MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, Dec. 2001, p. 497–522 Vol. 65, No. 4
1092-2172/01/$04.00+0 DOI: 10.1128/MMBR.65.4.497–522.2001

Aspergillus Enzymes Involved in Degradation of Plant Cell Wall Polysaccharides

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INTRODUCTION

This review summarizes our current knowledge on the different classes of enzymes involved in plant cell wall polysaccharide degradation produced by *Aspergilli*, the genes encoding these enzymes, and the regulation of these genes. The data from literature is presented in tables as much as possible to provide easy comparisons of the enzymes and genes reported so far. Only enzymes for which a detailed characterisation was published are presented this way, requiring at least a MW and one of three other characteristics (pI, pH optimum or T optimum). Enzymes that have been characterised in less detail are mentioned in the text when this provided additional information valuable to this review. The tables of genes list the assignment of the corresponding enzymes to the different glycosidase, polysaccharide lyase and carbohydrate esterase families (69, 145–147) as described by B. Henrissat at URL: http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html.

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Plant Cell Wall Polysaccharides

Plant cell wall polysaccharides are the most abundant organic compounds found in nature. They make up 90% of the plant cell wall and can be divided into three groups: cellulose, hemicellulose, and pectin (256). 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 (fibers), 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

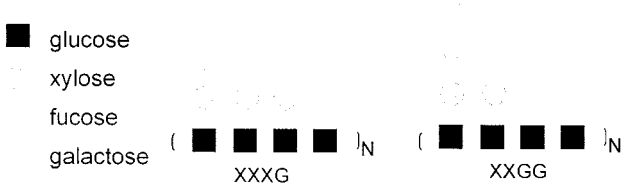

FIG. 1. Schematic presentation of the repeating units of the two major xyloglucan structures.

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 by different side groups such as L-arabinose, D-galactose, acetyl, feruloyl, *p*-coumaroyl, and glucuronic acid residues (400). A second hemicellulose structure commonly found in soft- and hardwoods is (galacto)glucomannan (369), which consists of a backbone of β-1,4-linked D-mannose and D-glucose residues with D-galactose side groups (see "Structural features of galacto(gluco)mannan" below). Softwoods contain mainly galactoglucomannan, whereas in hardwoods glucomannan is the most common form. Xyloglucans are present in the cell walls of dicotyledonae and some monocotylodonae (e.g., onion). Xyloglucans consist of a β-1,4-linked D-glucose backbone substituted by D-xylose. L-Arabinose and D-galactose residues can be attached to the xylose residues, and L-fucose has been detected attached to galactose residues in xyloglucan. Xyloglucans interact with cellulose microfibrils by the formation of hydrogen bonds, thus contributing to the structural integrity of the cellulose network (56).

Pectins form another group of heteropolysaccharides and consist of a backbone of α-1,4-linked D-galacturonic acid residues (see "Structural features of pectin" below). In specific "hairy" regions the galacturonic acid backbone is interrupted by α-1,2-linked L-rhamnose residues. Long side chains consisting mainly of L-arabinose and D-galactose residues can be attached to these rhamnose residues. In pectins of certain origins (e.g., sugar beet and apple), ferulic acid can be 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 fibrils, creating a rigid structure strengthening the plant cell wall. They also form covalent cross-links, which are thought to be involved in limiting cell growth and reducing cell wall biodegradability. Two types of covalent cross-links have been identified between plant cell wall polysaccharides and lignin (117). The cross-link formed by diferulic acid bridges is studied in most detail. Diferulic 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 (162), between pectin polymers in sugar beet (275), and between lignin and xylan in wheat (22). A second type of cross-link is the ester linkage between lignin and glucuronic acid attached to xylan, which was identified in beech wood (160, 359). Recently, indications of a third type of cross-linking have been reported involving a protein- and pH-dependent binding of pectin and glucuronoarabinoxylan to xyloglucan (311). This not yet fully characterized binding is dependent on the presence of fucose on the xyloglucan.

Structural Features of Cellulose and Xyloglucan

Cellulose consists of linear β-1,4-linked D-glucopyranose chains that are condensed by hydrogen bonds into crystalline structures, called microfibrils (205). These microfibrils consist of up to 250 glucose chains and are linked by hemicelluloses (56). In addition to this crystalline structure, cellulose contains noncrystalline (amorphous) regions within the microfibrils. The relative amounts of crystalline and noncrystalline cellulose vary depending on the origin (232).

Two major types of xyloglucans have been identified in the plant cell wall (Fig. 1). Xyloglucan type XXXG consists of repeating units of three β-1,4-linked D-glucopyranose residues, substituted with D-xylopyranose via an α-1,6-linkage, which are separated by an unsubstituted glucose residue. In xyloglucan type XXGG, two xylose-substituted glucose residues are separated by two unsubstituted glucose residues. The structural features of these, as well as some other types of xyloglucans, have been discussed in detail by Vincken et al. (391). The xylose residues in xyloglucan can be substituted with α-1,2-L-fucopyranose-β-1,2-D-galactopyranose and α-1,2-L-galactopyranose-β-1,2-D-galactopyranose disaccharides (138, 391). L-Arabinofuranose has been detected α-1,2-linked to main-chain glucose residues or xylose side groups (151, 157, 308, 409). In addition, xyloglucans can contain O-linked acetyl groups (243, 339). Xyloglucans are strongly associated with cellulose and thus add to the structural integrity of the cell wall. They are thought to play an important role in regulating cell wall extension. The length of the xyloglucan polymers enables them to cross-link many cellulose microfibrils, creating a rigid structure (248).

Structural Features of Xylan

The structure of xylans found in cell walls of plants can differ greatly depending on their origin, but they always contain a β-1,4-linked D-xylose backbone (101, 399). The schematic representation of xylan (Fig. 2) also lists the different structures which can be attached to the xylan backbone and which result

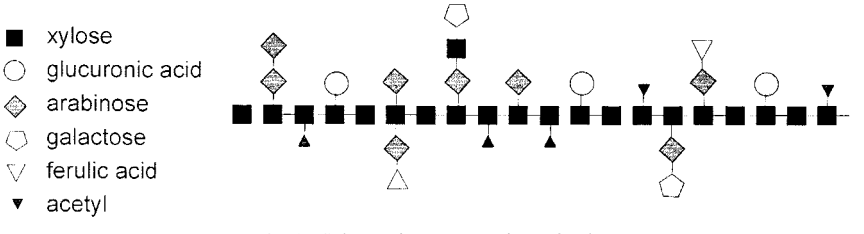

FIG. 2. Schematic presentation of xylan.

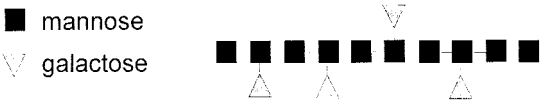

FIG. 3. Schematic presentation of galactomannan.

in the large variety of xylan structures found in plants. Although most xylans are branched structures, some linear polysaccharides have been isolated (102, 261). 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 large amount of D-glucuronic acid attached to the backbone.

Arabinose is connected to the backbone of xylan via an α-1,2- or α-1,3-linkage either 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 O-2 or O-3 of xylose in the backbone of xylan, but the degree of acetylation differs greatly amongst xylans from different origin. Glucuronic acid and its 4-O-methyl ether are attached to the xylan backbone via an α-1,2-linkage, whereas aromatic (feruloyl and *p*-coumaroyl) residues have so far been found attached only to O-5 of terminal arabinose residues (324, 343, 397). As a consequence of all these features, the xylans form a very heterogeneous group of polysaccharides (27, 47, 156, 328).

Structural Features of Galacto(gluco)mannan

Galactomannans and galactoglucomannans form a second group of hemicellulolytic structures present in plant cell walls. They are the major hemicellulose fraction of gymnosperms (20), in which they represent 12 to 15% of the cell wall biomass. Galactomannans are most commonly found in the family of Leguminoseae, in which they represent 1 to 38% of seed dry weight, but have also been identified in species of other plants such as Ebenaceae and Palmae (75, 97). They consist of a backbone of β-1,4-linked D-mannose residues, which can be substituted by D-galactose residues via an α-1,6-linkage (Fig. 3). Depending on the source of the polysaccharide, mannose/galactose ratios can vary from 1.0 to 5.3 (75, 97).

Galactoglucomannan is the major hemicellulolytic component of softwood. Two different structures can be identified within this group of polysaccharides (Fig. 4) (369). Both consist of a β-1,4-linked D-mannose backbone, which can be substituted by α-1,6-linked D-galactose. The galactoglucomannan backbone also contains β-1,4-linked D-glucose residues. Water-soluble galactoglucomannan has a higher galactose content than does water-insoluble galactoglucomannan and in addition

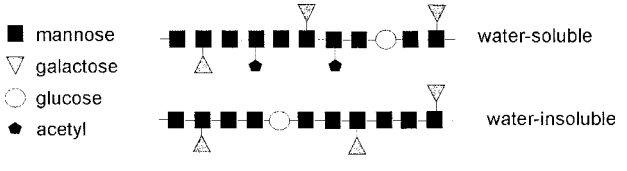

FIG. 4. Schematic presentation of the two galactoglucomannan structures.

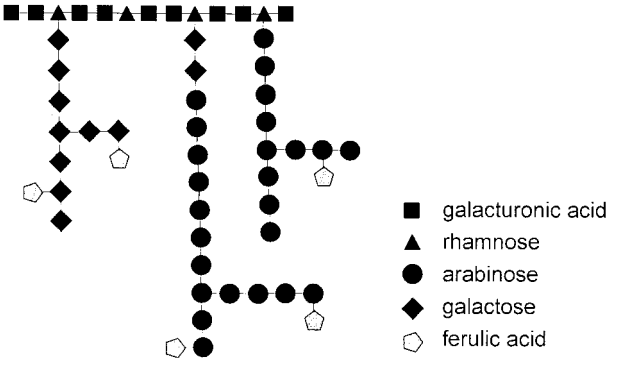

FIG. 5. Schematic presentation of the hairy region of pectin.

contains acetyl residues attached to the main chain (369). Approximately 20 to 30% of the backbone glucose and/or mannose residues are esterified with acetyl groups at C-2 or C-3 (233). Recently, the structure of a galactoglucomannan from *Nicotiana plumbaginifolia* was analyzed (338). Apart from side chains consisting of single α-1,6-linked galactose residues, this polysaccharide also contained a disaccharide consisting of a galactose residue β-1,2-linked to a galactose residue that is α-1,6-linked to the main chain.

Structural Features of Pectin

Pectins are complex heteropolysaccharides which contain two different defined regions (85, 290). The "smooth" regions consist of a backbone of α-1,4-linked D-galacturonic acid residues, which can be acetylated at O-2 or O-3 or methylated at O-6. In the "hairy" regions, two different structures can be identified, a xylogalacturonan consisting of a D-xylose-substituted galacturonan backbone and rhamnogalacturonan I. In rhamnogalacturonan I (Fig. 5), the D-galacturonic acid residues in the backbone are interrupted by α-1,2-linked L-rhamnose residues, to which long arabinan and galactan chains can be attached at O-4. The arabinan chains consist of a main chain of α-1,5-linked L-arabinose residues that can be substituted by α-1,3-linked L-arabinose and by feruloyl residues attached terminally to O-2 of the arabinose residues (66, 134). The galactan side chains contain a main chain of β-1,4-linked D-galactose residues, which can be substituted by feruloyl residues at O-6 (66, 134). Approximately 20 to 30% of the feruloyl residues in sugar beet pectin are attached to arabinan side chains, whereas the other feruloyl residues are attached to galactan side chains (134). Rhamnogalacturonan I also contains acetyl groups ester-linked to O-2 or O-3 of galacturonic acid residues of the backbone (326, 327). Rhamnogalacturonan II is a polysaccharide of approximately 30 monosaccharide units with a backbone of galacturonic acid residues that is substituted by four side chains. The structures of these side chains have been determined and have been shown to contain several uncommon sugars such as 2-*O*-methyl-L-fucose and 3-deoxy-D-*manno*-2-octulosonic acid (247). The structural arrangement in which these side chains are attached to the backbone of rhamnogalacturonan II have also been determined and have demonstrated two possible arrangements for this oligosaccharide (148). Whether rhamnogalacturonan II is covalently linked to the pectin main chain is not known.

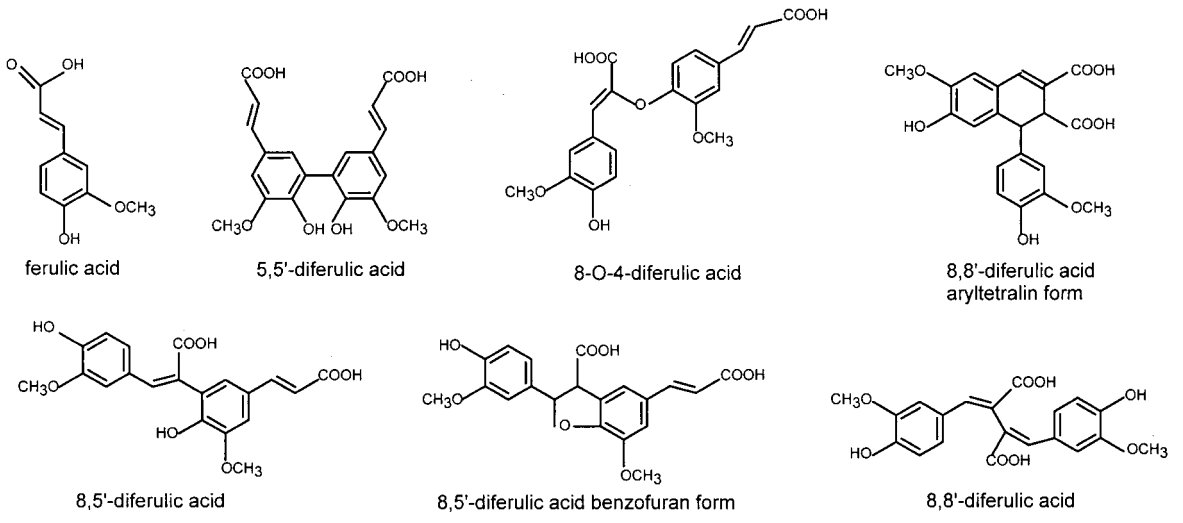

FIG. 6. Schematical presentation of ferulic acid and diferulic acid structures identified in plant cell walls.

Aromatic Residues in Plant Cell Wall Polysaccharides

Aromatic compounds are thought to play an important role in the structure and function of the plant cell wall. Ferulic acid can be linked to both the hemicellulose (343) and the pectin (314) fractions of plant cell walls and is able to cross-link these polysaccharides to each other as well as to the aromatic polymeric compound lignin (163, 230). 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 (118, 395). A role for these cross-links in preventing biodegradability of the plant cell wall by microorganisms has also been suggested. Indications for a limited enzymatic degradation of arabinoxylan due to ferulate cross-links have been obtained (104, 131). Additionally, the antimicrobial effects of these aromatic compounds (21) may contribute to to the plant defense mechanism against phytopathogenic microorganisms.

In cereals, cinnamic acids (mainly ferulic acid) are ester-linked to arabinose residues in arabinoxylan in the primary cell wall. Ferulic acid was detected both as terminal residues and as ferulate dimers linked in several ways (Fig. 6), such as 5,5′ or 5,8′ carbon-carbon bonds (163, 217).

BIODEGRADATION OF PLANT CELL WALL POLYSACCHARIDES

Aspergillus

The genus *Aspergillus* is group of filamentous fungi with a large number of species. The first record of this fungus can be found in Micheli's *Nova Plantarum Genera* (258), but a more detailed description of the aspergilli did not appear until the middle of the 19th century. In 1926 a first classification of these fungi was proposed describing 11 groups within the genus (366). A reexamination of the genus was published by Thom and Raper (367), identifying 14 distinct groups. Some of these groups consist of pathogenic fungi (e.g., *A. fumigatus*, *A. flavus*, and *A. parasiticus*), but most important for industrial applications 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 the reclassification of this group of aspergilli (137, 226, 257, 271, 283, 386). These analyses 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*) (283). 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 ideal organisms for industrial applications, such as good fermentation capabilities and high levels of protein secretion. In particular, the wide range of enzymes produced by *Aspergillus* for the degradation of plant cell wall polysaccharides are of major importance to the food and feed industry. Recently, several *Aspergillus* spp. have received increased interest as hosts for heterologous protein production (74).

Degradation of Cellulose and the Xyloglucan Backbone

Four classes of enzymes are involved in the biodegradation of cellulose. Endoglucanases (EC 3.2.1.4) (Table 1) hydrolyze cellulose to glucooligosaccharides. Cellobiohydrolases (EC 3.2.1.91) release cellobiose from crystalline cellulose. β-Glucosidases (EC 3.2.1.21) (Table 1) degrade the oligosaccharides to glucose. Exoglucanases (Table 1) release glucose from cellulose and glucooligosaccharides. The distinction between exoglucanases and cellobiohydrolases is not always clear due to differences in the methods used to study these enzymes. All four classes of enzymes have been identified in aspergilli, although the number of isozymes produced by different species or even strains of the same species can differ. An analysis of the production of endoglucanases by 45 *A. terreus* isolates not only revealed different electrophoretic mobilities for the enzymes of the different isolates but also indicated the absence of endoglucanase I in a number of the isolates (341). Endoglucanases and β-glucosidases are also able to degrade the backbone of xyloglucan. From *A. aculeatus* an endoglucanase has been purified that is specific for the substituted xyloglucan backbone (287). This enzyme was not able to hydrolyze cellulose, and

TABLE 1. Physical properties of endo- and exoglucanases and β-glucosidases from *Aspergillus*

Species and enzyme type	Enzyme	Mol mass (kDa)	pH_{opt}	T_{opt} (°C)	pI	Reference(s)
Endoglucanases						
A. aculeatus	XEG	23.6			3.4	287
A. aculeatus	FI-CMCase	25	4.5	50	4.8	263
A. aculeatus	FII-CMCase	66	5.0	70	4.0	358
A. aculeatus	FV-CMCase	38	4.0	65	3.4	358
A. aculeatus	hydrocellulase	68	2.5	60	3.5	358
A. aculeatus	FI-Avicelase	109	5.5	65	4.7	358
A. aculeatus	FIII-Avicelase	112	2.5	65	4.0	358
A. fumigatus		12.5	4.8	60	7.1	286
A. nidulans	Endo-I	25	6.0	65		24
A. nidulans	Endo-II	32.5	5.0	50		24
A. nidulans	EG A	35	6.5	50		58
A. niger		26	3.8–4.0	45		158
A. niger	A	43	2–7	60		389
A. niger	B	25	2–7	60		389
A. niger		40	6.0–7.0	70		7
A. niger		31	4.0	45–50	3.67	273
A. oryzae	CelA	31	5.0	55		193
A. oryzae	CelB	53	4.0	45		193
Exoglucanases						
A. nidulans	Exo-I	29	5.5	50		24
A. niger		52.5	5.5	50		340
β-Glucosidases						
A. aculeatus	β-Gluc1	133	4.5	55	4.7	358
A. japonicus		>240	5.0	65		323
A. nidulans	β-Gluco-I	26	6.0	35		24
A. nidulans	β-Gluco-II	14	5.0	65		24
A. nidulans	P-I	125	5.0	50	4.4	227
A. nidulans	P-II	50	5.5	60	4.0	227
A. niger		325	4.6	60		408
A. niger	I	96	5.1		4.6	401
A. niger	II	96	4.1		3.8	401
A. niger		120	4.5	55–60	4.0	396, 407
A. niger		137		3.8	373	
A. niger		117–122		4.2	150	
A. niger		100	4.8	65		120
A. niger	β-Glu I	49	5.0	55	3.2	406
A. oryzae	BGI	130			4.9	310
A. oryzae	HGT-BG	43	5.0	50	4.2	310
A. terreus		200	4.5	55–60	4.8	49, 313, 360

treatment of plant cell walls with the enzyme liberated only xyloglucan oligosaccharides.

Three exoglucanases have been purified from *A. nidulans* (24) but only exoglucanase I (Exo-I) was studied in detail. Exo-I, Exo-II, and Exo-III differed significantly in their molecular mass (29, 72.5, and 138 kDa, respectively). Exo-II and Exo-III had a had a higher affinity for cellulose than did Exo-I (24). Two exoglucanases have been identified in *A. terreus* (170). Two cellobiohydrolases have been purified from *A. ficuum* (143) and *A. terreus* (170). The two enzymes from *A. ficuum* have very different molecular masses (128 and 50 kDa, respectively), whereas the molecular masses of the *A. terreus* cellobiohydrolases are nearly identical (28.5 and 29.5 kDa, respectively).

Production of cellulolytic enzymes by aspergilli has been observed using the following carbon sources: cellulose (164, 285, 301), sophorose and 2-*O*-β-D-glucopyranosyl-D-xylose (155), and cellobiose, glucose and xylose (8). However, other factors were important as well. Production of both endo- and exoglucanases in *A. fumigatus* was much higher when ammonia was used as a nitrogen source instead of nitrate (347), whereas the production of β-glucosidase in *A. terreus* was higher on nitrate than on ammonia (301). In *A. nidulans*, an endoglucanase was identified that was developmentally regulated and that was produced only during cleisthothecial development (25). For several β-glucosidases, transglycosylation activity has been observed using cellobiose (33, 396), cellotriose, methyl-β-glucoside, and ethyl-β-glucoside (407) as substrates.

Based on the derived amino acid sequences, the gene products have been assigned to different glycosidase families. Endoglucanases are assigned mainly to families 5 and 12 (Table 2), with the exception of CelB from *A. oryzae*. This enzyme was assigned to family 7, which also contains the *Aspergillus* cellobiohydrolases (Table 2). The only exoglucanase gene cloned so far was assigned to family 74 (Table 2). All β-glucosidases from *Aspergillus* have been assigned to family 3 of the glycosidases (Table 2). All cellulose-degrading enzymes have a retaining mechanism. The exoglucanase from *A. aculeatus* (family 74) is the only enzyme for which the catalytic mechanism has not yet been determined.

Degradation of the Xylan Backbone

The biodegradation 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 then be degraded further to xylose by β-xylosidases (EC 3.2.1.37). Both classes of enzymes, as well as their encoding genes, have been characterized from many organisms. Various endoxylanases have been identified in *Aspergillus* (Table 3). 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 (168) to 9.0 (119). Endoxylanases

TABLE 2. Genes encoding endoglucanases, exoglucanases, cellobiohydrolases, and β-glucosidases from *Aspergillus* and their assignment to the glycosidase families

Species	Activity[a]	Gene	Glycosidase family	Database accession no.	Reference
A. aculeatus	EGL	*cel1*	5	AF054512	L. V. Kofod et al., unpublished data
A. aculeatus	EGL	*cmc2*	5	AB015510	M. Arai et al., unpublished data
A. aculeatus	EGL	*xeg*	12	AF043595	287
A. aculeatus	EGL		12	D00546	274
A. kawachii	EGL	*cekA*	12	D12901	322
A. nidulans	EGL	*eglA*	5	AB009402	58
A. niger	EGL	*eglA*	12	AJ224451	383
A. niger	EGL	*eglB*	5	AJ224452	383
A. oryzae	EGL	*celA*	12	D83732	193
A. oryzae	EGL	*celB*	7	D83731	193
A. aculeatus	EXG		74	AB015511	M. Arai et al., unpublished data
A. aculeatus	CBH	*cbhI*	7	AB002821	357
A. niger	CBH	*cbhA*, *cbhB*	7	AF156268, AF156269	124
A. aculeatus	BGL	*bgl1*	3	D64088	179
A. kawachii	BGL	*bglA*	3	AB003470	171
A. terreus	BGL		3	Z37722	D. B. Mitchell, unpublished data
A. wentii	BGL	*bglA-3*	3	P29090	31

[a] EGL, endoglucanase, EXG, exoglucanase; CBH, cellobiohydrolase; BGL, β-glucosidase.

TABLE 3. Physical properties of *Aspergillus* endoxylanases and β-xylosidases

Species and enzyme type	Enzyme	Mol mass (kDa)	pH_{opt}	T_{opt} (°C)	pI	Reference
Endoxylanases						
A. aculeatus	FIa	18	4.0	50	5.6	119
A. aculeatus	FIb	26	5.0	50	9.0	119
A. aculeatus	FIII	52	5.0	70	3.8	119
A. awamori	I	39	5.5–6.0	55	5.7–6.7	210
A. awamori	II	23	5.0	50	3.7	210
A. awamori	III	26	4.0	45–50	3.3–3.5	210
A. flavipes		45	5.0	55		334
A. foetidus	I	24			7.6	26
A. foetidus	II	26			4.6	26
A. fumigatus	I	22			>9.4	26
A. fumigatus	II	10			5.7	26
A. fumigatus	II	19	5.5	55		337
A. kawachii	XylA	35	5.5		6.7	168
A. kawachii	XylB	26	4.5		4.4	168
A. kawachii	XylC	29	2.0		3.5	168
A. nidulans		34	6.0	56	3.4	108
A. niger		33	4.0	50	4.2	130
A. niger	XYLI	20.8	5	55	6.7	115
A. niger	XYLII	13	6	45	8.6	116
A. niger	XYLIII	13	5.5	45	9.0	116
A. niger	XYLIV	14	4.9	45	4.5	333
A. niger	XYLV	28	5.0	42	3.65	114
A. oryzae	I	28	5.0		7.0	26
A. oryzae	II	26	5.0		4.9	26
A. oryzae		46.5	5.0	55	3.6	128
A. sojae	X-I	32.7	5.5	60	3.5	188
A. sojae	X-II-B	35.5	5.5	50	3.75	188
A. sydowii		30	5.5	60		123
A. tubingensis	XlnA	19			3.6	78
β-Xylosidases						
A. awamori		110	6.5	70	4.2	210
A. foetidus		83			4.4	26
A. fumigatus		90	4.5	75	5.4	199
A. fumigatus		60			4.3	26
A. nidulans	XlnD	85	5.0	50	3.4	222
A. niger			5.0	>75		370
A. niger		122	3.8–4.0	70	4.9	312
A. oryzae		62			4.5	26
A. oryzae	XylA	110	4.0	60		198
A. pulverulentus	β-Xyl I	65	2.5–3.5	60	4.7	350
A. pulverulentus	β-Xyl II	100	4.0–5.0	60	3.5	350

also differ in their specificity toward the xylan polymer. Some enzymes cut randomly between unsubstituted xylose residues, whereas the activity of other endoxylanases strongly depends on the substituents on the xylose residues neighboring the attacked residues.

In several aspergilli, three different endoxylanases have been identified (119, 168, 210). The best-studied *Aspergillus* endoxylanases, with respect to substrate specificity, are the three enzymes from *A. awamori* (210). Counting from the reducing end, *A. awamori* endoxylanase I is unable to remove one unsubstituted xylose residue adjacent to singly substituted xylose residues or two unsubstituted xylose residues adjacent to doubly substituted xylose residues (206). *A. awamori* endoxylanase III was not able to remove two unsubstituted xylose residues adjacent to singly or doubly substituted xylose residues toward the reducing end (206).

Hydrolysis of a glucuronoxylan by an endoxylanase from *A. niger* (130) resulted mainly in xylobiose, xylotriose, and xylose, but hydrolysis of an arabinoxylan by the same enzyme resulted mainly in oligosaccharides with a degree of polymerization of more than 3. This suggests that the action of this endoxylanase is reduced by the presence of arabinose residues on the xylan backbone. All xylanases that 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 below (see "Carbon catabolite repression").

Several genes encoding endoxylanases from aspergilli have been cloned. The encoded enzymes have been assigned to glycosidase families 10 and 11 (Table 4), and they all work via a retaining mechanism. 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 acidic endoxylanases are needed to verify this. Recently, a method was developed to experimentally determine whether an endoxylanase belongs to family 10 or 11 (272). This method is based on the irriversible inhibition of family 11 endoxylanases by epoxyl glycosides of D-xylose and xylooligosaccharides, whereas family 10 endoxylanases are unaffected (272).

β-Xylosidases have been identified in several aspergilli (Table 3). These enzymes are highly specific for small unsubstituted xylose oligosaccharides (degree of polymerization of up to 4), and their action results in the production of xylose. Although this activity is of major importance for the complete degradation of xylan, absence of the enzyme does not interfere with the induction of the xylanolytic system (382). The ability of an *A. awamori* β-xylosidase to release xylose from xylooligosaccharides was studied to determine its substrate specificity (206). This enzyme was able to release xylose from the nonreducing end of branched oligosaccharides only when two con-

TABLE 4. Genes encoding *Aspergillus* endoxylanases and β-xylosidases and their assignment to the glycosidase families

Species	Activity[a]	Gene	Glycosidase family	Database accession no.	Reference(s)
A. aculeatus	EXL	*Fla*	10	AB013110	M. Arai et al., unpublished data
A. awamori	EXL	*exlA*	11	X78115	149
A. kawachii	EXL	*xynA*	10	D14847	166
A. kawachii	EXL	*xynB, xynC*	11	D38070, S45138	167; K. Ito, unpublished data
A. nidulans	EXL	*xlnA, xlnB*	11	Z49892, Z49893	291
A. nidulans	EXL	*xlnC*	10	Z49894	238
A. niger	EXL		11	A19535	215
A. niger	EXL	*xynB*	11	D38071	192
A. niger	EXL	*xyn4, xyn5*	11	U39785, U39784	M. Luttig et al., unpublished data
A. oryzae	EXL	*xynG1*	11	AB003085	191
A. oryzae	EXL	*F1*	10	AB011212	197
A. tubingensis	EXL	*xlnA*	11	L26988	78
A. nidulans	BXL	*xlnD*	3	Y13568	292
A. niger	BXL	*xlnD*	3	Z84377	382
A. oryzae	BXL	*xylA*	3	AB013851	198
A. oryzae	BXL	*xyl-1*	3	AB009972	141

[a] EXL, endoxylanase; BXL, β-xylosidase.

TABLE 5. Physical properties of *Aspergillus* β-D-mannanases and β-D-mannosidases

Species and enzyme type	Enzyme	Mol mass (kDa)	pH_{opt}	T_{opt} (°C)	pI	Reference(s)
β-D-Mannanases						
A. aculeatus		45	5.0	60–70	4.5	59
A. aculeatus	FIIIa	39	4.0	70	4.2	356
A. niger		40–45	3.0–3.8	65	3.7–3.95	4, 6, 105, 251
A. niger			3.6	>80	4.1	405
A. niger		56	3.0	50	4.9	307
A. oryzae		110	6.0	40	3.5–4.5	307
A. tamarii		53	4.5			64
β-D-Mannosidases						
A. aculeatus		130	2.0	70	4.0	356
A. awamori		96–100	3.8	66	4.55	270
A. niger		120–130	3.5	55	4.7	46, 103
A. niger		135	2.4–5.0	70	5.0	3

tiguous unsubstituted xylose residues were present adjacent to singly or doubly substituted xylose residues.

Based on the sequence of the corresponding genes, β-xylosidases from *Aspergillus* spp. have all been assigned to glycosidase family 3 (Table 4) and have a retaining mechanism.

For some β-xylosidases, transxylosylation activity has been detected (26, 200, 335, 350), allowing the production of novel xylose containing oligosaccharides using these enzymes. Production of xylooligosaccharides from xylose using β-xylosidase in a condensation reaction was also demonstrated (159), suggesting a possible application for these enzymes in the synthesis of specific oligosaccharides.

Degradation of the Galacto(gluco)mannan Backbone

The degradation of the galacto(gluco)mannan backbone depends on the action of β-endomannanases (EC 3.2.1.78) and β-mannosidases (EC 3.2.1.25), which are commonly produced by aspergilli (Table 5). β-Endomannanases, generally referred to as β-mannanases, hydrolyze the backbone of galacto(gluco) mannans, resulting in mannooligosaccharides. The ability of β-mannanases to degrade the mannan backbone depends on several factors, such as the number and distribution of the substituents on the backbone and the ratio of glucose to mannose (250). β-Mannanase is most active on galactomannans with a low substitution of the backbone (64). The presence of galactose residues on the mannan backbone significantly hinders the activity of β-mannanase (252), but this effect is small if the galactose residues in the vicinity of the cleavage point are all on the same side of the main chain (251). β-Mannanases release predominantly mannobiose and mannotriose from mannan, confirming that they are true endohydrolases (4, 64, 105, 306). It has been shown that *A. niger* β-mannanase binds to four mannose residues during catalysis (249).

β-Mannosidases (EC 3.2.1.25) are exo-acting enzymes, which release mannose from the nonreducing end of mannooligosaccharides. The substrate specificity of *A. niger* β-mannosidase has recently been studied (3). The enzyme is able to completely release terminal mannose residues when one or more adjacent unsubstituted mannose residues are present. The presence of a galactose-substituted mannose residue adjacent to the terminal mannose residue reduces the activity of β-mannosidase to 18 to 43%, compared to unsubstituted substrates, depending on the size of the oligosaccharide (3). Both β-mannanase and β-mannosidase have transglycosylation activity (153, 169, 252) and can therefore be used for the synthesis of specific oligosaccharides.

Complete degradation of the galacto(gluco)mannan backbone to mannose by β-mannanase and β-mannosidase also depends on the action of β-glucosidase and α-galactosidase (see "α- and β-D-galactosidases" below). Galactomannan-degrading enzymes are produced when *Aspergillus* is grown on milled soybean (59), locust bean gum (4), galactomannan (64), and mannose (270). So far, only one β-mannanase-encoding gene (*A. aculeatus man1* [accession no. L35487]) (59) and two β-mannosidase -encoding genes (*A. aculeatus manB* [accession no. AB015509] and *A. niger mndA* [accession no. AJ251874]) (1, 356) have been reported. Based on the sequences of these genes, the *Aspergillus* β-mannanase and β-mannosidases are assigned to glycosidase families 5 and 2, respectively. Both types of enzymes use a retaining mechanism for catalysis.

Degradation of the Pectin Backbone

The structural differences between the main chain of the hairy and smooth regions of pectin have implications for the enzymes involved in the degradation of these regions. The backbone of the smooth region can be hydrolyzed 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 (51, 140). Several classes of enzymes are involved in the degradation of the hairy-region backbone.

The pectin main-chain-degrading enzymes can be divided into hydrolases (Table 6) and lyases (Table 7). Six types of hydrolases have been identified in aspergilli. Several endopolygalacturonases are produced that all cleave within the pectin smooth region (10, 185, 284), whereas exopolygalacturonases cleave at the nonreducing terminal end of this region (35, 139, 182, 259). The *A. aculeatus* and *A. tubingensis* exopolygalacturonases were able to release galacturonic acid from polygalacturonic acid, sugar beet pectin, and xylogalacturonan (35, 180, 182). It also released the dimer β-Xyl-(1,3)-GalA from xylogalacturonan, indicating that the action of the enzyme is not hindered by the presence of xylose on the terminal galacturonic acid residue. The seven endopolygalacturonases produced by *A. niger* differ in their specific activity (varying from 25 to 4,000 U/mg), sensitivity to methylation of the substrate (36, 183, 281, 284), and mode of action. Four of the enzymes (endopolygalacturonases I, A, C, and D) show processive behavior, also known as multiple attack on a single chain (36, 281, 284), whereas the other three enzymes (endopolygalacturonases II, B, and E) work via a single-attack mechanism (36, 281, 284).

Endorhamnogalacturonan hydrolases cleave within the main chain of rhamnogalacturonan and have been identified in several aspergilli (203, 325, 352). These enzymes are severely hindered in their activity by the presence of acetyl residues on the main chain and require the presence of rhamnogalacturonan acetyl esterase (see "Acetyl- and methylesterases" below) for efficient hydrolysis of the rhamnogalacturonan backbone (90). Also characterized are two exo-acting enzymes, rham-

TABLE 6. Physical properties of *Aspergillus* endo- and exopolygalacturonases and rhamnogalacturonan hydrolases

Species and enzyme type	Enzyme	Mol mass (kDa)	pH_{opt}	T_{opt} (°C)	pI	Reference
Endopolygalacturonases						
A. alliaceus	endoPG	40	5.5	35	5.9	259
A. carbonarius	PG-I	61	4.0	55		10
A. carbonarius	PG-II	42	4.1	50		10
A. carbonarius	PG-III	47	4.3	55		10
A. niger	E_1	35	4.1			68
A. niger	E_2	80	3.8			68
A. niger	Endo-I	55	4.9		3.2–3.5	185
A. niger	Endo-II	38	4.8		4.6–5.9	185
A. niger	Endo-III A	57	4.3		3.3	185
A. niger	Endo-III B	57	4.5		3.3	185
A. niger	Endo-IV	59	4.8		3.7	185
A. niger	PGA	35	4.0		3.43	281
A. niger	PGB	35	5.0		6.19	281
A. niger	PGD	51	4.2		4.1	284
A. oryzae	PGI		4.0	60		372
A. oryzae	PGA	41	5.0	45		195
A. oryzae	PGB	39	5.0	55		195
Exopolygalacturonases						
A. aculeatus		42			4.3	35
A. alliaceus	$exoPG_1$	40	3.5	45–50	5.7	259
A. alliaceus	$exoPG_2$	40	6.0	30–35	6.3	259
A. niger	exo-PG I	66	3.8	60	5.6	139
A. niger	exo-PG II	63	4.5	60	5.8	139
A. niger	Exo-I		4.0			185
Rhamnogalacturonan hydrolases						
A. aculeatus	RhgA	51	3–4	40–50		325
A. aculeatus	RGase A	59	3.5	30–50	4.5	203
A. aculeatus	RG-RH	84	4	60	4.9–5.4	265
A. aculeatus	RG-GH	66	4	50	5.12	264

nogalacturonan rhamnohydrolase (265) and rhamnogalacturonan galacturonohydrolase (264), that further degrade the oligosaccharides from the nonreducing end. Recently the activity of an endo-acting xylogalacturonase has been characterized (377) that is specific for a xylose-substituted galacturonic acid backbone. The stereochemical course of hydrolysis of several enzymes involved in the degradation of the main chain of the pectin hairy regions was studied recently (39, 297). Enzymes acting on the hairy regions of pectin, exogalacturonase, rhamnogalacturonan hydrolase, rhamnogalacturonan rhamnohydrolase, and α-rhamnosidase (297), as well as enzymes acting on the smooth regions, endopolygalacturonase I and II (39), all hydrolyzed the substrate via an inverting mechanism.

Sequencing of the corresponding genes grouped all *Aspergillus* endo- and exopolygalacturonases and rhamnogalacturonases in the same glycosidase family, which has an inverting mechanism of hydrolysis (Table 8).

Pectin, pectate, and rhamnogalacturonan lyases cleave the pectin backbone by β-elimination, which results in the formation of a Δ4,5-unsaturated nonreducing end. Pectin lyases prefer substrates with a high degree of methylesterification, whereas pectate lyases prefer those with a low degree of esterification. A clearer distinction between these two types of enzymes can be made based on the absolute requirement of Ca^{2+} ions for catalysis by pectate lyases versus the lack of Ca^{2+} ion requirement by pectin lyases (173). Six pectin lyase genes have been identified in *A. niger* (37), but so far no indications have been obtained for the presence of more than one pectate lyase (38, 76). The *A. niger* pectin lyases characterized (A, B, and II) prefer substrates with a high degree of esterification.

Only one rhamnogalacturonan lyase has been identified in aspergilli (203, 266). This enzyme has a higher molecular mass than the pectin and pectate lyases and was positively influenced by Ca^{2+} but did not require Ca^{2+} ions for catalysis (266). The activity of the enzyme was positively affected by the presence of galactose side chains and negatively affected by the presence of arabinose side chains and acetyl residues (266).

Lyases working on the smooth regions (pectin and pectate lyases) and on the hairy regions (rhamnogalacturonan lyases) of pectin have been assigned to two different glycosidase families (Table 8), indicating that the differences in the structure of the substrates require a different enzyme structure as well. In this respect, the lyases are different from the galacturonan hydrolases, which all belong to the same glycosidase family.

Crystal structures have been obtained for endopolygalacturonase II (385) and pectin lyases A and B (246, 393) from *A. niger* and for rhamnogalacturonan hydrolase A from *A. aculeatus* (293). All enzymes have the same β-helical topology.

Accessory Enzymes Involved in the Degradation of Plant Cell Wall Polysaccharides

In contrast to the enzymes described in the previous section, which act on the main chain of plant cell wall polysaccharides, accessory enzymes act on the substituents or the side chains of these structures. Some of these enzymes act on linkages between a main-chain residue and a substituent, whereas other enzymes cleave internal or terminal linkages of side chains. This section deals with the different classes of accessory enzymes produced by aspergilli that act on plant cell wall polysaccharides.

α-D-Xylosidases. α-D-Xylosidases can release α-linked xylose residues from xyloglucan. Only a limited number of α-xylosidases has been characterized from *Aspergillus* (Table 9). These enzymes are all highly specific for α-linked xylose residues (410, 411) but differ with respect to the type of glycoside they can hydrolyze. Both enzymes from *A. niger* were able to act on *p*-nitrophenyl-α-D-xylanopyranoside, isoprimeverose, and oligosaccharides derived from xyloglucan (244, 245). α-Xy-

TABLE 7. Physical properties of *Aspergillus* pectin, pectate, and rhamnogalacturonan lyases

Species and enzyme type	Enzyme	Mol mass (kDa)	pH_{opt}	T_{opt} (°C)	pI	Reference
Pectin lyase						
Aspergillus sp. strain CH-Y-1043			8.5–8.8	40–45		79
A. japonicus		32	6.0	55	7.7	161
A. niger	PL B	40	8.5–9.0		5.9	184
A. niger	PLI	37.5			3.65	380
A. niger	PLII	37.5			3.75	380
A. oryzae	PL		8.5	50–55		372
Pectate lyase						
A. nidulans	PL A	40			4.2	76
A. niger	PlyA	43	7.5–8.5			38
Rhamnogalacturonan lyase						
A. aculeatus	RGase B	55	6.0	50	5.2	203

TABLE 8. Genes encoding *Aspergillus* polygalacturonases, exopolygalacturonases, rhamnogalacturonases, endoxylogalacturonan hydrolases, pectin lyases, pectate lysases, and rhamnogalacturonan lyases and their assignment to the glycosidase families

Species	Activity[a]	Gene(s)	Glycosidase family	Database accession no.	Reference(s)
A. aculeatus	PG	*pgaI*	28	AF054893	S. Kauppinen et al., unpublished data
A. flavus	PG	*pecA*, *pecB*	28	U05015, U05020	398
A. niger	PG	*pgaI*, *pgaII*	28	X58892, X58893	50, 52
A. niger	PG	*pgaA*, *pgaB*	28	Y18804, Y18805	281
A. niger	PG	*pgaD*, *pgaE*	28	Y18806, Y14386	282, 284
A. niger	PG	*pgaC*	28	X64356	51
A. oryzae	PG	*pgaA*, *pgaB*	28	D14282, AB007769	194, 195
A. oryzae	PG	*pecA*	28	AF036848	122
A. parasiticus	PG	*pecA*	28	L23523	57
A. tubingensis	PG	*pgaII*	28	X58894, X54146	52, 321
A. tubingensis	XPG	*pgaX*	28	X99795	182
A. aculeatus	RHG	*rhgA*	28	L35499; X83525	203, 353
A. niger	RHG	*rhgA*, *rhgB*	28	X94220, X94221	352
A. tubingensis	XGH	*xghA*	28	AJ249460	377
A. niger	PEL	*pelA*, *pelB*	1	X60724, X65552	224, 225
A. niger	PEL	*pelD*	1	M55657	136
A. nidulans	PLY	*pelA*	1	U05592	152
A. niger	PLY	*plyA*		AJ276331	38
A. aculeatus	RGL	*rhgB*	4	L3550	203

[a] PG, polygalacturonase; XPG, exopolygalacturonase; RHG, rhamnogalacturonase; XGH, endoxylogalacturonan hydrolase; PEL, pectin lyase; PLY, pectate lyase; RGL; rhamnogalacturonan lyase.

losidase I from *A. flavus* is also able to act on all three types of substrates (410), but α-xylosidase II from this fungus is active only on *p*-nitrophenyl-α-D-xylanopyranoside and to a small extent on isoprimeverose (411). α-Xylosidase I from *A. flavus* is produced constitutively, whereas α-xylosidase II from this fungus is specifically induced by xylose (411).

α-L-Arabinofuranosidases and arabinoxylan arabinofuranohydrolases. Arabinose residues can be removed by α-L-arabinofuranosidases (EC 3.2.1.55) and arabinoxylan arabinofuranohydrolases. These enzymes and their corresponding genes from many different microorganisms have been studied and have been shown to differ strongly in substrate specificity. Several arabinofuranosidases and arabinoxylan arabinofuranohydrolases have been purified from *Aspergillus* spp. (Table 10) and studied with respect to their activity on polymeric and oligomeric substrates. The *A. niger* arabinofuranosidase purified by Kaneko et al. (174) was able to release only 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 (34). 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 (209). Wood and McCrae (403) reported the ability of an *A. awamori* arabinofuranosidase to release feruloylated arabinose residues from wheat straw arabinoxylan. Large differences can be observed when the molecular mass and pI of the arabinofuranosidases characterized are compared (Table 10).

Production of arabinofuranosidases has been observed on arabinoxylan (174), sugar beet pulp (110, 376), and L-arabinose and L-arabitol (303, 375). Arabinoxylan arabinofuranohydrolases were produced when *Aspergillus* was grown on oat straw (209) and birchwood xylan (126). The induction of these enzymes is discussed in more detail below (see "Expression of specific genes responding to different inducers").

The mode of action of AXH from *A. awamori* and two arabinofuranosidases (Arafurs A and B) from *A. niger* was studied on alkali-extractable wheat flour arabinoxylan (207). AXH specifically released α-1,2- and α-1,3-linked arabinose residues from singly substituted xylose residues. Whereas Arafur B was able to release arabinose only from terminal singly substituted residues, AXH and Arafur A were able to release arabinose from both terminal and nonterminal singly substituted xylose residues. AXH and Arafur B were able to release arabinose from the intact polysaccharide as well as from xylooligosaccharides, while Arafur A was able to release arabinose only from xylooligosaccharides. 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 Arafurs 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 glucuronoarabinoxylan as a substrate (387, 388). 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 (387, 388).

The difference between arabinoxylan arabinofuranohydrolases and arabinofuranosidases is also apparent with respect to the assignment to the glycosidase families (Table 11). Arabinofuranosidases are assigned to families 51 and 54, which both have a retaining mechanism, whereas arabinoxylan arabino-

TABLE 9. Physical properties of *Aspergillus* α-D-xylosidases

Species	Enzyme	Mol mass (kDa)	pH_{opt}	T_{opt} (°C)	pI	Reference
A. flavus	I	100	4.5	45		410
A. flavus	II	67	6.0	40		411
A. niger	I	123	2.5–3.0	45	5.6	245
A. niger	II	800	2.5–3.0	40–45		244

TABLE 10. Physical properties of *Aspergillus* arabinofuranosidases, arabinoxylan arabinofuranohydrolases, and endo- and exoarbinanases

Species and enzyme type	Enzyme	Mol mass (kDa)	pH_{opt}	T_{opt} (°C)	pI	Reference(s)
Arabinofuranosidases						
A. aculeatus	B1	37	3.0–3.5			34
A. aculeatus	B2	37	4.0–4.5			34
A. awamori		64	4.6	50	3.6, 3.2	403
A. nidulans	α-AFase	36	5.5	55	4.3	108
A. nidulans	AbfB	65	4.0	65	3.3	303
A. niger	AbfA	83	3.4	46	3.3	376
A. niger	AbfB	61–67	3.8–4.0	56–60	3.5	135, 174, 376
A. niger	A	128	4.1	50	6–6.5	315
A. niger	B	60	3.7	60	5.5–6	315
A. niger		53	3.8		3.6	355
A. niger var. Tieghem	α-L-AFS	64	4.5	50		267
A. sojae	X-II-A	34.3	5.0	50	3.9	188
A. terreus	AbfA	39	4.0		7.5	235
A. terreus	AbfB1	59	4.0		8.3	235
A. terreus	AbfB2	59	4.0		8.5	235
Arabinoxylan arabinofurano-hydrolases						
A. awamori	Axh	32	5.0			209
A. tubingensis	AxhA	32			3.6	126
Endoarabinases						
A. aculeatus	Endo-ara A	45	5.5			34
A. nidulans	Abn	40	5.5	68	3.25	303
A. niger		34.5	4.7	50–55	3.0	329
A. niger		35	5.0	50	4.5–5.5	315
A. niger	AbnA	43	4.6	51	3.0	376
Exoarabinase						
A. niger		67	4.0	60	2.85	M. Lahaye and J.-F. Thibault, unpublished results

furanohydrolases belong to family 62. Arabinofuranohydrolase from *A. sojae* was assigned to family 62 based on the amino acid sequence (189), but it has significantly different substrate specificity from that of AxhA from *A. niger* (126). The latter enzyme is active only on arabinoxylan, whereas arabinofuranohydrolase also releases arabinose from L-arabinan and arabinogalactan (189). For these enzymes, the hydrolysis mechanism has not yet been elucidated. AbfA from *A. niger* is assigned to a different family from AbfB from *A. niger*, which might reflect the differences in 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.

Endo- and exoarabinases. Endoarabinases (EC 3.2.1.99) hydrolyze the α-1,5-linkages of arabinan polysaccharides, which are present as side chains of pectin. Although some arabinofuranosidases are also able to hydrolyze polymeric arabinan (see the previous section), 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 10). The production of endoarabinases by *Aspergillus* spp. was observed on sugar beet pulp (376) and L-arabinose and L-arabitol (303, 375). In *A. niger*, induction of AbnA seems to occur simultaneously with the induction of AbfA and AbfB (111).

An analysis of the degradation patterns of linear (1–5)-α-L-arabino-oligosaccharides using *A. niger* endoarabinase demonstrated that the enzyme is not (or is hardly) able to release terminal residues but preferentially acts on internal linkages (100).

Only one endoarabinase-encoding gene has been found in *Aspergillus* spp. (Table 11). Based on the sequence of this gene, AbnA was assigned to family 43 of the glycosidases and has a inverting mechanism.

So far, only one exoarabinase has been purified from *Aspergillus* (228). This enzyme released mainly arabinobiose from sugar beet arabinan, although a small amount of arabinotriose was also liberated.

α- and β-D-galactosidases. The removal of D-galactose residues from plant cell wall polysaccharides requires the action of α-galactosidases (EC 3.2.1.22) and β-galactosidases (EC 3.2.1.23) (Table 12). β-Galactosidases release terminal galactose residues from the galactan side chains of pectins. α-Galactosidases are involved in the degradation of galacto(gluco) mannan, removing galactose from the mannose residues of the backbone. The presence of terminal β-linked galactose residues in certain galactoglucomannans (338) suggest that both α- and β-galactosidases may play a role in the degradation of these polysaccharides. Studies addressing the activity of α- and β-galactosidases on xylan have not been reported. However, the production of α- and β-galactosidases on crude substrates containing xylan indicates a putative role for these enzymes in the degradation of xylan. Production of α-galactosidases has been reported on arabinoxylan (242), glucose (412), locust bean gum (81), wheat and rice bran (345), lactose and galactose (309), galactomannan (64), and guar flour (5). *Aspergillus* spp. produce β-galactosidase during growth on arabinoxylan (242), polygalacturonic acid (254), wheat bran (129), and lactose (305). Several different α-galactosidases have been purified from *Aspergillus* spp. (Table 12), but there are no indications for the production of more than one β-galactosidase by any *Aspergillus* sp. The differences in molecular mass observed for the purified β-galactosidases (Table 12) are most probably

TABLE 11. Genes encoding *Aspergillus* arabinofuranosidases, AXH, and endoarabinanases and their assignment to the glycosidase families

Organism	Activity[a]	Gene	Glycosidase family	Database accession no.	Reference
A. nidulans	ABF	*abfB*	54	Y13759	125
A. niger	ABF	*abfA*	51	L29005	112
A. niger	ABF	*abfB*	54	X74777	110
A. niger	ABF	*abf2*	54	U39942	71
A. sojae	ABF		62	AB032289	189
A. niger	AXH	*axhA*	62	Z78011	126
A. tubingensis	AXH	*axhA*	62	Z78010	126
A. nigers	ABN	*abnA*	43	L23430	109

[a] ABF, arabinofuranosidase; ABN, endoarabinanase.

TABLE 12. Physical properties of *Aspergillus* α- and β-galactosidases and endo- and exogalactanases

Species and enzyme type	Enzyme	Mol mass (kDa)	pH_{opt}	T_{opt} (°C)	pI	Reference
α-Galactosidases						
A. ficuum		70.8	6.0	60		412
A. nidulans		87	4–5	50	6.3	309
A. niger	AglA	82			4.8	93
A. niger	AglB	54	4.5	50–55	4.2–4.6	242
A. niger		95	6.0			201
A. niger	AglA	82	4–4.5	50	3.73	330
A. niger	α-gal I	94	4.5	60	4.15	2
A. niger	α-gal II-IV	64	4.5	60	4.5–4.8	2
A. niger		45	4–4.5			5
A. niger		78, 69	5	50		345
A. oryzae		64	4.0	60		11
A. tamarii		88	4.2–4.3			63
A. tamarii		77.5	4.2–4.3			63
A. tamarii		56	4.8			64
β-Galactosidases						
A. fonsecaeus		124	4.5		4.2	129
A. niger		93	4	60–65	4.6	242
A. niger		117			4.9	133
A. phoenicis			4.0	70		305
Endo-β-1,4-galactanases						
A. aculeatus		42	4.25	50	4–6	374
A. aculeatus		38	3.5–4.0	50–55	2.8	229
A. aculeatus		43	4.0–4.5	40–65	<3.0	61
A. niger		46	4–5	55	2.9	329
A. niger		43	4.0	50–55	4–6	374
A. niger		32	3.5	55		404
A. niger var. Tieghem		44	3.6	55		267
A. sojae		39.7	4.5	50	3.6	190
Endo-β-1,6-galactanase						
A. niger	β-1,6-	60	3.5	60		48
Exo-β-1,3-galactanase						
A. niger	exo-β-1,3-	66	4.5	40–50		288
Exo-β-1,4-galactanase						
A. niger	exo-β-1,4-	90–120	3.5	60	3.8–4.1	43

due to strain differences and differences in glycosylation of the enzymes.

For one α-galactosidase from *A. niger*, α-galactosyltransferase activity has been detected (330). This enzyme transferred an α-galactosyl residue to the 4-position of a galactosyl receptor.

Several genes encoding α-galactosidases have been cloned and characterized from *Aspergillus niger* (2, 81, 94). Based on their sequence, AglA and AglB have been assigned to glycosidase family 27 (Table 13). AglC 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) (223), this enzyme has been assigned to family 35 of the glycosidases. All *Aspergillus* galactosidases work via a retaining mechanism.

TABLE 13. Genes encoding *Aspergillus* α-galactosidases, β-galactosidase, and β-1,4-endogalactanases and their assignment to the glycosidase families

Species	Activity[a]	Gene	Glycosidase family	Database accession no.	Reference
A. niger	AGL	*aglA*	27	X63348	81
A. niger	AGL	*aglB*	27	Y18586	94
A. niger	AGL	*aglC*	36	AJ251873	1
A. niger	LAC	*lacA*	35	L06037	223
A. aculeatus	GAL	*gal1*	53	L34599	61
A. niger	GAL	*galA*	53	AJ305303	R. P. de Vries et al., unpublished
A. tubingensis	GAL	*galA*	53	AJ012316	378

[a] AGL, α-galactosidase; LAC, β-galactosidase; GAL, β-1,4-endogalactanase.

TABLE 14. Physical properties of *Aspergillus* α-glucuronidases

Species	Enzyme	Mol mass (kDa)	pH_{opt}	T_{opt} (°C)	pI	Reference
A. niger 5–16	CM-I	130	4.8	60	5.3	371
A. niger 5–16	CM-II	150	4.8	60	5.3	371
A. tubingensis	AguA	107	4.5–6.0	70	5.2	93

Endo- and exogalactanases. The galactan side chains of pectin are hydrolyzed by endogalactanases (EC 3.2.1.89), exogalactanases, and β-galactosidases (see the previous section). Endogalactanases are able to hydrolyze the galactan polysaccharides, resulting in the liberation of galactobiose and galactose. Production of endogalactanases was observed on beet pulp (190), soybean (61), and locust bean gum (15). Differences between the enzymes exist with respect to their ability to hydrolyze β-1,3-, β-1,4- or β-1,6 linkages between galactose residues. Two types of arabinogalactans 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 12). Two exogalactanases have been purified from *A. niger*. The β-1,4-exogalactanase (43) was able to release galactose from galactooligosaccharides and potato galactan (44, 45). Additionally, this exogalactanase possessed galactose transferase activity (43–45), indicating a possible application for this enzyme in the production of specific galactooligosaccharides and a retaining mechanism of hydrolysis. The β-1,3-exogalactanase (288) was not active against native plant polysaccharides but had a high activity against a β-1,3-galactan obtained from gum arabic by partial acid hydrolysis and two Smith degradations. The enzyme was capable of releasing the β-1,6-side chains of type II arabinogalactans by hydrolyzing the β-1,3-linkages in the main chain adjacent to the branching point (288).

Genes encoding *Aspergillus* β-1,4-endogalactanase have been reported (Table 13). Based on these sequences, the enzymes were assigned to glycosidase family 53.

α-Glucuronidases. Glucuronic acid residues and their 4-*O*-methyl ethers can be removed from the xylan backbone by α-glucuronidases (EC 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 been purified from only a small number of organisms. α-Glucuronidases have been isolated from *A. niger* and *A. tubingensis* (Table 14). The enzyme is active mainly on small xylooligomers and therefore is dependent on the action of endoxylanases. α-Glucuronidases have the highest activity against oligosaccharides, whereas only low or no activity is observed against polymeric substrates (40,

TABLE 15. Physical properties of *Aspergillus* feruloyl, acetyl, and methyl esterases

Species and enzyme type	Enzyme	Mol mass (kDa)	pH_{opt}	T_{opt} (°C)	pI	Reference
Feruloyl esterases						
A. awamori	FE	112			3.7	253
A. awamori	CE	75			4.2	253
A. awamori		35	5.0	45	3.8	212
A. niger	Fae-I	63			3.0	107
A. niger	Fae-II	29			3.6	107
A. niger	FaeA (FAE-III)	36	5.0	60	3.3	92
A. niger	CinnAE	75.8	6.0	50	4.8	217
A. niger	CE	120				28
A. oryzae	FAE	30	4.5–6.0		3.6	363
A. tubingensis	FaeA	36	5.0	60	3.4	92
Acetylxylan esterase						
A. awamori	ACEA	31	7.0	40		213
A. niger		30.5	5.5–6.0	50	3.0–3.2	208
A. niger	AXE	35	7.0	35		234
Acetylgalactoglucomannan esterase						
A. niger		40	6	46	4.1	300
A. oryzae	AGME	36	5.0–5.5		4.6	364
Rhamnogalacturonan acetylesterases						
A. aculeatus		32–34	6.0	40		178
A. niger	RGAE	42	5.5	50	4.5–6	315
Pectin methylesterase						
A. aculeatus	PME	36.2	4.6	45	3.8	60
A. niger	PE	39	4.5	40		29
A. niger		43			3.6	187
Pectin acetyl esterase						
A. niger	PAE	60	5.5	50	4.1	332

93). Synergy between α-glucuronidases and endoxylanases and between α-glucuronidases and β-xylosidase has been reported (90, 93).

Two genes encoding *Aspergillus* α-glucuronidases have been reported (*A. niger agu*A [accession no. AJ290451] and *A. tubingensis aguA* [accession no. Y15405]) (93). These genes show significant sequence identity to other fungal and bacterial α-glucuronidases and are assigned to glycosidase family 67. Recently it has been shown that this enzyme has an inverting mechanism (40).

Feruloyl and *p*-coumaroyl esterases. Several types of feruloyl and *p*-coumaroyl esterases can be identified based on their physical properties as well as by substrate specificity (Table 15). All enzymes (except *A. awamori p*-coumaroyl esterase) are active on methylferulate, which is a synthetic substrate commonly used for feruloyl esterase assays. Studies of the activities of feruloyl esterases against natural substrates have focused mainly on xylan and xylan-derived oligosaccharides, from which most enzymes were able to release ferulic acid. Only two of these enzymes, FaeA (90, 92) and CinnAE (217), have been shown to release ferulic acid from pectin. A comparative study using *A. niger* FaeA and CinnAE (216) demonstrated a preference of FaeA for substrates with a methoxy group at position 3 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 position 3 of the aromatic ring, whereas additional methoxy groups at other positions of the aromatic 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 (302). FaeA was able to release ferulic acid, which was linked to O-5 of arabinose (as present in wheat arabinoxylan). FaeA was not able to release ferulic acid linked to O-2 of arabinose (as present in sugar beet pectin) but did release ferulic acid linked to O-6 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 (302). 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 hydrolyze feruloyl esters (253). A similar enzyme has not been reported for other organisms, but in nearly all purifications feruloyl esterase activity was monitored using methylferulate as a substrate. Coumaroyl esterase activity would therefore not be detected.

To date, several genes encoding feruloyl esterases have been cloned from *Aspergillus* (Table 16). A region of the amino acid sequence of FaeA from *A. niger* and *A. tubingensis* has homology to the active site of lipases (92). 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. No lipase activity could be detected for FaeA (9).

Acetyl- and methylesterases. Acetylesterases and methylesterases release acetyl and methyl residues from the backbone

TABLE 16. Genes encoding *Aspergillus* feruloyl, acetyl- and methylesterases and their assignment to the carbohydrate esterase families.

Species	Activity	Gene	Carbohydrate esterase family	Database accession no.	Reference
A. awamori	FE	*ferA*		AB032760	T. Koseki et al., unpublished results
A. niger	FE	*faeA*		Y09330	92
A. niger	FE	*faeB*		AJ309807	R. P. de Vries et al., submitted
A. tubingensis	FE	*faeA*		Y09331	92
A. awamori	AXE	*aceA*	1	D87681	213
A. niger	AXE	*axeA*	1	A22880	90
A. terreus	AXE	ORF1	3	AF141924	187
A. aculeatus	RGAE	*rha1*	12	X89714	178
A. niger	RGAE	*rgaeA*	12	AJ242854	90
A. aculeatus	PME	*pme1*	8	U49378	60
A. niger	PME	*pmeA*	8	X54145	187
A. oryzae	PME	*pmeA*	8	AB011211	196

[a] FE, feruloyl esterase; AXE, acetylxylan esterase; PME, pectin methylesterase.

of cell wall polysaccharides (Table 15). Acetylxylan esterases (EC 3.1.1.72) remove acetyl from O-2 or O-3 of xylose in the xylan-main chain. Although acetylxylan esterase activity has been detected in several aspergilli, such as *A. niger, A. japonicus*, and *A. nidulans* (41, 186, 342), only a limited number of acetylxylan esterases have been purified from *Aspergillus* spp. (208, 213, 351). Unlike most other accessory enzymes, acetylxylan esterases are highly active on the polymeric substrate and are thought to be important for efficient degradation of the xylan backbone by endoxylanases. The presence of the *A. niger* acetylxylan esterase enabled degradation of steamed birchwood xylan by three types of endoxylanase and a β-xylosidase, which could not degrade this substrate in the absence of the esterase (208), indicating the importance of this enzyme in xylan degradation.

Two acetylglucomannan esterases have been purified from *Aspergillus* (300, 364). The enzyme purified from *A. niger* (300) is highly specific for acetylated galacto(gluco)mannan, whereas the *A. oryzae* esterase is also active (to a lesser extent) on acetylated xylan (361, 364). Both esterases were active on polymeric and oligomeric substrates, and significantly influenced the activity of β-1,4-mannanase. However, the presence of β-1,4-mannanase had a greater influence on the activity of the *A. oryzae* esterase (364) than on the activity of the *A. niger* enzyme (300).

The acetyl and methyl residues in the smooth regions of pectins are removed by pectin acetylesterases (332) and pectin methylesterases (EC 3.1.1.11) (113, 187). Several pectin methylesterases have been purified from *Aspergillus* spp. (29, 60, 187). The ability of polygalacturonases and pectate lyases to degrade the pectin main chain depends on the activity of pectin methylesterase. Recently it has been shown that pectin methylesterase is unable to remove methyl residues from the nonreducing end of the pectin backbone and also cannot deesterify a methyl-esterified galacturonic acid dimer (181). Only one pectin acetyl esterase (PAE) from *Aspergillus* has been reported so far (332). ^{1}H nuclear magnetic resonance spectroscopy experiments identified differences in the acetyl residues attached to the pectin backbone, and showed that the activity of *A. niger* PAE did depend on these differences (332). PAE works synergistically with pectin methylesterase and pectin lyase.

A rhamnogalacturonan acetylesterase (RGAE) has been purified from *A. aculeatus* (178) and from *A. niger* (90, 332). This enzyme was found to be essential for the action of rhamnogalacturonan hydrolases (90, 178). ^{1}H nuclear magnetic resonance spectroscopy experiments also identified differences in the acetyl residues attached to the rhamnogalacturonan main chain, but RGAE was shown to randomly remove the different types of acetyl residues (332). Pectin acetylesterases can be easily distinguished from rhamnogalaturonan acetylesterases by their activity on triacetin, which cannot be hydrolyzed by the latter enzyme (332).

Only a limited number of genes encoding *Aspergillus* acetyl- or methylesterases have been reported so far (Table 16). Differences with respect to their substrate specificity are reflected by their assignment to the different carbohydrate esterase families (69).

Synergy between Polysaccharide-Degrading Enzymes

Efficient degradation of polysaccharides requires cooperative 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. In this section, some examples will be given relating to different plant cell wall polysaccharides demonstrating that synergy is in fact a general phenomenon.

Synergistic action has been observed between endoxylanase, β-xylosidase, arabinoxylan arabinofuranohydrolase, and acetylxylan esterase in the degradation of different (glucuronoarabino)xylans (211). Synergy has also been 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 (30, 90, 92). Similarly, both endoxylanase and β-xylosidase positively influenced the release of 4-*O*-methylglucuronic acid from birchwood xylan by *A. tubingensis* α-glucuronidase (93). The latter enzyme enhanced the activity of endoxylanase and β-xylosidase on this substrate. Synergy has also been demonstrated between an endoxylanase (XylI) and AXH from *A. awamori* in the degradation of sorghum glucuronoarabinoxylan (388). A recent study revealed that synergistic interactions in the degradation of xylan not only are present between main-chain-cleaving enzymes and accessory enzymes but also occur among accessory enzymes and that nearly all accessory enzymes positively influence the activity of the main-chain-cleaving enzymes (82). A strong synergistic effect has been observed for the role of *A. niger* acetylxylan esterase in the hydrolysis of steamed birchwood xylan by three endoxylanases from *A. niger* (208). The addition of acetylxylan esterase resulted in an increase in the release of xylose and short xylooligosaccharides by a factor of 1.9 to 4.4, 6.8 to 14.7, and 2.5 to 16.3 for endoxylanase I, II, and III, respectively, depending on the incubation time (208).

Only a limited number of studies demonstrating synergy between pectinolytic enzymes from *Aspergillus* have been reported. Pectin methylesterase from *A. aculeatus* strongly enhanced the degradation and depolymerization of pectin by polygalacturonases (60). Similarly, 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* (178). Although indications for inhibition of RGAE activity by the side chains of the hairy regions were obtained (178), pretreatment of pectin with arabinofuranosidase did not increase the acetyl release by RGAE (331). 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 (92). The release of ferulic acid from pectin by a second *A. niger* feruloyl esterase was positively affected by endoarabinase and arabinofuranosidase from *A. niger* (219), 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 (90). The positive effect of RGAE on the degradation of the hairy-region backbone also positively affected the activity of

feruloyl esterase A, β-galactosidase, and endogalactanase from *A. niger*. Additionally, synergistic effects among these three enzymes, an endoarabinase, and an arabinofuranosidase from *A. niger* were detected.

A similar effect was observed in the degradation of acetylgalactoglucomannan. The presence of galactose and acetyl residues on the backbone severely hindered the activity of β-mannanase (300). The presence of acetylmannanesterase and to a lesser extent α-galactosidase significantly increased the β-mannanase activity on this substrate. Additionally, the action of β-mannanase and α-galactosidase on acetylgalactoglucomannan was positively influenced by the removal of acetyl residues from the main chain by acetylgalactoglucomannan esterase (362, 364).

REGULATION OF GENE EXPRESSION

Coordinated Expression of Genes Encoding Xylanolytic and Cellulolytic 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 (e.g., cellulose and pectin) substrates. However, several cellulolytic enzymes are also produced in the presence of xylan and xylose. This suggests a general system of regulation of the genes encoding these enzymes. Several genes encoding xylanolytic enzymes have been studied with respect to regulation of expression, and all demonstrated expression in the presence of D-xylose, xylobiose, or xylan (78, 93, 108, 126, 222, 292, 382) but repression of expression in the presence of glucose (see "Carbon catabolite repression" below). Expression of some xylanolytic genes was also observed on L-arabinose (93, 95, 222). This might be due to the presence of small amounts of D-xylose in the L-arabinose preparations used, as detected in the L-arabinose preparation obtained from Sigma (R. P. de Vries, unpublished results). Additionally, induction of xylanolytic enzymes was observed on cellobiose and cellulose (154) and on a heterodisaccharide consisting of glucose and xylose (Glcβ1-2Xyl) (155) in *A. terreus*, which are compounds that also induce the cellulolytic system from this fungus. Sophorose and 2-*O*-β-D-xylopyranosyl-D-xylose specifically induced the synthesis of cellulolytic and xylanolytic enzymes, respectively, in this fungus (155). A xylose-induced, glucose-repressed, endoxylanase-encoding gene (*xynG1*) from *A. oryzae* was expressed in *A. nidulans*, resulting in expression of the gene on xylose as well as glucose (191). This indicates that regulation of the expression of xylanolytic genes is not identical in *A. oryzae* and *A. nidulans*, although identical regulation of xylanolytic genes has been reported for *A. niger* and *A. tubingensis* (78).

From *A. niger* a gene encoding a transcriptional activator has been isolated by complementation of a mutant unable to degrade xylan (384). Sequence analysis of this factor, XlnR, demonstrated that it is a member of the GAL4-like family of transcriptional activators. Characterization of XlnR showed that it was responsible for the expression of genes encoding endoxylanase and β-xylosidase. 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 (384). 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 does not activate only the expression of xylanolytic genes (383). Genes encoding two endoxylanases (*xlnB* and *xlnC*), a β-xylosidase (*xlnD*), an arabinoxylan arabinofuranohydrolase (*axhA*), an acetylxylan esterase (*axeA*), an α-glucuronidase (*aguA*), a feruloyl esterase (*faeA*), and two endoglucanase (*eglA* and *eglB*) were found to be regulated by XlnR (383). However, genes encoding α-arabinofuranosidase (*abfB*) and β-glucosidase (*bglA*) were not regulated by this protein. This indicates that in addition to its role as a xylanolytic activator, XlnR also regulates the expression of some, but not all, genes encoding cellulolytic enzymes. A subsequent study demonstrated that XlnR is also involved in the regulation of α- and β-galactosidase genes (*aglB* and *lacA* respectively) (94), two cellobiohydrolase-encoding genes (*cbhA* and *cbhB*) (124), and a gene encoding xylose reductase (142). Analysis of the promoter regions of the genes that are regulated by XlnR demonstrated that the third A in the consensus for the binding site is variable, and the consensus sequence was therefore shortened to GGCTAA (383). However, the presence of a putative XlnR binding site does not automatically imply regulation by XlnR. A putative XlnR binding site was detected in the promoter of an endoglucanase from *A. nidulans*, but no expression of this gene was detected on xylose (58). Introduction of a large number of copies of a xylanolytic gene or the promoter of a xylanolytic gene results in a decrease in the expression of other XlnR-regulated genes (197, 381). This indicates that the production of XlnR is tightly balanced with the number of genes this protein regulates. Indications for a transcription activator from *A. oryzae* binding to a similar DNA region have been obtained (176).

Recently a model was suggested for the role of XlnR in the regulation of (hemi)cellulose degradation by *A. niger* (Fig. 7) (87). XlnR is activated during growth of *A. niger* in the presence of arabinoxylan by monomeric xylose which is already present in the substrate or released by endoxylanase B and β-xylosidase that are present at low constitutive levels. XlnR then activates the expression of (hemi)cellulolytic genes (94, 124, 383). However if the concentration of xylose is high, this causes CreA-mediated repression, resulting in reduced expression of these genes (see "Carbon catabolite repression" below). This effect is stronger in the presence of glucose, which prevents the expression of (hemi)cellulolytic genes to a large extent (78, 93). XlnR-mediated expression of (hemi)cellulolytic genes results in the release of arabinose, cellobiose, ferulic acid, and release galactose by the enzymes encoded by these genes. These compounds induce the expression of other genes (see "Expression of specific genes responding to different inducers" below).

Expression of Pectinolytic Genes

The production of pectin main-chain-cleaving enzymes (polygalacturonases, pectin lyases, rhamnogalacturonan hydrolases, and lyases) has been detected on sugar beet pectin (53), apple pectin (140, 352), polygalacturonic acid (320), a combination of rhamnose and galacturonic acid (354), and soybean flour (204). However, some pectinolytic enzymes have also

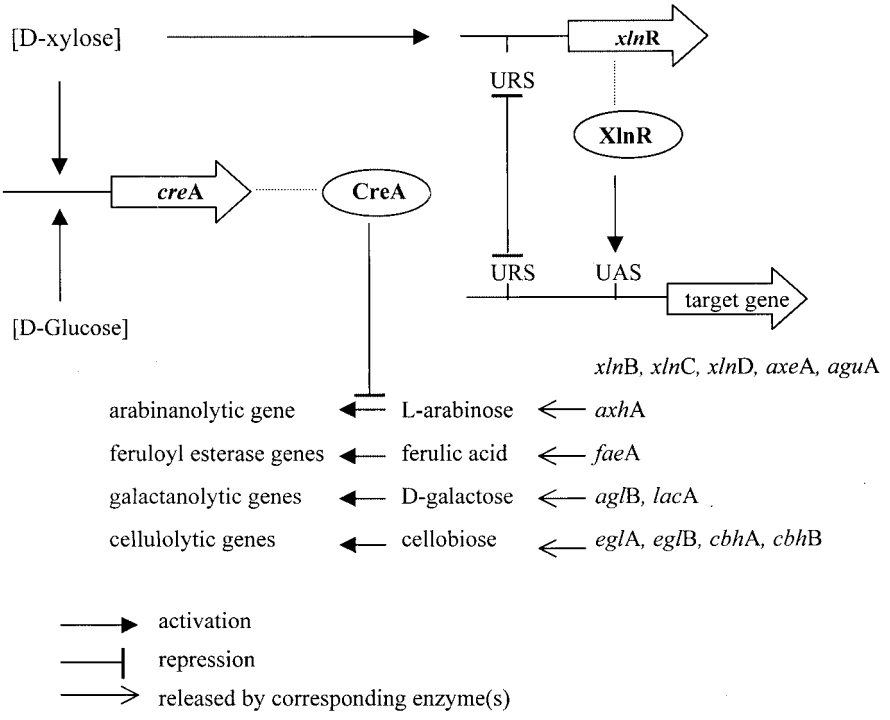

FIG. 7. Model for the role of XlnR and CreA in the regulation of the genes encoding (hemi)cellulose-degrading enzymes by *A. niger*. Other regulatory factors, such as the HAP complex and PacC, are not considered in this model. UAS, Upstream Activating Sequence; URS, Upstream Repressing Sequence. (Reprinted from reference 87 with permission of the publisher.)

been reported to be produced constitutively (259, 281). Expression of genes encoding pectinolytic main-chain-cleaving enzymes has been reported to occur on apple pectin (140, 224, 225), sugar beet pulp (140, 224, 225), galacturonic acid (182), and polygalacturonic acid (76). Strong differences in expression pattern are observed for the different polygalacturonase-encoding genes. Constitutively expressed polygalacturonase-encoding genes have been reported from *A. flavus* (398), *A. parasiticus* (57), and *A. niger* (281). In contrast, no expression could be detected on pectin, polygalacturonic acid, or galacturonic acid for the gene encoding endopolygalacturonase E from *A. niger* (282). 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 (5′-TYATTGGTGGA-3′) (53, 392). A region of high similarity was detected in the promoter of *pgaA* from *A. niger* (281). This gene showed increased expression on D-galacturonic acid, which might be attributed to this region. The expression of *pgaB*, which does not contain this region, is not increased on galacturonic acid, although it has a similar expression profile to that of *pgaA* on sucrose and pectin. Comparison of this region to the promoters of the other genes (*pgaI* and *pgaC*) (392) identified a consensus sequence, which is similar to an upstream activating sequence of the *Saccharomyces cerevisiae cycI* gene, to which the HAP2/3/4-activating complex binds (see "Regulation by a HAP-like CCAAT binding complex" below). Additionally, a hexanucleotide sequence was detected in the promoters of several pectin lyase-encoding genes (CCCTGA) (37). However, the functions of these sequences have not yet been studied in detail.

Recently, additional evidence has been obtained for the role of galacturonic acid as a general inducer for pectinolytic genes in *A. niger*. Several genes encoding pectin main-chain-cleaving enzymes (*pelA, plyA, pgaX*, and *rglA*) and a gene encoding pectin methylesterase (*pmeA*) are expressed in the presence of galacturonic acid (280). Genes encoding arabinofuranosidases (*abfA* and *abfB*), endoarabinase (*abnA*), endogalactanase (*galA*), and β-galactosidase (*lacA*), all of which act on the pectin side chains, are also expressed on galacturonic acid (de Vries, unpublished). In addition, a gene encoding a xylanolytic function (α-glucuronidase, *aguA*) is expressed on galacturonic acid and glucuronic acid (de Vries, unpublished). This may indicate a general system activating expression in response to the presence of galacturonic (and glucuronic) acid.

Expression of Specific Genes Responding to Different Inducers

Apart from the transcription activation by XlnR (xylan degradation) and the induction on galacturonic acid (pectin degradation), other monomeric compounds also induce the expression of specific sets of genes. As mentioned above, arabinofuranosidases and endoarabinases are induced when *Aspergillus* is grown on sugar beet pulp whereas AXH are produced during growth on xylan (see "α-L-Arabinofranosidases and arabinoxylan arabinofuranohydrolases" above). Expression of the genes encoding these enzymes was also observed on monomeric carbon sources, such as L-arabinose and L-arabitol (111, 125, 126, 303, 375). The expression of two arabinofuranosidase-encoding genes (*abfA* and *abfB*) and one

endoarabinase-encoding gene (*abnA*) has been studied in *A. niger* (111). 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 indicated that these genes are most probably under coordinated control of a common specific transcription factor. The reduced expression 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 from studies with an *A. nidulans* mutant defective in L-arabitol dehydrogenase activity (88). This mutant accumulated L-arabitol when grown on media containing glycerol and L-arabitol. Under these conditions, increased arabinofuranosidase, endoarabinase, and L-arabinose reductase activity was observed compared to a wild-type strain. These data also suggest coordinated 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) (375). In addition to the arabinases and the arabinose metabolic pathway functions, an endogalactanase-encoding gene (*galA*) and a β-galactosidase-encoding gene (*lacA*) are expressed in the presence of arabinose and arabitol (de Vries, unpublished), suggesting coordinated regulation of all pectin side-chain-cleaving functions in *A. niger*. Galactose and mannose are involved in the induction of α-galactosidases and β-mannosidases (1). However the expression pattern of α-galactosidases from *A. niger* varies significantly among the individual genes. den Herder et al. (81) demonstrated that the *A. niger* α-galactosidase A-encoding gene (*aglA*) was expressed on galactomannan. A recent study (94) compared the expression of this gene to two other α-galactosidase-encoding genes from *A. niger* (*aglB* and *aglC*) and the *A. niger* β-galactosidase-encoding gene (*lacA*). Of the three α-galactosidase -encoding genes, only *aglA* seemed to be specifically expressed on galactose and galactose-containing oligo- and polysaccharides. The *aglB* gene was constitutively expressed on all carbon sources tested but had increased expression on xylose and xylan. This has been attributed to regulation by the xylanolytic transcriptional activator XlnR (see "Coordinated expression of genes encoding xylanolytic and cellulolytic enzymes" above). Expression of *aglC* was detected only on glucose, which is surprising for an α-galactosidase-encoding gene. The expression of *lacA* was highest on xylose and xylan (regulated by XlnR [see above]), arabinose, and pectin (see "Expression of pectinolytic genes" above), whereas only low expression was observed on galactose. The expression on pectin is akin to the production of this enzyme by *A. oryzae* on polygalacturonic acid (254) and might indicate that this gene is coregulated with genes encoding pectin main-chain-degrading enzymes (see "Expression of pectinolytic genes" above).

The induction of *faeA* from *A. niger* by the product of the enzyme, ferulic acid, has recently been studied (95, 106). This gene is expressed during growth on xylan and xylose, which is mediated by XlnR (see "Coordinated expression of genes encoding xylanolytic and cellulolytic enzymes" above). The addition of ferulic acid to media containing xylan or xylose increased the expression of *faeA*, whereas other xylanolytic genes were unaffected, indicating a second regulatory system for the induction of *faeA*. Although the two systems positively influence each other with respect to the expression of *faeA*, ferulic acid-induced expression is not dependent on XlnR (95). In an XlnR-negative mutant, the level of expression of *faeA* was similar on ferulic acid and a combination of ferulic acid and xylose, while no expression of other xylanolytic genes was detected under these conditions.

Carbon Catabolite Repression

The major system responsible for carbon repression in *Aspergillus* is mediated by the carbon catabolite repressor protein CreA (99, 319). CreA is a zinc finger protein which binds to specific sites in the promoters (SYGGRG) of a wide range of target genes (221). In the presence of easily metabolizable substrates, such as glucose or fructose, CreA inhibits or decreases the expression of the target genes. Several *A. nidulans* and *A. niger* CreA mutants have been isolated which display (partially) derepressed phenotypes (19, 318, 336). Using these mutants, CreA-mediated repression of gene expression has been detected for the proline and alcohol gene clusters in *A. nidulans* (72, 278) and for several extracellular proteases in *A. niger* (172). Recently, the mechanism of CreA-mediated repression has been investigated in more detail (349). It appears that the *creA* gene is strongly expressed when monomeric repressing carbon sources are added to the culture, but CreA quickly down-regulates the expression of its own gene. This autoregulation has been demonstrated to be dependent on the formation of glucose-6-phosphate (349). Under carbon-derepressing conditions, a significantly higher *creA* 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 (349). 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 plant cell wall-degrading enzyme systems from *Aspergillus* has been extensively studied. CreA-mediated repression in *Aspergillus* was detected for genes encoding arabinases and L-arabinose catabolic enzymes (318), several endoxylanases (75, 108, 292, 296, 318), and other xylanolytic functions such as β-xylosidase (222), AXH (126), and feruloyl esterase (95). Some of the pectinolytic genes from *Aspergillus* are also repressed in the presence of glucose (52, 178, 182, 241, 344).

Removal of four putative CreA binding sites from the promoter of the *A. tubingensis xlnA* gene resulted in an increased expression of this gene (78), 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 (*pelA*) using a fusion protein containing the N-terminal part of CreA demonstrated binding of the fusion protein to this fragment (152) 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 xyl-

anolytic gene expression in *Aspergillus* (see "Coordinated expression of genes encoding xylanolytic and cellulolytic enzymes" above). Recently, the effect of different xylose concentrations on xylanolytic gene expression was studied (96), 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* (8). 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 (*faeA*) from *A. niger* was studied in combination with a number of different monomeric carbon sources (de Vries, unpublished). For nearly all combinations, higher *faeA* 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. Similarly, the addition of glycerol and glucose reduced the production of cellulolytic enzymes in *A. nidulans* (24) and *A. japonicus* (323), respectively. Addition of cyclic AMP relieved catabolite expression of cellulase production in glycerol-repressed *A. nidulans*, indicating a role for this compound in carbon catabolite repression (23).

pH-Dependent Expression

The major factor involved in pH-dependent expression in *Aspergillus* is the pH-regulatory protein PacC. At alkaline pH, PacC activates alkalin-expressed genes and represses acid-expressed genes; therefore, PacC has a dual function (368). The gene encoding this protein (*pacC*) has been cloned from *A. nidulans* (368) and was shown to complement *A. nidulans* pH-regulatory mutants, previously isolated as mutants defective in phosphatase expression (16, 18, 98). An *A. niger* homologue has also been cloned (240). PacC is activated under alkaline conditions by proteolytic modification of the C-terminal region, which enables it to bind to specific target sites (GCCA RG, with R being A or G) in the promoter of target genes (276). Six genes (*palA*, *palB*, *palC*, *palF*, *palH*, and *palI*) are believed to be involved in the signal transduction pathway leading to the activation of PacC (17, 54, 368). A more detailed description of pH regulation of gene expression in fungi can be found in a recent review (82).

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 pHs (204). From culture medium at pH 2, two polygalacturonases were purified, whereas only one polygalacturonase could be purified from culture medium at pH 5. The N-terminal amino acid sequences 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 (*abfB*) (125) and two endoxylanase-encoding genes (*xlnA* and *xlnB*) (239) is regulated by PacC. These two endoxylanase-encoding genes have opposite expression patterns with *xlnA* being expressed under alkaline conditions and *xlnB* being expressed under acidic conditions. Analysis of the promoter regions of *xlnA* and *xlnB* revealed the presence of two and one PacC consensus sites, respectively (239). Several putative PacC binding sites were also detected in the promoter of the *A. nidulans* β-xylosidase-encoding gene (*xlnD*), but no clear indications for PacC control of this gene were obtained (292).

The production of cellulases by *A. fumigatus* was reported to depend strongly on the pH (347). During growth on cellulose with ammonia as the nitrogen source, this species acidified the media, resulting in an increased cellulase production compared to cultures with nitrate as the nitrogen source.

Regulation by a HAP-Like CCAAT Binding Complex

The promoters of many *Aspergillus* genes, including genes encoding polysaccharide-degrading enzymes, contain CCAAT boxes. Recently, genes have been cloned from *A. nidulans* encoding homologues of the subunits of the HAP complex of *S. cerevisiae*. The *hapC* gene (279) has significant similarity to *HAP3* of *S. cerevisiae*, whereas *hapB* and *hapE* contain regions of high similarity to *S. cerevisiae HAP2* and *HAP5*, respectively. These three proteins have been produced in *Escherichia coli* and were shown to be necessary and sufficient for CCAAT binding in vitro (346). So far, no homologue of *S. cerevisiae HAP4* has been detected in *Aspergillus* or other filamentous fungi. The function of a HAP-like complex has been studied in *Aspergillus* for the expression of *amdS* (acetamidase) (379), *taaG2* (taka-amaylase) (269), and genes involved in penicillin biosynthesis (365), and the complex is involved in the enhancement of the expression levels of these genes. No studies of the role of HAP-like complexes in the regulation of genes encoding polysaccharide-degrading genes from *Aspergillus* have been reported, although CCAAT boxes have been identified in the promoters of many of the genes from *Aspergillus* encoding plant cell wall polysaccharide-degrading enzymes. However, the presence of CCAAT boxes and the involvement of HAP-like complexes in the regulation of xylanases (415) and cellulases (414) in *Trichoderma reesei* suggests that these systems might also be regulated by HAP-like complexes in *Aspergillus*.

INDUSTRIAL APPLICATIONS

The enzymes described in this review 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 provide a good alternative to chemical processing. In this section, examples of industrial applications of plant cell wall-degrading enzymes are given.

Applications of xylanolytic enzymes 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 biobleaching of pulps (62, 390). Enzymatic degradation of the hemicellulose-lignin complexes present in pulps leaves the cellulose fibers intact and strongly reduces the amount of bleaching chemicals (e.g., chlorine) required. This not only

results in a reduction in costs of chemicals 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 (62, 390), but the addition of other xylanolytic enzymes has also been shown to be effective (175). A second area in which the xylanolytic enzyme preparations are widely used is the bakery industry. In this context, their main effect is in solubilizing the arabinoxylan fraction of the dough, resulting in increased bread volume and an improved quality of the dough (237, 295, 298). Other applications in which xylanolytic enzymes are used include increasing the feed conversion efficiency of animal feed (32), clarifying juices (413), and producing xylose, xylobiose and xylooligomers (42, 289, 299). The oligosaccharides produced are used as functional food additives or alternative sweeteners with beneficial properties.

α-Galactosidases are used to improve the gelling capacity of galactomannans, which have applications in the food industry as well in the cosmetic and pharmaceutical industries (47, 70). Additionally, they reduce the concentration of raffinose and other oligosaccharides in soybean milk (262), cowpea meal (345), and sugar beet syrup (121).

Pectinases are of major importance in the beverage industry due to their ability to improve pressing and clarification of concentrated fruit juices (132). Pectin methylesterase and other pectinolytic enzymes are also used for the production of carrot puree (144). Whereas xylanolytic enzymes are used in the paper and pulp industry mainly for biobleaching, pectinolytic enzymes are used in enzymatic debarking (27, 304). Removal of the bark is the first step in all processing of wood and is traditionally a very energy-consuming process. A reduction in the amount of energy required can be obtained by using pectinolytic enzymes to aid in the process. Other applications for pectinolytic enzymes include increasing the yield of lemon peel oils (65) and the production of oligouronides (316).

The monosaccharides that are the building blocks of the pectin polymer all have different food and nonfood applications, and enzymatic release of these compounds is an important tool in industrial processes. Arabinose is a precursor of L-fructose and L-glucose, which can be used as sweeteners, and it can also be transformed to 5-deoxy-L-arabinose, a compound that has anti-Parkinson properties (394). Galacturonic acid can be enzymatically converted into L-ascorbic acid (220) or can be used to produce surface-active agents by esterification with various fatty acids (294). Chemical transformations of rhamnose result in aromas such as "furaneol," which is used in caramel and fruit flavors (402).

All industrial applications to date utilize the enzymes during the processing of the crude plant material. Recently, in vivo modifications of plant cell wall polysaccharides using *Aspergillus* enzymes has received increasing interest. Transgenic plants have been obtained containing the *A. aculeatus* endogalactanase-encoding gene (277). The galactosyl content of rhamnogalacturonan I in these plants was reduced by 70%, indicating the potential applications of introducing *Aspergillus* genes in plants for in vivo polysaccharide modifications.

CONCLUDING REMARKS

We have presented an overview of the large number of enzymes and genes from Aspergilli involved in the degradation of plant cell wall polysaccharides. Although the enzymes reported cover most of the functions necessary for the complete degradation of plant cell wall polysaccharides, some functions remain to be isolated (e.g., enzymes removing fucose residues from xyloglucan). Also, the substrate specificity and mode of action of many of the enzymes have not been studied in detail. A good example of this is β-galactosidase. So far, only a single β-galactosidase has been found in any *Aspergillus* species, although β-linked galactose residues are found in xylan, xyloglucan, and pectin side chains. Whether this single enzyme is able to hydrolyze all these linkages or whether other β-galactosidases are produced by aspergilli is unclear at this point. The availability of genome sequences for several *Aspergillus* species will accelerate the discovery in the near future of new genes encoding polysaccharide-degrading enzymes.

Only recently, plant proteins have been identified that are able to inhibit the action of some fungal plant cell wal polysaccharide-degrading enzymes. The polygalacturonase inhibitor protein (PGIP) has been studied in the most detail and has been proposed to form a part of the plant "immune system" (80). Evidence for the production of PGIPs in plants as a response to wounding but not to fungal infection was obtained (84). Fungal pathogens produce a family of polygalacturonases, suggesting the neccessity of several PGIPs with different specificities. Recent studies confirmed this by demonstrating that inhibition depends on the type of PGIP and the type of endopolygalacturonase (67, 83, 348). A glycoprotein was identified in kiwi fruit that inhibits the action of a different pectin-degrading enzyme, pectin methylesterase (55, 127). In addition, several plant proteins have been isolated that inhibit the activity of endoxylanases (77, 255, 317). One of these studies also produced evidence for the production of an arabinofuranosidase inhibitor by wheat (317). It is likely that future studies will identify other plant proteins capable of inhibiting the action of fungal plant cell wall polysaccharide-degrading enzymes.

A second topic that still requires intensive study is the regulation of the genes encoding plant cell wall polysaccharide-degrading enzymes. So far only one negatively acting factor (CreA [see "Carbon catabolite repression" above]) and one positively acting factor (XlnR [see "Coordinated expression of genes encoding xylanolytic and cellulolytic enzymes" above]) have been studied in detail. However, evidence for the existence of positively acting factors responding to galacturonic and glucuronic acid (see "Expression of pectiolytic genes" above) arabinose and arabitol, galactose, and ferulic acid (see "Expression of specific genes responding to different inducers" above) has been obtained. It has also become clear that these factors are not specific for the genes involved in the degradation of one particular polysaccharide. Expression profiling and proteomics will be powerful tools to further elucidate the complexity of these systems.

A number of studies report the production of *Aspergillus* plant cell wall polysaccharide-degrading enzymes in other organisms, in particular *S. cerevisiae* (see e.g., references 73, 231, and 268). Most plant cell wall-degrading enzymes as produced by *Aspergillus* are highly glycosylated proteins. When these enzymes are produced by *S. cerevisiae*, they are usually overglycosylated and one should keep in mind that the properties might differ from those of the native enzyme.

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