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Molecular characterization of side-chain cleaving hemicellulases of Trichoderma reesei

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ACADEMIC DISSERTATION

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ABSTRACT

The filamentous fungus *Trichoderma reesei* is one of the most potent microorganisms degrading cellulosic and hemicellulosic materials. To degrade the backbone of hemicelluloses such as xylans and mannans the fungus produces different xylanases and mannanases. However, natural hemicelluloses are substituted molecules and enzymes removing substituents are also needed for their total degradation. Several of these enzymes have been purified and characterized from *T. reesei*. The aim of the present investigation was to isolate and characterize genes of *T. reesei* encoding side-chain releasing enzymes.

The gene coding for α-glucuronidase, which releases glucuronic acid attached to xylose units of xylan, was isolated from an expression library of *T. reesei* RutC-30 using antibodies raised against the previously purified enzyme. The *glr*1 gene is the first α-glucuronidase gene hitherto cloned and characterised. The deduced amino acid sequence of the α-glucuronidase showed no similarity with any protein sequence available in the data bases. The *axe*1 gene encoding acetyl xylan esterase, which removes acetic acid bound to xylan was also isolated from the expression library using antibodies raised against the purified enzyme. Apparently *axe*1 codes for the two forms, pI 7 and pI 6.8, of acetyl xylan esterase previously characterized. The catalytic domain of AXEI had no amino acid similarity with any of the reported acetyl xylan esterases. However, it showed clear similarity with fungal cutinases, which are serine esterases. The highest similarity of AXEI with the cutinases was found in the region containing the active site serine. Inactivation of the enzyme with PMSF also indicated that AXEI is a serine esterase. AXEI has a modular structure carrying a cellulose binding domain (CBD) of fungal type at its C-terminus, separated from the catalytic domain by a serine, glycine, threonine and proline-rich region. Removal of the CBD from the catalytic domain by limited proteolysis abolished the capability of the enzyme to bind cellulose but the activity of the enzyme towards acetylated xylan was not affected.

Genes encoding α-arabinofuranosidase and α-galactosidase activities were cloned from a cDNA expression library of *T. reesei* RutC-30 constructed in the yeast *S. cerevisiae.* Two genes, *abf1* and *bxl1*, were isolated by screening the yeast library for extracellular α-L-arabinofuranosidase activity using the substrate p-

nitrophenyl-α-L-arabinofuranoside (PNPA). It was found that ABFI and BXLI corresponded to a previously purified α-L-arabinofuranosidase and a β-xylosidase from *T. reesei*, respectively. The deduced amino acid sequence of ABFI displayed high similarity with one of the α-L-arabinofuranosidases, ABF B, of *Aspergillus niger*, which has not been classified in the general classification of glycosyl hydrolases. These two enzymes can now form a new family of glycosyl hydrolases. The deduced amino acid sequence of BXLI showed no similarity with any of the known β-xylosidases, but was significantly similar to the β-glucosidases grouped into family 3. Three α-galactosidase encoding genes, *agl1*, *agl*2 and *agl3*, were isolated by screening the yeast cDNA expression library on plates containing the substrate 5-bromo-4-chloro-3-indolyl-α-D-galacto-pyranoside. The deduced amino acid sequences of AGLI and AGLIII showed similarity with the α-galactosidases of plant, animal, yeast and filamentous fungal origin which are classified into family 27 of the glycosyl hydrolases. On the other hand, the deduced amino acid sequence of AGLII showed similarity with the bacterial α-galactosidases of family 36, and was thus the first reported eukaryotic α-galactosidase to show similarity with the corresponding prokaryotic enzymes.

ABFI, BXLI, AGLI, AGLII and AGLIII were produced in yeast in order to test their action against different small synthetic and natural polymeric substrates. ABFI released L-arabinose from PNPA and arabinoxylans and showed some β-xylosidase activity towards p-nitrophenyl-β-D-xylopyranoside (PNPX). BXLI did not release L-arabinose from arabinoxylan. It showed α-L-arabinofuranosidase, α-L-arabinopyranosidase and β-xylosidase activities against PNPA, p-nitrophenyl-α-L-arabinopyranoside and PNPX, respectively, the latter activity being the highest. It was also able to hydrolyze xylobiose and slowly to release xylose from polymeric xylan. Both ABFI and BXLI produced in yeast displayed hydrolytic properties similar to those of the corresponding enzymes purified from *T. reesei*. AGLI, AGLII and AGLIII were able to hydrolyze the synthetic substrate p-nitrophenyl-α-D-galactopyranoside (PNPG) and the small galactose-containing oligosaccharides, melibiose and raffinose. They showed different efficiencies when acting on polymeric galacto(gluco)mannan. AGLI was the most active enzyme towards polymeric substrates and its action was enhanced by the presence of the endomannanase of *T. reesei.* The calculated molecular mass and the hydrolytic properties of AGLI indicated that it might correspond to the α-galactosidase previously purified from *T. reesei.* AGLII and AGLIII were less active on the intact polymer and showed synergy in galacto(gluco)mannan hydrolysis with the mannanase of *T. reesei* and a β-mannosidase of *Aspergillus niger*.

The new hemicellulase-encoding genes cloned in the present work will enable more detailed biochemical characterization and structure determination of the enzymes, which might improve the understanding of their catalytic mechanisms. The availability of the genes also increases the potential of their use for various applications.

PREFACE

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LIST OF PUBLICATIONS

This thesis is based on the following publications (Appendices I - IV), which are referred to in the text by their Roman numerals.

I Margolles-Clark, E., Saloheimo, M., Siika-aho, M. and Penttilä, M. 1996. The α-glucuronidase encoding gene of *Trichoderma reesei.* Gene. In press.

II Margolles-Clark, E., Tenkanen, M., Söderlund, H. and Penttilä, M. 1996. Acetyl xylan esterase from *Trichoderma reesei* contains an active site serine and a cellulose binding domain. Eur. J. Biochem. Vol. 237, No. 3, pp. 553 - 560.

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IV Margolles-Clark, E., Tenkanen, M., Luonteri, E. and Penttilä, M. 1996. Three α-galactosidase genes of *Trichoderma reesei* cloned by expression in yeast. Eur. J. Biochem. In press.

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ABBREVIATIONS

abf	*T. reesei* gene encoding α-L-arabinofuranosidase I
ABF	α-L-arabinofuranosidase I
axe	*T. reesei* gene encoding acetyl xylan esterase
AXE	acetyl xylan esterase
agl	*T. reesei* genes encoding α-galactosidase
AGL	α-galactosidase
bxl	*T. reesei* gene encoding β-xylosidase
BXL	β-xylosidase
glr	*T. reesei* gene encoding α-glucuronidase
GLR	α-glucuronidase
CBD	cellulose binding domain
HCA	hydrophobic cluster analysis
LBG	locust bean gum
PMSF	phenylmethylsulphonyl fluoride
PNPA	p-nitrophenyl-α-L-arabinofuranoside
PNPG	p-nitrophenyl-β-D-glucopyranoside
PNPX	p-nitrophenyl-β-D-xylopyranoside
TMP	thermomechanical pulp

1 INTRODUCTION

1.1 STRUCTURE OF HEMICELLULOSES

Hemicelluloses are the second most abundant polysaccharides in nature after cellulose. They form part of most plant materials and appear in close association with cellulose and lignin. In lignified tissue they often contribute to the rigidity of plant cell walls. The term hemicelluloses has been used mainly for those plant cell wall polysaccharides which are extractable by alkaline solutions. However, some hemicelluloses such as galactoglucomannans are also soluble in water (Sjöström, 1990). Hemicelluloses have a heterogeneous composition of various sugar units. Their primary structure depends on the type of plant and can vary even between different parts of the same plant. They are usually classified according oo the main sugar residues in the backbone of the polymer, e. g. as xylans (β-1,4-linked D-xylose units) and mannans (β-1,4-linked D-mannose units), which are the main group of hemicelluloses, and arabinans (α-1,5-L-linked L-arabinose unit) and galactans (β-1,3-linked D-galactose units) which are less abundant (Aspinall, 1959; Sjöström, 1981; Fengel and Wegener, 1984). A schematic presentation of the main hemicelluloses occurring in hardwood and softwood are presented in Fig. 1.

Xylans are present i all terrestrial plants. In annual plants such as grasses and cereals the content of xylan comprises up to 30 % of the cell wall material. They are also the main hemicellulose components of hardwood (15 - 30 %) and represent between 7 and 10 % of softwood cell wall polysaccharides (Wilkie, 1979; Sjöström, 1981). Xylans have very few branching points, but the degree of substitution with different side groups is high. Hardwood xylan (O-acetyl-4-methyl-glucuronoxylan) is substituted at irregular intervals with 4-O-methyl-α-D-glucuronic acid groups joined to xylose by α-1,2-glycosidic linkages. On average, every tenth xylose unit has a uronic acid side group attached at carbon 2 or 3 of the xylopyranose. About 60 to 70 % of the xylose residues in hardwood xylan are acetylated at the carbon 2 and/or 3 positions, whereas softwood xylan is not acetylated (Maekawa, 1976; Timell, 1967; Bouveng, 1961; Lindberg et al., 1973a). In annual plants, both xylans and glucomannans are reported to be acetylated (Maekawa, 1976; Buchala et al., 1974; Matsuo and Mizuno 1974; McCleary 1991).

The xylan of softwood is mainly arabino-4-O-methyl-glucuronoxylan. In addition to 4-O-methyl-glucuronic acid it also contains α-L-arabinofuranoside units which are α-1,3-linked to the xylan backbone an average molar ratio of arabinose: 4-O-methylglucoronic acid: xylose units of 1.3:2:10 (Timell, 1967; Sjöström, 1993). The xylans from annual plants are more heterogeneous than xylans from woody tissues. They contain both glucuronic acid and/or its 4-O-methyl ether and arabinose attached to positions 2 and 3 of the xylose residues, respectively (Aspinall, 1959). In cereal xylans the arabinose may also be linked to carbon 2 of the xylose. In some plant tissues such as endosperms, the xylose residues of xylan

may be substituted with arabinose residues at both C-2 and C-3 positions (Wilkie, 1979).

The presence of feruloyl (4-hydroxy-3-methoxycinnamic) and *p*-coumaroyl (4-hydroxycinnamic) acids in xylan has been verified in several studies (Mueller-Harvey et al., 1986; Kato and Nevis, 1985). These hydroxycinnamid acids are esterified to hydroxyl groups of the carbon 5 of arabinose bound to xylan as a side group. The amounts of these compounds are very small and normally represent less than 1 % of the cell walls. In grass cell walls they can however account for 2.5 % (Hartley and Jones, 1977; Hartley and Ford, 1989).

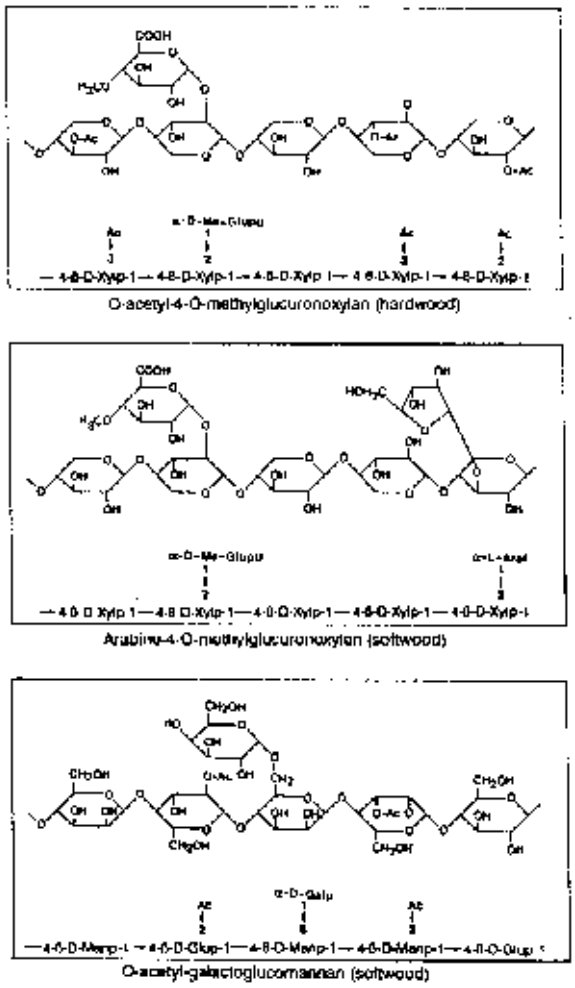

Figure 1. Schematic representation of typical wood hemicelluloses according to Kantelinen, 1992.

Galactoglucomannan is the most abundant hemicellulose in softwood (about 20 %), whereas its content in hardwood is only 2 - 5 % of the cell walls (Timell, 1967). The backbone glucomannan is formed by β-1,4-linked D-glucopyranose and D-mannopyranose units which are randomly distributed in the molecule. It is partially substituted with α-D-galactose side groups, which can be attached to both mannose and glucose units by an α-1,6-linkage (Sjöström, 1993). Two fractions of galactoglucomannan are present in softwood: a minor fraction with heavier galactose substitution (ratio of galactose:glucose:mannose of about 1:1:3) and a major fraction normally called glucomannan and with a low degree of galactose substitution (ratio of about 0.1:1:4) (Timell, 1967). Acetyl side groups are also present in galactoglucomannans at positions 2 or 3 in the hexose units (Lindberg et al., 1973b). In annual plants both glucomannans and galactomannans appear mainly in seeds, tubers and bulbs (Aspinall, 1959).

Arabinans, galactans, arabinogalactans, rhamnogalacturonans and D-galacturonans are regarded as pectic substances of plant cell walls and have a key role in mechanical strength and adhesion between cells (Aspinall, 1980). They are a complex mixture of colloidal polysaccharides that can be extracted from cell walls with water or chelating agents. Pectins are more abundant in the soft tissues of some fruits (15 - 30 % of the carbohydrates) as well as in sugar beet pulp (25 %), whereas in woody tissue they are present only in small amounts (Aspinall, 1970). The backbone arabinan is a polymer of α-1,5-linked arabinofuranose residues which are substituted mainly at position 3, but also at position 2, with one or two arabinose units (Aspinall, 1980). Arabinogalactan is composed mainly of a backbone of β-1,3-linked D-galactopyranose units, most of which are substituted at position 6 by β-1,6-linked D-galactopyranose and less frequently by 1,6-α-linked L-arabinose (Eriksson et al., 1990). The side chain β-D-galactosyl and α-L-arabinosyl residues may also be substituted by 1,6-linked β-D-galactopyranose and 1,3-linked β-L-arabinopyranose, respectively, forming two-unit side chains. More complex are the so-called “hairy” regions of pectins. These have a backbone of alternating galacturonic acid and rhamnose units with numerous polymeric side chains, mainly arabinan and galactan, usually attached to the rhamnose residues in the main chain. They appear interspersed with “smooth” regions of linear α-1,4-linked polygalacturonic acid residues which may also be partially esterified.

1.2 ENZYMATIC HYDROLYSIS OF HEMICELLULOSES

Although hemicelluloses are complex heteropolysaccharides, their enzymatic degradation is relatively well known, mainly on the basis of hydrolysis studies carried out with isolated soluble substrates (Dekker, 1985; Biely, 1985). Several different enzymes, collectively called hemicellulases, are needed for the degradation and modification of hemicellulose. In general, the complete hydrolysis of hemicelluloses is accomplished by the combined action of endo-enzymes, which cleave the main chain and produce oligosaccharides, ancillary

enzymes cleaving the side-chains from the main chain of the oligosaccharides and exo-enzymes leading to the final liberation of monomeric sugars.

The 1,4-β-D-xylosidic linkages in xylan are randomly hydrolysed by endo-1,4-β-D-xylanases (EC 3.2.1.8). These enzymes have a higher hydrolysis rate when acting on polymeric xylan than on xylo-oligomers. Their main hydrolysis products being xylobiose, xylotriose and substituted oligomers containing two to four xylosyl residues. Endoxylanases from several sources have been extensively studied, especially those produced by filamentous fungi such as *Trichoderma* and *Aspergillus* (Biely, 1985; Poutanen, 1988a; Wong et al., 1988; Senior et al., 1989; Eriksson et al., 1990; Tenkanen et al., 1992). The hydrolysis of xylo-oligosaccharides and xylobiose is carried out by exo-1,4-β-D-xylosidases (EC 3.2.1.37). Most microbial β-xylosidases have been isolated from filamentous fungi (Poutanen and Puls, 1988). They can remove single xylosyl residues from the non-reducing end of the substrate with different specificities. Some β-xylosidases have been reported to be active against xylobiose but inactive towards polymeric xylan (Rodionova et al., 1983; van Doorslaer et al., 1985). The β-xylosidase purified from *T. reesei* can release xylose from the non-reducing end of both xylo-oligosaccharides of different length (Poutanen and Puls, 1988) and from polymeric xylan (Herrmann et al., 1995).

The hydrolysis of the main chain of polymeric glucomannans is carried out by endo-1,4-β-mannanases (EC 3.2.1.78). These enzymes usually hydrolyse substrates with a degree of polymerization higher than three, the main products being mannobiose, mannotriose and various mixed oligosacharides. The hydrolysis yield of galactomannan with mannanases is dependent on the degree of substitution as well as on the distribution of the side-chain substituents and the glucose/mannose ratio (McCleary, 1991). Endo-mannanases have been purified and characterized from several fungi (Viikari et al., 1993), including *T. reesei* (Stålbrand et al., 1993; Arisan-Atac et al., 1993). It has been shown that some mannanases can hydrolyse the β-1,4-bonds between glucose and mannose as well as between adjacent mannose units in glucomannans (Takahashi et al., 1984; Kusakabe et al., 1988). β-Mannosidase (EC 3.2.1.25) catalyzes the hydrolysis of terminal, non-reducing β-D-mannose residues in manno-oligosaccharides. Their presence has been reported in a wide range of plant and animal tissues and in many microorganisms (Dey, 1978). However, only few β-mannosidases have been purified and characterized (Sone and Misaki, 1978; Elbein et al., 1977; Akino et al., 1988). Hydrolysis of glucomannan also requires the action of β-glucosidase (EC 3.2.1.21), which releases D-glucose β-1,4-linked to mannose.

Enzymes capable of removing side chain substituents are also needed for total hydrolysis of the polymers. It has been reported that the presence of side-chain L-arabinose, acetyl acid and 4-O-methylglucoronic acid in xylo-oligosaccharides can block further enzymatic hydrolysis by xylanases and β-xylosidases (Brice and Morrison, 1982; Tenkanen and Poutanen, 1992; Poutanen et al., 1990a). Total degradation of galacto(gluco)mannan requires an enzyme releasing the side chain

galactosyl groups, since these can also limit the action of endo-β-mannanase and β-mannosidase (Talbot and Sygusch, 1990; Gherardini and Salyers, 1987; McCleary and Matheson, 1983). A number of side chain removing enzymes including α-glucuronidases, acetyl xylan esterases, α-arabinofuranosidases and α-galactosidases have been isolated and characterized (see below). It has been shown that most of them act cooperatively with backbone degrading enzymes during the degradation of different substituted polymeric hemicelluloses (Dekker, 1985; Poutanen and Puls, 1989; Coughlan and Hazlewood, 1993; Greve et al., 1984; Bezalel et al., 1993; Poutanen, 1988b).

Enzymatic degradation of the neutral parts of pectins such as arabinan, galactan and arabinogalactan requires the action of endo- and exo-α-L-arabinases and α-galactosidases. Enzymes involved in the degradation of more complex pectin fractions have also been identified. This group includes endo- and exo-polygalacturonases, endopectinlyases and pectinesterases. Particular interest has been attached to those produced by *A. niger* (Rombouts and Pilnik, 1980).

1.3 OCCURRENCE AND CHARACTERIZATION OF SIDE-CHAIN CLEAVING HEMICELLULASES

Enzymes degrading the main chain of hemicelluloses, such as xylanases and mannanases, have been extensively studied. However, the importance of the side-chain cleaving enzymes in the complete hydrolysis of hemicelluloses is of relatively recent interest. These hemicellulases have been individually classified according to their substrate specificity.

1.3.1 α-Glucuronidases

The significance of 4-O-methylglucuronic acid side groups in the chemical hydrolysis of xylan has been known for some time (Roy and Timell, 1968). It has been shown that 4-O-methylglucuronic acid, α-1,2 linked to xylan, is stable under acidic conditions and can also have a stabilizing effect on the neighbouring xylosidic bonds. It has also been reported that this side-chain group can have a limiting effect in the enzymatic degradation of xylan (Puls et al., 1978). Partial hydrolysis of 4-O-methylglucuronoxylan has been obtained with crude hemicellulase preparations including β-xylosidase activity, but most of the uronic acid remained uncleaved (Sinner et al., 1972). Thus, an enzyme activity hydrolysing uronic acid and acting synergistically with xylanases is needed for total degradation of the polymer.

Table 1. Properties of purified α-glucuronidases.

Organism	M_r	pH[a]	pI	Activity against[b]		Remarks	Reference
				oligomer	polymer		
Bacteria							
Thermoanaero-bacterium sp.	74		4.65	++		dimer, cell associated	Shao et al., 1995
Piromonas communis	103[c]	5.5		++			Wood and Wilson, 1995
Fungi							
Trichoderma reesei	91	4.5-6.0	5.0-6.2	++	+		Siika-aho et al., 1994
Agaricus bisporus	>450[c]	3.3		++	-	partially purified	Puls et al., 1987; Korte, 1991
Thermoascus aurantiacus	107	4.5		++	++		Khandke et al., 1989
Aspergillus niger 5-16							
CI	130	4.8	5.3	++		intracellular	Uchida et al., 1992
CII	130	4.8	5.3	++		intracellular	
Phanerochaete chrysosporium	112	3.5	4.6	++	+		Castanares et al., 1995

[a] pH optimum
[b] Activity against 4-O-methylglucuronoxylan (polymer) and 4-O-methylglucurono-oligoxylan (oligomer)
[c] Determined by gel filtration. Other values were determined by SDS-PAGE

α-Glucuronidase activity was first detected in culture filtrates of *T. reesei* (Dekker, 1983) and was later verified by Poutanen et al., 1987. This activity has also been detected in several other fungal and bacterial culture filtrates (Korte, 1991). However, only a few α-glucuronidases have been purified and characterized (Table 1). The first α-glucuronidase was purified from *Thermoascus aurantiacus*. The enzyme was able to liberate glucuronic acid from both polymeric xylan and xylo-oligosaccharides at similar rates (Khandke et al., 1989). On the other hand, the reported α-glucuronidase of *Agaricus bisporus* was active only against oligomeric substrates (Puls et al., 1987). Only low activity against polymeric glucuronoxylan was detected even when high concentrations of the enzyme were used (Korte, 1991). The activity of the enzyme towards xylo-oligosacharides decreases with increasing chain length. The α-glucuronidase of *T. reesei* also prefers low molecular mass xylo-oligosaccharides as substrate and has only a minor activity against long chain glucuronoxylan. This enzyme seems to act almost exclusively on the 4-O-methylglucuronic acid bound to the terminal xylose at the non-reducing end of the xylose chain (Siika-aho et al., 1994). Analysis of the substrate specificities of other recently isolated α-glucuronidases has shown a similar behaviour. The reported enzymes of the fungus *Phanerochaete*

chrysosporium (Castanares et al., 1995) and those from the bacteria *Thermoanaerobacterium* sp. (Shao et al., 1995) and *Piromonas communis* (Wood and Wilson, 1995) were active only towards 4-O-methylglucuronoxylo-oligosaccharides and their activity decreased when the degree of polymerization increased (Shao et al., 1995; Wood and Wilson, 1995). The enzyme of *Phanerochaete chrysosporium* showed limited activity towards polymeric 4-O-methylglucuronoxylan (Castanares et al., 1995). Thus, it seems that most α-glucuronidases need to act synergistically with xylanases in order to liberate 4-O-methylglucuronic acid from 4-O-methylglucuronic acid substituted xylo-oligomers (Puls et al., 1987).

Most fungi secrete only low levels of α-glucuronidase and there is also evidence that some microorganisms are totally deficient in α-glucuronidase activity. For example, no 4-O-methylglucuronic acid was detected in the hydrolysis of 4-O-methylglucuronoxylan with extracellular enzymes produced by *Aspergillus awamori* (Poutanen et al., 1986). Furthermore, the bacterium *Clostridium thermocellum* was not able to ferment xylo-oligomers substituted with uronic acid, although it can ferment neutral xylo-oligomers (Weigel et al., 1985).

1.3.2 Acetyl Xylan Esterases

The effect of acetyl side groups on the enzymatic hydrolysis of natural hemicelluloses was first demonstrated by Biely et al. (1985a). Most of the previous enzymological studies on hemicellulose degradation had been carried out using substrates isolated by alkaline treatment. In these conditions the esterified side chain groups of hemicelluloses are removed by saponification and therefore the need for esterases in the enzymatic hydrolysis is not evident. However, there are also some near-neutral treatments for the separation of plant materials, such as steaming, aqueous phase thermomechanical treatment and organosolv pulping, which leave hemicellulose with most of its acetyl groups intact. Acetylated galactoglucomannan from softwood can also be isolated by water extraction (Sjöström, 1990).

Esterases are widely distributed in nature and have been classified on the basis of their substrate specificity (IUB, Enzyme Nomenclature, 1992). For instance, esterases highly active on esters of acetic acid have been classified as acetyl esterases (EC 3.1.1.6), and those acting on phenolic side groups as aryl esterases (EC 3.1.1.2). However, this classification is not very strict because of the wide substrate specificities of many esterases.Acetyl esterases acting on acetylated xylan were first detected in culture filtrates of different cellulolytic and hemicellulolytic fungi such as *Trichoderma reesei*, *T. viride*, *Aspergillus niger*, *Schizophyllum commune* and *Aerobasidium pullulans* (Biely et al., 1985b). It was found that these fungal esterases had higher specific activities towards acetylated glucuronoxylan than those of

Table 2. Properties of esterases acting on acetylated xylan and galactoglucomannan.

Organism	Enzyme[a]	M_r	pI	pH[b]	Activity against[c]		Remarks	Reference
					oligomer	polymer		
Bacteria								
Caldocellum saccharolyticum	AXE			6.0	+		Cloned and produced in *E. coli*, Not purified, thermotolerant	Lüthi et al., 1990a,b
Fibrobacter succinogens	AXE	55	4.0	7.0	++	+		McDermid et al., 1990
Pseudomonas fluorescens subsp. celulosa	AXE	59[d]		6.0	+	-	Cloned and produced in *E. coli*, Not purified, contains a CBD	Ferreira et al., 1993; Faulds et al., 1995
Streptomyces rubiginosus	AXE1	22	8.7	7.0		++		Keller, 1992
	AXE2	22	9.8	7.0		++		
Thermoanaerobacterium sp. strain JW/SL-YS485	AXEI	32	4.2	7.0		+	Hexamer, thermotolerant	Shao and Wiegel, 1995
	AXEII	26	4.3	7.5		+	Tetramer, thermotolerant	
Thermomonospora fusca	AXE1	80			++	+	Intracellular	Bachman and McCarthy, 1991
	AXE2	40			+		Not purified, extracellular	
Fungi								
Aspergillus niger	AXE	30	3.0-3.2	5.0-5.5	++	++		Kormelink et al.,1993
	AGME	40[d]	4.1	6.0		++[e]		Puls et al., 1992
Rhodotorula mucilaginosa	AXE			8-10		+	Partially purified	Lee and Forsberg, 1987
Schizophyllum commune	AXE	31	3.4	7.7	++	+		Biely et al., 1988 Halgasová et al., 1994
Trichoderma reesei	AXEI	34	7.0	5.0-6.0	++	++		Sundberg and Poutanen, 1991;
	AXEII	34	6.8	5.9-6.0	++	++		Poutanen et al., 1990b
Aspergillus oryzae	AGME	36	4.6	5.0-5.5	++[e]	+[e]		Tenkanen et al., 1995a

[a] AXE, acetyl xylan esterase; AGME, acetyl glucomannan esterase
[b] pH optimum
[c] Activity against acetylated xylan (polymer) and acetylated oligoxylan (oligomer)
[d] Determined by gel filtration. Other values were determined by SDS-PAGE
[e] Active on acetylated galactoglucomannan

plants and animals. Studies of fractionated culture filtrates from several microorganisms such as *T. reesei, T. viride, Streptomyces olivochromogens, S. rubiginosus* and *Fibrobacter succinogenes* also revealed that esterases can occur in multiple forms. These fractions showed different ratios of esterase activities against acetylated xylan and the artificial substrate 4-nitrophenyl acetate (Biely et al., 1987, 1988; Johnson et al., 1988; Keller et al., 1989; McDermid et al., 1990). On the basis of these studies it was concluded that acetyl xylan esterase activity is at least partially different from most non-specific acetyl esterase activities.

Several esterases acting with different specificities on esterified groups attached to hemicelluloses have been isolated and characterized. Some enzymes act more specifically on acetyl groups bound to xylan (acetyl xylan esterases) or mannan (acetyl glucomannan esterases) (Table 2). Others can act only on feruloyl groups but not on *p*-coumaroyl groups of xylan. Esterases with wider substrate specificity, which can liberate both phenolic and acetic acid from different hemicelluloses, have also been reported (for a review see Tenkanen, 1995).

Two acetyl xylan esterases (AXEI and AXEII) have been isolated from *T. reesei* (Sundberg and Poutanen, 1991). The two enzymes showed simila hydrolytic properties and differed only in their pI (7.0 and 6.8, respectively). Further studies were carried out with a mixture of both forms (Poutanen et al., 1990b). This preparation was capable of releasing all the acetic acid from acetylated beechwood xylan (Poutanen e al., 1990b). The preparation acted equally well on both polymeric and oligomeric substrates and showed only slightly higher activity when acting together with xylanases. Similarly, the acetyl xylan esterase of *A. niger* liberates most of the acetyl substituents of xylan when acting alone and does not show synergism with xylanases (Kormelink et al., 1993). Two acetyl xylan esterases from *S. rubiginosus*, which together corresponded to 90 % of the acetyl xylan activity of the microorganism, also showed high substrate specificity for polymeric xylan. They could liberate 70 % of the total acetic acid from native birchwood xylan. However, it was not reported whether xylanases influenced their action (Keller, 1992).

Other acetyl xylan esterases seem to have a preference for oligomeric substrates. The acetyl xylan esterases of *S. commune* has been reported to be responsible for most of the esterase activity secreted into the culture medium (Biely et al., 1987, 1988). The capability of the enzyme to degrade chemically acetylated xylan in the absence of xylanases was later reported (Halgasová et al., 1994). However, purification of the enzyme from xylanases remarkably increased the specific acetyl esterase activity, whereas the specific xylan esterase activity did not increase at a comparable ratio. This indicates that most probably the enzyme prefers short acetylxylo-oligosaccharides as substrate. On the other hand, it has earlier been reported that the presence of xylanases enhances the action of partially purified acetyl xylan esterases of *S. commune* (Biely et al., 1986). The actinomyces *Thermomonospora fusca* produces an acetyl xylan esterase which in

its intracellular form has a molecular mass of 80 kDa but can be processed and secreted in two active subunits of 40 kDa (Bachman and McCarthy, 1991). Crude preparations of both the intra- and extracellular forms of the enzyme were able to release approximately 70 % of the acetic acid linked to chemically acetylated xylan. The amounts of acetyl groups liberated by the purified intracellular form were considerably increased by addition of xylanases (Bachman and McCarthy, 1991). The *P. fluorescens* subsp. *cellulosa* esterase produced in *E. coli* was also able to liberate acetic acid from chemically acetylated xylo-oligosaccharides but not from polymeric xylan (Ferreira et al., 1993).

Only two acetyl esterases acting on acetylated galactoglucomannan have been reported. One was isolated from a commercial enzyme preparation "Celluzyme" of *A. niger* (Puls et al., 1992). The enzyme was highly specific against acetyl galactoglucomannan. Acting alone it could release over 90 % of the acetic acid bound to polymeric galactoglucomannan and its activity was slightly enhanced in the presence of α-galactosidase. The enzyme did not release acetic or ferulic acid from acetylated xylan or wheat straw xylan, respectively, and had very little activity towards the artificial substrates α-naphthyl acetate and p-nitrophenyl acetate (Puls et al., 1992). *A. oryzae* was found to produce several esterases able to act on acetylated galactoglucomannan and one of these enzymes was purified (Tenkanen et al., 1995a). It was able to liberate up to 60 - 70 % of the acetic acid in the substrate. The level of acetic acid liberated by the enzyme was enhanced in the presence of mannanase from *T. reesei*. Addition of α-galactosidase, β-glucosidase or β-mannosidase had no effect on the hydrolysis (Tenkanen et al., 1995a).

1.3.3 α-Arabinosidases

Enzymes releasing L-arabinose from arabinose-containing polysaccharides are very abundant in nature. They have mainly been isolated from microorganisms such as fungi and bacteria (Table 3). Most of the research with these enzymes acting on polymeric substrates has been focused on the hydrolysis of beet arabinan, whereas their role in the hydrolysis of arabinoxylans and arabinoglucuronoxylans has only been studied recently.

The enzymes hydrolysing L-arabinose linkages have been classified into two major groups: α-L-arabinofuranosidases (EC 3.2.1.55) that hydrolyze terminal non-reducing α-L-1,2, α-L-1,3 and α-L-1,5-linked arabinofuranosyl residues, and endo-1,5-α-L-arabinases (EC 3.2.1.99) that hydrolyze α-1,5-L-arabinofuranosidic linkages of arabinans (Kaji, 1984). On the basis of the specific activities of the enzymes purified from *Aspergillus niger* (Tagawa and Kaji, 1969) and *Streptomyces purpuracens* (Komae et al., 1982), the α-L-arabinofuranosidases have been further classified into two groups. The *A. niger* type of enzymes are active on the small synthetic substrates PNPA and arabino-oligosaccharides, and are able to hydrolyse arabinosyl side groups of arabinans, arabinogalactans and

arabinoglucuronoxylans. The *S. purpuracens* type act only on α-L-arabinosides of low molecular mass and oligosaccharides containing arabinose (Kaji, 1984).

There is still some contradiction in the classification of arabinose-releasing enzymes, and hydrolysis studies have shown that some enzymes can act in an unexpected manner. An α-L-arabinofuranosidase of *Erwinia carotova* IAM 1024 with a new exo-type activity was isolated by Kaji and Shimokawa (1984). This enzyme was active on beet arabinan and, contrary to other known enzymes, released only arabinotriose. The enzyme was not active against PNPA. No endo-type activity, however, was detected in the hydrolysis of linear α-1,5-linked arabinan (Kaji and Shimokawa, 1984). Hydrolysis studies with more defined substrates have also shown different specificities between enzymes that can be classified as *S. purpuracens* type (Table 3). The enzyme from *B. subtilis* 3-6, not active on polymeric arabinoxylan, was able to liberate arabinose from arabinan and arabinoxylobiose but also from xylotriose with an arabinose bound to the internal xylose unit (Kaneko et al., 1994). On the other hand, an intracellular enzyme from *A. niger* 5-16 degraded arabinan and arabinoxylobiose but could not release arabinose from xylotriose or xylotetraose with arabinose bound to an internal xylose unit (Kaneko et al., 1993). It was proposed that *S. purpuracens* type α-L-arabinofuranosidases should be classified at least into two groups, namely those hydrolysing arabinose from xylose at terminal positions in xylo-oligomers and those releasing arabinose from xylose at both terminal and internal positions in xylo-oligomers (Kaneko et al., 1994).

The *A. awamori* (1,4)-β-arabinoxylan arabinofuranosidase (AXH) is very specific for oligomeric and polymeric arabinoxylan and does not remove arabinose from linear or branched arabinan, galactan, arabinogalactan or PNPA. The enzyme can remove both α-1,2 and α-1,3 linked arabinose residues from xylan, but only from single substituted residues. No activity was detected on double substituted xylose (Kormelink et al., 1991). A more specific classification for arabinosidases has been proposed which is not only based on the capability of the enzyme to hydrolyse artificial substrates, arabino-oligosaccharides or polymeric substrates (Beldman et al., 1992). This classification also includes the ability of the enzymes to hydrolyse α-1,2, α-1,3 or α-1,5 linkages and to remove arabinose from single or double substituted xylose residues.

Some microorganisms produce multiple forms of arabinosidases. For example, *A. niger* produces three enzymes with different specific activities: α-L-arabinofuranosidase A active only on PNPA and oligomeric substrates, α-L-arabinofuranosidase B active on PNPA and both oligo and polymeric substrates, and an endo-α-1,5-arabinase (Kaji, 1984; Rombouts et al., 1988; van der Veen et al., 1991). On the other hand, *Penicillium capsulatum* (Filho et al., 1996) and *Aspergillus terreus* (Luonteri et al., 1995) produce two and three enzymes, respectively, with similar substrate specificities.

Table 3. Properties of microbial arabinose-releasing enzymes.

Organism	Class[a]	M_r	pI	pH[b]	Active on[c]	No/low activity on	Remarks	Reference
Bacteria								
Bacteroides xylanolyticus X5-1	AF(s)	61.0		5.5-6.0	PNPA, AXO	AX, AG	hexamer, cell wall attached	Schyns et al., 1994
Bacillus stearothermophilus	AF(s)	110.0[d]		7.0	PNPA, AXO	AX, AG	Dimer, 52,5 and 57.5 kDa	Bezalel et al., 1993
Bacillus stearothermophilus T-6	AF(s)	64.0	6.5	5.5-6.0	PNPA, BA, AXO	AX, AG	Tetramer	Gilead and Shoham, 1995
Bacillus subtilis F-11	EA			6.0	BA, DA	PNPA, AX, AG		Kaji and Saheki, 1975
Bacillus subtilis 3-6	AF(s)	61.0		7.0		AX, AG		Kaneko et al., 1994
Bacillus polymyxa	AF(s)	166.0	4.7	6.5	PNPA, AXO, DAO	AX, DA, AG	Dimer, 65 and 33 kDa	Morales et al., 1995
Streptomyces purpuracens IFO 3389	AF(s)	62.0	3.9	6.5	PNPA, AXO	AX, BA, AG		(Komae et al., 1982)
Streptomyces sp. strain 17-1	AF(a)	92.0	4.4	6.0	PNPA, AX, BA, DA, AG			Kaji et al., 1981
Streptomyces diastaticus	AF(a) C1 AF(a) C2	38.0 60.0	8.8 8.3	4.0 7.0	AX, DA AX, DA			Tajana et al., 1992
Streptomyces lividans 66	AF(s)	69.0	4.6	6.0	PNPA, AXO	AX, DA, AG	Intracellular	Manin et al., 1994
Ruminococcus albus 8	AF(a)	75.0	3.8	6.9	PNPA		Tetramer	Greve et al., 1984
Butyrivibrio fibrisolvens GS113	AF(a)	31.0	6.0	6.0-6.5	PNPA, AX, BA	AG	Hectamer, cytoplasmatic	Hespell and O'Bryan, 1992
Erwinia carotovora IAM 1024	AF(s)			6.0	BA	PNPA, AX, DA, AG		Kaji and Shimikawa, 1984
Pseudomonas fluorescens subsp. *cellulose*	AF(a)	59.0			AX		*E. coli* produced, contains a CBD	Kellett et al., 1990
Clostridium acetobutylicum ATCC 824	AF(s)	94.0	8.1	5.0-5.5	PNPA, BA, AXO	AG, AX		Lee and Forsberg, 1987
Clostridium stercorarium	AF(s)	53.3		7.0	PNPA	AX	Tetramer, *E. coli* produced	Sakka et al., 1993
Clostridium stercorarium	AF B(a)	52.0		5.5	AX, AXO		Tetramer, *E. coli* produced	Schwarz et al., 1990
Thermomonospora fusca	AF(s)	46.0		6.0	PNPA, AXO	AX	Dimer	Bachmann and McCarthy, 1991
Fungi								

Aspergillus niger	AF A(s)	83.0	3.3	3.4	PNPA, DAO	AX, BA, DA, AG		Rombouts et al., 1988; van der Veen et al., 1991; Dunkel and Amadó, 1994
	EA	43.0	3.0	4.6	DA, BA	PNPA, AX, AG		
Aspergillus niger 5-16	AF(s)	67.0	3.5	4.0	PNPA	AX, BA	intracellular	Kaneko et al., 1993
Aspergillus terreus	AF A(a)	39.0	7.5	3.5-4.5	PNPA, AX			Luonteri et al., 1995
	AF B1(a)	59.0	8.3	3.5-4.5	PNPA, AX			
	AF B2(a)	59.0	8.5	3.5-4.5	PNPA, AX			
Aspergillus nidulans	AF	36.0	4.3	5.5	PNPA			Fernández-Espinar et al., 1994
Aspergillus sojae	AF(a)	34.3	3.9	5.0	PNPA, AX, BA, AG		inactive towards birchwood xylan	Kimura et al., 1995
Aspergillus awamori	AXH[e]	32.0		5.0	AX	PNPA, BA, DA, AG		Kormelink et al., 1991
Termitomyces clypeatus	AF(a)			5.5	PNPA, AX, BA, AG, AXO			Sinha and Sengupta, 1995
Trichoderma reesei	AF(a)	53.0	7.5	4.5	PNPA, AX, BA			Poutanen, 1988b
Sclerotina sclerotiorum	AF(a)	63.0	7.5	4.0-4.5	PNPA, AX, BA, AG			Baker et al., 1979
Dichomitus squalens	AF(a)	60.0	5.1	3.5	PNPA, AX, BA			Brillouet and Moulin, 1985
Corticium rolfsii	AF(a)			2.5	PNPA, AX, BA, DA			Kaji and Yoshihara, 1971
Penicillium capsulatum	AF 1(a)	64.5	4.1	4.0	PNPA, AX, AXO			Filho et al., 1996
	AF 2(a)	62.7	4.5	4.0	PNPA, AX, AXO			

[a] Classification based on Kaji, 1984; AF, α-L-arabinofuranosidase; EA, endo-α-1,5-arabinase; (a) *A. niger* and (s), *S. purpuracens* α-L-arabinofuranosidase type (see text).
[b] pH optimum
[c] PNPA, p-nitrophenyl-arabinofuranoside; AX, arabinoxylan; BA, branched arabinan; DA, debranched arabinan; AG, arabinogalactan; AXO, arabinoxylo-oligosaccharide; DAO, branched arabino-oligosacharide
[d] Determined by gel filtration. Other values were determined by SDS-PAGE
[e] AXH, (1,4)-β-arabinoxylan arabinofuranosidase

Most fungal α-L-arabinofuranosidases are able to release arabinose from polymeric substrates acting alone (Table 3). The three enzymes of *A. terreus* can liberate up to 80 % of arabinose from polymeric arabinoglucuronoxylan (Luonteri et al., 1995). The one α-L-arabinofuranosidase purified from *T. reesei* can hydrolyse beet arabinan to arabinose and liberates 50 % of the arabinose in polymeric xylan (Poutanen, 1988b). However, the enzymes acting alone on polymeric substrates seem to prefer arabinose-substituted oligosaccharides as substrates, since in most of the cases the amounts of arabinose released by the enzymes are enhanced when xylanases are present (Sinha and Sengupta, 1995; Bezalel et al., 1993; Poutanen, 1988b; Bachmann and McCarthy, 1991; Hespell and O'Bryan, 1992; Kormelink et al., 1991; Greve et al., 1984).

Apparently α-L-arabinofuranosidases are more commonly found than endo-1,5-α-arabinases. Only a few enzymes with endo activity have been reported, including those from *Bacillus subtilis* F-11 (Kaji and Saheki, 1975) and *A. niger* (Kaji, 1984; Rombouts et al., 1988). One reason for this low occurrence might be that enzymatic activities are routinely assayed by monitoring the hydrolysis of PNPA and enzymes not active on this artificial substrate would be overlooked in screening procedures.

1.3.4 α-Galactosidases

α-Galactosidases (EC 3.2.1.22) catalyse the hydrolysis of α-1,6-linked α-galactoside residues from simple oligosaccharides such as melibiose, raffinose and stachyose, and also from polymeric galactomannans and galactoglucomannans. The enzyme was first known as melibiase, clasified on the basis of the enzyme activity produced by the yeasts *Saccharomyces cerevisiae* and *S. carlsbergensis* capable of hydrolysing melibiose (Bau, 1895). α-Galactosidases have been reported to occur widely in microorganisms (Table 4), plants and animals (Decker and Richards, 1976).

Most biochemical analyses of α-galactosidases have been carried out using small substrates such as p-nitrophenyl-α-D-galactopyranoside (PNPG), melibiose (α-Gal-1,6-Glc), raffinose (α-Gal-1,6-α-Glc-1,2-β-Fru) and stachyose (α-Gal-1,6-α-Gal-1,6-α-Glc-1,2-β-Fru). For example, the *S. cerevisiae* α-galactosidase has mainly been studied with respect to melibiose utilization by the yeast. The studies of α-galactosidases of *E. coli* (MelA and RafA) (Burstein and Kepes, 1971; Schmid and Schmitt, 1976) and *Streptococcus mutans* (Aduse-Opuku et al., 1991) have also been focused on their role in the uptake and metabolism of raffinose and melibiose. Other studies have been considered their potential use in the food industry, based on the ability of the enzymes to hydrolyse raffinose and stachyose (Annunziato and Mahoney, 1987; Elshafei et al., 1993; Wong et al., 1986). Thus, the capacity of these enzymes to hydrolyse galactose linkages from more complex polymeric substrates remains to be demonstrated.

The substrate specificities of different α-galactosidases against PNPG, melibiose, raffinose and stachyose can vary considerably. It has been reported that the activities of two enzymes purified from *Candida guilliermondii* decreased with the number of sugar units in the substrates (Hashimoto et al., 1991). Similar behaviour has been shown for the α-galactosidase of *Pseudomonas fluorescens* (Hashimoto et al., 1991). The transglycosylation activity of this enzyme was also demonstrated. It can produce Gal-α-1,6-Gal-α-1,6-Glu when incubated with melibiose.

α-Galactosidases acting with different substrate specificities on intact polymeric galactomannans have been isolated from several sources. Those isolated from plant seeds such as *Cyamopsis tetragonoloba* (guar) (Bulpin et al., 1990), *Phaseolus vulgaris* (French bean) (Dhar et al., 1994) and *Vigna radiata* (mung bean) (Dey, 1984) are very efficient in releasing galactose from polymeric galactomannan. The enzyme from guar is able to work at low water content and is also active against polygalactans (Bulpin et al., 1990). The enzyme purified from *T. reesei* can act on polymeric galactomannan but it releases higher amounts of galactose in the presence of mannanase (Zeilinger et al., 1993). *Aspergillus tamarii* produces an extracellular enzyme, GALIII, which can act on galactomannan. However, this fungus also produces two internal mycelial enzymes, GALI and GALII, which only hydrolyse galactose-containing oligosaccharides (Civas et al., 1984a, 1984b). Several enzymes showing different specific activities have also been isolated from *Aspergillus niger* (Table 4). Bahl and Agrawal (1969) isolated an α-galactosidase from a commercial enzyme preparation of *A. niger* (Rhozyme HP.150, Celite-free). This enzyme splits 37 - 40 % of the total terminal α-1,6 galactose of galactomannan from guar and locust bean gum (LBG). Enzymes with similar properties have been isolated from other strains of *A. niger* (Adya and Elbein, 1977; Kaneko et al., 1991). These enzymes showed only low activity against melibiose. *Penicillium ochrochloron* also produces an enzyme which is able to cleave galactose from different galactomannans more readily than from melibiose, raffinose or stachyose (Dey et al., 1993).

Some α-galactosidases, e. g. that of *Mortierella vinacea* (Kaneko et al., 1990), have no activity against galactomannan. Other enzymes, such as that from *Bacillus stearothermophilus* (Talbot and Sygusch, 1990) and two from *Bacteroides ovatus* (Gherardini et al., 1985), have limited activity on glucomannan but can release galactose from oligomers produced by mannanase hydrolysis. *Penicillium purpurogenum* also produces an enzyme releasing galactose from manno-oligosaccharides of copra galactomannan generated by mannanase. The activity of the enzyme against the intact polymer was not tested (Park et al., 1991).

A more detailed study of one of the *A. niger* α-galactosidases and the *Mortierella vinacea* enzyme was carried out by Kaneko et al. (1991) using substituted galactomanno-oligosaccharides of well defined structure. The enzyme of *A. niger* released galactose attached to mannose units at the inner or reducing position in galactomanno-oligosaccharides containing 2 to 5

Table 4. Properties of microbial α-galactosidases.

Organism	M_r	pI	pH[a]	Active on[b]	No/low activity on	Remarks	Reference
Bacteria							
Streptococcus mutans	82.0	6.1	6.5	PNPG, M, R		tetramer, not purified produced in *E. coli*	Aduse-Opuku et al., 1991
E. coli MelA	50,6		8.1	PNPG, M		dimer	Burstein and Kepes, 1971
E. coli RafA	82.0	5.1	7.2	PNPG, M, R		tetramer	Schmid and Schmitt, 1976;
Bacillus stearothermophilus	80		6.5				Ganter et al., 1988
Bacillus stearothermophilus	82		7.0-7.5	PNPG, M, R, S, GMO	GM	trimer	Talbot and Sygusch, 1990
Corynebacterium muricepticum	50		7.5	PNPG, M		tetramer, intracellular, partially purified	Nadkarni et al., 1992
Bacteroides ovatus							
GALI	85	5.6	5.8-6.4	PNPG, M, R, S, GMO	GM	trimer, intracellular	Gherardini et al., 1985
GALII	80.5	6.9	6.3-6.5	PNPG, M, R, S, GMO	GM	trimer, intracellular	
Pseudomonas fluorescens H-601	86.0	6.3	6.0-7.0	PNPG, M, R, S		tetramer	Hashimoto et al., 1991
Bifidobacterium logum			5.8	PNPG		intracellular, not purified	Garro et al., 1994
Fungi							
Aspergillus oryzae	64[c]		4.0	PNPG, R, S			Annunziato and Mahoney, 1987
Aspergillus niger			3.8-4.2	MGP, M, R, S, GM, GM, GGM			Bahl and Agrawal, 1969
Aspergillus niger 5-16	45			PNPG, GMO,	M		Kaneko et al., 1991; Adya and Elbein, 1977

Aspergillus niger	147[c]		5.0	M, S, R		dimer, 78 and 69 kDa	Somiari and Balogh, 1995
Aspergillus niger	82.0	4.8		PNPG			den Herder et al., 1992
Aspergillus nidulans	87	6.3	4.0-5.0	PNPG, M, R		tetramer, intracellular	Rios et al., 1993
Aspergillus tamarii							
GALI	88.0			ONPG, M, R, S	GM	trimer, intracellular,	Civas et al., 1984a
GALII	77.5			ONPG, M, R, S	GM	trimer, intracellular	
GALIII	56.0		4.8	ONPG, GM, R, S	M	extracellular	Civas et al., 1984b
Aspergillus ficcum	70.8		5.6-6.0	PNPG, M, R, S			Zapater et al., 1990
Penicillium ochrochloron	60.2		4.5	PNPG, M, R, S, GM			Dey et al., 1993
Penicillium janthinellum			4.5	PNPG			Elshafei et al., 1993
Penicilliun purpurogenum	63.0		4.0	PNPG, M, R, GMO			Park, et al., 1991
Humicola sp.			5.0	PNPG, R		not purified	Kotwal et al., 1995
Monascus pilosus	150[c]		4.5-5.0	PNPG, M, R, S		intracellular	Wong et al., 1986
Trichoderma reesei	50	5.2	4.0	PNPG, M, R, S, GM			Zeilinger et al., 1993
Cephalosporium acremonium 237	240[c]	4.96	5.0-6.0	PNPG, M, R			Zaprometova and Ulezlo, 1988
Saccharomyces serevisiae	300[c]			PNPG, M, R, S		oligomeric	Lazo et al., 1977
Candida guilliermondii H-404	64.0	6.16	4.5	PNPG, M, R, S		tetramer	Hashimoto et al., 1993
	64.0	6.21	4.5	PNPG, M, R, S		tetramer	

[a] pH optimum
[b] PNPG, p-nitrophenyl-galactopyranoside; ONPG, o-nitrophenyl-galacopyranoside; M, melibiose; R, raffinose; S, stachyose; GGM, galactoglucomannan; GM, galactomannan; GMO, galactomannan oligosaccharides.
[c] Determined by gel filtration. Other values were determined by SDS-PAGE.

units, but did not release terminal galactose attached to mannose at the non-reducing end. On the other hand the *Mortierella vinacea* α-galactosidase, previously shown to be inactive towards polymeric galactomannan, liberated terminal galactose from melibiose and galactose attached to mannose units at the non-reducing end of the galactomanno-oligosaccharides, but did not cleave galactose attached to mannose at internal or reducing-end positions in galactomanno-oligosaccharides (Kaneko et al., 1991).

1.4 MOLECULAR BIOLOGY OF SIDE-CHAIN CLEAVING HEMICELLULASES

The number of cloned genes encoding acetyl xylan esterases (Table 5), arabinose-releasing enzymes (Table 6) and α-galactosidases (Table 7) has increased significantly during the 1990s. On the other hand, before this work no genes encoding α-glucuronidase activity had been reported from any organism.

The increasing number of known amino acid sequences has allowed their analysis and comparison in order to obtain structural and/or functional information. Most analyses of protein sequences are carried out effectively with classical (1D) alignment methods, which are based on the maximisation of alignment scores with various comparison matrices. However, these methods are not reliable when the level of amino acid conservation is low. Furthermore, they may introduce deletions or insertions in the secondary structure elements which are usually well conserved during evolution.

A method of comparing protein sequences, Hydrophobic Cluster Analysis (HCA), has been developed which can reduce some of the limitations mentioned above (Gaboriaud et al., 1987; Lemesle-Varloot et al., 1990). HCA is not primarily based on the maximisation of similarity score, but rather on the detection and comparison of clusters of hydrophobic residues which are presumed to coincide with the structural segments constituting the hydrophobic core of globular proteins. The method represents the amino acid sequences to be compared as two-dimensional helical plots which are based on the assumption that the whole proteins would be in α-helical conformation. Clusters of hydrophobic residues with similar shapes, sizes, and relative positions are then localised in the plots and compared between the sequences. These two dimensional plots allow indistinct information to become visible more readily than with other 1D methods. This feature makes the HCA method particularly sensitive and efficient for the comparison of proteins displaying sequence identity lower than 15 %. HCA is also very effective in detecting homologous domains which are separated by variable segments of widely differing sizes (Henrissat et al., 1988). It can also allow accurate secondary structure predictions for numerous β-strands and α-helices present in globular proteins (Gaboriaud et al., 1987). However, HCA does have certain limitations. It is mainly visual and cannot be used directly to scan large

databases. Furthermore, the comparison of HCA plots relies partly on the experience and training of the user, being largely subjective (Lemesle-Varloot et al., 1990).

On the basis of the amino acid sequence similarities detected with the HCA method, used alone or in combination with classical (1D) methods, a number of glycosyl hydrolase protein sequences have been classified into different families (Henrissat, 1991; Henrissat and Bairoch, 1993). The growing number of glycosyl hydrolase genes that are being sequenced and the systematic comparison of the predicted primary protein sequences have allowed their grouping hitherto into 58 families (Henrissat and Bairoch, 1996). This classification of glycosyl hydrolases is intended to better reflect the structural features of these enzymes. It is complementary to the IUB classification, which is based primarily on substrate specificity but does not necessarily give structural information about the enzymes.

The classification of glycosyl hydrolases on the basis of their amino acid similarities has reflected evolutionary relatedness of the enzymes and has revealed evolutionary events such as divergent and convergent evolution, as evidenced for example by families that contain enzymes with several EC classifications and enzymes with similar substrate specificities but belonging to non-related families, respectively. The similarity between the members of one family most probably indicates the same folding characteristics, which can then enable homology modelling when the three-dimensional structure of one member is known.

1.4.1 Acetyl Xylan Esterases

The first gene encoding an acetyl xylan esterase activity was isolated from the thermophilic bacterium *Caldocellum saccharolyticum* (Lüthi et al., 1990a). The gene was identified in a recombinant λ bacteriophage containing five different ORFs which coded for xylanase and β-xylosidase activities detectable in *E. coli*. When expressed at a moderated level, the enzyme could be produced in soluble active form in *E. coli* and further characterized (Lüthi et al., 1990b). Analysis of the crude preparations showed that the enzyme was most active at 70 - 75 °C, but also very stable for 64 hours at 80 °C. It was determined that the enzyme preparation, free of xylanase activity, released acetic acid from acetylated xylan (Luthi et al., 1990b). No further purification or characterization of the enzyme have been reported.

An acetyl xylan esterase gene of *Pseudomonas flourescens* subs. *cellulosa* (*xylD*) has also been cloned and its corresponding protein analysed (Ferreira et al., 1993). The gene codes for a modular enzyme with a non-catalytic cellulose-binding domain (CBD) of bacterial type, separated from the catalytic domain by a serine-rich sequence. The gene was found when a genomic library of the microorganism was screened for new genes encoding hydrolases with a CBD specific probe. This region has been found in two

Table 5. Acetyl xylan esterase genes and the deduced proteins.

Organism	gene	ORF[a] (nt)	Signal[b] Sequence (aa)	Mature protein (aa)	M_r[c]	Remarks	References
Caldocellum saccharolyticum	*xylC*	798		266	30.6		Lüthi et al., 1990a
Pseudomonas fluorescens subs. *Cellulosa*	*xylD*	1752	36	548	60.6	CBD	Ferreira et al., 1993
Streptomyces lividans	*axeA*	1002	41	293	30.1	CBD	Shareck et al., 1995
Aspergillus aculeatus		1020	27	293			Christgau et al., 1995b
Aspergillus niger	*axe*A	918	27	279			de Graaff et al., 1992

[a] nt, nucleotide number
[b] aa, amino acid number
[c] Molecular mass estimated from the amino acid sequence of the mature protein

endoglucanases, two endoxylanases, a dextrinase and an arabinofuranosidase of *P. fluorescens* subs. *cellulosa* (Hazlewood and Gilbert, 1992). The CBD is found at the N-terminus, similarly to that in the xylanase (*xylB*) and arabinofuranosidase (*xylC*) of *P. fluorescens* (Ferreira et al., 1993). The esterase produced intracellularly in *E. coli* was able to bind onto cellulose, but not onto xylan. A truncated enzyme lacking the CBD retained the capacity to hydrolyse ester linkages, but did not bind cellulose. The complete recombinant enzyme was able to liberate acetic acid from chemically acetylated polymeric xylan, but only in the presence of xylanases. Together with xylanase, it was also able to liberate ferulic acid from plant structural polysaccharides (Ferreira et al., 1993).

The acetyl xylan esterase gene of *Streptomyces lividans* (*axeA*) was identified in a chromosomal region downstream from a previously isolated xylanase gene (*xlnB*) (Shareck et al., 1995). The work was carried out after detection of high acetyl xylan esterase activity in the culture filtrate of *S. lividans* IAF42 harbouring a plasmid containing both esterase and xylanase genes. This indicated that the acetyl xylan esterase was at least active against small acetylated xylo-oligosaccharides. The enzyme was not purified or characterized further. The enzyme encoded by *axeA* also consists of a catalytic and a substrate binding domain separated by a glycine-rich linker. The N-terminal catalytic domain has no amino acid similarity with the acetyl xylan esterases of *C. saccharolyticum* or *P. flourescens* subs. *cellulosa.* It shows some similarity with two enzymes involved in the liberation of acetic acid: a cytoplasmic rhizobial NodB protein and the chitin deacetylase (Cda) of the fungus *Mucor rouxii* (Shareck et al., 1995). NodB is involved in root nodulation in plants and diacetylates the non-reducing N-acetylglucosamine residues. Cda is involved in the synthesis of chitosan and hydrolyses the N-acetamino group of chitotetraose or large homopolymers. The C-terminus of *S. lividans* AXEA is highly similar to the C-terminus of *S. lividans* xylanase B (XlnB) and *Thermomonospora fusca* xylanase A (TFXA). The region in *T. fusca* was reported to represent a xylan binding domain sharing similarities with cellulose-binding domains (Irwin et al., 1994).

A gene of fungal origin encoding an acetyl xylan esterase was isolated from *A. niger* (de Graaff et al., 1992). This gene corresponded to the previously purified enzyme of *A. niger* (Kormelink *et al*., 1993; Table 2). A plasmid containing the complete chromosomal gene was transformed into another strain of *A. niger* and the overexpressed acetyl esterase was tested against acetylated xylan. The acetyl xylan esterase of *A. aculeatus* and its corresponding gene were recently isolated (Christgau et al., 1995a). The gene was isolated from a cDNA expression library using PCR fragments as probes, generated with oligonucleotides designed on the basis of the amino acid sequence of the purified protein. The gene was overexpressed in *A. niger* and *A. oryzae* (Christgau et al., 1995a). No similarities with other protein sequences were reported for the deduced protein.

1.4.2 α-Arabinosidases

The *Pseudomonas fluorescens* subsp. *cellulosa* α-L-arabinofuranosidase gene (*xynC*) was isolated from a chromosomal gene library established in *E. coli* (Kellett et al., 1990). The same plasmid contained both a xylanase (*xylB*) gene and the gene encoding the α-L-arabinofuranosidase, as determined by activity assays. The *E. coli*-produced enzyme did not degrade PNPA. The α-L-arabinosidase contains a non-catalytic CBD situated at the N-terminus immediately after a signal sequence, similar to those found in several other hydrolases of *Pseudomonas fluorescens* subsp. *cellulosa* (Kellett et al., 1990; Ferreira et al., 1993; Hazlewood and Gilbert, 1992). The truncated enzyme lacking the CBD was expressed in *E. coli*. This enzyme had lost the capacity to bind cellulose, but its activity was not affected.

Similarly, two α-L-arabinofuranosidase (*arfA* and *arfB*) genes of *Clostridium stercorarium* have been isolated from a genomic cosmid library by detection of α-L-arabinosidase activity in *E. coli* (Schwarz et al., 1990). The same DNA fragment contained a xylanase and two β-xylosidase genes, and two genes encoding enzymes termed celloxylanases on the basis of their activity towards both xylan and β-D-cellobiosides. The α-L-arabinofuranosidase encoded by *arfA* and expressed in *E. coli* was active against both the synthetic substrates PNPA and PNPX, particularly against the former. The enzyme encoded by the *arfB* gene exhibited higher specificity for PNPA, with little activity against other arylglycosides (Schwarz et al., 1990). No further characterization of the genes or the corresponding proteins has been reported.

The α-L-arabinofuranosidase gene of *Butyrivibrio fibrisolvens* was also cloned by screening a genomic bank constructed in *E. coli* for α-L-arabinofuranosidase activity (Utt et al., 1991). A single gene, *xylB*, was found flanked by other two incomplete ORFs. The three genes are probably part of a single operon. The deduced amino acid sequence of XYLB did not exhibit similarity with other xylan-degrading enzymes or glycosidases, but a conserved region was identified at the carboxyl end of the protein which was similar to the starch binding domain of *Aspergillus niger* glucoamylase. Similar to the enzymes of *C. stercorarium*, the *E. coli* produced XYLB showed both β-D-xylosidase and α-L-arabinofuranosidase activities against PNPX and PNPA, respectively. The specific arabinosidase activity was found to be approximately 1.6-fold higher than that of xylosidase (Utt et al., 1991). The chromosomal α-L-arabinofuranosidase gene of *Streptomyces lividans* 66 (*abfA*) was cloned by functional complementation of an *S. lividans* mutant strain which did not express either xylanase, cellulase or α-L-arabinofuranosidase activities (Manin et al., 1994). The protein sequence of ABFA did not show any similarity with the previously reported α-L-arabinofuranosidases from *Pseudomonas fluorescens* (Kellett et al., 1990) and *Butyrivibrio fibrisolvens* (Utt et al., 1991). The produced enzyme was localized in the cytoplasmic fraction. The authors correlated this with the fact that the enzyme exhibited limited activity against arabinoxylan, but

Table 6. L-Arabinose-releasing enzyme genes and their deduced proteins.

Organism	gene	ORF (nt)[a]	Signal Sequence (aa)[b]	Mature protein (aa)	M_r	Family[d]	Remarks	References
Pseudomonas fluorescens subs. *cellulosa*	*xynC*	1713	37	534	59.0[c]	nc[e]	CBD	Kellett et al., 1990
Clostridium stercorarium	*arfA*				49.0[c]	nc		Schwarz et al., 1990
	arfB				30.0[c]	nc		
Butyrivibrio fibrisolvens	*xylB*	1551		517	57.0	43		Utt et al., 1991
Streptomyces lividans 66	*abf*A	1986		662	73.0	51		Manin et al., 1994
Aspergillus niger	*abfA*	1884	25	603	65.4	51	7 introns, 10 CHO[f]	Flipphi et al., 1993a
	abfB	1500	18	481	50.7	nc	2 CHO	Flipphi et al., 1993c
	abnA	963	19	302	32.5	43	3 introns, 1 CHO	Flipphi et al., 1993b

[a] nt, nucleotide number
[b] aa, amino acid number
[c] Molecular mass determined by SDS-PAGE of the *E. coli* produced protein. Others were estimated from the amino acid sequences of the mature proteins.
[d] Family number in the general classification of glycosyl hydrolases
[e] nc, not classified
[f] CHO, number of potential *N*-glycocylation sites

hydrolysed efficiently short chain arabino-oligoxylosides (Manin et al., 1994).

Genes of fungal origin encoding enzymes hydrolysing L-arabinose linkages have only been isolated from *A. niger*. Three genes, encoding α-L-arabinofuranosidase A (*abfA*), α-L-arabinofuranosidase B (*abfB*) and endo-1,5-α-L-arabinase (*abnA*), were isolated which encoded the previously purified enzymes (Rombouts et al., 1988; van der Veen et al., 1991; Table 3). The α-L-arabinofuranosidase A (*abfA*) (Flipphi et al., 1993a) and the endo-1,5-α-L-arabinase (*abnA*) (Flipphi et al., 1993b) genes were isolated from a λ expression library. Both cDNA genes, *abfA* and *abnA*, were identified with antibodies prepared from the previously purified α-L-arabinofuranosidase A and endo-1,5-α-L-arabinase, respectively (van der Veen et al., 1991). The chromosomal *abfA* and *abnA* genes were also isolated from a genomic λ bacteriphage library. The gene (*abfB*) encoding α-L-arabinofuranosidase B was isolated from the genomic λ library using PCR probes amplified with oligonucleotides designed from peptide sequences of the previously purified enzyme (Flipphi et al., 1993c; van der Veen et al., 1991). Analysis of nucleotide sequences upstream from the coding region indicated the presence of necessary sequences to direct the expression of the three genes (Flipphi et al., 1993a; Flipphi et al., 1993b; Flipphi et al., 1994). The functionality of *abfA*, *abfB* and *abnA* genes was established by overexpression in strains of *A. niger* and *A. nidulans* (Flipphi et al., 1993a; Flipphi et al., 1993b; Flipphi et al., 1993c).

The authors reported that the deduced amino acid sequences of the *A. niger* ABFA, ABFB and ABNA showed no similarities between each other or with any of the reported α-L-arabinosidases of bacterial origin. Only short amino acid sequences of ABFA were shown to have similarity with pullulanases of family 13 of the glycosyl hydrolases, which contain only α-amylase-like hydrolyses and transferases (Flipphi et al., 1994). However, in the updated list of the general classification of glycosyl hydrolases (Henrissat and Bairoch, 1993), the *A. niger* endo-1,5-α-L-arabinase (ABN A) and the α-L-arabinofuranosidase of *B. fibrisolvens* (XYLB) appear grouped into family 43 together with several xylanases. On the other hand, the *A. niger* α-L-arabinofuranosidases A (ABF A) and the *S. lividans* α-L-arabinofuranosidase (ABFA) appear in the new family 51. The *A. niger* α-L-arabinofuranosidase B (ABFB), and also those from *C. stercorarium* (ARFA and ARFB) and *P. fluorescens*, have not been yet classified.

1.4.3 α-Galactosidases

Genes encoding α-galactosidases have been isolated from several sources including human, bacteria, plant seeds, yeasts and filamentous fungi. One of the first cloned and characterized α-galactosidase genes was the *MEL1* of *Saccharomyces cerevisiae* (Liljeström, 1985; Summer-Smith et al., 1985). The *MEL1* gene is part of the *GAL* regulon of yeast which confers the capability to utilize galactose and melibiose. The *MEL1* gene was isolated from a genomic

library of *S. cerevisiae* by complementation in a *mel1-18 S. cerevisiae* strain (Liljeström, 1985). Strains of *S. cerevisiae* can have from one to seven loci containing *MEL1* genes (Turakainen et al., 1993) and these have been described as a *MEL* family. *MEL* genes have also been isolated from other yeasts such as *Zygosaccharomyces cidri* and *S. carlsbergensis* NCYC393, and these show more than 70 % similarity wihh the *S. cerevisiae MEL* gene (Turakainen et al., 1991, 1994a, b). The *MEL1* gene has been integrated into commercial baker's yeast strains in order to construct strains capable of complete utilization of the raffinose present in beet molasses. The *MEL1* gene was also used as a marker to select transformants (Suominen, 1988).

Two non-related α-galactosidases genes, *melA* and *rafA*, have been cloned from *E. coli*. The *melA* is part of an operon also containing the *melB* gene encoding a melibiose permease (Schmid and Schmitt, 1976). The complete locus was cloned by selecting for growth of a mutant *E. coli* strain (*melA6*) on melibiose. The structural *rafA* gene was identified as part of the plasmid-borne *raf* operon which enables *E. coli* to utilize raffinose. The operon includes two other structural genes, *rafB* and *rafD*, encoding a raffinose permease and a sucrose hydrolase, respectively. It was suggested that the *raf* operon has a modular construction, having picked up the two genes from different sources (Aslanidis et al., 1989). No evident similarities were observed between the protein sequence of RafA and that ff the chromosomally encoded α-galactosidase MelA (Aslanidis et al., 1989).

The *Streptococcus mutans* α-galactosidase gene, *aga*, has been cloned from a genomic λ library. The clones producing α-galactosidase were identified on media containing raffinose which, once hydrolysed, allowed growth of *E. coli* cells surrounding the λ plaques (Aduse-Opoku et al., 1991). The *aga* gene was found to be linked to sucrose phosphorylase (*gtfA*) and dextran glucosidase genes (*dexB*). The chromosomal *aga* gene was expressed in *E. coli* and the produced intracellular enzyme was characterized (Section 1.3.4; Table 4). Inactivation of the gene in *S. mutans* eliminated the α-galactosidase activity of the strain and its capacity to ferment melibiose and raffinose. The capacity of the strain to ferment other sugars was not affected (Aduse-Opoku, et al., 1991). The protein sequence of AGA has some similarity with that of RafA of *E. coli*, but not with that of MelA. On the basis of its similarities with the *S. mutans* and *B. stearothermophilus* enzymes and its divergence from the chromosomal MelA of *E. coli*, it was suggested that the *E. coli* RafA might have originated from a Gram-positive organism. The human α-galactosidase A is a lysosomal hydrolase that cleaves terminal α-galactosyl residues from glycophingolipids and glycopeptides (Bishop and Desnick, 1981). It is expressed in all cells of normal individuals. The deficient activity of this enzyme causes Fabry's disease, which is an X-chromosomally linked recessive disorder leading to lysosomal accumulation of globotrioasylceramide, galabioasylceramide and blood group B substance. Progressive deposition of these substrates in lysosomes of vascular endothelial and smooth muscle cells provokes occlusive disease of the heart, kidneys and brain, leading to premature death

Table 7. α-Galactosidase genes and the deduced proteins.

Organism	gene	ORF (nt)[a]	Signal Sequence (aa)[b]	Mature protein (aa)	M_r[c]	Family[d]	Remarks	References
Saccharomyces cerevisiae	*MEL1*	1413	18	453	52.0	27	no introns, 8 CHO[e]	Liljeström, 1985; Summer-Smith et al., 1985
E. coli	*melA*	1353		451	50.6	4		Liljeström and Liljeström, 1987
	rafA	2124		708	81.1	36		Aslanidis et al., 1989
Streptococcus mutans	*aga*	2160		720	82.0	36		Aduse-Opoku, et al., 1991
Human	*GALA*	1287	31	398	45.3	27	6 introns, 4 CHO	Bishop et al., 1988
Cyamopsis tetragonaloba		1233	47	364		27		Overbeeke et al., 1989
Coffea arabica		1134	15	363	42.0	27	1 CHO	Zhu and Goldstein, 1994
Aspergillus niger	*aglA*	1635	23 or 31	514	60.0	27	no introns, 7 CHO	den Herder et al., 1992
Mortierella vinacea		1251	20	397	44.3	27	2 CHO	Shibuya et al., 1995

[a] nt, nucleotide number
[b] aa, amino acid number
[c] Molecular mass determined by SDS-PAGE of the *E. coli* produced protein. Other molecular mass were estimated from the amino acid sequences of the mature proteins.
[d] Family number in the general classification of glycosyl hydrolases
[e] CHO, number of potential *N*-glycocylation sites

of affected males. The α-galactosidase A gene was first isolated from a liver specific cDNA expression library by using oligonucleotides synthesized on the basis of a sequence derived from the purified enzyme (Calhoun et al., 1985). The α-galactosidase genes of many Fabry's disease patients have been isolated in order to identify mutations. A variety of single amino acid substitutions have been found mainly in the exons 1, 2 and 7 which can partially or completely inactivate the enzyme (Koide et al., 1990; Ishii et al., 1992; Eng et al., 1993; Ishii et al., 1994; Ishii et al., 1995). Small deletions and gene rearrangements have also been found (Eng et al., 1993; Bernstein et al., 1989). There is considerable interest in the structure of the human α-galactosidase. It has been crystallized and preliminary X-ray data is available (Murali et al., 1994), but the structure has not yet been reported. Due to its potential utilization in enzyme therapy, the α-galactosidase A has been expressed in the baculovirus system at a level of 5.6 mg/l and exhibited similar enzyme characteristics to those of the natural enzyme (Coppola et al., 1994).

The α-galactosidase gene of the plant *Cyamopsis tetragonaloba* (guar) was cloned from a cDNA bank constructed with mRNA isolated from aleurone cells of germinated seeds by screening with oligonucleotides as probes, designed on the basis of a sequence derived from the purified enzyme (Overbeeke et al., 1989). Previous studies had shown that the α-galactosidase gene is expressed along with a β-mannanase gene in the aleurone cells during seed germination. The enzymes are released into the endosperm, where they degrade storage polysaccharides (Hughes et al., 1988). The deduced protein sequence of the guar α-galactosidase showed a considerable degree of similarity with the enzymes of *S. cerevisiae* and human. As previously mentioned in Section 1.2.4, the guar α-galactosidase has efficient hydrolytic properties and there is considerable interest in producing the enzyme in large scale for various applications, e. g. for modification of galactomannans. The mature enzyme has been overexpressed in *B. subtilis* under the control of the *SPO2* promoter using the signal sequence of the α-amylase of *B. amyloliquefaciens* (Overbeeke et al., 1990). The *B. subtilis*-secreted enzyme (activity levels up to 1,700 U/liter) was active towards PNPA and galactomannan (guar gum), showing hydrolytic properties similar to those of the glycosylated natural enzyme. It was suggested that glycosylation has no essential function for the activity of the enzyme. The enzyme has also been expressed in the methylotrophic yeast *Hansenula polymorpha* under the control of the strong and methanol inducible *MOX* promoter and in *S. cerevisiae* under the *GAL7* promoter. The highest production levels obtained with *H. polymorpha* and *S. cerevisiae* were 42.0 and 22.4 mg/g of dry weight, respectively, using chemostat cultivations (Giuseppin et al., 1993).

The α-galactosidase gene of *Coffea arabica* (coffee bean) was also isolated from a cDNA library, prepared with mRNA from dried green coffee beans, and probed with oligonucleotides designed on the basis of a sequence derived from the purified enzyme (Zhu and Goldstein, 1994). The deduced protein sequence of the mature enzyme shared more than 80 % identity with the enzyme from guar.

Although the enzymes are very similar, they have significant differences in substrate specificity. The guar α-galactosidase preferably cleaves galactose which is α-1,6-linked to oligosaccharides (Giuseppin et al., 1993), whereas the coffee bean enzyme is more active on α-1,3 and α-1,4 linkages (Zhu and Goldstein, 1994). The main reason for interest in the coffee bean enzyme is its capability to cleave efficiently α-1,3-linked galactose residues from the cell surface of group B red cells (see Section 1.4). The enzyme was overexpressed in the baculovirus system in enzymatically active form (Zhu and Goldstein, 1994).

The *aglA* gene of *Aspergillus niger* was isolated from a λ genomic library using oligonucleotides designed from the N-terminal sequence of a purified 82 kDa α-galactosidase (den Herder et al., 1992). Highest similarity for the deduced protein sequence of AGLA was reported with the guar α-galactosidase (37 %). At the C-terminus of the enzyme a region preceded by a sequence rich in Ser and Thr was found, similar to those separating the catalytic domain and the CBDs of the cellulases of e. g. *T. reesei*. It was suggested that AGLA may have a similar domain structure to that of the cellulases, including a substrate binding domain which could be involved in binding of the enzyme to galactomannan. However, no data supporting this assumption was presented (den Herder et al., 1992). Deletion of the *aglA* gene in *A. niger* did not affect the total α-galactosidase activity produced by the fungus. The gene was also overexpressed in *A. niger* under the control of the glucoamylase promoter (*glaA*), but the α-galactosidase activity produced by the fungus increased only moderately. The authors concluded that *agaA* encodes an enzyme which represents a minor activity. The presence of at least three more α-galactosidases was detected (den Herder et al., 1992).

The α-galactosidase gene of the fungus *Mortierella vinacea* was recently cloned from a cDNA bank by screening with a PCR probe amplified from the DNA of the fungus using oligonuclotides designed from sequenced peptides of the purified enzyme (Shibuya et al., 1995). The deduced protein showed similarity with the enzymes of *S. serevisiae*, *Cyamopsis tetragonaloba* and human, with amino acid identities of 47, 43 and 34 %, respectively.

In the general classification of glycosyl hydrolases the α-galactosidases have been grouped into three well conserved families (Henrissat, 1991; Henrissat and Bairoch, 1993). The MelA of *E. coli* and a fragment of the α-galactosidase of *Salmonella typhimurium* (P30877) have been grouped into family 4, and the RafA of *E. coli* and the *Streptococcus mutans* enzyme into family 36. All the eukaryotic α-galactosidases have been grouped into family 27. In recent reports a distant relatedness between the protein sequences of families 27 and 36 was indicated (Dagnall et al., 1995; Henrissat and Romeu, 1995). It was suggested that these two families could comprise subclusters of a single superfamily which could have evolved from a common ancestor.

1.5 POTENTIAL APPLICATIONS OF SIDE-CHAIN CLEAVING HEMICELLULASES

Potential applications for hemicellulases could be found in industries in which hemicelluloses are the basic component of feedstocks, such as in the food, feed, and pulp and paper industries. In these industries total or partial enzymatic degradation and modification of hemicelluloses could improve existing processes or generate new products.

Agricultural, forestry and municipal solid waste residues represent a significant source of renewable resources, the disposal of which currently causes environmental problems. Exploitation of these low cost materials for bioconvertion processes will require maximal utilization of the various polymeric sugars including hemicelluloses. The presence of side-chain groups in hemicelluloses such as xylans and mannans can hamper the action of other depolymerizing enzymes (see Section 1.2). Thus, side-chain cleaving enzymes acting synergistically with depolymerizing enzymes would be essential for efficient degradation of hemicelluloses containing residues to obtain high yields of monosaccharide sugars.

Side-chain cleaving enzymes could be useful in the pulp and paper industry. They may have a role in assisting the action of xylanase and mannanases in the bleaching of Kraft pulps. Such a positive effect has been described for an α-arabinosidase of *A. niger* and a for the α-glucuronidase of *T. reesei* acting together with xylanase (Kantelinen, 1992). New processes for pulping are being studied in order to decrease the use of polluting chemicals. Enzymes hydrolysing side-chain groups from xylan and mannan could be especially beneficial, for example in mechanical pulping processes. The enzymes could be utilised in the partial degradation of the hemicellulose fractions containing side groups such as acetic and uronic acids, arabinose and galactose, which are completely or partially removed and modified in acid sulphite and alkaline sulphate processes, but not in mechanical pulping. On the other hand, the major polysaccharides dissolved in the thermomechanical pulping (TMP) of Norway spruce (*Picea abies*) are O-acetyl-galactoglucomannans. Their dissolution represents a significant yield loss and effluent load. It has been reported that treatment of TMP suspensions with an acetyl esterase of *A. oryzae* resulted in an increase of TMP yield by adsorption/deposition of the deacetylated galactoglucomannans onto the pulp fibres (Thornton et al., 1994). Hydrolysis of TMP suspensions with α-galactosidase might reduce further the solubility of galactoglucomannans, increasing their adsorption/deposition onto the pulp fibres.

The digestibility of animal feeds based on hemicellulosic materials has been reported to be enhanced by xylanase treatment (van Paridon et al., 1992). However, the presence of acetic acid, phenolic groups (Borneman et al., 1986) and arabinose (Brice and Morrison, 1982; Greve et al., 1984) as side groups restricts the utilization of hemicellulosic materials by ruminants. The use of enzymatic removal of these side groups together with xylanase could further improve digestibility of the feed.

In the food industry, hemicellulases used along with pectinases and cellulases might improve the extraction of juices during maceration of fruit and vegetable materials and also increase the rheological properties of these extracts. Juice concentrates derived from apple pulp treated with technical pectinase preparations can develop haze formation, generating a quality defect in the juice which is normally consumed as a clear product. The quality of fruit juices can be improved with the use of arabinases hydrolysing the arabinans and arabinogalactans which have been identified as the haze-forming materials in the product (Voragen et al., 1987).

Hemicellulases can be used for enzymatic modification of the properties of plant polysaccharides. The properties of guar gum and LBG as promoters of gelling have found extensive applications in the paper, food, cosmetics, pharmaceutical, explosive and mining industries. Guar gum is more available and significantly cheaper that LBG, but is a less effective gel promoter because of its higher galactose content. It has been reported that modification of guar galactomannan by enzymatic removal of the galactose yielded a polysaccharide with a galactose content comparable to that of LBG (Dey et al., 1993). Similarly, α-arabinosidases and acetyl xylan esterases could be used to control the degree of substitution and hence the water-binding capacity of acetyl arabinoxylans, which also form viscous solutions and gels. Soluble dietary fibre such as xylans are beneficial to humans and reduction of their water-binding capacity is desirable in order to increase diffusion of nutrients, improving the nutritional value of the food.

Soybean is an important source of protein for animal feed and human nutrition. The major limitation in the use of soya meal or soya milk is their high content of raffinose, stachyose and to a lesser extent verbascose (α-Gal-1,6-α-Gal-1,6-α-Gal-1,6-α-Glc-1,2-β-Fru), which can hinder digestion in humans due to the lack of α-galactosidase in the intestinal tract. Hydrolysis of these oligosaccharides in soya-based products using α-galactosidase could alleviate gastric distress caused by bacterial fermentation of carbohydrates in the large intestine (Cristofaro et al., 1974). This could also increase the use of soy meal in several other products.

In the beet-sugar industry, the presence of raffinose in beet sugar syrups inhibits sucrose crystallisation, causing economic losses by reduction of the sugar yield. Hydrolysis of raffinose using α-galactosidases can improve the quality and efficiency of the process of sugar crystallisation. Processes including the enzymatic hydrolysis of raffinose have been developed, for example the α-galactosidase of *M. vinacea* has been immobilized *in situ* in order to remove raffinose during sugar refining (Shimzu and Kaga, 1972).

In the medical field the α-galactosidases also have considerable potential. The α-galactosidase from coffee bean is capable of removing terminal α-1,3-linked galactose residues, responsible for blood group B specificity, from the surface of erythrocytes. Enzymatic treatment can convert these cells to the serological group O, with potential use in transfusion therapy (Zhu and Goldstein, 1994). The

human α-galactosidase A has also been overexpressed in the baculovirus system, with the intention of replacing the defective enzyme with the normal protein in Fabry's disease patients (Coppola et al., 1994).

Applications for hemicellulases can be also found in the field of synthetic chemistry. The transglycosylation activity of hemicellulases hydrolysing glycosidic bonds could lead to the synthesis of new biologically active oligosaccharides, which could improve health when used as food additives. For example, galacto-oligosaccharides, which promote growth of bifidobacteria in the human intestine, can be produced by the transglycosylating action of α-galactosidases (Hashimoto et al., 1991). Partial deacylation of sugars can be a valuable method for obtaining specifically protected intermediates in the synthesis of novel carbohydrates (Colquhoun et al., 1990; Haines et al., 1990). Production of partially acetylated sugars with conventional chemical methods can be difficult because of the abundance of multiple hydroxyl groups with similar reactivities. The use of enzymes for regioselective deacylation of sugar esters has attracted considerable interest and a number of lipases, proteases and esterases have been tested (Borneman et al., 1992).

Hemicellulases can be used as important tools in modern carbohydrate chemistry. Information concerning the structure of polysaccharides bound to glycoproteins as well as the structure and interconnections of cell wall polysaccharides can be deduced from analysis of the mono- and oligosaccharides produced by purified polysaccharide-degrading enzymes. Classical acidic or basic extraction procedures result in the simultaneous cleavage of a number of different types of bonds present in plant cell walls (Aspinall, 1980). These chemical procedures are less specific and might result in the removal of side groups, giving modified oligosaccharides which are not representative of the natural polysaccharides. Highly purified hemicellulases can split specific glycosidic or other linkages of the complex polymers present in the cell wall to a limited set of unmodified and identifiable oligosaccharides (Matheson and McCleary, 1985). Characterization of these oligomers will allow conclusive data about the structure of the polymers and also the pattern of action of the enzymes. This knowledge could then be used in more effective applications of both the polymers and the enzymes.

1.6 AIMS OF THIS STUDY

T. reesei produces high levels of extracellular enzymes which hydrolyse plant polysaccharides and it is an important industrial organism. The cellulolytic and xylanolytic system of *T. reesei* has been extensively studied. Many of its enzymes acting on polysaccharide backbones, such as several endoglucanases and cellobiohydrolases as well as two xylanases and a mannanase, have been characterised at the molecular level. A gene encoding a β-glucosidase has also been cloned. Recently, more attention has been paid to the side group releasing enzymes of *T. reesei* and some of them have been purified and characterized.

However, no knowledge of these enzymes is available at the molecular level. The aim of this work was to isolate and characterize genes encoding side-chain cleaving hemicellulases of *T. reesei* using different cloning methods. The specific aims of the study were:

- cloning and characterization of genes encoding an α-glucuronidase and an acetyl xylan esterase previously purified from *T. reesei*

- cloning and characterization of genes encoding α-arabinofuranosidase and α-galactosidase activities by expression in yeast

- study of the possibility of using the yeast *S. cerevisiae* as a host for expression cloning

- comparison of the derived protein sequences with other enzymes in order to search for functional important regions and to classify the enzymes

- preliminary characterization of the properties and substrate specificities of the yeast produced *T. reesei* enzymes by studying their α-arabinofuranosidase and α-galactosidase activities

2 RESULTS AND DISCUSSION

2.1 CLONING OF HEMICELLULASE GENES FROM A λ PHAGE cDNA EXPRESSION LIBRARY

Previously, a λ phage cDNA expression library of *T. reesei* RutC-30 had been prepared to isolate a β-mannanase gene (Stålbrand et al., 1995). The mRNA used to construct the library was isolated from mycelium of the fungus grown on a medium containing Solka flock cellulose, spent grain, galactomannan, acetylglucuronoxylan and arabinoxylan (Stålbrand et al., 1995). It was expected that the fungus cultivated on this complex mixture of plant polysaccharides would produce all possible hydrolases, and this previously constructed expression library was used in isolation of the α-glucuronidase and acetyl xylan esterase genes of *T. reesei*.

2.1.1 Isolation of the *glr1* gene

The major α-glucuronidase of *T. reesei* RutC-30 had previously been purified and characterized (Siika-aho et al., 1994). The purified enzyme was used to prepare polyclonal antibodies (see Materials and Methods/II). Five clones were identified by screening the λ phage cDNA expression library with anti-α-glucuronidase antibodies (Table 8). The λ clones were excised *in vivo* into phagemid pBluescript SK(-) forms. The five clones contained cDNA fragments corresponding to the same gene as determined by partial sequencing. The deduced protein sequence of the largest clone contained the N-terminal sequence determined from the purified protein (I) and three amino acids which could be part of the signal sequence, indicating that the clone was not complete. In order to find a full length gene, a 5´ end EcoRI/XbaI fragment of approximately 260 bp was used as probe to screen a larger cDNA library constructed in a yeast shuttle vector pAJ401 (III). Several new clones were partially sequenced and were found to code for the same gene. A single ORF coding for a typical eukaryotic signal peptide sequence (von Heijne, 1986) of 19 amino acids was identified preceding the N-terminus of the purified α-glucuronidase. The gene was named *glr1* (Table 8). The nucleotide and the deduced amino acid sequences are presented in I/Fig. 1. The mature protein had a length of 828 amino acids and a calculated molecular mass of 91.39 kDa. This value was in good agreement with the apparent molecular mass of the purified enzyme, which is 91 kDa when estimated by SDS-PAGE (Siika-aho et al., 1994). This would indicate that the enzyme is not substantially glycosylated although it contains eight potential N-glycosylation sites (Table 8; I).

The *glr1* gene is the first α-glucuronidase gene hitherto isolated and characterised. In order to examine the possible relation ship of the enzyme encoded by *glr1* with other protein sequences, similarity searches were performed in all the data banks available using different search programs (I).

Table 8. T. reesei hemicellulase genes isolated in this work and the deduced protein sequences.

Enzyme	Gene	Frequency[a]	ORF (nt)[b]	Signal sequence (aa)[c]	Mature protein (aa)	CHO[d]	M_r[e]	Similarity	Remarks	Reference
α-Glucuronidase	*glr*1	1/1.0x10^5	2541	19	828	8	91.39	none		I
Acetyl xylan esterase	*axe*1	1/1.0x10^3	906	20	271	1	27.5	Fungal cutinases	11 aa pro region, CBD	II
α-L-Arabinofuranosidase	*abf*1	1/2.5x10^3	1500	21	479	1	49.1	*A. niger* ABFB		III
β-Xylosidase	*bxl*1	1/4.0x10^5	2274	20	738	10	80.4	Family 3[f]		III
α-Galactosidases	*agl*1	1/3.4x10^3	1332	27	417	5	45.7	Family 27		IV
	*agl*2	1/7.8x10^4	2238	26	720	9	79.5	Family 36		IV
	*agl*3	1/5.6x10^3	1872	22	602	7	66.3	Family 27		IV

[a] Frequency of clones found in the screening of the cDNA banks
[b] nt, nucleotide number
[c] aa, amino acid number
[d] CHO, number of potential *N*-glycocylation sites
[e] Molecular mass estimated from the amino acid sequence of the mature protein.
[f] Family in the general classification of glycosyl hydrolases

The α-glucuronidase of *T. reesei* showed no significant amino acid sequence similarities with any other protein sequences in the data banks. Probably the α-glucuronidase represents a completely new group of glycosyl hydrolases.

2.1.2 Isolation and characterization of the *axe1* gene

Two pI forms of acetyl xylan esterases (pI 7 and pI 6.8) from *T. reesei* RutC-30 were previously purified and characterized, being capable of liberating acetic acid from polymeric acetyl xylan (Poutanen et al., 1990b; Sundberg and Poutanen, 1991). It was shown that both pI forms of the enzymes have very similar biochemical properties and thus they have been used unseparated for enzymatic studies of polysaccharide deacetylation (Poutanen et al., 1990b). However, the possibility that the isoenzymes are products of different genes was considered and polyclonal antibodies were prepared against both pI forms (II/Materials and Methods). They recognised the two pI forms of the enzyme as being alike (results not shown). The screening of the λ phage cDNA expression library using the antibodies resulted in the identification of a large number of putative clones. Expression and activity measurements in *E. coli* identified that only the clones giving a strong signal in antibody screening encoded acetyl xylan esterase activity (II). Partial DNA sequencing of both 5' and 3' ends indicated that all the were identical.

The nucleotide sequence of *axe1* and the deduced protein sequence are presented in II/Fig. 1. The *axe*1 gene codes for a protein of 302 amino acids. Analysis of the protein sequence indicated the presence of a putative signal sequence of 20 amino acids and a second processing site typical for a KEX2-like endopeptidase (Lys-30 and Arg-31) preceding a Glu (Julius et al., 1984). The fact that the N-termini of both forms, pI 7 and pI 6.8, of the purified acetyl xylan esterase are blocked indicates that most likely KEX2-like processing occurs in AXEI and that Gln is the first amino acid of the mature protein. Two peptides obtained by trypsin digestion of the purified enzyme (pI 7.0 and 6.8) were sequenced and their corresponding amino acid sequences were found in the deduced AXEI (II/Fig. 1).The mature AXEI had a length of 271 amino acids with a calculated molecular mass of 27,5 kDa. This value is in good agreement with the reported 34 kDa relative molecular mass of the two forms, pI 7 and pI 6.8, of the purified enzyme, which comigrate in SDS-PAGE (Sundberg and Poutanen, 1991). These results together with the previous biochemical data argue in favour of *axe*1 encoding both pI forms of the enzyme.

2.1.3 Characterization of AXEI

The deduced mature amino acid sequence of AXEI showed no similarity with the amino acid sequences of the published acetyl xylan esterase sequences of the bacteria *Caldocellum saccharolyticum* (Lüthi et al., 1990a), *Pseudomonas*

fluorescens subsp. *cellulosa* (Ferreira et al., 1993), or *Streptomyces lividans* (Shareck et al., 1995), or the fungi *Aspergillus niger* (de Graaff et al., 1992) and *A. aculeatus* (Christgau et al., 1995a). It has also been reported that these previous characterized acetyl xylan esterases are not similar to each other (Shareck et al., 1995; Ferreira et al., 1993; de Graaff et al., 1992; Christgau et al., 1995a). Only the amino acid sequence of the acetyl xylan esterase of *S. lividans* displays some relation with other protein sequences. It showed some similarity with the rhizobial NodB protein and with the chitin diacetylase (Cda) of the fungus *Mucor rouxii*, both of which are metabolic enzymes involved in the liberation of acetyl groups (Section 1.3.1; Shareck et al., 1995). An explanation for the variability of these enzymes could be the great diversity of ester bonds occurring in nature and which are cleaved by different esterases. Probably acetyl xylan esterases appeared later in evolution and were originated from different ancestral esterases adapted to the hydrolysis of acetic acid esters from polysaccharides.

Interestingly, the *T. reesei* AXEI showed some amino acid similarity with fungal cutinases, which are serine esterases hydrolysing cutin. Cutinases contain a classical catalytic triad Ser-Asp-His, found in all serine hydrolases (Köller and Kolattukudy, 1982). Although the overall similarity of AXEI with the cutinases was low (about 10 %), the protein alignment showed that the residues of the catalytic triad appeared well conserved in AXEI (see II/Fig. 2). The highest identity was found in the amino acids surrounding the active site serine, Ser-121. These residues match the consensus sequence (Gly-His/Tyr-Ser-X-Gly) found around the active site serine of cutinases as well as lipases of prokaryotic and eukaryotic origin (Brady et al., 1990; Winkler et al., 1990; Martinez et al., 1992). Many proteinases also have a similar catalytic center. However, AXEI showed no other evident similarities with any protease (II/Discussion). Another indication that AXEI is more closely related to cutinases than to other esterases is the fact that four cysteines appear in well conserved positions in the region of similarities with cutinases (II/Fig. 2). It has been determined that these four cysteine residues form two disulphide bridges critical for the catalytic activity of cutinases, and that reduction of the bridges results in complete inactivation of the enzyme (Ettinger et al., 1987). To test whether AXEI has a serine involved in the active site, the purified AXE (pI 7.0 and pI 6.8) was incubated with the strong serine enzymes inhibitor PMSF. Incubation with 5 mM PMSF resulted in reduction to 2 % of the original AXE activity (II). This strongly suggests that AXEI is a serine esterase.

Another interesting feature is that the C-terminus of AXEI shows strong similarity with fungal cellulose binding domains (CBD), for instance those present in the cellulases and a β-mannanase of *T. reesei* (Fig. 2). The AXEI CBD is preceded by a region rich in Ser, Thr, Pro and Gly, similar to the flexible and highly *O*-glycosylated linker regions separating the catalytic domains of cellulases from the CBDs (van Tilbeurgh et al., 1986; Tomme et al., 1988; Gilkes et al., 1991). The AXEI CBD is highly conserved and it contains all the amino acids typical for

other *T. reesei* CBDs, especially those found in the surface that binds cellulose (II/Results; Reinikainen et al., 1992; Linder et al., 1995a). The single divergence of AXEI CBD from the other *T. reesei* CBDs is a substitution of the conserved Asn295 by a Ser (Fig. 2). This Asn is strictly conserved in the other CBDs and could also be involved in cellulose attachment mediated by hydrogen bonding (Linder et al., 1995a).

The capability of AXE to bind to cellulose has been demonstrated (Tenkanen et al., 1995b). However, it was not clear whether the binding was mediated by a CBD. To test this, the AXE CBD was separated from the catalytic core by partial hydrolysis with papain (II/Results). Cellulose binding studies with the native enzyme and the purified catalytic core showed that the enzyme cannot bind cellulose without the CBD (II/Fig 4). On the other hand, the native enzyme bound cellulose in a similar manner to the *T. reesei* cellulases. It seems that the cellulose binding capacity of the AXEI CBD is not greatly affected by the replacement of Asn295 by Ser (Fig. 2). It has been suggested that this Asn is not critical for cellulose binding of CBHI CBD since its substitution by Ala reduces the binding capacity only moderately (Linder et al., 1995a).

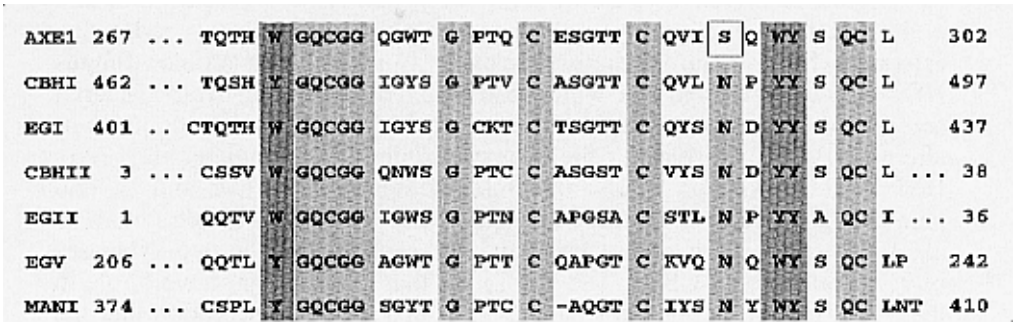

Figure 2. Alignment of the CBD of the T. reesei AXEI with the CBDs of the T. reesei cellulases CBHI (Shoemaker et al., 1983), CBHII (Teeri et al., 1987), EGI (Penttilä et al., 1987), EGII (Saloheimo et al., 1988), EGV (Saloheimo et al., 1994), and of the β-mannanase MANI (Stålbrand et al., 1995). Alignment was performed using the CLUSTAR program version 1.20. A one-letter amino acid code is used. Identical amino acids are shaded in light grey. The three tryptophan and tyrosine residues that interact with the substrate in the CBHI CBD are shaded dark grey. The serine (S) in the AXEI CBD differing from the other CBDs is shown in an open box. Hyphens indicate gaps and dots indicate continuation of the sequence.

Other amino acids in the AXEI CBD, such as the Trp271 and Trp297 (Fig. 2), might increase its affinity for cellulose. It has been shown that the EGI CBD has higher affinity for crystalline cellulose than the CBHI CBD and that the difference is probably caused by a Trp at position 406 in the EGI CBD instead of a Tyr (Fig.

2; Linder et al., 1995b). However, whether the presence of the Trp residues in AXEI CBD can compensate for the possible binding reduction effect of the substitution of Asn by a Ser has still to be tested.

An additional indication that AXEI has an similar organization similar to that of cellulases, with two separate functional domains, was the capability of AXEI core to release acetic acid from xylan to similar extent as the intact enzyme (II/Table 1). This also suggested that there is no apparent need for the CBD for the enzyme to act against polymeric substrates. The importance of CBDs for cellulases has been clearly demonstrated. The capability of truncated forms of the *T. reesei* cellulases CBHI and CBHII, lacking the CBD, to break down crystalline cellulose is severely impaired (Tomme et al., 1988). However, little is known about the mechanism by which CBDs function and whether they simply anchor the enzyme onto the substrate or have a more active role in disrupting the crystalline structure of cellulose.

The presence of cellulose binding motifs seems to be common in plant polysaccharide hydrolysing enzymes which do not hydrolyse cellulose. They have been reported for many bacterial hemicellulases (Tables 2, 3, 5, 6). Fungal hemicellulases carrying CBDs of fungal type have been reported in two xylanases of *Humicola insolens* (Dalbøge and Heldt-Hansen, 1994) and a β-mannanase of *T. reesei* (Stålbrand et al., 1995). Two acetyl xylan esterases have been recently isolated from the anaerobic fungus *Neocallimastix patricianum* which carry domains different from bacterial and fungal CBDs, which may represent novel cellulose binding domains or alternatively be involved in cellulosome assembly (Dalrymple et al., 1996). However, the role of CBDs in hemicellulases is not clear and several hemicellulases such as the acetyl xylan esterases of *A. niger* (de Graaff et al., 1994) and *A. aculeatus* (Christgau et al., 1995a) and the two xylanases of *T. reesei* have no CBDs. The possibility that CBDs might have a role in the hydrolysis of natural substrates is indicated by the observation that the bleaching enhancing role of the *T. reesei* mannanase is decreased when the CBD is removed (Tenkanen, personal communication). The presence of CBDs could be advantageous for the hemicellulases acting in natural conditions, since hemicelluloses are closely associated with cellulose in plant tissues (Timell, 1967) and the CBDs could aid in bringing the enzyme closer to its substrate.

2.2 CLONING OF HEMICELLULASE GENES BY EXPRESSION IN YEAST

One aim of this work was to isolate all possible *T. reesei* genes encoding α-L-arabinofuranosidase and α-galactosidase activities. The major α-L-arabinofuranosidase of *T. reesei* had been previously purified and characterized (Poutanen, 1988b). However, on the basis of fact that other fungi such as *A. niger* and *A. terreus* produce multiple α-L-arabinofuranosidases (Section 1.3.3; Table 3), it could be possible that *T. reesei* also produces additional enzymes, as in the

case of α-galactosidases. It has been suggested that *T. reesei* produces other α-galactosidases in addition to the one purified and characterized (Zeilinger et al., 1993) and other fungi such as *A. tamarii* and *A. niger* also produce more than one α-galactosidase (Section 1.3.4; Table 4).

Screening a λ phage expression library with specific antibodies was a good choice for a rapid identification of the *glr1* and *axe1* genes. However, isolation of genes encoding still unknown α-L-arabinofuranosidases and α-galactosidases using a similar method would have required identification, purification and characterization of the enzymes, and preparation of antibodies. Such a procedure would have been laborious and time consuming. Identification of the genes on the basis of their expression and activity detection was considered a better option. Unlike bacterial enzymes the fungal enzymes are not necessarily produced in active form in *E. coli*, and rapid cloning methods based on expression and activity screening in *E. coli* are not feasible. A methodology to clone hydrolase genes based on their expression in the yeast *Saccharomyces cerevisiae* has previously been developed in our laboratory (Saloheimo et al., 1994). This was based on earlier studies which had shown that several *T. reesei* cellulases were secreted in active form by yeast and in amounts which allowed detection of hydrolysis halos on plate assays (Penttilä et al., 1987, 1988). The method is simple and consists of the construction of a cDNA library into a yeast expression vector containing the strong phosphoglycerate kinase (*PGK*) promoter. The cDNA library is transformed into yeast and the yeast clones are screened for expression of the desired activity. This method has been used to isolate novel genes encoding the cellulase EGV (Saloheimo et al., 1994) and β-glucanase (Saloheimo and Penttilä, 1993) of *T. reesei* which were previously unknown at the protein and gene level. A similar procedure was used simultaneously by Novo Nordisk A/S to isolate several genes of *A. aculeatus* including genes encoding two rhamnogalacturonases (Kofod et al., 1994), a mannanase (Christgau et al., 1994) and a β-1,4-galactanase (Christgau et al., 1995b). The cloning of two exochitinases of *T. harzianum* using the procedure has also been reported (Draborg et al., 1995). In order to isolate α-L-arabinofuranosidase and α-galactosidase genes of *T. reesei* a yeast cDNA library was constructed using mRNA isolated from *T. reesei* RutC-30 cultivated in medium containing several plant polysaccharides (Section 2.1; III).

2.2.1 Isolation and characterization of an α-L-arabinofuranosidase gene *abf1* and a β-xylosidase gene *bxl1*

The yeast library was cultivated in pools in microtiter plate wells in medium containing the small synthetic substrate PNPA (III/Results). This substrate is very sensitive to most of the α-L-arabinofuranosidases releasing terminal L-arabinose units (Section 1.3.3). The screening of the library resulted in several clones producing α-L-arabinofuranosidase activity. Restriction analysis and partial DNA sequencing of the plasmids recovered from yeast indicated two different kinds of

clones. Each group contained inserts representing the same gene. A clone of each group was chosen for further characterization. On the basis of biochemical analyses (see below) of the enzymes produced in yeast one of the genes, *abf1*, was identified as encoding for an α-arabinofuranosidase. The other gene, *bxl1*, encoded for a β-xylosidase which is also active on PNPA.

The deduced protein sequences encoded by *abf1* and *bxl1* are shown in III/Figs. 1 and 2, respectively. The mature ABF protein has 479 amino acids and a calculated molecular mass of 49.1 kDa (Table 8). This is in good agreement with the 53 kDa molecular mass determined in SDS-PAGE for the α-L-arabinofuranosidase purified from *T. reesei* (Poutanen, 1988b). The N-terminal amino acid sequence determined from the purified enzyme was also found in the deduced ABFI protein sequence (Fig 1/III). This indicated that *abf*1 is most likely the corresponding gene for the previously purified enzyme.

The deduced mature BXL1 protein is 738 amino acids long and has a calculated molecular mass of 80.4 kDa. This value is in approximate agreement with the 100 kDa molecular mass reported for a β-xylosidase previously purified and characterized from *T. reesei* (Poutanen and Puls, 1988). The purified enzyme was subjected to amino acid sequencing and it appeared to have its N-terminus blocked. This is consistent with the fact that the first residue of the predicted mature BXLI is a Gln (III/Fig. 2). The purified enzyme was therefore digested with trypsin and the amino acid sequence of an isolated peptide was determined. The sequence of this peptide was found in the predicted protein sequence of BXLI (III/Fig. 2). This also indicated that BXLI corresponds to the previously purified β-xylosidase. Possibly the difference between the apparent molecular mass of the purified β-xylosidase and the calculated molecular mass of the deduced BXLI is due to glycosylation of the protein. The enzyme contains ten potential N-glycosylation sites (Table 8; III).

The screening of the yeast bank with the substrate PNPA resulted in a single α-L-arabinofuranosidase gene (*abf1*). We cannot exclude the possibility that *T. reesei* would produce additional enzymes with similar activity and expressed at a very low level in the presence of polysaccharides. In this case, screening of a larger number of yeast clones would be needed to isolate the corresponding genes. It is also possible that *T. reesei* produces enzymes hydrolysing arabinofuranose linkages but with different substrate specificities, such as endo-1,5-α-arabinase or (1,4)-β-arabinoxylan arabinofuranosidase activities (Section 1.3.3, Table 3). These enzymes do not act on the substrate PNPA and their corresponding genes were not expected to appear in the screening carried out in the present work. Isolation of such genes by expression and detection of activity would require other types of substrates.

2.2.2 Comparison of ABFI and BXLI with other protein sequences

Several genes encoding L-arabinose releasing enzymes have been isolated (Table 6). On the basis of the similarity of their deduced protein sequences and hydrophobic cluster analyses, some of them have been classified into families 43 or 51 in the general classification of glycosyl hydrolases (Henrissat and Bairoch, 1993; Table 6). Variability between enzymes also exits, and several have not yet been classified because they show no similarity with any other hydrolases. Similarity searches carried out with the protein sequence of ABFI showed no similarity with any of the bacterial α-L-arabinofuranosidases or the *A. niger* α-L-arabinofuranosidase A and endo-1,5-α-L-arabinase A (Table 6). However, ABFI showed more than 70 % identity with the protein sequence of the α-L-arabinofuranosidase B (ABF B) of *A. niger* (III/Fig 1), which has been shown not to be related in amino acid sequence to any other hydrolytic enzyme previously characterized. Now we can suggest that the ABFI of *T. reesei* and the ABF B of *A. niger* form a new family of glycosyl hydrolases.

Similarity searches were also carried out with BXLI. Interestingly, BXLI showed no amino acid similarity with any of the several reported β-xylosidases, classified into the families 39, 43 and 52. However, it showed significant similarity with the protein sequences of the β-glucosidases classified into family 3, which also includes a β-glucosidase (BGLI) previously isolated from *T. reesei* (Barnett et al., 1991). The family 3 is very conserved and consists only of β-glucosidases. The highest similarity of BXLI with the most similar β-glucosidases was found between the amino acids 69-314 and 487-643 (III/Fig. 2). Hydrolysis of glycosidic bonds carried out by glycosidases occurs by acid catalysis that requiring two critical carboxylic residues, one acting as a proton donor and the other as a nucleophile/base (McCarter and Withers, 1994; Davis and Henrissat, 1995). The active site Asp has been determined for the β-glucosidase from *Aspergillus wentii* (Bause and Legler, 1980). On the basis of sequence similarities, this residue has also been suggested to be the active site of the β-glucosidase of *T. reesei* and other organisms (Barnett et al., 1991). In BXLI the residue appears well conserved (Asp311, Fig. 3) and probably could take part in the catalytic activity of the enzyme. The β-glucosidases have a conserved motif (Ala/Ser-Gly-Leu-Asp-Met/Leu), which contains another Asp residue suggested to be the second carboxylic residue involved in the catalytic activity (Barnett et al., 1991) (Fig. 3). This motif, however, does not appear in BXLI and the position of the second possible active site carboxylic residue does not appear evident in the alignment. Nevertheless, the overall similarity of BXLI with the β-glucosidases is high and it could possibly be assigned to the family 3, being the only member with a different enzymatic activity.

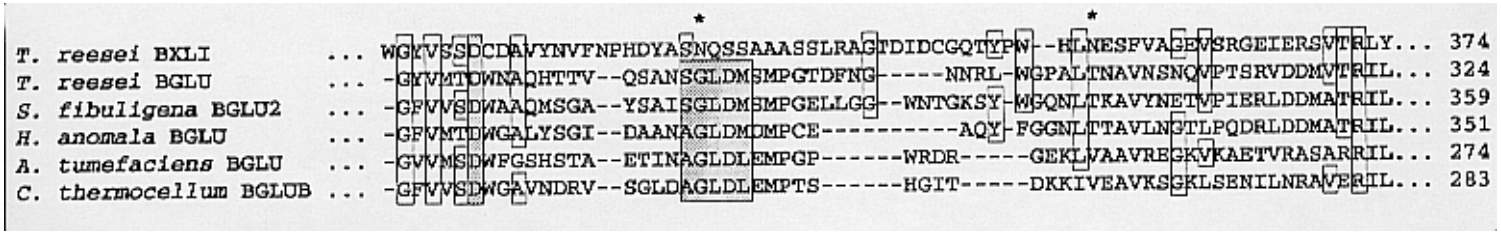

Figure 1

Figure 3. Alignment of the region containing the putative active site of the deduced protein sequence of BXLI with the β-glucosidases of T. reesei, Saccharomyces fibuligera, Candida pelliculosa, Agrobacterium tumefaciens and Clostridium thermocellum. Hyphens indicate gaps. Identical amino acids between BXLI and two or more of the other sequences are boxed. The conserved active site D and the motif (A/S-G-L-D-M/L) in the β-glucosidases are shadowed in grey. The putative N-glycosylation sites in BXLI are shown by asterisks.

2.2.3 Isolation and characterization of the α-galactosidase genes *agl1*, *agl2* and *agl3*

In order to, screen for α-galactosidase-encoding genes, the yeast library was plated on solid medium containing the substrate 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (IV). A number of yeast clones producing blue colour appeared gradually during several days of cultivation (IV/Results). On the basis of restriction analysis and partial DNA sequencing of the plasmids recovered from yeast, three different genes *agl*1, *agl*2 and *agl*3 were found, which appeared in the yeast library with different frequencies (Table 8). The fact that only a single *agl*2 clone was isolated indicates that perhaps not all different mRNA species were included in the yeast clones screened, and that the presence of other α-galactosidase genes expressed at lower level in *T. reesei* cannot be excluded.

The deduced protein sequences encoded by the genes *agl1*, *agl2* and *agl3* are shown in IV/Figs. 1 and 2. The predicted mature AGLI is 417 amino acids long with a calculated molecular mass of 45.7 kDa. This is in good agreement with the deduced 50 kDa molecular mass of an α-galactosidase purified from strain RutC-30 (Zeilinger et al., 1993) and proposed to have very little or no covalently bound carbohydrate. The hydrolytic properties of the yeast-produced AGLI were also comparable to those reported for the purified enzyme (see below), which suggested that *agl*1 is most likely its corresponding gene. On the other hand, *agl2* and *agl3* are genes encoding mature proteins of 720 and 602 amino acids, respectively, which were totally unknown previously. This finding demonstrated that the expression cloning strategy was advantageous for isolating genes encoding desired activities without previous knowledge of the enzymes.

2.2.4 Comparison of AGLI, AGLII and AGLIII with other α-galactosidase sequences

A number of α-galactosidase protein sequences from different origins are known. On the basis of protein similarities they have been classified into three well conserved families in the general classification of glycosyl hydrolases (Henrissat and Bairoch, 1993; Table 7). Computer analyses were carried out with the deduced amino acid sequences of AGLI, AGLII and AGLIII. AGLI and AGLIII showed high similarity with each other and with the enzymes grouped into family 27, which includes only eukaryotic α-galactosidases from plants, animals, yeasts and filamentous fungi. AGLI was more closely related to these sequences than AGLIII (IV/Fig. 1), which showed significant but only low similarity with the other eukaryotic enzymes. AGLIII is also the longest α-galactosidase and carries 230 extra amino acids at the N-terminus which show no similarity with any other protein sequence contained in the data banks. The alignment also indicates that perhaps the extra region in AGLIII is not directly involved in the catalytic activity of the enzyme. The possibility that it could be another functional domain such as a substrate binding domain (see Section 2.1.3) was considered. This kind of domain

structure has been suggested for the α-galactosidase of *A. niger* (AGLA), which has a C-terminal region of 100 residues of unknown function preceded by a linker similar to those found in fungal cellulases (den Herder et al., 1992). However, there are no indications that the extra region in AGLIII would be related to a substrate binding domain of any kind or that it would contain a linker region.

On the other hand, the protein sequence of AGLII was not related to AGLI, AGLIII or any of the α-galactosidases of family 27. Interestingly, it showed high similarity with the bacterial α-galactosidases grouped into family 36 (IV/Fig. 2). Furthermore, the size of AGLII is comparable with those of the bacterial enzymes, showing conserved regions throughout the sequence. AGLII is the first eukaryotic α-galactosidase found to be related to those of bacteria. The high similarity also suggests that AGLII was probably originated from a bacterial α-galactosidase and most likely from a Gram-positive bacterium, as indicated by its lack of relation with the chromosomal MelA of *E. coli* or the *Salmonella typhimurium* α-galactosidase, which are grouped into family 4, and by its similarity with the *E. coli* RafA (see Section 1.4.3; IV/Discussion).

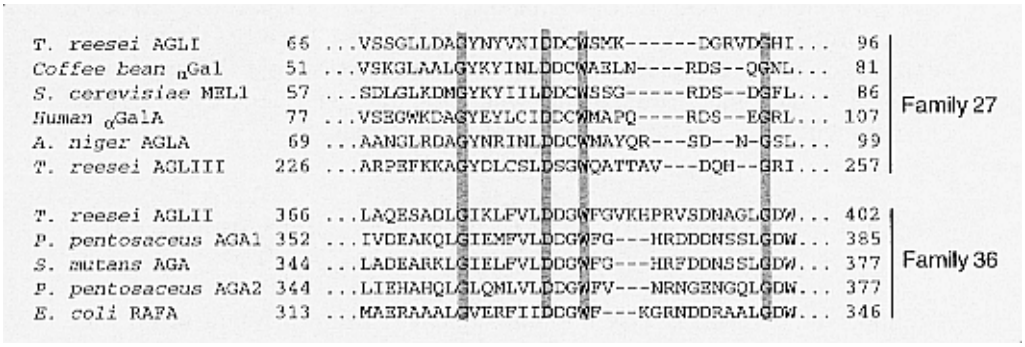

Figure 4. Alignment of the region conserved between members of α-galactosidase families 27 and 36. The sequences belonging to family 27 and 36 chosen for the analysis are those showing higher similarities to AGLI and AGLIII (Fig. 1/IV), and AGLII (Fig. 2/IV), respectively. Strictly conserved amino acids are shadowed in grey. Hyphen indicates gaps and dots indicate continuation of the sequence.

It has recently been suggested that the α-galactosidases of families 27 and 36 originated from a common ancestor and that they form two subclusters in a single superfamily (Dagnall et al., 1995; Henrissat & Romeu, 1995). There are clear divergences between the α-galactosidases of these two families, but multiple sequence alignments have revealed a conserved region between them (Fig. 4). The most conserved amino acids in the region are two glycines, a tryptophan and an

aspartic acid. Basic and acidic groups have been suggested to be present in the active site of α-galactosidases, for example two carboxylic groups have been identified by chemical modification of the α-galactosidase of coconut (Mathew and Balasubramaniam, 1987). It was also suggested that at or near the active site of the enzyme there is a tyrosine and a tryptophan, probably involved in the binding of the substrate to the active site. It is likely that the conserved region has functional significance for the α-galactosidases of both families 27 and 36. It is tempting to suggest that perhaps this region is part of the active site, based on the fact that all α-galactosidases catalyse the hydrolysis of α-galactosyl moieties and that a similar catalytic mechanism can be expected to take place involving analogous amino acids.

Several amino acids in eukaryotic α-galactosidases have been found to be important for the catalytic activity and they are not included in the conserved region shown in Fig. 4. Most of these amino acids are carboxylic or aromatic residues and many of them are well conserved in the sequences of AGLI, AGLIII, and other α-galactosidases (IV/Fig. 1). On the basis of linear protein alignments is not possible to know whether all these residues are directly involved in the active site of the enzymes or whether their replacements provoke structural changes which inactivate the enzymes. The crystallisation and preliminary X-ray analysis of human α-galactosidase A have been recently reported (Murali et al., 1994). Solving of the three-dimensional structure might answer these questions.

2.3 HYDROLYTIC PROPERTIES OF THE *T. REESEI* ENZYMES PRODUCED IN YEAST

Determination of the activities against both small synthetic and natural polymeric substrates of the enzymes encoded by the genes cloned by expression in yeast was an important part of this work in order to compare them with the different enzymes published before and to further evaluate their biotechnical potential.

S. cerevisiae does not itself produce many extracellular hydrolases and especially not those hydrolysing polymeric substrates, except e. g. a β-1,3-glucanase. This is an advantage since it allows evaluation of the properties of the enzymes encoded by the genes cloned with no interference from background activities and without the necessity for further purification of the enzymes. Although the expected production levels were not high, previous work (Penttilä et al., 1987, 1988) suggested that the amounts produced would be sufficient for enzyme analysis.

In order to produce ABFI, BXLI, AGLI, AGLII and AGLIII, the yeast strains transformed with the expression vectors containing their corresponding genes were cultivated in a fermentor. The yeast strain transformed with the vector pAJ401 was also included as control (III and IV). The amounts of enzymes

secreted by yeast were rather low and it was necessary to concentrate the growth medium. Due to the lack of specific antibodies against the enzymes, the amounts produced could not be well estimated. However, assuming the same specific activity against PNPA and PNPG for the yeast-produced ABFI and AGLI, respectively, as previously determined for the purified enzymes, it could be estimated that the levels produced varied between 3 and 30 mg/l.

2.3.1 Hydrolytic properties of ABFI and BXLI

The yeast-produced ABFI showed mainly α-L-arabinofuranosidase activity and some β-xylosidase activity against p-nitrophenyl derivative substrates (Table 9). No other significant activities were detected. These results were in accordance with those obtained with the α-L-arabinofuranosidase purified from *T. reesei* (Poutanen, 1988b), which showed significant β-xylosidase activity towards PNPX at a level of 1-2 % of the α-L-arabinofuranosidase activity towards PNPA. This dual activity against synthetic model substrates has been reported for a number of α-L-arabinosidases and β-xylosidases of different origin. However, the true activity of ABFI was observed when tested against natural polymeric substrates. The enzyme was able to liberate only L-arabinose from arabinoxylans of different sources (III/Table 2). No other mono- or oligosaccharides were released as determined by HPLC (data not shown). Similar results have been reported for the α-L-arabinofuranosidase purified from *T. reesei*. This enzyme was included in some of the hydrolysis experiments and the amounts of L-arabinose released were comparable with the amounts released by the enzyme produced in yeast (III/Fig. 3). The amounts of L-arabinose released by ABFI were not affected significantly when *T. reesei* xylanase was added into the reaction (III/Table 2). This could be a consequence of the high dosage of ABFI used. The synergistic effect of xylanase in the action of α-L-arabinofuranosidase of *T. reesei* was observed using a 10-fold lower enzyme dosage (Poutanen, 1988b).

The main activity of BXLI was β-xylosidase, but it also showed significant α-L-arabinofuranosidase activity towards PNPA (Table 9). Similar results have been reported for the β-xylosidase previously purified from *T. reesei* (Poutanen and Puls, 1988). The α-L-arabinofuranosidase activity of this enzyme was 70 % of the β-xylosidase activity. BXLI also showed clear α-L-arabinopyranosidase activity which was higher than the α-L-arabinofuranosidase activity. This preference for L-arabinose in pyranoside form was as expected, since xylose appears in this form. On the other hand, BXLI did not liberate L-arabinose from any of the arabinoxylans tested, even when used together with the *T. reesei* endoxylanase, although it did hydrolyse xylobiose (result not shown). BXLI was also able to slowly release xylose from polymeric xylan (III/Fig. 4). These hydrolytic properties of the yeast-produced BLXI are similar to those of the β-xylosidase purified from *T. reesei*, which hydrolyses xylose from xylo-oligosaccharides of different length (Poutanen and Puls, 1988) and also from polymeric xylan (Herrmann et al., 1995).

Table 9. Enzymatic activities of yeast-produced ABFI, BXLI, AGLI, AGLII and AGLIII. The activities produced by the host yeast strain are shown as a control. Abbreviations of activities in ml of 50-fold concentrated yeast culture media, measured as described in Activity assays (IV) are the following: αGal, α-galactosidase; αAraf, α-L-arabinofuranosidase; αArap, α-L-arabinopyranosidase; βArap, β-L-arabinopyranosidase; βXyl, β-xylosidase; βGlu, β-glucosidase; βGal, β-galactosidase; βMan, β-mannosidase.

Enzymes	Activity (nkat/ml)							
	αGal	αAraf	αArap	βArap	βXyl	βGlu	βGal	βMan
Control	0.0	0.0	0.0	0.00	2.5	6.5	0.3	0.0
ABFI	0.5	171.1	0.0	0.01	7.1	6.6	0.2	0.2
BXLI	0.0	2.4	4.0	0.03	16.3	5.2	0.2	0.0
AGLI	430.0	0.0	0.0	20.00	2.1	6.4	0.2	0.0
AGLII	17.3	0.0	0.0	0.03	2.1	6.5	0.1	0.0
AGLIII	1.1	0.0	0.0	0.02	1.9	6.1	0.1	0.0

2.3.2 Hydrolytic properties of AGLI, AGLII and AGLIII

The three yeast-produced enzymes showed mainly α-galactosidase activity against PNPG (Table 9). The enzymes also produced detectable β-arabinopyranosidase activity against p-nitrophenyl-β-L-arabinopyranoside, especially in the case of AGLI, at a level of approximately 5 % of the α-galactosidase activity (Table 9). The similarity between the stereochemical structures of α-D-galactopyranoside and β-L-arabinopyranoside could be an explanation for this activity.

The action of yeast-produced AGLI, AGLII and AGLIII against polymeric galacto(gluco)mannan was also tested. The three enzymes showed different substrate specificities (IV/Fig. 3). All three α-galactosidases released limited amounts of galactose when acting alone. AGLI produced the highest yields, being able to liberate 13 % and 25 % of the galactose side groups from LBG and pinewood galactoglucomannan, respectively, when high amounts of enzyme were used (IV/Figs. 3A and 3C). However, these values are low when compared with the results obtained for example with the α-galactosidases of *A. niger* (Bahl and Agrawal, 1969) and *P. ochrochloron* (Dey et al., 1993), which have been reported

to release 40 % and 85 %, respectively, of the galactose residues in LBG. Addition of *T. reesei* mannanase into the hydrolysis mixture clearly enhanced the action of AGLI. Under these conditions the enzyme could release up to 60 % of the galactose side groups from the polymers, indicating that it prefers oligomeric substrates. The presence of an *A. niger* β-mannosidase had no significant effect on the action of AGLI (IV/Fig. 3A).

AGLII was almost inactive against the intact polymers, but showed some synergism with the mannanase. However, the highest degree of hydrolysis was obtained by addition of the β-mannosidase (IV/Figs. 3B and 3D). The yeast culture medium containing AGLIII showed low activity against PNPG (Table 9). For this reason, the action towards polymeric galactomannan was evaluated in a single experiment using the maximum volume of the enzyme solution admissible in the reaction, which corresponded to a dosage of 600 nkat/g of substrate. AGLIII liberated only 4 % of galactose from the intact galactomannan. This value was comparable with the amounts of galactose liberated by similar dosages (500 nkat/g of substrate) of AGLI and AGLII. The action of AGLIII was also enhanced when mannanase and β-mannosidase were added into the reaction. However, the degree of galactose hydrolysis by AGLIII, 12 %, was clearly lower compared with that obtained with a similar dosage of AGLII (IV/Fig. 3B). The synergy of AGLII and AGLIII with β-mannosidase clearly showed their preference for small oligosaccharides carrying the galactose substitution in the mannose unit at the non-reducing end of the oligosaccharide (α-Gal-1,6-β-Man(-1,4-Man)$_n$).

Hydrolysis of the natural galactose-containing oligosaccharides melibiose and raffinose by the three α-galactosidases showed that AGLI was more efficient towards raffinose than melibiose (IV/Table 2). AGLII and AGLIII were more effective than AGLI and produced similar amounts of galactose from both substrates. Apparently AGLIII is more effective in hydrolysing both melibiose and raffinose. It was later found that AGLIII has low activity towards PNPG (IV/Table 3). This could be the reason for the high degree of hydrolysis by AGLIII in hydrolysis experiments using enzyme dosages based on PNPG activity. The preference of AGLII and AGLIII for galactose substitution at the non-reducing end of oligosaccharides such as melibiose (α-Gal-1,6-Glc) and raffinose (α-Gal-1,6-α-Glc-1,2-β-Fru) confirmed the results obtained with these enzymes in the hydrolysis of galactomannan.

3 CONCLUSIONS AND FUTURE PROSPECTS

The filamentous fungus *T. reesei* secretes a wide array of glycosyl hydrolases potentially useful for various industrial processes. The fungus produces several enzymes degrading the backbone of hemicelluloses and also versatile side-chain cleaving activities which make it more efficient than many other microorganisms in the hydrolysis of plant polysaccharides (Poutanen, 1988a). In this work the isolation and characterization of the *T. reesei* genes encoding four different side-chain activities using two different approaches are described. The genes encoding an α-glucuronidase (*glr1*) and an acetyl xylan esterase (*axe1*) were cloned using the standard method of screening a λ phage cDNA expression library with specific antibodies against the purified enzymes. The genes encoding α-arabinofuranosidase (*abf1*), β-xylosidase (*bxl1*) and three encoding α-galactosidase activities (*agl1, agl2* and *agl3*) were cloned on the basis of their expression in yeast. This second approach proved very convenient in cloning the genes in a single screening step and in further characterization of the corresponding enzymes produced in yeast. The functionality of the method was demonstrated by the fact that *bxl1* encoding an enzyme with only a minor α-L-arabinofuranosidase activity was cloned, and also that all the genes encoding relevant enzymes previously known to exist were obtained. These were the *abf1* and *bxl1* encoding an α-arabinofuranosidase and a β-xylosidase purified from *T. reesei*, respectively. The *agl1* gene encoded a previously known α-galactosidase. On the other hand, the methodology also allowed the isolation of *agl2* and *agl3*, encoding two previously unknown α-galactosidases.

The aim in the screening of the yeast expression bank was the cloning of all possible *T. reesei* genes encoding α-arabinofuranosidase and α-galactosidase activities. Whether the α-arabinofuranosidase and the three α-galactosidase genes hitherto isolated do in fact represent all the genes present in *T. reesei* remains to be seen. Screening of a larger number of yeast transformants of the cDNA library or construction of cDNA banks with mRNA obtained from mycelia of the fungus grown in other inducing conditions could be tried. Preliminary expression studies have indicated that the cloned α-arabinosidase and α-galactosidase genes could be more highly expressed in different conditions than those used to construct the yeast expression library (Margolles-Clark, Ilmén, Penttilä, manuscript in preparation). Enzymes acting preferably against polymeric substrates, not necessarily active against small oligomeric substrates, could be screened by plate assays containing e. g. β-glucan (Saloheimo and Penttilä, 1993; Saloheimo et al., 1994), xylan (Margolles-Clark, unpublished results), mannan (Christgau et al., 1994; Stålbrand et al., 1995) or arabinogalactan (Christgau et al., 1995b).

The set of genes isolated encode enzymes hydrolysing glucuronic acid, acetic acid, arabinose or galactose linkages, which are the most abundant side-chain groups bound to wood hemicelluloses. The availability of these genes, together with the previously isolated genes encoding depolymerizing enzymes, should enable a

better understanding of the molecular biology of *T. reesei* and the biological role of the enzymes in the process of hydrolysis of complex plant polysaccharides, as well as providing valuable tools for the analysis of polymer structure. However, there are still other side-chain releasing enzymes such as acetyl glucomannan, *p*-caumaroyl and feruloyl esterases, which are also needed for the complete hydrolysis of hemicelluloses. Furthermore, *T. reesei* might produce other arabinases, not active on the substrate PNPA, which have not yet been identified and that might play an important role in the hydrolysis of arabinose-containing polysaccharides.

A rather complete array of genes encoding plant polysaccharide hydrolysing enzymes is however now available from *T. reesei* including two cellobiohydrolases (Shoemaker et al., 1983; Teeri et al., 1987), four endoglucanases (Penttilä et al., 1986; Saloheimo et al., 1988; Ward et al., 1993; Saloheimo et al., 1994), one β-glucosidase (Barnett et al., 1991), two xylanases (Törrönen et al., 1992), a mannanase (Stålbrand et al., 1995), a β-glucanase (Saloheimo and Penttilä, 1993) and the seven genes isolated in this work. This will allow the possible common and separate regulatory patterns of the genes to be analysed and should provide insight into the mechanism by which the fungus regulates the production of optimal enzyme mixtures in varying environmental conditions in order to provide nutrients for its growth. Preliminary analyses at mRNA level (Margolles-Clark, Ilmén, Penttilä, manuscript in preparation) indicate that the genes isolated in this work are repressed by glucose and induced by sophorose to different extents, as in the case of the different *T. reesei* cellulases. More specific regulatory mechanisms also seem to exist, for example *bxl1* and *glr1* are particularly well expressed on media containing xylobiose, *agl1*, *agl2* and *axe1* on galactose-containing media, and *bxl1* and *abf1* on arabitol. Regulatory studies might provide useful information concerning the culture conditions which would lead to a certain desired mixture of desired activities.

In order to obtain enzyme mixtures enriched in side-chain cleaving activities or higher amounts of these enzymes for large scale application studies, overexpression in *T. reesei* is essential. Their natural production levels are in all cases low. High levels of expression can be obtained by using the strong *cbh1* promoter. Production in host strains lacking the main chain cleaving activities might be especially useful in applications in which the structure of the polymer needs to remain intact. Alternatively, since most of the hydrolases are repressed by glucose, production from a promoter functional on glucose-based medium (Nakari-Setälä and Penttilä, 1995) would provide a rather pure enzyme preparation. As shown in this work, and also previously (Penttilä et al., 1987, 1988; Stålbrand et al., 1995), extracellular enzymes of *T. reesei* can be produced in *S. cerevisiae* in enzymaticaly active form with unaltered substrate specificities. However, many of the enzymes previously characterized have been overglycosylated in *S. cerevisiae* when compared to the native *T. reesei*-produced

enzymes (Penttilä 1987, 1988; Stålbrand et al., 1995). This might severely affect for instance crystallization of the enzymes and for structure-function studies production in *T. reesei* is needed.

Knowledge of the primary structures of the enzymes and comparisons with other known protein sequences helped to reveal some relevant features of the enzymes. GLRI, however, showed no relation ship with any other hydrolase. Although GLRI is the first α-glucuronidase hitherto characterized at the molecular level, it can be anticipated that α-glucuronidases represent a new type of glycosyl hydrolases with a completely different structure from those previously described. The acetyl xylan esterase, AXEI, has an interesting organization. It contains a catalytic domain with a serine active site that, despite the low overall similarity, showed some conserved regions with fungal cutinases. The tertiary structure of the cutinase from *Fusarium solani* has been determined and has a high overall similarity to the structures of other serine esterases such as lipases, although their similarity in amino acid sequence is low (Martinez et al., 1992). The low amino acid similarity of AXEI with the cutinases might be sufficient to allow construction of three-dimensional models of its catalytic domain. This may lead to a better understanding of the structure and function of the enzyme. AXEI also contains a CBD, which on the basis of the preliminary analysis carried out in this study seems not to be needed for the activity of the enzyme against polymeric substrates. A CBD has previously been found also in the β-mannanase of *T. reesei*. Now it appears that CBDs are common in hydrolases of *T. reesei* which are not involved in cellulose degradation. The importance of this domain for hemicellulases is still a puzzle. Functional studies of CBDs would be needed in order to understand their role for these enzymes.

The glycosyl hydrolases have been classified into different families on the basis of comparisons of their amino acid sequences and hydrophobic cluster analyses. It was found that the β-xylosidase, BXLI, is more closely related to the β-glucosidases grouped into family 3 than to the β-xylosidases grouped into families 39, 43 and 52. The α-arabinofuranosidase, ABFI, is similar to the α-arabinofuranosidase B, ABFB, of *A. niger* which has not been classified before, and these two can now represent a new glycosyl hydrolase family. The α-galactosidases AGLI and AGLIII were classified into the family 27 and the AGLII into the family 36. It is expected that the enzymes grouped into a certain family have a common evolutionary background and a conserved overall structure, although in some cases the amino acid similarities are low. Very often, however, it can be assumed that rather accurate structural models of a protein could be constructed on the basis of the known structural data of another protein of the same family. This might facilitate structural and functional studies. There are no structural data available for any of the enzymes grouped into families 3, 27 or 36 that would allow structure modelling of BXLI, AGLI, AGLII or AGLIII. In the case of the α-galactosidases AGLI and AGLIII, the protein alignments revealed significant similarities between these two enzymes and with other sequences, and some conserved amino acids which are probably important for their catalytic

activity could be pointed out. However, AGLI and AGLIII have clear differences in substrate specificity. Corresponding differences have been found between other α-galactosidases of the same family, although the protein sequences are highly similar (more than 70 %). Thus, determination of the tertiary structures of the proteins will be needed in order to create precise data which would allow understanding of the structure-function relationships of the enzymes.

With the exception of AGLII and AGLIII, all the enzymes encoded by the genes isolated in this work are capable of releasing side-chain groups from natural polymeric hemicelluloses. AGLII and AGLIII were not able to release galactose from intact galacto(gluco)mannan and might not be suitable for applications involving partial modification of hemicelluloses. However, they could be useful for other purposes such as for example in the more complete hydrolysis of hemicelluloses or in the processes in which hydrolysis of melibiose and raffinose is required. Above all, the applicability of hemicellulases in general have not been fully investigated. The availability of their corresponding genes will facilitate high production levels of the individual enzymes, which could enable better evaluation of their potentialities in various applications in carbohydrate modification and analysis.

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