Accessory enzymes from *Aspergillus* involved in xylan and pectin degradation

Promotor: Dr. ir. J. A. M. de Bont
Hoogleraar Industriële Microbiologie

Copromotor: Dr. ir. J. Visser
Universitair hoofddocent, sectie Moleculaire Genetica van Industriële Micro-organismen

Ronald Peter de Vries

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aan mijn ouders

Contents

Chapter 1

General introduction

A modified verison of this chapter will be submitted for publication.

1. The plant cell wall

1.1. Plant cell wall polysaccharides

Plant cell wall polysaccharides are the most abundant organic compounds found in nature. They can be divided into three groups: cellulose, hemicellulose and pectin. Cellulose represents the major constituent of cell wall polysaccharides and consists of a linear polymer of β-1,4-linked D-glucose residues. The cellulose polymers are present as ordered structures (fibres) and their main function is to ensure the rigidity of the plant cell wall.

Hemicelluloses are more heterogeneous polysaccharides and are the second most abundant organic structure in the plant cell wall. The major hemicellulose polymer in cereals and hardwood is xylan. Xylan consists of a β-1,4-linked D-xylose backbone and can be substituted with different side-groups such as L-arabinose, D-galactose, acetyl, feruloyl and *p*-coumaroyl and glucuronic acid residues (Wilkie and Woo, 1977). A second hemicellulose structure commonly found in soft- and hardwoods is (galacto)glucomannan (Timell, 1967), which consists of a backbone of β-1,4-linked mannose and glucose residues and contains galactose side groups. Softwoods contain mainly galactoglucomannan whereas in hardwoods glucomannan is the most common form.

Pectins form another group of heteropolysaccharides, which consist of a backbone of α-1,4-linked galacturonic acid residues. In specific 'hairy' regions the galacturonic acid backbone is interrupted by α-1,2-linked rhamnose residues. Long side chains consisting mainly of L-arabinose and D-galactose residues can be attached to these rhamnose residues. In pectins of some origins (e.g. sugar beet and apple) ferulic acid is present as terminal residues attached to O-5 of the arabinose residues or O-2 of the galactose residues.

The hemicellulose and pectin polysaccharides, as well as the aromatic polymer lignin interact with the cellulose fibrilles creating a rigid structure strengthening the plant cell wall. They also form covalent crosslinks, which are believed to be involved in limiting cell growth and reducing cell wall biodegradibility. Two types of covalent crosslinks have been identified between plant cell wall polysaccharides and lignin (Fry, 1986). The crosslink studied in most detail is the formation of di-ferulic acid bridges. Di-ferulic (di-cinnamic) acid bridges between polysaccharides and lignin have been identified in many plants. They have been shown to occur between arabinoxylans from bamboo shoot cell walls (Ishii, 1991), between pectin polymers in sugar beet (Oosterveldt et al., 1997), and between lignin and xylan in wheat (Bach Tuyet Lam, 1992). A second type of crosslinks is the ester linkage between lignin and

glucuronic acid attached to xylan, which was identified in beech wood (Imamura et al., 1994; Takahashi et al., 1988).

1.2. Structural features of xylans

The structure of xylans found in cell walls of plants can differ strongly depending on the origin, but always contains a β-1,4-linked xylose backbone (Wilkie, 1979). The schematic representation of xylan (Fig. 1) also lists the different structures which can be attached to the xylan backbone and which cause the large variety of xylan structures found in plants. Although most xylans are branched structures, some linear polysaccharides have been isolated (Montgomery et al., 1956; Eda et al., 1976). Cereal xylans contain large quantities of L-arabinose and are therefore often referred to as arabinoxylans, whereas hardwood xylans are often referred to as glucuronoxylans due to the high amount of glucuronic acid attached to the backbone.

Arabinose is connected to the backbone of xylan via an α-1,2- or α-1,3-linkage as single residues or as short side chains. These side chains can also contain xylose, β-1,2-linked to arabinose, and galactose which can be either β-1,5-linked to arabinose or β-1,4-linked to xylose. Acetyl residues are attached to O2 or O3 of xylose in the backbone of xylan, but the degree of acetylation differs strongly amongst xylans from different origin. Glucuronic acid and its 4-O-methyl ether are attached to the xylan backbone via a α-1,2-linkage, whereas aromatic (feruloyl and *p*-coumaroyl) residues have so far only been found attached to O5 of terminal arabinose residues. As a consequence of all these features the xylans form a very heterogeneous group of polysaccharides (see e.g. Brillouet and Joseleau, 1987; Bajpai, 1997; Schooneveld-Bergmans et al., 1998).

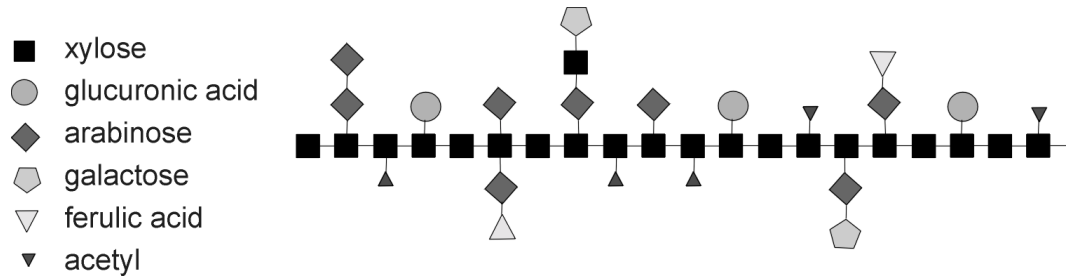

Fig. 1. Schematic presentation of the xylan.

1.3. Structural features of pectins

Pectins are complex heteropolysaccharides which contain two different defined regions (de Vries et al., 1982). The 'smooth' regions consist of a backbone of α-1,4-linked galacturonic acid residues, which can be acetylated or methylated. In the 'hairy' regions (Fig. 2) the galacturonic acid residues in the backbone are interrupted by α-1,2-linked rhamnose residues, to which long arabinan and galactan chains can be attached at O4. The arabinan chains consist of a main chain of α-1,5-linked arabinofuranosidase residues which can be substituted with α-1,3-linked arabinofuranoside and with feruloyl residues attached terminally to O2 of the arabinose residues (Guillon and Thibault, 1989). The galactan sidechains contain a main chain of β-1,4-linked galactopyranoside residues which can be substituted by feruloyl residues at O6 (Guillon and Thibault, 1989). Approximately 20-30% of the feruloyl residues in sugar beet pectin are attached to arabinan side chains, while the other feruloyl residues are attached to galactan side chains (Guillon and Thibault, 1989). The hairy regions also contain acetyl groups ester-linked to galacturonic acid residues of the backbone (Schols and Voragen, 1994).

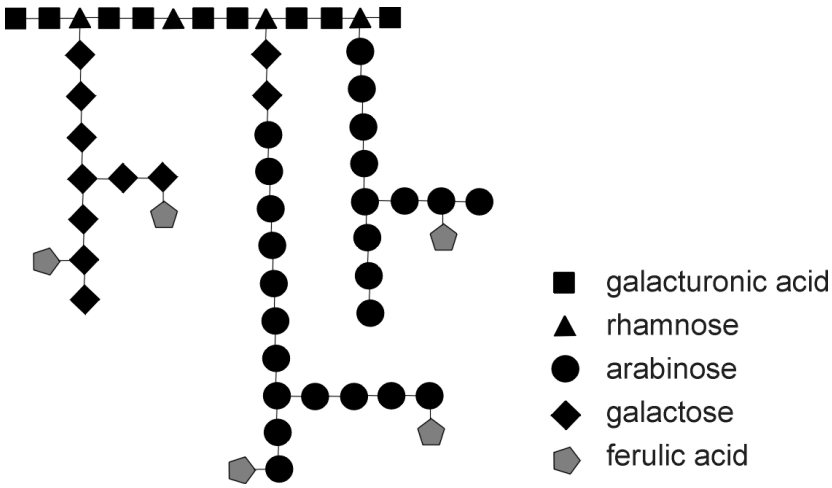

Fig. 2. Schematic presentation of the hairy region of pectin. Based on Schols and Voragen (1996).

1.3. Aromatic residues in plant cell wall polysaccharides

The aromatic acids commonly found in nature can be divided into two classes of compounds: cinnamates and cinnamate conjugates (Clifford et al., 1999). The most common cinnamate is ferulic acid, which is the major aromatic compound present in sugar beet fibre and cereal brans. Other cinnamates are *p*-coumaric acid (spinach, sugar beet fibre, cereal brans), caffeic acid (coffee beverage, blueberries, apples, ciders) and sinapic acid (broccoli, kale, citrus

juices). The group of cinnamate conjugates consists of *p*-coumaroylquinic and feruloylquinic acids, tartaric and malic conjugates, rosmarinic acids and cell wall conjugates (Clifford et al., 1999). The latter compounds are believed to play an important role in the structure and function of the plant cell wall. Ferulic acid can be linked to both the hemicellulolytic (Smith and Hartley, 1983) as well as the pectic (Rombouts and Thibault, 1986) fraction of plant cell walls and is able to cross-link these polysaccharides to each other as well as to the aromatic polymeric compound lignin (Lam et al., 1994; Ishi, 1997). This cross-linked structure results in an increased rigidity of the cell wall. An increase in ferulic acid cross-links during ageing of the plant cell, suggests a function for these cross-links in limiting cell growth (Fry, 1979; Wakabayashi et al., 1997). A role for these cross-links in preventing bio-degradability of the plant cell wall by micro-organisms has also been suggested. Indications for a limited enzymatic degradation of arabinoxylan due to ferulate cross-links have been observed (Eraso and Hartley, 1990; Grabber et al., 1998). Additionally, the antimicrobial effects of these aromatic compounds (Aziz et al., 1998) could add to the plant defense mechanism against fythopathogenic micro-organisms.

In cereals, cinnamic acids (mainly ferulic acid) were identified to be ester-linked to arabinose residues in arabinoxylan in the primary cell wall. Ferulic acid was detected both as terminal residues and ferulate dimers linked in several ways (Fig. 3), such as 5,5’ or 5,8’ carbon-carbon

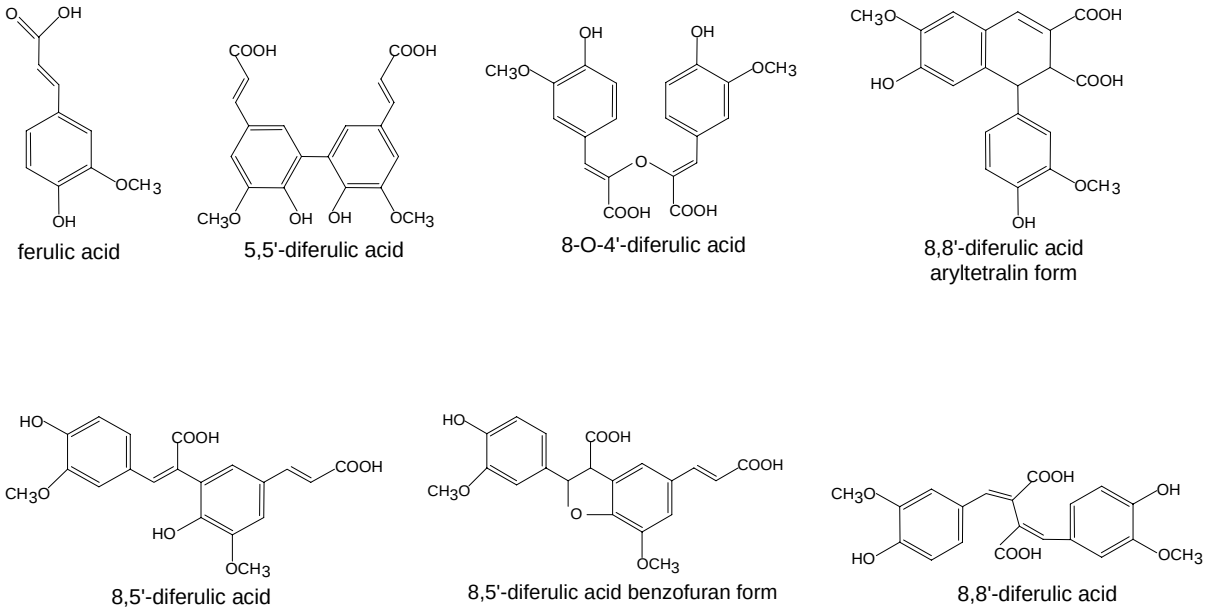

Fig. 3. Schematical presentation of ferulic acid and di-ferulic acid structures identified in plant cell walls. Based on Kroon et al. (1999).

bonds (Kroon et al., 1996b; 1996; Ishii, 1997). Analysis of feruloylated oligosaccharides isolated from several crude plant products indicated that ferulic acid is linked to C5 of arabinose residues attached to xylan (Smith & Hartley, 1983; Saulnier et al., 1995; Wende & Fry, 1997) whereas in pectin ferulic acid is attached to either C2 of arabinose residues or C6 of galactose residues of the pectic sidechains (Colquhoun et al., 1994).

2. Bio-degradation of plant cell wall polysaccharides

2.1. *Aspergillus*

The genus *Aspergillus* is a group of filamentous fungi which consists of a large number of species. The first record of this fungus can be found in Micheli's *Nova Plantarum Genera* (1729), but a more detailed description of the *Aspergilli* didn't appear until the middle of the 19th century. In 1926 a first classification of these fungi was proposed describing 11 groups within the genus (Thom and Church, 1926). A re-examination of the genus was published by Thom and Raper (1945), identifying 14 distinct groups. Some of these groups consist of pathogenic fungi (e.g. *A. fumigatus*, *A. flavus* and *A. parasiticus*), but most important in the context of this thesis are some members of the group of black *Aspergilli* (*A. niger* and *A. tubingensis*). In addition to the morphological techniques traditionally applied, new molecular and biochemical techniques have been used in recent years in the re-classification of this group of *Aspergilli* (Kusters-van Someren et al., 1991a; Megnegneau et al., 1993; Varga et al., 1994; Nikkuni et al., 1996; Hamari et al., 1997; Parenicová et al., 1997). This analysis resulted in the clear distinction of eight groups of black *Aspergilli* (*A. niger*, *A. tubingensis*, *A. foetidus*, *A. carbonarius*, *A. japonicus*, *A. aculeatus*, *A. heteromorphus*, and *A. ellipticus*; Parenicová et al., 1997). Products of several of these species have obtained a GRAS (Generally Regarded As Safe) status, which allows them to be used in food and feed applications. The black *Aspergilli* have a number of characteristics which make them interesting organisms for industrial applications, such as good fermentation capabilities and high levels of protein secretion. In particular the wide range of enzymes *Aspergillus* produces for the degradation of plant cell wall polysaccharides are of major importance to the food and feed industry. Recently, several *Aspergillus* sp. have received an increasing interest as hosts for heterologous protein production (Davies, 1994).

2.2. Degradation of the xylan backbone

The bio-degradation of the xylan backbone depends on two classes of enzymes. Endoxylanases (EC 3.2.1.8) are able to cleave the xylan backbone into smaller oligosaccharides, which can than be further degraded to xylose by β-xylosidases (EC 3.2.1.37). Both classes of enzymes, as well as their encoding genes, have been characterised from many organisms. Several different endo-xylanases have been identified in *Aspergillus*. Although variation is detected in their molecular mass or pH optimum, the major difference between the enzymes is in their pI which ranges from 3.5 (Ito et al., 1992a) to 9.0 (Fujimoto et al., 1995). Endoxylanases also differ in their activity

Table 1. Physical properties of *Aspergillus* endoxylanases.

Organism	Enzyme	Mw	pH_{opt}	T_{opt} (°C)	pI	Reference
A. aculeatus	FIa	18	4.0	50	5.6	Fujimoto et al., 1995
A. aculeatus	FIb	26	5.0	50	9.0	Fujimoto et al., 1995
A. aculeatus	FIII	52	5.0	70	3.8	Fujimoto et al., 1995
A. awamori	I	39	5.5-6.0	55	5.7-6.7	Kormelink et al., 1993a
A. awamori	II	23	5.0	50	3.7	Kormelink et al., 1993a
A. awamori	III	26	4.0	45-50	3.3-3.5	Kormelink et al., 1993a
A. flavipes		45	5.0	55		Sherief, 1990.
A. foetidus	I	24			7.6	Bailey et al., 1991
A. foetidus	II	26			4.6	Bailey et al., 1991
A. fumigatus	I	22			>9.4	Bailey et al., 1991
A. fumigatus	II	10			5.7	Bailey et al., 1991
A. kawachii	XylA	35	5.5		6.7	Ito et al., 1992a
A. kawachii	XylB	26	4.5		4.4	Ito et al., 1992a
A. kawachii	XylC	29	2.0		3.5	Ito et al., 1992a
A. nidulans		34	6.0	56	3.4	Fernandez-Espinar et al., 1994
A. niger		33	4.0	50	4.2	Gorbacheva and Rodionova, 1977a
A. niger		20.8	4-6	55	6.7	Frederick et al., 1981
A. niger		28	5.0	40-45	3.65	Fournier et al., 1984
A. ochraceus		48	6.0			Biswas et al., 1990
A. oryzae	I	28	5.0		7.0	Bailey et al., 1991
A. oryzae	II	26	5.0		4.9	Bailey et al., 1991
A. oryzae		46.5	5.0	55	3.6	Golubev et al., 1993
A. sojae	X-I	32.7	5.5	60	3.5	Kimura et al., 1995
A. sojae	X-II-B	35.5	5.5	50	3.75	Kimura et al., 1995
A. sydowii		30	5.5	60		Ghosh & Nanda., 1994
A. tubingensis	XlnA	19			3.6	de Graaff et al., 1994

towards the xylan polymer. Some enzymes cut randomly between unsubstituted xylose residues, whereas the activity of other endo-xylanases strongly depends on the substituents on the xylose residues neighbouring the attacked residues. Another distinction between endoxylanases is in the liberation of L-arabinose. Based on this criteria endoxylanases were divided into two groups by Dekker and Richards (1976), those which did not release L-arabinose and those which did release L-arabinose.

In several *Aspergilli*, three different endoxylanases have been identified (Ito et al., 1992a; Kormelink et al., 1993a; Fujimoto et al., 1995). The best studied *Aspergillus* endoxylanases, with respect to substrate specificity, are the three enzymes from *A. awamori* (Kormelink et al., 1993a). Counting from the reducing end, *A. awamori* endoxylanase I is unable to remove one unsubstituted xylose residue adjacent to single substituted xylose residues or two unsubsituted xylose residues adjacent to doubly substituted xylose residues (Kormelink et al., 1993b). *A. awamori* endoxylanase III was not able to remove two unsubstituted xylose residues adjacent to singly or doubly substituted xylose residues towards the reducing end (Kormelink et al., 1993b).

Table 2. Genes encoding *Aspergillus* endoxylanases and their assignment to the glycosyl hydrolase families.

Organism	Gene	Glycosyl hydrolase family	Database access. no.	Reference
A. aculeatus	FIa	10	AB013110	Arai et al., in press
A. awamori	*exl*A	11	X78115	Hessing et al., 1994
A. kawachii	*xyn*A	10	D14847	Ito et al., 1992b
A. kawachii	*xyn*B	11	D38070	Ito, unpublished
A. kawachii	*xyn*C	11	S45138	Ito et al., 1992c
A. nidulans	*xln*A	11	Z49892	Perez-Gonzalez et al., 1996
A. nidulans	*xln*B	11	Z49893	Perez-Gonzalez et al., 1996
A. niger		11	A19535	Krengel and Dijkstra, 1996
A. niger	*xyn*B	11	D38071	Ito, unpublished
A. niger	*xyn*4	11	U39785	Luttig et al., unpublished
A. niger	*xyn*5	11	U39784	Luttig et al., unpublished
A. oryzae	*xyn*G1	11	AB003085	Kimura et al., 1998b
A. oryzae	F1	10	AB011212	Kitamoto, unpublished
A. tubingensis	*xln*A	11	L26988	de Graaff et al., 1994

Hydrolysis of a glucuronoxylan by an endoxylanase from *A. niger* str. 14 (Gorbacheva and Rodionova, 1977b) resulted mainly in xylobiose, xylotriose, and xylose, but the hydrolysis of an arabinoxylan by the same enzyme resulted mainly in oligosaccharides with a degree of polymerisation of over 3. This suggest that the action of this endoxylanase is strongly inhibited

by the presence of arabinose residues on the xylan backbone. All xylanases which have been purified to date are produced when *Aspergillus* is grown on xylan. Most of these enzymes are also produced when xylose was used as a carbon source, but all at lower levels than on xylan. This is discussed in more detail in section 3.4.

Several genes encoding endoxylanases from *Aspergillus* sp. haven been cloned. Based on the derived amino acid sequence the gene products have been assigned to the different glycosyl hydrolase families (Henrissat, 1991; Henrissat and Bairoch, 1993 & 1996) as described by B. Henrissat at URL: http://afmb.cnrs-mrs.fr/~pedro/cazy/ (Table 2). *Aspergillus* endoxylanases can be found in families 10 and 11 of the glycosyl hydrolases. Based on the data of the *A. kawachii* endoxylanases it would appear that the acidic endoxylanases belong to family 11, whereas the neutral endoxylanases belong to group 10. However, more data on other neutral and basic endoxylanases is needed to verify this.

β-Xylosidases have been identified in several *Aspergilli* (Table 3). This enzyme is highly specific for small unsubstituted xylose oligosaccharides (up to DP 4) and results in the production of xylose. Although this enzyme is of major importance for the complete degradation of xylan, absence of the enzyme does not interfere with the induction of the xylanolytic system (van Peij et al., 1997).

Table 3. Physical properties of *Aspergillus* β-D-xylosidases.

Organism	Enzyme	Mw	pH_{opt}	T_{opt} (°C)	pI	Reference
A. awamori		110	6.5	70	4.2	Kormelink et al., 1993a
A. foetidus		83			4.4	Bailey et al., 1991
A. fumigatus		90	4.5	75	5.4	Kitpreechavanich et al., 1986
A. fumigatus		60			4.3	Bailey et al., 1991
A. nidulans	XlnD	85	5.0	50	3.4	Kumar and Ramon, 1996
A. niger			5.0	>75		Uchida et al., 1992b
A. niger		122	3.8-4.0	70	4.9	Rodionova et al., 1983
A. oryzae		62			4.5	Bailey et al., 1991
A. pulverulentus	β-Xyl I	65	2.5-3.5	60	4.7	Sulistyo et al., 1995
A. pulverulentus	β-Xyl II	100	4.0-5.0	60	3.5	Sulistyo et al., 1995

The ability of an *A. awamori* β-xylosidase to release xylose from xylo-oligosaccharides was studied to determine its substrate specificity (Kormelink et al., 1993b). This enzyme was only able to release xylose from the non-reducing end of branched oligosaccharides when two

contiguous unsubstituted xylose residues were present adjacent to singly or doubly substituted xylose residues. The genes encoding β-xylosidases from *Aspergillus* sp. have all been assigned to glycosyl hydrolase family 3 (Table 4), unlike some bacterial β-xylosidases which have been assigned to family 39.

Table 4. Genes encoding *Aspergillus* β-D-xylosidases and their assignment to the glycosyl hydrolase families.

Organism	Gene	Glycosyl hydrolase family	Database access. no.	Reference
A. nidulans	*xln*D	3	Y13568	Perez-Gonzalez et al., 1998
A. niger	*xln*D	3	Z84377	van Peij et al., 1997
A. oryzae	*xyl*-1	3	AB009972	Hashimoto et al., unpublished

For some β-xylosidases transxylosylation activity has been detected (Kizawa et al., 1991; Bailey et al., 1991; Shinoyama et al., 1991; Sulistyo et al., 1995), allowing the production of novel xylose containing oligosaccharides by using this enzyme. Production of xylo-oligosaccharides from xylose using β-xylosidase in a condensation reaction was also demonstrated (Iizuka et al., 1992), suggesting a possible application for these enzymes in the synthesis of specific oligosaccharides.

2.3. Degradation of the pectin backbone

The structural differences between the main chain of the hairy and the smooth regions of pectin have implications for the enzymes involved in the degradation of these regions. The smooth region backbone can be hydrolysed by pectin lyases (EC 4.2.2.10), pectate lyases (EC 4.2.2.2) and polygalacturonases (EC 3.2.1.15 and EC 3.2.1.67). In *Aspergillus*, families of genes encoding these types of enzymes have been identified (Harmsen et al., 1990; Bussink et al., 1992a; Parenicová et al., 1998). Several classes of enzymes are involved in the degradation of the hairy region backbone. Rhamnogalacturonan hydrolases and rhamnogalacturonan lyases have been identified in *Aspergilli* (Mutter et al., 1994a, 1994b & 1996; Suykerbuyk et al., 1995) and were found to be highly specific for the hydrolysis of the hairy regions of pectin.

2.4. Accessory enzymes involved in the degradation of plant cell wall polysaccharides

2.4.1. α-L-Arabinofuranosidases and arabinoxylan arabinofuranohydrolases

Arabinose residues can be removed by arabinofuranosidases and arabinoxylan arabinofuranohydrolases. These enzymes and their corresponding genes have been widely studied from many different micro-organisms and differ strongly in substrate specificity. Several

arabinofuranosidases and arabinoxylan arabinofuranohydrolases have been purified from *Aspergillus* sp. (Table 5), and studied with respect to their activity on polymeric and oligomeric substrates. The *A. niger* arabinofuranosidase purified by Kaneko et al. (1993) was only able to release terminal α-1,3-linked arabinose residues, whereas arabinofuranosidase B from *A. niger* was able to release terminal α-1,2-, α-1,3- and α-1,5-linked arabinose residues (Beldman et al., 1993). Unlike some of the arabinofuranosidases, the arabinoxylan arabinofuranohydrolase (AXH) from *A. awamori* was not able to release arabinose from pectin or pectin derived oligosaccharides but is highly specific for arabinose residues linked to xylan (Kormelink et al., 1991). Wood and McCrae (1996) reported the ability of an *Aspergillus awamori* arabinofuranosidase to release feruloylated arabinose residues from wheat straw arabinoxylan. Large differences can be observed when comparing the molecular mass and pI of several arabinofuranosidases (Table 5).

Table 5. Physical properties of *Aspergillus* arabinofuranosidases and arabinoxylan arabinofuranohydrolases.

Organism	Enzyme	Mw	pH_{opt}	T_{opt} (°C)	pI	Reference
A. aculeatus	B1	37	3.0-3.5			Beldman et al., 1993
A. aculeatus	B2	37	4.0-4.5			Beldman et al., 1993
A. awamori		64	4.6	50	3.6, 3.2	Wood and McCrae, 1996
A. nidulans	α-AFase	36	5.5	55	4.3	Fernandez-Espinar et al., 1994
A. nidulans	AbfB	65	4.0	65	3.3	Ramon et al., 1993
A. niger	AbfA	83	3.4	46	3.3	van der Veen et al., 1991
A. niger	AbfB	67	3.8	56	3.5	van der Veen et al., 1991
A. niger	A	128	4.1	50	6-6.5	Rombouts et al., 1988
A. niger	B	60	3.7	60	5.5-6	Rombouts et al., 1988
A. niger		53	3.8		3.6	Tagawa and Kaji, 1988
A. niger		61	3.9	60	<3.7	Gunata et al., 1990
A. niger 5-16		67	4.0	60	3.5	Kaneko et al., 1993
A. sojae	X-II-A	34.3	5.0	50	3.9	Kimura et al., 1995
A. terreus	AbfA	39	4.0		7.5	Luonteri et al., 1995
A. terreus	AbfB1	59	4.0		8.3	Luonteri et al., 1995
A. terreus	AbfB2	59	4.0		8.5	Luonteri et al., 1995
A. awamori	Axh	32	5.0			Kormelink et al., 1991
A. tubingensis	AxhA	32			3.6	Gielkens et al., 1997

Production of arabinofuranosidases has been observed on arabinoxylan (Kaneko et al., 1993), sugar beet pulp (van der Veen et al., 1991; Flipphi et al., 1993b), and L-arabinose and L-arabitol (van der Veen et al., 1993; Ramon et al., 1993). Arabinoxylan arabinofuranohydrolases were produced when *Aspergillus* was grown on oat straw (Kormelink et al., 1991) and birchwood xylan (Gielkens et al., 1997). The induction of these enzymes is discussed in more detail in section 3.3.

The mode of action of AXH from *A. awamori*, and two arabinofuranosidases (Arafur A and B) from *A. niger*, was studied on alkali-extractable wheat-flour arabinoxylan (Kormelink et al., 1993c). AXH specifically released α-1,2- and α-1,3-linked arabinose residues from singly substituted xylose residues. While Arafur B was only able to release arabinose from terminal single substituted residues, AXH and Arafur A were able to release arabinose from both terminal and non-terminal single substituted xylose residues. AXH and Arafur B were able to release arabinose from the intact polysaccharide as well as from xylo-oligosaccharides, while Arafur A was only able to release arabinose from oligosaccharides. Additionally, AXH was not able to release arabinose from arabinan, sugar beet pulp, or pectin, whereas Arafur A and B were active on these substrates. Based on this information it can be concluded that AXH is specifically involved in arabinoxylan degradation, while Arafur A and B are more general arabinose releasing enzymes. Additional information about the substrate specificity of AXH was obtained from a study using a sorghum glucuronarabinoxylan as a substrate (Verbruggen et al., 1998a & 1998b). It was demonstrated that AXH was not able to release arabinose from xylose residues adjacent to glucuronic acid substituted xylose residues. The enzyme was also not able to remove arabinobiose side chains (Verbruggen et al., 1998a & 1998b).

The difference between arabinoxylan arabinofuranohydrolases and arabinofuranosidases is also apparent with respect to the assignment to the glycosyl hydrolase families (Table 6). Arabinofuranosidases are assigned to family 51 and 54, whereas arabinoxylan arabinofuranohydrolases belong to family 62. AbfA from *A. niger* is assigned to a different family than AbfB from *A. niger*, which might reflect the substrate specificity of the enzymes. Both enzymes are able to release arabinose from arabinan and sugar beet pulp, but only AbfB is able to release arabinose from xylan.

Table 6. Genes encoding *Aspergillus* arabinofuranosidases and arabinoxylan arabinofuranohydrolases and their assignment to the glycosyl hydrolase families.

Organism	Gene	Glycosyl hydrolase family	Database access. no.	Reference
A. nidulans	*abf*B	54	Y13759	Gielkens et al., 1999
A. niger	*abf*A	51	L29005	Flipphi et al., 1993a
A. niger	*abf*B	54	X74777	Flipphi et al., 1993b
A. niger	*abf*2	54	U39942	Crous et al., unpublished
A. niger	*axh*A	62	Z78011	Gielkens et al., 1997
A. tubingensis	*axh*A	62	Z78010	Gielkens et al., 1997

2.4.2. Endoarabinases

Endoarabinases hydrolyse the α-1,5-linkages of arabinan polysaccharides which are present as side chains of pectin. Although some arabinofuranosidases are also able to hydrolyse polymeric arabinan (see 2.4.1), endoarabinases strongly enhance the efficiency of arabinan degradation and positively influence the action of arabinofuranosidases. So far, no indications have been obtained for the presence of more than one endoarabinase in any *Aspergillus* sp. (Table 7). The production of endoarabinases by *Aspergillus* sp. was observed on sugarbeet pulp (van der Veen et al., 1991), and L-arabinose and L-arabitol (Ramon et al., 1993; van der Veen et al., 1993). In *A. niger*, induction of AbnA seems to occur simultaneously with the induction of AbfA and AbfB (Flipphi et al., 1994).

Table 7. Physical properties of *Aspergillus* endoarabinases.

Organism	Enzyme	Mw	pH_{opt}	T_{opt} (°C)	pI	Reference
A. aculeatus	Endo-ara A	45	5.5			Beldman et al., 1993
A. nidulans	Abn	40	5.5	68	3.25	Ramon et al., 1993
A. niger		34.5	4.7	50-55	3.0	Schopplein and Dietrich, 1991
A. niger		35	5.0	50	4.5-5.5	Rombouts et al., 1988
A. niger	AbnA	43	4.6	51	3.0	van der Veen et al., 1991

An analysis of the degradation patterns of linear (1-5)-α-L-arabino-oligosaccharides using *A. niger* endo-arabinase, demonstrated that the enzyme is not (or hardly) able to release terminal residues, but preferentially acts on internal linkages (Dunkel and Amado, 1995).

Only one endoarabinase encoding gene has been reported from *Aspergillus* sp. (Flipphi et al., 1993c; Access. No. L23430). Based on the sequence of this gene, AbnA was assigned to family 43 of the glycosyl hydrolases.

2.4.3. α- and β-D-Galactosidases

The removal of D-galactose residues requires the action of α-galactosidases (EC 3.2.1.22) and β-galactosidases (EC 3.2.1.23). β-Galactosidases release terminal galactose residues from the galactan side chains of pectins. Studies addressing the activity of α- and β-galactosidases on xylan have not been reported. However, the production of α- and β-galactosidases on xylan containing crude substrates indicates a putative role for these enzymes in the degradation of xylan. Production of α-galactosidases has been reported on arabinoxylan (Manzanares et al., 1998), glucose (Zapater et al., 1990), locust bean gum (den Herder et al., 1992), wheat and rice bran (Somiari and Balogh, 1995), lactose and galactose (Rios et al., 1993) and guar flour (Adya and Elbein, 1977). *Aspergillus* sp. produce β-galactosidase during growth on arabinoxylan (Manzanares et al, 1998), polygalacturonic acid (McKay, 1991), wheat bran (Gonzalez and Monsan, 1991), and lactose (Reczey et al., 1992). Several different α-galactosidases have been purified from *Aspergillus* sp. (Table 8), but there are no indications for more than one β-galactosidase produced by any *Aspergillus* sp. The differences in molecular mass observed for

Table 8. Physical properties of *Aspergillus* α- and β-galactosidases

Organism	Enzyme	Mw	pH_{opt}	T_{opt} (°C)	pI	Reference
α-galactosidases						
A. ficuum		70.8	6.0	60		Zapater et al., 1990
A. nidulans		87	4-5	50	6.3	Rios et al., 1993
A. niger	AglA	82			4.8	den Herder et al., 1992
A. niger	AglB	54	4.5	50-55	4.2-4.6	Manzanares et al., 1998
A. niger	AglC	95	6.0			Knap et al., 1994
A. niger		45	4-4.5			Adya and Elbein, 1977
A. niger		78, 69	5	50		Somiari and Balogh, 1995
A. oryzae		64	4.0	60		Annunziato and Mahoney, 1987
A. tamarii		88	4.2-4.3			Civas et al., 1984a
A. tamarii		77.5	4.2-4.3			Civas et al., 1984a
A. tamarii		56	4.8			Civas et al., 1984b
β-galactosidases						
A. fonsecaeus		124	4.5		4.2	Gonzalez and Monsan, 1991
A. niger		93	4	60-65	4.6	Manzanares et al., 1998
A. niger		117			4.9	Greenberg and Mahoney, 1981
A. phoenicis			4.0	70		Reczey et al., 1992

the purified β-galactosidases (Table 8) are most likely due to strain differences and differences in glycosylation of the enzymes.

Several genes encoding α-galactosidases have been cloned and characterised from *Aspergillus niger* (den Herder et al., 1992; Knap et al., 1994; Chapter 8). Based on the sequence, AglA (den Herder et al., 1992; Acces. Nr. X63348) has been assigned to glycosyl hydrolase family 27. AglB (Chapter 8) shares significant sequence identity with AglA and other eukaryotic α-galactosidases from family 27, suggesting that this enzyme also belongs in this family. AglC (Knap et al., 1994) is highly homologous to *Trichoderma reesei* Agl2, a member of family 36 which consists mainly of bacterial α-galactosidases. Based on the sequence of the β-galactosidase encoding gene (*lac*A; Kumar et al., 1992; Access. No. L06037) this enzyme has been assigned to family 35 of the glycosyl hydrolases.

2.4.4. Endo- and exogalactanases

The galactan side chains of pectin are hydrolysed by endogalactanases (EC 3.2.1.89), exogalactanases and β-galactosidases. Endogalactanases are able to hydrolyse the galactan polysaccharides resulting in the liberation of galactobiose and galactose. Production of endogalactanases was observed on beet pulp (Kimura et al., 1998a), soy bean (Christgau et al., 1995), and locust bean gum (Araujo and Ward, 1990). Differences between the enzymes exist with respect to their ability to hydrolyse β-1,3-, β-1,4- or β-1,6-linkages between galactose residues. Two types of arabino-galactans are present as side chains of pectins. Type I consists of a backbone of β-1,4-linked galactopyranose residues, while type II consists of a backbone of β-1,3-linked galactopyranose residues that can be branched by β-1,6-linked galactopyranose residues. For the complete degradation of these polysaccharides all three types of endogalactanases would be required, but so far mainly β-1,4-endogalactanases have been reported (Table 9). So far, only one exogalactanase has been purified from *Aspergillus* (Bonnin et al., 1995). This enzyme was able to release galactose from galacto-oligosaccharides and potato galactan (Bonnin and Thibault, 1996; Bonnin et al., 1997). Additionally, exogalactanase possessed galactose transferase activity (Bonnin et al., 1995 & 1997; Bonnin and Thibault, 1996), indicating a possible application for this enzyme in the production of specific galacto-oligosaccharides.

Table 9. Physical properties of *Aspergillus* endo- and exogalactanases.

Organism	linkage	Mw	pH_{opt}	T_{opt} (°C)	pI	Reference
A. aculeatus	β-1,4-	42	4.25	50	4-6	van de Vis et al., 1991
A. aculeatus	β-1,4-	38	3.5-4.0	50-55	2.8	Lahaye et al., 1991
A. aculeatus	β-1,4-	43	4.0-4.5	40-65	<3.0	Christgau et al., 1995
A. niger	β-1,4-	46	4-5	55	2.9	Schopplein and Dietrich., 1991
A. niger	β-1,4-	43	4.0	50-55	4-6	van de Vis et al., 1991
A. niger	β-1,4-	32	3.5	55		Yamaguchi et al., 1995
A. sojae	β-1,4-	39.7	4.5	50	3.6	Kimura et al., 1998a
A. niger	β-1,6-	60	3.5	60		Brillouet et al., 1991
A. niger	exo-β-1,4-	90-120	3.5	60	3.8-4.1	Bonnin et al., 1995

A gene encoding an *A. aculeatus* β-1,4-endogalactanase has been reported (Christgau et al., 1995; Acces. Nr. L34599) and the sequence of a similar gene from *A. tubingensis* has been submitted to the GenBank database (Van der Vlugt-Bergmans and van Ooijen, unpublished results; Acces. Nr. AJ012316). Based on these sequences both enzymes were assigned to glycosyl hydrolase family 53.

2.4.5. α-Glucuronidases

Glucuronic acid residues and their 4-O-methyl ethers can be removed from the xylan backbone by α-glucuronidases (3.2.1.131). The activity of this enzyme has been detected in a large number of fungal and bacterial culture filtrates, but α-glucuronidases have only been purified from a small number of organisms (Table 10). All eukaryotic α-glucuronidases so far identified are present as monomeric enzymes with the exception of the α-glucuronidase from *Agaricus bisporus* (Puls et al., 1987). All enzymes are mainly active on small xylo-oligomers and therefore depend on the action of endoxylanases. α-Glucuronidases have the highest activity against aldotriouronic acid ($MeGlcAXyl_2$), whereas only low or no activity is observed against aldobiouronic acid (MeGlcAXyl) or aldotetrauronic acid ($MeGlcAXyl_3$). Synergy between α-glucuronidases and endoxylanases, and α-glucuronidases and β-xylosidase has been reported for several of these enzymes (Castanares et al., 1995; Chapter 7 and 10). The α-glucuronidase from *Helix pomatia* is the only α-glucuronidase for which activity was detected against *p*-nitrophenyl-α-D-glucuroniside (Kawabata et al., 1995).

Table 10. Physical properties of fungal and bacterial α-glucuronidases.

Organism	Enzyme	Mw	pH_{opt}	T_{opt} (°C)	pI	Reference
Agaricus bisporus		180	3.3	52		Puls et al., 1987
A. niger 5-16	CM-I	130	4.8	60	5.3	Uchida et al., 1992a
A. niger 5-16	CM-II	150	4.8	60	5.3	Uchida et al., 1992a
A. tubingensis	AguA	107	4.5-6.0	70	5.2	Chapter 7
Phanerochaete chrysosporium		112	3.5	50	4.6	Castanares et al., 1995
Piromonas communis		103	5.5	50		Wood and Wilson, 1995
Thermoascus aurantiacus		118	4.5	65		Khandke et al., 1989
Trichoderma reesei		91	4.5-6.0		5.0-6.2	Siika-Aho et al., 1994
Helix pomatia (snail)	PNP-Gaase	97	3.0	50	6.8	Kawabata et al., 1995
Thermotoga maritima	AguA	78	6.3	85	4.7	Ruile et al., 1997
Thermoanearobacterium sp.		74	5.4	60	4.65	Shao et al., 1995

Only five genes encoding α-glucuronidases have so far been reported (Table 11). These genes show significant sequence identity and the corresponding enzymes are therefore all assigned to glycosyl hydrolase family 67. Additionally, genome sequencing of *Neurospora crassa* revealed the presence of a putative α-glucuronidase encoding gene in this fungus (Nelson et al., 1997).

Table 11. Genes encoding α-glucuronidases and their assignment to the glycosyl hydrolase families.

Organism	Gene	Glycosyl hydrolase family	Database access. no.	Reference
Aeromonas caviae	*xyg*	67	AB022788	Kitagawa et al., in press
A. tubingensis	*agu*A	67	Y15405	Chapter 7
Bacillus stearothermophilus	*agu*A	67	AF098273	Shulami et al., 1999
Thermotoga maritima	*agu*A	67	Y09510	Ruile et al., 1997
Trichoderma reesei	*glr*I	67	Z68706	Margolles-Clark et al., 1996

2.4.6. Feruloyl and *p*-coumaroyl esterases

Feruloyl and *p*-coumaroyl residues are found attached to O5 of arabinose residues in xylans, and to O3 of arabinose residues and O6 of galactose residues in pectin. Due to the ability of these residues to crosslink xylan and pectin polysaccharides to each other and to lignin they are important for the structural integrity of the plant cell wall. Although some prokaryotic feruloyl esterases have been purified, the majority of these enzymes have been studied from eukaryotic sytems (Table 12). Several types of enzymes can be identified based on their physical properties

as well as by substrate specificity. Based on molecular mass feruloyl esterases can be divided in small monomeric enzymes (20-36 kDa), large dimeric enzymes (monomeric residues of 60-120 kDa), and monomeric enzymes with an intermediate molecular mass (57-75 kDa). All enzymes (except *A. awamori* CE) are active on methyl-ferulate, which is a synthetic substrate commonly used for feruloyl esterase assays. Studies of the activities of feruloyl esterases against natural substrates have focussed mainly on xylan and xylan derived oligosaccharides, from which most enzymes were able to release ferulic acid. Only two of these enzymes, FaeA (Chapter 3 and 12) and CinnAE (Kroon et al., 1996a) have been shown to release ferulic acid from pectin.

Aspergillus species produce several feruloyl esterases with different substrate specificities. A comparative study using *A. niger* FaeA and CinnAE (Kroon et al., 1997) demonstrated a preference of FaeA for substrates with a methoxy group at O3 of the aromatic ring and an increase in activity was observed when the number of methoxy groups on the aromatic ring increased. The activity of CinnAE was low or absent on substrates containing a methoxy group at O3 of the aromatic ring, while additional methoxy groups at other positions of the aromatic

Table 12. Physical properties of fungal and bacterial feruloyl esterases.

Organism	Enzyme	Mw	pH_{opt}	T_{opt} (°C)	pI	Reference
A. awamori	FE	112			3.7	McCrae et al., 1994
A. awamori	CE	75			4.2	McCrae et al., 1994
A. niger	Fae-I	63			3.0	Faulds and Williamson, 1993
A. niger	Fae-II	29			3.6	Faulds and Williamson, 1993
A. niger	FaeA	36	5.0	55	3.3	Chapter 3
A. niger	CinnAE	75.8	6.0	50	4.8	Kroon et al., 1996a
A. niger	CE	120				Barbe and Dubordieu, 1998
A. oryzae	FAE	30	4.5-6.0		3.6	Tenkanen et al., 1991
A. tubingensis	FaeA	36				Chapter 3
Neocallimastix		11	7.2		4.7	Borneman et al., 1991
Neocallimastix MC-2	FAE-I	69				Borneman et al., 1992
Neocallimastix MC-2	FAE-II	24				Borneman et al., 1992
Penicillium expansum		57.5	5.6	37		Donaghy and McKay, 1997
Penicillium pinophilum	p-CAE / FAE	57	6.0	55	4.6	Castanares et al., 1992
Clostridium thermocellum	XynZ	45				Blum et al., 1998
Streptomyces olivochromogenes	FAE	29	5.5	30	7.9	Faulds and Williamson, 1991
Streptomyces avermitilis	FAE		6	50		Garcia et al., 1998

ring reduced CinnAE activity compared to unsubstituted compounds. Hydroxy substitutions on the aromatic ring increased the activity of CinnAE, but reduced FaeA activity. These two enzymes were also studied with respect to their ability to release ferulic acid from oligosaccharides derived from sugar beet pulp and wheat bran (Ralet et al., 1994). FaeA was able to release ferulic acid, which was linked to O5 of arabinose (as present in wheat arabinoxylan). FaeA was not able to release ferulic acid linked to O2 of arabinose (as present in sugar beet pectin), but did release ferulic acid linked to O6 of galactose (also present in sugar beet pectin) suggesting a specificity for the linkage rather than the polymeric compound. CinnAE (FAE-I) was able to release ferulic acid from all oligosaccharides tested, but was more active against arabinose linked ferulic acid (Ralet et al., 1994). These data suggest that the different feruloyl esterases from *A. niger* have complementary functions in the degradation of cell wall polysaccharides. Although this has not been studied in detail for other organisms, differences in substrate specificity have been identified for other feruloyl esterases. *A. awamori* produces a coumaroyl esterase which is unable to hydrolyse feruloyl esters (McCrae et al., 1994). A similar enzyme has not been reported for other organisms, but in nearly all purifications feruloyl esterase activity was followed using methylferulate as a substrate. Coumaroyl esterase activity would therefore not be detected.

Table 12. Genes encoding enzymes with feruloyl esterase activity.

Organism	Gene	Database access. no.	Reference
A. niger	*fae*A	Y09330	Chapter 3
A. tubingensis	*fae*A	Y09331	Chapter 3
Butyrivibrio fibrisolvens	*cin*I	U44893	Dalrymple et al., 1996
Butyrivibrio fibrisolvens	*cin*II	U64802	Dalrymple et al., 1997
Clostridium thermocellum	*Xyn*Y	X83269	Blum et al., 1998
Clostridium thermocellum	*Xyn*Z	M22624	Blum et al., 1998
Pseudomonas fluorescens	*xyn*D	X58956	Ferreira et al., 1993

To date genes encoding enzymes with feruloyl esterase activity have only been cloned from *A. niger*, *A. tubingensis*, *Butyrivibrio fibrisolvens*, *Clostridium thermocellum* and *Pseudomonas fluorescens* (Table 13). The level of sequence identity between these genes is very low. A region of the amino acid sequence of FaeA from *A. niger* and *A. tubingensis* has homology to the active site of lipases (Chapter 3). In lipases the active site is a catalytic triad, which consists of a serine, an aspartic acid and a histidine residue. The spacing between these residues in the amino acid

sequences of lipases is conserved and is also present in FaeA, suggesting a similar active site for this enzyme. However, no lipase activity could be detected for FaeA (Aliwan et al., 1999).

2.4.7. Acetyl- and methylesterases

Acetylesterases (EC3.1.1.72) and methylesterases (EC 3.1.1.11) release acetyl- and methyl-residues from the backbone of cell wall polymers (xylan and pectin). Acetylxylan esterases remove acetyl from O3 of xylose in the xylan main chain. Although acetylxylan exterase activity has been detected in several *Aspergilli*, such as *A. niger*, *A. japonicus*, and *A. nidulans* (Khan et al., 1990), only a limited number of acetylxylan esterases have been purified from *Aspergillus* sp. (Sundberg et al., 1990, Kormelink et al., 1993d). Unlike most other accessory enzymes acetylxylan esterases are highly active on the polymeric substrate, and are believed to be important for an efficient degradation of the xylan backbone by endoxylanases.

The acetyl- and methylresidues in the smooth regions of pectins are removed by pectin acetylesterases (Searle-van Leeuwen et al., 1996) and pectin methylesterases (Forster et al., 1988; Khanh et al., 1991). So far, two pectin methylesterases have been purified from *Aspergillus* sp. (Baron et al., 1980; Khanh et al., 1991). A rhamnogalacturonan acetylesterase has been purified from *A. aculeatus* (Kauppinen et al., 1995) and from *A. niger* (Searle-van Leeuwen et al., 1996). This enzyme removes the acetylresidues from the backbone of the hairy regions of pectin and was found to be essential for the action of rhamnogalacturonan hydrolase (Chapter 11).

Table 13. Genes encoding *Aspergillus* acetyl- and methylesterases and their assignment to the carbohydrate esterase families. Abbreviations: RGAE, rhamnogalacturonan acetylesterase; AXE, acetylxylan esterase; PME, pectin methylesterase.

Organism	Enzyme	Gene	Carbohydrate esterase family	Database access. no.	Reference
A. aculeatus	RGAE	*rha*1	12	X89714	Kauppinen et al., 1995
A. niger	RGAE	*rgae*A	12	AJ242854	Chapter 11
A. awamori	AXE	*ace*A	1	D87681	Koseki et al., 1997
A. niger	AXE	*axe*A	1	A22880	de Graaff et al., 1992
A. aculeatus	PME	*pme*1	8	U49378	Kauppinen et al., unpublished
A. tubingensis	PME	*pme*A	8	X54145	Khanh et al., 1991
A. oryzae	PME	*pme*A	8	AB011211	Kitamoto et al., 1999

Only a limited number of genes encoding *Aspergillus* acetyl- or methylesterases have been reported so far (Table 13). Their difference with respect to their substrate specificity is reflected by their assignment to the different carbohydrate esterase families (Coutinho and Henrissat., 1999).

2.5. Synergy between enzymes involved in the degradation of xylan and pectin

Efficient degradation of heteropolysaccharides requires co-operative or synergistic interactions between the enzymes responsible for cleaving the different linkages. Synergy has been reported for many enzymes from *Aspergillus* involved in xylan degradation, usually between a main chain cleaving enzyme and one or more accessory enzymes. Synergistic action was observed between endoxylanase, β-xylosidase, arabinoxylan arabinofuranohydrolase and acetylxylan esterase in the degradation of different (glucuronoarabino-)xylans (Kormelink and Voragen, 1993). Synergy was also observed between these enzymes and some of the other xylanolytic enzymes. The release of ferulic acid from xylan by a feruloyl esterase from *A. niger* was strongly enhanced by the addition of endoxylanases (Bartolome et al., 1995; Chapter 3). Similarly, both endoxylanase and β-xylosidase positively influenced the release of 4-O-methyl-glucuronic acid from birchwood xylan by *A. tubingensis* α-glucuronidase (Chapter 7). The latter enzyme in turn enhanced the activity of endoxylanase and β-xylosidase on this substrate. Synergy was also demonstrated between an endoxylanase (XylI) and arabinoxylan arabinofuranohydrolase (AXH) from *A. awamori* in the degradation of sorghum glucuronoarabinoxylan (Verbruggen et al., 1998a). A recent study revealed that synergistic interactions in the degradation of xylan are not only present between main chain cleaving enzymes and accessory enzymes, but also occur amongst accessory enzymes and that nearly all accessory enzymes positively influence the activity of the main chain cleaving enzymes (see Chapter 10).

Only a limited number of studies demonstrating synergy between pectinolytic enzymes from *Aspergillus* have been reported. Pectin methyl esterase from *A. aculeatus* strongly enhanced the degradation and depolymerization of pectin by polygalacturonases (Christgau et al., 1996). Similarly, rhamnogalacturonan acetyl esterase (RGAE) from *A. aculeatus* had a positive effect on the hydrolysis of the backbone of pectic hairy regions by rhamnogalacturonase A and rhamnogalacturonan lyase from *A. aculeatus* (Kaupinnen et al., 1995). Although indications for inhibition of RGAE activity by the side chains of the hairy regions were obtained (Kaupinnen et al., 1995), a pre-treatment of pectin with arabinofuranosidase did not increase the acetyl release by RGAE (Searle-van Leeuwen et al., 1992). Pectin lyase positively influenced the release of

ferulic acid from sugar beet pectin by a feruloyl esterase from *A. niger*, but only to a small extent (Chapter 3). The release of ferulic acid from pectin by a second *A. niger* feruloyl esterase was positively affected by endoarabinase and arabinofuranosidase from *A. niger* (Kroon and Williamson, 1996), indicating that synergy also occurs amongst pectinolytic accessory enzymes. Recently, synergy in the degradation of hairy regions from sugar beet pectin was studied using six accessory enzymes and a main chain cleaving enzyme (see Chapter 11). The positive effect of rhamnogalacturonan acetyl esterase on the degradation of the hairy region backbone also positively effected the activity of feruloyl esterase A, β-galactosidase, and endogalactanase from *A. niger*. Additionally, synergistic effects amongst these three enzymes, an endoarabinase and an arabinofuranosidase from *A. niger* were detected.

3. Regulation of expression of genes encoding cell wall degrading enzymes in *Aspergillus*

3.1. Co-ordinated expression of genes encoding xylanolytic enzymes

Xylanolytic enzymes from *Aspergilli* have all been found to be produced on xylose, xylan and crude xylan containing substrates, but not on other monomeric (e.g. glucose, galactose) or polymeric substrates (e,g, cellulose, pectin). This suggests a general system of regulation of the genes encoding these enzymes. Several genes encoding xylanolytic enzymes have been studied with respect to induction of expression, all demonstrating expression in the presence of D-xylose, xylobiose or xylan (de Graaff et al., 1994; Fernandez-Espinar et al., 1994; Kumar et al., 1996; van Peij et al., 1997; Gielkens et al., 1997; Perez-Gonzalez et al., 1998; Chapter 7), but repression of expression in the presence of glucose (see 3.4). Expression of some xylanolytic genes was also observed on L-arabinose (Kumar et al., 1996; Chapter 5 & 7). This might be due to small amounts of D-xylose present in the L-arabinose preparations used, as was detected for the L-arabinose preparation obtained from Sigma (de Vries, unpublished results). Additionally, induction of xylanolytic enzymes was also observed on cellobiose and cellulose (Hrmová et al., 1989) and on a heterodisaccharide consisting of glucose and xylose (Glcβ1-2Xyl) (Hrmová et al., 1991) in *A. terreus*, which are compounds that also induce the cellulolytic system from this fungus. A xylose induced, glucose repressed endoxylanase encoding gene (*xyn*G1) from *A. oryzae* was expressed in *A. nidulans*, resulting in expression of the gene on xylose as well as glucose (Kimura et al., 1998b). This indicates that the regulation of the expression of xylanolytic genes is not identical in *A. oryzae* and *A. nidulans* as was reported for *A. niger* and *A. tubingensis* (de Graaff et al., 1994).

From *A. niger*, a gene encoding a transcriptional activator has been isolated by complementation of a mutant unable to degrade xylan (van Peij et al., 1998a). Characterisation of this factor, XlnR, showed that it was responsible for the expression of genes encoding endo-xylanase and β-xylosidase. Sequence analysis demonstrated that XlnR is a member of the GAL4-like family of transcriptional activators. Analysis of the promoter region of these genes identified a putative XlnR binding site, GGCTAAA, of which the second G was determined to be essential for XlnR binding by band mobility shift assays and *in vivo* (van Peij et al., 1998a). A more detailed analysis of the role of XlnR in the regulation of genes involved in xylan, arabinan, and cellulose degradation indicated that this protein activates the expression of other genes than xylanolytic genes (van Peij et al, 1998b). Two endoxylanase (*xln*B and *xln*C), a β-xylosidase (*xln*D), an arabinoxylan arabinofuranohydrolase (*axh*A), an acetylxylan esterase (*axe*A), an α-glucuronidase (*agu*A), a feruloyl esterase (*fae*A) and two endoglucanase (*egl*A and *egl*B) encoding genes were found to be regulated by XlnR (van Peij et al., 1998b). However, an α-arabinofuranosidase (*abf*B) and a β-glucosidase (*bgl*A) encoding gene were not regulated by this protein. This indicates that besides its role as a xylanolytic activator, XlnR also regulates the expression of some, but not all, genes encoding cellulolytic enzymes. A later study demonstrated that XlnR is also involved in the regulation of an α- and a β-galactosidase gene (*agl*B and *lac*A, Chapter 8). Analysis of the promoter regions of the genes which are regulated by XlnR demonstrated that the 3rd A in the consensus for the binding site is variable and the consensus sequence was therefore altered to GGCTAA (van Peij et al., 1998b). Introduction of a high number of copies of a xylanolytic gene or the promoter of a xylanolytic gene results in a decrease in expression of the other genes (Kitamoto et al., 1998). This indicates that the production of XlnR is tightly balanced with the amount of genes this protein has to regulate.

3.2. Induction of expression of pectinolytic genes

The production of main chain cleaving enzymes (polygalacturonases, pectin lyases, and rhamnogalacturonan hydrolases and lyases) has been detected on sugar beet pectin (Bussink et al, 1992b), apple pectin (Harmsen et al., 1990; Suykerbuyk et al., 1997), polygalacturonic acid (Ruttkowski et al., 1991), a combination of rhamnose and galacturonic acid (Suykerbuyk et al., 1996), and soybean flour (Kojima et al., 1999). Expression of genes encoding pectinolytic main chain cleaving enzymes has been reported on apple pectin (Harmsen et al., 1990; Kusters-van Someren et al., 1991b & 1992), sugar beet pulp (Bussink et al., 1990 & 1991; Suykerbuyk et al., 1995), galacturonic acid (Kester et al., 1996), and polygalacturonic acid (Dean and Timberlake,

1989). The expression of these genes differs from the expression of the genes encoding accessory enzymes (see 3.3.), indicating the absence of a general system regulating the expression of pectinolytic genes as was described for the xylanolytic system (see 3.1.) Strong differences are observed in the expression of polygalacturonase encoding genes. Cary et al. (1995) reported a constitutively expressed polygalacturonase encoding gene from *A. parasiticus* which was highly expressed on both pectin and glucose, whereas for the gene encoding endopolygalacturonase E from *A. niger*, no expression could be detected on pectin, polygalacturonic acid or galacturonic acid (Parenivová et al., 1998). Three other polygalacturonase encoding genes from *A. niger* were induced in the presence of sugar beet pulp, and a promoter deletion analysis of one of these genes (*pga*II) identified a region which was important for high-level gene expression (Bussink et al., 1992b; Visser et al., 1994). Comparison of this region to the promoters of the other genes (*pga*I and *pga*C) identified a consensus sequence which is similar to an upstream activating sequence of the *S. cerevisiae cyc*I gene, to which the HAP2/3/4 activating complex binds (Visser et al., 1994). Additionally, a hexanucleotide sequence was detected in the promoters of several pectin lyase encoding genes (CCCTGA; Benen et al., 1996). However, the function of these sequences have not yet been studied in detail.

The accessory enzymes involved in pectin degradation all have specific induction patterns. Endoarabinases and arabinofuranosidases are induced by sugar beet pulp, arabinose and L-arabitol (see 3.3.). Endogalactanases and β-galactosidases are induced by galactose and several galactose containing oligo- and polysaccharides. Induction of these types of enzymes on pectin may be due to the release of small amounts of arabinose and galactose from the polymer. An exception is the *A. oryzae* β-galactosidase which is also induced during growth on polygalacturonic acid (McKay, 1991) and could therefore be co-regulated with some of the genes encoding pectin main chain degrading enzymes.

3.3. Regulatory factors involved in the regulation of specific genes

Certain xylanolytic and pectinolytic genes can also be induced by specific compounds. Frequently, the inducing compounds are products of the encoded enzymes or metabolites derived from the enzyme products.

As mentioned before arabinofuranosidases and endoarabinases are induced when *Aspergillus* is grown on sugar beet pulp, whereas arabinoxylan arabinofuranohydrolases are produced during growth on xylan (see 2.4.1.). Induction was also observed on monomeric carbon sources, such as

L-arabinose and L-arabitol (Ramon et al., 1993; van der Veen et al., 1993; Flipphi et al., 1994; Gielkens et al., 1997 & 1999). The expression of two arabinofuranosidase encoding genes (*abf*A and *abf*B) and one endoarabinase encoding gene (*abn*A) was studied in *A. niger* (Flipphi et al., 1994). Transformants containing additional copies of one of the genes showed reduced expression levels of the other two genes compared to a wild type strain. This indicates that these genes are most likely under co-ordinated control of a specific transcription factor. The reduction in expression observed would then be caused by titration of the transcription factor by the additional copies of one of the genes. Indications that L-arabitol, and not L-arabinose, is the true inducing compound of this regulatory system were obtained by using an *A. nidulans* mutant defective in L-arabitol dehydrogenase activity (de Vries et al., 1994). This mutant accumulated L-arabitol when grown on media containing glycerol and L-arabitol. Under these conditions an increase in the arabinofuranosidase, endoarabinase, and L-arabinose reductase activity was observed compared to a wild-type strain. These data also suggest co-ordinated induction of extracellular arabinose releasing enzymes and enzymes involved in L-arabinose catabolism. This was confirmed in *A. niger* where induction of arabinofuranosidases and endoarabinases occurred simultaneously with the induction of intracellular enzyme activities involved in L-arabinose catabolism (L-arabinose reductase and L-arabitol dehydrogenase; van der Veen et al., 1993).

Induction of α- and β-galactosidases from *Aspergillus* was observed on wheat and oat bran (Park et al., 1979; Somiari and Balogh, 1995), polygalacturonic acid (McKay, 1991), lactose (Rios et al., 1993; Manzanares et al., 1998), and galactose (Rios et al., 1993), but few papers describe studies of the expression of *Aspergillus* α- and β-galactosidase encoding genes at the molecular level. Den Herder et al. (1992) demonstrated that the *A. niger* α- galactosidase A encoding gene (*agl*A) was expressed on galactomannan. A recent study (see Chapter 8) compared the expression of this gene to two other α-galactosidase encoding genes from *A. niger* (*agl*B and *agl*C) and the *A. niger* β-galactosidase encoding gene (*lac*A). Strong differences were observed in the expression of the individual genes on monomeric, oligomeric and polymeric substrates. Of the three α- galactosidase encoding genes, only *agl*A seemed to be specifically expressed on galactose and galactose-containing oligo- and polysaccharides. The *agl*B gene was constitutively expressed on all carbon sources tested, but had increased expression on xylose and xylan. This could be attributed to regulation via the xylanolytic transcriptional activator XlnR (see 3.1.). Expression of *agl*C was only detected on glucose, which is surprising for an α-galactosidase encoding gene. The expression of *lac*A was highest on arabinose, xylose and xylan (regulated by

XlnR, see 3.1.) and pectin, whereas only low expression was observed on galactose. The expression on pectin is supported by the production of this enzyme from *A. oryzae* on polygalacturonic acid (McKay, 1991) and might indicate that this gene is co-regulated with genes encoding pectin main chain degrading enzymes (see 3.2.).

The induction of *fae*A from *A. niger* by the product of the enzyme, ferulic acid, has recently been studied (Chapter 4 and 5). This gene is expressed during growth on xylan and xylose, mediated by XlnR (see 3.1.). The addition of ferulic acid to media containing xylan or xylose increased the expression of *fae*A whereas other xylanolytic genes were unaffected, indicating a second regulatory system for the induction of *fae*A. Although the two systems positively influence each other, ferulic acid induced expression is not dependent on XlnR (Chapter 5). In an XlnR negative mutant, expression of *fae*A was similar on ferulic acid or a combination of ferulic acid with xylose, while no expression of other xylanolytic genes was detected.

3.4. Carbon catabolite repression

The major system responsible for carbon repression in *Aspergillus* is mediated via the carbon catabolite repressor protein CreA (Dowzer and Kelly, 1991; for a recent review see Ruijter and Visser, 1997). CreA is a zinc finger protein which binds to specific sites in the promoters (SYGGRG) of a wide range of target genes (Kulmburg et al., 1993) in the presence of easy metabolisable substrates, such as glucose or fructose, and inhibits or decreases the expression of these genes. Several *A. nidulans* and *A. niger* CreA mutants have been isolated which display in (partially) de-repressed phenotypes (Arst et al., 1990; Shroff et al., 1996, Ruijter et al., 1997). Using these mutants, CreA mediated repression of gene expression has been detected for the alcohol and proline gene clusters in *A. nidulans* (Cubero and Scazzochio, 1994; Panozzo et al., 1998) and for several extracellular proteases in *A. niger* (Jarai and Buxton, 1994). Recently, the mechanism of CreA mediated repression has been investigated in more detail (Strauss et al., 1999). It appeared that the *cre*A gene is strongly expressed when monomeric repressing carbon sources are added to the culture, but CreA quickly down-regulates the expression of its encoding gene. This autoregulation was demonstrated to be dependent on the formation of glucose-6-phosphate (Strauss et al., 1999). Under carbon de-repressing conditions a significantly higher *cre*A expression was observed, but this did not result in the formation of the CreA-DNA complex. Formation of this complex was dependent on *de novo* protein synthesis (Strauss et al., 1999). At this point it is not clear whether the change from active to inactive CreA and *vice versa* is caused by covalent modification of CreA or by protein degradation.

The influence of CreA on the cell wall degrading enzyme systems from *Aspergillus* has been widely studied. CreA mediated repression in *Aspergillus* was detected for genes encoding arabinases and L-arabinose catabolic enzymes (Ruiter et al., 1997), several endoxylanases (de Graaff et al., 1994; Fernandez-Espinar et al., 1994; Pinaga et al., 1994; Perez-Gonzalez et al., 1998; Orejas et al., 1999) and other xylanolytic functions, such as β-xylosidase (Kumar and Ramon, 1996), arabinoxylan arabinofuranohydrolase (Gielkens et al., 1997) and feruloyl esterase (Chapter 5). Some of the pectinolytic genes from *Aspergillus* are also repressed in the presence of glucose (Maldonado, 1989; Bussink et al., 1991; Solis-Pereira, 1993; Kester et al., 1996).

Removal of four putative CreA binding sites from the promoter of the *A. tubingensis xln*A gene resulted in an increased expression of this gene (de Graaff et al., 1994), indicating that at least one of these sites is involved in CreA binding. Gel mobility shift analysis of a fragment of the promoter of the *A. nidulans* pectate lyase encoding gene (*pel*A) using a fusion protein containing the N-terminal part of CreA demonstrated binding of the fusion protein to this fragment (Ho et al., 1995) and a potential role for CreA in the regulation of (some) pectinolytic genes.

CreA mediated repression is not restricted to glucose and fructose alone. Other monomeric carbon sources also result in (weaker) CreA mediated repression of gene expression. Xylose is commonly regarded as the monomeric inducer of xylanolytic gene expression in *Aspergillus* (see 3.1). Recently, the effect of different xylose concentrations on xylanolytic gene expression was studied (Chapter 9) demonstrating that high concentrations of xylose result in CreA mediated repression of the expression of xylanolytic genes. At lower xylose concentrations higher expression levels were observed. A similar phenomenon was observed for the regulation of cellulase biosynthesis in *A. terreus* (Ali and Sayed, 1992). At high concentrations of glucose, xylose and cellobiose a decrease in cellulase production was observed.

The ferulic acid induced expression of the feruloyl esterase A encoding gene (*fae*A) from *A. niger* was studied in combination with a number of different monomeric carbon sources (Chapter 6). For nearly all combinations, higher *fae*A expression was detected in a CreA derepressed mutant than in a wild-type strain, indicating that CreA mediated repression occurred in the presence of all these carbon sources.

3.5. pH dependent expression

The major factor involved in pH dependent expression in *Aspergillus* is the pH regulatory protein PacC. At alkaline pH PacC activates alkaline expressed genes and represses acid expressed genes, therefore PacC has a dual function (Tilburn et al., 1995). The gene encoding

this protein (*pac*C) has been cloned from *A. nidulans* (Tilburn et al., 1995) and *A. niger* (MacCabe et al., 1996), and was shown to complement *A. nidulans* pH regulatory mutants, previously isolated as mutants defective in phophatase expression (Dorn, 1965; Arst and Cove, 1970; Arst et al., 1980). PacC is activated under alkaline conditions by proteolytic modification of the C-terminal region, after which it is able to bind to specific target sites (GCCARG, with R being A or G) in the promoter of target genes (Orejas et al., 1995). Six genes (*pal*A, *pal*B, *pal*C, *pal*F, *pal*H and *pal*I) are involved in the signal transduction pathway leading to the activation of PacC (Caddick et al., 1986; Arst et al., 1994; Tilburn et al., 1995).

pH regulation of genes encoding cell-wall degrading enzymes has not been studied in detail in *Aspergillus*. However, indications for pH dependent expression of xylanolytic and pectinolytic genes have been obtained. The production of different polygalacturonases was studied in *A. kawachii* using culture media with different pH (Kojima et al., 1999). From culture medium at pH 2, two polygalacturonases were purified, whereas one polygalacturonase was purified from culture medium at pH 5. The N-terminal amino acid sequence of these enzymes were different, demonstrating that the pH of the culture medium influences which polygalacturonase is produced by *A. kawachii*. Using PacC mutants, it was shown that in *A. nidulans* the expression of the major arabinofuranosidase encoding gene (*abf*B; Gielkens et al., 1999) and two endoxylanase encoding genes (*xln*A and *xln*B; MacCabe et al., 1998) is regulated by PacC. These two endoxylanse encoding genes have opposite expression patterns, *xln*A being expressed under alkaline conditions and *xln*B under acidic conditions. Analysis of the promoter regions of *xln*A and *xln*B revealed the presence of two and one PacC consensus sites, respectively (MacCabe et al., 1998). Several putative PacC binding sites were also detected in the promoter of the *A. nidulans* β-xylosidase encoding gene (*xln*D), but no clear indications for PacC control of this gene were obtained (Perez-Gonzalez et al., 1998).

4. Industrial applications

The enzymes discribed described in this chapter are involved in the degradation of complex plant cell wall polysaccharides. Plant cell walls are a major part of the crude biomass which is used in a wide variety of industrial processes. A first step in the industrial processing of biomass frequently involves (partial) degradation of the polymeric fraction. It is therefore obvious that enzymes capable of degrading the plant cell wall can be applied in many of these processes and are a good alternative to chemical processing. In this section, examples of industrial applications of xylanolytic and pectinolytic enzymes are given.

Applications of xylanolytic systems can be found in a variety of industrial processes. In the pulp and paper industry cellulase free xylanolytic enzyme preparations can be of great value in the bio-bleaching of pulps (Viikari et al., 1994). Enzymatic degradation of the hemicellulose-lignin complexes present in pulps leaves the cellulose fibres intact and strongly reduces the amount of bleaching chemicals (chlorine) needed. This not only results in a reduction in chemical costs, but also reduces the environmental problems caused by the use of chlorine. The most important enzyme that is used in enzyme-aided bleaching is endoxylanase (Viikari et al., 1994), but the addition of other xylanolytic enzymes has also been shown to be effective (Kantelinen et al., 1988). A second area in which the xylanolytic enzyme preparations are widely used is the bakery industry. In this context their main effect is in solubilising the arabinoxylan fraction of the dough, resulting in an increased bread volume and an improved quality of the dough (Maat et al, 1992; Poutanen, 1997; Petit-Benvegnen et al., 1998). Other applications for xylanolytic enzymes can be found, such as in increasing the feed conversion efficiency of animal feeds (Bedford and Classen, 1992), clarifying juices (Zeikus et al., 1991), and the production of xylose, xylobiose and xylo-oligomers (Puls et al, 1988; Biely et al, 1991; Pellerin et al., 1991). The oligosaccharides produced can be used as functional food additives or alternative sweeteners with beneficial properties.

Pectinases are of major importance in the beverages industry, due to their ability to improve pressing and clarification of concentrated fruit juices (Grassin and Fauquembergue, 1996). Pectin methyl esterase and other pectinolytic enzymes are also used for the gelation of oranges and the production of carrot puree (Heldt-Hansen et al., 1996). Whereas xylanolytic enzymes are mainly used in the paper and pulp industry for bio-bleaching, pectinolytic enzymes are applied in enzymatic debarking (Rättö and Viikari, 1996; Bajpai, 1997). Removal of the bark is the first step in all processing of wood and is traditionally a very energy consuming process. Reduction of the amount of energy required can be obtained by using pectinolytic enzymes to aid in the process.

Other applications for pectinolytic enzymes have been found in increasing the yield of lemon peel oils (Coll et al., 1996), and the production of oligouronides (Ross et al., 1996).

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Chapter 2

Aim and outline of the thesis

This study focuses on accessory enzymes from *Aspergillus* involved in the degradation of two types of plant cell wall heteropolysaccharides: xylan and pectin. At the start of this project, enzymes cleaving the main chains of xylan and pectin were already being studied in detail. However, relatively little was known about the molecular characteristics of most of the accessory enzymes and their encoding genes, or the influence these enzymes have on the action of the main chain cleaving enzymes. The (partial) degradation of xylan and pectin is of major importance for industrial processes in which crude plant materials are converted into food or feed products. However, the amount of plant cell wall degrading enzymes produced by *Aspergillus* is often insufficient to test applications at an industrial scale. To be able to rationally apply these enzymes in food and feed processes it is important to study the characteristics of the enzymes in more detail, both with respect to substrate specificity and synergistic effects with other cell wall degrading enzymes. This thesis reports work in which meeting the industrial need for larger quantities of better defined enzymes is combined with a detailed analysis of the molecular characteristics of the genes encoding these enzymes, their regulation and the activity of the enzymes on polymeric and oligomeric substrates.

One of the key-enzymes of this project was feruloyl esterase A (FaeA). This enzyme was purified from *Aspergillus niger* and *Aspergillus tubingensis* and the genes (*fae*A) encoding this enzyme were cloned from both fungi (Chapter 3). The genes were overproduced in different fungi and the stability of the gene products was compared (Chapter 3). The production of *A. niger* FaeA was studied on xylan and crude plant cell wall preparations (Chapter 4), and on pectin (Chapter 6), as well as the influence of the product of this enzyme, ferulic acid, on the induction on xylan (Chapter 4). FaeA from *A. niger* was tested for activity on xylan and pectin (Chapter 3) and a model for the 3-dimensional structure of this enzyme was proposed (Chapter 6). The expression of *fae*A from *A. niger* was analysed in order to identify both positively and negatively acting transcription factors regulating this gene (Chapter 5 and 6).

A second accessory enzyme with a central role in this project was α-glucuronidase (AguA), which was purified from *A. tubingensis* (Chapter 7). The activity of this enzyme on xylan was studied as well as the synergy with an endoxylanase and β-xylosidase in xylan degradation (Chapter 7). The gene (*agu*A) encoding this enzyme was cloned and the expression of this gene on xylan and some monomeric carbon sources was analysed (Chapter 7). The balance between induction and repression of the expression of *agu*A, *fae*A, an endoxylanase (*xln*B) and a β-xylosidase (*xln*D) encoding gene from *A. niger* on xylose has been studied to

determine whether the xylose concentration influences the expression of the different xylanolytic genes (Chapter 9).

Chapter 8 describes the cloning and characterisation of another gene (*agl*B) encoding an accessory enzyme from *A. niger*, α-galactosidase B. The expression of this gene on momomeric, oligomeric and polymeric carbon sources is compared with the expression of a β-galactosidase and two other α-galactosidase encoding genes from *A. niger*.

In Chapters 10 and 11 the synergy between the xylanolytic enzymes in the degradation of wheat arabinoxylan, and between some pectinolytic enzymes in the degradation of sugar beet pectin has been described. Synergy between individual main chain cleaving and accessory enzymes has been reported in literature and also in this thesis (Chapters 3 and 7). These chapters focus on synergy between accessory enzymes as well as on the combined effects of a large number of accessory enzymes and some main chain cleaving enzymes in the degradation of xylan and pectin.

Finally, the main observations of this project are discussed, with an emphasis on the work which is still needed to increase the understanding of the microbial systems involved in the degradation of plant cell wall polysaccharides (Chapter 12).

Chapter 3

The *fae*A genes from *Aspergillus niger* and *Aspergillus tubingensis* encode ferulic acid esterases involved in degradation of complex cell wall polysaccharides

R. P. de Vries, B. Michelsen, C. H. Poulsen, P. A. Kroon, R. H. H. van den Heuvel, C. B. Faulds, G. Williamson, J. P. T. W. van den Hombergh and J. Visser

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Abstract

We report the cloning and characterisation of a gene encoding a ferulic acid esterase, *fae*A, from *Aspergillus niger* and *Aspergillus tubingensis.* The *A. niger* and *A. tubingensis* genes have a high degree of sequence identity and contain one conserved intron. The gene product, FaeA, was overexpressed in wild-type *A. tubingensis* and a protease-deficient *A. niger* mutant. Overexpression of both genes in wild type *A. tubingensis* and an *A. niger* protease-deficient mutant showed that the *A. tubingensis* gene product is more sensitive to degradation than the equivalent gene product from *A. niger*. FaeA from *A. niger* was identical to *A. niger* Fae-III (Faulds and Williamson, 1994), as assessed by molecular mass, pH and temperature optima, pI, N-terminal sequence and activity on methyl ferulate. The *fae*A gene was induced by growth on wheat arabinoxylan and sugar beet pectin, and its gene product (FaeA) released ferulic acid from wheat arabinoxylan. The rate of release was enhanced by the presence of a xylanase. FaeA also hydrolysed smaller amounts of ferulic acid from sugar beet pectin but the rate was hardly affected by addition of an endo-pectin lyase.

Introduction

Cinnamic acids have been demonstrated to be covalently bound to polysaccharides (arabinoxylans and pectins) in cell walls of gramineous plants (Fry, 1982; Kato and Nevins, 1985, Rombouts and Thibault, 1986, Smith and Hartley, 1983). Here, they have a possible function in decreasing cell wall biodegradability (Borneman et al., 1986) and in regulating cell growth (Fry, 1979) by covalently cross-linking cell wall polymers. To degrade these cell wall polymers, many bacteria and fungi produce a wide range of hemicellulases, as well as cinnamic acid esterases (Christov and Prior, 1993). Several cinnamic acid esterases have been isolated from *Aspergillus niger* (Faulds and Williamson, 1993 and 1994; Kroon et al., 1996; Tenkanen et al., 1991). Most of these esterases are active on methyl esters of ferulic and *p*-coumaric acids, but other hemicellulases increase the hydrolysis of these cinnamic acids from xylan. So far, only a cinnamoyl esterase (CinnAE) from *A. niger* has been shown to release ferulic acid from sugar beet pectin (Kroon and Williamson, 1996).

We isolated a ferulic acid esterase (FaeA) from a commercial pectinase preparation which is similar to Fae-III (Faulds and Williamson, 1994). We used the partial amino acid data to clone the FaeA-encoding genes from *A. niger* and *Aspergillus tubingensis* and subsequently

overexpressed these genes. To our knowledge, this is the first paper that describes the isolation and characterisation of a gene encoding a specific ferulic acid esterase.

Materials and methods

Strains, libraries and plasmids

The *A. niger* N402 (*csp*A1) and NW219 (*nic*A1 *leu*A1 *pyr*A6) were derived from strain N400 (=CBS 120.49). The *prt*F mutation present in *A. niger* NW154 (*pyr*A6 *prt*F28) was previously described (van den Hombergh et al., 1995). The *A. tubingensis* strains used were NW756 and NW241 (*pyr*A2 *fwn*A1). *Escherichia coli* DH5aF' (BRL, Life Technologies Inc., Gaithersburg, USA) was used for routine plasmid propagation. *E. coli* LE392 was used as a host for phage λEMBL3. pBluescript (Short et al., 1988) was used for subcloning. The genomic libraries of *A. niger* and *A. tubingensis* and the cDNA library of *A. niger* have been described previously (de Graaff et al., 1994, Gielkens et al., 1997, Harmsen et al., 1990).

Chemicals

Methyl ferulate was obtained from APIN Chemicals Ltd. Ferulic acid esterase was purified from a commercial product, Pectinase PII^R from Amano. Nitrophenyl acetate, phenylmethylsulfonyl fluoride, ferulic acid, oat spelt xylan, and endoproteinase lysC were obtained from Sigma. Sugar beet pectin was obtained from Danisco Ingredients. Lyophilised bovine plasma gamma globulin was obtained from Bio-Rad Laboratories. Endoproteinase V8 was obtained from Boehringer Mannheim. Xylanase 1 was purified from the commercial product Grindamyl H121 (Danisco Ingredients) and is equivalent to XlnA from *A. tubingensis* (de Graaff et al., 1994). Pectin lyase was purified from the commercial product Pektolase CA (Danisco Ingredients). Water insoluble pentosan (WIP) from wheat arabinoxylan was isolated by the method of Gruppen et al. (1989).

Media and culture conditions

Minimal medium (MM) contained (per litre): 6.0 g of $NaNO_3$, 1.5 g of KH_2PO_4, 0.5 g of KCl and 0.5 g of $MgSO_4$ as well as trace elements (Vishniac and Santer, 1957) and 1 to 3 % glucose as carbon source unless otherwise indicated. For complete medium MM was supplemented with 0.2% (wt/vol) tryptone / 0.1% (wt/vol) yeast extract / 0.1% (wt/vol) Casamino Acids / 0.05% (wt/vol) RNAs. Liquid cultures were inoculated with 10^6 spores/ml and incubated at 30°C in a Gallenkamp orbital shaker at 250 rpm. Agar was added at a concentration of 1.5% for solid medium. For the growth of strains with auxotrophic mutations, the corresponding supplements were added to the medium.

For characterisation, transformants were grown for 6 days on MM containing a crude wheat arabinoxylan fraction at a concentration of 1% (wt/vol). Supernatant samples were analysed by Western analysis and activity measurement. For production of recombinant FaeA, transformant NW154::pIM3207.7 was grown at 30°C in three batches of 300 ml of MM containing 0.5 g of ferulic acid per litre and 10 g of oat spelt xylan per litre.

Purification of ferulic acid esterases

FaeA was purified from 10 g of Pectinase PII which was dissolved in 300 ml of 50 mM sodium phosphate buffer-1 mM EDTA (pH 7.0), stirred for 30 min, centrifuged (10,000 x g for 30 min), and filtered (pore size, 0.8 mM). Fae-III was purified from culture supernatants (10 l) of *A. niger* CBS 120.49 grown on oat spelt xylan as described previously (Faulds and Williamson, 1994). Recombinant FaeA

was purified from 600 ml of culture filtrate from a 3-day-old culture of NW154::pIM3207.7 grown on MM containing 0.5 g of ferulic acid per litre and 10 g of oat spelt xylan per litre. For all three sources of enzyme, the ferulic acid esterase was purified by ammonium sulphate precipitation, hydrophobic interaction chromatography, and anion-exchange chromatography as described previously for the purification of Fae-III (Faulds and Williamson, 1994). The purity of isolated ferulic acid esterases was assessed by sodiumdodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Characterisation of ferulic acid esterases

Molecular masses were determined by SDS-PAGE (Novex 4 to12% Tris-glycine gel with the electrophoresis conditions given by the manufacturer) by using a Pharmacia LMW electrophoresis calibration kit and by mass spectrometry performed with a MALDI-TOF mass spectrometer (Voyager, Perceptive) as described previously (Vestal et al., 1995). The protein concentrations of purified pectinase ferulic acid esterase, recombinant FaeA and Fae-III were determined by measuring the A_{280} and using the extinction coefficient for FaeA at 280 nm (43,660 M^{-1} cm^{-1}), which was estimated from the amino acid sequence as previously described (Gill and von Hippel, 1989), and the molecular weight obtained by mass spectrometry. pI values were determined on a Novex isoelectric focussing gel (pH 3-7) using the IEF calibration kit from Pharmacia. The temperature optimum was determined by measuring activity over a temperature range from 35 to 70°C. The pH optimum was determined by measuring activity at 37°C in 100 mM sodium acetate buffer with pH values ranging from 4.2 to 5.8 and in 100 mM morpholineethanesulfonic acid (MES) buffer with pH values ranging from 5.5 to 7.0. Kinetic measurements for methyl ferulate were performed in 100 mM morpholinepropanesulfonic acid (MOPS) (pH 6.0) at 37^{o}C with substrate concentrations ranging from 0.1 to 2.0 mM. The assay mixtures had a total volume of 0.5 ml and contained 20 ng protein. At this protein concentration, reactions were linear up to 60 min; reactions were terminated after 15 min by the addition of 0.5 ml of 11.3% acetic acid in methanol. Hydrolysed samples were assayed by high performance liquid chromatography (HPLC) as described below. Raw data were analysed by the method of Wilkinson (1961).

Western analysis and protein determination

Western analysis of supernatant samples from *Aspergillus* cultures was performed by using polyclonal antibodies raised in mice against purified FaeA. Protein concentrations in supernatants and throughout purification procedures were estimated with the Bio-Rad protein assay reagent by using lyophilised bovine serum albumin as a standard (Bio-Rad Bulletin 1177). The method is based on protein-dye binding (Bradford, 1976).

Enzyme assays

Ferulic acid esterase activities in supernatant samples and throughout enzyme isolation were measured by using methyl ferulate as a substrate. Samples (0.02 ml) were incubated in 100 mM sodium acetate buffer (0.48 ml, pH 4.8) containing methyl ferulate (2 mg/ml). After 15 min, the incubation mixture (0.1 ml) was transferred to an Eppendorf tube containing methanol (0.5 ml) and MilliQ water (0.4 ml). This effectively stopped the enzyme reaction. An aliquot (0.05 ml) of this mixture was analysed by HPLC with a type RP-8 reverse phase column (Spectra-Physics, Santa Clara, Ca, USA). Separation of substrate (methyl ferulate) and product (ferulic acid) was achieved by using a linear gradient starting with 32% (vol/vol) methanol-0.3% (vol/vol) acetic acid-67.7% (vol/vol) water and ending with 64% (vol/vol) methanol-0.3% (vol/vol) acetic acid-35.7% (vol/vol) with detection at 325 nm. Endo-xylanase, β-xylosidase, and pectin lyase activities were determined as described previously (Bailey et al., 1992; Rodionova et al., 1983; van Houdenhoven, 1975).

Analysis of hydrolysis products from wheat arabinoxylan and sugar beet pectin

Hydrolysis by FaeA of sugar beet pectin and arabinoxylan was measured in 0.1 M sodium acetate buffer (pH 4.8) containing 1% (wt/vol) WIP (isolated from wheat flour) and 1% (wt/vol) sugar beet pectin, respectively, as the substrate. To 10 ml of the solutions enzymes were added in a total volume of 1 ml. Samples were incubated for 1 and 24 h, respectively, at 50°C, after which a 5-ml sample was transferred to a boiling water bath and incubated for 15 min to stop the reaction. The samples were filtered (pore size, 45 mm) and subsequently analysed by HPLC.

Determination of N-terminal and internal peptide sequences of pectinase ferulic acid esterase

Amino acid sequences were determined by traditional procedures (Allen, 1989; Stone and Williams, 1986). Purified ferulic acid esterase was digested with endoproteinases lysC and V8 and with CNBr. The digests were applied to reverse-phase columns (lysC and V8 digests were applied to C18 [Vydac] and the CNBr digest was applied to C2 [Brownlee]). The columns were equilibrated with 0.1% (vol/vol) trifluor acetic acid (TFA) in water, and peptides were separated by elution with 0.1% (vol/vol) TFA in acetonitrile in a 10 to 40% (vol/vol) gradient. Individual peptides were applied to micro-TFA filters and analysed with an Applied Biosystems amino acid sequencer, (model 476A).

PCR cloning of a specific fragment of the *fae*A genes

Two degenerate oligonucleotides (5'-CARACIGAYATHAAYGGNTGGAT-3' and 5'-CRTARTCNGGRTAYTG-3', with Y, R, N, H, and I representing C/T, A/G, A/C/G/T, A/C/T, and ionisine, respectively) were designed and synthesised with an Applied Biosystems model 392 DNA synthesiser. PCR were performed with a Sensa model 949E DNA processor by using these oligonucleotides at 55°C and chromosomal DNA from *A. niger* N402 and *A. tubingensis* NW756. The resulting fragments were cloned into the pGEM-T vector system (Promega), and sequence analysis was performed as described below.

Isolation, cloning and characterisation of the *fae*A genes

Plaque hybridisation by using nylon replicas was performed as described by Benton and Davis (1977). Hybridizations were performed overnight at 65°C by using the PCR fragment as a probe. Filters were washed with 0.2x SSC-0.5% (wt/vol) SDS (1x SSC is 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.6]. Positive plaques, identified on duplicate replicas after autoradiography, were recovered from the original plates and were purified by re-screening at low plaque density. Standard methods were used for other DNA manipulations, such as Southern analysis, subcloning, DNA digestions and l phage and plasmid DNA isolations (Sambrook et al., 1989). In vivo excision of positive cDNA clones was performed as recommended by the supplier (Stratagene). Chromosomal DNA was isolated as previously described (de Graaff et al., 1988). Sequence analysis was performed with both strands of DNA by using the Sequenase DNA sequencing kit (United States Biochemical Corp.) and a T7 sequencing kit (Pharmacia LKB) with additional oligonucleotides. Nucleotide and amino acid sequences were analysed by using the computer programmes of Devereux et al. (1984). *Aspergillus* transformations were performed as described by Kusters-van Someren et al. (1991) by using 1 µg of a construct carrying the *pyr*A gene for selection of transformants and 20 µg of the plasmid with the gene of interest. Contour-clamped homogeneous electric field-Southern analysis of the *A. niger fae*A was performed as described by Verdoes et al. (1994) by using modifications described by van den Hombergh et al. (1996).

Nucleotide sequence accession numbers

The EMBL accession numbers for *fae*A from *A. niger* and *A. tubingensis* are Y09330 and Y09331 respectively.

Results

Purification and amino acid sequence analysis of FaeA from Pectinase PII

FaeA was purified approximately 100-fold to electrophoretic homogeneity with a recovery of 34% from Pectinase PII, a commercial *A. niger* product. Peptides were prepared by endoproteinase lysC and V8 and CNBr digestion of the purified sample. Sequencing of the N-terminal part of the protein and several internal peptides resulted in the sequences shown in Fig 2. FAE-1 is a combination of the N-terminal sequence and a LysC peptide. FAE-2 is a combination of LysC, V8 and CNBr peptides. FAE-3 is a LysC peptide. A total of 132 amino acids were sequenced.

Cloning and sequence analysis of the ferulic acid esterase (*fae*A) gene from *A. niger* and *A. tubingensis*

Using two degenerate oligonucleotides based on peptides FAE-1 and FAE-2 a 256 bp fragment was generated and subcloned for both *A. niger* and *A.tubingensis*. Translation of the DNA sequence of the two PCR fragments identified all three amino acid sequences (FaeA 1, 2 and 3), as determined by amino acid sequencing. Screening of the genomic libraries of *A. niger* and *A. tubingensis* by using the PCR fragments as probes resulted in four and three positive l clones, respectively. Restriction analysis of these clones was performed, and a 1.5-kb *Pst*I/*Hind*III fragment and a 4-kb *Kpn*I/*Bam*HI fragment from an *A. niger* clone and a 2-kb *Pst*I/*Bam*HI fragment and a 3-kb *Pst*I/*Xho*I fragment from an *A. tubingensis* clone were subcloned into pBluescript SK+ resulting in plasmids pIM3202, pIM3204, pIM3205, and pIM3206, respectively. Sequence analysis demonstrated that the *A. niger* and *A. tubingensis* structural genes contain 900 bp (Fig. 1) and 897 bp, respectively. Sequencing of the cDNA clones confirmed the presence in both genes of an intron of 57 bp starting at position 461 of the structural gene. Alignment of the DNA sequences of the two genes showed an identity of 87.1%.

Translation of the DNA sequences from *A. niger* and *A. tubingensis* resulted in amino acid sequences containing 281 and 280 amino acids, respectively (Fig 2). Comparison of the deduced amino acid sequences to the determined amino acid sequences of the mature *A. niger* protein indicated that there was a signal peptide of 21 amino acids. This peptide has all of the characteristics of a typical signal peptide in fungi (von Heijne, 1986).

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- ATGAAGCAATTCTCTGCAAAATACGCCCTCATTCTTTTGGCGACTGCAGGACAAGCCCTCGCGGCCTCCACGCAA - 75
- GGCATCTCCGAAGACCTCTACAATCGCTTGGTAGAGATGGCCACTATCTCCCAAGCCGCCTACGCCGACCTATGC -150
- AATATTCCATCGACTATTATCAAAGGAGAGAAAATTTACAACGCTCAAACTGATATCAACGGATGGATCCTCCGC -225
- GACGACACCAGCAAAGAAATTATCACCGTCTTCCGTGGCACTGGCAGTGACACAAACCTACAGCTCGATACTAAC -300
- TACACGCTCACGCCATTCGACACTCTACCTCAATGCAACGATTGCGAGGTACACGGTGGATACTATATTGGATGG -375
- ATCTCAGTCCAAGACCAAGTCGAGTCGCTTGTCAAACAACAGGCTAGCCAGTATCCGGACTATGCGCTTACCGTG -450
- ACAGGCCATAggtatgcgtgcttaccgtcttacaatttgttgtatgatatatactaactttatgataGTCTGGGA -525
- GCGTCGATGGCAGCACTCACTGCCGCCCAGCTGTCCGCGACATACGACAACGTCCGTCTGTACACATTCGGCGAA -600
- CCGCGCAGCGGCAACCAGGCCTTCGCGTCGTACATGAACGATGCGTTCCAGGTCTCGAGCCCGGAGACGACCCAG -675
- TACTTCCGGGTCACTCATTCCAACGACGGCATCCCAAACTTGCCCCCGGCGGACGAGGGTTACGCCCATGGTGGT -750
- GTAGAGTACTGGAGCGTTGATCCTTACAGCGCCCAGAACACGTTTGTCTGTACTGGGGATGAAGTACAGTGCTGT -825
- GAGGCACAGGGCGGACAGGGGGTGAATGATGCGCATACTACTTATTTTGGGATGACGAGCGGAGCTTGTACTTGG -900
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Fig.1. Nucleotide sequence of the *fae*A structural gene from *A. niger*. The intervening region (position 461 to 517) is given in lower case letters.

The enzymes from *A. niger* and *A. tubingensis* contain a single putative N-glycosylation site, and alignment of their amino acid sequences showed an identity of 92.5%. The calculated molecular mass is 28 kDa for both proteins. The *A. niger* and *A. tubingensis* sequences differ at five and three positions, respectively, from the determined amino acid sequences of pectinase FaeA. The asparagine residue of the putative N-glycosylation site is likely to be glycosylated as this residue could not be determined experimentally.

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FAENIG      - mkqfsakyalillatagqalaASTQGISEDLYNRLVEMATISQAAYADLCNIPSTIIKGEKIYNAQTDINGWILR – 75
FAETUB      - mkqfsakyaiavvvtaghalaASTQGISEDLYSRLVEMATISQAAYADLCNIPSTIIKGEKIYNSQTDINGWILR - 75
FAE-1                             -ASTQGISEDLYTRLVEMATISQAAYADLCNIPSTIIKGEKIYNSQTDINGWILR

FAENIG      - DDTSKEIITVFRGTGSDTNLQLDTNYTLTPFDTLPQCNDCEVHGGYYIGWISVQDQVESLVKQQASQYPDYALTV -150
FAETUB      - DDSSKEIITVFRGTGSDTNLQLDTNYTLTPFDTLPQCNSCEVHGGYYIGWISVQDQVESLVQQQVSQFPDYALTV -150
FAE-1/FAE-2 - DDSSK                                                    -SLVKQQVSQYPDYALTV
FAE-3             -EIITVFRGTGSDTNLQLDTXYTLTPFD-

FAENIG      - TGHSLGASMAALTAAQLSATYDNVRLYTFGEPRSGNQAFASYMNDAFQVSSPETTQYFRVTHSNDGIPNLPPADE -225
FAETUB      - TGHSLGASLAALTAAQLSATYDNIRLYTFGEPRS-NQAFASYMNDAFQASSPDTTQYFRVTHANDGIPNLPPADE -224
              - TGHSLGASLAALTAAQLSATYDNIRLYTF- FAEA-2

FAENIG      - GYAHGGVEYWSVDPYSAQNTFVCTGDEVQCCEAQGGQGVNDAHTTYFGMTSGACTW -281
FAETUB      - GYAHGVVEYWSVDPYSAQNTFVCTGDEVQCCEAQGGQGVNNAHTTYFGMTSGHCTW -280
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Fig. 2. Alignment of the deduced amino acid sequences for FaeA from *A. niger* and *A. tubingensis*. The sequence identity is 92.5%. The determined amino acid sequences of the pectinase ferulic acid esterase (FAE-1, FAE-2 and FAE-3) are also aligned with the protein sequences. The putative N-glycosylation site is in boldface type and underlined. The signal sequence is given in lower case letters. The region homologous to the putative lipase serine active site is underlined. FAENIG, *A. niger* FaeA; FAETUB, *A. tubingensis* FaeA.

Analysis revealed high homology of a region of the enzymes to a putative lipase serine active site (Fig. 3). This region is aligned with the putative serine active sites of *Rhizopus delemar* (Haas et al., 1991), *Mucor miehei* (Brady et al., 1990), *Humicola lanuginosa*, and *Penicillium camembertii* (Yamaguchi et al., 1991). A search of gene and protein libraries did not produce any other genes or proteins with significant homology to these esterases. Contour-clamped homogeneous electric field-Southern analysis demonstrated that the *A. niger fae*A gene is located on chromosome I.

Mucor miehei lipase	-VAVTGHSLGG...59...D...53...H-
Humicola lanuginosa lipase	-VVFTGHSLGG...55...D...57...H-
Penicillium camembertii lipase	-LVVVGHSLGA...55...D...60...H-
Aspergillus niger FaeA	-LTVTGHSLGA...61...D...53...H-
consensus	-..VT**GHSLG**.....**D**.....**H**-

Fig. 3. Alignment of the putative serine active sites of a number of lipases with a region of the *A. niger* FaeA. Only the regions containing the putative active site are shown. In the consensus sequence, amino acids which are conserved in at least four of the five enzymes are shown. Amino acids which are conserved in all five enzymes are indicated by boldface type.

Overexpression of *fae*A from *A. niger* and *A. tubingensis*

Combination of fragments from plasmid pIM3202 and pIM3204 and plasmids pIM3205 and pIM3206 resulted in functional constructs for *fae*A from *A. niger* and *A. tubingensis*, respectively (plasmids pIM3207 and pIM3208) (Fig 4). These constructs were transformed to *A.*

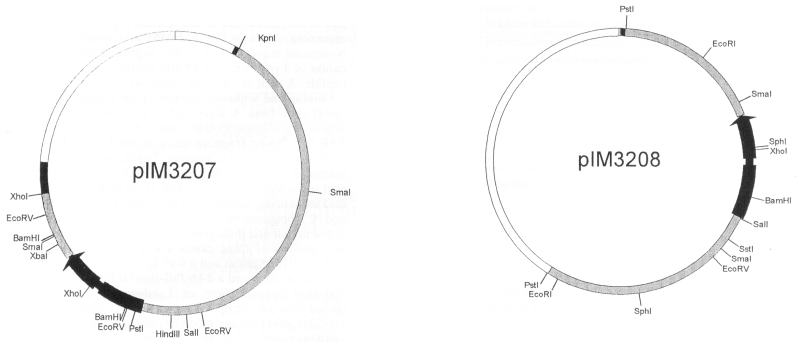

Fig. 4. Expression constructs containing the *fae*A gene from *A. niger* (pIM3207) and *A. tubingensis* (pIM3208). The genes are depicted as black arrows. The narrow regions in the arrows indicate the intervening regions. Chromosomal regions flanking the gene are stippled. The polylinker regions of the plasmid are depicted as black boxes. pIM3207 is a combination of the 1.5-kb *Hind*III-*Pst*I fragment from pIM3202 and the 3.9-kb *Kpn*I-*Pst*I fragment from pIM3204. pIM3208 is a combination of the 2-kb *Bam*HI-*Pst*I fragment of pIM3205 and the 2.2-kb *Bam*HI-*Pst*I fragment of pIM3206.

niger NW219 and *A. tubingensis* NW241 and to an *A. niger prt*F mutant (NW154). Western analysis performed with a specific antibody against the pectinase FaeA was applied to select transformants with a high level of FaeA production. Six high-producing transformants were chosen, and the copynumber and FaeA activity after 6 days of growth on MM containing a crude wheat arabinoxylan preparation at a concentration of 1% (wt/vol) were determined(Table 1). The activity in the *prt*F strain was higher than the activity in the wild-type *A. niger* strain for the *A. niger* gene product, while the copy numbers were comparable. For the *A. tubingensis* gene product the same difference was observed, with a lower number of copies in the *prt*F strain. Similarly, the *A. tubingensis* strain also exhibited higher activities than the wild-type *A. niger* strain for both gene products.

Table 1. FaeA activity and copynumber of six transformants.

strain[a]	FaeA activity[b]	copy-number[c]	strain description
NW219	0.06	1	
NW154	0.13	1	
NW241	0.00	1	
NW219::pIM3207.28	1.60	20	*A. niger fae*A in *A. niger* NW219
NW154::pIM3207.7	7.71	25	*A. niger fae*A in *A. niger* NW154 (*prt*F mutant)
NW241::pIM3207.15	3.16	10	*A. niger fae*A in *A. tubingensis* NW241
NW219::pIM3208.10	0.00	6	*A. tubingensis fae*A in *A. niger* NW219
NW154::pIM3208.5	0.97	8	*A. tubingensis fae*A in *A. niger* NW154 (*prt*F mutant)
NW241::pIM3208.6	0.80	7	*A. tubingensis fae*A in *A. tubingensis* NW241

[a] Transformant designations are given as follows: strain::plasmid.transformant number.

[b] FaeA activity is expressed as micromoles of ferulic acid produced per minute per millilitre of supernatant

[c] Copynumbers were determined by hybridisation with the *fae*A gene, after which intensities were compared.

Transformant NW154::pIM3207.7 was used to compare the levels of induction on wheat arabinoxylan and sugar beet pectin. The highest levels of FaeA activity in supernatant samples were observed when this transformant was grown on a crude wheat arabinoxylan preparation (Table 2). FaeA activity was also found in supernatant samples from media containing WIP and sugar beet pectin, demonstrating that the *fae*A gene is not specifically induced on only xylan but also on pectin.

Table 2. Ferulic acid esterase activities in culture supernatants of transformant NW154::pIM3207.7 after 6 days of growth.

Substrate[a]	Activity[b]
Crude wheat arabinoxylan	3.08
WIP	1.39
Sugar beet pectin	0.19

[a] Substrates were added at a concentration of 0.5% (wt/vol).

[b] Ferulic acid esterase activity is expressed as micromoles of ferulic acid produced per millilitre of supernatant per minute.

FaeA from *A. tubingensis* is more sensitive to degradation than FaeA from *A. niger*

Four of the transformants described above were grown for 6 days on MM containing 1% (wt/vol) oat spelt xylan. Supernatant samples were isolated and concentrated five times and aliquots (0.02) of the concentrated samples were subjected to Western analysis. Several low-molecular-weight bands were detected (Fig 5). N-terminal amino acid sequencing showed identical sequences for the mature protein and the second-largest protein, presumably produced by truncation, indicating that this peptide originated by C-terminal degradation. In both backgrounds (*A. niger prt*F and *A. tubingensis*) the amount of degraded protein compared to the amount of mature protein was much higher in the transformant containing the *A. tubingensis* construct than in the transformant containing the *A. niger* construct, indicating that the *A. niger* gene product is less sensitive to degradation.

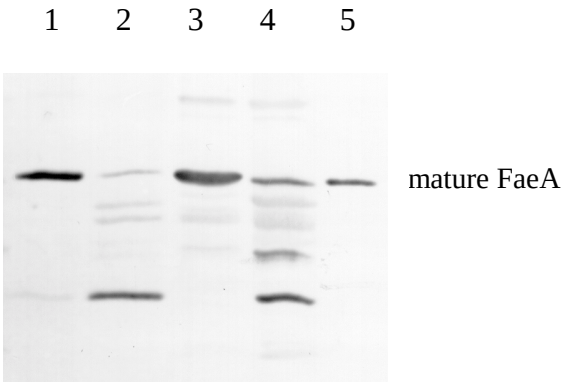

Fig. 5. Degradation patterns for FaeA from *A. niger* and *A. tubingensis* in the *A. niger prt*F mutant and in *A. tubingensis*. Lane 1, transformant NW154::pIM3207.7 (*A. niger fae*A in *A. niger*); Lane 2, NW154::pIM3208.5 (*A. tubingensis fae*A in *A. niger*); Lane 3, NW241::pIM3207.15 (*A. niger fae*A in *A. tubingensis*); Lane 4, NW241::pIM3208.6 (*A. tubingensis fae*A in *A. tubingensis*); Lane 5, purified mature FaeA from *A. niger*.

Recombinant A. niger FaeA is identical to *A. niger* Fae-III.

Recombinant FaeA (from transformant NW154::pIM3207.7), Fae-III, and the pectinase FaeA were purified to electrophoretic homogeneity (see above). None of the purified preparations contained detectable endo-xylanase, b-xylosidase, or endo-pectin lyase activity. Fae-III and recombinant FaeA were identical in all respects (Table 3). All three enzymes were identical with respect to apparent M_r as determined by SDS-PAGE, pH optimum and temperature optimum. However, the pectinase FaeA had a slightly higher pI than FaeA and Fae-III and a lower V_{max} for methyl ferulate.

Table 3. Comparison of Fae-III, FaeA from *A. niger* and FaeA isolated from a commercial pectinase preparation.

Enzyme	M_r as determined by		pI	Optimum pH	Optimum temp (°C)	V_{max} methyl ferulate	K_m methyl ferulate
	SDS-PAGE	Mass spectrometry					
Fae-III	36,000	29,740 ± 16	3.3	5.0	55-60	147	0.72
FaeA from A. niger	36.000	29,738 ± 50	3.3	5.0	60	143	0.76
FaeA from pectinase	36.000	ND	3.4	5.0	60	86.0	1.00

[a] Total protein content was determined by measuring the A_{280} of the purified enzyme solutions and using the extinction coefficient for recombinant FaeA (43,660 M^{-1} cm^{-1}) calculated from the amino acid sequence and the molecular mass determined by mass spectrometry.

FaeA from *A. niger* is able to release ferulic acid from wheat arabinoxylan and sugar beet pectin

WIP (1%, wt/vol) from wheat arabinoxylan was incubated with recombinant FaeA and xylanase 1 for 1 and 24 h at 50°C. HPLC analysis of the samples showed that FaeA was able to release ferulic acid from arabinoxylan in the absence of other hemicellulases (Table 4). Addition of xylanase resulted in a strong increase in the amount of ferulic acid released. Depending on the conditions, a two- to sevenfold increase could be obtained. The alkali-extractable ferulic acid content of the WIP was estimated to be 0.86% (wt/wt). In the presence of xylanase 1, FaeA was able to release approximately 4.9% of the esterified ferulic acid in the WIP sample.

In a similar experiment 1% (wt/vol) sugar beet pectin was incubated with recombinant FaeA and a pectin lyase. FaeA was able to release ferulic acid from sugar beet pectin in the absence of other enzymes, but at a much lower level than it released ferulic acid from xylan (Table 3). Addition of pectin lyase had only a minor effect on the liberation of ferulic acid. The alkali-

extractable ferulic acid content of the sugar beet pectin was estimated to be 1.30% (wt/wt). FaeA was able to release approximately 0.16% of the esterified ferulic acid in the sugar beet pectin sample.

Table 4. FaeA activity on wheat arabinoxylan and sugar beet pectin[a]

Substrate	Amount of FaeA (µl)	Amount of xylanase 1 (µl)	Amount of pectin lyase (µl)	Amount of ferulic acid released (ng/ml) 1 h	24 h
WIP from wheat arabinoxylan	0	0		0	17
	5	0		0	155
	50	0		94	314
	500	0		324	1,356
	0	500		12	0
	5	500		29	597
	50	500		196	2,273
	500	500		1,065	4,211
Sugar beet pectin	0		0	20	16
	5		0	21	22
	50		0	63	95
	500		0	84	166
	0		500	14	15
	5		500	16	43
	50		500	31	102
	500		500	95	202

[a] The FaeA solution contained 0.6 U/ml (0.6 mmol of ferulic acid liberated from methyl ferulate·min^{-1}·ml^{-1}), the xylanase 1 solution contained 0.18 mU/ml (nmol xylose liberated from oat spelt xylan/min/ml), and the pectin lyase solution contained 6 units/ml. 1 pectin lyase unit was the amount of activity which gave rise to a change in A_{235} of 0.01/min when the preparation was incubated with highly esterified pectin.

Discussion

Faulds and Williamson (1994) purified a ferulic acid esterase (Fae-III) from *A. niger* with high activity against the methyl esters of several cinnamic acids. We purified an enzyme from a commercial enzyme preparation which was similar to Fae-III. Using reverse genetics we cloned the corresponding gene (*fae*A) from *A. niger* and *A. tubingensis* and overexpressed both gene products.

The characteristics of the product from the *A. niger fae*A gene are identical to those of Fae-III (Faulds and Williamson, 1994), indicating that these enzymes are in fact the same enzyme. The differences observed in the molecular mass, K_m, and V_{max} are well within the expected experimental variations. This conclusion was strengthened by the identical N-terminal amino acid sequences of the two enzymes. The characteristics of FaeA isolated from the commercial pectinase preparation are very similar but not identical to those of the other two enzymes. This is

probably due to strain differences as the origin of the preparation is unknown. The differences in the determined amino acid sequences of the enzyme from the commercial preparation and the amino acid sequence of the recombinant FaeA also indicate that the *A. niger* strain used for the production of the pectinase preparation was not *A. niger* N400 (=CBS120.49). Further evidence for the identity of FaeA and Fae-III was obtained by heterologous Southern hybridisation of *A. niger* N400 chromosomal DNA, which showed only one band for all individual restriction digests tested when *fae*A was used as a probe, thus demonstrating the presence of a single *fae*A gene (data not shown).

The differences in molecular mass observed when different methods of determination (SDS-PAGE and mass spectrometry) were used could be the result of a combination of two effects. FaeA is a very acidic protein (pI = 3.3) which may lead to reduced binding of SDS and reduced mobility on SDS-PAGE. Second, the fact that the asparagine residue of the putative glycosylation site could not be determined experimentally indicates that the enzyme is probably glycosylated, which results in a higher molecular mass on SDS-PAGE gels.

Overexpression of FaeA showed that the *A. tubingensis* enzyme was more sensitive to degradation than the *A. niger* enzyme. This was clearly demonstrated in two different genetic backgrounds, and it can therefore be assumed that the difference is due to the nature of the two enzymes, even though they are nearly 93% identical in sequence. Whether this difference in stability is due to the primary structure of these enzymes or to other factors is not clear at this moment. In wild- type *A. niger* carrying the *A. tubingensis fae*A gene, hardly any mature FaeA from *A. tubingensis* can be detected. Since *A. tubingensis* does not acidify the medium as strongly as wild-type *A. niger* acidifies the medium, this could indicate that FaeA from *A. tubingensis* is more sensitive to acid proteases than FaeA from *A. niger*. However, in the *A. niger* prtF mutant, which is strongly reduced in acid protease activity, the difference between the two gene products is also very obvious. In this strain the amount of degradation for both gene products is much lower than the amount of degradation in the *A. niger* wild-type strain, but degraded bands can still be observed. If the degradation of FaeA from *A. tubingensis* in the *prt*F strain is due to residual protease activity, this enzyme could be a useful target for further studies to decrease protease activity.

FaeA is able to release ferulic acid from wheat arabinoxylan without the aid of other xylan degrading enzymes. Addition of xylanase from *A. niger* increased the amount of released ferulic acid two- to sevenfold. Bartolomé et al. (1995) showed that the source of the xylanase has a strong effect on the level at which the liberation of ferulic acid is increased. They studied the

effects of various xylanases on the liberation of ferulic acid from destarched wheat bran by Fae-III and showed that the *A. niger* xylanase had a relatively small effect compared to some other xylanases. Addition of other xylanases could therefore increase the amount of ferulic acid liberated even more.

FaeA is also able to release ferulic acid from sugar beet pectin, without the aid of other enzymes. With Fae-III, no activity was detected on sugar beet pulp. This is because of differences in the experiments. The hydrolysis with FaeA was performed with purified sugar beet pectin, whereas the Fae-III hydrolysis was performed with crude sugar beet pulp, which leads to a lower substrate concentration and low substrate accessibility. Second, due to the amount of enzyme available the amount of enzyme added was much lower in the Fae-III experiment, which may have resulted in activity which is below the detection limit. Fae-III was active on feruloylated galactose, but not on feruloylated arabinose isolated from sugar beet pulp (Ralet et al., 1994). The activity on sugar beet pectin observed for FaeA was therefore probably activity on the ferulic acid linked to the galactose residues of sugar beet pectin. Addition of a pectin lyase from *A. niger* had only a minor effect on the amount of ferulic acid which was released. This could indicate that most of the feruloyl groups on which FaeA is active are already sufficiently accessible for FaeA to release them. Alternatively, this pectin lyase may not produce any oligomeric compounds with accessible ferulic groups, other than the ones that FaeA can already release from the polymer.

A region of FaeA has high homology to a putative lipase serine active site, which could be a result of similarities in substrate characteristics and reaction mechanism for these two types of enzymes. Both act on large molecules which contain polar and apolar residues. Additional indications that there is a lipase-like serine active site in FaeA are found when the spacing within the catalytic triade from lipases, which consists of a serine, an aspartic acid and a histidine, is compared with the putative serine active site in FaeA. The spacing for the serine and the aspartic acid is between 55 and 59 residues and for the aspartic acid and the histidine between 53 and 60 residues for the four lipases in figure 3. FaeA has an aspartic acid at 61 residues after the putative active site serine and a histidine at 53 residues after that aspartic acid, which is a spacing similar to that of the lipases. No significant homology was found with any other gene in the database including *cin*I, a gene coding for a cinnamoyl esterase from the bacterium *Butyrivibrio fibrisolvens* (Dalrymple et al., 1996).

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Chapter 4

Influence of ferulic acid on the production of feruloyl esterases by *Aspergillus niger*

C. B. Faulds, R. P. de Vries, P. A. Kroon, J. Visser and G. Williamson

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Abstract

Extracellular feruloyl esterases from the filamentous fungus *Aspergillus niger* are induced by growth on oat spelt xylan (OSX), which contains no detectable esterified ferulic acid. FaeA accounted for most of the feruloyl esterase activity. Addition of free ferulic acid to OSX at the start of the culture induced FaeA secretion a further 2.3-fold, and also induced other feruloyl esterases which could not be ascribed to FaeA. Wheat bran (WB)-grown cultures, containing 1% (m/v) ester-linked ferulic acid, gave almost identical FaeA and total feruloyl esterase activities as the cultures grown on OSX plus ferulic acid. De-esterification of WB yielded less total feruloyl esterase, and 2.4-fold less FaeA, compared to untreated WB. A slightly modified form of FaeA was produced on de-esterified WB. These results show that production of FaeA does not absolutely require ferulic acid. However, production is stimulated by the presence of free ferulic acid through increased expression, and is reduced by the removal of esterified ferulic acid from the growth substrate.

Introduction

Cereals and sugarbeet contain high concentrations of ferulic acid (FA):- wheat bran (0.5-1% m/w dry weight) (Hartley and Jones, 1977), maize bran (3%) (Saulnier et al., 1995), sugarbeet pulp (0.6-1%) (Rombouts and Thibault, 1986). In the plant cell-wall, FA is mainly associated with either hemicellulose (Smith and Hartley, 1983) or highly branched pectin (Rombouts and Thibault, 1986), where it is ester-linked to arabinose or galactose depending on the cell-wall source (Ralet et al., 1994; Colquhoun et al., 1994). Microbes, both fungal and bacterial, have been identified which secrete specific esterases to cleave the FA from the plant polymers, and some of these feruloyl esterases have been purified and partially characterised (Faulds and Williamson, 1994; McCrae et al., 1994; Kroon et al., 1996). These act synergistically with other carbohydrate-degrading enzymes (Bartolome et al., 1994). Feruloyl esterases from *Aspergillus niger* are produced during growth on cell-wall derived growth substrates, but not by glucose (Faulds and Williamson, 1994; Kroon et al., 1996). Enzymic hydrolysis of complex plant cell-wall polysaccharides, such as arabinoxylan, is regulated by the micro-organism. Induction of individual extracellular enzymes, such as xylanases (Pinaga et al., 1994), arabanases and arabinofuranosidases (van der Veen et al., 1993) have previously been examined in *Aspergillus* species. In this paper, we have measured the changes in both total

feruloyl esterase and FaeA levels in culture supernatants during growth of *A. niger* on different plant cell-wall preparations, both feruloylated and non-feruloylated.

Materials and methods

Strains and growth conditions

A. niger CBS 120.49 was grown on 1% (m/v) plant cell-wall preparations, supplemented with 0.1% (m/v) D-glucose, at 25^{o}C and 120 rpm agitation speed, as described previously (Faulds and Williamson, 1994). *A. niger* NW154 (*pyr*A6, *prt*F28) was derived from *A. niger* N400 (CBS 120.49). The *prt*F mutation present in this strain was previously described (van den Hombergh et al., 1995). The multicopy *faeA* transformant used for the induction experiment (NW154::pIM3207.7) was described previously (Chapter 3), and grown as above. Cultures were prepared in duplicate, and aliquots (3 ml) removed daily.

Chemicals

De-starched wheat bran (WB) was obtained from Agro-Industrie Research et Developpements, Pomacle, France. Xylans from oat spelt, birchwood and larchwood were obtained commercially. Soluble xylan was prepared by the method of He et al. (1993), methyl ferulate (MFA) by the method of Borneman et al. (1990), and pure FaeA prepared as previously described (Faulds and Williamson, 1994). De-esterified WB was prepared by incubating WB (1g, containing 1% FA, m/m, alkali-extractable ferulic acid) overnight in 100 ml NaOH (0.1M) in a covered flask, under gentle agitation. The whole reaction mixture was dialysed extensively against water. This material was then supplemented with 0.1% (m/v) D-glucose, in growth media (Faulds and Williamson, 1994). WB in water was used as a control throughout the dialysis procedure and subsequent media preparation.

Partial purification of culture filtrates

Culture filtrates (100 ml) of *A. niger* CBS 120.49, obtained by filtering through miracloth, were subjected to 80% saturation $(NH_4)_2SO_4$ fractionation. The pellet was resuspended in 50 mM sodium phosphate (pH 7)/ 1 mM EDTA/ 0.6 mM $(NH_4)_2SO_4$, syringe filtered (0.45 mm), and fractionated on an FPLC hydrophobic interaction chromatography (HIC) column (High-Load phenyl Sepharose HR 26/10) with a flow rate of 5 ml/min. After elution of unbound material with 50 mM $NaPO_4$ (pH 7)/ 1 mM EDTA/ 1.7 M $(NH_4)_2SO_4$ (buffer A; 50 ml), bound proteins were eluted with a four-step gradient:- 100%-60% buffer A in 150 ml, 60-30% buffer A in 150 ml, and 30-0% buffer A in 100ml. A further 100 ml buffer B (buffer A without $(NH_4)_2SO_4$) was run through the column, before re-equilibrating the column in buffer A. Fractions of 5 ml were collected.

Enzyme assays

Feruloyl esterase activity against methyl ferulate (MFA) was determined by the spectrophotometric method of Ralet *et al* (1994), using an e_{335} of 14000 M^{-1} cm^{-1} for MFA and 4300 M^{-1} cm^{-1} for free FA. Activity (U) is expressed as mmol FA released/min at 37^{o}C and pH 6. Xylanase activity was determined by the release of reducing sugars from 1% (m/v) soluble xylan (Kellet et al, 1990). Activity is expressed as mmol xylose equivalents released/min at 37^{o}C and pH 5.4. Pyruvate kinase and phosphoglucose isomerase levels were determined as described by Ruijter et al. (1997). Residual FA in culture filtrates was measured by HPLC (Kroon and Williamson, 1996). Extracellular and intracellular protein levels were determined using Coomassie-Plus reagent , bovine serum albumin

used as a protein standard. Immunoblotting of culture supernatants (Brezillon et al., 1996), and two-site ELISA (Mills et al., 1997) were performed as previously described.

Results and discussion.

Effect of carbon source on the production of feruloyl esterase

A. niger (CBS 120.49) was grown on either 1% OSX, or on 1% WB, over a 10-day period. Profiles of total feruloyl esterase activity on MFA in the culture filtrates are shown in Fig. 1. On OSX, activity showed a maximum at 0.13 U/mg protein after 4 days growth. On WB, activity showed a maximum after 3 days (0.34 U/mg protein), which was 2.6-fold higher than the OSX-grown cultures. However, maximum activity may be reached after 3.5 days for both cultures. Multiple forms of feruloyl esterase may be secreted during growth on cereals (Faulds and Williamson, 1994; Kroon et al., 1996; Johnson et al., 1989; Linden et al., 1994; Kormelink et al., 1993), and so not all of the esterase activity may be attributed to one enzyme. No growth was observed when cultures were grown on FA (0.03%, m/v) alone.

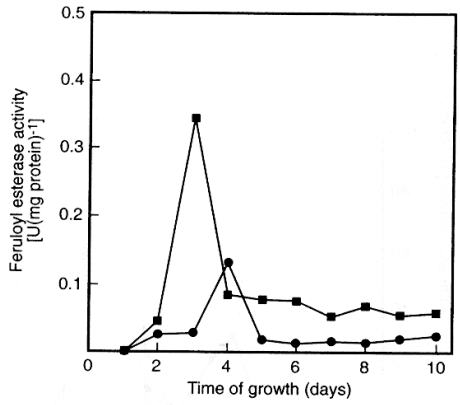

Fig. 1. Feruloyl esterase activities from culture supernatants of *A. niger* CBS 120.49 grown over a 10-day period on 1% (m/v) OSX (•) or wheat bran (■).

Xylanase activity was detected in the supernatant after 24 h growth on WB and after 48 h growth on OSX, maximum activity occurring 48 h after activity being first detected in the culture filtrates on both growth substrates (704 and 703 U/mg protein, respectively).

Influence of ferulic acid on enzyme production

1. Addition of ferulic acid

As there was higher feruloyl esterase activity in WB-grown cultures compared to OSX-grown cultures, the effect of adding free FA to a non-feruloylated growth substrate was examined. OSX-containing medium was supplemented with either 0, 10 or 30 mg FA per 100 ml, which is comparable to the alkali-extractable levels of ferulic acid found in OSX, WB and maize

bran, respectively. OSX contained <0.0007% FA (m/m) in the cell-wall material (based on a limit of detection of FA on the HPLC of 4.85 ng FA/ml soluble extract , after alkali-treatment and HPLC analysis). Since highest feruloyl esterase levels on OSX were reached over the first 5 days (Fig. 1), cultures containing the supplementary FA were examined over the same period (Fig. 2a). Addition of free FA very clearly stimulated feruloyl esterase production by the cultures, the response being dependent on the amount of FA added, with the highest activity (0.3 U/mg protein) obtained in cultures grown in the presence of 30 mg FA. This reponse was not due to changes in fungal biomass, as determined by measuring intracellular protein, pyruvate kinase and phosphoglucose isomerase. Ferulic acid was very quickly lost from the medium during the first 24 h of growth. By day 5, residual levels of the free acid were below the limits of detection by HPLC (4.85 ng/ml culture medium). Xylanase activity (Fig. 2b) was suppressed by the addition of FA, the degree of suppression increasing with higher levels of free acid. This effect is the opposite to that observed with total feruloyl esterase activity, showing that the fungal regulatory mechanisms involved in the production and secretion of feruloyl esterases and xylanases are different.

2. Removal of ferulic acid

In a separate experiment, the effect of the removal of ferulic acid, and other ester-linked groups, from wheat bran was examined. Alkali-treated WB was used as the carbon source, as described in Materials and Methods. Use of de-esterified WB as a growth substrate decreased feruloyl esterase levels in the culture filtrate compared to the control, from 0.24 U/mg protein to 0.14 U/mg protein (Fig. 2c). Thus, feruloyl esterase activity is diminished but not completely lost. This is consistent with the results above, which show that ferulic acid either stimulated or de-repressed feruloyl esterase production by *A. niger*, but FA is not absolutely essential for feruloyl esterase production. It also shows that other ester-linked groups, such as acetyl or methoxy-groups, are not essential for feruloyl esterase production.

Effect of carbon source on the production of FaeA

1. FaeA production from transformed *A. niger*

The role of FA was further elucidated using *A. niger* NW154:: pIM3207.7 (Chapter 3). The multi-copy *A. niger* transformant was grown on a variety of cell-wall derived growth substrates for up to 6 days, and activity against MFA was measured. Table 1 shows the activities after day 4 of growth. FaeA production is clearly regulated by both FA and a sugar-

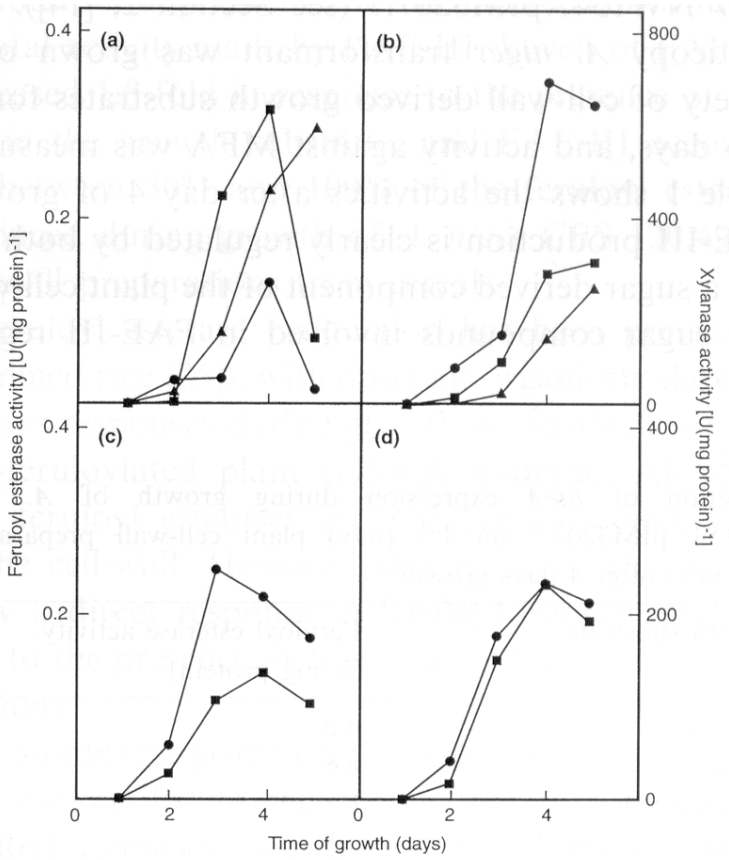

Fig. 2. Influence of ferulic acid on feruloyl esterase and xylanase production. Feruloyl esterase (a) and xylanase (b) activities from *A. niger* CBS 120.49 grown on OSX without supplementation (●) or supplemented with either 0.01% (m/v) FA (■) or 0.03% (m/v) FA (▲). Feruloyl esterase (c) and xylanase (d) activities from *A. niger* CBS 120.49 grown on either WB (●) or de-esterified WB (■).

derived component of the plant cell-wall. The sugar compounds involved in FaeA regulation do not necessarily have to be short linear xylo-oligosaccharides, which arise during growth on different xylan preparations. There may also be more branched oligomers containing FA, which are released from the xylan preparation during fungal growth, and which act on *faeA* regulation, the gene encoding FaeA (Chapter 3). FA clearly stimulates FaeA production, but the mechanism has yet to be elucidated. During growth of the transformant on xylose, FaeA activity was detected, but at a lower level than during growth on more complex substrates. This suggests that during growth of the wild-type fungus on xylose, activity was below the detection limit of the assays used (0.6 mU/ml), and that the higher level produced by the multi-copy transformant is thus detectable. No feruloyl esterase activity was detected during growth of NW154::pIM3207.7 on glucose.

Table 1. Induction of *faeA* expression during growth of *A. niger* NW154::pIM3207.7 on 1% (m/v) plant cell-wall preparations (1 %, m/v) after 4 days growth. n.d. = not detected

Growth substrate	Feruloyl esterase activity (U/mg protein)
Glucose	n.d
Xylose	0.6
Oat spelt xylan	1.5
Birchwood xylan	0.5
Larchwood xylan	2.6
Wheat bran	5.5
OSX + 0.1% glucose	3.6
OSX + 0.01% FA	6.4
OSX + 0.03% FA	7.3
OSX + 0.05% FA	5.2

2. Characterisation of secreted FaeA

Other enzymes which hydrolyse MFA are also secreted by the fungus. Assays using MFA, therefore, only give an estimate of total feruloyl esterase activity, so we determined the amount of FaeA secreted in cultures using purification, immunoblotting and ELISA. From the time course of feruloyl esterase production on the various growth substrates, cultures were harvested on the days corresponding to the time of maximum activity for the relevant culture, *ie* day 3 for WB and de-esterified WB, and day 4 for OSX, OSX/FA and xylose-grown cultures. SDS-PAGE of the culture supernatants showed multiple bands after staining. Immunoblotting of the same culture filtrates and development with anti-FaeA antiserum showed only one band corresponding to FaeA in each of the samples which exhibited feruloyl esterase activity. Glucose- or xylose-grown culture supernatants, with no detectable feruloyl esterase activity, do not contain an antibody-reactive protein, confirming that FaeA is not produced in detectable levels by wild-type *A. niger* during growth on simple sugars. An immuno-reactive band, corresponding to FaeA, was seen in the de-esterified WB-grown culture filtrate, so the removal of FA from the growth substrate does not completely inhibit FaeA production.

Immunoblotting cannot be used to quantify the contribution that FaeA makes to overall feruloyl esterase activity. The use of ELISAs on the crude culture supernatants was precluded because of complete blocking of the antibody-FaeA interaction by the culture supernatant. FaeA was therefore quantified using purification and ELISA. Feruloyl esterase activities against MFA for five different growth substrates are shown as elution peaks after fractionation

by HIC (Fig. 3). The peak of activity at 415 ml corresponds to FaeA. A different retention time for activity (385 ml) was observed for the supernatant from the de-esterified WB-grown culture. No activity was detected in any of the fractions from the xylose-grown wild-type culture filtrate. Both peaks at 385 ml and 415 ml reacted in an ELISA with anti-FaeA antiserum, the peak at 385 ml presumably being a post-translationally modified form of FaeA.

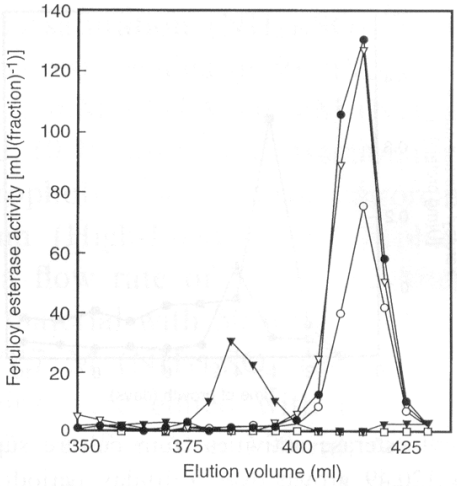

Fig. 3. Feruloyl esterase activity against MFA of $(NH_4)_2SO_4$-treated culture supernatants after growth for maximum activity on xylose (□), OSX (o), OSX + 0.03% FA (•), WB (∇) or de-esterified WB (▼).

FaeA has previously been shown to be precipitated by 80% $(NH_4)_2SO_4$, and the activity values from Fig. 3 allow quantification of FaeA in each culture supernatant (Table 2). These results confirm that there is more than one feruloyl esterase with activity on MFA produced by *A. niger*. The ratio of activities against the methyl esters of ferulate, sinapate, caffeate and *p*-coumarate in the peak fractions was comparable to pure FaeA (1:4:0:~0) for both the 385 ml and the 415 ml peaks. This confirms that the active peaks after HIC are FaeA, since different feruloyl esterases give different ratios for these substrates (Faulds and Williamson, 1994; Kroon et al., 1996). Table 1 shows both the total feruloyl esterase levels and the contribution to total activity made by FaeA. Levels of FaeA increased 1.8-fold in response to the presence of free FA in the growth substrate, and FaeA accounts for between 30% and 100% of the feruloyl esterase produced during growth of *A. niger* CBS 120.49 on cell-wall preparations from cereals.

Feruloyl esterase production has been previously examined (Christov and Prior, 1993) with different organisms showing various responses during growth on feruloylated and non-feruloylated plant cell-wall material. All agree that feruloyl esterases are induced by

components of the cell-wall. However, this report is the first to show a direct response in feruloyl esterase production to the presence of free ferulic acid in the growth medium.

Table 2. Contribution of FaeA to overall feruloyl esterase activity in cultures of *A. niger* CBS 120.49 grown on plant cell-wall preparations (1 %, m/v).

Growth Substrate	Total Feruloyl Esterase activity (U)	Total Feruloyl Esterase activity after HIC corresponding to FaeA (U)	Estimated percentage contribution of FaeA in total activity
Oat spelt xylan	0.4	0.4	81
Oat spelt xylan + FA	1.7	0.6	38
Wheat bran	1.7	0.6	36
De-esterified wheat bran	1.1	0.2	15

In summary, growth of *Aspergillus niger* on plant cell-wall preparations led to the production of a number of feruloyl esterases, which are not detected during growth on monosaccharides. The addition of FA stimulates feruloyl esterase production. The production of a known feruloyl esterase, FaeA, is also stimulated by FA. Xylanase levels in the cultures were repressed by the addition of FA, while fungal growth was not affected.

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Chapter 5

Regulation of the feruloyl esterase (*fae*A) gene from *Aspergillus niger*

R. P. de Vries and J. Visser

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Abstract

Feruloyl esterases can remove aromatic residues (e.g. ferulic acid) from plant cell wall polysaccharides (xylan, pectin) and are essential for their complete degradation. The expression of the feruloyl esterase encoding gene (*fae*A) from *Aspergillus niger* depends on D-xylose, mediated by XlnR, the xylanolytic transcriptional activator, and on a second system that responds to aromatic compounds with a defined ring structure such as ferulic acid and vanillic acid. From the compounds tested, all inducing compounds contain a benzene ring, which is substituted at C_3 with a methoxy group and at C_4 with a hydroxy group, but is not substituted at C_5. Various aliphatic groups may occur at C_1. *fae*A expression on xylose or ferulic acid is repressed in the presence of glucose, mediated by CreA the carbon catabolite repressor protein of *A. niger*. *fae*A expression on combinations of ferulic acid and xylose is higher than on either compound alone, indicative of a physiologically relevant releationship. The different inducing systems enable *A. niger* to produce feruloyl esterase not only during growth on xylan, but also on other ferulic acid containing cell wall polysaccharides such as pectin.

Introduction

Feruloyl esterases are enzymes that release aromatic residues, such as ferulic acid, from plant cell wall polymers (de Vries et al., 1997). Ferulic acid residues in plant cell walls are mainly linked to xylan and pectin and may crosslink cell wall polymers to increase rigidity, inhibit cell elongation, and reduce the biodegradability by micro-organisms (Ishii, 1997). In xylans ferulic acid is attached to arabinose residues, which are linked to the xylan backbone (Saulnier et al., 1995; Wende and Fry, 1997). In pectins, ferulic acid can be attached to galactose or arabinose residues present in the side chains (Ishii, 1997). Ferulic acid can be enzymatically converted into vanillin, a major flavour compound (Falconnier et al., 1994; Lesage-Meessen et al., 1996; Gasson et al., 1998).

Feruloyl esterases have been isolated from a wide range of micro-organisms (Christov and Prior, 1993; Faulds and Williamson, 1994; Kroon et al., 1996; Donaghy and McKay, 1997) when they were cultured on complex substrates such as xylan, pectin, wheat bran or sugarbeet pulp. Most of these enzymes are active on feruloylated oligomers from xylan or pectin, but have little or no activity towards the polymeric substrates. The feruloyl easterase A (FaeA) encoding gene (*fae*A) from *Aspergillus niger* has been cloned and induction of the gene has been studied at the protein level in an highly overproducing transformant (Chapter 3). High FaeA activity in the culture

filtrate was detected when this transformant was grown on medium containing wheat arabinoxylan. Low levels of FaeA activity were detected when *A. niger* was grown on sugarbeet pectin (Chapter 3). Addition of ferulic acid to media containing xylan increased the level of FaeA activity in the culture filtrate (Chapter 4).

Two regulatory systems of *A. niger* affect the expression of genes encoding enzymes involved in the degradation of cell wall polymers. The carbon catabolite repressor protein, CreA, prevents transcription of these genes in the presence of easy metabolisable substrates such as glucose or fructose (Drysdale et al., 1993; Ruijter and Visser, 1997). The xylanolytic transcriptional activator, XlnR (van Peij et al., 1998a), is required for the expression of all xylanolytic genes (including *fae*A) and some cellulolytic genes when *A. niger* is grown on xylose or xylan (van Peij et al., 1998b). These two systems are not sufficient to account for the induction pattern observed for *fae*A (Chapter 3 and 4). Induction on pectin and the enhanced induction on xylan in the presence of ferulic acid suggest a more complex system of regulation. In this paper we have studied the regulation of *fae*A at the molecular level, using *A. niger* strains with mutations in different regulatory systems.

Materials and Methods

Strains

All strains were derived from *A. niger* N400 (=CBS120.49). A low sporulating mutant, N402 (*csp*A1), was used as a wild-type strain in all experiments. Mutations were obtained by UV mutagenesis. The *A. niger cre*A mutant NW200 (*bio*A1, *csp*A1, *cre*Ad4, *pyr*A13::pGW635, *are*A1::pAREG1), with a derepressed phenotype, was selected in an *are*A1 background (Ruijter et al., 1997) and co-transformed with pAREG1 (containing the *A. niger are*A gene; McCabe et al., 1998) and pGW635 (containing the *pyr*A selection marker). *A. niger* NXA1-4 (*argB*13, *cspA*1, *nicA*1, *pyrA*6, UAS(*xlnA*)-*pyrA*, *xlnR*1) has previously been described (van Peij et al., 1998a). No expression of xylanolytic genes was observed on xylose in this loss of function mutant (van Peij et al., 1998b). *Escherichia coli* DH5αF' (BRL, Life Technologies Inc., Gaithersburg, Md.) was used for routine plasmid propagation.

Chemicals

Caffeic acid, cinnamic acid, p-coumaric acid, ferulic acid, 4-hydroxybenzoic acid, 3,4-dimethoxycinnamic acid, protocatechuic acid, sinapic acid, syringic acid, vanillic acid, veratric acid, veratryl alcohol, vanillyl alcohol and anisyl alcohol were obtained from Acros (Oxon, UK). D-Glucose, D-fructose and D-xylose were obtained from Merck (Darmstadt, Germany). L-Arabinose was from Sigma (Zwijndrecht, The Netherlands). All other standard chemicals were either obtained from Sigma or from Merck. 3-4-hydroxy-methoxy-phenyl propionic acid was a gift from Dr. Gary Williamson, Institute of Food Research (Norwich, UK).

Media and culture conditions

Minimal medium (MM) contained per litre: 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, trace elements (Vishniac and Santer, 1957) and 1% (mass/vol) glucose as a carbon source unless

otherwise indicated. For complete medium (CM), MM was supplemented with 0.2% (mass/vol) tryptone, 0.1% (mass/vol) yeast extract, 0.1% (mass/vol) casamino acids and 0.05% (mass/vol) yeast ribonucleic acids. Liquid cultures were inoculated with 10^6 spores/ml and incubated at 30°C in an orbital shaker at 250 rpm. Bacto-agar at 1.5% (mass/vol) was used to solidify the media. For the growth of auxotrophic strains, the necessary supplements were added to the media at the following concentrations: biotine, 4 µg/l; uridine, 200 mg/l; arginine, 200 mg/l; nicotinamide, 1 mg/l.
For all transfer experiments, the strains were pre-grown in CM (2% fructose) for 16 h at 30°C in an orbital shaker at 250 rpm. Mycelium was harvested by suction over a nylon membrane and washed with MM or CM without carbon source. Aliquots (1.5 g, wet weight) of the mycelium were transferred to MM or CM containing different carbon sources and incubated at 30°C for the time indicated. The mycelium was again harvested by suction over a nylon membrane, frozen in liquid nitrogen and stored at -70°C.

DNA manipulations

Standard methods were used for DNA manipulations, such as subcloning, DNA digestions, and plasmid DNA isolation (Sambrook et al., 1989). Sequence analysis was performed on both strands of DNA using either the Cy™5 AutoCycle™ Sequencing kit or the Cy™5 Thermo Sequenase™ Dye Terminator Kit (Pharmacia Biotech, Uppsala, Sweden). The reactions were analysed with an ALFred™ DNA Sequencer (Pharmacia Biotech). Nucleotide sequences were analysed with computer programs based on Devereux *et al*. (1984).

Northern analysis

Mycelium was powdered using a Micro-dismembrator (Braun, Melsungen, Germany). Total RNA was isolated from powdered mycelium using TRIzol Reagent (Life Technologies), according to the supplier's instructions. For northern analysis, 5 µg total RNA was incubated with 3.3 µl 6 M glyoxal, 10 µl DMSO and 2 µl 0.1 M phosphate buffer (pH=7) in a total volume of 20 µl for 1 h at 50°C to denature the RNA. The RNA samples were separated on a 1.5% agarose gel using 0.01 M phosphate buffer (pH=5) and transferred to Hybond-N filters (Amersham, Little Chalfont, UK) by capillary blotting. Filters were hybridised at 42°C in a solution of 50% (vol/vol) formamide, 10% (mass/vol) dextran sulphate, 0.9 M NaCl, 90 mM Na_3-citrate, 0.2% (mass/vol) ficoll, 0.2% (mass/vol) polyvinylpyrolidone, 0.2% (mass/vol) bovine serum albumin, 0.1% (mass/vol) SDS and 100 µg/ml single stranded herring sperm DNA. Washing was performed under similar stringency conditions: 30 mM NaCl, 3 mM Na_3-citrate and 0.5% (mass/vol) SDS at 68°C. A 0.6 kb cDNA fragment of *fae*A, a 0.8 kb *Eco*RV/*Kpn*I fragment of *agu*A, encoding an α-glucuronidase (de Vries et al., 1998) and a 0.7 kb *Eco*RI fragment of the 18S rRNA subunit (Melchers et al., 1994) were used as probes. The *agu*A gene was used to compare the expression of *fae*A to another gene regulated by XlnR and the 18S rRNA probe was used as a RNA loading control.

Results

Identification of putative regulatory boxes in the promoter region of *fae*A

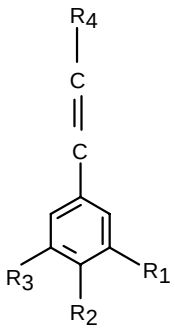
Sequence analysis was performed on the promoter region of *fae*A (EMBL Access. No. Y09330). A consensus sequence for the CreA repressor protein, SYGGRG (with S, Y and R representing C/G, C/T and A/G, respectively; Kulmburg et al., 1993), was identified at position -376 upstream from the ATG in the 3'-5' orientation, and for the xylanolytic

transcriptional activator XlnR, GGCTAA (van Peij et al., 1998a) at positions -265 upstream from the ATG in the 5'-3' orientation. A XlnR-like sequence (GGCTAG) was detected at –225 upstream from the ATG in the 5'-3' orientation.

Expression of *fae*A on aromatic compounds

Mycelia of *A. niger* N402 were harvested 4 h after transfer to 0.03% (mass/vol) of the various aromatic compounds shown in Fig. 1 or 0.03% (mass/vol) fructose.

cinnamic acid:	$R_1=R_2=R_3=H$; $R_4=COOH$
coniferyl alcohol:	$R_1=OCH_3$; $R_2=OH$; $R_3=H$; $R_4=CH_2OH$
p-coumaric acid:	$R_1=R_3=H$; $R_2=H$; $R_4=COOH$
caffeic acid:	$R_1=R_2=OH$; $R_3=H$; $R_4=COOH$
ferulic acid:	$R_1=OCH_3$; $R_2=OH$; $R_3=H$; $R_4=COOH$
sinapic acid:	$R_1=R_3=OCH_3$; $R_2=OH$; $R_4=COOH$
3,4-dimethoxy cinnamic acid:	$R_1=R_2=OCH_3$; $R_3=H$; $R_4=COOH$



anisyl alcohol:	$R_1= R_3=H$; $R_2=OCH_3$; $R_4=CH_2OH$
benzoic acid:	$R_1=R_2=R_3=H$; $R_4=COOH$
protocatechuic acid:	$R_1=R_2=OH$; $R_3=H$; $R_4=COOH$
syringic acid:	$R_1=R_3=OCH_3$; $R_2=OH$; $R_4=COOH$
vanillic acid:	$R_1=OCH_3$; $R_2=OH$; $R_3=H$; $R_4=COOH$
vanillin:	$R_1=OCH_3$; $R_2=OH$; $R_3=H$; $R_4=CHO$
vanillyl alcohol:	$R_1=OCH_3$; $R_2=OH$; $R_3=H$; $R_4=CH_2OH$
veratric acid:	$R_1=R_2=OCH_3$; $R_3=H$; $R_4=COOH$
veratryl alcohol:	$R_1=R_2=OCH_3$; $R_3=H$; $R_4=CH_2OH$
4-hydroxy benzoic acid:	$R_1=R_3=H$; $R_2=H$; $R_4=COOH$
3-methoxy-4-hydroxy-phenylpropionic acid:	$R_1=OCH_3$; $R_2=OH$; $R_3=H$; $R_4=C_2H_4COOH$

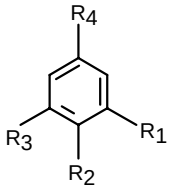


Fig. 1. Aromatic compounds used in this study.

High levels of expression were observed on ferulic acid, vanillic acid, vanillyl alcohol, coniferyl alcohol and 4-hydroxy-3-methoxy-phenyl propionic acid (Fig. 2). Lower expression levels were observed on vanillin, veratric acid and veratryl alcohol. No expression was detected on CM with any of the other aromatic compounds.

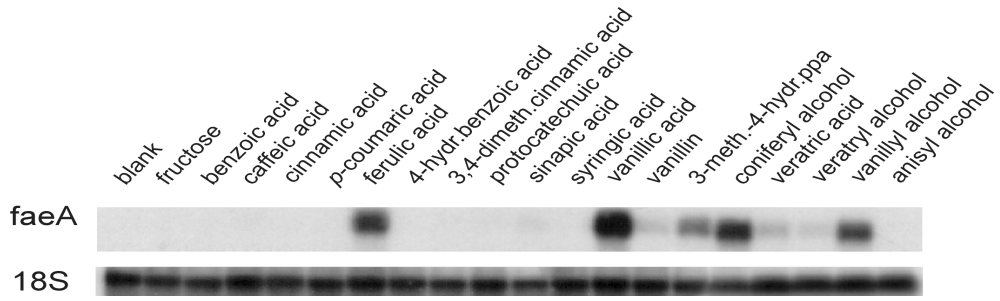


Fig. 2. Expression of *fae*A on aromatic compounds. *A. niger* N402 mycelium was incubated for 4 h in CM (blank), CM containing 0.03% (mass/vol.) fructose and CM containing 0.03% (mass/vol.) of the different aromatic compounds. The 18S rRNA served as a RNA loading control. Abbreviations: hydr. = hydroxy; meth. = methoxy; ppa = phenylpropionic acid.

CreA represses the expression of *fae*A

Mycelia from *A. niger* N402 and NW200 were transferred to MM containing different carbon sources and harvested after 4 h. The *fae*A gene was expressed on xylose, xylan and ferulic acid at similar levels in both strains (Fig. 3). Glucose strongly reduced the expression of *fae*A on xylose in the wild-type strain, but only a small decrease was observed in the CreA mutant. The presence of glucose abolished expression of *fae*A on ferulic acid and xylan in the wild-type strain, but a low level of expression on ferulic acid was detected in the CreA mutant.

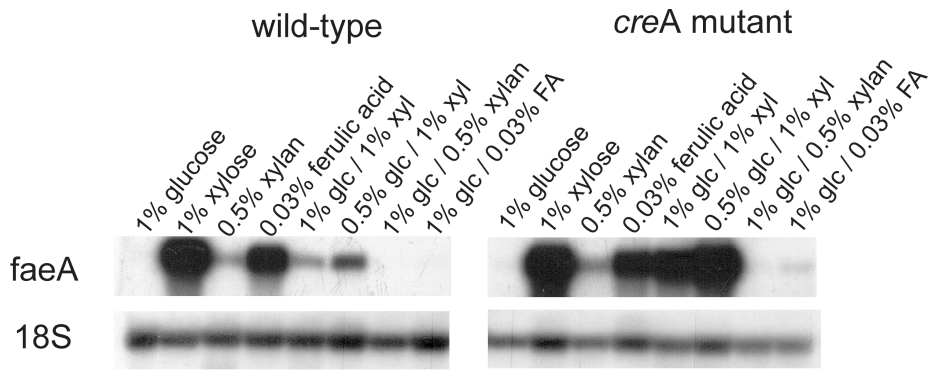


Fig. 3. CreA repression of *fae*A expression. To determine the influence of the carbon catabolite repressor protein CreA on *fae*A expression mycelium from *A. niger* N402 and NW200 (CreA mutant) was incubated for 4 h in MM containing different carbon sources. The 18S rRNA served as a RNA loading control. Abbreviations: glc = glucose; xyl = xylose; FA = ferulic acid.

Interaction between the different systems for induction of *fae*A expression

We compared expression of *fae*A to expression of *agu*A, which encodes an α-glucuronidase, and also is regulated by XlnR (van Peij et al., 1998b) in wild type and an XlnR-deficient mutant 2 h after induction. Expression of both *fae*A and *agu*A was higher on 0.03% xylose

than on 1% xylose in the wild type strain (Fig. 4). Addition of ferulic acid to the carbon sources strongly increased *fae*A expression, but had no significant effect on *agu*A expression. *agu*A expression was not observed on any carbon source in the XlnR mutant, but *fae*A expression was detected in this mutant in the presence of ferulic acid, especially in combination with arabinose.

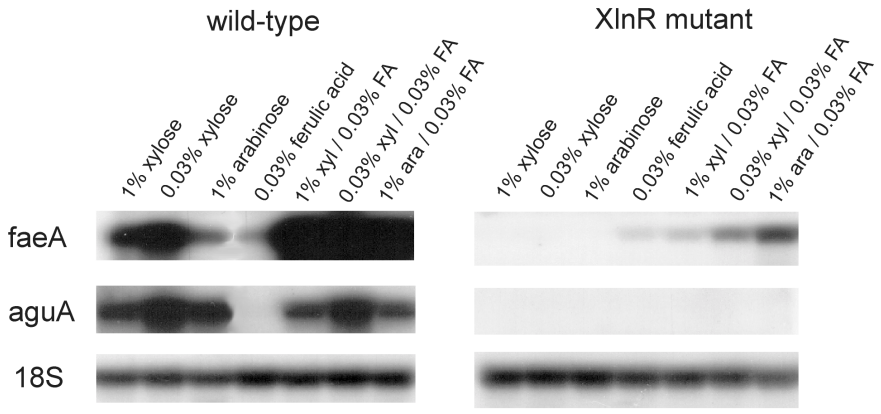


Fig. 4. Interaction of different systems for induction on *fae*A expression. Relations between XlnR and ferulic acid induction of *fae*A expression were studied by incubating mycelium of *A. niger* N402 and NXA1-4 (XlnR mutant) for 2 h in MM different carbon sources. The 18S rRNA served as a RNA loading control. Abbreviations: xyl = xylose; ara = arabinose; FA = ferulic acid.

Discussion

The data presented in this paper reveal that regulation of *fae*A transcription is complex. *faeA* like other genes encoding plant cell-wall degrading enzymes is subject to carbon catabolite repression. Expression of *faeA* on xylose in the presence of glucose was strongly reduced in the *A. niger* wild-type strain, but only slightly in the *cre*Ad4 mutant indicating that CreA regulates the expression of *faeA*. The *cre*Ad4 mutant is not a complete loss of function mutant and the degree of derepression is target and allele specific (unpublished data). This might explain the reduced *faeA* expression on ferulic acid in the *creA* mutant when glucose is present in the medium. The xylose concentration influences the XlnR induced expression levels of xylanolytic genes, which can be observed when comparing the expression of *faeA* and *agu*A on 1% and 0.03% xylose. This phenomenon has been assigned to modulation of gene expression via CreA (de Vries et al., 1999). *faeA* and *aguA* expression on arabinose is most likely due to a small amount of xylose present in the arabinose preparation obtained from Sigma (de Vries et al., 1998). The addition of ferulic acid to xylose or arabinose resulted

in increased expression levels on these carbon sources for *faeA*, but not for *aguA* and for two other xylanolytic genes *xlnB* and *xlnD* (data not shown), indicating that addition of ferulic acid does not influence the overall regulation of the xylanolytic enzyme spectrum.

The *faeA* gene is expressed on aromatic compounds which have common features with respect to their aromatic structure: the aromatic ring is substituted at C_3 with a methoxy group and at C_4 with a hydroxy group, whereas C_5 is not substituted. This indicates the presence of a specific transcriptional activator which responds to these compounds. The nature of the group attached to C_1 of the aromatic ring seems to be of less importance for the expression level. From this group of aromatic compounds, only vanillin results in low *faeA* expression. One explanation for this might be that the aldehyde function on C_1 results in lower induction of *faeA*. Alternatively, the low level of expression may be caused by the strong toxic effect of vanillin on *A. niger*. All aromatic compounds tested so far are to some extent toxic for *A. niger* (Guiraud et al., 1995; López-Malo et al., 1997). Vanillin is the most toxic of the compounds used in this study as tested by plate growth experiments (data not shown). The low levels of expression observed on veratric acid and veratryl alcohol in which both C_3 and C_4 are substituted with a methoxy group, suggest that the regulatory system is also activated to some extent by other aromatic compounds. The regulatory system described here differs from other systems responding to the presence of aromatic compounds. Catabolism of benzoic acid in *A. niger* was demonstrated to occur via 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid to protocatechuic acid (Boschloo et al., 1990). Expression of the gene encoding benzoate-para-hydroxylase (*bph*A), responsible for the first step in the catabolism was induced by benzoic acid (van Gorcom et al., 1990), but induction of the pathway was also reported in the presence of 4-hydroxybenzoic acid (Boschloo et al., 1990). Since *faeA* expression is not induced by benzoic acid or 4-hydroxybenzoic acid, this indicates the presence of at least two independent regulatory systems responding to induction by aromatic compounds in *A. niger*. Milstein *et al.* (1988) suggested a model for the degradation of certain aromatic compounds in *Aspergillus japonicus*. In this model four groups of aromatic compounds can be distinguished with individual catabolic pathways all converging at protocatechuic acid, followed by ring-opening via a common pathway. One group consists of ferulic acid and derived compounds, which are catabolized via vanillic acid. Cinnamic acid, coumaric acid and benzoic acid are catabolized via 4-hydroxybenzoic acid. This suggests the presence of specific regulatory systems for the different branches of aromatic catabolism. Recently, the expression of the *Penicillium simplicissimum vaoA* gene, encoding a vanillyl-alcohol oxidase, has been studied

in *A. niger* on a set of aromatic compounds (Benen et al., 1998). The highest expression of *vaoA* was detected on compounds with an alcohol function on C_1 of the aromatic ring and a methoxy group on C_4. Compounds with a hydroxy group at C_4 did induce *vaoA* expression but only at a low level. This is different from the expression pattern described for *faeA* in this paper, indicating that the expression of *vaoA* involves a different system.

The expression of the *faeA* gene from *A. niger* is influenced by at least three regulatory systems. It is co-expressed with other genes involved in xylan degradation and under the control of the transcriptional activator XlnR. As xylose is not only the inducer of this system but also causes carbon catabolite repression, mediated by the CreA protein, *faeA* expression levels will strictly depend on the inducer concentration. The response of an additional regulatory system to the release of ferulic acid from xylan and pectin might provide a mechanism to make these plant cell wall polymers more rapidly accessible for degradation even at higher xylose concentrations

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Chapter 6

Induction and structural characterisation of FaeA from *A. niger*

R. P. de Vries, C. B. Faulds, F. O. Aliwan, R. W. Pickersgill, J. Benen, and J. Visser

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Abstract

Previously, *fae*A expression was detected on xylose, ferulic acid, xylan, pectin and crude carbon sources, such as wheat bran and sugar beet pulp. A comparison of FaeA activity, when an *A. niger fae*A multicopy transformant was grown on different polymeric and crude carbon sources revealed that the highest FaeA production occurred when wheat bran was used as a carbon source. Crude wheat arabinoxylan, sugar beet pulp and oat spelt xylan also resulted in high levels of FaeA activity. Low levels of FaeA activity were detected when sugar beet or apple pectin was used as a carbon source. Expression of *fae*A was also studied on different monomeric carbon sources and ferulic acid in both a wild-type and an CreA derepressed mutant. Only xylose and arabinose were able to induce *fae*A expression in the absence of ferulic acid. For combinations of ferulic acid with all carbon sources, except galacturonic acid and rhamnose, an increase in expression was observed in the CreA mutant compared to the wild-type.

A model for the 3-dimensional structure of FaeA is proposed, in which the putative lipase-like catalytic triade and three disulfide bridges are visible. Most of the 15 amino acid differences between *A. niger* and *A. tubingensis* FaeA can be found on the outside of the structure, suggesting that they may be responsible for the increased sensitivity of *A. tubingensis* FaeA for proteolytic degradation.

Introduction

Feruloyl esterase A from *Aspergillus niger* has been characterised at the enzymatic and molecular level (Chapters 3, 4 and 5). The *fae*A gene has been cloned and overexpressed (Chapter 3) and induction of the gene has been detected on crude plant cell derived substrates, xylan, sugar beet pectin, ferulic acid and xylose (Chapters 3, 4 and 5). Repression of *fae*A expression of xylose and ferulic acid was observed in the presence of glucose and could be assigned to the carbon catabolite repressor protein CreA (Chapter 5).

The enzyme is active on methylferulate (Chapter 3), feruloyl-arabinofuranoside-xylopyranoside-xylopyranose (FAXX, Faulds and Williamson, 1994), wheat arabinoxylan (Chapter 3 and 4) and sugar beet pectin (Chapter 3). Using oligosaccharides isolated from wheat bran and sugar beet pulp (Ralet et al., 1994) it was shown that FaeA was able to release ferulic acid linked to O-5 of arabinose (as present in xylan), but that it was not able to release ferulic acid linked to O-2 of arabinose (as present in pectin).

Despite high amino acid sequence identity for *A. niger* and *A. tubingensis* FaeA, a remarkable difference in stability was observed for the two proteins (Chapter 3). *A. tubingensis* demonstrated a strongly increased sensitivity to proteolytic degradation resulting in multiple discrete degradation bands on a SDS-PAGE gel.

This chapter provides additional data on the induction of *fae*A expression on polymeric substrates and a more detailed analysis on the relation of ferulic acid induced *fae*A expression with different monomeric carbon sources and CreA. Furthermore, it presents the 3-dimensional model which is proposed for *A. niger* FaeA and possible implications of this model in the difference in stability observed for *A. niger* and *A. tubingensis* FaeA.

Materials and Methods

Strains and growth conditions

Strain NW154::pIM3207.7 (*csp*A1, *pyr*A6::pGW635, *prt*F28) is a transformant containing multiple copies of the *A. niger fae*A gene (Chapter 3). The *prt*F mutation present in this strain was previously described (van den Hombergh et al., 1995). The *A. niger* CreA mutant NW200 (*bio*A1, *csp*A1, *cre*Ad4, *pyr*A13::pGW635, *are*A1::pAREG1) showing a derepressed phenotype was selected in an *are*A1 background (Ruijter et al., 1997) and subsequently co-transformed with pAREG1 (containing the *A. niger are*A gene; McCabe et al., 1998) and pGW635 (containing the *pyr*A selection marker).

Minimal medium (MM) contained per litre: 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, trace elements (Vishniac and Santer, 1957) and 1% (mass/vol) glucose as a carbon source unless otherwise indicated. For complete medium (CM), MM was supplemented with 0.2% (mass/vol) tryptone, 0.1% (mass/vol) yeast extract, 0.1% (mass/vol) casamino acids and 0.05% (mass/vol) yeast ribonucleic acids.

All cultures were inoculated at 10^6 spores/ml and grown in MM in a rotary shaker at 250 rpm and 30°C, unless otherwise indicated. For the growth of strains with auxotrophic mutations, the corresponding supplements were added to the medium. Transfer experiments were performed as described previously (Chapter 5).

Enzyme assay

Ferulic acid esterase activities in culture filtrate samples were measured by using methyl ferulate as a substrate as previously described (Chapter 3).

Determination of N-terminal amino acid sequences

Amino acid sequences were determined from electroblots of an SDS-PAGE separation of purified *A. tubingensis* FaeA on an Applied Biosystems Procise Sequencer.

Modelling of FaeA

The *A. niger* FaeA model was generated using the program MODELLER (Sali and Blundell, 1993) with four aligned lipases giving the input protein scaffold and the sequence alignment giving the relationship between this scaffold and the *A. niger* FaeA sequence. The molecules were displayed on a Silicon Graphics Indigo 2 with program O (Jones et al., 1991) and the protein cartoon was generated using Insight II '98 (Molecular Dynamics, Inc).

Results and Discussion

Induction of *fae*A on polymeric substrates

Using an *A. niger* multicopy *fae*A transformant (NW156::pIM3207.7; Chapter 3) induction of *fae*A on polymeric substrates was studied using Fae activity measurements. The highest activity was measured in cultures containing wheat bran (Table 1), which is consistent with previous experiments (Chapter 4). Cultures containing sugar beet pulp result in higher extracellular Fae activities than cultures containing a crude wheat arabinoxylan preparation or oat spelt xylan. Low levels of Fae activity were detected in cultures containing sugar beet or apple pectin. The higher activity on wheat bran, sugar beet pulp and xylan is in part due to the presence of xylose, which induces xylanolytic genes via the xylanolytic activator XlnR (van Peij et al, 1998a). The *fae*A gene is regulated by XlnR (van Peij et al., 1998b) and is also induced by ferulic acid (Chapter 4). A positive interaction between ferulic acid induction and XlnR regulation has been demonstrated (Chapter 5). In the crude substrates (wheat bran, sugar beet pulp, wheat arabinoxylan) both xylose and ferulic acid are present resulting in a high induction of *fae*A expression. Since both pectins contain low amounts of ferulic acid (data not shown) this could mean that on pectin induction occurs only through ferulic acid. Alternatively, an inducing component from pectin may not be able to induce *fae*A to the same extent as xylose, resulting in lower Fae levels. *A. niger* produces several feruloyl esterases (Brezillion et al., 1996). However, it is unlikely that the activity measured in the pectin cultures should be assigned to another enzyme than FaeA, since cultures using an *A. niger* wild-type strain and apple or sugar beet pectin as substrate resulted in lower levels of Fae activity (data not shown).

Table 1: Induction of *Aspergillus niger* ferulic acid esterase A on polymeric substrates.
All substrates were added at the amount of 1% (mass/vol). Fae activity is defined as the amount of ferulic acid produced per min per ml supernatant (μmoles×min^{-1}×ml^{-1}).

substrate	activity
wheat bran	4.58
crude wheat arabinoxylan	3.08
oat spelt xylan	2.05
sugarbeet pulp	3.51
sugarbeet pectin	0.19
apple pectin	0.16

Influence of the carbon source on ferulic acid induction of *fae*A expression

A transfer experiment was performed using an *A. niger* wild-type (N402) and a CreA derepressed mutant (NW200) to study the effect of the carbon source on the ferulic acid induced expression of *fae*A. Both strains were grown for 16 h in CM containing 2% (mass/vol) fructose after which the mycelium was harvested, washed and aliquots were transferred to MM with different carbon sources. Mycelium samples were harvested after 4 h and a northern analysis was performed on RNA isolated from the samples. In the absence of ferulic acid, *fae*A expression was only observed on xylose and arabinose (Fig. 1). The expression on arabinose is probably due to the presence of a low amount of xylose present in the arabinose preparation obtained from Sigma (Chapter 7). Expression of *fae*A was observed on combinations of all carbon sources except galacturonic acid with ferulic acid in the CreA mutant, but at different levels. For all of these carbon sources except rhamnose, reduced levels of expression were observed in the wild-type.

The differences observed could be caused by several factors. Growth of *A. niger* is poor on some of the carbon sources tested in this study (e.g. galacturonic acid) which may lead to a reduction in expression of *fae*A. Secondly, the CreA mutant is not a loss of a function mutation but has a derepressed phenotype. A low level of CreA repression is still present in this strain. The presence of a strong repressing carbon source (e.g. glucose) could therefore have a more significant effect in this background than carbon sources resulting in weaker repression. Although other carbon sources than glucose and fructose have been demonstrated to result in CreA mediated gene expression (e.g. xylose, Chapter 9), little is known about the extent of the CreA effect of the different monomeric sugars. From the data presented here it

can be concluded that nearly all monomeric carbon sources tested are to some extent able to repress gene expression of *fae*A mediated via CreA. Only for the combination of rhamnose with ferulic acid, no difference in *fae*A expression was observed between the wild-type and the CreA mutant.

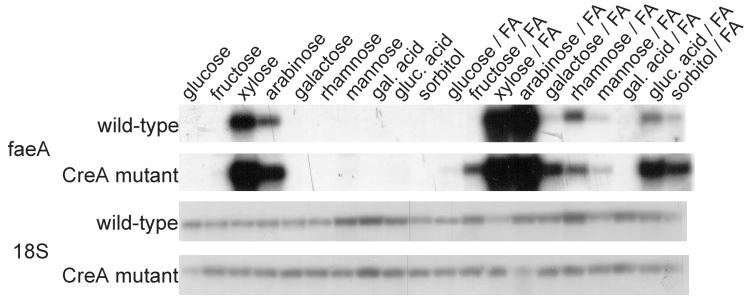

Fig. 1. Expression of *fae*A on 1% (mass/vol) monomeric carbon sources alone and in the presence of 0.03% (mass/vol) ferulic acid. The 18S probe (Melchers et al., 1994) was used as a RNA loading control. Abbreviations: gal. acid, galacturonic acid; gluc. acid, glucuronic acid; FA, ferulic acid.

Putative structure and stability of FaeA from *A. niger* and *A. tubingensis*

Based on the amino acid sequence of *A. niger* FaeA and its homology to four fungal lipases, a model for the 3-dimensional structure of this enzyme was designed (Fig. 2). Three disulfide bridges can be detected in the model (indicated in black). The three amino acids possibly forming the active site, S^{133}, D^{194} and H^{247} (Chapter 3) are also indicated. When comparing the amino acid sequences of FaeA from *A. niger* and *A. tubingensis*, 15 amino acid differences and 1 amino acid deletion (in *A. tubingensis* FaeA) can be detected (Fig. 4). Only one of the amino acid changes and the deletion are located in the core of the enzyme. All other amino acid changes are on the outside of the enzyme structure.

The stability of *A. niger* FaeA compared to *A. tubingensis* FaeA as established by SDS-PAGE is much higher (Chapter 3). Since discrete degradation products are visible, the reduced stability *of A. tubingensis* FaeA is most likely due to an increased sensitivity to specific proteases. However, when the two proteins are kept under non-denaturing conditions, a single protein is present with the molecular mass of mature FaeA as determined by massspectrometry (*A. niger* FaeA: 29724 Da and *A. tubingensis* FaeA: 29756 Da). This suggests that, although *A. tubingensis* FaeA is partially digested by proteases, the disulfide bridges are capable of maintaining the structural integrity of the enzyme.

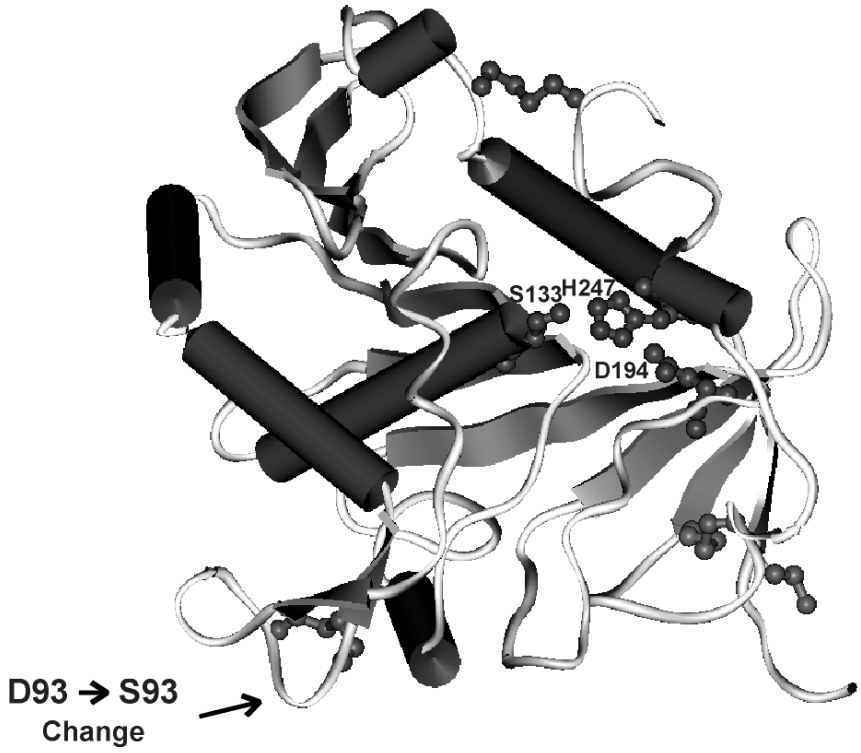

Fig. 2. Suggested model for the 3-dimensional structure of FaeA from *A. niger*. The putative active side residues (S133, D194, H247) and di-sulfide bridges are indicated by stick and ball structures (Based on Faulds et al., 1998).

The first few residues of three degradation products from *A. tubingensis* FaeA have been N-terminally sequenced. The largest product (26 kDa) had an identical sequence as the mature FaeA (A S T Q C), indicating that this product originates from C-terminal truncation of the mature protein. A well separated band of 22 kDa contained two proteins, which were both N-terminally sequenced. One of the two proteins again had an identical N-terminal amino acid sequence as the mature enzyme, indicating a further C-terminal truncation. The N-terminal sequence of the other protein (X E V H) corresponds to a sequence 95 residues from the N-terminus. In the amino acid sequence of FaeA X corresponds to a cysteine which is involved in a disulfide bridge. The degradation product starts immediately after one of the amino acid changes between the two enzymes: *A. niger* (**D** ♦C E V H) and *A. tubingensis* (**S** ♦C E V H). Thus, it appears that an aspartic acid residue instead of a serine at position 93 may contribute to the proteolytic stability of FaeA. This residue is located on the outside of the 3-dimensional model (Fig. 2), which would make it easily accessible for proteases. Whether the other degradation products of *A. tubingensis* FaeA are also caused by amino acid changes compared

to *A. niger* FaeA is not clear at this point. The position of most of these residues on the outside of the 3-dimensional structure makes them highly accessible for proteases, suggesting that some of these residues may be involved in the decrease in stability of *A. tubingensis* FaeA compared to *A. niger* FaeA.

```
A. nig.   ASTQG ISEDL YNRLV EMATI SQAAY ADLCN IPSTI IKGEK IYNAQ TDING  50
A. tub.   ASTQG ISEDL YSRLV EMATI SQAAY ADLCN IPSTI IKGEK IYNSQ TDING  50
                     *                                       *

A. nig.   WILRD DTSKE IITVF RGTGS DTNLQ LDTNY YTLTP FDTLP QCNDC EVHGG 100
A. tub.   WILRD DSSKE IITVF RGTGS DTNLQ LDTNY YTLTP FDTLP QCNSC EVHGG 100
               *                                         *

A. nig.   YYIGW ISVQD QVESL VKQQA SQYPD YALTV TGHSL GASMA ALTAA QLSAT 150
A. tub.   YYIGW ISVQD QVESL VQQQV SQFPD YALTV TGHSL GASLA ALTAA QLSAT 150
                          *  *   *                 *

A. nig.   YDNVR LYTFG EPRSG NQAFA SYMND AFQVS SPETT QYFRV THSND GIPNL 200
A. tub.   YDNIR LYTFG EPRS- NQAFA SYMND AFQAS SPDTT QYFRV THAND GIPNL 199
             *            *                  *    *           *

A. nig.   PPADE GYAHG GVEYW SVDPY SAQNT FVCTG DEVQC CEAQG GQGVN DAHTT 250
A. tub.   PPADE GYAHG VVEYW SVDPY SAQNT FVCTG DEVQC CEAQG GQGVN NAHTT 249
                     *                                          *

A. nig.   YFGMT SGACTW -261
A. tub.   YFGMT SGHCTW -260
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Fig. 3. Alignment of the deduced amino acid sequence of mature FaeA from *A. niger* (*A. nig*.) and *A. tubingensis* (*A. tub*.). Putative active site residues are indicated in boldface underlined. Sequence differences are indicated by an asterix below the sequence.

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Chapter 7

*agu*A, the gene encoding an extracellular α-glucuronidase from *Aspergillus tubingensis*, is specifically induced on xylose and not on glucuronic acid

R. P. de Vries, C. H. Poulsen, S. Madrid, and J. Visser

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Abstract

An extracellular α-glucuronidase was purified and characterised from a commercial *Aspergillus* preparation and from culture filtrate of *Aspergillus tubingensis*. The enzyme has a molecular mass of 107 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 112 kDa as determined by mass spectrometry, has a determined pI just below 5.2, and is stable at pH 6.0 for prolonged times. The pH optimum for the enzyme is between 4.5 and 6.0, and the temperature optimum is 70°C. The α-glucuronidase is active mainly on small substituted xylo-oligomers but is also able to release a small amount of 4-*O*-methylglucuronic acid from birchwood xylan. The enzyme acts synergistically with endoxylanases and β-xylosidase in the hydrolysis of xylan. The enzyme is N glycosylated and contains 14 putative N-glycosylation sites. The gene encoding this α-glucuronidase (*agu*A) was cloned from *A. tubingensis*. It consists of an open reading frame of 2,523 bp and contains no introns. The gene codes for a protein of 841 amino acids, containing an eukaryotic signal sequence of 20 amino acids. The mature protein has a predicted molecular mass of 91,790 Da and a calculated pI of 5.18. Multiple copies of the gene were introduced in *A. tubingensis*, and expression was studied in a highly overproducing transformant. The *agu*A gene was expressed on xylose, xylobiose, and xylan, similarly to genes encoding endoxylanases, suggesting a co-ordinate regulation of expression of xylanases and α-glucuronidase. Glucuronic acid did not induce the expression of *agu*A and also did not modulate the expression on xylose. Addition of glucose prevented expression of *agu*A on xylan but only reduced the expression on xylose.

Introduction

Xylan is the most abundant hemicellulose structure present in plant cell walls. It consists of a β-1,4-linked backbone of xylose residues which can be substituted with a number of different functions such as acetyl, arabinosyl, ferulic acid and 4-*O*-methyl-α-glucuronic acid residues. To ensure cell wall rigidity, xylan is linked to other cell wall polymers, such as pectin and lignin. Two residues attached to xylan are involved in these linkages. Ferulic acid, connected to the xylan backbone through arabinose, can form a covalent linkage with other phenolic acid residues present in pectin or lignin (Iiyama et al., 1994; Ishii, 1991; Ralph et al., 1995). The other residue so far identified to be involved in cross-linking cell wall polymers is 4-*O*-

methyl-glucuronic acid. Indications for an ester linkage between lignin and glucuronoxylan through 4-*O*-methyl-glucuronic acid have been found in beech wood (Takahashi and Koshijima, 1988). Calculations indicated that approximately one third of the glucuronic acid residues attached to the xylan backbone are involved in this linkage.

Many bacteria and fungi are capable of degrading polymeric structures from plant cell walls by producing a large number of enzymes which specifically cleave certain linkages in these polymers. Endoxylanases (EC 3.2.1.8) cleave the xylan backbone, whereas β-xylosidase (EC 3.2.1.37) cleaves off xylose monomers from the non-reducing end of xylo-oligomers. To remove the side groups from the xylose backbone, arabinofuranosidases (EC 3.2.1.55), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases, and α-glucuronidases (EC 3.2.1.139) are needed. A complex synergy exists between these enzymes, resulting in an efficient degradation of the xylan polymer. α-Glucuronidase releases 4-O-methyl-α-glucuronic acid from xylan. Although many organisms have been reported to produce extracellular α-glucuronidases (Bronnenmeier et al., 1995; Ishihara et al., 1990; Khandke et al., 1989; Puls et al., 1987; Shao et al., 1995; Siika-aho et al., 1994), for *Aspergillus* only two intracellular α-glucuronidases have been described (Uchida et al., 1992). These intracellular α-glucuronidases have slightly different properties than extracellular α-glucuronidases from other fungi (Bronnenmeier et al., 1995; Khandke et al., 1989; Shao et al., 1995; Siika-aho et al., 1994), which all have molecular masses between 90 and 130 kDa and a slightly acidic pI and which are active mainly on small xylo-oligomers. So far, the molecular structure of α-glucuronidase encoding genes has been described for only two organisms. An activity screening of *Trichoderma reesei* cDNA clones resulted in the isolation of a clone which contained the α-glucuronidase encoding gene (Margolles-Clark et al., 1996). A gene encoding α-glucuronidase was also isolated from the hyperthermophilic bacterium *Thermotoga maritima* (Ruile et al., 1997).

We have purified an extracellular α-glucuronidase from *Aspergillus tubingensis* and, using reverse genetics, cloned the corresponding gene.

Materials and methods

Strains, libraries, and plasmids

The *A. tubingensis* strains used were NW756 and NW241 (*pyr*A2 *fwn*A1). *Escherichia coli* DH5αF' (BRL, Life Technologies Inc., Gaithersburg, Md.) was used for routine plasmid propagation. *E. coli*

LE392 was used as a host for phage λ. pBluescript was used for subcloning. The genomic library from *A. tubingensis* was previously described (de Graaff et al., 1994).

Media and culture conditions

Minimal medium (MM) contained the following (per litre): 6.0 g of $NaNO_3$, 1.5 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4$ and trace elements (Vishniac and Santer, 1957) and 1% (wt/vol) glucose as a carbon source unless otherwise indicated. For complete medium (CM), MM was supplemented with 0.2% (wt/vol) tryptone, 0.1% (wt/vol) yeast extract, 0.1% (wt/vol) casamino acids and 0.05% (wt/vol) yeast ribonucleic acids. Liquid cultures were inoculated with 10^6 spores/ml and incubated at 30°C in an orbital shaker at 250 rpm. Agar at 1.5% (wt/vol) was added for solid medium. For the growth of strains with auxotrophic mutations, the necessary supplements were added to the medium.

Chemicals

D-Glucuronic acid was obtained from Fluka (Buchs, Switzerland). p-Nitrophenol-β-D-xylopyranoside, D-xylose, D-glucose, L-arabinose, 3,5-dimethoxy-4-hydroxycinnamic acid, and birchwood xylan were obtained from Sigma (St.Louis, Mo.). Aldotriouronic acid, xylo-oligosaccharides and Xylazyme tablets were obtained from Megazyme International (Dublin, Ireland). Endoproteinase Lys-C and bovine serum albumin were from Boehringer (Mannheim, Germany). N-Glycosidase F was from Oxford GlycoSystems (Oxon, United Kingdom). *Taq*-polymerase, Q-Sepharose FF, Phenyl Sepharose FF, Superdex 200 PG, Butyl Sepharose FF, protein molecular weight markers, and the fast protein liquid chromatography Mono Q HR 5/5 and Superose 6 HR 10/30 columns were purchased from Pharmacia (Uppsala, Sweden). Poros 10 HQ medium was obtained from PerSeptive Biosystems (Cambridge, Mass.). Sumizyme AC was obtained from Sumitomo (Osaka, Japan). A PA 100 column was obtained from DionexCorp (Sunnyvale, Calif.).

α-Glucuronidase assay

The incubation mixture for the α-glucuronidase assay (total volume, 0.2 ml) contained 0.16 ml of substrate (2 mg/ml of aldotriouronic acid-aldobiuronic acid [80:20] in 0.05 M sodium acetate buffer [pH 5.0] and 0.04 ml of enzyme solution to be assayed. The incubation was started by addition of the enzyme. After 30 min of incubation at 40°C, the reaction was stopped by boiling the samples for 4 min. Precipitates were removed by centrifugation (10.000 × *g*) after which the supernatant was transferred to a new tube. To each tube, 0.6 ml of copper reagent prepared as described by Milner and Avigad (1967) was added, and then the sample was boiled for 10 min and cooled on ice. Subsequently, 0.4 ml of arsenomolybdate reagent prepared as described by Nelson (1944) was added. The samples were mixed gently, 0.8 ml of H_2O was added, and the absorbance at 600 nm was measured against H_2O. Controls were prepared by boiling a complete assay mixture at time zero, before incubation at 40°C. A substrate control was made by adding water instead of enzyme solution. A standard curve was prepared by using D-glucuronic acid. One α-glucuronidase unit is the amount of enzyme liberating 1 μmol of glucuronic or 4-*O*-methylglucuronic acid per min under standard assay conditions.

β-Xylosidase assay

The β-xylosidase assay mixture contained 600 μl of substrate (5.5 mg of p-nitrophenyl-β-D-xylopyranoside in 6 ml 50 mM sodium acetate, pH 4.2) and 100 μl of purified β-xylosidase. The assay mixture was incubated at 40°C. At 0, 7, 15 and 22 min, a 100-μl sample was removed and added to 600 μl of stop reagent (0.13 M Na_2CO_3), after which the absorbance at 405 nm was measured. A

substrate blank was prepared by adding water instead of enzyme solution. One β-xylosidase unit is the amount of enzyme which liberates 1 μmol of xylose per min at 40°C.

Endoxylanase assay

Xylanase activity was determined by the amount of blue colour liberated from azurine-dyed cross-linked birchwood xylan (Xylazyme tablets) under conditions recommended by the manufacturer.

HPLC analysis of monomeric and oligomeric residues from xylan

A 2.5-ml solution of birchwood xylan (0.5%) in 50 mM sodium acetate (pH 5.0) was incubated with 0.45 U of purified α-glucuronidase, 7.0 mU of purified xylanase A from *A. tubingensis*, 5.8 mU of xylanase complex (Sumizyme AC), and 0.48 U of purified β-xylosidase. The four enzymes were incubated alone and in all possible combinations in a total volume of 3.25 ml for 3 h at 45°C. Boiling the samples for 3 min stopped the incubation. The samples were analysed on a Dionex high-performance liquid chromatography system equipped with a Dionex PA 100 column an a pulsed electrochemical detector with a pH reference electrode. Elution was carried out with a 12 min linear gradient from 0.02 to 0.05 M followed by a 33 min linear gradient from 0.05 to 0.12 mM sodium acetate in 0.1 M NaOH at a flow rate of 1 ml/min.

Protein determination

During the α-glucuronidase purification, the protein concentrations were determined by measuring the absorbance at 280 nm. Protein concentrations in the pooled samples were determined in microtiter plates by a sensitive method (Bradford, 1976) performed according to instructions given by Bio-Rad (Bio-Rad Bulletin 1177 EG). Bovine serum albumin was used as a standard.

PAGE and Western analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), native gel electrophoresis, and isoelectric focusing were carried out by using the Novex (San Diego, Calif.) system with precast gels. Both electrophoresis and silver staining of the gels were done according to the instructions of the manufacturer. Western analysis of supernatant samples from *A. tubingensis* cultures was performed with polyclonal antibodies raised in mice against purified α-glucuronidase A (AguA) from *A. tubingensis*.

Purification

α-Glucuronidase was isolated from a commercial enzyme preparation, Pektinase 146 (Danisco Ingredients, Brabrand, Denmark), derived from *Aspergillus niger* and from culture filtrate of *A. tubingensis* NW241::pIM3212.8. All procedures were performed at room temperature.

$(NH_4)_2SO_4$ was added to 200 ml of Pektinase 146 or 20 ml of *A. tubingensis* culture filtrate to 45% saturation. After 30 min of stirring, the precipitated protein was recovered by centrifugation for 20 min at 11,000 × *g*. The pellet was solubilised in 120 ml of Phenyl Sepharose buffer consisting of 20 mM sodium acetate (pH 5.0) and 1.5 M $(NH_4)_2SO_4$. The sample was applied to a 155-ml Phenyl Sepharose FF column equilibrated in Phenyl Sepharose buffer. α-Glucuronidase was eluted with a 1,320 ml linear gradient from 1.5 to 0 M $(NH_4)_2SO_4$ in 20 mM sodium acetate (pH 5.0) with a flowrate of 4 ml/min during which 12 ml fractions were collected. Fractions 60 to 101 (500 ml) were pooled, concentrated, and desalted in Q-Sepharose buffer (20 mM triethanolamine, pH 7.3) by ultrafiltration in an Amicon 8400 unit equipped with a 10-kDa membrane. The resulting sample was applied to a 106-ml Q-Sepharose FF column equilibrated with Q-Sepharose buffer. After the column was washed with 240 ml of Q-Sepharose buffer, the α-glucuronidase was eluted with a 420 ml linear

gradient from 0 to 0.4 M sodium chloride in Q-Sepharose buffer at a flowrate of 3 ml/min, during which 7.5 ml fractions were collected. Fractions 23 to 36 (105 ml) were pooled and concentrated by ultrafiltration. The concentrated sample (7 ml) was loaded onto a Superdex 200 PG column (180 ml) equilibrated in 20 mM sodium acetate (pH 5.0)-0.1 M sodium chloride. α-Glucuronidase was eluted from the column with a flowrate of 1 ml/min, during which fractions of 2 ml were collected. Fractions 22 to 41 were pooled (18 ml), concentrated, and desalted. This sample was separated on a Mono Q HR 5/5 column in six runs with 20 mM triethanolamine buffer (pH 7.3). The column was washed with an 18 ml sodium chloride gradient from 0 to 0.1 M, and then α-glucuronidase was eluted at a constant concentration of 0.1 M sodium chloride in the same buffer at a flowrate of 1.5 ml/min, during which fractions of 0.75 ml were collected. The α-glucuronidase containing fractions were pooled (27 ml), and $(NH_4)_2SO_4$ was added to a final concentration of 1.5 M. This sample was loaded on a 30 ml Butyl Sepharose FF column equilibrated with Phenyl Sepharose buffer. After the column was washed with 50 ml of this buffer, the α-glucuronidase was eluted with a 160 ml linear gradient from 1.5 to 0 M $(NH_4)_2SO_4$ in Phenyl Sepharose buffer at a flowrate of 2 ml/min, during which 4-ml fractions were collected. Fractions 22 to 26 (20 ml) were pooled, concentrated, and desalted as described above. A final purification was achieved by loading the sample on a 4 ml Poros 10 HQ column equilibrated in Q-Sepharose buffer; 5 ml was loaded per run. Elution was performed with a 22-ml linear gradient of sodium chloride from 0 to 0.3 M in Q-Sepharose buffer at a flowrate of 2 ml/min. Fractions of 1 ml were collected and screened for α-glucuronidase activity.

Determination of N-terminal and internal peptide sequences of AguA

The purified freeze-dried enzyme (100 μg) was dissolved in 50 μl of a solution containing 8 M urea and 0.4 M NH_4HCO_3 (pH 8.4). After the solution was flushed with N_2, 5 μl of 45 mM dithiothreitol was added, and the protein was denatured and reduced for 15 min at 50°C under N_2. After the solution had cooled to room temperature, 5 μl of 100 mM iodoacetamide was added for the cysteines to be derivatised for 15 min at room temperature in the dark under N_2. Subsequently, 135 μl of water and 5 μg of endoproteinase Lys-C in 5 μl of water were added and the sample was incubated at 37°C under N_2 for 24 h. The resulting peptides were separated by reverse phase HPLC on a VYDAC C18 column (0.46 by 15 cm; 10 μm; The Separation Group, Hesparia, Calif.) using solvent A (0.1% trifluoroacetic [TFA] in water) and solvent B (0.1% TFA in acetonitrile). Selected peptides were re-chromatographed on a Develosil C18 column (0.46 by 10 cm, Novo Nordisk, Bagsværd, Denmark) with the same solvent system, prior to N-terminal sequencing. Sequencing was done on a 476A sequencer using pulsed-liquid fast cycles according to the instructions of the manufacturers (Applied Biosystems, Foster City, Calif.). For direct N-terminal sequencing, the purified protein was passed through a Brownlee C2 Aquapore column (0.46 by 3 cm, 7 μm, Applied Biosystems) with the same solvent system as above. N-terminal sequencing was performed as described above.

Deglycosylation

Deglycosylation of the pure α-glucuronidase was performed with N-Glycosidase F (Oxford Glycosystems, Oxon, UK) according to the procedure recommended by the manufacturer, with denaturation of the protein before addition of the N-Glycosidase F.

Characterisation of the α-glucuronidase

The molecular masses of the native and the recombinant α-glucuronidase were determined by gel permeation chromatography on a Superose 6 column at a flow rate of 0.5 ml/min with 20 mM

triethanolamine (pH 7.3) as the eluent and RNase A (13.7 kDa), ovalbumin (43 kDa), aldolase (158 kDa), and catalase (232 kDa) as size standards.
The optimum temperature was measured using the assay described above, with incubation for 10 min in 0.05 M sodium acetate buffer (pH 5.0) at different temperatures. The optimum pH was determined by using 0.1 M sodium acetate in a pH range from 3.5 to 6.7; pH values were determined for the assay tubes at room temperature. Temperature stability was determined by incubating 200 µl of purified α-glucuronidase in 50 mM sodium acetate buffer (pH 5.0) at different temperatures for 20 h, after which the α-glucuronidase activity was determined as described above. pH stability was determined by incubating 150 µl of purified α-glucuronidase in 500 µl of 0.2 M sodium acetate (pH 4.0)-0.2 M bis-tris (pH 6.0) or 0.2 M Tris (pH 8.0) for 3, 10, 13, 28, and 62 days at room temperature. Residual activities were measured as described above.

Determination of the molecular mass using mass spectrometry
Samples containing 10 µl of native and recombinant α-glucuronidase were mixed with 1 µl of 10% acetonitrile and desalted for 2 h at room temperature by use of VSWP013 filters (Millipore). MALDI/TOF mass spectrometry was performed with a Voyager Biospectrometry Work Station (Perseptive Biosystems). Samples were prepared by mixing 1 µl of desalted proteins and 2 µl of matrix solution (saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid in 60% acetonitrile with 0.1% TFA). A 1 µl sample of the mixture was spotted into a well of the MALDI sample plate and allowed to air dry prior to introduction into the mass spectrometer. Data for 100 3-ns laser pulses were averaged for each spectrum, and linear, positive ion TOF detection was performed with an accelerating voltage of 20,000V. Spectra were smoothed with a 19-point Savitzky-Golay filter.

PCR cloning of a specific fragment of the *agu*A gene
Several degenerate oligonucleotides were designed and synthesised on an Applied Biosystems 392 DNA Synthesiser. PCRs were performed with a Biometra Personal Cycler using these oligonucleotides at 55°C and chromosomal DNA from *A. tubingensis* NW756. A PCR using oligonucleotides 5 and 9 (5'-GGNCCNATHGAYTTYCARGT-3' and 5'-ARRTCRTARTTNACNCC-3', with H, Y, and R representing A/C/T, C/T, and A/G, respectively) resulted in a fragment of 1,142 bp which was cloned into the pGEM-T vector system (Promega). Sequence analysis was performed as described below.

Isolation, cloning, and characterisation of the *agu*A gene
Plaque hybridisation using nylon replicas was performed as described by Benton & Davis (1977). Hybridizations were performed overnight at 65°C using the PCR fragment as a probe. The filters were washed with SSC/SDS (final concentrations, 0.2 ×and 0.5%, 1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.6]). Positive plaques, identified on duplicate replicas after autoradiography, were recovered from the original plates and purified by re-screening at a low plaque density. Standard methods were used for other DNA manipulations, such as Southern and Northern analysis, subcloning, DNA digestions, and λ phage and plasmid DNA isolations (Sambrook et al., 1989). Chromosomal DNA was isolated as previously described (de Graaff et al., 1988). Sequence analysis was performed on both strands of DNA with a Sequenase DNA sequencing kit (United States Biochemical Corporation, Cleveland, Ohio) and a ^{T7}Sequencing Kit (Pharmacia LKB), using additional oligonucleotides. Nucleotide and amino acid sequences were analysed with the computer program of Devereux et al. (1984). *Aspergillus* transformations were performed as described by Kusters-van Someren et al. (1991).

Nucleotide sequence accession number

The EMBL accession numbers for *agu*A from *A. tubingensis* is Y15405.

Results

Purification of α-glucuronidase

α-Glucuronidase was purified as described in Materials and Methods. A summary of the purification from the Pektinase 146 preparation is shown in Table 1. Throughout the purification α-glucuronidase always eluted as a single peak. The enzyme was purified 371-fold, with a yield of 5.8%. SDS-PAGE patterns showing the different steps of the purification are given in Fig. 1. The purified α-glucuronidase had no β-xylosidase activity or endoxylanase activity.

Table 1. Purification of the *A. niger* α-glucuronidase.

Fraction	Protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg of protein)	Purification factor	Yield (%)
Pektinase 146	2,740	380	0.14	1	100
45% $(NH_4)_2SO_4$	1,899	300	0.16	1.1	79
Phenyl Sepharose	619	195	0.31	2.2	51
Q-Sepharose	88	162	1.83	13.1	42
Superdex 200 PG	57	102	1.80	12.9	27
Mono Q	11	69	6.20	44.3	18
Butyl Sepharose	2.2	54	24.3	175	14.2
Poros 10 HQ	0.42	22	52.3	371	5.8

Enzyme properties

The apparent molecular mass of α-glucuronidase was 107,000 Da as determined by SDS-PAGE (Fig 1) and 100,000 Da as determined by gel filtration, indicating that the native enzyme consists of a single peptide chain. For the recombinant enzyme an apparent molecular mass of 115 kDa was determined by SDS-PAGE. After N deglycosylation, a molecular mass of 95,000 Da was observed for both the native and the recombinant enzyme by SDS-PAGE. The molecular mass was also determined by mass spectrometry resulting in values of 112,079 Da for the native enzyme and 116,488 for the recombinant enzyme.

The isoelectric point for the α-glucuronidase was just below pH 5.2. The pH and temperature optima were 4.5 to 6 and 70°C, respectively. At pH 6.0, the α-glucuronidase was completely

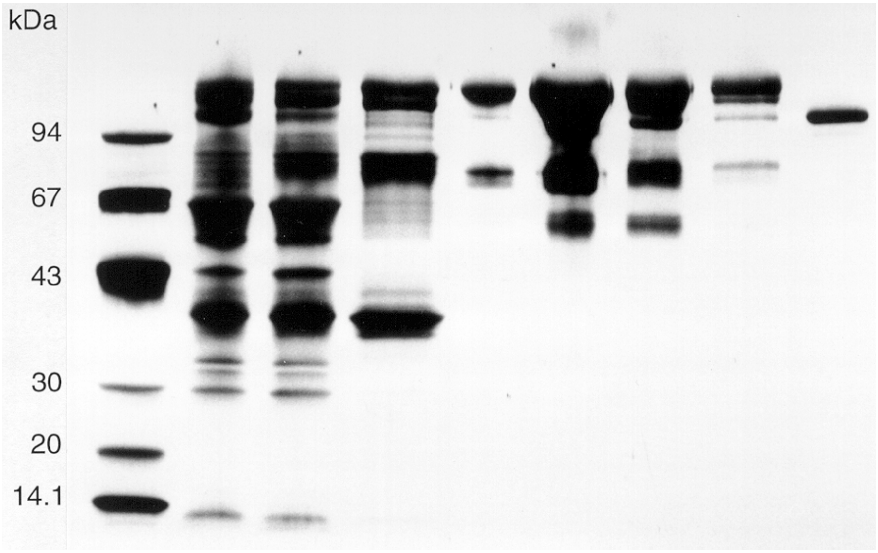

Fig. 1. SDS-PAGE of the different steps in the purification of α-glucuronidase. Lane 1, low molecular mass standard proteins; lane 2, starting material; lane 3, 45% ammonium sulphate precipitate; lane 4, Phenyl Sepharose FF pool; lane 5, Q-Sepharose pool; lane 6, Superdex 200 PG pool; lane 7, Mono Q pool; lane 8, Butyl Sepharose pool; lane 9, Poros 10 HQ pool.

stable for at least 62 days at room temperature. A loss of 15% of the activity was observed after 13 days at pH 4, but even after 62 days, 68% of the activity was recovered. pH 8 was the least favourable of the tested pH values. After 13 days, 82% of the activity was recovered; after 62 days, this value had dropped to 45%. At pH 5, the α-glucuronidase was 100% stable at 10°C for 20 h. At the same pH at 45, 50, 55, and 60°C the recoveries after 20 h were 88, 70, 52, and 10%, respectively. Loss in activity during freezing was not observed. With aldotriouronic acid-aldobiuronic acid as a substrate the K_m for α-glucuronidase was determined to be 0.14 ± 0.03 mg/ml (mean ± standard deviation). For the recombinant enzyme, similar results were obtained.

Cloning and overexpression of *agu*A from *A. tubingensis*

Amino acid sequences were obtained for AguA as described in Materials and Methods. In total, seven fragments containing 201 amino acids were sequenced (with six uncertainties). On the basis of these amino acid sequences, nine degenerate oligonucleotides were designed and used in PCRs with *A. tubingensis* chromosomal DNA. Although several DNA fragments were generated, only one combination (primers 5 and 9, based on peptides 5 and 4, respectively; see Materials and Methods) resulted in a fragment in which both the amino acid sequences on which the primers were based could be identified. The size of this fragment is 1142 bp.

A genomic library of *A. tubingensis* was screened by using this fragment as a probe, and four hybridising phage λ clones were isolated and purified. From one of these phages, a 7-kb *Xho*I/*Bam*HI fragment and a 4-kb *Kpn*I fragment containing part of the *agu*A gene and some flanking regions were cloned (pIM3210 and pIM3211). These fragments were combined, resulting in plasmid pIM3212 (Fig. 2), which was used to generate *A. tubingensis* multicopy transformants. Transformation of this construct resulted in a number of transformants with elevated levels of AguA as determined by Western analysis (data not shown). Transformant NW241::pIM3212.8 had the highest level of α-glucuronidase activity (10 times the wild-type activity) and was selected for further experiments. Slot blot analysis indicated the presence of 11 copies of the *agu*A gene in this transformant.

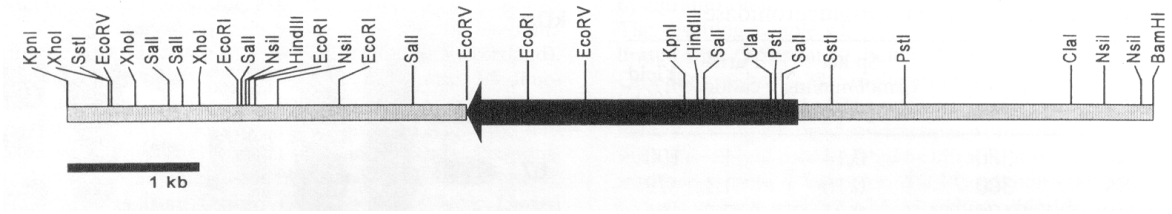

Fig. 2. Restriction map of the insert containing the *agu*A gene (arrow) which is present in the functional construct pIM3212.

Analysis of the nucleotide sequence of *agu*A and the derived amino acid sequence of the enzyme

Subclones were made from pIM3210 and pIM3211 and sequenced. Additional sequence data were obtained by using specific oligonucleotides. The *agu*A gene consists of an open reading frame of 2,523 bp which contains no introns and codes for a protein of 841 amino acids (Fig. 3). Analysis of the derived amino acid sequence indicated a putative eukaryotic signal sequence of 20 amino acids, which was confirmed by the N-terminal amino acid sequence of the mature protein starting at position 21. The mature protein contains 14 putative N-glycosylation sites, of which 4 were confirmed by the presence of an unidentifiable amino acid residue in the sequenced peptides. The enzyme has a calculated pI of 5.18, which is identical to the measured value. The calculated molecular mass of the mature protein is 93,904 Da, which is similar to the value determined by mass spectrometry. In the promoter region of the gene, several boxes possibly involved in transcription and regulation were identified. A TATA box was found 65 bp upstream from the ATG, and CAAT boxes were found at positions -106, -161, and -313.

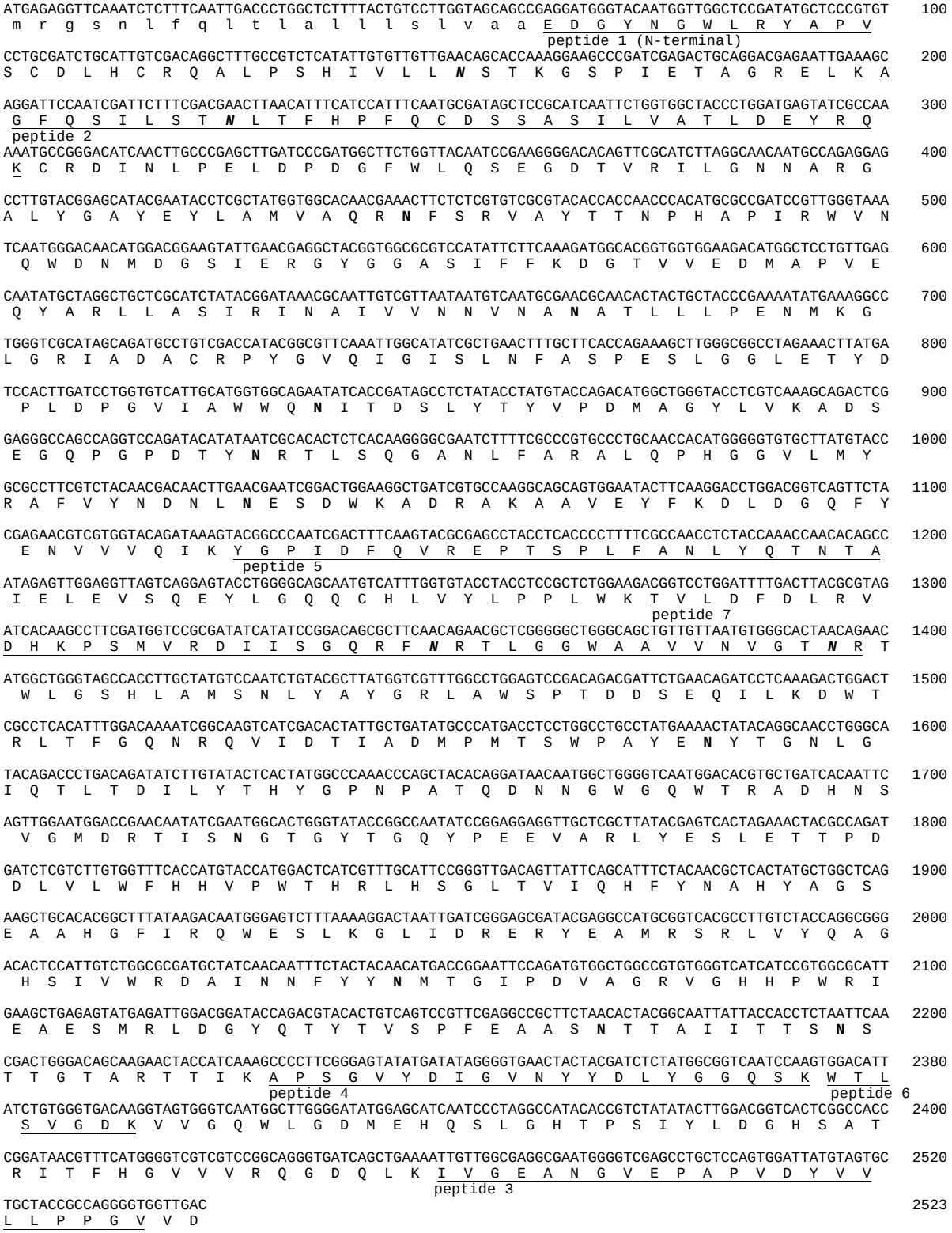

Fig. 3. Nucleotide sequence of *agu*A and derived amino acid sequence. The signal peptide (lowercase letters), putative (boldface roman letters) and confirmed (boldface italics) N-glycosylation sites and the determined amino acid sequences (underlined) are indicated.

Putative binding sites for the CreA protein (Kulmburg et al., 1993), involved in carbon catabolite repression, were found at positions -100, -123, -247 and -440. Only the first site is present in the upper strand, the others are in the complementary strand.

```
A. tubingensis  MRGSNLFQLTLALLLSLVAAEDGYNGWLRYAPVSCDLHCRQ--ALPSHIVLLNSTKGSPIETAGRELKAGFQSIL  73
T. reesei       MVIRSLLLLLLAAIVPVFA-ESGIDAWLRYARLPSSATRGHLTSFPDRIVVLNASKNGPLASASSELHKGIKGIL  74
T. maritima     MDYRM--------------------CWLEYRGLPADVA-GKLKDWFSSVSIL--------EPGSSVLKDEIRRFS  46
                M                         WL Y                     L             L

A. tubingensis  STNLTFHP-F---QCDSSASILVATLDEYRQKCRDINLP-ELDPDGFWLQS---EGDTVRILGNNARGAL-YGAY 139
T. reesei       GLDLDVSS-RGGKHCSTQKSIVISTLDTYQSACGKLSPKLNLKEDGYWLST---KGGSVQIIGQNERGAL-YGAF 144
T. maritima     ERSIGITPRFYSRPLKKEKYIMVGRLESLP-----IKLDVNLGEEGFMLRTIEWNGSKILLVTGETKKALVYGIF 116
                                    I    L               L   G  L      G            AL YG

A. tubingensis  EYLAMVAQR-NFSRVAYTTNPHAPIRWVNQWDNMD------GSIERGYGGASIFFKDGTVVEDMAPVEQYARLLA 207
T. reesei       QYLSYLGQG-DFSGKAFASNPSAPVRWSNQWDNLNAATAAHGSIERGYGGPSIFFENGLIKEDLSRVPLYGRLLA 218
T. maritima     DLMKRIRLGEDIEKMNVLAKPKAKFRMLNHWDNLD------GTIERGYAGNSIFFKDNRIIIN-QRTKDYARLLA 184
                                    P A  R  N WDN        G IERGY G SIFF              Y RLLA

A. tubingensis  SIRINAIVVNNVNANAT---LLLPENMKGLGRIADACRPYGVQIGISLNFASPESLGGLETYDPLDPGVIAWWQN 279
T. reesei       SVGLNGIVINNVNADAN---LLNETNLQGLKRIADLFRPWGVNVGISLNFASPQVLGDLSTFDPLDDSVIKWWTD 290
T. maritima     SIGINGVVINNVNVKKREVYLIDSIYLKKLKKLADIFREYGIKIYLSINFASPVYLGGLDTADPLDERVARWWRE 259
                S   N  V NNVN       L        L   AD  R  G     S NFASP  LG L T DPLD  V  WW

A. tubingensis  ITDSLYTYVPDMAGYLVKADSEGQPGPDTYNRTLSQGANLFARALQPHGGVLMYRAFVYNDNLNESDWKADRAKA 354
T. reesei       KTDRIYQLVPDLAGYLVKANSEGQPGPLTYNRTLAEGANLFAKAVQPHGGIVVFRAFVY-DQLNETDWKADRANA 364
T. maritima     KARGIYDYIPDFGGFLVKADSEFNPGPHMFGRTHAEGANMLARALAPFGGVVIWRAFVYNCLQDWRDYKTDRAKA 334
                     Y   PD  G LVKA SE  PGP    RT   GAN  A A  P GG    RAFVY       D K DRA A

A. tubingensis  AVEYFKDLDGQFYENVVVQIKYGPIDFQVREPTSPLFANLYQTNTAIELEVSQEYLGQQCHLVYLPPLWKTVLDF 429
T. reesei       AVDFFKSLDGQFDDNVLVQIKYGPIDFQVREPASPLFANLPKTAVSIELEVTQEYLGQQSHLVYLPPLWQTVLGF 439
T. maritima     AYDNFKPLDGQFDDNVIIQIKYGPMDFQVREPVNPLFGGMEKTNQILELQITQEYTGQQIHLCFLGTLWKEILEF 409
                A   FK LDGQF  NV  QIKYGP  FQVREP  PLF     T    EL   QEY GQQ HL  L  LW   L F

A. tubingensis  DLRVDHKPSMVRDIISGQRFNRTLGGWAAVVNVGTNRTWLGSHLAMSNLYAYGRLAWSPTDDSEQILKDWTRLTF 504
T. reesei       DMRYNNRQSYVRDIISGEVFGHKLGGYAGVINVGMDDTWLGSHLAMSNMFAYGRLAWNPRADSRDIVEEWTRLTF 514
T. maritima     DTFAKGEGSYVKRIVDGTLFDRENNGFAGVSNVGDSVNWTGHDLAQANLYAFGRLAWNPDEEIERIVEEWIKLTF 484
                D       S V  I  G  F     G A V NVG    W G  LA  N  A GRLAW P      I   W  LTF

A. tubingensis  GQNRQVIDTIADMPMTSWPAYENYTGNLGIQTLTDILYTHYGPNPATQDNNGWGQWTRADHNSVGMDRTISNGTG 579
T. reesei       GLDRDVVSTIADMSLKSWPAYEGYSGNLGIQTLTDILYTHYGANPASQDNNGWGQWTRADSKTIGMDRTVSNGTG 589
T. maritima     GDDEKVLENVSYMLMKSHRTYEKYTTPFGLGWMVNPGH-HYGPNPEGYEYSKWGTYHRANWEAIGVDRT-SRGTG 557
                G    V      M   S   YE Y    G          HYG NP       WG   RA     G DRT S GTG

A. tubingensis  YTGQYPEEVARLYESLETTPDDLVLWFHHVPWTHRLHSGLTVIQHFYNAHYAGSEAAHGFIRQWESLKGLIDRER 654
T. reesei       NAGQYPKEVAARFEHTQTTPDDLMLWFHHVPYTFRLHSGKSVIQHFYDAHYTGAATVQRFPAAWKSLKSKIDTER 664
T. maritima     YTLQYHSPWKEIYDDINTCPEDLLLFFHRVRYDHRLKSGKTLLQTMYDLHFEGVEEVEEFIKKWEELKDRVSPDI 628
                   QY            T P DL L FH V    RL SG    Q  Y  H   G      F   W  LK

A. tubingensis  YEAMRSRLVYQAGHSIVWRDAINNFYYNMTGIPDVAGRVGHHPWRIEAESMRLDGYQTYTVSPFEAASNTTAIIT 729
T. reesei       YNAVLYKLQYQTGHSLVWRDAITEFYRNLSSIPDQLNRVRNHPHRIEAEDMDLSGFTVVNVSPTECASKYKAIAT 739
T. maritima     FERVKERLHMQLEHAKEWRDVINTYFYRRTGIPDEKGRKIYP                                  671
                      L  Q  H   WRD I         IPD   R

A. tubingensis  TSNSTTGTARTTIKAPSGVYDIGVNYYDLYGGQSKWTLSVGDKVVGQWLGDMEHQSLGHTPSIYLDGHSATRITF 804
T. reesei       NG---TGTATTRLNVPSGKYTVAVNYYDVINGTASYDVLLNGKSLGKWKGDSETH-LGHDFSTFLDCHSAIRITF 810

A. tubingensis  HGVVVRQGDQLKIVGEANGVEPAPVDYVVLLPPGVVD                                       841
T. reesei       EGVRISRGDKLTIRGTGNAQEQAAIDYVSILPQGVVD                                       847
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Fig. 4. Alignment of the amino acid sequences of α-glucuronidase from *A. tubingensis, T. reesei,* and *T. maritima*. Identical amino acids are depicted below the amino acid sequences.

Alignment of the amino acid sequences of AguA and two other α-glucuronidases

The deduced amino acid sequence of AguA from *A. tubingensis* was aligned with the deduced amino acid sequences of AguA from *T. maritima* (Ruile et al., 1997) and GlrI from *T. reesei*

(Margolles-Clark et al., 1996) as shown in Fig. 4. AguA was 59.3% identical to GlrI from *T. reesei* and 39.3% identical to AguA from *T. maritima*. No clear highly identical boxes could be identified, although the level of identity is highest in the middle region of the enzymes.

The *agu*A gene is induced by xylan and xylose, but not by glucuronic acid

The induction of *agu*A was studied by a transfer experiment. Transformant NW241::pIM3212.8 was grown for 16 h in CM (3% fructose). The mycelium was harvested, washed with MM, and transferred to MM with different carbon sources. After 6 h, the mycelium was harvested and stored at -70°C. A Northern analysis was performed using RNA isolated from the mycelium samples. Induction of *agu*A was observed on xylose, arabinose, xylobiose, and birchwood xylan alone but not on glucose, fructose, glycerol, or glucuronic acid (Fig 5). The presence of glucose completely inhibited the expression on birchwood xylan, but only reduced the expression on xylose. Addition of glucuronic acid to the monomeric carbon sources did not result in an increase in the expression of *agu*A.

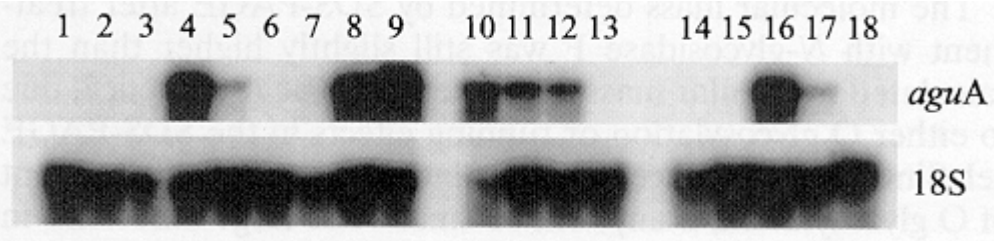

Fig 5. Northern analysis of the induction of *agu*A on different carbon sources. The top panel was probed with the internal 2-kb *Sal*I fragment of *agu*A, and the bottom panel was probed with a 700-bp *Eco*RI fragment from the *A. niger* 18S ribosomal DNA and served as a loading control. Lane 1, mycelium from the pre-culture on fructose; other lanes: mycelium transferred to the following carbon sources: lane 2, 1% glucose; lane 3, 1% fructose; lane 4, 1% xylose; lane 5, 1% arabinose; lane 6, 1% glycerol; lane 7, 1% glucuronic acid; lane 8, 0.2% xylobiose; lane 9, 0.5% birchwood xylan; lane 10, 1% xylose-0.2% glucose; lane 11, 1% xylose-1% glucose; lane 12, 1% xylose-2% glucose; lane 13, 0.5% birchwood xylan-1% glucose; lane 14, 1%glucose-1% glucuronic acid; lane 15; 1%fructose-1% glucuronic acid; lane 16, 1% xylose-1% glucuronic acid; lane 17, 1% arabinose-1%glucuronic acid; lane 18, 1% glycerol-1% glucuronic acid.

The presence of endoxylanase or β-xylosidase enhances the activity of α-glucuronidase on xylan

The purified native α-glucuronidase was able to liberate minor amounts of 4-*O*-methylglucuronic acid from birchwood xylan and wheat bran (data not shown) but had a much higher activity on xylan derived oligomers. The influence of endoxylanase and β-xylosidase on

α-glucuronidase activity was studied by incubating combinations of these enzymes with birchwood xylan as described in Materials and Methods. Addition of xylanase A, xylanase complex, and β-xylosidase increased the amount of 4-*O*-methylglucuronic acid liberated (Table 2). The amount of small oligomers (xylobiose and xylotriose) is larger when a combination of α-glucuronidase and endo-xylanase is used than when endoxylanase is used alone, indicating a positive effect of α-glucuronidase on the activity of endoxylanase on xylan. The most efficient degradation of birchwood xylan was achieved when a combination of α-glucuronidase, endoxylanase, and β-xylosidase was used.

Table 2. Synergistic effects of α-glucuronidase, endo-xylanase and β-xylosidase activity on release of 4-*O*-methylglucuronic acid, xylose and xylooligomers from birchwood xylan.

	Amt released (μg/ml)[a]			
Enzyme(s) used in reaction mixtures	4-*O*-Methyl-glucuronic acid	Xylose	Xylobiose	Xylotriose
None (control)	63	0	0	0
α-Glucuronidase	62	0	0	0
Xylanase complex	127	1260	440	129
Xylanase A	95	1300	<50	<50
β-Xylosidase	63	270	0	0
α-Glucuronidase plus:				
Xylanase complex	379	1210	680	210
Xylanase A	206	1360	60	70
β-Xylosidase	187	1060	<50	<50
Xylanase A + β-xylosidase	107	1320	<50	<50
Xylanase complex + β-xylosidase	146	1410	<50	<50
α-Glucuronidase + xylanase A + β-xylosidase	398	1500	<50	<50

[a] The amount of 4-O-methylglucuronic acid was determined by the colorimetric method. The amounts of the other compounds were determined by HPLC analysis.

Discussion

Purification of the α-glucuronidase required a complex procedure. The ammonium sulphate precipitation applied as the first step did not give much purification with respect to the increase in specific activity but was important because it removed much of the coloured contaminants (possibly phenolic compounds), which would otherwise have interfered with later column

chromatography steps. Gel filtration always gave a large loss in activity but could not be omitted in the procedure. The most essential step was the Poros 10 HQ column, which had a high selectivity for the α-glucuronidase. However, the separation capacity of Poros 10 HQ was poor compared to that of Mono Q, and it was not possible to eliminate the Mono Q column.

The molecular mass determined by SDS-PAGE after treatment with N-glycosidase F was still slightly higher than the calculated molecular mass from the amino acid sequence, due to either O glycosylation or running effects in the SDS-PAGE gel. Since the difference in molecular mass is small, the amount of O glycosylation, if any, will be small. The large difference in molecular masses observed between the mature and the deglycosylated enzyme (107 and 95 kDa, respectively, for the native enzyme) suggests that most of the 14 putative N-glycosylation sites are actually involved in glycosylation. The difference in molecular masses for the native and the recombinant enzyme is probably due to differences in the degree of glycosylation.

The molecular mass is similar to those of some other fungal α-glucuronidases (Ishihara et al., 1990; Siika-aho et al., 1994), but is lower than those of the *Agaricus bisporus* α-glucuronidase (Puls et al., 1987), which has a molecular mass of 160,000 Da as determined by SDS-PAGE, and the two internal α-glucuronidases from *A. niger* (Uchida et al., 1992), which have molecular masses of 130 and 150 kDa, respectively. The pI of AguA is similar to the pI of the *A. niger* α-glucuronidases.

The amino acid sequence of AguA had a high level of identity to the amino acid sequences of the α-glucuronidases from *T. reesei* and *T. maritima* (Margolles-Clark et al., 1996; Ruile et al., 1997) as shown in Fig. 4. The homology was present throughout the sequence until the end of the *T. maritima* amino acid sequence. The additional amino acid sequence from the two fungal α-glucuronidases (starting at the end of the *T. maritima* sequence) also showed a high level of identity, indicating that this region might be specific for fungal α-glucuronidases. Screening of the databases did not detect any other enzymes which had a significant level of identity with the α-glucuronidase from *A. tubingensis*.

The *agu*A gene was induced on xylose, xylobiose, and xylan, which resembles the induction of *xln*A, encoding an *A. tubingensis* endoxylanase (de Graaff et al., 1994) and *xln*D from *A. niger*, encoding a β-xylosidase (van Peij et al., 1997). These data suggest that *agu*A is regulated by a xylan- or xylose-specific system, which induces genes coding for xylan-degrading enzymes in the presence of xylose or xylan. The low level of expression on arabinose has also been observed for other xylanolytic genes (unpublished data). Since a minor amount of xylose is present in the

arabinose purchased from Sigma, this could explain the low level of expression, rather than a possible inducing effect of L-arabinose itself. Glucose repressed the expression of *agu*A completely in the presence of xylan but only partly in the presence of xylose. The presence of four putative CreA binding sites in the region directly upstream from the structural part of *agu*A suggests that glucose repression occurs through this regulator protein. The reason for the leaky repression in the presence of xylose is not clear and requires further study.

The hydrolysis of birchwood xylan by xylanases was enhanced in the presence of α-glucuronidase, but complete hydrolysis to xylose was not observed. Although the enzymes tested in this study clearly have a synergistic effect, other enzymes are needed as well to completely degrade xylan. A synergistic effect of the addition of (endo)xylanase to an α-glucuronidase incubation mixture was found. The activity of α-glucuronidases on polymeric substrates is very low compared to the activity on small-oligomeric substrates, indicating that the presence of a xylanase is essential for efficient α-glucuronidase activity in vivo. Siika-aho et al (1994) found that α-glucuronidase seemed to act exclusively on bonds between the terminal xylose at the non reducing end and 4-*O*-methylglucuronic acid attached to it. From this data, a synergistic effect of the addition of β-xylosidase to an incubation with α-glucuronidase (and xylanase) was expected, which was confirmed by the experiments described in this paper. Although the xylanase complex already contains β-xylosidase, addition of this enzyme resulted in an increase in the amount of liberated 4-*O*-methylglucuronic acid and a further degradation of xylobiose and xylotriose to xylose.

In this investigation, we have studied the hydrolysis of only hardwood xylan. A comparison with softwood xylan or deacetylated xylan could elucidate whether the acetylation present in hardwood is of any influence on the hydrolysis by α-glucuronidase. Hardwood xylan is a linear xylan, while wheat bran xylan is highly branched. The influence of branching requires further investigation in relation to the activity of α-glucuronidase on wheat bran xylan and applications thereof.

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Chapter 8

Differential expression of three α-galactosidase genes and a single β-galactosidase gene from *Aspergillus niger*

R. P. de Vries, H. C. van den Broeck, E. Dekkers, P. Manzanáres, L. H. de Graaff and J. Visser

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Abstract

A gene encoding a third α-galactosidase (AglB) from *Aspergillus niger* has been cloned and sequenced. The gene consists of an open reading frame of 1750 bp containing six introns. The gene encodes a protein of 443 amino acids, which contains an eukaryotic signal sequence of 16 amino acids and seven putative N-glycosylation sites. The mature protein has a calculated molecular mass of 48835 Da and a predicted pI of 4.6. An alignment of the AglB amino acid sequence with those of other α-galactosidases revealed that it belongs to a subfamily of α-galactosidases that also includes *A. niger* AglA. *A. niger* AglC belongs to a different subfamily that consists mainly of prokaryotic α-galactosidases. The expression of *agl*A, *agl*B, *agl*C and *lac*A, the latter of which encodes an *A. niger* β-galactosidase, has been studied by using a number of monomeric, oligomeric and polymeric compounds as growth substrates. Expression of *aglA* is only detected on galactose and galactose-containing oligomers and polymers. The *aglB* gene is expressed on all of the carbon sources tested, including glucose. Elevated expression was observed on xylan, which could be assigned to regulation via XlnR, the xylanolytic transcriptional activator. Expression of *aglC* was only observed on glucose, fructose, and combinations of glucose with xylose and galactose. High expression of *lacA* was detected on arabinose, xylose, xylan, and pectin. Similar to *aglB*, the expression on xylose and xylan can be assigned to regulation via XlnR. All four genes have distinct expression patterns, which seem to mirror the natural substrates of the encoded proteins.

Introduction

α-Galactosidases (EC 3.2.1.22) and β-galactosidases (EC 3.2.1.23) are enzymes, which are commonly found in nature and which are able to release α- or β-linked D-galactose from a wide range of compounds. Several fungal α-galactosidases have been purified, in particular from *Aspergillus* sp., and these enzymes have different physicochemical and kinetic properties (Adya and Elbein, 1977; den Herder et al., 1992; Manzanares et al., 1998; Neustroev et al., 1991; Riós et al., 1993; Somiari and Balogh, 1995). These enzymes can be divided into different classes based on their characteristics. Some of these α-galactosidases have molecular masses of 70 to 95 kDa (den Herder et al, 1992; Neustroev et al, 1991; Somiari and Balogh, 1995), whereas others are in the range of 45 to 56 kDa (Adya and Elbein, 1977; Civas et al., 1984; Manzanares et al., 1998). The pI of *Aspergillus* α-galactosidases is between 4.5 and

5.0, and all of the enzymes have activity against galactose containing oligosaccharides such as raffinose. Induction of α-galactosidases has been observed on arabinoxylan (Manzanares et al., 1998), galactose (Riós et al., 1993), galactomannan (den Herder et al., 1992), and wheat and rice bran (Somiari and Balogh, 1995). Up to now two α-galactosidase encoding genes have been cloned from *Aspergillus niger*. Den Herder et al. (1992) purified an α-galactosidase, which was expressed on galactomannan and cloned the corresponding gene (*aglA*). A second gene encoding an α-galactosidase from *A. niger* with activity against galactose containing oligosaccharides has been described more recently (Knap et al., 1994). We have designated this gene *aglC*. An extracellular β-galactosidase has also been purified from *A. niger*, and the corresponding gene (*lacA*) has been cloned (Kumar et al., 1992). There are no indications of other genes encoding additional extracellular β-galactosidases in *A. niger*. One paper reported the purification of three forms of *A. niger* β-galactosidase (Widmer and Leuba, 1979), but these are most likely different glycoforms of the same enzyme. *A. niger* β-galactosidase is induced during growth on polygalacturonic acid (McKay, 1991) and arabinoxylan (Manzanares et al., 1998).

Galactose is present in different oligosaccharides (e.g., raffinose, stachyose, and melibiose) and polysaccharides (galactomannan, pectin, and xylan) from plants. In pectin, galactose is mainly present as branched sidechains (O'Neill et al., 1990), whereas in arabinoxylan, single galactose residues are attached to xylose or arabinose (Ebringerová et al., 1990; Wilkie and Woo, 1977).

A. niger has a very efficient extracellular enzyme spectrum specialised in degrading plant-derived oligo- and polysaccharides, including those hydrolysing α- and β-linked galactosides. Some of these enzymes have a high substrate specificity, resulting in the production of a number of enzymes with similar functions. α-Galactosidases from *Aspergillus* have been shown to catalyse the hydrolysis of α-1,6-linked galactose residues from oligomeric (e.g., melibiose, raffinose, and stachyose) and polymeric (e.g., galactomannan) compounds (Manzanares et al., 1998; Riós et al., 1993). The potential of *A. niger* to produce several α-galactosidases could indicate that these enzymes are active on different substrates and might therefore have different expression patterns.

β-Galactosidase (lactase) is able to cleave β-linked galactose residues from various compounds and is commonly used to cleave lactose into galactose and glucose (Wigley, 1996). The role of this enzyme of *A. niger* in nature is more likely in removing β-linked

galactose residues from plant-derived oligo- and polysaccharides than in the hydrolysis of lactose.

Here we describe the cloning and characterisation of the *aglB* gene encoding a third α-galactosidase from *A. niger*, which was previously purified in our laboratory (Manzanares et al., 1998). Also, we studied the expression patterns of the three α- and the β-galactosidase genes from *A. niger* in detail.

Materials and methods

Strains libraries and plasmids

All strains were derived from wild type *A. niger* N400 (=CBS 120.49). N402 has short conidiophores (*csp*A1). CreA mutant strain NW200 (*bio*A1, *csp*A1, *cre*Ad4, *pyr*A13::pGW635, *are*A1::pAREG1) was selected in an *are*A1 background (Ruijter et al., 1997) and subsequently cotransformed with pAREG1 (containing the *A. niger are*A gene; MacCabe et al., 1998) and pGW635 (containing the *pyr*A selection marker) to restore the *areA* wild type. The *prt*F mutation present in strain NW156 (*leu*A1, *pyr*A6, *prt*F28) was previously described (van den Hombergh et al., 1995) as was *A. niger* NXA1-4 [*arg*B13, *csp*A1, *nic*A1, *pyr*A6, UAS(*xln*A)-*pyr*A, *xln*R1], which has a defect in the xylanolytic transcriptional activator gene *xln*R (van Peij et al., 1998a). *Escherichia coli* DH5αF' was used for routine plasmid propagation. *E. coli* LE392 was used as a host for phage λEMBL3. pBluescript (Short et al., 1988) and pGEM-T (Promega) were used for subcloning. The genomic and cDNA libraries of *A. niger* have previously been described (Gielkens et al., 1997; Harmsen et al., 1990).

Media and culture conditions

Minimal medium (MM) contained (per litre): 6.0 g of $NaNO_3$, 1.5 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4$, trace elements (Vishniac & Santer, 1957), and 1% (mass/vol) glucose as a carbon source unless otherwise indicated . For complete medium (CM) MM was supplemented with 0.2% (mass/vol) tryptone, 0.1% (mass/vol) yeast extract, 0.1% (mass/vol) casamino acids, and 0.05% (mass/vol) yeast RNAs. Liquid cultures were inoculated with 10^6 spores/ml and incubated at 30°C in an orbital shaker at 250 rpm. Agar was added at 1.5% (mass/vol) for solid medium. For the growth of strains with auxotrophic mutations, the necessary supplements were added to the medium.

In transfer experiments, strains were pregrown in CM containing 2% (mass/vol) fructose as a carbon source. After 16 h mycelium was harvested and washed with MM without carbon source, and aliquots of 1 g (wet weight) were transferred to 50 ml of MM containing carbon sources as indicated in Results. After 4 h (unless stated otherwise) of incubation in a rotary shaker at 250 rpm and 30°C, mycelium was harvested, frozen in liquid nitrogen, and stored at -70°C.

Chemicals

D-Xylose, D-glucose, D-fructose, D-galactose, D-mannose and lactose were obtained from Merck (Darmstadt, Germany). D-Glucuronic and D-galacturonic acid were from Fluka (Buchs, Switzerland). Melibiose, raffinose, stachyose, L-arabinose, gum arabic, gum karaya, locust bean gum, methyl-umbelliferyl-α-D-galactoside and beechwood xylan were from Sigma (St. Louis, Mo.). Potato pectic galactan was from Megazyme International (Bray, Ireland).

PCR cloning of specific fragments of *lacA*, *aglA*, *aglB*, and *aglC*

Based on the nucleotide sequences of *lacA*, *aglA*, and *aglC*, specific oligonucleotides were designed (5'-GGTCTCTCTGAGGCAGGC-3' and 5'-TAGTATGCACCCTTCCGC-3' for *lacA*, 5'-ACGGCTCTATCGAGCAGCCC-3' and 5'-CTCCCCGTATATCGGGACCC-3' for *aglA*, and 5'-ATGATCGGTCTTCCCATGCTG-3' and 5'-TCGTCCATGACAAAGAGGTGG-3' for *aglC*) and used in PCRs under the following conditions: melting at 95°C, annealing at 50°C, and elongation at 72°C. Chromosomal DNA of *A. niger* N402 was used as the template. This resulted in specific DNA fragments of 373, 593, and 1276 bp for *lacA*, *aglA*, and *aglC*, respectively. For the isolation of a specific fragment of *aglB*, one oligonucleotide was designed based on the N-terminal amino acid sequence of AglB (23) and one was based on a highly conserved region in a number of α-galactosidases (5'-GGNTGGAAYTCNTGGAAYGC-3' and 5'-CATNCCNCCRTTNCCNACYTC-3', with Y and R representing C/T and A/G, respectively). PCRs at 42°C using these two oligonucleotides and total cDNA from the *A. niger* cDNA library resulted in a fragment of 762 nucleotides. All three fragments were cloned in the PGEM-T vector system (Promega). Sequence analysis was performed as described below.

Isolation, cloning and characterisation of the *aglB* gene

Plaque hybridisation using nylon replicas was performed as described by Benton & Davis (1977). Hybridizations were performed overnight at 65°C using the *aglB* PCR fragment as a probe. Filters were washed in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M Na_3-citrate, pH 7.6)-0.5% (mass/vol) sodium dodecyl sulphate (SDS). Positive plaques, identified on duplicate replicas after autoradiography, were recovered from the original plates and purified by rescreening at low plaque density. Standard methods were used for other DNA manipulations, such as Southern and Northern analyses, subcloning, DNA digestions, and lambda phage and plasmid DNA isolations (Sambrook et al., 1989). Chromosomal DNA was isolated as previously described (de Graaff et al., 1988). Sequence analysis was performed on both strands of DNA by using either the Cy5 AutoCycle Sequencing kit or the Cy5 Thermo Sequenase Dye Terminator Kit (Pharmacia Biotech, Uppsala, Sweden). The reactions were analysed with an ALFred DNA Sequencer (Pharmacia Biotech). Nucleotide sequences were analysed with computer programs based on Devereux *et al*. (1984). *Aspergillus* cotransformations were performed as described by Kusters-van Someren *et al.* (1991) by using the *pyr*A gene as a selection marker.

Northern analysis

Total RNA was isolated from powdered mycelium using TRIzol Reagent (Life Technologies) in accordance with the supplier's instructions. For Northern analysis, 5 μg of total RNA was incubated with 3.3 μl 6 M glyoxal-10 μl dimethyl sulfoxide-2 μl 0.1 M phosphate buffer (pH 7) in a total volume of 20 μl for 1 h at 50°C to denature the RNA. The RNA samples were separated on a 1.5% agarose gel using 0.01 M phosphate buffer (pH 5) and transferred to Hybond-N filters (Amersham) by capillary blotting. Filters were hybridised at 42°C in a solution of 50% (vol/vol) formamide, 10% (mass/vol) dextran sulfate, 0.9 M NaCl, 90 mM Na_3-citrate, 0.2% (mass/vol) ficoll, 0.2% (mass/vol) polyvinylpyrrolidone, 0.2% (mass/vol) bovine serum albumin, 0.1% (mass/vol) SDS, and 100 μg of single stranded herring sperm DNA per ml. Washing was performed under homologous conditions in 30 mM NaCl-3 mM Na_3-citrate-0.5% (mass/vol) SDS at 68°C. A 0.7-kb *Eco*RI fragment of the 18S rRNA subunit (Melchers et al., 1994) was used as a probe for RNA loading control.

Sequence alignments

Amino acid sequence alignments were performed by using the Blast programs (Altschul et al., 1990) at the server of the National Centre for Biotechnology Information (Bethesda, Md.).

Nucleotide sequence accession number. The EMBL accession number for *aglB* from *A. niger* is Y18586.

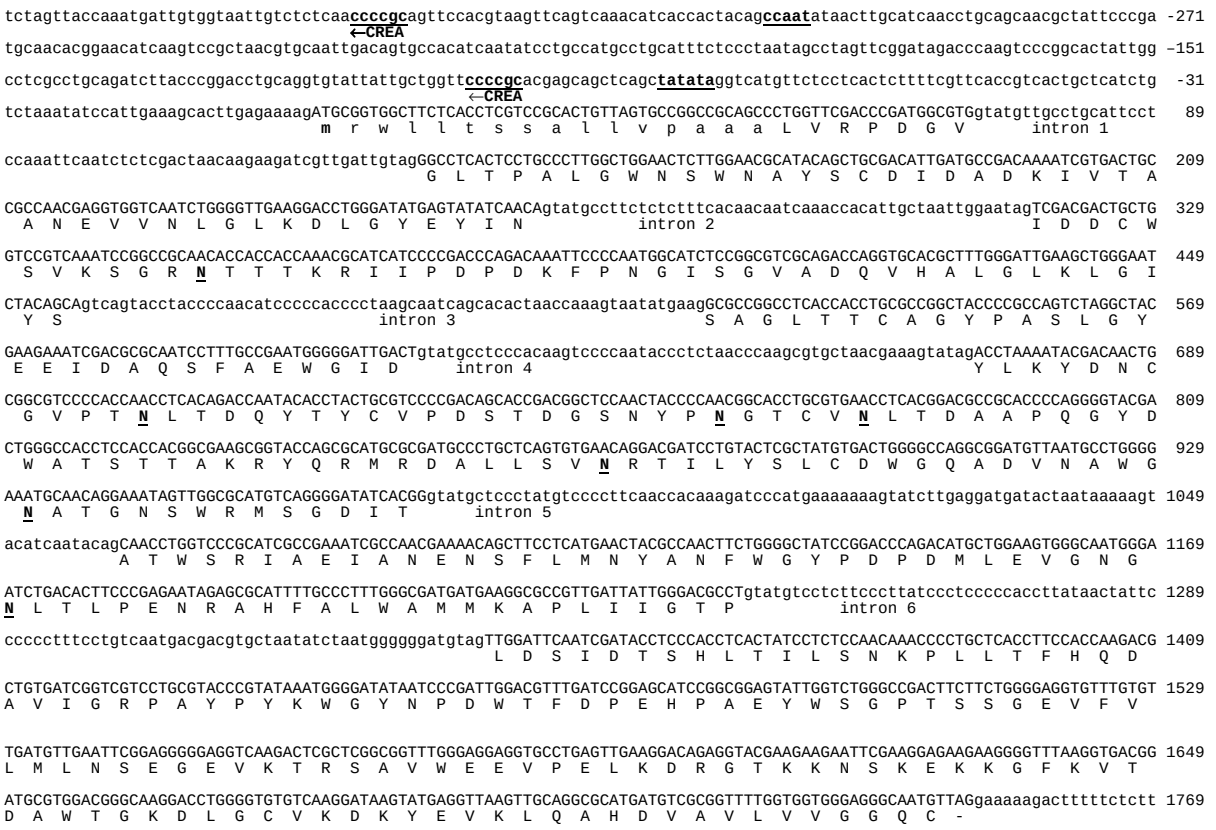

Fig. 1. Nucleotide and derived amino acid sequences of *agl*B. The introns (lowercase letters), putative regulatory sequences (boldface and underlined), signal peptide (lowercase letters) and the putative N-glycosylation sites (boldface capital letters) are indicated.

Results

Cloning and sequence analysis of *aglB* from *A. niger*

Based on the N-terminal amino acid sequence (LVRPDGVGLTPALGWNSWNAY) of AglB (Manzanares et al., 1998) and a highly conserved region present in a number of α-galactosidases, two degenerate oligonucleotides were designed and a specific fragment of *aglB* cDNA was isolated as described in Materials and Methods.

A genomic library of *A. niger* N400 was screened by using this fragment as a probe, and four hybridising phage λ clones were isolated and purified. From one of these phage clones, a 6-kb *Sst*I fragment containing the *aglB* gene and flanking regions was cloned in pBluescript SK^+

(pIM3214). Subclones were made from this construct, and sequence analysis was performed, resulting in the genomic sequence of *aglB* and its flanking regions (Fig. 1). A specific oligonucleotide was designed for the region containing the start of the coding sequence of the gene (5'-ATGCGGTGGCTTCTCAC-3'), and another was designed for the region containing the putative stop (5'-CTAACATTGCCCTCCCAC-3'), based on homology with other α-galactosidases. PCRs at 50°C using these oligonucleotides and total cDNA from the *A. niger* cDNA library resulted in a fragment of 1332 bp. A comparison of the sequence of this fragment with the genomic sequence identified six introns (Fig. 1).

Analysis of the derived amino acid sequence indicated that the *aglB* gene encodes a protein of 443 amino acids containing a putative eukaryotic signal sequence of 16 amino acids. This signal sequence was confirmed by the N-terminal amino acid sequence of the mature protein (23). The mature protein has a calculated pI of 4.6 and a calculated molecular mass of 48,835 Da. In the amino acid sequence of the mature protein, seven putative N-glycosylation sites could be identified.

Sequence analysis of the promoter region of *aglB* revealed several sequences possibly involved in transcription and regulation. A CCAAT box was identified at position -308 from the ATG and a TATA box was found at position -78. Putative binding sites for the CreA regulatory protein that mediates carbon catabolite repression (19) were identified at positions -98 and -350.

Overexpression of *aglB*

A. niger NW156 was transformed with plasmid pIM3214 to generate multicopy transformants. A total of 20 transformants were selected and inoculated on MM plates containing 1% (mass/vol) xylose and methylumbelliferyl-α-D-galactoside. Five transformants were identified which showed increased α-galactosidase activity, and these were purified. These transformants and wild-type N402 were grown overnight on CM containing 1% (mass/vol) fructose and transferred to MM containing 1% (mass/vol) xylose. After 4 h, mycelium was harvested and RNA and chromosomal DNA were isolated. Southern and Northern analyses were performed, and autoradiographs were scanned by using an LKB Ultroscan XL laser densitometer and subsequently normalised for the loading control (18S expression) to determine copynumbers, and expression levels. The estimated copynumbers of the transformants ranged from 3 to 40 (Table 1), and expression levels were between 2.5 and 40 times wild type expression.

Table 1. Copynumber and expression levels of *A. niger agl*B multicopy transformants.

strain	Copy-number	Relative expression level
Wild type	1	1
NW156::pIM3214.16	40	38
NW156::pIM3214.20	15	20
NW156::pIM3214.21	3	2.5
NW156::pIM3214.23	32	39
NW156::pIM3214.30	5	7

Comparison of the amino acid sequence of AglB to other α-galactosidases

The deduced amino acid sequence of AglB was compared to the amino acid sequences of other α-galactosidases. AglB showed the highest overall similarity to an α-galactosidase from *Penicillium purpurogenum* and Agl1 from *Trichoderma reesei* (62 and 54% amino acid sequence identity, respectively). Two regions, in particular, were highly similar to those of a number of other α-galactosidases from various organisms, including *A. niger* AglA, but not to *A. niger* AglC (Fig. 2). The highest similarity is found in a region of approximately 120 amino acids starting just C terminal of the signal peptide of AglB. High sequence similarity between the enzymes is also observed in a region of approximately 70 amino acids which starts at residue 233. As already stated, AglC had no significant similarity to *A. niger* AglA and AglB but was found to be related to *T. reesei* Agl2 (64% amino acid sequence identity) and to a number of bacterial α-galactosidases (Fig. 3).

Differential expression of *A. niger* galactosidases

The expression of *aglA*, *aglB*, *aglC* and *lacA* was studied by carrying out transfer experiments. *A. niger* N402 was grown for 16 h in CM containing 2% (mass/vol) fructose. The mycelium was harvested and washed with MM without a carbon source, and aliquots were transferred to MM with different carbon sources and incubated for 4 h as described in Materials and Methods. A Northern analysis was performed using RNA isolated from the mycelium samples and using the PCR fragments of *aglA*, *aglB*, *aglC*, and *lacA* and a fragment of the 18S rRNA gene (Melchers et al, 1994) as probes (Fig. 4).

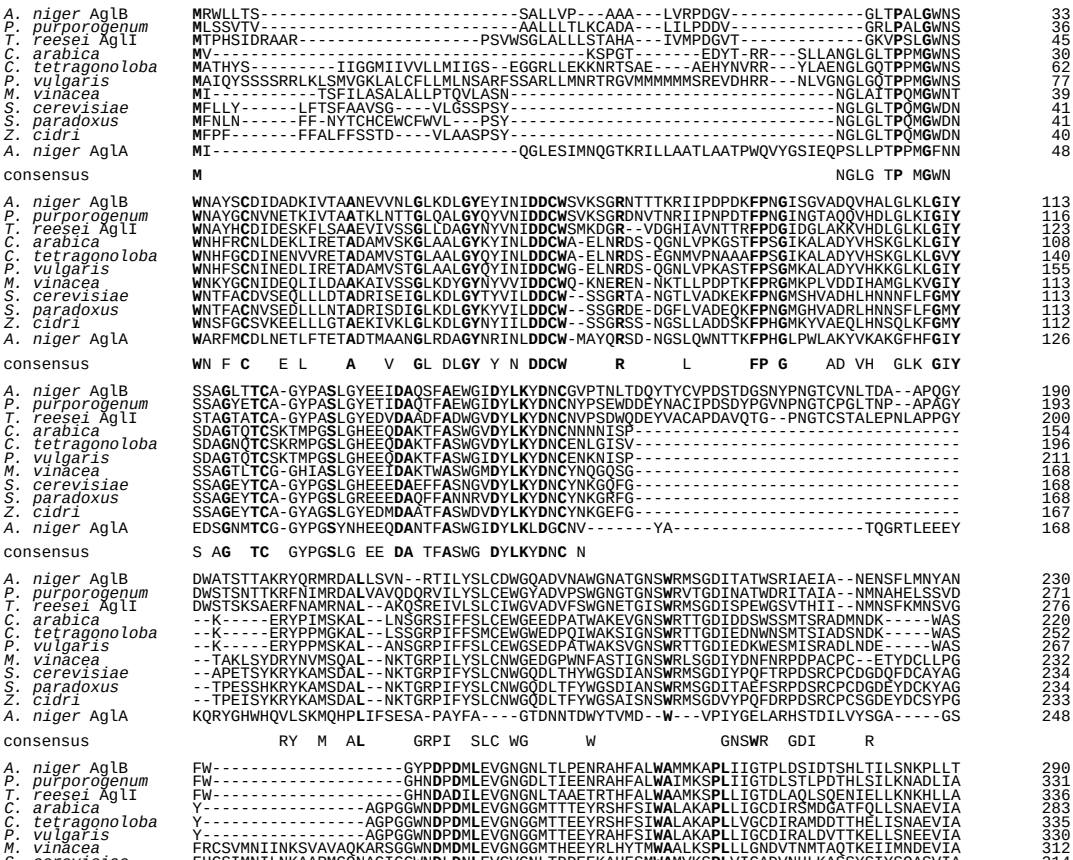

Fig. 2. Alignment of the amino acid sequences of α-galactosidases from *A. niger* (AglB and AglA; de Herder et al., 1992), *P. purpurogenum* (Shibuya et al.), *T. reesei* (AglI; Margolles-Clark et al., 1996), *Coffea arabica* (Zhu & Goldstein, 1994), *Cyamopsis tetragonoloba* (Overbeeke et al., 1989), *Phaseolus vulgaris* (Davis et al., 1997), *Mortierella vinacea* (Shibuya et al., 1995), *Saccharomyces cerevisiae* (Turakainen et al, 1991), *S. paradoxus* (Naumova et al., 1996), and *Zygosaccharomyces cidri* (Turakainen et al., 1994). In the consensus sequence, amino acids are depicted which are conserved in at least 7 of the 11 α-galactosidases. Amino acids, which are identical in all 11 α-galactosidases are in boldface type.

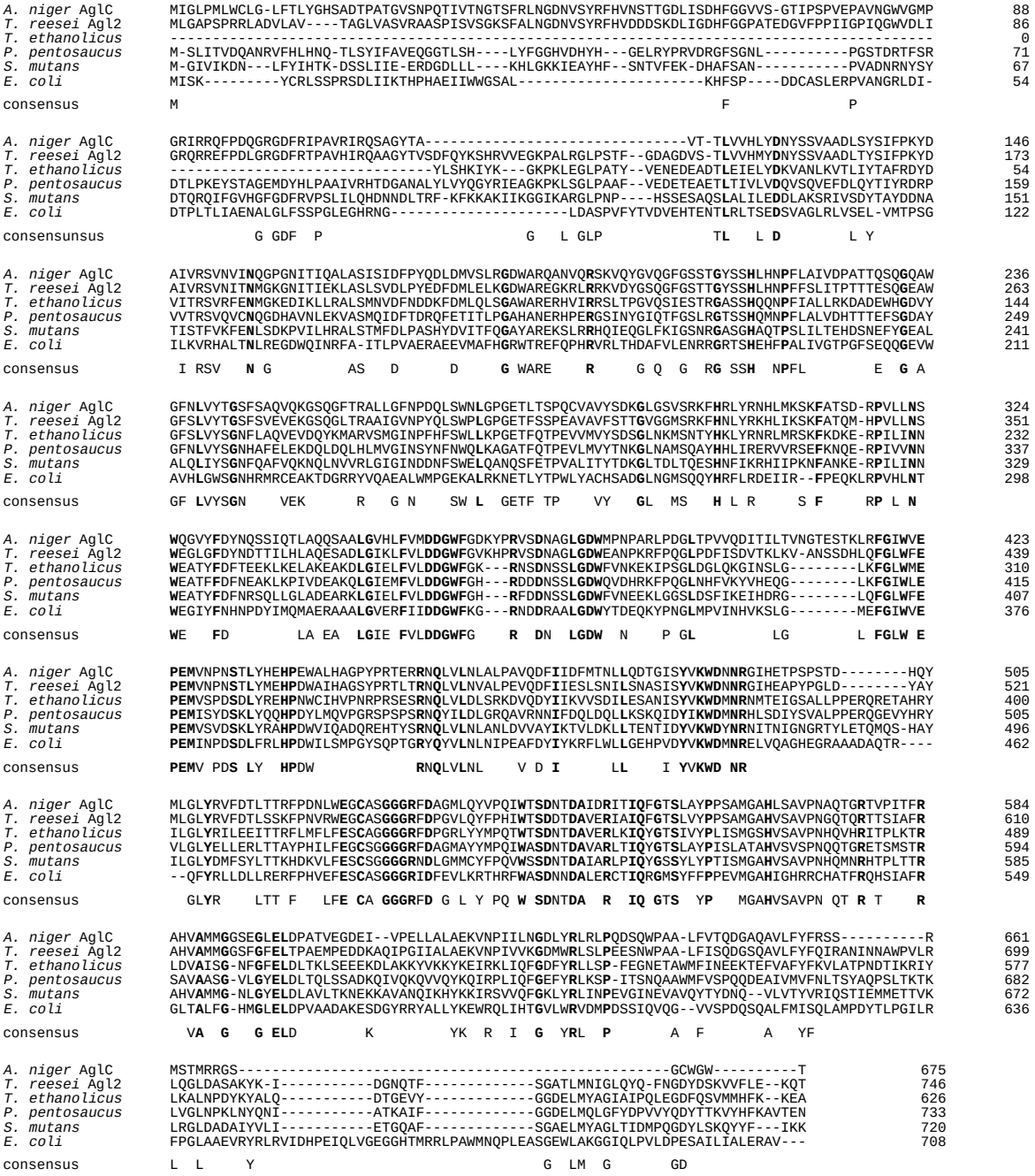

Fig. 3. Alignment of the amino acid sequences of α-galactosidases from *A. niger* (AglC; Knap et al., 1994), *T. reesei* (AglII; Margolles-Clark et al., 1996), *Thermoanaerobacter ethanolicus* (Zerlov), *Pediococcus pentosaceus* (Leenhouts et al.), *Streptococcus mutans* (Aduse-Opoku et al., 1991) and *Escherichia coli* (Aslanidis et al., 1989). In the consensus sequence, amino acids are depicted which are conserved in at least four of the six α-galactosidases. Amino acids, which are identical in all six α-galactosidases are in boldface type.

Expression of *aglA* was observed on galactose and galactose-containing oligosaccharides (lactose, melibiose, raffinose and stachyose) and polysaccharides (pectin, xylan, gum arabic, gum karaya, and locust bean gum). A low level of expression was observed on arabinose. The presence of glucose repressed the expression of *aglA* on galactose. The *aglB* gene was

expressed on all of the carbon sources tested, including glucose and fructose. Expression on xylan was very high, whereas elevated expression levels were detected on galactose and xylose. Expression of *agl*C was observed on glucose and fructose alone and on combinations of glucose with xylose and galactose.

High expression of *lacA* was observed on arabinose, xylose, xylan and pectin. Low levels of expression were detected on galactose and galactose-containing oligosaccharides (lactose, melibiose, raffinose, and stachyose) and polysaccharides (gum arabic, gum karaya, and locust bean gum). The presence of glucose reduced *lacA* expression on xylose.

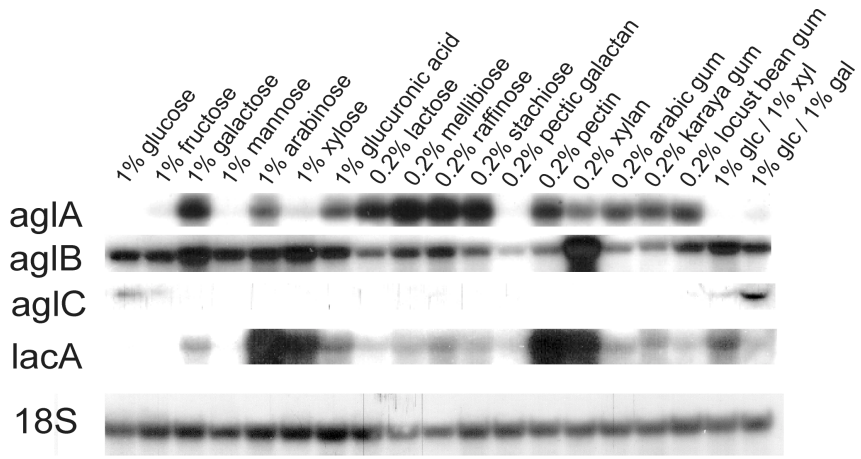

Fig. 4. Expression patterns of galactosidase genes from *A. niger* on different compounds after 4 h of transfer. The 18S rRNA served as a RNA loading control. Percentages are in mass per volume. Abbreviations: glc, glucose; xyl, xylose; gal, galactose.

Influence of XlnR on the expression of *aglB* and *lac*A

Based on the high expression of *lacA* on xylose, arabinose, xylan and pectin and of *aglB* on xylan, a second transfer experiment was performed to study the influence of the xylanolytic transcriptional activator (XlnR; van Peij et al, 1998a) on the expression of *aglB* and *lacA*. *A. niger* N402 and an XlnR$^-$ mutant (NXA1-4) were grown for 16 h in CM containing 2% (mass/vol) fructose as a carbon source at 30°C. The mycelium was harvested and washed with MM without a carbon source, and aliquots were transferred to MM containing different levels of xylose (1 and 0.03%, mass/vol) or arabinose (1%, mass/vol) as a carbon source. After a 2-h incubation period, mycelium was harvested and a Northern analysis was performed by using RNA isolated from the samples. Both the β-galactosidase- and α-galactosidase B-encoding genes were expressed on all three carbon sources in the wild-type strain (Fig. 5). The expression on 1-% xylose was lower than the expression on 0.03% xylose for both genes. In

the XlnR$^-$ mutant, no expression of *lacA* was detected, but low levels of *aglB* expression were still observed on xylose.

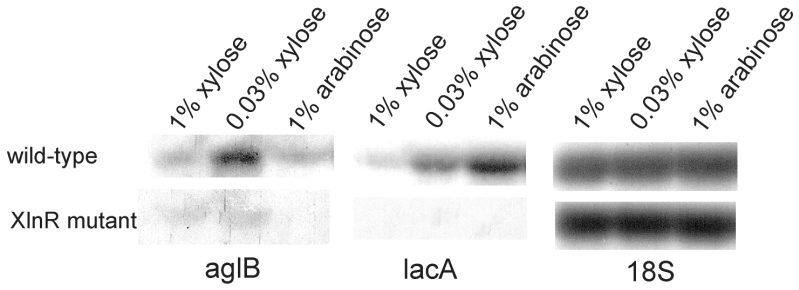

Fig. 5. Influence of XlnR on the expression of *lac*A and *agl*B. Northern blot analysis was performed after 2 h of transfer. The 18S rRNA served as a RNA loading control. Percentages are in mass per volume.

Discussion

Analysis of the derived amino acid sequence of AglB resulted in a molecular mass of 48,835 Da, whereas the experimentally determined molecular mass is 54-kDa (Manzanares et al., 1998). The difference in molecular mass suggests that AglB is a glycoprotein and that several of the putative N-glycosylation sites identified in the amino acid sequence are, in fact, glycosylated. The predicted pI of 4.6 is in good agreement with the experimentally determined pI value of 4.2 to 4.6 (Manzanares et al., 1998). The variation observed for the purified enzyme is most likely caused by different glycoforms of the enzyme.

The amino acid sequence of AglB is similar to the amino acid sequences of a number of other α-galactosidases of eukaryotic origin, including another α-galactosidase (AglA) from *A. niger* (den Herder et al., 1992) and an α-galactosidase (Agl1) from *T. reesei* (Manzanares et al., 1998). All of these enzymes belong to family 27 of the glycosyl hydrolases (Henrissat and Bairogh, 1996). Two regions with a high level of similarity can be identified by comparing the amino acid sequences of these enzymes, suggesting that they belong to a subfamily of α-galactosidases. AglC belongs to a different subfamily of α-galactosidases, together with *T. reesei* Agl2 (Margolles-Clark et al., 1996) and a number of bacterial α-galactosidases, which have been assigned to family 36 of the glycosyl hydrolases (Henrissat and Bairogh, 1996). A third α-galactosidase isolated from *T. reesei* (Agl3; Margolles-Clark et al., 1996) does not contain the conserved regions of either of these subfamilies and might therefore belong to yet another subfamily. Den Herder et al. (1992) suggested the presence of four different α-galactosidases in *A. niger* based on the analysis of α-galactosidase activities, which could be an indication of the presence of an *A. niger* homologue of *T. reesei* Agl3. However, several

glycoforms of AglB were previously isolated (Manzanares et al., 1998), indicating that the total number of α-galactosidases could be lower than four.

The expression of the four galactosidases studied here is specific for each gene. The carbon sources resulting in the highest expression of β-galactosidase-encoding *lacA* are xylose, arabinose, xylan, and pectin. The expression on arabinose is probably caused by the presence of a small amount of xylose in the arabinose that is commercially available (de Vries, unpublished data). Expression levels of a number of xylanolytic genes on xylose have been shown to be the result of a balance between XlnR mediated induction and CreA mediated repression of expression (Chapter 9). This appears also to be the case for *lacA* and would explain the different expression levels observed for the different xylose concentrations. The absence of *lacA* expression in the XlnR deficient mutant indicates that the expression of this gene on xylose and xylan is regulated by XlnR, as has been shown for a number of other genes (van Peij et al., 1998b). Production of β-galactosidase has been performed by using wheat bran (Park et al., 1979) that is rich in arabinoxylan, confirming the expression data obtained in this study. The function of LacA as a member of the xylanolytic spectrum may therefore be in removing β-linked galactose residues from xylan. The expression of *lacA* on galactose is much lower than the expression on arabinose or on xylose. Although β-galactosidase is commonly used for the hydrolysis of lactose (Wigley, 1996), plant β-galactosidases have been suggested to play a role in pectin degradation (Gross et al., 1995) and production of *Aspergillus* β-galactosidase on polygalacturonic acid has been reported (McKay, 1991). The expression of *lacA* on pectin observed in this study confirms that a pectin related compound is also able to induce *lacA* gene expression. As for xylan, this could indicate a role for LacA in the degradation of pectin by *A. niger*.

The expression of *aglA* was high on galactose and galactose-containing oligosaccharides but was fully repressed in the presence of glucose. No expression was observed on other carbon sources except arabinose and glucuronic acid, whereas moderate expression was also observed on galactose-containing gums. In contrast, *aglB* was expressed on all of the carbon sources tested. This suggests a basic level of expression of the gene, which is confirmed by the fact that the increase in expression in multicopy transformants is similar to the increase in copynumber. High levels of expression of *aglB* were observed on galactose, xylose, and beechwood xylan but not on galactose-containing oligo- and polysaccharides (other than xylan). The expression on glucose and fructose suggests that although the promoter of *aglB*

contains two putative CreA binding sites, *aglB* is not, or is only to a small extent, subject to CreA-mediated repression of gene expression. Previous studies demonstrated induction of α-galactosidases on galactomannan (Civas et al., 1984; den Herder et al., 1992), lactose (Manzanares et al., 1998; Riós et al., 1993), locust bean gum (Margolles-Clark et al., 1996), wheat and rice bran (Somiari and Balogh, 1995), and galactose (Riós et al., 1993). The *aglA* gene seems to represent an α-galactosidase, which is specifically induced on galactose. The high expression levels on galactose-containing oligosaccharides could indicate a preference for these structures (stachyose, melibiose, and raffinose) as the natural substrates. The *aglB* gene is expressed on all carbon sources at a high basal level. The product of this gene might therefore be important for the induction of other α-galactosidases by releasing small amounts of galactose from polymeric compounds. The high level of expression on xylan suggests a role for the xylanolytic activator XlnR in the expression of *aglB*. The results from the experiment with the XlnR$^-$ mutant confirm that XlnR has a function in the expression of *aglB*, although it is different from the effects observed for other xylanolytic genes (van Peij et al, 1998b). These genes are not induced on other sugars than xylose, and expression on xylose in the XlnR$^-$ mutant is abolished. The expression of *aglB* on xylose is decreased in the XlnR$^-$ mutant but not abolished. Thus, the expression of this gene does not exclusively depend on XlnR. The effect of XlnR does suggest a role for AglB in the xylanolytic spectrum, indicating that AglB might be involved in releasing α-linked galactose from the xylan backbone. The different expression levels at different xylose concentrations can't be explained as described above for *lacA*, since no indications for CreA-mediated repression were observed for aglB. The difference might be caused by a more indirect effect, possibly mediated by CreA, in xylanolytic induction of gene expression. The xylanolytic genes tested for modulation of expression on xylose (Chapter 9) also did not have identical expression patterns at different xylose concentrations, but for all of the genes expression decreased with increasing xylose concentrations. The results in this paper demonstrate that the xylanolytic activator XlnR is also involved in the regulation of an α- and a β-galactosidase gene of *A. niger*, emphasising its key role in hemicellulose degradation.

The expression pattern of *aglC* is remarkable for a gene encoding an α-galactosidase. Expression was only observed on glucose, fructose, or combinations containing glucose. Similar results have been obtained for *T. reesei agl3*, when expression of the latter and of a number of other hemicellulase genes was studied on a set of different carbon sources (Margolles-Clark et al., 1997). Expression was only observed on cellulose, sorbitol and

glucose but not on galactose, xylose or other monomeric or polymeric compounds. The *agl*C gene of *A. niger* has been clearly demonstrated to encode α-galactosidase activity by using *p*-nitrophenyl-α-D-galactoside, raffinose, and stachyose as substrates, although no activity was found by using guar gum (Knap et al., 1994). This could be an indication that AglC activity is specific for galactose residues linked to glucose or fructose in galactose-containing oligosaccharides such as raffinose and stachyose and that the gene is therefore only expressed in the presence of glucose.

Whether the expression of the four genes tested indeed mirrors the substrate preferences of the encoded enzymes requires further study, involving activity measurements of the enzymes against oligo- and polysaccharides. It is clear that the differences in expression of the genes will result in specific enzyme spectra on different polymeric substrates. The polymeric substrate used to produce *A. niger* enzyme preparations will therefore reflect its composition.

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Chapter 9

CreA modulates the XlnR induced expression on xylose of *Aspergillus niger* genes involved in xylan degradation

R. P. de Vries, J. Visser and L. H. de Graaff

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Abstract

The expression of the feruloyl esterase gene *fae*A, the α-glucuronidase gene *agu*A, the endo-xylanase gene *xln*B and the β-xylosidase gene *xln*D from *Aspergillus niger* on xylose has been studied in a wild-type strain and in a CreA mutant. A decrease in expression of all four genes was observed with increasing xylose concentrations in the wild-type strain, whereas expression levels in the CreA mutant were not influenced. The results in the wild type indicate that xylose concentrations higher than 1 mM result in repression of the expression of the xylanolytic genes tested mediated by the carbon catabolite repressor protein CreA. On xylose the expression levels of the xylanolytic genes are therefore not only determined by induction via XlnR but also by repression via CreA. The genes tested are not influenced to the same extent by XlnR or CreA resulting in specific expression levels and patterns for each individual gene.

Introduction

Degradation of xylan by filamentous fungi is widely studied especially in *Aspergillus* and *Trichoderma* species (see for example Visser et al., 1992). These fungi have a wide spectrum of genes encoding xylanolytic enzymes, such as endoxylanases (Törrönen et al., 1992; de Graaff et al., 1994) β-xylosidase (Margolles-Clark et al., 1996a; van Peij et al., 1997) α-glucuronidase (Margolles-Clark et al., 1996b; Chapter 7), feruloyl esterase (Chapter 3) and acetylxylan esterase (de Graaff et al., 1992a). All these genes are expressed on xylan and xylose and repressed in the presence of glucose. In *A. niger* the transcriptional activator XlnR (van Peij et al., 1998a) directs the expression of these genes and of some cellulolytic genes (van Peij et al., 1998b). Repression of xylanolytic genes in the presence of glucose has been assigned to the carbon catabolite repressor protein CreA in *A. niger* (de Graaff et al., 1994) and to its homologue Cre1 in *T. reesei* (Strauss et al., 1995). This protein is the major factor responsible for carbon catabolite repression in *Aspergillus* and it's structure and mechanism has been studied in detail in *Aspergillus nidulans* (Cubero and Scazzocchio, 1994; Shroff et al., 1996; Panozzo et al., 1998).

Glucose and fructose strongly repress the expression of many genes including the xylanolytic genes. However, carbon catabolite repression is not restricted to these sugars alone and other monosaccharides such as xylose and arabinose have also been shown to have a repressing effect on the expression of specific genes (Ruijter et al., 1997). We have studied whether the expression of the xylanolytic genes in *A. niger* is influenced by a xylose mediated CreA effect

by comparing the expression of four of these genes (*fae*A, *agu*A, *xln*B and *xln*D) on different xylose concentrations in an *A. niger* wild-type strain and in a CreA mutant. This mutant is not a complete loss of function mutant, but has a severely derepressed phenotype with respect to the regulation of arabinofuranosidases and functions involved in arabinose catabolism (Ruijter et al., 1997).

Materials and Methods

Strains and growth conditions

All strains were derived from *A. niger* N400 (=CBS 120.49). *A. niger* N402 (*csp*A1) was used as a wild type. *A. niger* NW200 (*cre*Ad4, *bio*A1, *are*A1::pAREG1, *pyr*A13::pGW635) was derived from *A. niger* NW145 (Ruijter et al., 1997) by restoring the *are*A1 defect via a co-transformation with pGW635 (containing the *pyr*A gene as a selection marker) and pAREG1 (containing the *are*A gene; McCabe et al., 1998) and has a derepressed phenotype for CreA function. Cultures were grown in minimal medium (MM) containing per litre: 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, trace elements (Vishniac and Santer, 1957), pH 6.0 or complete medium (CM = MM + 2 g/l peptone, 1 g/l casamino acids, 1 g/l yeast extract, 0.5 g/l yeast ribonucleic acids, pH 6.0) with carbon sources as indicated in the text, in a rotary shaker at 250 rpm and 30°C. For the growth of strains with auxotrophic mutations, the necessary supplements were added to the medium.

Transfer experiments were performed by growing the strains for 16 h in 300 ml CM containing 2% (mass/vol) fructose as carbon source. The mycelium was harvested by suction over a filter and washed with MM without a carbon source. One gram aliquots (wet weight) of the mycelium were transferred to 50 ml MM with different carbon sources and incubated for 2 h. The mycelium was harvested again, dried between paper and frozen in liquid nitrogen. The mycelium samples were stored at -70°C to be used for RNA isolation.

Table 1. Probes used in Northern analysis.

Gene	EMBL	Enzyme	Fragment	Reference
*agu*A	Y15405	α-glucuronidase A	0.8 kb *Eco*RV/*Kpn*I	de Vries *et al*, 1998
*fae*A	Y09330	feruloyl esterase A	0.5 kb *Eco*RV/*Xho*I	de Vries *et al*, 1997
*xln*B	D38071	endoxylanase B	0.9 kb *Eco*RI/*Xho*I	Kinoshita *et al*, 1995
*xln*D	Z84377	β-xylosidase	2.8 kb *Pst*I/*Nsi*I	van Peij *et al*, 1997
18S	X78538	18S rRNA subunit	0.7 kb *Eco*RI	Melchers *et al*, 1994

Northern blot analysis

Total RNA was isolated from powdered mycelium using TRIzol Reagent (Life Technologies), according to the supplier's instructions. For northern blot analysis 5 µg total RNA was incubated with 3.3 µl 6 M glyoxal, 10 µl DMSO and 2 µl 0.1 M sodium phosphate buffer pH 7 in a total volume of 20 µl for 1 h at 50°C to denature the RNA. The RNA samples were separated on a 1.5% agarose gel using 0.01 M sodium phosphate buffer pH 5 and transferred to Hybond-N filters (Amersham) by capillary blotting. Filters were hybridised at 42°C in a solution of 50% (vol/vol) formamide, 10% (mass/vol) dextran sulphate, 0.9 M NaCl, 90 mM Na_3-citrate, 0.2% (mass/vol) ficoll, 0.2% (mass/vol) polyvinylpyrolidone, 0.2% (mass/vol) bovine serum albumin, 0.1% (mass/vol) SDS and 100 µg/ml

single stranded herring sperm DNA. Washing was performed under homologous conditions to 30 mM NaCl, 3 mM Na_3-citrate and 0.5% (mass/vol) SDS at 68°C. The probes used are described in Table 1. The 18S probe was used as a RNA loading control.

Concentration determination of xylose and glucose by HPLC

Culture filtrates were diluted with water to equal a 1 mM starting concentration and analysed by high-performance anion-exchange chromatography (HPAEC) system (Dionex Corp., Sunnyvale, Calif.) equipped with a pulsed amperometric detector (PAD. Samples were injected in a Carbopac PA 100 column (25 × 4 mm; Dionex Corp., Sunnyvale, Calif.) and eluted with 0.05 M NaOH for 10 min at a flow of 1 ml/min. Concentrations were calculated using xylose and glucose as the standards.

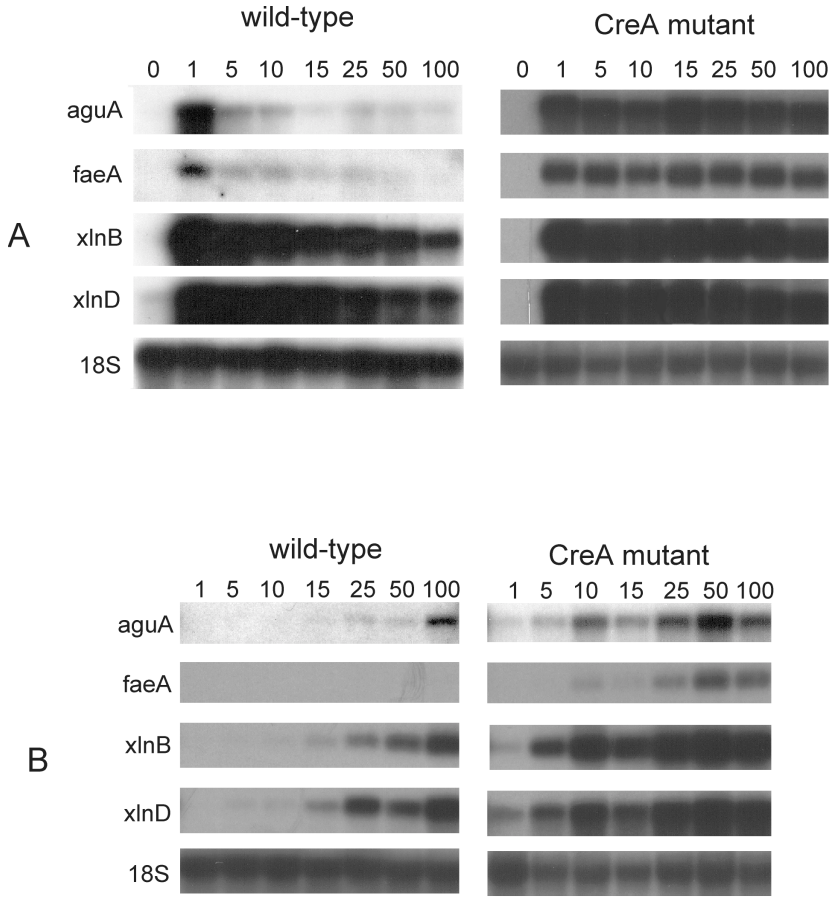

Fig.1. Expression of *A. niger* xylanolytic genes at different xylose concentrations.
The expression of four xylanolytic genes was compared at different xylose concentrations in the absence and presence of 50 mM glucose. Panel A: expression at different xylose concentrations (mM); panel B: expression at different xylose concentrations (mM) in the presence of 50 mM glucose.

Results and discussion

The expression of the genes encoding the accessory enzymes feruloyl esterase and α-glucuronidase has been compared with that of two genes encoding functions involved in degradation of the xylan backbone *viz* endoxylanase B and β-xylosidase. In the wild-type strain

the mRNA levels of the four genes tested were highly dependent on the xylose concentration. The highest expression of all genes was observed at the lowest xylose concentration tested (1 mM). A decrease in expression was observed with an increasing xylose concentration, which was gradual for *xln*B and *xln*D but more abrupt for the side chain removing functions (Fig. 1A). In the CreA mutant, no significant differences in expression level were observed over the xylose concentration range tested (1 - 100 mM). The consumption of xylose over the 2 h incubation period is comparable for the different xylose concentrations, indicating that the differences observed are not caused by starvation effects (Table 2).

Table 2. Extracellular concentrations of xylose and glucose after a 2 h transfer of *Aspergillus niger* pregrown on fructose.

	wild-type (N402)		CreA-mutant (NW200)	
	xylose	glucose	xylose	glucose
Initial concentration	(mM)		(mM)	
0 mM xylose	0	0	0	0
1 mM xylose	0.3	0	0.5	0
5 mM xylose	4.4	0	3.8	0
10 mM xylose	9.0	0	8.7	0
15 mM xylose	13.7	0	13.4	0
25 mM xylose	23.8	0	22.6	0
50 mM xylose	46.8	0	42.9	0
100 mM xylose	91.4	0	92.1	0
1 mM xylose – 50 mM glucose	0.7	48.9	0.6	42.5
5 mM xylose – 50 mM glucose	4.6	45.8	4.3	38.2
10 mM xylose – 50 mM glucose	9.8	44.9	9.9	34.0
15 mM xylose – 50 mM glucose	14.7	42.9	13.5	34.2
25 mM xylose – 50 mM glucose	23.9	44.7	21.4	38.7
50 mM xylose – 50 mM glucose	48.7	45.3	39.6	42.4
100 mM xylose – 50 mM glucose	95.8	43.7	71.5	35.9

The results described here indicate that high xylose concentrations cause a significant repression of the expression of xylanolytic genes in particular of the *agu*A and *fae*A genes. This is mediated by the carbon catabolite repressor CreA. The actual level of expression will therefore be influenced by the balance between induction by XlnR and repression by CreA. The level of expression due to induction by XlnR varies for the individual genes. Similarly, the degree of repression by CreA varies. Both effects will depend on number and the position of the functional

regulatory sequences in the promoters of the xylanolytic genes. As a result of this, not only the absolute expression levels of the genes are different, but also the decrease in expression with increasing xylose concentrations can be different for each gene.

In previous studies xylose has mainly been regarded as an inducing carbon source and expression of xylanolytic genes has always been studied in general at relatively high xylose concentrations (approximately 30-70 mM; de Graaff et al., 1992b and 1994; Gouka et al., 1996; Mach et al., 1996; Gaspar et al., 1997; van Peij et al., 1997; Chapter 3 and 7). The expression level of these genes on xylan was usually higher than on xylose. For *A. niger* this can now be explained based on the results reported in this paper. Xylose is released gradually from xylan resulting in a low xylose concentration in the medium. Under these conditions, repression by xylose mediated by CreA is low and high expression levels are detected. However, in the medium containing approximately 70 mM xylose, repression through CreA plays a more important role, resulting in decreased expression levels. It is possible that the same modulation occurs in other filamentous fungi, such as *T. reesei* as a homologue of CreA (Cre1)was detected (Strauss et al., 1995).

The expression of the same genes was also studied at different xylose concentrations in the presence of 50 mM glucose (Fig 1B). In the case of *xln*B and *xln*D, expression in the wild type (*A. niger* strain N402) is observed in the range of 10-15 mM xylose, for *agu*A this starts at 25 mM whereas no *fae*A expression is detected at any xylose concentration in the wild type. In the CreA mutant a gradual increase in expression is observed with increasing xylose concentrations. The expression of *agu*A and *fae*A remains much weaker than in the presence of xylose alone. The observed effects can be explained by residual CreA repression due to the fact that the CreA mutant used is not a complete loss of function mutation. An alternative and more likely explanation could be that glucose and xylose compete for the same transport system. When the xylose concentration increases, the balance will shift and more xylose will be taken up resulting in an increased expression of the xylanolytic genes. Results obtained previously using protoplasts and different concentrations of glucose and xylose (de Graaff et al., 1992b) demonstrate the same effect. The glucose consumption is much higher in the CreA mutant than in the wild-type strain (Table 2), indicating that CreA may be involved in down regulating glucose transport systems.

Although the molecular details for our observations are not precisely known yet it is important to notice that expression studies on the xylanolytic system in fungi should not only focus on the role of xylose as an inducer, but also as a factor triggering carbon catabolite repression.

Depending on the set-up of the experiment (growth conditions, incubation time) xylose concentrations of 30 to 70 mM can be too high to study the induction of the xylanolytic system.

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Chapter 10

Synergy between accessory enzymes of *Aspergillus* in the degradation of wheat arabinoxylan

R. P. de Vries, H. C. M. Kester, C. H. Poulsen, and J. Visser

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Abstract

Synergy in wheat arabinoxylan degradation was studied using two main chain cleaving enzymes (endo-xylanase and β-xylosidase) and six accessory enzymes (α-L-arabinofuranosidase, arabinoxylan arabinofuranohydrolase, α-glucuronidase, α-galactosidase, β-galactosidase and feruloyl esterase) from *Aspergillus*. These enzymes were used, alone and in combinations, in incubations using water insoluble pentosan (WIP) isolated from wheat flour. Synergy was observed between most enzymes tested, although not always to the same extent. Degradation of the xylan backbone by endoxylanase (XlnA) and β-xylosidase (XlnD) was influenced most strongly by the action of α-L-arabinofuranosidase (AbfB) and arabinoxylan arabinofuranohydrolase (AxhA), whereas α-glucuronidase (AguA) and feruloyl esterase (FaeA) had the largest influence on arabinose release by AbfB and AxhA. Ferulic acid release by FaeA and the release of 4-O-methyl glucuronic acid by AguA depended largely on the degradation of the xylan backbone by XlnA.

Introduction

Xylans are the major hemicellulolytic structures found in nature, and are important for the structural integrity of the plant cell wall. Although all xylans contain a β-1,4-xylose backbone, large differences exist with respect to the sidegroups attached to the backbone. The major substituent of wheat arabinoxylan is L-arabinose, but D-galactose, D-(4-O-methyl-) glucuronic acid, and ferulic acid residues are also present (Brillouet and Joseleau, 1987; Schooneveld-Bergmans et al., 1998). Arabinose can be present as single α-1,3- or α-1,2-linked residues (Kormelink et al., 1993a) or as short arabino-oligosaccharides. Galactose has been detected β-1,5-linked to single arabinose residues as well as β-1,4-linked to xylose in short arabinoxylan side chains (Ebringerova et al., 1990; Wilkie and Woo, 1977) Feruloyl esters are detected at C5 of the arabinose residues (Borneman et al., 1986) and glucuronic acid and its 4-O-methyl ether are attached to the xylan main chain via an α-1,2-linkage (Wilkie and Woo, 1977). Acetyl residues have not been detected in wheat xylans.

Due to heterogeneity in the composition and structure of wheat arabinoxylan, a wide range of enzymes is required for the biodegradation of this polysaccharide. Xylanolytic enzymes have been purified from many micro-organisms, especially from species of the fungus *Aspergillus*. Previously synergism between endo-xylanases and some accessory enzymes of *Aspergillus*

has been demonstrated (Chapter 3 and 7; Wood and McCrae, 1996). However synergy between accessory enzymes involved in xylan degradation has not been studied in detail.

In this paper we have studied 8 xylanolytic enzymes (Table 1), with respect to their individual activity on wheat arabinoxylan and the synergism between these enzymes. The genes encoding these enzymes are all expressed when *A. niger* is grown on xylan. Except for AbfB, all genes are regulated by the xylanolytic transcriptional activator XlnR (van Peij et al., 1998). We have chosen water insoluble pentosan (WIP) from wheat flour as a substrate for our studies, because this polysaccharide contains all the sidegroups (accept acetyl residues) commonly found in xylans. A comparison has been made between incubations with intact WIP and incubations with WIP which was pre-treated with endoxylanase A to determine whether there was a difference between simultaneous and sequential activity of main-chain cleaving and accessory enzymes.

Table 1. Origin and amounts of the enzymes used in this study.

Enzyme	symbol	origin	amount used (μg[1])	reference
Endoxylanase A	XlnA	*A. tubingensis*	1.0	Danisco A/S
β-Xylosidase	XlnD	*A. niger*	2.5	van Peij et al., 19997
Arabinofuranosidase B	AbfB	*A. niger*	10.1	van Veen et al., 1991
Arabinoxylan arabinofuranohydrolase A	AxhA	*A. tubingensis*	2.5	Danisco A/S Gielkens et al., 1997
α-Glucuronidase	AguA	*A. tubingensis*		Chapter 7
α-Galactosidase	AglB	*A. niger*	0.5	Manzanares et al., 1998
β-Galactosidase	LacA	*A. niger*	2.1	Manzanares et al., 1998
Feruloyl esterase A	FaeA	*A. niger*	0.5	Chapter 3

[1] As added to 500 μl of 0.5% WIP in the enzyme incubations.

Experimental

Sugar and ferulic acid analysis of water insoluble pentosan from wheat arabinoxylan

Water insoluble pentosan (WIP) from wheat arabinoxylan was isolated by the method of Gruppen et al (1989). The sugar analysis was based on the work of Englyst and Cummings (1984) and involved acid hydrolysis of WIP with 1M sulphuric acid after which the released monomers were determined by HPLC using a Carbopac PA1 column (Dionex Corp., Sunnyvale, Ca) and a pulsed electrochemical detector. The ferulic acid content was determined as described previously (Faulds et al., 1995).

Xylan incubations

The amount of enzyme necessary when acting alone to obtain the maximum amount of released monomeric compound after 24 h of incubation was determined by incubating WIP with the individual enzymes and carrying out a time-dependent analysis of the amount of monomeric compound released. The amounts of the pure enzymes which were applied are indicated in Table 1. Enzyme incubations were all performed in duplicate, using 500 µl of a 0.5% WIP suspension in 50 mM sodium acetate buffer (pH 4.5) at 30°C for 24 h. The enzymes were inactivated by heating them in a boiling water bath for 5 min. Undissolved material was precipitated by centrifugation (14.000 × g, 10 min) and the supernatant was transferred to a new tube and stored at -20°C.

Sugar and ferulic acid analysis of the incubations

Ferulic acid concentrations in the incubations were analysed by HPLC with a type RP-8 reverse phase column (Supelco, Bellefonte, Pa). Separation was achieved by using a linear gradient starting with 32%, 0.3%, 67.7% (vol/vol) methanol, acetic acid, water and ending with 64%, 0.3%, 35.7% (vol/vol) methanol, acetic acid, water with detection at 325 nm. 4-O-methyl-glucuronic acid concentrations were also determined by HPLC using a Carbopac PA-100 column (Dionex Corp., Sunnyvale, Ca). Separation was achieved by using a linear gradient starting with 100 mM NaOH, 5 mM Na-acetate and ending with 100 mM NaOH, 400 mM Na-acetate over a 20 min period at a flow of 1 ml/min. Xylose, arabinose and galactose concentrations were determined using a Carbopac MA-1 column (Dionex Corp., Sunnyvale, Ca.) and isocratic elution with 0.48 M NaOH. Detection of both 4-O-methyl-glucuronic acid and neutral sugars was carried out using a pulsed amperometric detector (PAD).

Results and discussion

Analysis of the composition of water insoluble pentosan (WIP) from wheat flour

The sugar and ferulic acid content of wheat arabinoxylan were analysed as described in Experimental. Xylose and arabinose were the major neutral sugars present in the arabinoxylan sample (Table 2) and only a small amount of galactose was detected (2.1%). The sample also contained ferulic acid (0.49%) and 4-O-methyl glucuronic acid (6%). No acetyl residues were detected in the WIP sample. The isolation of WIP did not prevent low amounts of glucose and cellulose to be present in the sample.

Table 2. Sugar and ferulic acid analysis of water insoluble pentosan (WIP) from wheat flour.

Component	% of total WIP (mass)	Component	% of total WIP (mass)
Xylose	32.1	Ferulic acid	0.49
Arabinose	20.7	Glucose	11.2
Galactose	2.1	Cellulose	9.4
4-O-methyl glucuronic acid	6.0		

Degradation of the xylan backbone is highly dependent on the removal of arabinose residues by AbfB and AxhA

XlnA alone was able to release 4.4% of the total amount of xylose from WIP but in combination with XlnD 52.4% was released (Table 3). AbfB and AxhA acted synergistically with both XlnA and XlnD resulting in increased xylose release, while in the absence of XlnC or XlnD they did not release any xylose. The presence of AglB, LacA, AguA and FaeA resulted in a minor increase in the amount of xylose released by XlnA (data not shown). Since all enzymes increased the amount of xylose liberated by XlnA, it can be concluded that XlnA prefers unsubstituted regions of xylan as a substrate. In particular removal of the arabinose residues from the xylan backbone enables XlnA to further degrade the polymer resulting in increased amounts of xylose. This observation suggests that XlnA is able to cleave the linkage between a terminal xylose residue and the adjacent residue. Whether this action is from the reducing or non-reducing end of the polymer can not be concluded from these results. Studies of the action of the homologous enzyme purified from *Aspergillus awamori* have demonstrated that, counting from the reducing end, at least two unsubstituted xylose residues adjacent to singly or doubly substituted xylose cannot be removed by XlnA (Kormelink et al., 1993a and 1993b). The high proportion of side groups present in the wheat arabinoxylan studied in this paper explains the large effect of the addition of accessory enzymes on XlnA

Table 3. Influence of accessory enzymes on the release of xylose by XlnA and XlnD from water insoluble pentosan (WIP) from wheat flour. Values are the percentage of the total amount of xylose present in the WIP sample. N.D. means not determined. Absolute variations between duplicate incubations were all between 0.1% and 0.6%.

Enzymes	Intact WIP	WIP treated with XlnA
XlnA	4.4	N.D.
XlnA / AbfB	10.9	6.5[1]
XlnA / AxhA	8.9	4.8[1]
XlnA / AbfB / AxhA / FaeA	16.8	N.D.
XlnA / XlnD	52.4	N.D.
XlnD	12.6	21.9
XlnD / AbfB	19.6	45.2
XlnD / AxhA	23.3	71.6
XlnA / XlnD / AbfB / AxhA / AguA / AglB / LacA / FaeA	98.7	99.6[1]

[1] XlnA was only used for the pre-treatment, but is not present in the incubations.

activity. A pre-treatment with XlnA followed by incubations with AbfB and AxhA released less xylose than incubations of WIP with a combination of XlnA with AbfB or AxhA, indicating synergy when both enzymes act simultaneously. However, incubation of WIP with eight enzymes or a pre-treatment with XlnA followed by incubations with the other seven enzymes both resulted in complete degradation of the xylan backbone. This implies that XlnD is able to release all the xylose from the oligosaccharides which arose from the XlnA pre-treatment. XlnD cleaves terminal xylose residues from the non-reducing end of xylo-oligomers. The increase observed when accessory enzymes are added indicates a preference for unsubstituted terminal xylose residues, and illustrates the necessity for the presence of accessory enzymes for the complete degradation of the xylan backbone.

The release of arabinose from water insoluble pentosan (WIP) from wheat flour, by both AbfB and AxhA, is not only influenced by the main chain cleaving enzymes (XlnA and XlnD), but also by other accessory enzymes

AbfB is able to release 40.9% and AxhA 23.7% of the arabinose present in wheat arabinoxylan (Table 4). Incubation with both enzymes releases 62.2% of the arabinose present, suggesting that the action of the enzymes is complimentary and liberate different arabinose residues. Previous studies indicated that AxhA is able to release α-1,2- and α-1,3-linked arabinose residues from both terminal and non-terminal singly-substituted xylose residues, whereas AbfB can only release arabinose from terminal singly substituted xylose residues (Kormelink et al., 1993a) The data presented in this study suggest that AbfB is capable of removing arabinose residues from non-terminal xylose residues, since incubations on intact arabinoxylan with this enzyme result in release of 40% of the total amount of arabinose present. Arabinose release by AxhA is strongly increased in the presence of XlnA, but this is not the case for AbfB. The addition of AguA and FaeA result in similar relative increases in arabinose released by both AbfB and AxhA, demonstrating that the presence of 4-O-methyl glucuronic acid or ferulic acid residues limits the activity of both enzymes. In the case of ferulic acid this suggests that AbfB and AxhA are not able to release feruloylated arabinose residues from arabinoxylan. 4-O-methyl glucuronic acid is attached directly to the xylan backbone. The negative effect of this sidegroup is most likely due to a steric effect. XlnD positively influences the activity of AxhA on both intact wheat arabinoxylan and arabinoxylan pre-treated with XlnA. Although the presence of XlnD increases arabinose

Table 4. Arabinose release from water insoluble pentosan (WIP) from wheat flour by AbfB and AxhA. The values are the percentage of the total amount of arabinose present in the WIP sample. N.D. means not determined. Absolute variations between duplicate incubations were all between 0.1% and 1.0%.

	Intact WIP			WIP pre-treated with XlnA		
Additional enzymes	AbfB	AxhA	AbfB / AxhA	AbfB	AxhA	AbfB / AxhA
-	40.9	23.7	62.2	54.3	35.6	99.8
XlnA	43.6	37.8	86.4	N.D.	N.D.	N.D.
XlnD	52.0	29.2	83.1	49.8	38.5	92.7
AguA	54.5	29.3	N.D.	59.4	40.6	N.D.
AglB	42.9	28.4	N.D.	58.4	41.5	N.D.
LacA	46.1	29.8	N.D.	55.2	40.0	N.D.
FaeA	52.5	28.3	67.9	58.9	42.1	97.3
XlnA / FaeA	53.9	30.5	91.4	N.D.	N.D.	N.D.

release by AbfB from intact arabinoxylan, it reduces the amount of arabinose released from pre-treated arabinoxylan. Incubations of pre-treated WIP with a AbfB alone released 54.3% of the total amount of arabinose, whereas addition of XlnD resulted in a release of 49.3% (Table 4). Similarly, incubations of pre-treated WIP with a combination of AbfB, AxhA, AguA, AglB, LacA and FaeA released 99.6% of the total amount of arabinose, while addition of XlnD to this combination resulted in a release of 87.1%. These data suggest a preference of AbfB for longer xylo-oligosaccharides. Although this is not the case for AxhA, this enzyme is not able to compensate for the reduced AbfB activity in mixed incubations, indicating again that AbfB and AxhA act on different arabinose residues of wheat arabinoxylan.

LacA and AglB positively influence the arabinose release by AbfB and AxhA. LacA and AglB alone released only 0.4% and 1.2% respectively, of the total amount of galactose present in the WIP sample (data not shown). Incubations combining these enzymes with XlnA, XlnD, AbfB, AxhA, AguA and FaeA released 5.6% of the galactose present. It is possible that the WIP sample contains a small amount of arabinogalactan, reducing the amount of galactose actually attached to arabinoxylan. Galactose has been demonstrated to be β-1.4-linked to arabinose in arabinoxylan (Wilkie and Woo, 1977), which would be consistent with the effect observed for LacA in this study. No reports about α-linked galactose in arabinoxylan have been published. The data described here, combined with the fact that AglB and LacA are strongly induced on xylan (de Vries ate al, 1999), suggest that wheat arabinoxylan contains both α- and β-linked galactose and that AglB and LacA are indeed members of the xylanolytic

spectrum of *A. niger*. Whether α-linked galactose is also attached to arabinose is not clear. The positive influence of AglB on arabinose release could also be caused by a steric effect as is suggested above for AguA .

Ferulic acid release by FaeA depends largely on the degradation of the arabinoxylan backbone

FaeA alone was able to release 14.4% of the total amount of ferulic acid present in wheat arabinoxylan (Table 5). The addition of AbfB or AxhA resulted in only a minor increase in ferulic acid release, whereas addition of XlnA increased the amount of ferulic acid released to 55%. After a XlnA pre-treatment FaeA alone was able to release 40.8% of the ferulic acid and up to 50.4% in combination with other accessory enzymes. Incubations with FaeA, XlnA, AbfB and AxhA on intact xylan released more than 96% of the total amount of ferulic acid. This demonstrates that a combined enzymatic treatment results in a higher release than consecutive treatments with individual enzymes. FaeA was shown previously to be active on an arabinose-ferulic acid dimer as well as on small xylo-oligosaccharides containing arabinose linked ferulic acid (Ralet et al., 1994). Our data indicate that the action of endo-xylanase A is a crucial step to allow efficient removal of ferulic acid residues by FaeA, which is then further enhanced by AbfB and AxhA.

Table 5. Influence of main chain cleaving enzymes and accessory enzymes on ferulic acid release by FaeA from water insoluble pentosan (WIP) from wheat flour. Values are the percentage of the total amount of ferulic acid present in the WIP sample. N.D. means not determined. Absolute variation between duplicate incubations were all between 0.1% and 0.9%.

Enzymes	Intact WIP	WIP treated with XlnA
FaeA	14.4	40.8
FaeA / AbfB	16.2	44.6
FaeA / AxhA	16.9	43.4
FaeA / AbfB / AxhA	16.6	45.1
FaeA / XlnA	55.0	N.D.
FaeA / AbfB / AxhA / XlnA	96.9	N.D.
FaeA / AbfB / AxhA / AguA / XlnD /AglB / LacA	17.2	50.4
FaeA / AbfB / AxhA / AguA / XlnD / AglB / LacA / XlnA	97.5	N.D.

The release of 4-O-methyl glucuronic acid by AguA depends on an interaction with XlnA, as well as on the activity of other accessory enzymes

AguA alone released 0.6% of the total amount of 4-O-methyl glucuronic acid from intact wheat arabinoxylan and 6.7% from wheat arabinoxylan pre-treated with XlnA. Incubations with a combination of AguA and XlnA on intact arabinoxylan released 86.7% of the total amount of 4-O-methyl glucuronic acid. Previously, a low level of synergy between AguA and a endoxylanase A from *Aspergillus tubingensis* has been demonstrated (Chapter 7).

Table 6. Influence of XlnA and accessory enzymes on the release of 4-O-methyl glucuronic acid by AguA from water insoluble pentosan (WIP) from wheat flour. Values are the percentage of the total amount of 4-O-methyl glucuronic acid present in the WIP sample. N.D. means not determined. Absolute variations between duplicate measurements were all between 0.1% and 0.9%.

Enzymes	Intact WIP	WIP treated with XlnA
AguA	0.6	6.7
AguA / XlnA	86.7	N.D.
AguA / AbfB	3.2	16.2
AguA / AxhA	1.6	12.3
AguA / AglB / LacA / AbfB / AxhA / FaeA	3.8	59.2
AguA / AglB / LacA / AbfB / AxhA / FaeA / XlnD	6.2	72.8
AguA / AglB / LacA / AbfB / AxhA / FaeA / XlnD / XlnA	88.9	N.D.

The strong effect observed in this study can be attributed to the ability of XlnA to remove terminal xylose residues from xylan, which becomes an important factor when the incubation time is longer (24 h in this study compared to 30 min; Chapter 7). The addition of other accessory enzymes to such an incubation does not significantly increase the amount of 4-O-methyl glucuronic acid released. In incubations using pre-treated arabinoxylan, the addition of other accessory enzymes has a strong positive effect on the release of 4-O-methyl glucuronic acid by AguA, although the release does not reach the level of the simultaneous XlnA / AguA treatment. This indicates that the oligosaccharides which result from an incubation with XlnA alone are different from those arising when AguA is present. This also implies that the presence of 4-O-methyl glucuronic acid residues on the xylan backbone limits the degradation of this polymer by XlnA. Mainly the release of 4-O-methyl glucuronic acid is strongly influenced in combination with XlnA. Nearly all the ferulic acid, arabinose, and xylose can be released from arabinoxylan pre-treated with XlnA.

The data in this study demonstrate that the synergy between the various xylanolytic enzymes in the degradation of wheat arabinoxylan is not just based on the interactions between main chain cleaving enzymes and accessory enzymes, but is a more complex phenomenon. Synergy also occurs between the accessory enzymes resulting in an efficient and complete degradation of this heterogeneous polysaccharide. These data are in agreement with the induction of the genes encoding the xylanolytic enzymes which are under the co-ordinated regulation of the xylanolytic transcriptional activator XlnR (van Peij et al., 1998; Chapter 8).

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Chapter 11

Synergy between accessory enzymes of *Aspergillus* involved in the degradation of sugar beet pectin

R. P. de Vries, H. C. M. Kester, J. A. E. Benen and J. Visser

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Abstract

Synergy in the breakdown of the hairy regions of sugar beet pectin was studied using enzymes from *Aspergilli* involved in the degradation of the pectin side chains (arabinofuranosidase, endoarabinase, β-galactosidase, endogalactanase, and feruloyl esterase). Enzyme incubations, using these five side chain degrading enzymes alone and in combinations, were performed using sugar beet pectin and sugar beet pectin pre-treated with rhamnogalacturonan hydrolase and rhamnogalacturonan acetyl-esterase and analysed for the release of arabinose, galactose, and ferulic acid. The pre-treatment resulted in a two-fold increase in the release of galactose by β-galactosidase and endogalactanase, but did not significantly influence the arabinose release by arabinofuranosidase and endoarabinase. Galactose release by β-galactosidase and endogalactanase was positively influenced by the presence of feruloyl esterase, endoarabinase, and arabinofuranosidase. The release of ferulic acid by feruloyl esterase alone was only detected using the pre-treated pectin. Ferulic acid release from pre-treated sugar beet pectin by feruloyl esterase was affected most strongly by the presence of β-galactosidase raising the amount of ferulic acid released from 6.3 to 14.7% of the total amount of ferulic acid present in sugar beet pulp. Incubations using a combination of arabinofuranosidase, endoarabinase, β-galactosidase, and endogalactanase with feruloyl esterase released 58.4% of the total amount of ferulic acid from pre-treated sugar beet pectin and 7.9% from untreated sugar beet pectin.

Introduction

Pectins are heteropolysaccharides present in the primary cell wall of plants. In the backbone of this polymer, smooth regions consisting of α-D-1,4-linked galacturonic acid residues are interrupted by ramified hairy regions. Side chains consisting mainly of L-arabinose and D-galactose are attached to the α-1,2-linked L-rhamnose residues present in the backbone of the hairy regions (de Vries et al., 1982). Especially sugar beet pectin contains high levels of ferulic acid, which can be attached to O-2 of α-1,5-linked arabinose residues of the arabinan side chains, or to O-6 of β-1,4-linked galactose in the galactan side chains (Guillon and Thibault, 1989a and 1989b; Ralet et al., 1994; Colquhoun et al., 1994). *Aspergilli* produce a wide range of enzymes for the degradation of pectin. Several polygalacturonases, pectin lyases, pectate lyases, and rhamnogalacturonases degrade the main chain of the pectin

polymer. Additionally, acetyl- and methylesterases remove the acetyl and methyl groups from the main chain residues. Some of these enzymes were studied in detail (Benen et al., 1999; Mutter et al., 1996; Parenicová et al., 1998), and it has been shown that the combination of these enzymes is necessary for the complete degradation of the pectin backbone. Degradation of the side chains from the hairy regions requires additional enzymes. The arabinan polysaccharides are hydrolysed by arabinofuranosidases and endoarabinases (Rombouts et al., 1988), whereas galactan is degraded by beta-galactosidases and endogalactanases (van de Vis et al., 1991). The feruloyl residues are removed by feruloyl esterases (Kroon et al., 1996; Chapter 3). We have studied the synergy between five *Aspergillus* enzymes involved in the degradation of pectic side chains (Table 1), and the influence of a pre-treatment of the pectin, with rhamnogalacturonan hydrolase and rhamnogalacturonan acetylesterase, on the hydrolysis of the side chains.

Table 1. Origin and amounts of the enzymes used in this study.

Enzyme	symbol	origin	amount used (μg)[1]	reference
Endoarabinase	AbnA	*A. niger*	20.0	Ramon et al., 1993
Arabinofuranosidase B	AbfB	*A. niger*	22.1	van der Veen et al., 1991
β-galactosidase	LacA	*A. niger*	4.2	Manzanares et al., 1998
Endogalactanase	GalA	*A. niger*	19.7	Megazyme
Feruloyl esterase A	FaeA	*A. niger*	0.9	Chapter 3
Rhamnogalacturonan hydrolase	RhgA	*A. aculeatus*	12.0	Suykerbuyk et al., 1995
Rhamnogalacturonan acetylesterase	RgaeA	*A. niger*	16.7	This chapter

[1] Per 500 μl 1% sugar beet pectin in the enzyme incubations.

Materials and Methods

Sugar and ferulic acid analysis of sugar beet pectin

The sugar analysis involved acid hydrolysis of WIP with 1M sulphuric acid after which the released monomers were determined by HPLC using a a Carbopac PA1 column (Dionex Corp., Sunnyvale, Ca) and a pulsed electrochemical detector. The ferulic acid content was determined as described previously (Faulds et al., 1995).

Cloning, sequencing and overexpression of the *A. niger rgae*A gene encoding rhamnogalacturonan acetylesterase (RgaeA)

The *rgae*A gene was cloned from an *A. niger* N400 λ_{EMBL4} genomic library as described previously for the *fae*A gene encoding feruloyl esterase (Chapter 3) using part of the *A. aculeatus rgae*A gene as a probe. The *A. aculeatus rgae*A cDNA sequence (acc. nr. X89714) was used to design two oligonucleotides (5'-ACCGCCGTCTTGCACC-3' and 5'-CCGTGCATACCACCGCC-3') which were used in PCR with the *A. aculeatus* CBS 101.43 DNA as a template. The resulting 692 bp (0.7 kb) DNA

fragment was sequenced to verify its identity and used as a probe. The sequence of the *A. niger rgae*A gene has been established as described before (Chapter 3) and has been deposited in the EMBL nucleotide database (acc. nr. AJ242854). Overexpression of the *A. niger rgae*A gene was accomplished using a translational promoter fusion in which the constitutive promoter of the *A. niger pep*E gene, which encodes peptidase E, a vacuolar aspartic protease (Jarai et al, 1994). The fusion of the *pep*E promoter and the *rgae*A gene followed the same strategy as outlined by Benen et al. (Benen et al, 1999) for the fusion of the *pki*-promoter of the pyruvate kinase gene and several *A. niger* endopolygalacturonases encoding genes, and resulted in plasmid pIM3874. This plasmid was used to transform the *A. niger* strain NW188 (*csp*A1, *pyr*A6, *leu*A1, *gox*C17, *prt*F28) as previously described (Kusters-van Someren et al., 1991). The *A. niger pep*E-*rgae*A transformant NW188::pIM3874.43, which produced the highest amount of RgaeA, was used for large scale production and purification of the enzyme.

RgaeA purification

*A. niger pep*E-*rgae*A transformant NW188::pIM3874.43 was cultured using a 2.5 l fermentor in minimal medium (Chapter 3) with 4g/l NH_4Cl as N-source instead of $NaNO_3$, supplemented with trace elements (Visniac and Santer, 1957), 2 g/l yeast extract, 0.5 g/l casamino acids and 90 g/l glucose, as carbon source. The culture medium was inoculated with 10^6 spores/ml and the fermentation was carried out for 20 h at 700 rpm and 30°C. Relative O_2-saturation was maintained at 30 %. The pH was regulated at pH 3.5 by the addition of 5M NaOH. Culture fluid was collected by filtration, diluted five-fold with water and adjusted to pH 6. Next, 50 g DEAE-Streamline (Pharmacia-Biotech) was added to the filtrate and stirred for 1 h, after which the matrix was collected by filtration. RgaeA was recovered by elution with 10 mM piperazine/HCl buffer pH 6.0, 1 M NaCl, followed by extensive dialysis against the same buffer without the salt. The enzyme was loaded onto a Q-Sepharose Fast Flow column (5x5 cm) (Pharmacia- Biotech) equilibrated in 20 mM piperazine/HCl pH 6.0 and eluted with a linear 1500 ml NaCl gradient (0-1 M). RgaeA containing fractions were pooled and dialysed against 20 mM piperazine/HCl pH 5.2 prior to storage at -20^{o}C,. From 2 l of culture fluid 270 mg RgaeA was purified.

Enzyme assay

RgaeA activity was routinely assayed at 30^{o}C in 20 mM piperazine / HCl pH 5.0, using 1% (mass/vol) sugarbeet pectin as substrate. Samples (100 µl) were taken from the reaction mixture, and the pectin was precipitated by the addition of 100 µl 2-propanol followed by centrifugation. The supernatant was evaporated under vacuum and the residual material was dissolved in 50 µl of water. Acetate release was analysed by HPLC using an Aminex HPX-87H (Biorad) column eluted with 25 mM HCl at 50°C and using UV (210 nm) and RI detection. Using these standard conditions the activity of RgaeA was 0.3 U/mg (=5 nkat/mg).

Synergism with rhamnogalacturonan hydrolase (RhgA)

The synergistic effect between RgaeA and *A. aculeatus* RhgA was investigated by incubation of a 500 µl of 1% (mass/vol) sugar beet pectin solution in 20 mM piperazine/HCl pH 5.0 for 16 h at 30^{o}C with RgaeA (100 µg) and RgaeA and RhgA (40 µg), respectively. Reactions were stopped by boiling for 5 min. The synergistic effect was monitored by determining the molecular mass distribution of the sugar beet pectin before and after enzymatic treatment. Samples (50 µl) were analysed by high performance size exclusion chromatography (HPSEC) as described before (Kester et al., 1999).

Pre-treatment with RhgA and RgaeA

To 100 ml of a 1% pectin solution in 10 mM sodium acetate buffer (pH 4.5) 2.4 mg RhgA and 4.4 mg RgaeA was added. The mixture was incubated for 72 h at 30°C to degrade the rhamnogalacturonan backbone, after which the enzymes were inactivated by heating the mixture for 10 min in a boiling water bath.

Pectin incubations

The amount of enzyme necessary to obtain the maximum amount of released monomeric compound after 24 h of incubation was determined by incubating pectin with the individual enzymes alone and a time-dependent analysis of the amount of monomeric compound released (Table 1). Enzyme incubations were all performed using 500 µl of a 1% pectin solution in 10 mM sodium acetate buffer (pH 4.5) at 30°C for 24 h. The enzymes were inactivated by heating them in a boiling water bath for 5 min. Undissolved material was precipitated by centrifugation (14.000 × G, 10 min) and the supernatant was transferred to a new tube and stored at -20°C.

Enzyme assays

Ferulic acid concentrations in the incubations were analysed by HPLC with a type RP-8 reverse phase column (Supelco, Bellefonte, Pa). Separation was achieved by using a linear gradient starting with 32% (vol/vol) methanol-0.3% (vol/vol) acetic acid-67.7% (vol/vol) water and ending with 64% (vol/vol) methanol-0.3% (vol/vol) acetic acid-35.7% (vol/vol) water, with detection at 325 nm. Galactose and arabinose concentrations were also determined by HPLC using a Carbopac PA-100 column (Dionex Corp., Sunnyvale, Ca). Separation was achieved by using a linear gradient, starting with 100 mM NaOH, 5 mM sodium acetate and ending with 100 mM NaOH, 400 mM sodium acetate, over a 20 min period at a flow of 1 ml/min. Detection was carried out using a pulsed amperometric detector (PAD).

Results and discussion

Analysis of the composition of sugar beet pectin

The arabinose, galactose and ferulic acid content of sugar beet pectin was analysed as described in Materials and Methods. Galactose was found to be the major neutral sugar component of sugar beet pectin, followed by arabinose and rhamnose (Table 2). Small amounts of other sugars (fucose, glucose, xylose, and mannose) were also present, but each of these constituted less then 0.1% by mass.

Table 2. Sugar and ferulic acid analysis of sugar beet pectin.

Component	% of total pectin (mass)
arabinose	5.0
galactose	9.6
rhamnose	2.4
ferulic acid	0.63

Rhamnogalacturonan acetylesterase (RgaeA) positively influences the degradation of the rhamnogalacturonan backbone by rhamnogalacturonan hydrolase (RhgA)

The molecular mass distribution of sugar beet pectin was determined, before and after incubations with RhgA alone and in combination with RgaeA, using size exclusion chromatography. RhgA alone only resulted in a minor decrease in the molecular mass of the pectin polymer (Fig. 1), whereas the combination of RhgA and RgaeA resulted in a large decrease of the molecular mass. These results demonstrate that the removal of acetylesters by RgaeA is essential for the action of RhgA.

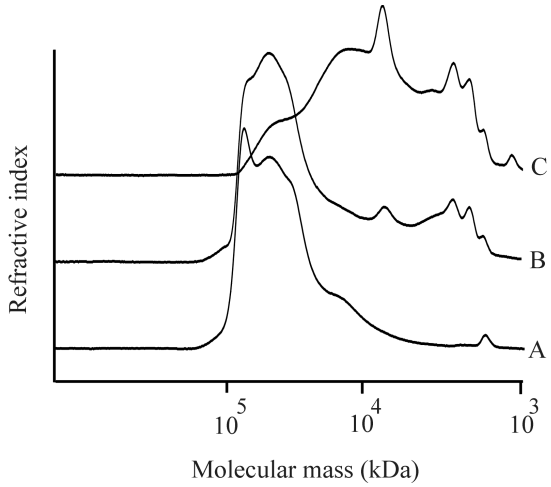

Fig. 1. Molecular mass distribution of sugar beet pectin before (A) and after a treatment with RhgA (B) and a combination of RhgA and RgaeA (C).

The release of ferulic acid from sugar beet pectin by feruloyl esterase (FaeA) strongly depends on the pre-treatment with RhgA and RgaeA and is increased by the addition of other accessory enzymes

FaeA alone was able to release some ferulic acid from RhgA/RgaeA-treated sugar beet pectin (6.3%), but not from untreated sugar beet pectin (Table 3). Ferulic acid is involved in cross-linking cell wall polysaccharides (Fry, 1979) and will therefore not be readily accessible to ferulic acid esterases. As demonstrated in this paper, degradation of the pectin backbone increases the accessibility of the ferulic acid residues resulting in an increased release of ferulic acid by the esterases. In the past it has been reported that FaeA is not active against sugar beet pulp (Brézillon et al., 1996; Faulds and Williamson, 1994). However, these measurements were done on sugar beet pulp which was not treated with rhamno-galacturonan degrading enzymes. The results described in this paper reveal a strong synergy between FaeA and these rhamnogalacturonan degrading enzymes.

Table 3. Influence of the RhgA and RgaeA pre-treatment and the addition of other accessory enzymes on ferulic acid release from sugar beet pectin by FaeA. Values are in percentage of the total amount of ferulic acid present in sugar beet pectin.

Enzyme	untreated pectin	treated pectin
FaeA	0	6.3 ± 0.6
FaeA / AbfB	2.4 ± 0.1	9.4 ± 0.2
FaeA / AbnA	0	7.4 ± 0.2
FaeA / LacA	0	14.7 ± 0.2
FaeA / GalA	0	7.9 ± 0.1
FaeA / AbfB / AbnA	4.7 ± 0.1	9.7 ± 0.5
FaeA / LacA / GalA	0.5 ± 0.1	20.1 ± 0.6
FaeA / LacA / AbfB	1.4 ± 0.1	23.9 ± 0.2
FaeA / LacA / AbnA	0	13.4 ± 0.1
FaeA / LacA / GalA /AbfB	4.2 ± 0.1	38.4 ± 1.1
FaeA / LacA / GalA / AbnA	2.6 ± 0.1	19.5 ± 0.1
FaeA / LacA / AbfB / AbnA	5.5 ± 0.1	22.4 ± 0.1
FaeA / LacA / GalA / AbfB / AbnA	7.9 ± 0.2	58.4 ± 1.3

Incubations with arabinofuranosidase (AbfB), endoarabinase (AbnA), β-galactosidase (LacA), and endo-galactanase (GalA), alone or in combinations, in the absence of FaeA did not result in detectable amounts of ferulic acid released from either untreated or treated pectin (data not shown), but these enzymes did influence the release of ferulic acid in the presence of FaeA. The amount of ferulic acid released from enzyme incubations using pre-treated sugar beet pectin was only slightly increased in individual combinations of FaeA with AbfB, AbnA, and GalA, or with a combination of AbfB and AbnA (Table 3), but the presence of LacA had a strong effect on ferulic acid release (14.7-24%). Incubations with FaeA, AbfB, AbnA, LacA, and GalA resulted in the highest release of ferulic acid from RhgA/RgaeA treated pectin (58%). Previous reports demonstrated that FaeA was not active against arabinose (O-2) linked ferulic acid, but was able to release galactose linked ferulic acid from sugar beet pectin derived oligosaccharides (Ralet et al., 1994). This is in agreement with the data shown above. Synergy was mainly observed between FaeA and LacA indicating a preference of the esterase for galactose linked ferulic acid. Using untreated pectin, a small amount of ferulic acid was released by FaeA when a combination of LacA and GalA was added (Table 3). Higher

synergistic effects were observed in the presence of AbfB or in combinations of FaeA and AbfB with other enzymes. Since FaeA was not able to release arabinose linked ferulic acid from pectin oligomers (Ralet et al., 1994), this increase in released ferulic acid is most likely due to the degradation of the arabinan side chains by AbfB resulting in an increased accessibility of the galactose linked ferulic acid residues.

A different feruloyl esterase from *A. niger* (cinnamoyl esterase) able to release ferulic acid from sugar beet pulp (Kroon and Williamson, 1996) was shown to be mainly active on arabinose linked ferulic acid. This enzyme acted synergistically with endoarabinase in the release of ferulic acid, but no positive interaction was observed with endogalactanase or β-galactosidase (Kroon and Williamson, 1996). The importance of β-galactosidase and arabinofuranosidase in the release of ferulic acid from sugar beet pectin is supported by the data obtained by Micard et al. (1994). When comparing different commercial pectinolytic enzyme preparations the highest release of ferulic acid from sugar beet pectin was obtained using Pektolase LM, which contained the highest β-galactosidase and arabinofuranosidase activity of all the enzyme preparations tested.

The high amount of ferulic acid released by FaeA, in combination with rhamnogalacturonan and side chain degrading enzymes indicates an important role for this enzyme in the complete degradation of sugar beet pectin.

Arabinose and galactose release from sugar beet pectin

Arabinose release by AbnA was higher in untreated pectin than in RhgA / RgaeA treated pectin (Table 4), whereas no significant increase was observed using AbfB or a combination of AbnA and AbfB. No significant amounts of arabinose were released by any of the other enzymes from treated or untreated pectin nor did the addition of LacA and GalA result in an increase in the amount of arabinose released by AbfB and AbnA (data not shown). However, the amount of galactose released by LacA, GalA, and a combination of these enzymes was higher from treated pectin than from untreated pectin (Table 5). The strongest effect was observed in incubations with only LacA. Incubations with a combination of LacA and GalA resulted in an 3-fold increase in the amount of galactose released compared to incubations with either of these enzymes alone. Addition of AbfB and AbnA to incubations with LacA and GalA resulted in a further increase in the amount of galactose released. Hairy regions from pectins are dense structures in which the individual side chains are close together. This

Table 4. Influence of the RhgA and RgaeA pre-treatment and the addition of FaeA on arabinose release from sugar beet pectin by AbfB and AbnA. Values are in percentage of the total amount of arabinose present in sugar beet pectin.

enzyme	untreated pectin	treated pectin	treated pectin with FaeA
AbfB	64.7 ± 1.1	62.4 ± 0.2	68.4 ± 0.8
AbnA	2.7 ± 0.1	5.9 ± 0.3	10.4 ± 0.1
AbfB / AbnA	68.6 ± 0.7	64.8 ± 0.5	69.5 ± 0.5

Table 5. Influence of the RhgA and RgaeA pre-treatment and the addition of FaeA on galactose release from sugar beet pectin by LacA and GalA. Values are in percentage of the total amount of galactose present in sugar beet pectin.

enzyme	untreated pectin	treated pectin	treated pectin with FaeA
LacA	0.9 ± 0.1	8.2 ± 0.6	10.4 ± 0.3
GalA	8.3 ± 0.6	10.0 ± 0.8	12.4 ± 0.1
LacA / GalA	15.2 ± 0.3	30.4 ± 0.1	34.9 ± 0.5
LacA / GalA / AbfB / AbnA	26.7 ± 0.6	44.1 ± 0.3	50.4 ± 0.6

may cause steric hindrance, resulting in a decreased activity of the enzymes acting on these side chains. A structure has been proposed for sugar beet pectic side chains in which short galactan side chains connect the longer arabinan chain to the rhamnogalacturonan backbone (Albersheim, 1975; Sakamoto and Sakai, 1995). This is in agreement with the data described above. In such a structure the long arabinan side chains which extend away from the pectin backbone would be accessible for AbfB and AbnA, whereas the shorter galactan chains would be shielded by the arabinan chains, preventing efficient hydrolysis by LacA and GalA. By hydrolysing the rhamnogalacturonan backbone, the galactan chains become more accessible, resulting in an increase in the amount of galactose released by LacA and GalA. The positive effect of AbfB and AbnA on galactose release by LacA and GalA can also be explained by an increase in the accessibility of the shorter galactan chains to LacA and GalA.

The addition of FaeA to incubations using AbfB, AbnA, LacA, and GalA, alone or in combination did not influence the release of arabinose or galactose from untreated pectin (data not shown). Addition of FaeA to incubations of treated pectin with AbnA alone increased the amount of arabinose two-fold, but only a minor effect was observed using AbfB or a

combination of AbfB and AbnA (Table 4). The release of galactose from RhgA / RgaeA treated pectin was increased by the addition of FaeA in all enzyme incubations (Table 5). The highest release of galactose was observed in incubations with all five enzymes. It is most likely that the effects of the addition of FaeA on galactose release by LacA and GalA are due to the ability of the enzyme to remove ferulic acid residues from the galactan side chains. This would make these side chains more accessible to the galactan degrading enzymes. The inability of FaeA to increase the arabinose release by AbfB confirms the observations that FaeA is not able to hydrolyse the link between arabinose and ferulic acid in sugar beet pectin, and would therefore not be able to remove the ferulic acid crosslinks. The other feruloyl esterase purified previously (Kroon and Williamson, 1996) might have a stronger effect on the release of arabinose by AbfB. Adding this enzyme to incubations with the five enzymes used in this study (AbfB, AbnA, LacA, GalA and FaeA) could result in a further increase in the release of both arabinose and ferulic acid from sugar beet pectin.

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Chapter 12

Concluding remarks

The xylanolytic and pectinolytic enzyme systems from *Aspergillus* are widely studied due to the wide range of industrial applications of these enzymes. This thesis adds information about the expression of xylanolytic and pectinolytic genes and about the action of the encoded enzymes on the polymeric substrates (xylan and pectin) to the data present in the literature. There still is a need to understand these systems in more detail with respect to their production (gene expression, post-transcriptional regulation and secretion), the activity of the enzymes on polymeric and oligomeric substrates, their kinetic parameters and the synergy between the enzymes. Some of these issues will be discussed in this chapter.

1. Regulation of xylanolytic genes

The regulation of the xylanolytic system has been studied in detail and several factors involved in the expression of xylanolytic genes have been detected. Xylanolytic genes are expressed in the presence of xylose, xylobiose or xylan (Chapters 3 to 9). The xylanolytic transcriptional activator protein XlnR plays a central role in the induction of xylanolytic gene expression (see Chapter 1). Although it is obvious that intact xylan will not be the actual inducer of the system, it is at this point unclear whether induction is only initiated by the monomeric sugar xylose or if small xylo-oligosaccharides (xylobiose, xylotriose) are also capable of inducing the system without first being degraded to xylose.

A consensus site (GGCTAA) has been proposed for the XlnR binding site in the promoters of xylanolytic genes. This consensus or a slight variation of it can be found in all XlnR regulated genes. Only the second G has thus far been confirmed to be essential by band shift assays and *in vivo*. This residue was also conserved in all putative XlnR binding sites identified so far. However, analysis of the promoter regions of several xylanolytic genes identified sequences which are highly similar to this consensus, but in which one of the two adenosine residues is replaced by a different residue. The promoter of *A. niger fae*A contains one putative XlnR binding site and a sequence in which the second adenosine residue is replaced by a guanosine. The XlnR consensus site is absent in the promoter of *A. tubingensis fae*A, but a similar sequence (GGCTAT) is present at the same position as the GGCTAG sequence in *A. niger*. This *fae*A gene from *A. tubingensis* is expressed on xylose and xylan, suggesting that a T at position 6 does not diminish expression. Both a XlnR consensus sequence and the GGCTAG sequence are present at conserved positions in the promoters of *A. niger* and *A. tubingensis agu*A. A more detailed analysis using site-directed mutagenesis to change individual

nucleotides will be needed to fully determine the variations which still result in a functional binding site for XlnR. In such an approach it will be possible to eliminate the (wild-type) XlnR consensus site by mutating the second G, allowing a functional study of the sequences similar to the XlnR consensus.

A second factor which plays a central role in the expression of xylanolytic genes is the carbon catabolite repressor protein CreA. The promoters of all xylanolytic genes isolated so far contain one or more putative CreA binding sites and expression of these genes is repressed in the presence of glucose. The extent of CreA repression is not limited to glucose. Other monomeric carbon sources also result in repression of the expression of xylanolytic genes. Xylose not only induces the expression of the xylanolytic genes via XlnR, but also results in repression of expression via CreA (Chapters 8 and 9). An increase in the xylose concentration resulted in a decrease in the expression of xylanolytic genes. CreA mediated repression was also observed in the presence of other carbon sources. Ferulic acid induced expression of *fae*A in the presence of these carbon sources was lower in an *A. niger* wild type strain than in a CreA derepressed mutant (Chapter 6). These results have implications for the industrial production of xylanolytic enzymes from *Aspergillus* on (crude) polymeric substrates. During growth of *Aspergillus* a mixture of carbon sources will be released, of which some might lead to significant CreA mediated repression, resulting in a reduced production of the enzymes. To be able to predict the levels of expression of xylanolytic genes on these substrates it will be important to determine the strength of CreA mediated repression by other monomeric carbon sources present in or liberated from polymeric substrates. Xylose is not used for the production of xylanolytic enzyme preparations, since the commonly used concentration at lab scale (between 30 and 70 mM) would lead to a substantial increase in the production costs of the preparations. The data presented in this thesis demonstrate that a significant increase in xylanolytic gene expression can be achieved at very low xylose concentrations (1 mM). If the production of the xylanolytic enzymes is similarly influenced, adding a low amount of xylose to the crude substrate will probably lead to a quicker and higher production of the enzymes.

In *A. nidulans*, pH regulation of some xylanolytic genes has been reported. Opposite expression patterns for two endoxylanase encoding genes with respect to ambient pH, are mediated by the pH regulatory protein PacC (see Chapter 1). This phenomenon has so far not been studied in detail in other *Aspergilli*, but might have implications for the production of

these enzymes by *Aspergillus* sp., since some species (e.g. *A. niger*) strongly acidify the culture medium during growth. More detailed studies will be needed to determine the influence of the pH of the culture medium on the production of xylanolytic enzymes.

Apart from these general regulatory systems a system specific for the feruloyl esterase A encoding gene (*fae*A) has been identified. This system responds to the product of the enzyme (ferulic acid) and acts synergistically with XlnR in inducing the expression of *fae*A (Chapter 5). Several other feruloyl esterases have been purified from *Aspergillus* sp. (Chapter 1). Whether the corresponding genes of these enzymes are controlled by the same ferulic acid responsive system as *fae*A is unclear at this point.

No data has been reported in which additional regulatory systems have been studied for the other xylanolytic genes. It would be interesting to see if other accessory enzymes are also induced by the product of the enzyme reaction resulting in increased gene expression in the presence of XlnR induction. Adding small amounts of these specific inducers might enable the production of differently balanced mixtures of xylanolytic enzymes.

2. Regulation of pectinolytic genes

Little is known about the regulation of pectinolytic genes from *Aspergillus*. Data about the production of the pectinolytic enzymes indicates that unlike for the xylanolytic system, genes encoding main chain cleaving pectinolytic enzymes and genes encoding accessory pectinolytic enzymes do not have an overall inducing system (see Chapter 1). Some of the genes encoding main chain cleaving enzymes seem to be constitutively expressed, whereas others are only expressed in the presence of pectin or polygalacturonic acid. Although it is unlikely that these polymeric compounds actually enter the cell and induce pectinolytic gene expression, monomeric or low oligomeric compounds which are inducers of the pectinolytic system have not been identified with certainty. The expression of some of the accessory enzymes encoding genes has been studied in more detail and galactose and arabinose have been identified as inducers of genes encoding galactose releasing and arabinose releasing enzymes, respectively (see Chapter 1). Some of these genes are also part of the xylanolytic system, indicating the presence of several regulatory boxes in the promoters of these genes responding to different environmental conditions. Since it appears that not all pectinolytic genes are coordinately regulated, it might be important to perform an expression study in which the influence of the wide domain regulatory proteins is minimised. This can be achieved by using regulatory

mutant strains (CreA, PacC, AreA) or by specifically mutating putative regulatory sequences in the promoters of these genes. Such a study might reveal whether more than one pectinolytic transcriptional activator is present.

3. Mode of action of pectinolytic and xylanolytic enzymes

The mode of action of most pectinolytic and xylanolytic main chain cleaving enzymes is being studied in detail. Especially for the polygalacturonases and pectin lyases data is available with respect to the kinetics, number of sub-sites and substrate specificity of the enzymes. In order to be able to rationally select enzymes for industrial applications it will be useful to set up a database for the xylanolytic main chain cleaving enzymes and for the accessory enzymes involved in xylan and pectin degradation based on their mode of action on substrates of different origin. These data will enable a rational choice between several similar enzyme functions for a specific application involving a particular substrate. Using such a strategy will increase the amount of desired product formed and will reduce the amount of undesired by-products. Additionally, this might also lead to an understanding why *Aspergillus* produces several enzymes for one function (e.g. endoxylanase, polygalacturonase, α-galactosidase), whereas single enzymes are produced for other functions (e.g. α-glucuronidase, β-galactosidase).

4. Synergy between cell wall degrading enzymes

Synergy has been observed between main chain cleaving and accessory enzymes in both the xylanolytic and pectinolytic degrading system of *Aspergillus* and also between accessory enzymes involved in these systems (Chapters 10 and 11). Using different combinations of enzymes a different spectrum of products will be produced. Crude enzyme preparations containing several enzyme functions are most commonly used in industrial processes, resulting in good yields of products especially when complete degradation of the polysaccharide to monosaccharides is required. However, in the production of tailor made products (e.g. specific oligosaccharides) it might be more efficient to use a defined combination of enzymes which will increase the amount of desired product formed, but will not further degrade this product to monosaccharides. In order to design rational strategies for such processes, it will be necessary to study the synergy of the xylanolytic and pectinolytic enzymes in more detail. Analysis of the reaction products should then not only focus on the

formation of monosaccharides, but also on the structure of the oligosaccharides. This will require elaborate and detailed studies using NMR and mass spectroscopy and HPLC analysis. Additionally, a more detailed study of synergy between the xylanolytic and pectinolytic enzymes and other enzymes potentially able to act on xylan or pectin might result in the identification of additional enzymes involved in the degradation of these polysaccharides. A first example of this strategy is FaeA, which was stated not to be active on pectin. However, in combination with some pectinolytic main chain cleaving and accessory enzymes, FaeA was able to release 58% of the total amount of ferulic acid present in sugar beet pectin (Chapter 11). α- and β-galactosidases positively influenced the action of other xylanolytic enzymes (Chapter 10), although no detectable amounts of galactose were released. This suggests that the release of small amounts of specific residues might already have a significant impact on the total degradation of the polysaccharide. In order to determine the action of specific enzymes alone and in combinations, the availability of pure samples of all enzymes involved in the degradation of xylan and pectin is required. At this point, it seems clear that not all enzymes involved in the degradation of heteropolysaccharides have been identified. Therefore, it can also be expected that additional synergistic effects will be observed as the number of xylan and pectin degrading enzymes purified increases.

Summary

The xylanolytic and pectinolytic enzyme systems from *Aspergillus* have been the subject of study for many years. Although the main chain cleaving enzymes and their encoding genes have been studied in detail, little information is available about most of the accessory enzymes and their corresponding genes. This thesis describes the purification and characterisation of two accessory enzymes from *Aspergillus*, feruloyl esterase A (FaeA) and α-glucuronidase A (AguA), and the activities of these enzymes on polymeric substrates in relation to other accessory enzymes. Furthermore, the characterisation and regulation of the FaeA and AguA encoding genes (*fae*A and *agu*A), and some genes encoding other accessory enzymes is studied.

FaeA is the major feruloyl esterase produced when *Aspergillus niger* is grown on xylan or crude substrates such as wheat bran or sugar beet pulp. Addition of ferulic acid, the product of FaeA, to media containing xylan increases the production of this enzyme. FaeA is able to release ferulic acid from xylan and pectin oligosaccharides, as well as from synthetic substrates such as methylferulate. The *fae*A gene was cloned from *A. niger* and *Aspergillus tubingensis*. A blast of the deduced amino acid sequence of FaeA revealed no significant homology to other proteins, except for a small region of FaeA which was highly similar to the active site of lipases. Based on this homology, a 3-dimensional model for FaeA was proposed by Pickersgill et al. Although only 16 amino acid differences were observed between FaeA from *A. niger* and *A. tubingensis*, the latter enzyme was found to be much more sensitive to proteolytic degradation.

AguA from *A. tubingensis* was able to release (4-O-methyl-) glucuronic acid from xylan derived oligosaccharides, but had very little activity towards the intact polysaccharide. The *agu*A gene was cloned, and database analysis of the deduced amino acid sequence revealed homology to the α-glucuronidases from *Trichoderma reesei* and *Thermotoga maritima*.

Regulation of *fae*A and *agu*A expression was studied in *A. niger* and compared to other xylanolytic genes. Both genes were found to be under the control of the xylanolytic transcriptional activator protein XlnR, which also regulates endoxylanase, β-xylosidase, acetylxylan esterase, arabinoxylan arabinofuranohydrolase, and endoglucanase gene expression. In a XlnR negative mutant no expression of *fae*A and *agu*A was observed on xylose or xylan. Expression of *fae*A in this mutant was observed in the presence of ferulic

acid, indicating the presence of a second system for the induction of this gene. This system seems to be specific for *fae*A, since no expression of *agu*A or other xylanolytic genes was observed under these conditions. In a wild-type *A. niger* strain, expression levels of *fae*A were higher on a combination of xylose and ferulic acid than the sum of the expression levels on xylose and ferulic acid alone, suggesting a synergistic effect of these two inducing systems.

The carbon catabolite repressor protein CreA is involved in the repression of xylanolytic gene expression in the presence of easy metabolisable carbon sources, such as glucose or fructose. Expression of *agu*A and *fae*A on xylose and xylan, as well as expression of *fae*A on ferulic acid was repressed in the presence of glucose. Depending on the concentration of xylose present in the medium, this sugar also triggers CreA mediated repression of xylanolytic gene expression. Using a concentration range from 1 to 100 mM, it was shown that expression levels of *fae*A, *agu*A, and genes encoding endoxylanase B and β-xylosidase decreased with increasing xylose concentrations in an *A. niger* wild type strain. In a CreA derepressed mutant constant levels of XlnR induced gene expression were observed indicating that the xylose concentration has a modulating effect via CreA.

A gene (*agl*B) encoding an α-galactosidase, which was produced when *A. niger* was grown on crude wheat arabinoxylan, was cloned and the expression of this gene was compared with the expression of two other α-galactosidase encoding genes (*agl*A and *agl*C) and a β-galactosidase encoding gene (*lac*A) from *A. niger*. All four genes had specific expression profiles with respect to monomeric sugars, galacto-oligosaccharides and polymeric substrates. High expression on xylan was only observed for *agl*B and *lac*A, suggesting that these genes may be part of the xylanolytic system from *A. niger*. This was confirmed using a XlnR negative mutant, which showed no (*lac*A) or reduced (*agl*B) expression of these genes on xylose.

Synergy was studied between the accessory enzymes from *Aspergillus* involved in xylan degradation and two main chain cleaving enzymes, endoxylanase A (XlnA) and β-xylosidase (XlnD). Except for α-L-arabinofuranosidase B (AbfB), the activity of all accessory enzymes on xylan was increased in the presence of XlnA. Similarly, the presence of accessory enzymes increased the activity of XlnA on xylan, indicating synergy between these enzymes. Synergy was also observed between the accessory enzymes, resulting in more efficient degradation of xylan. These results confirm that the gene products of XlnR regulated genes are in fact all part of the xylanolytic enzyme system of *Aspergillus*.

Similarly, the synergy of enzymes involved in the degradation of the hairy regions in sugar beet pectin was studied. Degradation of the pectin backbone did not influence the activity of the arabinose releasing enzymes, AbfB and endoarabinase (AbnA), but had a strong effect on the release of ferulic acid by FaeA and the release of galactose by endogalactanase (GalA) and β-galactosidase (LacA). Synergy was also observed between galactose- and ferulic acid-releasing enzymes.

The accessory enzymes from *Aspergillus* involved in the degradation of xylan and pectin form a diverse group of enzymes which actively co-operate in polysaccharide degradation. Common factors have been identified in the regulation of the genes encoding these enzymes, but the expression patterns of the different genes also indicate the presence of other factors influencing specific genes. This most likely enables *Aspergillus* to modulate the production of these enzymes to obtain an efficient mixture for the degradation of the variety of substrates it encounters.

Samenvatting

De schimmel *Aspergillus* produceert een groot scala aan enzymen om de plant celwand polysacchariden xylan en pectine af te breken. Deze xylanolytische en pectinolytische enzymsystemen worden al vele jaren bestudeerd en er is al veel informatie beschikbaar over de enzymen die de hoofdketen van deze polysacchariden afbreken. Echter, de enzymen die zijketens van xylan en pectine afbreken en de coderende genen zijn in mindere mate bestudeerd. In dit proefschrift is de zuivering en de karakterisering van twee zijketen afsplitsende enzymen, feruloyl esterase A (FaeA) en α-glucuronidase (AguA), beschreven en is de activiteit van deze enzymen op polymere substraten bestudeerd in relatie tot andere zijketen afsplitsende enzymen. Daarnaast zijn de genen die coderen voor deze enzymen (*fae*A en *agu*A) gekloneerd en is de regulatie van deze genen en een aantal andere voor zijgroep afsplitsende enzymen coderende genen bestudeerd.

FaeA is het voornaamste feruloyl esterase dat door *Aspergillus niger* geproduceerd wordt bij groei op xylan of ruwe substraten zoals tarwe zemelen of suikerbieten pulp. De productie van dit enzym door *A. niger* wordt nog verhoogd als er ferulazuur, het product van FaeA, wordt toegevoegd aan het groeimedium. FaeA is in staat om ferulazuur af te splitsen van oligosacchariden afgeleid van xylan en pectine, maar ook van synthetische substraten zoals methyl-ferulaat. Het *fae*A gen is geïsoleerd uit *A. niger* en *Aspergillus tubingensis*. De afgeleide aminozuur sequentie van FaeA heeft geen significante homologie met andere eiwitten. Een uitzondering hierop is een kleine regio van FaeA dat zeer hoge homologie vertoond met de ‘active site’ van lipases. Op grond van deze overeenkomst is een 3-dimensionaal model voor FaeA voorgesteld door Pickersgill et al. Een vergelijking van de aminozuur sequentie van *A. niger* en *A. tubingensis* FaeA toonde aan dat er slechts 16 aminozuur verschillen zijn tussen de twee enzymen. Desondanks is het *A. tubingensis* enzym veel gevoeliger is voor proteolytische afbraak dan *A. niger* FaeA.

AguA van *A. tubingensis* is in staat om (4-O-methyl-)glucuronzuur af te splitsen van oligosacchariden van xylan, maar is slecht in geringe mate actief op het intacte polysaccharide. Het *agu*A gen is gekloneerd en de afgeleide aminozuur sequentie van AguA is vergeleken met andere eiwitten uit de database. AguA van *A. tubingensis* vertoonde sequentie overeenkomst met α-glucuronidases van de schimmel *Trichoderma reesei* en de bacterie *Thermotoga maritima*.

Regulatie van de expressie van *fae*A en *agu*A is bestudeerd in *A. niger* en vergeleken met die van andere xylanolytische genen. Het xylanolytisch transcriptie activatie eiwit XlnR reguleert de expressie van *fae*A en *agu*A op xylose en xylan. XlnR reguleert verder ook de expressie van genen coderend voor endoxylanase, β-xylosidase, acetylxylan esterase, arabinoxylan arabinofuranohydrolase en endoglucanase. In een XlnR negatieve mutant werd geen expressie gedetecteerd van *fae*A en *agu*A op xylose of xylan, maar *fae*A kwam wel tot expressie in aanwezigheid van ferulazuur. Dit geeft aan dat er een apart inducerend systeem bestaat dat specifiek is voor *fae*A, aangezien er onder deze condities geen expressie waargenomen werd voor *agu*A of andere xylanolytische genen. In een wild type stam is de *fae*A expressie op een combinatie van xylose en ferulazuur hoger dan de som van de expressie op beide substraten apart, wat suggereert dat de beide systemen een synergistisch effect hebben.

Het koolstof kataboliet repressie eiwit CreA is betrokken bij de repressie van xylanolytische genexpressie in de aanwezigheid van makkelijk afbreekbare koolstofbronnen zoals glucose en fructose. De expressie van zowel *fae*A als *agu*A op xylan en xylose, alsmede de expressie van *fae*A op ferulazuur werd gerepresseerd in de aanwezigheid van glucose. Ook xylose is in staat om de expressie van xylanolytische genen te represseren via CreA, afhankelijk van de concentratie waarin deze suiker aanwezig is. De expressie van *fae*A, *agu*A en de genen coderend voor endoxylanase B (*xln*B) en β-xylosidase (*xln*D) nam af naarmate de xylose concentratie toenam van 1 naar 100 mM. In een CreA negatieve mutant werd een constant expressie niveau waargenomen, wat aangeeft dat de afname in de wild type stam een gevolg is van CreA repressie.

A. niger produceert een α-galactosidase bij de groei op ruw tarwe arabinoxylan. Het gen coderend voor dit enzym (*agl*B) is gekloneerd en de expressie van dit gen is vergeleken met de expressie van twee andere α-galactosidase coderende genen (*agl*A en *agl*C) en met een β-galactosidase coderend gen (*lac*A) van *A. niger*. De vier genen hadden elk specifieke expressie patronen met betrekking tot monomere suikers, galacto-oligosacchariden en polymere substraten. Hoge expressie van *agl*B en *lac*A werd waargenomen op xylan, wat aangeeft dat deze genen deel uit maken van het xylanolytisch systeem van *A. niger*. Dit werd bevestigd doordat geen (*lac*A) of verlaagde (*agl*B) expressie werd waargenomen in een XlnR negatieve mutant.

De synergie is bestudeerd tussen zijketen afsplitsende enzymen en twee hoofdketen splitsende enzymen, endoxylanase A (XlnA) en β-xylosidase (XlnD) bij de afbraak van tarwe xylan. De activiteit van alle zijketen afsplitsende enzymen, met uitzondering van α-L-arabinofurano-

sidase B (AbfB), werd verhoogd in de aanwezigheid van XlnA. Omgekeerd hadden de zijketen afsplitsende enzymen ook een positief effect op de activiteit van XlnA, wat aangeeft dat er synergie bestaat tussen deze enzymen. Daarnaast werd ook synergie waargenomen tussen de zijgroep afsplitsende enzymen, resulterend in een meer efficiënte afbraak van xylan. De synergie van enzymen betrokken bij afbraak van de 'hairy regions' van suikerbieten pectine is eveneens bestudeerd. Afbraak van de hoofdketen van pectine had geen effect op de activiteit van de arabinose afsplitsende enzymen, AbfB en endoarabinase (AbnA), maar had wel een positieve invloed op de activiteit van FaeA en de galactose afsplitsende enzymen endogalactanase (GalA) en LacA. Verder werd ook synergie waargenomen tussen galactose en ferulazuur afsplitsende enzymen.

De xylanolytische en pectinolytische zijketen afsplitsende enzymen van *Asergillus* vormen een gevarieerde groep enzymen die samenwerken in de afbraak van xylan en pectine. Een aantal algemene transcriptie factoren zijn betrokken bij de regulatie van de genen die coderen voor deze enzymen. Echter, de expressie patronen van de verschillende genen geven ook indicaties voor specifieke transcriptie factoren betrokken bij de regulatie van specifieke genen. Via deze factoren is *Aspergillus* waarschijnlijk in staat om de productie van de verschillende enzymen te beïnvloeden om een mengsel te verkrijgen dat leidt tot een efficiënte afbraak van de verschillende substraten waarmee de schimmel in aanraking komt.

Curriculum Vitae

Ronald Peter de Vries werd op 15 oktober 1967 te Wageningen geboren. In 1986 behaalde hij het VWO diploma aan het Heldring College te Zetten en begon in september van datzelfde jaar met de studie Moleculaire Wetenschappen aan de Landbouw Universiteit Wageningen. Deze studie werd in augustus 1992 afgesloten met de hoofdvakken organische chemie en bacteriële genetica. Daarnaast werd een moleculair biologische stage van 6 maanden afgelegd bij het Instituut voor Bodemvruchtbaarheid (IB-DLO). Na een korte onderbreking in verband met de dienstplicht begon hij in juli 1993 met een na-doctoraals onderzoeksproject (NOP) bij de sectie Moleculaire Genetica van Industriële Micro-organismen (MGIM). Gedurende drie maanden werd onderzoek gedaan aan een *Aspergillus nidulans* mutant gestoord in het arabinose catabolisme, waarna een overstap werd gemaakt naar het binnen de sectie lopende protease onderzoek in *Aspergillus niger*. In oktober 1994 begon hij met een AIO project bij MGIM gefinancierd door het Deense bedrijf Danisco waarvan dit proefschrift het resultaat is. Deze aanstelling werd verlengd tot 1 januari 1999. Vanaf die datum is hij werkzaam als toegevoegd onderzoeker bij MGIM in het kader van een EG project dat zich richt op het bestuderen van het glycerol en trehalose metabolisme in *Aspergillus nidulans*. Het doel van dit project is het ophelderen van de rol hiervan in de regulatie van de glycolytische flux, deresistentie tegen stress en de morfologie van deze schimmel.

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