en *Aspergillus nidulans*. Deze saprofiete filamenteuze schimmels maken een extra-cellulair hydrolytisch enzym-systeem om het plante-celwand polysaccharide L-arabinan af te kunnen breken. **Hoofdstuk 1** geeft een overzicht van de eigenschappen, verspreiding en toepassingen van L-arabinanolytische enzymen (arabinases). Het enzym-systeem van *A. niger* bestaat uit een endolytisch endo-1,5-*α*-L-arabinase (ABN A) en twee verschillende *α*-L-arabinofuranosidases (ABF A en ABF B). De drie *A. niger* arabinases zijn geglycosyleerd. Dit systeem is in het verleden veelvuldig onderzocht en is tot op heden het best gekarakteriseerde L-arabinanolytische enzym-systeem. Alles dat bekend is over de inductie van arabinase expressie in schimmels is geïnventariseerd in dit Hoofdstuk. Daarnaast is de structuur van het substraat L-arabinan en zijn functie in de plante-celwand matrix geïntroduceerd.

In de Hoofdstukken 2 tot en met 5 is de klonering van de structurele genen uit *A. niger*, welke coderen voor de drie bovengenoemde glycosyl hydrolases, beschreven en zijn deze genen gekarakteriseerd. *abf*A en *abf*B zijn de eerste ABF-coderende genen van eukaryote oorsprong, waarvan de isolatie en de DNA-sequentie is gepubliceerd, terwijl *abn*A het eerste ABN-coderende gen is, dat ooit is gekloneerd. De isolatie van het *abf*A gen, dat codeert voor het ABF dat extra-cellulair het minst voorkomt, is beschreven in **Hoofdstuk 2**. Het gen kon worden gekloneerd door ABF A-specifiek cDNA te gebruiken als "probe". Dit cDNA is met behulp van antiserum immunochemisch geïdentificeerd en geïsoleerd uit een cDNA-bank, die is gemaakt van RNA afkomstig van mycelium van een mutante *A. niger* stam die niet kan groeien op L-arabitol, een verbinding die arabinase produktie induceert. Dit pentitol is een intermediair uit de verwerkingsroute van L-arabinose, het ultieme produkt van L-arabinan afbraak. Na transfer van mycelium naar medium met daarin L-arabitol als enige koolstofbron, scheidt de mutant méér arabinases uit dan het wildtype. In **Hoofdstuk 3** is de klonering van het ABN-coderende *abn*A gen beschreven en zijn het gen en het gen-produkt gekarakteriseerd. Bij de isolatie van dit gen is eenzelfde strategie toegepast als in geval van het *abf*A gen, maar hiervoor diende eerst een nieuwe cDNA-bank te worden gemaakt. Om de juiste inductie condities voor die nieuwe bank vast te stellen, is het verloop van het inductieproces van ABN A expressie bestudeerd. Van zowel het geïsoleerde DNA als van het cDNA zijn de nucleotide sequenties bepaald. Daarnaast zijn van het eiwit en van een peptide, ontstaan na cyanogeen-bromide (CNBr) splitsing van ABN A, de N-terminale aminozuur-sequenties bepaald. Meerdere startpunten van gen-transcriptie en één polyadenylerings plaats konden worden aangetoond. Het structurele gebied van het gen codeert

voor voor een gen-produkt van 321 aminozuren en bevat drie intronen. Het uitgescheiden eiwit bevat 302 aminozuren en heeft een afgeleid molecuulgewicht van 32,5 kDa. De theoretische waarde voor het iso-elektrisch punt (pI) is 3,5. Voor ABN A zijn eerder experimenteel waarden van respectievelijk 43 kDa en 3,0 vastgesteld. **Hoofdstuk 4** behandelt de klonering van het *abf*B gen, dat codeert voor het meest prominente extra-cellulaire ABF. Hiertoe zijn eerst de N-terminale aminozuur sequenties van ABF B en van een aantal peptiden, ontstaan na CNBr-splitsing van dit eiwit, bepaald. Daarna konden een drietal deoxyoligo-nucleotide mengsels worden gemaakt die coderen voor drie van de bepaalde aminozuur-sequenties. Als die oligonucleotide mengsels worden benut als "primers" in een "polymerase chain reaction" (PCR) ontstaan ABF B-specifieke amplificatie-produkten. Eén van die produkten is gebruikt als "probe" voor het *abf*B gen. Van het geïsoleerde gen is de DNA sequentie bepaald. Dit is ook gebeurd voor ABF B-specifiek cDNA afkomstig uit de cDNA-bank beschreven in Hoofdstuk 3. Meerdere startpunten van gen-transcriptie en één polyadenylerings plaats konden worden aangetoond. Het structurele gebied wordt niet onderbroken door intronen en codeert voor een primair gen-produkt van 499 aminozuren. Het uitgescheiden enzym is 481 aminozuren groot en heeft een afgeleid molecuulgewicht van 50,7 kDa, terwijl de theoretische waarde van 3,8 voor het pI is berekend. Eerder zijn experimenteel een molecuulgewicht van 67 kDa en een pI van 3,5 vastgesteld. Het gen-produkt ABF B is waarschijnlijk identiek aan het ABF, dat is gezuiverd door Kaji en Tagawa (Biochim Biophys Acta **207**: 456-464 (1970)). Rekening houdende met het gehalte aan bestanddelen die géén aminozuren zijn, zoals is bepaald voor het laatstgenoemde eiwit, kan voor ABF B een molecuulgewicht van 64 kDa worden afgeleid. In **Hoofdstuk 5** is ondermeer de karakterisatie van het *abf*A gen en haar gen-produkt ABF A, beschreven. Hiertoe zijn de nucleotide sequenties van het gekloneerde gen en het cDNA, waarvan de isolatie is beschreven in Hoofdstuk 2, bepaald. Daarnaast zijn van het eiwit en van een peptide, ontstaan na CNBr-splitsing van ABF A, de N-terminale aminozuur-sequenties bepaald. Eén gen-transcriptie startpunt en twee polyadenylerings plaatsen konden worden aangetoond. Het structurele gebied van het gen codeert voor een gen-produkt van 628 aminozuren en bevat zeven intronen. Het extra-cellulaire eiwit bevat 603 aminozuren en heeft een afgeleid molecuulgewicht van 65,4 kDa. De theoretische waarde voor het pI is 3,7. Voor ABF A zijn eerder experimenteel waarden van respectievelijk 83 kDa en 3,3 vastgesteld.

Hoewel alle drie de enzymen dezelfde (1→5)-α-glycosidische bindingen tussen L-arabinofuranoses splitsen, zijn ze niet genetisch verwant. In tegenstelling tot ABF B en ABN A, blijkt ABF A *N*-geglycosyleerd te zijn. Ter identificatie zijn voor elk van de drie genen, arabinase-overproducerende schimmelstammen gemaakt door met behulp van de cotransformatietechniek, meerdere kopieën van de structurele genen in het genoom van uridine-behoeftige *A. niger* en *A. nidulans*

mutanten te introduceren. Hierbij zijn getransformeerde schimmels primair geselecteerd op groei zonder toegevoegde uridine. Door overproduktie van enzym als gevolg van de aanwezigheid van extra gen-kopieën aan te tonen, kon de identiteit van de genen geverifieerd worden na groei van de recombinante schimmelstammen op suikerbietenpulp, een koolstofbron die arabinase produktie induceert. Deze overproduktie is met behulp van antisera immunochemisch aangetoond, en bevestigd door de enzym-activiteit te meten. Het *abf*A gen blijkt tot expressie gebracht te kunnen worden in de heterologe gastheer *A. nidulans*, ondanks het feit dat dit organisme géén gelijkwaardig gen heeft. De uitscheiding van extra-cellulair eiwit door *A. niger* blijkt te zijn verstoord als er veel *abf*B gen-kopieën in het genoom aanwezig zijn. Introductie van extra *abn*A gen-kopieën heeft in *A. niger* slechts een beperkte overproduktie van ABN A tot gevolg, maximaal zo'n vijf keer het wildtype niveau. Deze beperking is niet gevonden in de heterologe gastheer *A. nidulans*, en ook niet in recombinante schimmels met extra kopieën van de ABF-coderende genen.

In Hoofdstukken 5 en 6 is de regulatie van het L-arabinan-afbrekende enzym-systeem bestudeerd. De expressie lijkt voornamelijk te worden gereguleerd op het niveau van de gen-transcriptie. De aanwezigheid van extra kopieën van elk van de ABF-coderende genen in het *A. niger* genoom, bleek een reductie van het expressieniveau van het, in enkelvoud aanwezige, wildtype *abn*A gen tot gevolg te hebben (**Hoofdstuk 5**). Alle geteste stammen maakten echter nog steeds ABN A. De reductie van het expressieniveau van het *abn*A gen was gecorreleerd aan het aantal extra *abf* gen-kopieën. De repressie als gevolg van extra *abf*B gen-kopieën was stringenter dan de repressie die werd uitgeoefend in aanwezigheid van meerdere *abf*A kopieën, zowel in omvang als in duurzaamheid. Deze effecten zijn waargenomen op zowel suikerbietenpulp als op L-arabitol, twee totaal verschillende koolbronnen die beiden het enzym-systeem induceren. Echter de repressie op suikerbietenpulp was sterker en langduriger. Daarnaast bleken extra *abf* gen-kopieën de expressie van de aanwezige enkelvoudige *abf*A, respectievelijk *abf*B genen op eenzelfde manier, doch minder stringent, te beïnvloeden in deze transformante schimmels. Dit kan worden verklaard door te veronderstellen dat de *abf* genen twee verschillende activerende factoren titreren, die beide een rol spelen bij de coördinatie van de expressie van arabinases. Omdat de drie genen verschillend reageerden wanneer schimmelmycelium werd overgebracht naar cultuurmedium met L-arabitol als enige koolstofbron, moet worden aangenomen dat hierbij ook gen-specifieke regulatoire factoren een belangrijke rol spelen. In de promoter gebieden van de drie bestudeerde genen zijn vier verschillende sequentie-elementen gevonden, die mogelijk betrokken zijn bij de coördinatie van arabinase expressie. Eén van deze elementen is identiek aan het *A. nidulans* CREA-motief, dat is betrokken bij de koolstof kataboliet repressie van een aantal enzym-systemen in *A. nidulans*. Arabinase expressie in *A. niger* is inderdaad gerepresseerd in aanwezigheid van D-glucose. Twee andere elementen zijn homoloog aan functionele "cAMP-responsive elements"

beschreven in andere organismen. Voor het vierde element zijn géén functionele analogen gevonden, maar dit element bleek ook aanwezig in het stroomopwaarts gelegen niet-coderende gebied van een aantal andere schimmelgenen, die zeker niet betrokken zijn bij de afbraak van L-arabinan. Het is daarom redelijk te veronderstellen dat géén van de genoemde sequentie-elementen een rol speelt bij de systeem-specifieke regulatie.

Het vermoeden omtrent de betrokkenheid van L-arabitol in het inductie-proces van L-arabinan afbraak, kon verder onderbouwd worden na bestudering van de inductie-karakteristieken van een *A. nidulans* mutant, die niet kan groeien op L-arabinose, het eindprodukt van de L-arabinan afbraak, en ook niet op L-arabitol (**Hoofdstuk 6**). L-Arabitol is een intermediair van het L-arabinose katabolisme in *Aspergilli*. In de mutant ontbreekt een NAD^+-afhankelijke L-arabitol dehydrogenase activiteit, en de aanwezigheid van L-arabinose leidt tot ophoping van L-arabitol, zowel intra- als extra-cellulair. Bij submerse groei in vloeibaar medium op verschillende koolstofbronnen in aanwezigheid van L-arabinose, bleek er in de mutant sprake te zijn van verhoogde expressie van de enzymen, die zijn betrokken bij het L-arabinose katabolisme, én van arabinases. De koolstofbronnen die aanleiding gaven tot het uitscheiden van grote hoeveelheden L-arabitol, produceerden veel arabinase terwijl ook de groei van de mutant was vertraagd. Excretie van het pentitol alsmede enzym-produktie vond ook plaats op een gemengde koolstofbron van L-arabinose en D-glucose, maar de groei was hier normaal. Dus als L-arabinose aanwezig is, wordt de D-glucose kataboliet repressie, die arabinase expressie in het wildtype vrijwel volledig onderdrukt, omzeild in de mutant.

Zoals beschreven in **Hoofdstuk 7**, is beperkte sequentie homologie aangetoond tussen *A. niger* ABN A en een viertal bacteriële xylanolytische glycosyl hydrolases (drie β-D-xylosidases en een endo-1,4-β-D-xylanase), waarvan er drie *para*-nitrophenyl-α-L-arabinofuranoside kunnen splitsen. Dit kunstmatige substraat wordt algemeen gebruikt om ABF activiteit te kunnen meten, terwijl het bekend staat als een remmer van het vierde enzym. De homologie werd pas herkend na meervoudige sequentievergelijkingen en "hydrophobic cluster analysis". Voorgesteld is dat al deze enzymen een bindingsplaats voor een terminaal niet-reducerend en α-gebonden L-arabinofuranosyl residu hebben, en dat zij deel uit maken van glycosyl hydrolase familie 43. De gevolgen van deze toekenning zijn nader geanalyseerd. De beide ABFs konden niet worden ondergebracht in een bestaande glycosyl hydrolase familie.

Uitgaande van het L-arabinanolytische enzym-systeem van de schimmel *Monilinia fructigena*, en gegeven de sequentie homologie zoals die is gevonden tussen ABF A en bacteriële pullulanan-afbrekende enzymen, én de ABF produktie onder hongercondities en op D-glucose als koolstofbron, zijn voor ABF A en ABF B verschillende functies in L-arabinan en plante-celwand afbraak voorgesteld. ABF A zou primair celwandgebonden kunnen zijn en zorg dragen voor de afbraak van,

door ABN A gegenereerde fragmenten. Daarentegen zou ABF B activiteit van belang zijn voor het vrijmaken van kleine hoeveelheden L-arabinose teneinde verschillende endolytische enzym-systemen te induceren, en dus voor het traceren van potentiële polymere koolstofbronnen voor groei ("substrate sensing"). In verband hiermee is de betrokkenheid van andere, nog niet gekarakteriseerde glycosyl hydrolases bij L-arabinan afbraak gesuggereerd.

De inductie en repressie van arabinase gen-expressie zijn onderwerp van verdere discussie in het vervolg van **Hoofdstuk 7**. Hierbij zijn de resultaten van Hoofdstuk 5 (*A. niger*) en Hoofdstuk 6 (*A. nidulans*) integraal geïnterpreteerd. De identiteit van de verbinding die klein genoeg is om direct inductie te kunnen bewerkstelligen, is onder de loep genomen. Zowel L-arabinose als L-arabitol kunnen de rol van directe "inducer" vervullen. Het bleek echter niet mogelijk onderscheid tussen deze twee stoffen te maken vanwege hun *in vivo* convertibiliteit en de repressieve eigenschappen van het pentose. Onderlinge competitie voor een dergelijke verbinding is geïntroduceerd als een alternatieve verklaring voor de waargenomen effecten beschreven in Hoofdstuk 5. De rol van de transcriptie repressor CREA in arabinase expressie is niet beperkt tot de directe repressie van de transcriptie van de structurele en systeem-afhankelijke regulatoire genen. Zij speelt ook een rol bij "inducer exclusion" en "end-product repression", twee processen die de L-arabinanolysis in de wildtype *A. nidulans* stam blijken te beïnvloeden. De groeisnelheid van de schimmel is mogelijk gerelateerd aan derepressie van het L-arabinan-afbrekende enzym-systeem. De mogelijke betrokkenheid van cAMP bij arabinase gen-expressie, zoals wordt gesuggereerd door de aanwezigheid van mogelijke "cAMP-responsive elements" in de structurele genen, is geïnventariseerd. De verschillende routes waarlangs cAMP de arabinase produktie zou kunnen beïnvloeden, zijn nader toegelicht.

Curriculum vitae

Michel Flipphi werd geboren op 30 september 1964 te Bergh (Gld). Na het behalen van het VWO diploma aan het Rijnlands Lyceum te Sassenheim, begon hij in 1982 met de studie Moleculaire Wetenschappen aan de toenmalige Landbouwhogeschool te Wageningen. In 1988 studeerde hij *cum laude* af met als afstudeervakken Moleculaire Biologie (Prof dr A. van Kammen), Moleculaire Genetica (Dr J. Visser) en Celbiologie (Dr E. Egberts). In juni 1989 werd hem de scriptieprijs van het Wageningenfonds toegekend. Van november 1988 tot januari 1994 was hij verbonden aan de sectie Moleculaire Genetica van Industriële Micro-organismen van de Landbouwuniversiteit Wageningen, eerst als assistent in opleiding, later als gastmedewerker. In deze periode voerde hij ondermeer het onderzoek uit waarvan in dit proefschrift verslag wordt gedaan.

Publicaties

Flipphi MJA, Heuvel M van, Veen P van der, Visser J, Graaff LH de (1993) Cloning and characterization of the *abf*B gene coding for the major *α*-L-arabinofuranosidase (ABF B) of *Aspergillus niger*. Curr Genet **24**:525-532

Flipphi MJA, Panneman H, Veen P van der, Visser J, Graaff LH de (1993) Molecular cloning, expression and structure of the endo-1,5-*α*-L-arabinase gene of *Aspergillus niger*. Appl Microbiol Biotechnol **40**:318-326

Flipphi MJA, Visser J, Veen P van der, Graaff LH de (1993) Cloning of the *Aspergillus niger* gene encoding *α*-L-arabinofuranosidase A. Appl Microbiol Biotechnol **39**:335-340

Flipphi MJA, Visser J, Veen P van der, Graaff LH de (1994) Arabinase gene expression in *Aspergillus niger*: indications for coordinated regulation. Microbiology **140**:2673-2682

Heuvel M van, Bakhuis JG, Coutel Y, Harder A, Graaff LH de, Flipphi MJA, Veen P van der, Visser J, Andreoli PM (1992) Cloning and expression of genes encoding arabinan-degrading enzymes of fungal origin. Eur Pat Appl EP 506,190 (Cl. C12N15/56). European Patent Office Bulletin 92/40 n^0. EP 0 506 190 A1 (30.09.1992); 75 pp.

Kusters-van Someren M, Flipphi M, Graaff L de, Broeck H van den, Kester H, Hinnen A, Visser J (1992) Characterization of the *Aspergillus niger pel*B gene: structure and regulation of expression. Mol Gen Genet **234**:113-120

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1991) Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. Arch Microbiol **157**:23-28

Veen P van der, Flipphi MJA, Voragen AGJ, Visser J (1993) Induction of extracellular arabinases on monomeric substrates in *Aspergillus niger*. Arch Microbiol **159**:66-71

Veen P van der, Arst HN Jr, Flipphi MJA, Visser J (1994) Extracellular arabinases in *Aspergillus nidulans*: the effect of different *cre* mutations on enzyme levels. Arch Microbiol **162**:433-440

Vries RP de, Flipphi MJA, Witteveen CFB, Visser J (1994) Characterization of an *Aspergillus nidulans* L-arabitol dehydrogenase mutant. FEMS Microbiol Lett **123**:83-90

Nawoord

Dit proefschrift beschrijft de wetenschappelijke resultaten van mijn promotie-onderzoek, verricht bij de sectie Moleculaire Genetica van Industriële Micro-organismen van de Landbouw Universiteit Wageningen. Hoewel alleen mijn naam boven dit werkstuk staat hebben velen bijgedragen aan de totstandkoming ervan. In de eerste plaats Jaap Visser, mijn co-promotor. Hij bood mij de mogelijkheid mijn kennis en vaardigheden verder te ontwikkelen. Ik ben dankbaar voor het vertrouwen dat hij mij heeft geschonken. Daarnaast ook Leo de Graaff, die lange tijd leiding heeft gegeven aan het practische gedeelte van het onderzoek. Zijn inzet heeft het mogelijk gemaakt dat ik nu dit proefschrift kan verdedigen. Ab van Ooyen wil ik bedanken omdat hij mijn promotor wilde zijn. Zijn kritische oog heeft het nodige bijgedragen aan de uiteindelijke vorm van het manuscript. Sabine Cuypers, Michiel Kotterman, Marianne Linkels, Henk Panneman, Peter van der Veen, Peter van de Vondervoort, Ronald de Vries en Cor Witteveen; bedankt voor jullie hulp op de werkvloer. Daarnaast wil ik Margreet van Heuvel en de andere collega's van Gist brocades bedanken voor hun bijdragen. I want to thank Bernard Henrissat for his interest in my work and his help in connection with hydrophobic cluster analysis.
Next to science I've experienced lots of other things during my stay at MGIM. I'm proud of my Dutch and foreign friends and grateful for their patience, attention and support, providing motivation and inspiration. May our friendship persist regardless time and distance. Without all you it would not have been possible to finish this thesis. Dit geldt zeker ook voor mijn ouders: Pa en Ma, bedankt voor alles.