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Fungal α-arabinofuranosidases and α-galactosidases acting on polysaccharides

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Abstract

Various natural polymers, including hemicelluloses and pectic polysaccharides, contain arabinose and galactose residues either as main or side chain components. These side-groups affect the form and the functional properties of the polymers and they can be specifically removed by α-L-arabinofuranosidases and α-galactosidases, respectively. The properties and functions of three *Aspergillus terreus* α-L-arabinofuranosidases and three α-galactosidases of *Penicillium simplicissimum* were studied in this work. In addition, the effects of their action on different fibre matrices were evaluated.

The three *A. terreus* α-L-arabinofuranosidases (αAra), A, B1 and B2, had very similar molecular and functional properties and almost identical N-terminal amino acid sequences. All three enzymes had broad substrate specificity, and they liberated arabinose as the only product from various arabinans, arabinogalactans and arabino(glucurono)xylans. Pectic polymers, including both branched arabinans and arabino-β-1,4-D-galactans, appeared to be the best substrates for all these enzymes. In addition, the enzymes were also capable of releasing arabinose from softwood kraft pulp and from different wheat and rye flour fractions. Simultaneous application with xylanase had only minor effect on the activity. However, the purified enzymes were only poorly capable of removing arabinose residues attached to the internal xylose moieties of short arabinoxylo-oligosaccharides of DP 3-5, and they were unable to hydrolyse arabinose linked to C-2 of non-reducing terminal xylose residues. Feruloyl substituents limited the hydrolysis of arabinoxylan and arabinan oligosaccharides, but only if the feruloyl group was esterified to the terminal non-reducing arabinose. Surprisingly, a model lignin-carbohydrate compound, in which an arabinose residue linked to C-3 of methyl-xylopyranoside was substituted with a bulky dilignol group at O-5, was afficiently degraded by αAra B2. This suggests that the enzyme was able to approach this linkage from the xylose side.

P. simplicissimum produces at least four α-galactosidases (AGL), of which three were characterized in this study. α-Galactosidases AGLI and AGLIII had rather similar molecular properties and N-terminal amino acid sequences, whereas the third enzyme, AGLII, differed from the other two

enzymes in both respects. In addition, both AGLI and AGLIII were inactiviated at rather low substrate (*p*-nitrophenyl-α-D-galactopyranoside) and galactose concentrations, whereas AGLII was highly resistant to both substrate and end product inhibition. The gene encoding the α-galactosidase AGLI was cloned and sequenced. On the basis of the similarities of the complete amino acid sequence with those of some other glycosyl hydrolases, AGLI was classified into the glycosyl hydrolase family 27, which includes several α-galactosidases of both pro- and eukaryotic origins. AGLIII had a similar N-terminal aa sequence to that of AGLI and other members of the family 27, but that of AGLII had no any similarity with any of the published sequences of glycosyl hydrolases. The purified enzymes had clearly different hydrolytic properties. AGLI efficiently removed galactose side groups from polymeric galacto(gluco)mannans even in the absence of backbone-degrading enzymes, but it was also active for various oligomeric substrates. AGLII was highly specific towards the liberation of galactose residues attached to the non-reducing ends of various oligomers, including melibiose, raffinose-family and short galactomanno-oligo-saccharides. AGLIII had inferior activity towards the substrates tested and its action was clearly augmented by the depolymerizing enzymes.

The ability of the purified enzymes to liberate monosaccharides was not restricted to the isolated substrates, as they were also able to act on different fibre matrices. The α-L-arabinofuranosidases were able to release 20–25% of arabinose residues, and α-galactosidase AGLI about 10% and 22% of the galactose residues alone and in combination with mannanase, respectively, from softwood kraft pulp. These amounts are comparable to those released by the polymer backbone-degrading enzymes. Surprisingly, the α-L-arabinofuranosidase treatment decreased the extractability of lignin and the pulp bleachability. In addition, the beneficial effects obtained with xylanase were diminished when the two enzymes were applied simultaneously. The overall effects of α-L-arabinofuranosidase treatment on the other pulp properties were of minor importance.

In wheat bread baking, α-L-arabinofuranosidase had only a slight effect on the dough properties or on the specific volume of the baked loafs. The xylanase and combined treatments resulted in softer and stickier doughs, but beneficial changes, such as improved loaf volume, were observed in the bread appearence. The major effect obtained by α-L-arabinofuranosidase addition was the more homogeneous pore structure and distribution without loss of the volume increase caused by xylanase.

Preface

This work was carried out at VTT Biotechnology and Food Research during the period of 1.7.1993–31.12.1997. I am very grateful to Professor Matti Linko and Research Director, Professor Juha Ahvenainen for providing the excellent working facilities at VTT. I also express my warm thanks to Professor Simo Laakso, Professor Katharina Nordström and Docent Heikki Rosenqvist for their support during my studies. I am grateful to Docent Ilari Suominen and Dr. Richard Fagerström for the critical reading and valuable comments of the thesis.

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List of publications

This work is based on the following publications (Appendices I–VI), which are referred to in the text by their Roman numerals. Additional unpublished data is also presented.

I Luonteri, E., Siika-aho, M., Tenkanen, M. and Viikari, L. 1995. Purification and characterization of three α-arabinosidases from *Aspergillus terreus*. J. Biotechnol. 38, pp. 279–291.

II Luonteri, E., Beldman, G. and Tenkanen, M. 1998. Substrate specificities of *Aspergillus terreus* α-arabinofuranosidases. Carbohydr. Polym. 37, pp. 131–141.

III Luonteri, E., Kroon, P.A., Tenkanen, M., Teleman, A. and Williamson, G. 1998. Activity of *Aspergillus terreus* α-arabinofuranosidase on phenolic-substituted oligosaccharides. J. Biotechnol. In press.

IV Luonteri, E., Alatalo, E., Siika-aho, M., Penttilä, M. and Tenkanen, M. 1998. α-Galactosidases of *Penicillium simplicissimum*; production, purification and characterization of the gene encoding AGLI. Biotechnol. Appl. Biochem. Vol. 28, pp. 179–188.

V Luonteri, E., Tenkanen, M. and Viikari, L. 1998. Substrate specificities of *Penicillium simplicissimum* α-galactosidases. Enzyme Microb. Technol. 22, pp. 192–198.

VI Luonteri, E., Tenkanen, M., Siika-aho, M., Buchert, J. and Viikari, L. 1996. α-Arabinosidases of *Aspergillus terreus* and their potentials in pulp and paper applications. In: Srebotnik, E. and Messner, K. (Eds.) Biotechnology in the pulp and paper industry – Recent advances in applied and fundamental research, Vienna: WUV Universitätsverlag. Pp. 119–122.

The author of the present thesis had the main responsibility for the practical work in all publications, except the cloning of AGLI in publication IV, which was carried out by Edward Alatalo and the NMR analysis in publication III, which was carried out by Anita Teleman.

Contents

Appendices of this publication are not included in the PDF version. Please order the printed version to get the complete publication (http://www.inf.vtt.fi/pdf/publications/1998/)

Abbreviations

αAra	α-L-arabinofuranosidase
aa	amino acid
A_2F	*O*-[-2-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→5)-L-arabinofuranose
A_3F	*O*-α-L-arabinofuranosyl-(1→3)-*O*-[-2-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→5)-L-arabino-furanose
AGL	α-galactosidase
AXH	(1,4)-β-D-arabinoxylan arabinofuranohydrolase
CBD	cellulose binding domain
cDNA	complementary DNA
CinnAE	cinnamoyl esterase
D	chlorine dioxide
Da	Dalton
DP	degree of polymerization
E	alkaline extraction
ECF	elemental chlorine free
EX	xylanase
FAE	ferulic acid esterase
FAX	*O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-D-xylopyranose
FAX_2	*O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose
FPLC	fast protein liquid chromatography
G’	storage modulus
HexA	hexenuronic acid
HPAEC	high-performance anion-exchange chromatography
HPLC	high-performance liquid chromatography
IEF	isoelectric focusing
LBG	locust bean gum
LCC	lignin carbohydrate complex
M_r	molecular mass
MW	molecular weight
mRNA	messenger RNA
NMR	nuclear magnetic resonance
O	oxygen

P	hydrogen peroxide
PAD	pulsed amperometric detection
PAGE	polyacrylamide gel electrophoresis
PAX_2	*O*-[5-*O*-(*trans*-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose
pI	isoelectric point
pNPA	*p*-nitrophenyl-α-L-arabinofuranoside
pNPG	*p*-nitrophenyl-α-D-galactopyranoside
Q	chelation
SDS	sodium dodecyl sulphate
TCF	totally chlorine free
TMP	thermomechanical pulp
XBD	xylan binding domain
Z	ozone
δ	phase angle

1 Introduction

1.1 Plant cell wall polysaccharides

The plant cell wall has two biologically important functions. Firstly, it determines the morphology and to some extent also the function of the cell. Secondly, it forms a limit for the cell and is obviously involved in the regulation of cellular expansion. The chemical and physical structure of the wall varies depending on the plant group and cell type. However, there are some general characteristics that are common to all plant cell walls: they are not chemically homogeneous but are composed of several different substances; and they contain distinct layers. Cell walls contain microfibrillar polysaccharides (cellulose) and matrix polysaccharides, which are divided into hemicelluloses (xylans, mannans, 1,3- and 1,4-β-D-glucans, galactans) and pectins (polyuronic acids, arabinans and galactans), lignin, proteins, encrusting substances (cutin, suberin, inorganic compounds) and water (Aspinall, 1980; Goodwin and Mercer, 1983).

Cellulose is the most common polysaccharide found in the plant kingdom. The unbranched chains of β-1,4-linked D-glucopyranose residues are thousands of units long. The repeating unit in the cellulose molecule is cellobiose. Polymers are organized to form crystalline microfibrils interrupted by regions with relatively low crystallinity (amorphous or paracrystalline cellulose) (Aspinall, 1980; Goodwin and Mercer, 1983).

Hemicelluloses are low-molecular weight polysaccharides, which are closely associated with cellulose. They can be removed from plant cell walls by extraction with aqueous alkali or in some cases with water. Hemicelluloses are composed of two or more different monosaccharide species and are frequently highly branched. Furthermore, structural differences exist within one type of polysaccharide isolated from different plant groups or even from different tissues or wall layers of the same plant (Timell, 1967; Aspinall, 1980; Goodwin and Mercer, 1983). Non-hemicellulosic reserve polysaccharides, with structural elements similar to those of hemicelluloses, are found predominantly in seeds, roots, rhizomes, tubers, bulbs, shoot axes and, to a lesser extent, in leaves. These include glucomannans, galactomannans, xyloglucans and galactans (Meier and Reid, 1981).

Pectins are not only important cell wall matrix polysaccharides, but are also abundantly present in soft tissues of land plants, such as the rinds of citrus fruit (about 30%), sugar beet pulp (25%) and apples (15%). Woody tissues contain only minor amounts of pectins (Aspinall, 1970a; Goodwin and Mercer, 1983). The principal constituent of pectins is D-galacturonic acid, but various proportions of neutral arabinans and galactans also occur.

1.1.1 Arabinans and arabinogalactans

L-Arabinan has been recognized as one of the three pectic homo-polysaccharides in addition to D-galacturonan and D-galactan. Arabinans have been isolated from e.g. sugar beet, peanut, apple, citrus pectins, carrot, cabbage, rape and mustard seeds, the wood of maritime pine (*Pinus pinaster*) and the inner bark of *Rosa glauca* (Aspinall, 1970 a, b; Aspinall, 1980; Joseleau *et al.*, 1977, Voragen *et al.*, 1987; Churms *et al.*, 1983). They are highly branched polysaccharides and consist predominantly of α-L-arabinofuranose residues (Table 1). Some arabinans isolated under strongly basic conditions have contained significant proportions of other sugar residues, such as D-galactopyranose and galacturonic acid, linked to the arabinofuranose residues (Joseleau *et al.*, 1977, Aspinall, 1970a; Aspinall, 1980).

Arabinogalactans have been classified into three types: arabino-1,4-β-D-galactans, arabino-1,3/6-β-D-galactans and a related but distinct group of cell wall glycoproteins containing arabinose and galactose. In all cases, the backbone is composed of D-galactopyranose residues substituted mainly at C-6 by single L-arabinose and/or D-galactose residues or longer branched arabinans or arabinogalacto-oligomers. Different members of the same arabinogalactan group may have considerable structural differences (Clarke *et al.*, 1979; Aspinall, 1980) (Table 1).

Sources of arabino-1,4-β-D-galactans include pectic complexes in e.g. seeds, bulbs and leaves, and coniferous compression wood. Arabino-1,3/6-β-D-galactans have been found in mosses, coniferous woods, gums, saps and exudates of angiosperms, in the seeds, leaves, roots and fruits of higher plant families, and in suspension cultured plant cells. These arabino-galactans are also constituents of many exudate gums, both of angiosperms (e.g. *Acacias*) and of gymnosperms (e.g. genus *Larix*), and of pectic complexes. They are branched polysaccharides, highly water-soluble and form solutions with low viscosity (Clarke *et al.*, 1979). The different members of the genus *Larix* (larch) contain 10–25% of water-soluble arabinogalactan, which is the most complex wood polysaccharide. It occurs in high concentrations especially in the heartwood. In hardwoods and conifers arabinogalactan is present in only very low concentrations. Arabinogalactans isolated from different pines, spruces and from Douglas fir (*Pseudotsuga menziesii*) contain a higher amount of arabinofuranose residues as end groups than larch arabinogalactan (Timell, 1967; Clarke *et al.*, 1979).

Structures containing ferulic acid have been isolated from the neutral side-chains of sugar beet pulp pectin. Ferulic acid was found to be linked to the 1,5-linked α-L-arabinofuranoside residues in the arabinan backbone and to

the main core of arabino-1,4-β-D-galactan with ratios of 1:56 and 1:16, respectively (Colquhoun *et al.*, 1994; Ralet *et al.*, 1994).

Table 1. Compositions of arabinans and arabinogalactans.

Polysaccharide	Components	Linkage		Sources	Reference
		Main-chain	Side-chain		
Arabinan	α-L-Ara*f* α-L-Ara*f*	1,5	 1,3 1,2	*Rosa glauca* arabinan I DP 100 arabinan II DP 34	Aspinall, 1980; Joseleau *et al.*, 1977
Arabino-1,4-β-D-galactan	β-D-Gal*p* α-L-Ara*f*	1,4	1,6 1,5 (1,3) (1,2)	Potato Gal:Ara 6.4 Onion Gal:Ara 9.5 Soy Gal:Ara 1.5 Citrus Gal:Ara 0.2	van de Vis, 1994
Arabino-1,3/6-β-D-galactan	β-D-Gal*p* α-L-Ara*f* β-L-Ara*p*	1,3	1,6 1,5 (1,3) (1,2) 1,5 (1,3) (1,2)	coffee bean Gal:Ara 2.7 stractan Gal:Ara 4.9 larch Gal:Ara 2.6–7.8 MWs 37 000–100 000; 7 500-18 000 pine, spruce, Douglas fir Gal:Ara 7–13 exudate gums Gal:Ara 0.3–12	van de Vis, 1994 Clarke *et al.*, 1979 Timell, 1967 Clarke *et al.*, 1979

1.1.2 Arabino(glucurono)xylans

Arabinose-containing xylans are found mainly as the secondary wall components in gymnosperms and monocotyledonous plants (Aspinall, 1980). They consist of a linear 1,4-β-D-xylopyranose backbone which is variably substituted depending on the origin. Cereal and grass arabinoxylans have a more complex structure than softwood arabinoglucuronoxylan. Although they contain less uronic acids, they are more highly branched with L-arabinofuranosyl sidegroups. Some of the arabinofuranose residues are esterified with ferulic acid and less frequently with *p*-coumaric acid (Mueller-Harvey *et al.*, 1986; Puls and Schuseil, 1993).

Arabinoxylans constitute the major fraction of the cereal cell wall polysaccharides. The starchy endosperm cell walls of cereal grains are mainly composed of arabinoxylans (60–70%), except in the case of barley (~20%) and rice (27–40%) (Fincher and Stone, 1986). The main differences in the chemical structure of the arabinoxylans isolated from various cereals and/or plant tissues occur in the ratio of arabinose to xylose, in the relative

proportions and sequence of the various linkages between these two sugars and in the presence of other substituents (Izydorczyk and Biliaderis, 1995). Compositions of some arabinoxylans isolated from annual plants are presented in Table 2.

Phenolic acid and acetyl substituents are found at intervals on cereal arabinoxylans and they are known to modify to some extent the solubility and interaction between the arabinoxylans and solvents (Fincher and Stone, 1986). According to Rybka *et al.* (1993), the dominant form of phenolic acid in both rye and wheat grain was ferulic acid, although some other phenolic compounds were also detected. Rye grain had a significantly higher ferulic acid content than wheat. In both cereal species about 80% of the alkali-labile ferulic acid was located in the bran. However, according to Vinkx *et al.* (1991), wheat contains about twice as much ferulic acid as rye.

The distribution of substituents along the 1,4-β-D-xylan backbone is not even and several models for cereal arabinoxylans have been proposed (Bengtsson *et al.*, 1992; Gruppen *et al.*, 1993; Viëtor *et al.*, 1994). In highly branched regions single- and double-substituted residues are clustered together and are interrupted by less substituted or unsubstituted regions. It is also possible that two structurally distinct polymers exist.

Molecular weight values reported for cereal arabinoxylans have varied extensively depending on the fraction, source of the sample and the analysis method. Molecular weights ranging from about 2×10^4 to 1.7×10^6 have been obtained by ultracentrifugation and osmometry, whereas extremely high values of up to 5×10^6 were estimated using gel filtration (Fincher and Stone, 1986).

Softwoods contain 10–15% arabino-4-*O*-methylglucuronoxylan. The backbone is substituted by 1,3-linked α-L-arabinofuranose and 1,2-linked 4-*O*-methyl-α-D-glucuronic acid residues with ratios to xylopyranose residues of 1:8–9 and 1:5–6, respectively (Timell, 1967). In comparison to the other softwood arabinoglucuronoxylans, the majority of the arabinofuranose sidegroups in larchwood arabinoxylan are attached to the C-2 of the xylopyranose residues instead of the C-3. In addition, a minor amount of double substituted xylopyranoses exists (Kormelink and Voragen, 1993).

Table 2. Compositions of arabinoxylans isolated from some annual plants.

Source	Extraction	Ara : Xyl	Substitution of Xyl*p* (mol %) [a]			Other components	Reference
			single	s-3 : s-2	d-2,3		
Wheat							
Endosperm	Water	0.45-0.65	24.5–26.6	26.7–32.0	9.3–10.6		Izydorckzyk and Biliaderis, 1993
		0.67-0.82	12.5–13.6	6.7–7.8	22.5–24.2		
		0.85–0.97	12.1–14.3	4.8–4.9	23.7–26.3		
Endosperm	Alkali	0.52–0.56	13.2	8.8	9.5		Gruppen *et al.*, 1992a
Rye							
Grain	Water	0.48	47.2		2.8		Bengtsson and Åman, 1990
	Water	0.56	52.1		5.6		
Flour	Water	0.86					Meuser *et al.*, 1986
Bran	Water	0.79					
Flour	Alkali	0.65					Nilsson *et al.*, 1996
Bran	Alkali	0.54					
Wholemeal	Water	0.55	47	46	3		Vinkx *et al.*, 1995a
		1.09	22	2	41		
		1.42	19	0.3	60		
Wholemeal	Alkali	0.55–0.79	22–27	10–13	9–11		Vinkx *et al.*, 1995b
		1.1	31–35	6–7	25–26		
		0.2	8–11	7–10	3–7		
Barley							Viëtor *et al.*, 1992
Endosperm		0.72	23	1.6	18		
Malt							Viëtor *et al.*, 1992
Endosperm		0.68	25	1.3	16		
Sorghum	Alkali	>1	53–60	3–6	16–17	Uronic acids (10%)	Verbruggen *et al.*, 1995
Corn cobs	Water	0.03	14		2.2	2-O-β-D-Xyl*p*-α-L-Ara*f* - side-chains	Ebringerová *et al.*, 1992

[a] single — sum of single-substituted Xyl*p*, C-3 or C-2;

s-3 : s-2 — ratio of C-3 single-substituted to C-2 single-substituted Xyl*p*;

d-2,3 — double-substituted Xyl*p*, both C-2 and C-3

1.1.3 Galacto(gluco)mannans

The main group of mannose-containing polysaccharides from the cell walls of higher plants are glucomannans and galactoglucomannans, which are the major cell wall components (12–15%) of gymnosperms (Aspinall, 1980). The richest sources of galactomannans are the members of the family *Leguminoseae* (1–38% of seed dry weight). In this family, galactomannan has been located in the seed endosperm, except for *Gymnocladus dioica* (Kentacky coffee bean), in which the polysaccharide lies in the inner side of the seed coat, and *Glycine max*, in which it occurs in the hulls. Galactomannans have also been found in species of *Annonaceae* (custard apple family), *Convolvulaceae* (morning glory family), *Ebenaceae* (ebony family), *Loganiaceae* (Buddleia family) and *Palmae* (palm family) (Dea and Morrison, 1975; Dey, 1978). Compositions of some galacto(gluco)-mannans are presented in Table 3.

Table 3. Compositions of galacto(gluco)mannans.

Polysaccharide	Components	Linkage		Remarks	Reference
		Main-chain	Side-chain		
Galactomannan	β-D-Man*p* α-D-Gal*p*	1,4 (1,3) (1,2)	 1,6 (1,2)	*Medicago sativa* Man:Gal 1.0 *Sophora japonica* Man:Gal 5.3 *Cyamopsis tetragonoloba* (guar) Man:Gal 1.5 *Ceratonia siliqua* L. (locust bean gum) Man:Gal 3.8	Dea and Morrison, 1975; Dey, 1978
Galactogluco-mannan	β-D-Man*p* β-D-Glc*p* α-D-Gal*p* Acetyl	1,4 1,4	 1,6	Softwood Water-soluble Man:Glc:Gal:acetyl 3:1:1:0.24 Alkali-soluble Man:Glc:Gal 3:1:0.1	Timell, 1967

Galactomannan is a reserve polysaccharide which is utilized during germination. The distribution of the side-groups along the backbone has only been studied in a few species, but great differences between polymers with similar D-mannose to D-galactose ratio have been observed. It has been suggested that galactomannans with completely regular or more random structures might exist (Dea and Morrison, 1975). Determination of the molecular weights of galactomannans has been problematic due to

difficulties in preparing true solutions and in the evaluation of the molecular shape. However, the determined molecular weights have ranged from $6x10^3$ to 1.9×10^6, corresponding to DPs of 25–1500, depending on the source of the polysaccharide and the method used (Dea and Morrison, 1975).

Galactoglucomannans are the main group of hemicelluloses in all softwoods. The water-soluble polysaccharide isolated with potassium hydroxide and the alkali-soluble polymer compose 5–10% and 10–15% of wood, respectively. The DPs of both polymers have been reported to be over 150 (Timell, 1967).

1.2 Degradation of hemicelluloses

Hemicelluloses and pectins are complex heteropolysaccharides and a vast variety of synergistically acting main-chain and side-chain cleaving enzymes are needed for their complete hydrolysis. Several enzymes hydrolysing plant cell walls been identified and characterized from both fungal and bacterial sources, as reviewed by e.g. Dekker and Richards (1976), Coughlan *et al.* (1993) and Viikari *et al.* (1993). In addition to the molecular and hydrolytic properties, several amino acid sequences encoding cell wall hydrolases, especially xylan-degrading enzymes, have been determined (Hazlewood and Gilbert, 1993). Some years ago a glycosyl hydrolase classification system based on similarities in amino acid sequences and hydrophobic cluster analysis was proposed by Henrissat (1991). Originally about 350 sequences were grouped into 35 families, but presently the number of hydrolase families has increased to 65, which in total contain almost 1000 sequences (SWISS-PROT Protein Sequence Data Bank, Release 35.0 and updates to February 1998).

The breakdown of the neutral components of pectic polysaccharides, i.e. arabinans, galactans and arabinogalactans, requires the action of several enzymes. Endo-1,5-α-L-arabinanases (EC 3.2.1.99) degrade the backbones of both linear and branched arabinans, but their action can be limited by the high degree of substitution of the substrate. The fungal endo-arabinanases are known to produce arabinobiose and -triose as end-products, whereas arabinose and arabinobiose accumulate in the reaction mixture when bacterial enzyme is used. Exo-α-L-arabinanases probably attack the α-1,5-linked side-chains of beet arabinan exo-wise, releasing predominantly oligosaccharides with three arabinose residues. However, linear arabinan is not degraded. α-L-Arabinofuranosidases (EC 3.2.1.55), which are discussed in greater detail in Section 1.3, release single L-arabinose units from both arabinans and arabinogalactans (McCleary, 1991; Beldman *et al.*, 1993; Beldman *et al.*, 1997).

Endo-acting galactanases form two distinct groups: endo-1,4-β-D-galactanases (EC 3.2.1.89) are specific for 1,4-β-D-galactopyranosyl residues, whereas endo-1,3-β-D-galactanases (EC 3.2.1.90) degrade the backbone of 1,3-linked β-D-galactans and arabinogalactans. They have been reported to produce β-1,4-linked galacto-oligosaccahrides of DP 1-3 and several β-1,3- and β-1,6-linked galacto-oligosaccharides, some of which may contain arabinose, as major hydrolysis products, respectively (Dekker and Richards, 1976; Ward and Moo-Young, 1989). In addition, β-galactosidases (EC 3.2.1.23) hydrolyse β-D-galactobiose, galactotriose and galacto-oligosaccharides by releasing D-galactose from the non-reducing end (Ward and Moo-Young, 1989).

Xylans are the most common hemicelluloses, and thus the enzyme systems needed for their degradation are relatively well known. Endo-1,4-β-D-xylanases (EC 3.2.1.8) randomly attacking the backbone of the β-1,4-linked xylans, have been isolated from various origins including fungi (e.g. A*spergillus* and *Trichoderma*), bacteria and plants. In most of these sources multiple forms of the enzymes are found, with different specificities (Coughlan *et al.*, 1993; Coughlan and Hazlewood, 1993; Viikari *et al.*, 1993). Xylo-oligosaccharides formed by the xylanases are further degraded by exo-1,4-β-D-xylosidases (EC 3.2.1.37), which remove D-xylose residues from the non-reducing end of the substrate. Their activity normally decreases with increasing DP of the substrate (Coughlan *et al.*, 1993).

Depending on the origin of the xylan, different side-group cleaving enzymes are needed. Arabinose side-groups in arabino(glucurono)xylans of softwood and annual plants are removed by α-L-arabinofuranosidases (Section 1.3). α-D-Glucuronidases (EC 3.2.1.131) hydrolyze the α-1,2-glycosidic linkage between D-glucuronic acid or its 4-*O*-methyl ether and the main-chain xylose residue. Several fungi and bacteria are known to produce α-glucuronidase, but only a few of these enzymes have hitherto been characterized (Poutanen *et al.*, 1991; Margolles-Clark, 1996). In addition, esterases (EC 3.1.1) liberating the acetic and phenolic components are required for the complete hydrolysis of xylans from hardwood and annual plants (Christov and Prior, 1993; Tenkanen, 1995).

The backbone of polymeric (gluco)mannans is degraded by endo-β-1,4-D-mannanases (EC 3.2.1.78). The action of mannanases on galacto(gluco)-mannans is dependent on the degree of substitution and the distribution of the substituents along the backbone, as well as on the ratio of glucose to mannose and the physical state of the polysaccharide (McCleary, 1991). Mannanases have been reported to be produced by various species of bacteria, fungi, marine algae, germinating terrestrial plant seeds and invertebrates (Dekker and Richards, 1976; McCleary, 1991; Viikari *et al.*, 1993). β-D-Mannosidases (EC 3.2.1.25) are exo-hydrolases catalyzing the removal of β-1,4-linked D-mannosyl residues from the non-reducing end of

oligosaccharides containing D-mannose (Dey, 1978). They are known to be present in a range of plant and animal tissues and in some microorganisms. However, only a few of these enzymes have been isolated (Dey, 1978; Viikari *et al.*, 1993). α-Galactosidases (EC 3.2.1.22), which liberate terminal, non-reducing α-D-galactose residues, are discussed in detail in Section 1.4. β-Glucosidase (EC 3.2.1.21), releasing 1,4-β-D-glucopyranose residues from the non-reducing end of the substrate, is also required for the hydrolysis of glucomannan.

1.3 α-L-Arabinofuranosidases

1.3.1 Induction of α-L-arabinofuranosidases

The induction of α-arabinofuranosidases has been studied using various carbon sources including monomeric sugars and complex polysaccharides. L-Arabinose has been the best monosaccharide inducer for α-L-arabinofuranosidase production, e.g. in *Aspergillus niger* (van der Veen *et al.*, 1991), *A. nidulans* (Fernández-Espinar *et al.*, 1994) and S*treptomyces olivochromogenes* (Higashi *et al.*, 1983). In the case of the cell-associated α-L-arabinofuranosidase of *Bacteroides ovatus* L-arabinose was required for optimal production, although the production of other glycosidases was generally repressed by simple monosaccharide sugars (Macfarlane *et al.*, 1990). However, the presence of arabinose in the carbon source is not obligatory: e.g. the major xylanolytic ruminal species *Butyrivibrio fibrisolvens* produced α-L-arabinofuranosidase when grown on xylose or different xylans containing essentially no arabinose, whereas only a minor amount of activity was detected when L-arabinose was used (Hespell and Cotta, 1995).

The complex pectin- and arabinan-containing carbon sources, e.g. sugar beet pulp, sugar cane bagasse, apple pulp and beet arabinan, have efficiently induced α-L-arabinofuranosidase production in *A. nidulans* (Ramón *et al.*, 1993; Fernández-Espinar *et al.*, 1994), *A. niger* (van der Veen *et al.*, 1991), various strains of *Streptomyces* (Kaji *et al.*, 1981; Higashi *et al.*, 1983; Johnson *et al.*, 1988) and the phytopathogenic fungus *Sclerotinia sclerotiorum* (Riou *et al.*, 1991). The utilization of lignocellulosic materials, such as wheat bran and Solka floc cellulose, or isolated arabinose-containing xylans, e.g. oat spelt xylan, has also resulted in the production of notable amounts of α-L-arabinofuranosidase by *Aspergillus* strains (Bailey and Poutanen, 1989), yeast *Aureobasidium pullulans* (Myburgh *et al.*, 1991), *Bacillus macerans* (Williams and Withers, 1985) and different S*treptomyces* strains (MacKenzie *et al.*, 1987; Johnson *et al.*, 1988; Zimmerman *et al.*, 1988).

In batch cultures maximal activities of both the cell-associated and extracellular α-L-arabinofuranosidases of *Bacillus macerans* were reached at the beginning of the stationary growth phase (Williams and Withers, 1985). Furthermore, the enzyme production was largely independent of growth rate in continuous culture, although the activities were reduced at higher dilution rates. Similarly, α-L-arabinofuranosidase production by *Bacteroides ovatus* decreased at high growth rates (Macfarlane *et al.*, 1990).

Solid-state fermentation has not been commonly used in the production of hemicellulose-degrading enzymes. *Trichoderma reesei* and a thermophilic fungus *Thermoascus aurantiacus* have been reported to produce extracellular α-L-arabinofuranosidases when grown in solid-state cultures on sugar beet pulp (Roche *et al.*, 1995; Roche *et al.*, 1994). In the case of *Penicillium capsulatum*, the medium was also supplemented with wheat bran (Filho *et al.*, 1996).

1.3.2 Characterization of α-L-arabinofuranosidases

Most of the fungal α-L-arabinofuranosidases have been isolated from different strains of *Aspergillus*, especially *A. niger* (Table 4). The molecular masses of these arabinose-hydrolysing enzymes range from 32 kDa to 128 kDa. All *Aspergillus* α-L-arabinofuranosidases, except the two enzymes isolated from Pectinase 29, were proteins with isoelectric points around 3.5. The optimal conditions for the action of the α-L-arabinofuranosidases were also in the acidic pH range. α-L-Arabinofuranosidase B of *A. niger* N400 (van der Veen *et al.*, 1991) and the enzymes purified from *A. niger* KI (Kaji *et al.*, 1969; Kaji and Tagawa, 1970; Tagawa and Kaji, 1988), Hemicellulase REG2 (Gunata *et al.*, 1990), the intracellular enzyme from strain 5–16 (Kaneko *et al.*, 1993) and an enzyme isolated from *A. nidulans* CECT2663 (Ramón *et al.*, 1993) all had very similar molecular properties. The *A. nidulans* CECT2663 α-L-arabinofuranosidase also cross-reacted with the antibody raised against the α-L-arabinofuranosidase B of *A. niger* N400 (Ramón *et al.*, 1993). On the other hand, the two enzymes B1 and B2 purified from Pectinex Ultra SP produced by *A. aculeatus* reacted with the antibody raised against the α-L-arabinofuranosidase B of *A. niger* from Pectinase 29 (Beldman *et al.*, 1993).

The molecular properties of the α-L-arabinofuranosidases isolated from other fungal strains are rather similar to those of *Aspergillus* enzymes (Table 4). The molecular masses of these enzymes range between 53 and 65 kDa and their isoelectric points from 3.9 to 5.1, except for the slightly basic enzymes produced by *Sclerotinia sclerotiorum* (Baker *et al.*, 1979) and *Trichoderma reesei* (Poutanen, 1988). The optimal pH for the enzymes ranged from 2.5 to 5.0. α-L-Arabinofuranosidase of *Corticium rolfsii* IFO

4878 had an unusually broad pH-stability range from pH 1.5 to 10 (Kaji and Yoshihara, 1971).

Most of the α-L-arabinofuranosidases of actinomycetes and bacteria have been isolated from various strains of *Bacillus* and *Streptomyces* (Table 5). In comparison to the monomeric fungal enzymes, several large multimeric enzymes have been isolated with molecular masses ranging from 110 kDa for the α-L-arabinofuranosidase from *B. stearothermophilus* T-6 (Gilead and Shoham, 1995) up to 495 kDa for the enzyme from *S. purpurascens* IFO 3389 (Komae *et al.*, 1982). Furthermore, the range of isoelectric points has been broader (3.8-9.0) and the optimal reaction pH values more basic (4.0–7.0) compared to those of the fungal α-L-arabinofuranosidases.

Two α-L-arabinofuranosidases have been reported to contain a polymer-binding domain. The *Pseudomonas fluorescens* α-L-arabinofuranosidase (XYLC) has a cellulose-binding domain (CBD) located in the N-terminal portion of the amino acid sequence (Kellett *et al.*, 1990). An α-L-arabinofuranosidase (AbfB) of *Streptomyces lividans* strain 1326 is composed of two distinct domains: a catalytic domain at the N-terminus and a xylan-binding domain at the C-terminus of the protein (Vincent *et al.*, 1997). AbfB resembled the monomeric low molecular mass proteins of *A. awamori* (Kormelink *et al.*, 1991) and *P. fluorescens* (Kellett *et al.*, 1990), which specifically attack arabinoxylans but not other arabinose-containing substrates.

Table 4. Fungal α-L-arabinofuranosidases.

Organism	M_r [a] (kDa)	pI	pH-optimum	Activity against [b]	Remarks	Reference
Aspergillus aculeatus						Beldman *et al.*, 1993
B1	37		3.0–3.5	pNPA, sugar beet arabinan, linear apple arabinan, UFR arabinan		
B2	37		4.0–4.5			
Aspergillus awamori	64-66	3.2	4.6	Sugar beet arabinan, larchwood arabinogalactan, wheat straw arabinoxylan (water-soluble), oat spelt, oat straw, barley straw, rye-grass, Timothy grass and larchwood arabinoxylans	Two isoenzymes Retaining Hydrolysis of ferulic acid - substituted arabinose residues	Wood and McCrae, 1996 Pitson *et al.*, 1996
IMI (CMI) 142717	64-66	3.6				
	32		5.0	Wheat and oat spelts arabinoxylans	(1,4)-β-D-Arabinoxylan arabinofuranohydrolase (AXH) Inverting Specific for arabinoxylans Hydrolysis of α-1,2- and α-1,3-linked single arabino-furanosyl groups.	Kormelink *et al.*, 1991 Kormelink *et al.*, 1993a Pitson *et al.*, 1996
Aspergillus nidulans	36	4.3	5.5	pNPA		Fernández-Espinar *et al.*, 1994
Aspergillus nidulans CECT 2663	65	3.3	4.0	pNPA		Ramón *et al.*, 1993

Aspergillus niger Hemicellulase REG2	61	3.7	3.7–4.0	monoterpenyl arabinofuranosyl-glucosides		Gunata *et al.*, 1990
Aspergillus niger KI	53	3.6	3.8–4.0	pNPA, beet arabinan, 1,5-α-L-arabinan, arabinoxylan, gum arabic	Glycoprotein Amino acid composition Crystallised	Kaji *et al.*, 1969 Kaji and Tagawa, 1970 Tagawa and Kaji, 1988
Aspergillus niger 5-16	67	3.5	4.0	pNPA, A_1X_2, arabinan	Intracellular Amino acid composition Only terminal arabinose residues from arabinoxylo-oligosaccharides	Kaneko *et al.*, 1993
Aspergillus niger						Rombouts *et al.*, 1988
ABFA	128	6.0–6.5	4.1	pNPA, 1,5-α-L-arabino-oligosaccharides UFR arabinan, beet arabinan	Hydrolysis of terminal and non-terminal α-1,3-linked single arabinofuranosyl groups	Pitson *et al.*, 1996 Kormelink *et al.*, 1993a
ABFB	60	5.5–6.0	3.7	pNPA, 1,5-α-L-arabino-oligosaccharides, 1,5-α-L-arabinan, UFR arabinan, beet arabinan, potato and citrus galactans, oat spelt xylan	Hydrolysis of single non-reducing terminal arabino-furanosyl groups Retaining (ABF A, ABF B)	
Aspergillus niger N400 (CBS12049)					Amino acid compositions Genes cloned (*abf*A, *abf*B)	van der Veen *et al.*, 1991 Flipphi *et al.*, 1993a Flipphi *et al.*, 1993b
ABFA	83	3.3	3.4	pNPA		
ABFB	67 50.7 (aa)	3.5	3.8	pNPA, sugar beet arabinan		

Table 4. continued.

Organism	M_r [a] (kDa)	pI	pH-optimum	Activity against [b]	Remarks	Reference
Aspergillus sojae no. 3	34.3	3.9	5.0	pNPA, beet pulp arabinan, rice straw arabinoxylan, soybean arabino-galactan	Amino acid composition	Kimura *et al.*, 1995
Aureobasidium pullulans NRRL Y-12974	210 (gf)		4.0–4.5	pNPA, arabinan, debranched arabinan, oat spelt xylan, wheat and rye arabinoxylans	Dimer: 105 kDa (SDS) Thermostable (half-life 8 h at 75°C)	Saha and Bothast, 1998
Cochliobolus carbonum	62		3.5–4.0	pNPA, sugar-beet arabinan, wheat arabinoxylan, xylobiose, oat spelt, larchwood and birchwood xylans	Apparent mass 33 kDa by gel filtration and 62 kDa by SDS-PAGE	Ransom and Walton, 1997
Dichomitus squalens CBS 432.34	60	5.1	3.5	pNPA, sugar-beet arabinan, wheat straw and oat spelts arabinoxylans	Glycoprotein	Brillouet *et al.*, 1985
Penicillium capsulatum						Filho *et al.*, 1996
I	64.5	4.2	4.0	pNPA, arabinan, wheat straw and oat spelts arabinoxylans, arabinoxylo-oligosaccharides preferred (II)		
II	62.7	4.5	4.0			
Sclerotinia fructigena			4.0	pNPA, arabinan, apple fibre	Partially purified	Fielding and Byrde, 1969
Sclerotinia sclerotiorum	62 64 (uc)	7.5	4.0–4.5	pNPA, arabinan, bean and rice cell walls		Baker *et al.*, 1979

Trichoderma reesei	53 49.1 (aa)	7.5	4.0	pNPA, beet arabinan, wheat, wheat straw, oat spelt and rye arabinoxylans, pine kraft pulp arabinoglucuronoxylan	Gene cloned (*abf1*)	Poutanen, 1988 Margolles-Clark *et al.*, 1996a

[a] determined by SDS unless otherwise indicated; aa molecular mass calculated from amino acid sequence; gf molecular mass determined by gel filtration; uc molecular mass determined by ultracentrifugation

[b] pNPA, p-nitrophenyl-α-L-arabinofuranoside; MUAf, methylumbelliferyl-α-L-arabinofuranoside; MUAp, methylumbelliferyl-α-L-arabinopyranoside; pNPGl p-nitrophenyl-α-D-glucoside; UFR arabinan ultrafiltration retentate arabinan

Table 5. α-L-Arabinofuranosidases from actinomycetes and other bacteria.

Organism	M_r [a] (kDa)	pI	pH-optimum	Activity against [b]	Remarks	Reference
Bacillus polymyxa CECT 153	166 (gf)	4.7	6.5	pNPA, MUAp, 1,5-α-L-arabino-oligosaccharides, arabinoxylo-oligosaccharides	Trimer: 33, 65 and 65 kDa (SDS)	Morales *et al.*, 1995a
Bacillus polymyxa					Genes cloned	Morales *et al.*, 1995b
AF64	64	8.7	6.5	pNPA, oat spelt and wheat flour arabinoxylans	Identical N-terminal amino acid sequences	
AF53	53	9.0	6.5			
Bacillus stearothermophilus L1	110 (gf)		7.0	pNPA, arabinoxylo-oligosaccharides	Dimer: 52.5 and 57.5 kDa (SDS)	Bezalel *et al.*, 1993
Bacillus stearothermophilus T-6	256 (gf)	6.5	5.5–6.0	pNPA, sugar beet arabinan, oat spelt xylan	Tetramer: 64.0 kDa (SDS)	Gilead and Shoham, 1995
Bacillus subtilis 3-6	61		7.0	pNPA, sugar-beet arabinan, A_1X_2, A_1X_3	Amino acid composition Both terminal and stub arabinose residues hydrolysed	Kaneko *et al.*, 1994 Kaneko and Kusakabe, 1995
Bifidobacterium adolescentis DSM 20083	100 (gf)		6.0	Wheat flour arabinoxylan	Inverting Hydrolysis of α-1,3-linked arabinofuranosyl residues from double substituted xylose residues Specific for arabinoxylans	Van Laere *et al.*, 1997 Pitson *et al.*, 1996

Bacteroides xylanolyticus X5-1	364 (gf)		5.5–6.0	pNPA, arabinoxylo-oligosaccharides	Hexamer: 61.0 kDa (SDS) Cell-associated	Schyns *et al.*, 1994
Clostridium acetobutylicum ATCC 824	94	8.15	5.0–5.5	pNPA, pNPGl, arabinan, oligosaccharides from oat spelts arabinoxylans	Terminal, nonreducing α-L-arabinofuranosyl groups	Lee and Forsberg, 1987
Clostridium stercorarium	195 (gf)		5.0	pNPA, oat spelt arabinoxylan, arabinogalactan	Tetramer: 52 kDa Gene cloned Thermostable	Schwarz *et al.*, 1995
Cytophaga xylanolytica XM3 (DSM 6779)	160–210 (gf)	6.1	5.8	MUAf, sugar beet arabinan, rye, wheat, corn cob and oat spelt arabinoxylans	Tri- or tetramer: 56 kDa (SDS) Largely cytoplasmic	Renner and Breznak, 1998
Ruminococcus albus 8	305 (gf)	3.8	6.9	pNPA, arabinose-containing alfalfa cell wall polymers	Tetramer: 75.0 kDa (SDS) Glycoprotein	Greve *et al.*, 1984
Streptomyces sp. no. 17-1	92	4.4	6.0	pNPA, beet arabinan, 1,5-arabinan, arabinoxylan, arabinogalactan		Kaji *et al.*, 1981
Streptomyces diastaticus C1 C2	 38 60	 8.8 8.3	 4–7 4–7	pNPA, oat spelt xylan, debranched sugar beet 1,5-β-arabinan (carboxy-methylarabinan)		Tajana *et al.*, 1992
Streptomyces lividans 66 strain 1326	380 (gf)	4.6	6.0	pNPA, branched and linear beet arabinan, rye and wheat flour arabinoxylans, arabinoxylo-oligo-saccharides	Polymeric: 69 kDa (SDS) Gene cloned (*abf*A)	Manin *et al.*, 1994

Table 5. continued.

Organism	M_r [a] (kDa)	pI	pH-optimum	Activity against [b]	Remarks	Reference
Streptomyces lividans IAF10-164 (*msiK*$^-$)	43 47 (aa)	7.4	6.0	wheat, rye and oat spelt arabinoxylans	Specific xylan-binding domain	Vincent *et al.*, 1997
Streptomyces purpurascens IFO 3389	495 (gf)	3.9	6.5	pNPA, arabino-oligomers,	Octamer: 62.0 kDa (SDS)	Komae *et al.*, 1982
Thermomonospora fusca BD21	92 (gf)			pNPA, oat spelt xylan oligosaccharides	Dimer: 46.0 kDa (SDS)	Bachmann and McCarthy, 1991

[a, b] See Table 4.

1.3.3 Molecular properties of α-L-arabinofuranosidases

Several genes encoding L-arabinose-releasing enzymes have been sequenced, and they have been classified into four distinct glycosyl hydrolase families (Table 6). Members of the family 43 originate from both eukaryotic and prokaryotic sources and are known to invert the anomeric configuration of the substrate (SWISS-PROT Protein Sequence Data Bank, Release 35.0 and updates to February 1998). They are mainly bifunctional β-xylosidases (EC 3.2.1.37) able to hydrolyze terminal non-reducing D-xylose residues, but also α-L-arabinofuranoside residues from α-L-arabinosides. Moreover, two genes encoding endo-acting enzymes, *abnA* from *Aspergillus niger* (Flipphi *et al.*, 1993c) and *arbA* from *Pseudomonas fluorescens* subsp. *cellulosa* (McKie *et al.*, 1997), have been classified into this family. Families 51 and 54 contain genes from both taxonomic ranges and only from eukaryotes, respectively, but all enzymes catalyze only the hydrolysis of L-arabinofuranose residues. The anomeric configuration of the substrate is retained during the reaction with enzymes belonging to these families (SWISS-PROT Protein Sequence Data Bank, Release 35.0 and updates to February 1998). The *A. niger ABF2* nucleotide sequence encoding α-L-arabinofuranosidase 2 protein has not been classified, but it was found to be 94% identical to the sequence of the α-L-arabinofuranosidase B of *A. niger* N400 (Flipphi *et al.*, 1993b) belonging to the family 54 (Crous *et al.*, 1996).

Family 62 consists at the moment only of one gene: *xynC* from *Ps. fluorescens* subsp. *cellulosa* (Kellett *et al.*, 1990). It is composed of a putative catalytic domain in the C-terminal portion and a cellulose binding domain in the N-terminal portion of the sequence. The catalytic domain of *Streptomyces lividans* IAF10-164 AbfB, encoded by an unclassified gene, is highly similar (66%) to XynC of *Ps. fluorescens*; however, their substrate-binding domains are clearly different (Vincent *et al.*, 1997). On the other hand, the N-terminal amino acid sequence of AbfB exhibited extensive homology (63% identity) to the C-terminus of *S. lividans* XlnA, which is the substrate binding domain of the enzyme on xylan (Vincent *et al.*, 1997). On the basis of amino acid sequence similarities, the binding domains of *S. lividans* AbfB and XlnA could not be allocated to any of the known binding-domain families (Dupont *et al.*, 1998). Both were able to bind to insoluble xylan, even in the precense of non-hydrolysing protein, but neither of them adsorbed to Avicel cellulose. The proteins were bound specifically to their natural substrate, and thus they could be considered as a new family of true xylan-binding domains.

Table 6. Genes coding for L-arabinose-releasing enzymes.

Organism	Gene	Signal sequence	Mature protein	M_r kDa	Hydrolase family	Remarks	Reference
Aspergillus niger	*abnA* [a]	19 aa	302 aa	34.454	43	1 N-glycosylation site	Flipphi *et al.*, 1993c
Pseudomonas fluorescens subs. *cellulosa*	*arbA* [a]	31 aa	316 aa	39.438 (- signal seq.)	43	2 N-glycosylation sites	McKie *et al.*, 1997
Clostridium stercorarium	*xylA*		473 aa	53.340	43	1 N-glycosylation site	Sakka *et al.*, 1993
Bacteroides ovatus			325 aa	37.245	43		Whitehead, 1995
Butyrivibrio fibrisolvens	*xylB*		517 aa	58.222 or 62.040	43	2 N-glycosylation sites	Utt *et al.*, 1991
Bacillus polymyxa	*XynD*	26 aa	609 aa	67.8	43	8 N-glycosylation sites	Gosalbes *et al.*, 1991
Aspergillus niger	*abfA*	25 aa	603 aa	65.4	51	10 N-glycosylation sites	Flipphi *et al.*, 1994
Bacteroides ovatus	*asdII*		514 aa	57.979	51		SWISS-PROT Q59219
Bacillus subtilis			495 aa	56.509	51		SWISS-PROT P94552
Bacillus subtilis			500 aa	57.060	51		Wipat *et al.*, 1996 Sa-Nogueira *et al.*, 1997

Clostridium stercorarium	*arfB*		491 aa	56.163	51		Zverlov *et al.*, 1998
Streptomyces lividans 66 strain 1326	*abfA*		662 aa	72.492	51	3 N-glycosylation sites	Manin *et al.*, 1994
Cytophaga xylanolytica	*arfI*		509 aa	57.7	n.c. (51)[b]		Kim *et al.*, 1998
	arfII	48 aa	486 aa	59.2	n.c. (51)[b]		
Aspergillus niger	*abfB*	18 aa	481 aa	50.7	54	2 N-glycosylation sites	Flipphi *et al.*, 1993b
Trichoderma reesei	*abf1*	21 aa	479 aa	49.1	54	1 N-glycosylation site	Margolles-Clark *et al*., 1996a
Trichoderma koningii	*xyl1*	21 aa	479 aa	51.129	54	1 N-glycosylation site	SWISS-PROT P48792
Aspergillus niger MRC11624	*ABF2*	18 aa	481 aa	52.6	n.c. (54) [b]	1 N-glycosylation site	Crous *et al.*, 1996
Pseudomonas fluorescens subs. *cellulose*	*xynC*	37 aa	534 aa	59.0	62	Cellulose binding domain	Kellett *et al*., 1990
Streptomyces lividans IAF10-164	*abfB*	37 aa	441 aa	50.826 47.051 (mature)	n.c.	2 distinct domains	Vincent *et al.*, 1997

[a] encoding endo- and/or exo-acting enzyme

[b] n.c. not classified

1.3.4 Substrate specificities of α-L-arabinofuranosidases

Arabinan-degrading enzymes can be classified on the basis of their mode of action into exo-acting α-L-arabinanofuranoside arabinofuranohydrolases (EC 3.2.1.55), also called α-arabinofuranosidases or α-arabinosidases, and endo-acting 1→5-α-L-arabinan 1→5-α-L-arabinanohydrolases (EC 3.2.1.99), more commonly called endo-1,5-α-L-arabinanases. Kaji (1984) subdivided the α-L-arabinofuranosidases into two groups on the basis of the source and the substrate specificity of the enzyme. *Aspergillus niger* type α-L-arabinofuranosidases are active on side-chain L-arabinosyl residues of L-arabinan, L-arabinoxylan and L-arabinogalactan, but also hydrolyse simple synthetic substrates. *Streptomyces purpurascens* type α-L-arabino-furanosidases act only on low-molecular weight substrates, such as *p*-nitrophenyl-α-L-arabinofuranoside (pNPA) and L-arabino-oligosaccharides, and are inactive towards polymers. Beldman *et al.* (1997) based the classification of arabinan-degrading enzymes not only on the substrate specificity but also on the mode of action of the enzyme (Table 7). The six groups were α-L-arabinofuranosidases, which are not active towards polymers (EC 3.2.1.55), α-L-arabinofuranosidases active towards polymers, α-L-arabinofuranohydrolases specific for arabinoxylans, exo-α-L-arabinanases, which are not active on pNPA, β-L-arabinopyranosidases and endo-1→5-α-L-arabinanases (EC 3.2.1.99). The enzymes belonging to the three latter groups are not discussed in this work (review: Beldman *et al.*, 1997). Data about the substrate specificities of various fungal and bacterial α-L-arabinofuranosidases are presented in Tables 4 and 5.

Aspergillus awamori has been reported to produce two α-arabino-furanosidases and a (1,4)-β-D-arabinoxylan-arabinofuranohydrolase (AXH) with novel specific hydrolytic properties (Wood and McCrae, 1996; Kormelink *et al.*, 1991). The α-L-arabinofuranosidases were able to release arabinose from polymeric beet arabinan, larchwood arabinogalactan and arabinoxylan and from several cereal arabinoxylans. In addition, they were able to release ferulic or *p*-coumaric acid substituted arabinofuranose residues (Wood and McCrae, 1996). AXH was highly specific towards arabinoxylans and did not show any activity towards pNPA or other arabinose-containing polysaccharides (Kormelink *et al.*, 1991). However, all three enzymes acted synergistically with xylanase (Wood and McCrae, 1996; Kormelink *et al.*, 1991).

Table 7. Classification of arabinan-degrading enzymes according to Beldman et al. (1997).

Enzyme	Linkage	pNPA	Arabinan		Arabino-galactan		Arabino-xylan	
			o [a]	p	o	p	o	p
α-L-Arabinofuranosidase (EC 3.2.1.55)	1→3 1→5 (1→2)	+	+	-	+	-	+	-
α-L-Arabinofuranosidase	1→3 1→5	+	+	+	+	+	+	+
α-L-Arabinofuranohydrolase	1→3 1→2	-	-	-	-	-	+	+
Exo-α-L-arabinanase	1→5	-	-	+[b]	-	-	-	-
β-L-Arabinopyranosidase		+[c]	-	-	-	-	-	-
Endo-1→5-α-L-arabinanase (EC 3.2.1.99)	1→5	-	+	+	-	-	-	-

[a] o oligomer; p polymer

[b] exo-attack on side-chains of branched arabinan

[c] *p*-nitrophenyl-β-L-arabinopyranoside

Isolated arabinoxylo-oligosaccharides have been used to study the mode of action of some α-L-arabinofuranosidases in more detail. According to Kormelink *et al.* (1993a), the α-L-arabinofuranosidase A from *A. niger* (Rombouts *et al.*, 1988) was able to remove all single α-1,3-linked arabinofuranosyl side-groups, irrespectively of whether the group was attached to the terminal or non-terminal xylopyranose residue. The enzyme isolated from *Bacillus subtilis* 3-6 acted similarly to α-L-arabinofuranosidase A (Kaneko *et al.*, 1994). In addition, it hydrolysed different methyl α-L-arabinofurano-biosides to arabinose and methyl α-L-arabinofuranoside in the order of 1,2- > 1,3- > 1,5-linkages and preferred the 1,3-linkage of the methyl α-L-arabinofuranotrioside over the 1,5-linkage (Kaneko and Kusakabe, 1995). On the other hand, α-L-arabinofuranosidase B produced by *A. niger* (Rombouts *et al.*, 1988) and the enzyme isolated from *A. niger* 5-16 could only remove arabinofuranosyl groups from singly substituted non-reducing terminal xylopyranosyl residues (Kormelink *et al.*, 1993a; Kaneko *et al.*, 1993).

A. awamori AXH was able to hydrolyse not only α-1,3-linked but also α-1,2-linked arabinofuranose residues both from terminal and non-terminal

singly substituted xylopyranosyl residues (Kormelink *et al*., 1993a). An arabinose-releasing enzyme, AXH-d_3, with a novel property was recently isolated from *Bifidobacterium adolescentis* DSM 20083 (van Laere *et al*., 1997). Similarly to the AXH isolated from *A. awamori*, it was highly specific towards arabinoxylans. AXH-d_3 was the first and only enzyme hitherto purified which was able to hydrolyse α-1,3-linked arabinofuranosyl residues from double substituted xylose residues.

Pitson *et al.* (1996) studied the stereochemical course of hydrolyses catalyzed by various enzymes acting on arabinofuranosyl linkages. The α-L-arabinofuranosidases produced by *A. aculeatus* (Beldman *et al.*, 1993) and *A. niger* (Rombouts *et al.*, 1988) retained the stereochemical structure of the substrate after hydrolysis. α-L-Arabinofuranosidases of *Penicillium capsulatum*, *Humicola insolens*, *Bacillus subtilis* and *Monilinia fructigena* have also been reported to retain the configuration of the substrate during catalysis. On the other hand, *A. awamori* AXH and the enzymes from *Bifidobacterium adolescentis* (van Laere *et al.*, 1997) and *Trichoderma reesei*, which were able to attack arabinofuranose residues from double-substituted xylose residues, acted in an inverting manner (Pitson *et al.*, 1996).

The ability of some α-L-arabinofuranosidases to release arabinose from different fibres has also been tested. α-L-Arabinofuranosidase from *Bacillus stearothermophilus* L1 showed only low activity against high molecular weight substrates such as arabinoxylan or arabinogalactan (Bezalel *et al.*, 1993). However, when the enzyme was applied to a semi-bleached Kraft pulp together with xylanase, improved removal of lignin from the pulp was observed. The amount of delignification obtained by the combined enzyme treatment exceeded the sum of the amounts obtained by the individual enzymes separately, indicating that the two enzymes were acting synergistically. According to Margolles-Clark *et al.* (1996a), *Trichoderma reesei* α-L-arabinofuranosidase I could also liberate about 60% of the arabinose from arabinoglucuronoxylan isolated from pine kraft pulp, but its action in pulp was not studied.

The extracellular α-L-arabinofuranosidase from *Ruminococcus albus* 8 was able to liberate arabinose from alfalfa cell wall polymers in the presence of xylanase and polygalacturonase (Greve *et al.*, 1984). On the other hand, *Sclerotinia sclerotiorum* enzyme hydrolysed arabinan and released significant amounts of arabinose from isolated bean or rice cell walls in the absence of other polysaccharide-degrading enzymes (Baker *et al.*, 1979). The partially purified α-L-arabinofuranosidase from *S. fructigena* was also able to hydrolyse arabinan and apple fibre with arabinose as the only hydrolysis product (Fielding and Byrde, 1969).

1.4 α-Galactosidases

1.4.1 Induction of α-galactosidases

The carbon source used for the induction of α-galactosidases has been found to have a marked effect on the properties of the α-galactosidase produced. *Bacteroides ovatus* produced two inducible α-galactosidases: α-galactosidase I, which is able to degrade galactomannan, was induced by guar gum but not by other galactosides, whereas synthesis of α-galactosidase II, incapable of acting on guar gum, was induced by galactose, melibiose, raffinose and stachyose (Gherardini *et al.*, 1985). The production of α-galactosidase I appeared to be regulated coordinately with the mannanase activity, which degrades the backbone of the substate. Another galactomannan, locust bean gum, induced α-galactosidase production by *Trichoderma reesei* Rut C-30 (ATCC 56765) (Zeilinger *et al.*, 1993) and *Bacillus stearothermophilus* (Talbot and Sygush, 1990). As in the case of *B. ovatus* α-galactosidase I, in *B. stearothermophilus* the maximum α-galactosidase activity did not occur until 5 days after inoculation, suggesting that the mannanase was required to depolymerize the galactomannan to oligosaccharides before α-galactosidase was significantly expressed (Talbot and Sygush, 1990). In *T. reesei* a low consititutive amount of α-galactosidase is present and it has been suggested that this enzyme releases galactose from locust bean gum and thereby triggers the production of inducible α-galactosidase (Zeilinger *et al.*, 1993). α-Galactosidase has also been reported to be produced constitutively by the thermophilic fungus *Humicola* sp. (Kotwal *et al.*, 1995), S*treptomyces mutans* (Aduse-Opoku *et al.*, 1991a) and *Bacillus* sp. No. 7-5 (Akiba and Horikoshi, 1976), although notable increases in activity have been observed after e.g. melibiose, raffinose or lactose supplementation. Wong-Leung and co-workers (1993) used extracts of sugarcane and soybean wastes as carbon sources for *Monascus anka* M9 IAM. Both supported the growth of the fungus, but sugarcane waste was superior for the production of α-galactosidase (Wong-Leung *et al.*, 1993).

Galactose and several galactose-containing oligosaccharides, such as melibiose, raffinose and stachyose, have commonly been used for the induction of α-galactosidases. The low molecular weight compounds have been effective inducers for the production of intracellular enzymes by e.g. *Monascus* sp. (Imanaka *et al.*, 1972), *Monascus pilosus* (Wong *et al.*, 1986), *Corynebacterium murisepticum* ATCC 21474 (Nadkarni *et al.*, 1992) and *Micrococcus* sp. No. 31-2 (Akiba and Horikoshi, 1976), and for both cell-associated and extracellular enzymes of the yeast *Torulaspora delbrueckii* IFO 1255 (Oda and Tonomura, 1996). In addition to galactose, L-arabinose and corresponding polyols induced α-galactosidase in *T. reesei* (Zeilinger *et al.*, 1993).

Foda *et al.* (1995) screened 38 fungal strains for α-galactosidase production using Czapek-Dox agar medium supplemented with melibiose or galactose. Only five strains produced appreciable amounts of enzyme, *Penicillium janthinellum* being superior for the formation of both intra- and extracellular α-galactosidase. In further studies carried out with this fungus, galactose, lupin seed powder and soybean were shown to be the best carbon sources for α-galactosidase production. Srinivas *et al.* (1994) used a Plackett-Burman design for the screening of five sources of nitrogen, six minerals, six enzyme inducers and some growth as well as product promotors for their effects on the production of α-galactosidase by *Aspergillus niger* MRSS 234 in solid-state culture. On the basis of the results obtained, urea, corn steep liquor, guar flour, soybean flour and citric acid were selected as the most promising inducers for further optimization studies.

1.4.2 Characterization of α-galactosidases

α-Galactosidases have been isolated from several strains of fungi, bacteria and yeast, but also from plants such as *Cyamopsis tetragonoloba* (guar) and coffee bean, as well as from humans (Dey and Pridham, 1972; Dey and Del Campillo, 1984; Müller and Köhler, 1985). Most of the α-galactosidases produced by fungi have been isolated from different strains of *Aspergillus* and *Penicillium* (Table 8). *Aspergillus* α-galactosidases form a heterogeneous group with highly variable molecular properties. The molecular masses have ranged from 45 to 93 kDa monomers up to 147–318 kDa multimers (Table 8). Despite the heterogenity in the molecular masses, the isoelectric points determined for *Aspergillus* α-galactosidases have been rather similar, ranging from 4.2 to 4.8. *A. tamarii* α-galactosidases I and II were purified from the mycelium (Civas *et al.*, 1984a). *Penicillium* α-galactosidases form a more homogeneous group with molecular masses between 55 and 67 kDa as determined by SDS-PAGE and isoelectric points between 4.0 and 4.3 (Table 8). The optimal pH range for the action of the *Penicillium* α-galactosidases is pH 4.0–6.0, the enzymes of *P. chrysogenum* DSM 3214 and *P. notatum* DSM 3216 having the highest pH optima (Pat. EP 0 192 401 B1, 1993).

Four glycosylated α-galactosidases have been purified from *Mortierella vinacea* (Suzuki *et al.*, 1970; Galas and Miskiewicz, 1996; Shibuya *et al.*, 1997). The mycelial enzyme purified by Suzuki *et al.* (1970) and the secreted α-galactosidase I isolated by Shibuya *et al.* (1997) had similar physicochemical properties, substrate specificities and N-terminal amino acid sequences, although the latter probably existed as a tetramer. *Cephalosporium acremonium* 237 (Zaprometova and Ulezlo, 1988) and *Monascus pilosus* (Wong *et al.*, 1986) also produced large α-galactosidases

with molecular masses of 240 and 150 kDa, respectively, as determined by gel filtration (Zaprometova and Ulezlo, 1988).

Zeilinger and co-workers (1993) isolated an α-galactosidase from *Tricoderma reesei* RutC-30, which is known to produce several hemi-cellulolytic, especially xylanolytic enzymes (Poutanen and Puls, 1989). The genes encoding three α-galactosidases in the same strain were recently cloned (Margolles-Clark *et al.*, 1996b). On the basis of the molecular mass calculated from the amino acid sequence and the hydrolytic properties, it was concluded that AGLI was most probably the same enzyme as isolated by Zeilinger and co-workers. The two other enzymes, AGLII and AGLIII are somewhat larger than AGLI. An α-galactosidase of *T. reesei* has also been isolated by Kachurin *et al.* (1995). The enzyme was a glycoprotein containing O- and N-linked carbohydrate chains (Savel'ev *et al.*, 1997). The protein existed in two forms, with similar amino acid compositions and identical N-terminal aa sequences. However, the forms differed in the number of N-carbohydrate chains per molecule and in their monosaccharide compositions. Despite the similar molecular mass and pI to those of AGLI of the strain RutC-30, the enzyme isolated by Kachurin and co-workers is obviously a different enzyme because the N-terminal amino acid sequences of the enzymes are not identical (determined at VTT, unpublished results).

In comparison to the fungal α-galactosidases, all isolated bacterial enzymes, except the α-galactosidase of the hyperthermophilic eubacterium *Thermotoga neapolitana* 5068 (TN5068) (Duffaud *et al.*, 1997), have been large, 195–390 kDa proteins and most commonly composed of 3–4 subunits (Table 9). The only intracellular bacterial α-galactosidase has been isolated from *Bifidobacterium longum* CRL 849 (Garro *et al.*, 1994). The isoelectric points range from 4.5 to 6.9. Bacterial α-galactosidases had somewhat higher pH optima than the fungal enzymes, being between pH 5.0 and 7.5. The *T. neapolitana* 5068 (TN5068) α-galactosidase is the most thermostable α-galactosidase hitherto isolated, with a temperature optimum of 100–103°C (activity assay) and a half-life of 2 h at 90°C and 3 min at 100 °C (Duffaud *et al.*, 1997).

α-Galactosidases from yeast have been purified only recently. Analogously to the bacterial α-galactosidases, the enzymes purified from yeasts have been large multimeric proteins (Table 10). The molecular masses of the *Saccharomyces* α-galactosidases have been calculated from the amino acid sequences (Liljeström, 1985; Sumner-Smith *et al.*, 1985; Turakainen *et al.*, 1991), and thus the sizes of the native proteins are unknown. At least the *Torulaspora delbrueckii* enzyme was a glycoprotein (Oda and Tonomura, 1996). The isoelectric point has been determined only for the two *Candida guillermondii* H-404 enzymes, which had pI values around 6.2 (Hashimoto *et al.*, 1993). The pH optima of the yeast α-galactosidases were similar to the optimum values of the fungal enzymes.

Table 8. Fungal α-galactosidases.

Organism	M_r [a] (kDa)	pI	pH-optimum	Activity against[b]	Remarks	Reference
Aspergillus awamori X100/D27	91–93	4.7	4.5		Glycoprotein Amino acid composition	Neustroyev *et al.*, 1991
Aspergillus ficuum NRRL3135	70.8 74.1 (gf)		5.6–6.0	pNPG, oNPG, mNPG, melibiose, raffinose, stachyose	Glycoprotein	Zapater *et al.*, 1990
Aspergillus niger						Ademark *et al.*, 1997
I	318	4.2		melibiose, raffinose, stachyose	Trimer: 101 kDa	
II–IV	75	4.5, 4.7, 4.8		melibiose, raffinose, stachyose including the α-1,2-linkages, galacto-mannan, branched galactomanno-oligosacchrides		
Aspergillus niger	45		4.0–4.5	pNPG, raffinose, stachyose, melibitol, guar flour, locust bean gum	Glycoprotein	Adya and Elbein, 1977
Aspergillus niger				pNPG, melibiose		Agnantiari *et al.*, 1991
Aspergillus niger (Rhozyme HP-150)			3.8–4.2	pNPG, methyl-α-galactopyranoside, melibiose, raffinose, stachyose, guar and locust bean gum galactomannans		Bahl and Agrawal, 1969
Aspergillus niger			4.0		Thermostable	Christakopoulos *et al.*, 1990
Aspergillus niger N402	82	4.8			Gene cloned (*aglA*)	den Herder *et al.*, 1992

Aspergillus niger 5-16				pNPG, $Gal^{1}Man_{2}$, $Gal^{3}Man_{4}$, $Gal^{1,3}Man_{4}$, $Gal^{3,4}Man_{4}$, $Gal^{3,4}Man_{5}$	Liberates the stub galactose residues attached to the inner mannoses of the main-chain galactomanno-oligosaccharides	Kaneko *et al.*, 1991
Aspergillus niger	147		5.0	raffinose, stachyose, oligosaccharides also from cowpea meal	Dimer: 78 and 69 kDa	Somiari and Balogh, 1995
Aspergillus oryzae	64 (gf)		4.0	pNPG, raffinose, oligosaccharides in soy milk		Annunziato and Mahoney, 1987
Aspergillus oryzae	200 (gf)		4.0	pNPG, melibiose		Cruz *et al.*, 1991
Aspergillus saitoi	290 (gf)		5.0–5.5	raffinose-family oligosaccharides from soy milk	Partially purified.	Sugimoto and van Buren, 1970
Aspergillus tamarii IP 1017-10					Glycoproteins Mycelial	Civas *et al.*, 1984a
I	265 (ef)		4.2–4.3	oNPG, melibiose, raffinose, stachyose	Multimer: 88 kDa (SDS)	
II	254 (ef)		4.2–4.3	oNPG, melibiose, raffinose, stachyose	Multimer: 77.5 kDa (SDS)	
III	56		4.8	oNPG, raffinose, stachyose, galactomannobiose, galactomannan	Glycoprotein Extracellular	Civas *et al.*, 1984b
Cephalosporium acremonium 237	240 (gf)	4.4 / 4.96	5.0–6.0	pNPG, melibiose, raffinose, methyl-α-D-galactopyranoside, galactosyl-α-(1,3)-galactoside, blood group B substance, ceramide trihexoside, galactomannan	Multimer: 40 kDa Glycoprotein Amino acid composition Hydrolyses Galα(1,3)-, Galα(1,4)- and Galα(1,6)-linkages	Zaprometova and Ulezlo, 1988 Zaprometova *et al.*, 1990
Humicola sp			5.0	pNPG	Not purified	Kotwal *et al.*, 1995
Monascus pilosus	150 (gf)		4.5–5.0	pNPG, melibiose, raffinose, stachyose		Wong *et al.*, 1986

Table 8. continued.

Organism	M_r [a] (kDa)	pI	pH-optimum	Activity against[b]	Remarks	Reference
Mortierella vinacea	52 44.4 (aa)		3.0–6.0	oNPG, pNPG, methyl-αD-galactopyranoside, galactinol, melibiose, manninotriose, raffinose, stachyose, $Gal^{3}Man_3$, $Gal^{3,4}Man_4$, 4-*O*-α-D-galactopyranosyl-D-galactose, 6-*O*-α-D-galactopyranosyl-*O*-β-D-galactopyranosyl-1-glycerol	Glycoprotein Mycelial Crystallized Amino acid sequence Liberates terminal galactose residues from galactomanno-oligosaccharides	Suzuki *et al.*, 1970 Kaneko *et al.*, 1990 Shibuya *et al.*, 1995b
Mortierella vinacea						Shibuya *et al.*, 1997
I	240 (gf)	5.4	3.0–4.0	pNPG, melibiose, raffinose, stachyose, $Gal^{3}Man_3$,	Tetramer: 50–56 kDa (SDS) Glycoprotein N-terminal amino acid sequence	
II	51–62 60 (gf) 41.3 (aa)	8.5	3.0–4.0	pNPG, melibiose, raffinose, stachyose, $Gal^{3}Man_3$, $Gal^{3}Man_4$, guar gum and locust bean gum galacto-mannans	Glycoprotein Gene cloned	
Mortierella vinacea IBT-3	68	5.95	4.0–5.0	oNPG, pNPG, melibiose, raffinose	Glycoprotein	Galas and Miskiewicz, 1996
Penicillium chrysogenum DSM 3214	55–60	4.3	5.0–6.0	pNPG, guar flour		Pat. EP 0 192 401 B1, 1993
Penicillium islandicum DSM 3215	62–67	4.3	4.0–5.5	pNPG, guar flour		Pat. EP 0 192 401 B1, 1993
Penicillium janthinellum NRRL 5576			4.5	pNPG		Elshafei *et al.*. 1993

Penicillium notatum DSM 3216	55–60	4.3	5.0–6.0	pNPG, guar flour		Pat. EP 0 192 401 B1, 1993
Penicillium ochrochloron IMI 061271	60.2 57.5 (gf)		4.5	pNPG, melibiose, raffinose, stachyose, guar, tara and locust bean gum galactomannans		Dey *et al.*, 1993
Penicillium purpurogenum	63–67	4.0–4.1	4.5	pNPG, melibiose, raffinose, copra galactomannan oligosaccharides	N-terminal amino acid sequence	Park *et al.*, 1991 Shibuya *et al.*, 1995a
Trichoderma reesei RutC-30 AGLI AGLII AGLIII	 45.7 (aa) 79.5 (aa) 66.3 (aa)			 pNPG, melibiose, raffinose, locust bean galactomannan, pinewood galactoglucomannan (I–III)	Genes cloned (*agl1*, *agl2*, *agl3*) Hydrolysis of polymers enhanced by endo-1,4-β-mannanase (I–III) and β-mannosidase (II, III)	Margolles-Clark *et al.*, 1996b
Trichoderma reesei RutC-30	50 ± 3	5.2	4.0	pNPG, melibiose, raffinose, stachyose, locust bean gum galactomannan	Hydrolysis of polymers enhanced by endo-1,4-β-mannanase	Zeilinger *et al.*, 1993
Trichoderma reesei	54	5.25		pNPG, methyl-α-galactopyranoside, melibiose, raffinose, stachyose	Glycoprotein Transgalactosylation activity	Kachurin *et al.*, 1995 Savel'ev *et al.*, 1996 Savel'ev *et al.*, 1997

[a] See Table 4. ef molecular mass determined by electrophoresis

[b] pNPG, p-nitrophenyl-α-D-galactopyranoside; oNPG, o-nitrophenyl-α-D-galactopyranoside

Table 9. Bacterial α-galactosidases.

Organism	M_r [a] (kDa)	pI	pH-optimum	Activity against[b]	Remarks	Reference
Bacillus stearothermophilus	247 ± 13.4 (ef)		7.0–7.5	pNPG, melibiose, raffinose, stachyose, limited activity on guar and locust bean gums	Trimer: 82 kDa (SDS)	Talbot and Sygusch, 1990
Bacteroides ovatus 0038-1						Gherardini *et al.*, 1985
I	250 (gf)	5.6	5.9–6.4	oNPG, pNPG, melibiose, raffinose, stachyose, guar gum galactomannan oligosaccharides (I, II)	Trimer: 85 kDa (SDS)	
II	250 (gf)	6.9	6.3–6.5		Trimer: 80.5 kDa (SDS)	
Bifidobacterium longum CRL 849			5.8	pNPG	Intracellular Not purified	Garro *et al.*, 1994
Bifidobacterium adolescdntis DSM 20083	340 (sec)		5–6	pNPG, melibiose, α-1,3-D-galacto-biose, α-1,4-D-galactobiose, raffinose, stachyose, locust bean gum, Gal-α-1,3-Gal-ß-1,4-Gal, Gal-α-1,3-Gal-ß-1,4-Gal-α-1,3-Gal	Intracellular Multimer: 79 kDa (SDS) Produced anaerobically	Leder *et al.*, 1997
Corynebacterium murisepticum ATCC 21374	320		7.5	pNPG	Tetramer: 83 kDa (SDS) Amino acid composition	Nadkarni *et al.*, 1992
Escherichia coli K12	329 (sed)	5.1	7.2	pNPG, methyl-α-D-galactoside, melibiose, raffinose	Tetramer: 82 kDa (SDS) Amino acid composition	Schmid and Schmitt, 1976

Lactobacillus fermentum CRL 251	195 (gf)		5.0–6.5	pNPG	Tetramer: 45 kDa (SDS)	Garro *et al.*, 1996
Pseudomonas fluorescens H-601	390 (gf)	6.3	6.0–7.0	pNPG, oNPG, melibiose, raffinose, stachyose	Multimer: 86 kDa (SDS) Trasgalactosylation activity	Hashimoto *et al.*, 1991
Streptococcus mutans	~300 (gf)		6.5	pNPG, melibiose, raffinose	Tetramer: 82 kDa (SDS) Gene cloned	Aduse-Opoku *et al.*, 1991
Thermotoga neapolitana TN5068	61	4.6 ± 0.1	7.3	pNPG, guar gum galactomannan	Extremely thermostable	McCutchen *et al.*, 1996 Duffaud *et al.*, 1997
Thermotoga neapolitana NS-E	61		7.0	pNPG, melibiose, α-1,6-galacto-oligosaccharides from soy molasses	Extremely thermostable	King *et al.*, 1998

[a, b] See Table 8. sec molecular mass determined by size exclusion chromatography; sed molecular mass determined by high-speed sedimentation analysis

Table 10. Yeast α-galactosidases.

Organism	M_r [a] (kDa)	pI	pH-optimum	Activity against[b]	Remarks	Reference
Candida guilliermondii H-404					Transgalactosylation activity	Hashimoto *et al.*, 1993
I	270 (gf)	6.2	4.5	pNPG, oNPG, mNPG, melibiose, stachyose, raffinose (I, II)	Multimeric: 64 kDa (SDS)	
II	270 (gf)	6.2	4.5		Multimeric: 64 kDa (SDS)	
Saccharomyces carlsbergensis	50 (aa)				Native protein dimer Gene cloned	Sumner-Smith *et al.*, 1985
Saccharomyces carlsbergensis NCYC396	52 (aa)				Gene cloned	Turakainen *et al.*, 1991
Saccharomyces cerevisiae	50 (aa)					Liljeström, 1985
Torulaspora delbrueckii IFO 1255	530 (gf)		4.5–5.5	pNPG, oNPG, mNPG, melibiose, raffinose, stachyose	Hexameric: 88 kDa (SDS) Glycoprotein	Oda and Tonomura, 1996

[a] See Table 8.

[b] See Table 8; mNPG, m-nitrophenyl-α-D-galactopyranoside

1.4.3 Molecular properties of α-galactosidases

Genes encoding α-galactosidases have been isolated from various sources, including fungi, bacteria, yeast, plant seeds and humans (Table 11). On the basis of their amino acid sequence similarities, α-galactosidase genes have been classified into three different glycosyl hydrolase families, of which family 4 contains genes of prokaryotic origin and families 27 and 36 genes of both eukaryotic and prokaryotic origins (SWISS-PROT Protein Sequence Data Bank, Release 35.0 and updates to February 1998). The sequences belonging to the two latter families have been found to be related and they have been proposed to form a single superfamily (Henrissat and Bairoch, 1993; Dagnall *et al.*, 1995; Henrissat and Bairoch, 1996).

α-Galactosidase A of *Aspergillus niger* represents apparently a minor α-galactosidase activity of the fungus, and it has been suggested that at least three more α-galactosidases are present (den Herder *et al.*, 1992). The gene *aglA* showed considerable amino acid sequence similarity with various α-galactosidases, the best overall similarity (37%) being with the *C. tetragonoloba* enzyme, although the protein encoded by *aglA* is about 100 amino acids larger than any of the other α-galactosidases hitherto studied. *aglA* contains a serine- and threonine-rich C-terminal extension similar to the O-glycosylated Ser/Thr-rich domains present e.g. in the extracellular cellobiohydrolases CBH-I and CBH-II from *Trichoderma reesei* (Penttilä *et al.*, 1986). In these enzymes this domain separates the N-terminal catalytic domain from the C-terminal substrate binding domain. Thus it has been speculated that the extension of *aglA* could also be involved in binding insoluble substrates.

It has been suggested that some of the residues present in the homologous regions might be part of the activite site. Enzymatic hydrolysis of the glycosidic bond proceeds via general acid catalysis which requires two critical residues: a proton donor and a nucleophile/base. In general, replacement of the catalytic general acid (Asp, Glu or Tyr) results in inactivation of the enzyme, whereas low activity often remains after mutation of other residues involved in catalysis (Svensson and Søgaard, 1993). It has also been proposed that the glycosylation of the protein might have an effect on enzyme activity (Overbeeke *et al.*, 1989).

The deduced amino acid sequences of α-galactosidases from *Cyamopsis tetragonoloba* (guar) (Overbeeke *et al.*, 1989) and *Coffea arabica* (coffee bean) (Zhu and Goldstein, 1994) belonging to the family 27 share approximately 80% homology. Moreover, guar and mouse α-galactosidases are highly homologous (53% and 79%, respectively) with human α-galactosidase (Overbeeke *et al.*, 1989; Ohshima *et al.*, 1995). Although these enzymes have considerable similarities in their amino acid sequences, their substrate specificities differ significantly, e.g. guar α-galactosidase

Table 11. Genes coding for α-galactosidases.

Organism	Gene	Signal sequence	Mature protein	M_r kDa	Hydrolase family	Remarks	Reference
Eschericia coli K-12	*melA*		450 aa	50.6	4		Liljeström and Liljeström, 1987
Aspergillus niger	*aglA*	31 aa	514 aa	60.148	27	7 N-glycosylation sites Possible O-glycosylation sites in the C-terminal Ser/Thr-rich region	den Herder *et al.*, 1992
Mortierella vinacea	*(agl2)*	20 aa	376 aa	41.334	27	9 N-glycosylation sites	Shibuya *et al.*, 1997
Mortierella vinacea	*(agl1)*	20 aa	397 aa	44.350	27	5 N-glycosylation sites	Shibuya *et al.*, 1995b
Saccharomyces carlsbergensis	*MEL1*	18 aa	453 aa	52.102 50.104 (mature)	27	8 N-glycosylation sites	Sumner-Smith *et al.*, 1985
Saccharomyces carlsbergensis NCYC396	*MEL1*	18 aa	453 aa	52.006	27	9 N-glycosylation sites	Turakainen *et al.*, 1991
Saccharomyces cerevisiae	*MEL1*	18 aa	453 aa	52.044	27	9 N-glycosylation sites	Liljeström, 1985
Trichoderma reesei	*agl1*	27 aa	417 aa	45.7	27	5 N-glycosylation sites	Margolles-Clark *et al.*, 1996b

Trichoderma reesei	*agl3*	22 aa	602 aa	66.3	27	7 N-glycosylation sites	Margolles-Clark *et al*., 1996b
Coffea arabica		15 aa	363 aa	41.310	27	1 N-glycosylation site Trp-16 and Tyr-108 important for catalytic activity	Zhu and Goldstein, 1994 Zhu *et al*., 1995 Zhu *et al*., 1996
Cyamopsis tetragonoloba (guar)		47 aa (24 + 23)	364 aa	45.135 39.777 (mature)	27	3 N-glycosylation sites Asp-272 probably involved in catalytic reaction	Overbeeke *et al.*, 1989
Homo sapiens (human)	*GALA*	31 aa	398 aa	48.766 45.356 (mature)	27	4 N-glycosylation sites	Bishop *et al.*, 1986 Bishop *et al.*, 1988
	αGalA	31 aa	388 aa		27	4 N-glycosylation sites	Ohshima *et al.*, 1995
Escherichia coli	*rafA*		708 aa	81.188	36		Aslanidis *et al*., 1989
Streptococcus mutans	*aga*		720 aa	82.022	36	8 N-glycosylation sites	Aduse-Opoku *et al*., 1991a, b
Thermotoga neapolitana	*aglA*		552 aa	61	36		King *et al.*, 1998
Trichoderma reesei	*agl2*	26 aa	720 aa	79.5	36	9 N-glycosylation sites	Margolles-Clark *et al*., 1996b

primarily cleaves α-1,6-linked galactose residues (Overbeeke *et al.*, 1990), whereas the coffee bean α-galactosidase prefers α-1,3- and α-1,4-linkages (Zhu and Goldstein, 1994). An aspartic acid at position 272 in guar α-galactosidase might be an important residue for the activity of the enzyme, as it is located in a region which shows a strong homology with other α-galactosidases (Overbeeke *et al.*, 1989).

Tyrosine and tryptophan residues are often found at the active sites of glycosidases, e.g. in coconut α-galactosidase, which is a common feature shared with other sugar-binding proteins (Mathew and Balasubramaniam, 1986; McCarter and Withers, 1994). The roles of two tyrosine residues at positions 108 and 158, and of a tryptophan residue at position 16 on the activity of coffee bean α-galactosidase were studied using a baculovirus expression system and expression in the yeast *Pichia pastoris*, respectively (Zhu *et al.*, 1995; Zhu *et al.*, 1996). All these residues are located in the well conserved regions of the amino acid sequences of α-galactosidases. Replacement of the Tyr-158 with phenylalanine resulted in a mutant α-galactosidase which retained approximately 88% of the activity of the wild-type enzyme, whereas the substitution of Tyr-108 almost abolished the enzymatic activity (Zhu *et al.*, 1995). The mutant protein, in which the residue Trp-16 of the wild-type α-galactosidase had been replaced with an alanine residue, was produced in the yeast but the mutant enzyme sufferred more than a 10^4 -fold decrease in its specific activity (Zhu *et al.*, 1996). Thus the residues Trp-16 and Tyr-108 are most probably involved in the catalysis.

Most of the work involved in the understanding of the roles of individual amino acids in the activity of α-galactosidases has been performed with human α-galactosidase. Fabry disease is an X-chromosome linked inborn error of glycolipid metabolism caused by a deficiency of the lysosomal α-galactosidase A (Desnick and Bishop, 1989). The disease occurs in two different forms: classic and atypical, which have dissimilar symptoms. Several mutations in the α-galactosidase genes have been identified and characterized from patients with Fabry disease (e.g. Bernstein *et al.*, 1989; Koide *et al.*, 1990; Sakuraba *et al.*, 1990; Ishii *et al.*, 1992). hitherto, all point mutations have been found either in exons 1, 2 or 6. Koide and co-workers (1990) identified two nucleotide substitutions in exon 1: one of the mutations (A24G) was silent, but the other (C118T) resulted in an amino acid substitution of proline at position 40 by serine. In the *in vitro* mutagenesis experiments Pro-40 was individually replaced by threonine and glycine residues, resulting in gross secondary structures similar to the wild-type and mutant proteins, respectively. In both cases, the observed α-galactosidase activity was very low, suggesting that the activity of the enzyme was dependent on the Pro-40 residue *per se* and not on the secondary structure of the protein. The two combined point mutations in

exon 2 (Glu66Gln; Arg112Cys) and a third mutation in exon 6 (Gly328Arg) were identified from patients with classic Fabry disease (Ishii *et al.*, 1992). The mutations in the atypical cardiac type cases have been localized in the upstream region of exon 6: Gln279Glu (Ishii *et al.*, 1992) and Arg301Gln (Sakuraba *et al.*, 1990).

Other amino acid residues important for the activity of human α-galactosidase were identified when five individual amino acid substitutions were introduced with an oligonucleotide-directed *in vitro* mutagenesis kit into human α-galactosidase and the mutant enzymes were expressed in a baculovirus (Ishii *et al.*, 1995). Two mutants obtained by substitution of glutamine at position 280 with serine (Glu280Ser) and threonine at position 282 with alanine (Thr282Ala) showed increased K_m values and decreased thermostabilities as compared to the wild-type enzyme. Moreover, both mutants showed reduced enzyme activity at acidic pH. Thus it was suggested that both residues are in or near the active site of the α-galactosidase.

1.4.4 Substrate specificities of α-galactosidases

α-Galactosidases can be divided into two groups on the basis of their hydrolytic properties (Dey and Pridham, 1972; Dey and Del Campillo, 1984). One group is specific for low-molecular weight α-galactosides, such as alkyl- and arylgalactosides, melibiose and the raffinose-family oligosaccharides. The other type of enzymes act on polymeric galactomannans; however they are also able to hydrolyse lower-molecular weight substrates to various extents. In general, it appears that the low-molecular mass α-galactosidases from both fungal and bacterial sources are able to liberate galactose residues not only from low-molecular weight substrates but also from intact polymeric galacto(gluco)mannans (Tables 8-10). The action of the high-molecular mass enzymes appears to be limited to the small synthetic α-galactosides, melibiose and raffinose-family oligosaccharides and terminal galactose residues in galactomanno-oligosaccharides.

Aspergillus niger (Ademark *et al.*, 1997) and *A. tamarii* IP 1017-10 (Civas *et al.*, 1984a, b) are good examples of organisms producing both types of α-galactosidases. *A. niger* α-galactosidase I and the *A. tamarii* α-galactosidases I and II, which were all large proteins, catalysed the hydrolysis of terminal α-1,6-linked galactose residues from linear oligomeric substrates but did not attack polymeric galactomannan or branched galactomanno-oligosaccharides (Ademark *et al.*, 1997; Civas *et al.*, 1984a). The three smaller *A. niger* α-galactosidases, II, III and IV, were highly active towards branched galactomanno-oligosaccharides and also released galactose side-groups from galactomannan polymers (Ademark *et*

al., 1997). The linear substrates were less efficiently degraded, but surprisingly all the enzymes were also able to cleave the linkage between glucose and fructose from raffinose and stachyose, obviously after the removal of galactose. *A. tamarii* α-galactosidase III also had significantly lower affinity towards small substrates than the two larger enzymes, but it was able to degrade galactomannobiose and to remove galactose residues from the polymeric galactomannans (Civas *et al.*, 1984b).

Trichoderma reesei α-galactosidases hydrolysed pNPG, melibiose, raffinose and stachyose, but their activity towards polymeric galacto(gluco)-mannans was lower (Zeilinger *et al.*, 1993; Margolles-Clark *et al.*, 1996b). *T. reesei* AGLI expressed in yeast liberated 13% and 25% of the galactose present in locust bean gum galactomannan and the less substituted pinewood galactoglucomannan, respectively, and its action was clearly enhanced by the presence of mannanase (Margolles-Clark *et al.*, 1996b). The synergistic action of these enzymes was also observed when the α-galactosidase isolated from the culture filtrate was used (Zeilinger *et al.*, 1993). The other *T. reesei* α-galactosidases, AGLII and AGLIII, were almost inactive towards polymeric substrates. They acted synergistically with the mannanase and the degree of hydrolysis was further increased by the addition of β-mannosidase (Margolles-Clark *et al.*, 1996b).

Various *Penicillium* α-galactosidases have also been able to hydrolyse both oligomeric and polymeric substrates. *P. ochrochloron* IMI 061271 α-galactosidase cleaved α-galactose residues from galactomannans more readily than from α-galactose-containing oligosaccharides (Dey *et al.*, 1993). During prolonged incubation of the enzyme with guar, tara and locust bean gums, with galactose to mannose ratios of 1:2, 1:3 and 1:4, respectively, 37%, 75% and 84% of the galactose side-groups were liberated, indicating that the high degree of substitution limited the hydrolysis of side-groups. Analogously, the locust bean gum galactomannan was less efficiently hydrolysed by *Mortierella vinacea* α-galactosidase than the less substituted guar galactomannan (Shibuya *et al.*, 1997). *P. chrysogenum* DSM 3214, *P. islandicum* DSM 3215 and *P. notatum* DSM 3216 were found to release galactose efficiently from guar flour and to improve the gelling properties of the polymer (Pat. EP 0 192 401 B1, 1993).

Cephalosporium acremonium α-galactosidase possessed an exceptionally broad aglycone specificity (Zaprometova *et al.*, 1990). In addition to the common oligomeric substrates, it was able to release terminal α-1,3-, α-1,4- and α-1,6-linked galactose residues from digalactosides, glycoproteins and glycolipids.

Table 12. Substrate specificities of Aspergillus niger 5-16, Mortieralla vinacea and Penicillium purpurogenum α-galactosidases (Kaneko et al., 1990; Kaneko et al., 1991; Shibuya et al., 1995a).

Substrate	Structure [a]	*Aspergillus niger* 5-16	*Mortieralla vinacea*	*Penicillium purpurogenum*
$Gal^{1}Man_2$	Gal | Man-Man	+[b]	-	n.d.[c]
$Gal^{3}Man_3$	Gal | Man-Man-Man	-	+	-
$Gal^{3}Man_4$	Gal | Man-Man-Man-Man	+	-	+
$Gal^{1,3}Man_4$	Gal Gal | | Man-Man-Man-Man	+ / -[d]	- / -	n.d.
$Gal^{3,4}Man_4$	Gal Gal | | Man-Man-Man-Man	- / +	+ / -	n.d.
$Gal^{3,4}Man_5$	Gal Gal | | Man-Man-Man-Man-Man	+ / +	- / -	n.d.

[a] Gal galactose; Man mannose; Man reducing end; | α-1,6-linkage; – β-1,4-linkage

[b] + galactose released; – galactose not released

[c] n.d. not determined

[d] + / - ; - / + ; - / - ; + / + removal of the first / second residue from the non-reducing end

The modes of action of the α-galactosidases from *A. niger* 5-16 (Kaneko *et al.*, 1991), *M. vinacea* (Kaneko *et al.*, 1990) and *P. purpurogenum* (Shibuya *et al.*, 1995a) have been studied in more detail using isolated galactomanno-oligosaccharides with known structures (Table 12). *A. niger* 5-16 α-galactosidase hydrolysed the stub galactose residues attached to the inner mannoses of the main-chain of galactomanno-oligosaccharides, but did not release the terminal galactoses attached to the non-reducing end mannoses of the main-chain. On the other hand, the galactose residue attached to the reducing-end mannose of $Gal^{1}Man_2$ was hydrolysed, but the residue attached to that of $Gal^{1,3}Man_4$ remained unhydrolysed, even after hydrolysis of the galactose attached to the inner mannose residue. Thus the hydrolysis was probably restricted by the length of the mannose backbone rather than by a steric hindrance by the other substituent. Similarly, the α-galactosidase of *P. purpurogenum* showed preference for the stub α-galactosyl residue

attached to the inner mannose of the main-chain, but it was not able to hydrolyse the terminal galactose attached to the non-reducing end mannose of galactomanno-oligosaccharides (Shibuya *et al.*, 1995a). The mode of action of the *M. vinacea* α-galactosidase was quite different from that of the two previous enzymes, as it liberated the galactosyl residues attached to the non-reducing end mannose of galactomanno-oligosaccharides, but was unable to cleave the stub or reducing-end galactosyl residues (Kaneko *et al.*, 1990).

α-Galactosidases of *T. reesei* (Savel'ev *et al.*, 1996), *Pseudomonas fluorescens* H-601 (Hashimoto *et al.*, 1991) and *Candida guillermondii* H-404 (Hashimoto *et al.*, 1993) have been reported to possess trans-galactosylation activity. *T. reesei* α-galactosidase was able to produce transgalactosylation products when galactose was used as a donor and pNPG, oNPG, 4-methylumbelliferyl α-D-galactopyranoside or methyl α-D-galactopyranoside, which inhibited the enzyme at high concentrations, were used as acceptors (Savel'ev *et al.*, 1996). On the other hand, no transgalactosylation was observed when the enzyme was incubated with melibiose, raffinose or stachyose. *P. fluorescens* H-601 α-galactosidase had a broad acceptor specificity as pNPG, D-galactose, D-glucose, D-mannose, D-fructose, sucrose and glycerol were all effective acceptors in the reaction (Hashimoto *et al.*, 1991). The transgalactosylation to melibiose occurred selectively at the C-6 hydroxyl group of galactosyl residue.

1.5 Potential applications of arabinose- and galactose-hydrolysing enzymes

Possible applications for α-L-arabinofuranosidases and α-galactosidases may be found in various industries which use hemicelluloses or related polysaccharides as raw materials. These include the food and feed, pulp and paper, cosmetic and pharmaceutical industries.

For the complete enzymatic hydrolysis of lignocellulosic materials the cooperative action of several cellulose- and hemicellulose-degrading enzymes is required. The role of main-chain hydrolysing glycanases in different applications has been studied rather thoroughly. However, synergistic action of side-group removing enzymes with the depolymerases is required for efficient total hydrolysis of the polysaccharides. The use of a single accessory enzyme for partial or specific modification of the substrate might offer new interesting options for utilization of these low cost raw materials.

Pectinases are widely used in the processing of fruit and vegetables, with beneficial effects on the extraction and maseration resulting in increased juice yields, better colour and clarity. In apple and pear juice production

haze formation is a problem arising from solubilized arabinans, which are linearized from the native branched stage obviously as a result of both the acidity of the juice and the heat treatment during processing as well as by the α-L-arabinofuranosidase activity present in the enzyme preparations used in processing. The precipitates can most probably be avoided by including sufficient amounts of α-L-arabinofuranosidase and endo-arabinanase activities or, alternatively, using enzyme preparations completely devoid of α-L-arabinofuranosidase activity (Whitaker, 1984; Voragen *et al.*, 1987; McCleary *et al.*, 1988).

Terpenols are strongly aromatic molecules that represent an important part of grape aromas. In addition to the free volatile forms, odorless glycosidic precursors of the aromas occur. A major portion of these monoterpenols in grapes are linked to disaccharide moieties, in which the major terminal non-reducing sugar is α-L-arabinofuranose. α-L-Arabinofuranosidase treatment followed by the addition of other glycosidases can be used for the enhancement of wine flavour by release of the free terpenols (Gunata *et al.*, 1990; Grassin and Fauquembergue, 1996).

Enzymatic modification of non-starch polysaccharides improves both the physical properties of bread dough and bread quality, including increased bread volume, improved crumb structure and prolonged shelf life (Poutanen, 1997). Beneficial effects in the baking process have been obtained both with commercial enzyme mixtures (Rouau *et al.*, 1994) and with single purified enzymes (Maat *et al.*, 1992; Autio *et al.* 1996), but their mechanisms are still mostly unknown. The effects of the expression of some xylanolytic enzymes in yeast used in bread making have also been studied (Monfort *et al.*, 1997). Exogenous enzymes from microbial sources might also be added to improve the efficiency and versatility of the brewing process (Bamforth, 1986). In general, arabinoxylans have received little attention in studies on the effects of non-starch polysaccharides on the brewing process. It is known that they may retard wort separation (Barrett *et al.*, 1975) and cause haze formation (Coote and Kirshop, 1976). However, very little is known about the role of xylanases and α-L-arabinofuranosidases as processing aids, whereas a wide range of microbial endo-glucanases for this purpose is available (McCleary *et al.*, 1988).

The utilization of cell wall polysaccharides by poultry and pigs has been shown to be improved by the addition cellulases, pectinases and xylanases (Chesson, 1987). However, phenolic compounds and arabinose side-groups restrict the action of glycanases. Enzymatic removal of these groups together with xylanase or polygalacturonase could further promote the hydrolysis of solubilized cell wall polysaccharides (Greve *et al.*, 1984; Hespell and O'Bryan, 1992).

The use of hemicellulases in bleaching is considered as one of the most important large-scale applications of these enzymes. Several commercial xylanase preparations for bleaching are already available and they are used in industrial scale (Viikari *et al.*, 1994). It has also been claimed that application of α-L-arabinofuranosidase would further enhance the delignification of pulp (Pat. Appl. PCT WO 93/20192, 1993; Bezalel *et al.*, 1993). On the other hand, mannanase treatment alone has little effect on the bleachability of conventional softwood kraft pulps, but mannanases can be applied to enhance the effect of xylanases (Buchert *et al.*, 1996; Suurnäkki *et al.*, 1996a). Enzymatic deacetylation of *O*-acetyl-galactoglucomannan has been found to increase the yield of the thermo-mechanical pulping (TMP) process by as much as 1% (Thornton *et al.*, 1994), but the potential of α-galactosidases, removing the α-galactose side-groups, in pulp and paper applications is still unknown.

Microbial α-galactosidase preparations can be used in the modification of various agricultural products. Locust bean gum can form gels both in the native stage and in combination with other polysaccharides such as agar, carrageenan and xanthan. As it is a rather expensive raw material, guar gum, which has poorer gelling properties than LBG, has been modified with α-galactosidases to produce galactomannan with improved gelling capacity. The ability to retain activity even at high substrate concentrations is a prerequisite for the enzymes used in this type of applications. The modified galactomannans can be used in food products as well as in cosmetic and pharmaceutical applications (Pat. Appl. EP 0 121 960 A2, 1984; Critchley, 1987; Bulpin *et al.*, 1990; Pat. EP 0 192 401 B1, 1993). Thermostable α-galactosidases might also be used in the hydrolysis of guar gum for viscosity reduction of the guar-based hydraulic fracturing fluids used in oil and gas well stimulation (McCutchen *et al.*, 1996; Duffaud *et al.*, 1997).

The raffinose-family oligosaccharides in various legumes induce flatulence in humans and many animals because their digestive tracks lack the enzyme required for the hydrolysis of the α-galactosyl linkages present in these oligosaccharides. The application of an α-galactosidase to hydrolyse these compounds prior to utilization could make it possible to use legumes as protein supplements in foods with low protein contents. Several α-galactosidases have been reported to reduce the concentration of these oligosaccharides from e.g. soybean milk (Sugimoto and van Buren, 1970; Thananunkul *et al.*, 1976; Mulimani and Ramalingam, 1995) and cowpea meal (Somiari and Balogh, 1995). A thermostable α-galactosidase has also been used successfully for the removal of D-raffinose from sugar beet syrup (Ganter *et al.*, 1988). In the sugar manufacturing process from beets, D-raffinose accumulates during sucrose crystallization and at a certain concentration prevents the crystallization process.

In addition to using α-galactosidases for the reduction of antinutritional factors of food raw materials, interest in the other reactions catalyzed by these enzymes has recently increased. α-Linked galacto-oligosaccharides could be produced from hydrolysates of lactose, an inexpensive raw material, using the reverse reaction of α-galactosidase or by transgalactosylation reaction with different saccharides or alcohols as acceptors. The synthesised oligosaccharides could serve as growth factors for prebiotic organisms, e.g. bifidobacteria (Hashimoto *et al.*, 1995a, b).

α-Galactosidases also have potential applications in medicine. In studies of blood group conversion, coffee bean α-galactosidase has been used for removing the terminal α-1,3-linked galactose residues responsible for blood group B specificity from the glycoconjugates at the red cell surface. The enzyme has been successfully expressed in a baculovirus system, which may prove to be a valuable system for obtaining enzymatically active α-galactosidase in large quantities for use in B→O blood type conversion (Zhu and Goldstein, 1994). Human Fabry disease patients have an X-linked inborn error in glycolipid metabolism caused by a deficiency of the lysosomal enzyme α-galactosidase A. High levels of catalytically active human α-galactosidase have been produced by insect cells infected with a baculovirus recombinant vector for physical characterization and for the development of new approaches for enzyme therapy (Coppola *et al.*, 1994).

Different glycanases have considerable potential for use in the structural analysis of complex polysaccharides (McCleary and Matheson, 1986). Commercial preparations are often mixtures of both endo- and exo-acting enzymes, which can limit their use in structural studies. However, several different purified enzymes with varying specificities are currently available. Purified glycanases have been used e.g. in the analysis of the fine structures of galactomannans (McCleary, 1994) and pectic polysaccharides (Lerouge *et al.*, 1993). Improved understanding of the mode of action of the enzymes and the structures of various polysaccharides widens the possibilities for their utilization in different applications.

1.6 The background and aims of this study

Various enzymes attacking arabinose and galactose side-groups have been purified and characterized from different microorganisms. Their substrate specificities have been studied using both oligomeric and polymeric substrates. However, the effects of side-group removal on the properties of the substrate in a matrix environment have only been studied in a few cases.

The specific aims of this study were

- production and purification of α-L-arabinofuranosidases and α-galactosidases able to act on polymeric substrates
- characterization of the molecular properties of the purified enzymes
- characterization of the substrate specificities and the modes of action of the purified enzymes
- preliminary characterization of the effects of the purified enzymes in different applications.

2 Materials and methods

Only a summary of the materials and methods used in this work is presented in this section. More detailed information is given in the original publications I–VI.

2.1 Microbial strains and cultivation conditions

2.1.1 Production of α-L-arabinofuranosidases by *Aspergillus terreus*

The culture filtrate used as starting material for the purification of *Aspergillus terreus* VTT-D-82209 α-L-arabinofuranosidases was produced in a laboratory bioreactor (Chemap CF 2000, working volume 15 l) on a medium containing sugar beet extraction waste (30 gl^{-1}), KH_2PO_4 (15 gl^{-1}) and $(NH_4)_2SO_4$ (5 gl^{-1}). Cultivation conditions were: temperature 30°C, pO_2 ≥ 20% (controlled by agitation), aeration 5 l min^{-1} and cultivation time 90 h. The pH was not controlled (I).

2.1.2 Production of α-galactosidases by *Penicillium simplicissimum*

The production of α-galactosidases by *Penicillium simplicissimum* VTT-D-78090 was studied in shake flask cultivations (200 min^{-1}, 30°C) using various carbon sources (insoluble, unsubstituted beechwood xylan, birchwood 4-*O*-methyl-glucuronoxylan, wheat bran, oat spelt xylan, steam-exploded oat husks and oat husk meal) alone (20 gl^{-1}) or in combination with locust bean gum (10 + 10 gl^{-1}). Corn steep solids (10 gl^{-1}) were used as nitrogen source. The reference medium contained peptone (6 gl^{-1}) and locust bean gum (10 gl^{-1}). The basal salts added were KH_2PO_4 (15 gl^{-1}) and $(NH_4)_2SO_4$ (5 gl^{-1}). The initial pH was 4.5 ± 0.1 and cultivation time 4 d (IV).

The culture filtrate for the purification of α-galactosidases was produced in a laboratory bioreactor (Chemap CF 2000, working volume 10 l) on steam-exploded oat husks (10 gl^{-1}), spent grain (10 gl^{-1}) and the basal salts. Cultivation conditions were: 30°C, agitation 400 min^{-1}, aeration 5 l min^{-1}, pH > 3.5 (controlled with NH_4OH). Cultivation time was 77 h (IV).

The production of starting material for the mRNA isolation from *P. simplicissimum* was carried out as described above but on a medium containing oat husk meal (20 g l^{-1}) and spent grain (5 g l^{-1}) instead of steam-exploded oat husks (IV).

Table 13. Compositions of the isolated polymeric substrates used in hydrolysis experiments.

Substrate	Total sugar (%)	Composition (mol-% of monosaccharides)[a]							Obtained from	Use	Reference
		Ara	Xyl	Gal	Man	Glc	Rha/ Fuc	AUA	Ac			
α-1,5-L-Arabinan											
Sugar beet, linear	>99	97	-[b]	0.4	-	-	0.1/-	2.1	-	Megazyme	I	Megazyme
Sugar beet, linear	>90	>90	-	-	-	-	-	-	-	British Sugar	II	Pat. Appl. GB2282920.9, 1989
Sugar beet, branched	>80	>80	-	-	-	-	-	-	-	British Sugar	II	Pat. Appl. GB2282920.9, 1989
Arabino(glucurono)xylan											
Oat spelt	?[b]	9.7	81	1.1	-	3.4	-	4.3	-	Sigma, X-0376	I	Kormelink and Voragen, 1993
Rye flour	>98	46	47	-	6.7	-	-	-	-	Megazyme	I	Megazyme
Wheat flour	>98	41	59	-	-	-	-	-	-	Megazyme	II	Megazyme
Wheat bran BE.RT30	84	41	50	1.4	-	3.2	-	3.7	-	M.Bergmans, WAU	II	Bergmans *et al.*, 1996
Sorghum BE1.2	>95	44	39	2.3	0.6	3.5	0.3	9.9	-	M.Verbruggen, WAU	II	Verbruggen *et al.*, 1995
Sorghum 1K4	>95	25	30	2.7	-	32	0.7	11	-	M.Verbruggen, WAU	II	Verbruggen *et al.*, 1995
Larchwood	?	11	56	3.1	-	26	-	4.2	-	St.Regis Paper Co. USA	II	Kormelink and Voragen, 1993
Softwood kraft pulp	98	10	79	trace	trace	trace	-	11	-	J.Jansson, Central Laboratory	I, II	J. Jansson (personal comm.)
Bleached softwood kraft pulp	100	7.8	92	-	-	-	-	0.2	-	T.Vuorinen, HUT	I	T. Vuorinen (personal comm.)
Arabino-β-1,3/6-D-galactan											
Coffee	85	24	-	65	3.9	2.1	0.4	4.1	-	J. van de Vis, WAU	II	van de Vis, 1994
Larchwood	88	17	-	83	-	-	0.4	1.3	-	J. van de Vis, WAU	II	van de Vis, 1994
Larchwood	>98	17	-	83	-	trace	-	-	-	Sigma, A-2012	V	Acid hydrolysis
Arabino-β-1,4-D-galactan											
Onion	71	7.5	trace	71	5.2	15	0.2	1.0	-	J. van de Vis, WAU	II	van de Vis, 1994
Soya	75	38	1.4	57	-	-	1.8	2.4	-	J. van de Vis, WAU	II	van de Vis, 1994
β-1,4-D-Galactan											
Lupin	?	2.0	0.2	91	-	-	1.8	5.0	-	Megazyme	V	Megazyme

Galactomannan												
Guar gum	?	-	-	38	60	-	0.2	-	-	Sigma, G-4129	V	Acid hydrolysis
Locust bean gum	?	1.5	-	19	76	1.8	1.8	-	-	Sigma, G-0753	V	Acid hydrolysis
Galacto(gluco)mannan												
Radiata pine	91	0.9	3.8	3.4	74	18	-	-	-	T. Clark, Papro, NZ	V	Acid hydrolysis
Oxygen-delignified Radiata pine kraft pulp	?	0.9	22	2.4	65	9.6	-	-	-	T. Clark, Papro, NZ	V	Acid hydrolysis
Spruce TMP	80	2.4	1.1	12	41	11	-	-	33	J. Thornton, SCA	V	J. Thornton (personal comm.)

[a] Ara arabinose; Xyl xylose; gal galactose; Man mannose; Glc glucose; Rha/Fuc rhamnose or fucose; AUA uronic acids (galacturonic acid; (4-O-methyl)-glucuronic acid; hexenuronic acid); Ac acetyl groups

[b] ? unknown; - not detected or not determined

2.2 Substrates

Sugar beet extraction waste (Glc:Ara:Gal:Xyl in a molar ratio of 12:12:4:1) used as a carbon source for the production of *Aspergillus terreus* α-L-arabinofuranosidases was obtained from Cultor Ltd., Finland (I). Medium components used in the production of α-galactosidases by *Penicillium simplicissimum* were Bacto-peptone (Difco, 0118-77-0) corn steep solids (Sigma, C-8160), beechwood xylan (Lenzing AG, Austria), birch xylan (Carl Roth GmbH, Karlsruhe, Germany, No. 7500) and locust bean gum galactomannan (Sigma, G-0753). Steam exploded oat husks, containing 74.5% (w/w) sugars of which 59.9% was glucose, 35.9% xylose, 3.3% arabinose and 0.9% galactose, were kindly supplied by Dr. Jürgen Puls (BFH, Hamburg, Germany). In addition, wheat bran, oat husk meal and spent grain were used as culture media components (IV).

Compositions and sources of the polymeric substrates used in different hydrolysis experiments (I, II and V) are listed in Table 13. In addition, full corn rye flour from Finnish Voima rye (Table 1/I) and pine kraft pulp (kappa 26.9, 8.1% of pulp dw arabinoglucuronoxylan; 7.3% galactoglucomannan) produced in a laboratory digester (Suurnäkki *et al.*, 1996b; V, VI) were used as substrates.

Arabinoxylo-oligosaccharides were isolated and purified from pine pulp (Teleman *et al.*, 1996; Tenkanen *et al.*, 1996) and from wheat flour (van Laere *et al.*, 1997) (II). The ferulic acid substituted oligosaccharides FAX_2 and A_nF (n = 2,3) were isolated from wheat bran (Borneman *et al.*, 1990) and from sugarbeet pulp (Ralet *et al.*, 1994; Kroon *et al.*, 1997), respectively (Fig. 1/III). Their de-esterified counterparts, AX_2 and A_3, were produced by enzymatic hydrolysis as described in III. Arabinobiose (A_2) was purchased from Megazyme. A model lignin-carbohydrate complex (LCC), methyl-3-*O*-(5´-[1´´-(R/S)-C-(3´´´-methoxy-4´´´-hydroxyphenyl)-2´´-(R/S)-*O*-(2´´´-methoxyphenyl)-glyceryl]-α-L-arabinofuranosyl-β-D-xylopyranoside (dilignol-Ara-MeXyl) (Fig. 1/III), was synthesized by Merja Toikka at the University of Helsinki (Toikka *et al.*, 1995). Galactose-containing oligosaccharides used as substrates were melibiose (Fluka AG, 63630), raffinose (Merck, 7549), stachyose (Sigma S4001), verbascose (Megazyme), 6^1-α-D-galactosyl mannotriose ($GalMan_3$, Megazyme) and 6^3,6^4-di-α-D-galactosyl mannopentaose (Gal_2Man_5, Megazyme) (V).

The chemical compositions of the rye and wheat flours and brans used in extraction and baking studies (Section 2.9.2) are presented in Tables 14 and 15.

Table 14. Chemical compositions (g / 100 g dw) of flours and brans used in extraction and baking experiments.

	Wheat flour (extraction)	Wheat flour (baking)	Wheat bran (extraction)	Rye flour (extraction)	Rye bran (extraction)
Protein (N x 6.25)	17	13	19	8.1	16
Fat	- [a]	3.0	-	1.1	4.2
Ash	0.68	0.77	5.2	0.8	4.6
Fibre	1.7	-	42	6.8	31
Starch	74	77	26	-	-
Pentosans (Total / Soluble)	2.0 / 0.45	1.7 / 0.44	20 / 0.45	3.2 / 1.7	18 / 2.4
β-Glucan	0.11	0.36	0.79	0.6	3.8

[a] not detected

Table 15. Monosaccharide compositions (g / 100 g dw) of flours and brans used in extraction studies.

	Wheat flour	Wheat bran	Rye flour	Rye bran
Total monosaccharides	80	57	89	64
Arabinose	0.96	8.0	1.6	6.1
Galactose	0.32	1.1	trace	0.96
Glucose	77	36	86	47
Xylose	1.1	12	1.6	10
Mannose	- [a]	-	0.39	-
Rhamnose	-	-	-	-
Arabinose : Xylose	0.87	0.66	1.0	0.60

[a] not detected

2.3 Enzyme activity assays

2.3.1 α-L-Arabinofuranosidase activity

α-L-Arabinofuranosidase activity was determined using 2 mM *p*-nitrophenyl-α-L-arabinofuranoside (pNPA; Sigma, N-3641) as substrate (Poutanen *et al.*, 1987; Poutanen, 1988). 0.9 ml of the 2 mM substrate in 50 mM sodium citrate buffer, pH 4.0 was incubated with 0.1 ml of enzyme sample at 50°C for 10 min. The reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3. The liberated *p*-nitrophenol was measured spectrophotometrically at 400 nm. *p*-Nitrophenol (Merck 6798) was used as standard.

For screening the α-arabino-furanosidase activity from the chromatographic runs, the method was modified for the microtiter plates (I). In this case, substrate concentration was 1 mM and the pH 5.0, and the absorbance was measured at 405 nm.

2.3.2 α-Galactosidase activity

α-Galactosidase activity was assayed analogously to α-L-arabino-furanosidase activity using 1 mM *p*-nitrophenyl-α-D-galactopyranoside (pNPG; Sigma, N-0877) as substrate at pH 4.0 (Rättö and Poutanen, 1988). For screening the α-galactosidase activity from chromatographic runs, the method was modified on microtiter plates (IV).

2.3.3 Other enzyme activities

Endo-1,4-β-xylanase activity was measured using 1% (w/v) soluble birchwood 4-*O*-methylglucuronoxylan (Roth 7500, Germany) as substrate as described previously by Bailey *et al.* (1992). Endo-1,5-α-arabinanase activity was assayed analogously to the xylanase assay using 0.5% (w/v) raw arabinan (Südzucker, Mannheim, Germany) as substrate.

Endo-1,4-β-mannanase activity was determined as described by Stålbrand *et al.* (1993) using 0.5% (w/v) locust bean gum (Sigma, G-0753) as substrate.

All activities are expressed in SI units, katals (1 kat = 1 mol s^{-1} substrate conversion).

2.4 Purification of enzymes

Three extracellular α-L-arabinofuranosidases of *A. terreus* were purified using bentonite adsorbtion, ion exchange chromatography (CM Sepharose FF, DEAE Sepharose FF, Pharmacia, Sweden), gel filtration (Sephacryl S-100 HR, Pharmacia) and ion exchange chromatography in FPLC (Mono S, HR 5/5, Pharmacia) (Fig. 2/I).

Three extracellular α-galactosidases of *P. simplicissimum* were purified using ion exchange (DEAE Sepharose FF, CM Sepharose FF, Pharmacia), hydrophobic interaction (Octyl Sepharose 4FF, Pharmacia) and gel filtration chromatography (Sephacryl S-100 HR, Pharmacia) (Fig. 1/IV).

2.5 Biochemical characterization of enzymes

The molecular masses of the purified enzymes were determined by SDS-PAGE on PhastSystem (Pharmacia) (I) or according to Laemmli (1970) using a midget electrophoresis unit (Pharmacia) (IV) and by gel filtration chromatography in a Sephacryl S-100 HR (Pharmacia) column (I, IV). The isoelectric points were determined by isoelectric focusing (PhastSystem, Pharmacia) (I, IV). Possible glycosylation of the proteins was detected on IEF gel stained with Schiff's reagent as described by Zacharius *et al.* (1969).

The pH and temperature optima of the purified enzymes were determined by performing the activity assay at different pH values and temperatures. Enzyme stabilities were estimated by incubating the purified enzyme protein at different pH values (pH stability) or at different temperatures (thermal stability) and analyzing the residual activity (I, IV).

The kinetic parameters K_m, V_{max} and k_{cat} for α-L-arabinofuranosidases and α-galactosidases were determined from Lineweaver-Burk plots using the activity assay with 2–10 mM pNPA and 1–50 mM pNPG (IV) as substrate, respectively. In each case, enzyme concentration was kept constant. The end product inhibition was studied by performing the activity assay with substrate solutions (5 and 7 mM pNPA; 2 or 7 mM pNPG (IV)) to which various concentrations of inhibiting sugars were added. The inhibitors used for α-L-arabinofuranosidases and α-galactosidases were 0–300 mM L-arabinose (Merck, 1492) and 0–20 mM D-galactose (Merck, 4058) (IV), respectively.

The N-terminal amino acid sequences of the purified proteins were determined by automated Edman degradation at the University of Kuopio, Finland, using an Applied Biosystems Inc. 477A Pulsed Liquid Phase Protein Sequencer equipped with a 120A Analyzer (HPLC with C18 reverse phase column) (Edman and Begg, 1967; Tempst and Riviere, 1989).

2.6 Cloning and sequencing of the *agl1* gene

The polyclonal antibodies against the purified *P. simplicissimum* AGLI were produced in rabbits at the University of Kuopio, Finland. The antibody was purified from the serum using a Protein A-Sepharose CL-4B column (Pharmacia) as described by Harlow and Lane (1988). cDNA was synthetized from mRNA isolated from the culture supernatant of *P. simplicissimum* according to Chirgwin *et al.* (1979). The *P. simplicissimum* cDNA library was prepared using λ Uni-Zap^{TM} XR vector according to the manufacturer's instructions (Stratagene). The *Eschericia coli* strains XL1 Blue MRF and SORL (Stratagene) were used as hosts. The amplified cDNA

library was screened with the AGLI antibody (Margolles-Clark *et al.*, 1996c) and the positive λ clones were excised *in vivo* into phagemid pBluescriptSK(-) form as described by the manufacturer. Partial DNA sequencing of putative AGLI-encoding plasmids was performed using the Sanger dideoxy-nucleotide method, T7 DNA polymerase (Pharmacia) and T3 or T7 primers. The *agl1* cDNA was sequenced from both strands using synthetic oligomers as primers (IV).

2.7 Hydrolytic properties of the purified enzymes

2.7.1 α-L-Arabinofuranosidases

Substrate specificities of the *A. terreus* α-L-arabinofuranosidases were determined using various isolated oligomeric and polymeric substrates. Hydrolysis experiments were carried out either at pH 4.0 or 5.0 and at 30°C or 40°C. The concentrations of enzymes and substrates varied as indicated in the original publications (I–III).

Other hemicellulolytic enzymes used in the experiments were *Trichoderma reesei* xylanase pI 9.0 (Tenkanen *et al.*, 1992), β-xylosidase (Poutanen and Puls, 1988) and α-L-arabinofuranosidase (Poutanen, 1988). α-L-Arabinofuranosidases A and B were from *Aspergillus niger* (Rombouts *et al.*, 1988). (1,4)-β-D-Arabinoxylan arabinofuranohydrolase (AXH; Kormelink *et al.*, 1991) and xylanases I and III (EXI and EXIII; Kormelink *et al.*, 1993b) were from *A. awamori*. Cinnamoyl esterase (CinnAE; Kroon *et al.*, 1996) and ferulic acid esterase (FAE-III; Faulds and Williamson, 1994) were from *A. niger* strains CS-180 and CBS-120.49, respectively.

2.7.2 α-Galactosidases

Several polymeric and oligomeric substrates were used in order to determine the substrate specificities of the *P. simplicissimum* α-galactosidases. Hydrolysis experiments were carried out at pH 4.0 and 40°C using the enzyme and substrate concentrations indicated in the original publication (V).

Other enzymes used in the experiments were *T. reesei* mannanase (Rättö *et al.*, 1993) and β-mannosidase purified from a commercial mannanase preparation, Gamanase (Novo Nordisk, Bagsvaerd, Denmark) (Tenkanen *et al.*, 1995).

2.8 Chemical analyses

Soluble protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumine as standard. The amount of free galactose (V) and free arabinose (Section 2.9.2) in polysaccharide and flour hydrolysates, respectively, were determined enzymatically (Boehringer Mannheim Test Combination 176 303).

The oligosaccharide and polysaccharide hydrolysates after α-L-arabinofuranosidase and α-galactosidase treatments were analysed either by HPLC (I, III) or by high-performance anion-exchange chromatography (HPAEC) (II, III, V) according to Gruppen *et al.* (1992b), Hausalo (1995), Kroon and Williamson (1996) or Tenkanen *et al.* (1997) as described in the individual publications.

2.9 Use of the purified enzymes in pulp treatment and wheat bread baking

2.9.1 Pulp treatments with *A. terreus* α-L-arabinofuranosidase

The ability of *A. terreus* α-L-arabinofuranosidase A to release arabinose from pine kraft pulp and from pulps bleached with different bleaching sequences was studied using different enzyme dosages (200–2 000 nkat g^{-1} dry pulp) as indicated in VI. In order to examine the effects of arabinose removal on bleaching and lignin extraction, the enzyme was applied to untreated and oxygen-treated pine kraft pulps at two dosages (200 and 2 000 nkat g^{-1} dry pulp) alone and in combination with *T. reesei* xylanase pI 9 (10 nkat g^{-1} dry pulp) (Tenkanen *et al.*, 1992) (VI). After the enzyme treatment, one step peroxide bleaching (Suurnäkki *et al.*, 1994) was performed and the Kappa number (SCAN C1:1977) and brightness (ISO 3688-1977) of the bleached pulps were determined. Pulp properties (tear index, tensile index, stretch, light absorbtion coefficient, opacity, air resistance (Gurley) and density) were determined from handsheets made from industrial ECF softwood pulp treated with α-L-arabinofuranosidase (1 000 nkat g^{-1} dry pulp). In addition, viscosity and fibre length were determined.

2.9.2 Potential of *A. terreus* α-L-arabinofuranosidases in wheat bread baking

Effects of α-L-arabinofuranosidase treatment on the extractability of arabinoxylans and β-glucans from wheat and rye flours (compositions: see Tables 14 and 15) and on the wheat dough and bread properties were studied using an enzyme preparation containing *A. terreus* α-L-

arabinofuranosidases B1 and B2. As the enzymes possessed similar hydrolytic properties, they were not separated from each other for these experiments. In addition, *T. reesei* xylanase pI 9 (Tenkanen *et al.*, 1992) was used.

Extractabilities of arabinoxylans and β-glucans

α-L-Arabinofuranosidase (100 and 1 000 nkat g^{-1} flour) and xylanase (20 nkat g^{-1} flour) were added alone and in combination to rye and wheat flour and bran fractions (10% w/v in 50 mM sodium acetate, pH 4.5). Samples were incubated with mixing at 30°C for 24 h, after which they were centrifuged (2 800 x *g*, 15 min, Hettich Rotanta/P). The supernatant was filtered through twofold wire mesh and heated for 5 min at 100°C to inactivate the enzymes. The extracts were analysed for free arabinose (Boehringer Mannheim Test Combination 176 303), soluble pentosans (Douglas, 1981; Rouau and Surget, 1994), β-glucans (McCleary and Codd, 1991; Enzymatic assay kit No. BBG 8/91, Megazyme (Aust.) Pty. Ltd) and proteins (Kjeldahl; nitrogen x 6.25).

Wheat bread baking

The baking experiments were carried out using α-L-arabinofuranosidase (100 nkat g^{-1} flour) and xylanase (60 nkat g^{-1} flour) alone and in combination. Each dough consisted of wheat flour containing 1% freeze-dried baker's yeast (based on flour weight), 2% sugar and 1.5% salt. The amount of water added (56.5% based on the flour weight) was determined from a farinograph. In the enzyme treatments, part of the water was replaced by the enzyme solution. Enzymes were mixed with water (41°C) before the addition of flour and other components. The doughs were mixed in a fork mixer (John Holmström, Stockholm, Sweden) at 1 200 min^{-1} for 15 min. After a floor time of 20 min at 28°C the dough was cut into pieces (50 g flour) and proofed (35 min, 34°C, 80% humidity) in small pans. The breads were baked at 200°C for 18 min. After cooling the visual appearances of the breads were compared. Loaf volume was measured by rapeseed displacement and the porosity of the breads was evaluated using the Dallman porosity scale (Dallman, 1969).

The bread crumb firmness during storage (20°C, 72 h) was determined as a maximum compression force (40% compression with a 20 mm diameter plunger, compression rate 1.7 mm s^{-1}) (AACC 74-09) using the Texture Profile Analysis (TPA) test. The maximum force needed for compression was calculated from the curves and the results were expressed as means of nine measurements from three bread slices (thickness 1 cm). The variation

of the values within each measurement series was small, whereas greater differences were observed between different measurement series.

Rheological measurements

Doughs in the rheological measurements consisted of 10 g of wheat flour and 56.5% (based on the flour weight) of water containing the appropriate enzymes (α-L-arabinofuranosidase, xylanase or both enzymes; dosages as in the baking studies). Doughs were mixed in a mixograph (Mixograph, National Mfg. Co. USA) for 3.5 min, after which they were incubated in plastic bags at 35°C for 45 min and temperated at 24°C for 10 min before the measurements. Viscoelastic measurements were made using a StressTech constant stress rheometer (ReoLogica Instruments AB, Sweden) with the plate-plate measuring system. The measurement was performed at 1 Hz frequency and a stress of 10 Pa at 24°C. Silicon oil was applied to the plate edges to prevent the sample from drying. Three replicate doughs were made for the reference and each enzyme combination. Five samples (á 0.96-0.98 g) were taken from each dough, and five replicate measurements were made at 10 sec intervals. The first and the last value of each series were neglected and the mean values of the three middle measurements were calculated. The results (storage modulus (G') and phase angle (δ)) were expressed as the average of the five mean values of the three replicate doughs.

3 Results and discussion

3.1 Production of enzymes

3.1.1 Production of α-L-arabinofuranosidases by *A. terreus* (I)

Aspergillus terreus was cultivated on a medium containing sugar beet pulp extraction waste as the sole carbon source. This efficiently induced endo-arabinanase and α-L-arabinofuranosidase production, whereas xylanase production remained low compared to the activity levels obtained when the fungus was grown e.g. on different xylans (Bailey and Poutanen, 1989). This was as expected, because the substrate contained only minor amounts of xylose (see Section 2.2). The production of α-L-arabinofuranosidase was slightly delayed when compared with the endo-arabinanase and xylanase activities (Fig. 1/I), indicating that induction of this enzyme was dependent on the production of arabino-oligosaccharides by the endo-acting arabinanase. The maximum α-L-arabinofuranosidase activity (6 nkat ml^{-1}) was reached after 40 h of cultivation, whereas the highest arabinanase and xylanase activities (20–30 nkat ml^{-1}) were measured after 50 h.

Sugar beet pulp and other materials with high arabinan contents have been quite commonly used as the carbon source when producing arabinan-degrading enzymes e.g. in various strains of *Aspergillus* (Ramón *et al.*, 1993; Fernandez-Espinar *et al.*, 1994; van der Veen *et al.*, 1991) and *Streptomyces* (Kaji *et al.*, 1981; Higashi *et al.*, 1983; Johnson *et al.*, 1988). When xylan-rich media have been used, α-L-arabinofuranosidase production has been dependent on the prior action of xylanase, e.g. in *Streptomyces olivochromogenes* (Johnson *et al.*, 1988).

3.1.2 Production of α-galactosidases by *P. simplicissimum* (IV)

The production of α-galactosidases by *Penicillium simplicissimum* was studied using rather complex and undefined, but readily obtainable and assimilable carbon sources, which were known to induce the production of different hemicellulose-degrading enzymes. As the main aim was to identify an α-galactosidase capable of hydrolysing polymeric galacto(gluco)-mannans, low mannanase activity in the culture broth was desired. The best production of α-galactosidase (28 nkat ml^{-1}) was obtained on steam exploded oat husks supplemented with corn steep solids (Table 1/IV), although steam exploded oat husks contain only a small amount of galactose (0.7% w/w). This medium also had the advantage of not inducing mannanase production, which was produced in all media containing locust bean gum galactomannan. α-Galactosidase production was induced notably by locust bean gum only when isolated xylans or wheat bran were used as supplementary carbon source. In the larger cultivation, performed on a

medium containing steam exploded oat husks supplemented with spent grain, α-galactosidase activity became detectable after 20 h of cultivation and increased until 72 h. The activity at the end of cultivation (77 h) was 35 nkat ml^{-1}. Mannanase activity was already detected in the beginning of the cultivation. It was greater than the α-galactosidase activity until about 55 h, after which it remained rather constant (25 nkat ml^{-1}).

Dey *et al.* (1993) used guar galactomannan to induce α-galactosidase production in *Penicillium ochrochloron*, and the induction of mannanase by galactomannan was also observed. They reported that guar gum induced only guar galactomannan-degrading α-galactosidase, whereas melibiose and raffinose-family oligosaccharides induced an enzyme incapable of hydrolysing galactose from guar gum. A similar relationship between the carbon source and the substrate specificity of the α-galactosidase produced was observed in *Bacteroides ovatus* (Gherardini *et al.*, 1985). However, the induction of α-galactosidase by galactomannans has often been associated with the production of mannanase depolymerizing the substrate to oligo-saccharides (Gherardini *et al.*, 1985; Talbot and Sygush, 1990).

3.2 Characterization of the purified enzymes

3.2.1 Molecular properties of *A. terreus* α-L-arabinofuranosidases (I and unpublished results)

Three α-L-arabinofuranosidases (αAra) were purified to homogeneity from *A. terreus* as judged by IEF-electrophoresis (Fig. 3A/I). The molecular properties of the purified enzymes are presented in Table 16. Two of the enzymes, αAra B1 and B2, could only be separated after FPLC-chromatography in a cation-exchange column. They had the same molecular mass as determined by SDS-PAGE (59 kDa) (Fig. 3B/I), but they had different mobilities in IEF gel electrophoresis (pI 8.3 and pI 8.5; Fig. 3A/I). αAra A was smaller (39 kDa) and less basic (pI 7.5) than the other two enzymes (Fig. 3/I). αAra A was unglycosylated, whereas αAra B1 and B2 gave a colour reaction when stained with Schiff's reagent. The carbohydrate compositions of these enzymes were not determined further. All enzymes were optimally active at pH 3.5–4.5. αAra B2 was stable in a pH range from 4.5 to 7 and at temperatures up to 55–60°C, whereas αAra A and αAra B1 were stable at lower and narrower pH ranges and at lower temperatures.

Table 16. Properties of the three A. terreus α-L-arabinofuranosidases.

	αAra A	αAra B1	αAra B2
pI[a]	7.5	8.3	8.5
M_r[b] (kDa)	39	59	59
pH-optimum[c]	3.5–4.5	3.5–4.5	3.5–4.5
pH-stability[d]	3.5–5.0	3.5–5.5	4.5–7.0
T-stability[e]	40°C	45°C	55–60°C
Specific activity[f] (nkat mg^{-1})	1 210	2 220	1 980
k_{cat} [g] (s^{-1})	57	174	162
K_m [g] (mM)	0.43	0.65	0.76
k_{cat} / K_m [g] (s^{-1} mM^{-1})	132	268	213

[a] Isoelectric point determined by IEF

[b] Molecular mass determined by SDS-PAGE

[c] Normal activity assay

[d] The pH range within which the enzyme retained more than 80% of its activity for 24 h.

[e] The highest temperature at which the enzyme retained more than 80% of its activity for 24 h.

[f] Based on the normal activity assay (2 mM *p*-nitrophenyl-α-L-arabinofuranoside, pNPA)

[g] Against pNPA, calculated from Lineweaver-Burk plots

The maximum velocities V_{max} for αAra A, αAraB1 and αAra B2 were 1 460, 2 950 and 2 740 nkat mg^{-1}, respectively, when determined with pNPA as substrate. The turnover numbers (k_{cat}) of αAraB1 and αAraB2 were higher than that of αAraA indicating higher efficiency of their active sites. αAra B1 had the highest specificity towards pNPA as based on the ratio of k_{cat}/K_m. In order to obtain information about end product inhibition of the enzymes, L-arabinose was added to the assay and the inhibition constants were determined from Dixon plots. The K_I values determined for the three enzymes were of the same magnitude (αAra A: 31 mM; αAra B1: 21 mM and α-Ara B2: 18 mM), but it appeared that αAra A was slightly more resistant to the inhibition. Furthermore, the arabinose used as an inhibitor was only 99% pure and thus the impurities may also have contributed to the inhibition, especially at high inhibitor concentrations.

The K_m values of various fungal α-L-arabinofuranosidases, determined using pNPA as substrate, have ranged from 0.18 mM for *Penicillium*

capsulatum α-L-arabinofuranosidase I (Filho *et al.*, 1996) up to 5 mM determined for the enzyme of *A niger* KI (Tagawa and Kaji, 1988). For bacterial enzymes the range is even greater, from 0.08 mM for *Streptomyces purpurascens* α-L-arabinofuranosidase (Komae *et al.*, 1982) to 12.5 mM for α-L-arabinofuranosidase C2 of *Streptomyces diastaticus* ET (Tajana *et al.*, 1992). The values closest to those of the *A. terreus* enzymes studied in this work were those of *A. niger* α-L-arabinofuranosidases A and B: 0.6 mM and 0.5 mM, respectively (Rombouts *et al.*, 1988; van der Veen *et al.*, 1991). However, the comparison of these values is uncertain due to the different incubation conditions used.

A. terreus αAra A	1	GSCDI YSAGG KPCVA AHSKT	20
A. terreus αAra B1	1	GPCDI YSSGG TPCVA AHSTT	20
A. terreus αAra B2	1	GPCDI YSSGG TPCVA AHSTT	20
T. reesei ABFI	22	GPCDI YSSGG TPCVA AHSTT	41
A. niger Arafur B	19	GPCDI YEAGD TPCVA AHSTT	38
A. niger Arafur A	26	ISLKV STQGG NSSSP ILYGF	45
S. lividans	38	AGSGA LRGAG SNRCL DVLGG	57

Figure 1. Comparison of N-terminal amino acid sequences of Aspergillus terreus α-L-arabinofuranosidases with those of A. niger (Flipphi et al., 1993 a,b), Trichoderma reesei (Margolles-Clark et al., 1996a) and Streptomyces lividans (Vincent et al., 1997) α-L-arabinofuranosidases. Identical amino acids are boxed.

The N-terminal amino acid sequences (20 amino acids) of the *A. terreus* α-L-arabinofuranosidases B1 and B2 (Fig. 1) were found to be identical with that of *T. reesei* α-L-arabinofuranosidase ABFI, which is rather similar to the amino acid sequence of *A. niger* α-L-arabinofuranosidase B (Margolles-Clark *et al.*, 1996a; Flipphi *et al.*, 1993b). *T. reesei* ABFI and *A. niger* α-arabinosfuranosidase B belong to the glycosyl hydrolase family 54. Furthermore, the N-terminus of αAra A had only four different amino acids in the sequence of 20 aa as compared with the other two *A. terreus* enzymes. α-L-Arabinofuranosidase A of *A. niger* (Flipphi *et al.*, 1993a) and *Streptomyces lividans* α-L-arabinofuranosidase (Vincent *et al.*, 1997) belong to the glycosyl hydrolase families 51 and 62, respectively, and their N-terminal amino acid sequences were unlike the sequences of the *A. terreus* α-L-arabinofuranosidases. The 124 amino acids in the N-terminal

sequence of *S. lividans* α-L-arabinofuranosidase starting from aa 38 are known to code a xylan binding domain (Vincent *et al.*, 1997).

Both the physical characteristics and the N-terminal amino acid sequences indicated high similarity between the three *A. terreus* enzymes, and most probably the αAras B1 and B2 are encoded by a single gene. Their molecular properties resembled those of other α-L-arabinofuranosidases isolated from various *Aspergillus* and other fungal strains, although their isoelectric points were more alkaline (Table 4, page 22). Similarly to *A. terreus*, several other *Aspergillus* strains have produced at least two α-L-arabinofuranosidases with slightly different molecular and physical characteristics. In addition, some of these strains have produced an endo-arabinanase degrading the backbone of polymeric arabinans especially when grown on sugar beet pulp or arabinan (Beldman *et al*., 1993; Rombouts *et al.*, 1988; van der Veen *et al.*, 1991). In this work the presence of an endo-acting enzyme was also observed, but its properties were not studied further.

3.2.2 Substrate specificities of *A. terreus* α-L-arabinofuranosidases (I–III)

The purified *A. terreus* α-L-arabinofuranosidases possessed the ability to release arabinose from various polymeric substrates (Table 3/I, Tables 3 and 4/II). The degrees of hydrolysis of the different polymers by α-arabino-furanosidases B1 and B2 were slightly higher than by α-arabino-furanosidase A. During an extensive treatment, up to 80% of the arabinose residues present in kraft pulp arabinoglucuronoxylan and about one third of those in cereal arabinoxylans were liberated, whereas the hydrolysis of α-1,5-linked arabinose from linear arabinan was clearly lower (Table 3/I). In cereal arabinoxylans, the xylose residues are heavily substituted with arabinose at C-3 and/or at C-2 even at adjacent backbone residues, thus restricting the accessibility of the substrate to enzymes. As the enzyme dosage was increased in a 24 h hydrolysis of softwood kraft pulp arabinoglucurono-xylan, the rate of hydrolysis increased and the maximum amount of arabinose, about 80% of theoretical, was liberated already after 5–10 hours of incubation (Fig. 5/I). Thus it appears that the remaining arabinose residues are located in such positions, in adjacent xylose residues or next to a (methyl)glucuronic acid residue, that the action of α-L-arabino-furanosidases is hindered. When the treatment was performed in combination with xylanase, the action of α-L-arabinofuranosidase was slightly improved, but the hydrolysate clearly contained increased amounts of xylose and short-chain xylo-oligosaccharides as compared to those obtained by xylanase alone (Table 3/I; Fig. 6/I), indicating that the removal of arabinose side-groups created new reaction sites for xylanase. Synergism between α-L-arabinofuranosidase and xylanase has also been observed in

several other fungi, e.g. *A. awamori* (Kormelink *et al.*, 1991; Wood and McCrae, 1996) and *Trichoderma reesei* (Poutanen, 1988).

Highly branched pectic substrates, sugar beet arabinan and soya arabino-1,4-β-D-galactan appeared to be the best substrates for all three α-L-arabinofuranosidases on the basis of the amount of arabinose released per mg protein added (Tables 3 and 4/II). As in the extensive treatment, during the short incubation the degradation of linear arabinan was clearly slower than that of the branched arabinan. This was probably due to the lower concentration of end-groups susceptible to hydrolysis rather than to inability of the α-L-arabinofuranosidases to cleave the α-1,5-linked arabinose residues. The less efficient hydrolysis of arabino-1,3/6-β-D-galactans compared to that of arabino-1,4-β-D-galactans might be due to the higher degree of branching of the backbone by the galactose side-chains. The arabinose residues in these polymers may also have been further substituted with other sugar residues (van de Vis, 1994). Surprisingly, a higher amount of arabinose was released from the more branched cereal arabinoxylans than from the polymers isolated from larchwood or softwood pulp. This might be explained by the higher absolute amount of arabinose present in cereal arabinoxylans and thus susceptible for hydrolysis. However, the high degree of substitution and the double-substituted xylose residues restrict the action of the enzymes.

A. terreus α-L-arabinofuranosidases appeared to act in a similar way against the arabinoxylo-oligosaccharide mixtures obtained by xylanase treatment (Table 5/II, Fig. 2/II). They preferred substrates with arabinose attached to the non-reducing end xylose residue, whereas the oligosaccharides with arabinose attached to internal xylose residues were only poorly degraded (Table 6/II; Table 1/III). Furthermore, the enzymes were unable to release α-1,2-linked arabinose residues even from the non-reducing end xylose (Table 6/II). On the other hand, linear α-1,5-arabinobiose and arabinotriose with an α-1,3-linked residue at the non-reducing end were at least partially hydrolysed to arabinose (Table 1/III). In this respect, the *A. terreus* enzymes resembled the α-L-arabinofuranosidase B of *A. niger* (Kormelink *et al.*, 1993a) and the α-L-arabinofuranosidase of *A. niger* 5-16 (Kaneko *et al.*, 1993), which were only able to release arabinose residues attached to C-3 of single-substituted non-reducing end xylose residues of arabinoxylo-oligosaccharides. Ferulic acid substitution prevented the release of arabinose when it was attached to the residue to be liberated (FAX_2, FA_2), whereas the precense of the feruloyl substituent at C-2 of the arabinose residue to which the "target" arabinose was attached (C-3) did not affect the hydrolysis (FA_3; Tables 2 and 3/III). The α-L-arabinofuranosidase of *Butyrivibrio fibrisolvens* was unable to release phenolic acid substituted arabinose residues from FAX_2 and PAX_2 (Hespell and O'Bryan, 1992), whereas the enzyme isolated from *A. awamori* was capable of removing

considerable amounts of feruloylated arabinose residues from intact and xylanase-hydrolysed wheat arabinoxylan (Wood and McCrae, 1996).

Although the esterified ferulic acid subsitutent prevented the action of α-L-arabinofuranosidase B2, the enzyme was able to hydrolyse the linkage between arabinose and xylose in the LCC model compound despite the bulky dilignol subtituent ether-linked to *O*-5 of arabinose (Fig. 2/III). The degradation of the LCC model compound by αAra B2 to dilignol-L-arabinose, methyl-3-*O*-xylopyranoside and arabinose was detected by NMR spectroscopy (Fig. 2/III). Furthermore, as no methyl-3-*O*-(α-L-arabino-furanosyl)-β-D-xylopyranoside was detected, it was concluded that the enzyme did not hydrolyse the linkage between the dilignol and carbohydrate moieties. Thus it is probable that the enzyme was able to attack the linkage between arabinose and xylose residues from the xylose side. Unfortunately, the oligosaccharides (e.g. FAX or LCC-model compound with two xylose residues) needed for the confirmation of this theory were not available.

On the basis of the results obtained, the three *A. terreus* α-L-arabinofuranosidases all belong to the group of arabinofuranosidase B-type enzymes, as classified by Beldman *et al.* (1997), possessing a broad specificity towards polymeric substrates including arabinans, arabino-galactans and arabinoxylans, and being able to remove arabinose only from the non-reducing terminal xylose residues of arabinoxylo-oligosaccharides. Although the majority of the purified α-L-arabinofuranosidases appear to be enzymes of this type notable differences in the rates of hydrolysis have been observed between them (Table 4, page 22). The enzymes isolated in the present study showed highly similar hydrolytic properties to those of *A. niger* α-L-arabinofuranosidase B (Rombouts *et al.*, 1988), as indicated in publication II.

3.2.3 Molecular properties of *Penicillium simplicissimum* α-galactosidases (IV)

Three α-galactosidases were purifiedfrom *Penicillium simplicissimum*. The molecular properties of the purified enzymes are presented in Table 17. AGLI and AGLIII were monomeric proteins with similar molecular masses (60–62 kDa) as determined by SDS-PAGE (Fig. 2/IV), but AGLI was a more acidic protein (pI 5.2) than AGLIII (pI 7.0). The molecular mass of AGLI obtained by gel filtration corresponded to the value on SDS-PAGE, but AGLIII was adsorbed to the gel filtration column and delayed, and thus its molecular mass could not be determined using this method. Both proteins were glycosylated, but the amount and type of glycosylation were not studied further. The optimal pH for their action was around pH 4. AGLIII had a slightly broader pH stability range and it retained its activity

at somewhat higher temperatures than AGLI. AGLII was clearly different from the other two enzymes. It had a molecular mass of 83–85 kDa as determined by SDS-PAGE (Fig. 2/IV), and was either agglomerative or composed of several subunits (400 kDa by gel filtration). Similarly to the other two enzymes, AGLII was glycosylated. Furthermore, it was stable even under slightly alkaline conditions (pH 4–8) and retained more than 80% of its activity at pH 5 and 60°C and even at pH 8 and 50°C for 24 h.

AGLII had considerably higher specific activity, when determined using pNPG as substrate, than AGLI and AGLIII. The two latter enzymes were inhibited already at relatively low substrate concentrations (Fig. 3B/IV) and therefore the kinetic parameters K_m,V_{max} and k_{cat} could only be determined for AGLII. The obtained values (0.75 mM, 26 600 nkat mg^{-1} and 10 650 s^{-1}, respectively) demonstrated that the enzyme had high affinity and specificity towards small substrates (Fig. 3A/IV; Table 17). This has also been typical for other large α-galactosidases isolated from fungal (Ademark *et al.*, 1997; Civas *et al.*, 1984a; Shibuya *et al.*, 1997) or bacterial strains (Aduse-Opoku *et al.*, 1991a; Gherardini *et al.*, 1985). On the other hand, inhibition of α-galactosidase by higher concentrations of pNPG has also been observed and has been proposed to be due to the formation of a complex of the enzyme with two substrate molecules (Dey *et al.*, 1983). AGLII was also most resistant towards end product inhibition by galactose (Fig. 3C/IV). AGLI and AGLIII lost 80–90% of their activity when 20 mM (3.6 gl^{-1}) galactose was added, whereas AGLII retained about 65% of its activity at this galactose concentration. However, the impurities in the galactose used (98% purity) have possibly also contributed to the inhibition. D-Galactose is known to be a powerful and competitive inhibitor for α-galactosidases (Dey and Pridham, 1972). In addition, the structural analogues of D-galactose, such as L-arabinose and D-fucose, can also inhibit the enzyme.

During the purification process, a fourth α-galactosidase was also detected and separated (results not shown). It was assumed to be an isomeric or degraded form of AGLIII, because of its slightly smaller molecular mass and identical N-terminal amino acid sequence. However, the protein appeared to be unstable during storage, and thus the enzyme was not studied further.

The molecular masses of *P. simplicissimum* α-galactosidases AGLI and AGLIII were rather similar to those of α-galactosidases isolated from other *Penicillium* strains (Dey *et al.*, 1993; Pat. EP 0 192 401 B1, 1993; Elshafei *et al.*, 1993; Park *et al.*, 1991) and from *Trichoderma reesei* (Zeilinger *et al.*, 1993; Margolles-Clark *et al.*, 1996b; Kachurin *et al.*, 1995), but the *P. simplicissimum* enzymes were more basic proteins. Similarly to *P. simplicissimum* some other fungal strains have produced α-galactosidases with highly versatile molecular properties. For example,

Table 17. Properties of the three P. simplicissimum α-galactosidases.

	AGLI	AGLII	AGLIII
pI[a]	5.2	4.4	7.0
M_r[b] (kDa)	60–62[b1]	83–85[b1]	60–62[b1]
	55[b2]	400[b2]	-[h]
pH-optimum[c]	3.5–4.5	4.0–5.0	3.5–4.5
pH-stability[d]	3.0–4.5	4.0–8.0	4.0–6.0
T-stability[e]	40°C (pH 4.5)	60°C (pH 5.0)	45°C (pH 4.5)
		50°C (pH 8.0)	
Specific activity[f] (nkat mg^{-1})	3 200	13 900	2 400
k_{cat} [g] (s^{-1})	-[i]	10 650	-[i]
K_m [g] (mM)	-[i]	0.75	-[i]
k_{cat} / K_m[g] (s^{-1} mM^{-1})	-[i]	14 200	-[i]

[a] Isoelectric point determined by IEF

[b] Molecular mass determined by b1) SDS-PAGE and b2) gel filtration

[c] Normal activity assay

[d] The pH range within which the enzyme retained more than 80% of its activity for 24 h

[e] The highest temperature at which the enzyme retained more than 80% of its activity for 24 h

[f] Based on the normal activity assay (1 mM *p*-nitrophenol-α-D-galactopyranoside (pNPG))

[g] Against pNPG, calculated from Lineweaver-Burk plots

[h] Could not be determined. Protein was bound to the column

[i] Could not be determined

Aspergillus niger (Ademark *et al.*, 1997), *A. tamarii* (Civas *et al.*, 1984a,b) and *Mortierella vinacea* (Suzuki *et al.*, 1970; Kaneko *et al.*, 1990; Shibuya *et al.*, 1995b; Shibuya *et al.*, 1997) produced α-galactosidases of which at least one differed considerably from the other enzymes (Table 8, page 38). The bacterial α-galactosidases have typically been larger and composed of several subunits compared to fungal enzymes (Table 9, page 42). Thus the *P. simplicissimum* AGLII resembled more the bacterial or *Aspergillus* than the other *Penicillium* α-galactosidases.

The N-terminal amino acid sequences were determined for the three purified α-galactosidases (Fig. 4/IV). α-Galactosidases have been classified into three glycosyl hydrolase families; 4, 27 and 36 (SWISS-PROT database, Release February 13, 1998). The N-terminus of AGLIII (17 aa) showed similarity with the N-termini of α-galactosidases belonging to the family 27. The highest similarities were found with the sequences encoding α-galactosidases from *Mortierella vinacea* (Shibuya *et al.*, 1995b), *Saccharomyces cerevisiae MEL1* (Liljeström, 1985) and *Cyamopsis tetragonoloba* (guar) (Overbeeke *et al.*, 1989). In addition, AGLIII shared some similarity with AGLI. Interestingly, the N-terminal aa sequence (20 aa) of AGLII was completely different from the other sequences and no similarity was found with the published amino acid sequences of α-galactosidases or other glycosyl hydrolases (SWISS-PROT database, Release February 13, 1998).

One of the aims of this study was to isolate enzymes active against polymeric substrates. *P. simplicissimum* AGLI was found to be able to release galactose from polymeric galacto(gluco)mannans (see Section 3.2.4), and thus the gene encoding AGLI was cloned. A polyclonal antibody was raised against the purified AGLI protein and the cross-reactivities of the other two enzymes were tested using ELISA. AGLIII, which had partial N-terminal amino acid sequence similarity with AGLI, gave about 50-fold weaker response than AGLI, whereas AGLII did not react with the antibody.

The deduced protein sequence of AGLI encoded by the *agl1* gene was composed of 435 aa including a predicted signal sequence of 18 aa (Fig. 5/IV). The mature protein was 417 aa long, corresponding to a molecular mass of 45 867 Da, which was about 25% lower than that obtained for the purified glycosylated protein (60–62 kDa). Five possible N-glycosylation sites (Asn-X-Ser/Thr; X not Pro) were found in the sequence. The other characterized genes encoding microbial or plant α-galactosidases have contained signal sequences of 18 to 31 amino acids and several putative N-glycosylation sites (Table 11, page 46). The aa sequence of AGLI showed significant similarity (73%) with the sequence encoding AGLI of *T. reesei* (Margolles-Clark *et al.*, 1996b). Furthermore, it shared 57–58% similarity with α-galactosidases isolated from the yeast *Saccharomyces cerevisiae* (*MEL5*; SWISS-PROT P41946) and from the plants *Cyamopsis tetragonoloba* (guar; Overbeeke *et al.*, 1989) and *Coffea arabica* (Zhu and Goldstein, 1994). All these enzymes have been classified into the glycosyl hydrolase family 27. The most highly conserved regions were located in the amino terminal halves of the sequences. The tyrosine residues at positions 108 and 158 of the sequence of *C. arabica* α-galactosidase (Zhu *et al.*, 1995) and the aspartic acid at position 272 of the guar (Overbeeke *et al.*, 1989) and the proline at position 40 of human (Ishii *et al.*, 1992) α-

galactosidase sequences, which have been suggested to be essential residues for the actvity of the enzyme, were also conserved in the *P. simplicissimum* AGLI sequence.

3.2.4 Substrate specificities of *P. simplicissimum* α-galactosidases (V)

The three *Penicillium simplicissimum* α-galactosidases showed clear differences in their hydrolytic properties. AGLI liberated galactose residues from both oligomeric and polymeric substrates (Figs. 1A, 2A and 2B/V). The poorer activity towards longer raffinose-family oligosaccharides was probably partially due to end product inhibition by the galactose released rather than to inability of the enzyme to liberate all the galactose residues present in the substrate (Fig. 1A/V). AGLI was highly active against different polymeric galacto(gluco)mannans (Figs. 2A and 2B/V). Even with a small enzyme dosage (500 nkat g^{-1}), about half of the galactose residues present in these polymers could be released, except in the case of highly substituted guar galactomannan (Fig. 2A/V). When a tenfold enzyme dosage was applied, the amount of free galactose increased to 60–90% of theoretical (Fig. 2B/V). The addition of mannanase facilitated the action of AGLI to some extent, especially in the case of low α-galactosidase dosage, and when β-mannosidase was also included the degree of hydrolysis increased to almost 100%. However, the Radiata pine galactoglucomannan was more resistant to hydrolysis even when the mixture of enzymes was used.

The steric hindrance of the enzyme action caused by heavy substitution of the mannan backbone was also observed when isolated galactomanno-oligosaccharides were used as substrates (Table 1/V). AGLI was able to release galactose residues attached to the reducing end mannose units, although the degradation of galactomannotriose ($GalMan_3$) was improved by the addition of β-mannosidase (Table 1/V; Fig. 2). When two galactose residues were linked to adjacent mannose residues (Gal_2Man_5) the degree of hydrolysis was lower, presumably due to the steric hindrace (Table 1/V). However, the addition of β-mannosidase also enhanced the liberation of galactose from Gal_2Man_5, obviously because removal of the unsubstituted mannose residue from the non-reducing terminus allowed the galactose substituent attached to C-6 of the now terminal mannose residue to turn to a spatially more favourable position. Furthermore, AGLI and β-mannosidase were shown to act synergistically, as the highest degree of hydrolysis was obtained when both enzymes were applied simultaneously (Table 1/V).

The ability of AGLI to liberate galactose was not restricted to isolated galacto(gluco)mannans. It released about 10% and 22% of the galactose present in pine kraft pulp alone and in combination with mannanase,

respectively, corresponding to about 42% and 92%, respectively, of the galactose present in the galactoglucomannan accessible to mannanase alone (Suurnäkki, 1996). Recently it has been reported that the action of enzymes might be either chemically or physically limited by the relatively high content of lignin present in the surface material of fibres in pulp (Suurnäkki *et al.*, 1996c). The effect of galactose removal on bleaching or on pulp properties was not studied further. No other reports concerning the action of α-galactosidases in pulp have been published.

AGLII was clearly distinct from the other two α-galactosidases studied, as it released only terminal non-reducing galactose residues. It degraded melibiose and raffinose-family oligosaccharides extensively and its action was only slightly affected by the chain length of the oligosaccharide (Fig. 1B/V). On the other hand, AGLII was able to release only minor amounts of galactose from polymeric galacto(gluco)mannans even at high enzyme dosages (Fig. 2D/V). Its action was considerably facilitated by the backbone-degrading enzymes. However, degradation of the highly substituted locust bean gum and especially guar gum galactomannans remained low even after addition of the endo-acting enzymes, obviously because of the lack of unsubstituted reaction sites. The pine and pine pulp galactoglucomannans are less densely substituted and thus the depolymerizing enzymes were able to act and create oligosaccharides substituted by a galactose residue, which could then be attacked by AGLII. The requirement of the terminal non-reducing end position for the galactose residue to be released by AGLII was further proved by the hydrolysis of 6^1-α-D-galactosyl mannotriose ($GalMan_3$), which was degraded only in combination with β-mannosidase (Table 1/V; Fig. 2).

Despite its rather similar physical properties to those of AGLI, AGLIII was less efficient in the liberation of galactose from either oligomeric or polymeric substrates (Figs. 1 and 2/V; Table 1/V). The activity of AGLIII on oligosaccharides with galactose attached to the non-reducing end terminus decreased with increasing chain-length, comparably to AGLI, but the total degree of hydrolysis was clearly lower (Fig. 1/V). Furthermore, AGLIII was unable to degrade the isolated galactomanno-oligosaccharides. The degradation of polymeric galacto(gluco)mannans by AGLIII was also less efficient than by AGLI and its action was notably facilitated by the addition of backbone-degrading enzymes (Fig. 2D/V). As the fungus *P. simplicissimum* is a rather efficient producer of enzymes for the degradation of both polymeric and oligomeric substrates, the biological function of AGLIII, which possessed relatively poor activity towards both types of substrates, remains unclear.

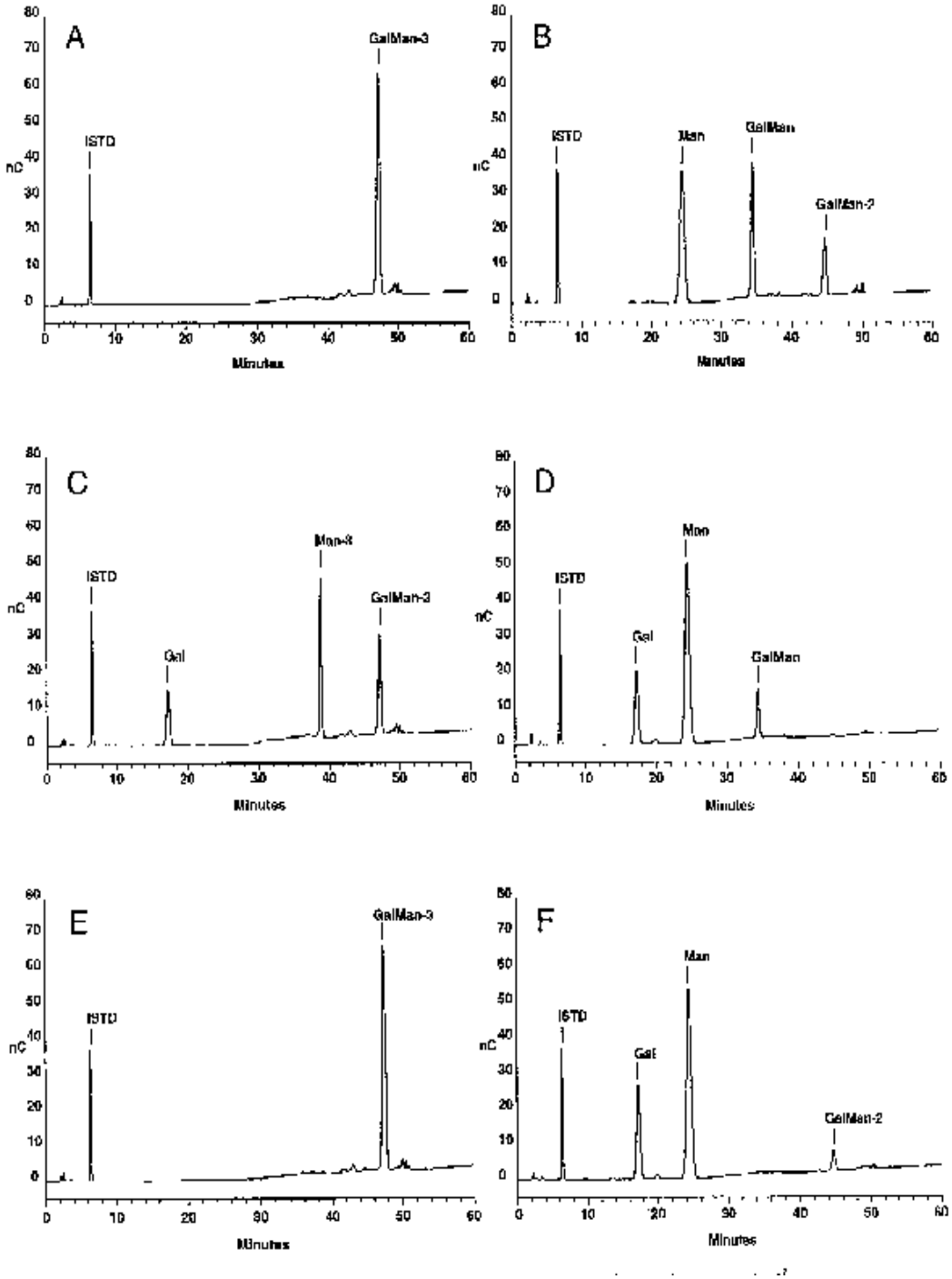

Figure 2. HPAEC-PAD chromatograms of the degradation of 6^1-α-D-galactosyl mannotriose ($GalMan_3$). A) Reference; B) ß-Mannosidase (ßM); C) AGLI; D) AGLI + ßM; E) AGLII; F) AGLII + ßM. Incubation: α-Galactosidase 1 000 nkat g^{-1}, ß-mannosidase 500 nkat g^{-1}, substrate concentration 0.5 mg ml^{-1}, pH 4.0, 40°C, 24h.

Several α-galactosidases belonging to the glycosyl hydrolase family 27 have been reported to be able to release galactose residues from polymeric substrates. Some of these, e.g. α-galactosidases of guar (Overbeeke *et al.*, 1989) and coffee bean (Zhu and Goldstein, 1994) and AGLI of *T. reesei* (Margolles-Clark *et al.*, 1996b), shared homologous regions with *P. simplicissimum* AGLI and AGLIII. Unfortunately, the amino acid sequences of α-galactosidases of *A. niger* (Bahl and Agrawal, 1969), *A. tamarii* (Civas *et al.*, 1984b) and various *Penicillium* species (Pat. EP 0 192 401 B1, 1993; Dey *et al.*, 1993) also able to release galactose from intact galactomannans have not been published. Despite the genetical similarity, some members of the family 27, such as *Mortierella vinacea* AGL1 (Kaneko *et al.*, 1990; Shibuya *et al.*, 1995b), could rather be classified as melibiases, preferably hydrolysing melibiose and other low molecular weight compounds. *T. reesei* AGLII and AGLIII (Margolles-Clark *et al.*, 1996b) and α-galactosidases from S*treptococcus mutans* (Aduse-Opoku *et al.*, 1991a) and *Escherichia coli* (Aslanidis *et al.*, 1989) possessing this type of activity have been classified to the glycosyl hydrolase family 36. Surprisingly, the N-terminal amino acid sequence of *P. simplicissimum* AGLII, which had similar hydrolytic properties to these enzymes, showed no similarity with their aa sequences. However, a complete amino acid sequence would be required before further conclusions about the amino acid similarity of AGLII could be drawn. α-Galactosidases belonging to different glycosyl hydrolase families still contain highly conserved regions and it has been suggested that they might all have a common ancestor from which they have developed (Dagnall *et al.*, 1995; Henrissat and Bairoch, 1996).

3.3 Potential applications of α-L-arabinofuranosidases

3.3.1 Effects of α-L-arabinofuranosidase treatment on kraft pulp properties (VI and unpublished results)

The effects of α-L-arabinofuranosidase treatment on pulp properties were studied using various bleached and unbleached pulps. In addition to being able to release about 70% of the arabinose present in arabinoglucuronoxylan isolated from pine kraft pulp, *A. terreus* α-L-arabinofuranosidase A released 20–25% of the arabinose from unbleaced and peroxide, ozone or chlorine dioxide bleached (TCF:QPPP; ECF: OZEP, ODEDED) softwood kraft pulps (Fig. 3). The bleaching sequence used or the amount and ratio of uronic acid substituents (MeGlcA and HexA) in the pulps (Table 1/VI) did not appear to affect the removal of arabinose.

When the α-L-arabinofuranosidase-treated pulps (pine kraft and oxygen-treated pine kraft) were bleached with one-step peroxide bleaching, only minor effects on the kappa number and brightness of the pulp were

observed (Fig. 2/VI). However, in a combined treatment, the addition of α-L-arabinofuranosidase clearly decreased the positive bleaching effects obtained with xylanase, although the release of free arabinose and reducing sugars by α-L-arabinofuranosidase and xylanase were not affected as compared with the individual treatments. The removal of arabinose groups appeared to reduce the extraction of lignin from unbleached pulp. It is possible that xylan from which arabinose side-groups have been removed can form more crystalline or less soluble structures, consequently reducing the extractability of lignin. Contradictory results were obtained by Bezalel *et al.* (1993) and Rosenberg and Shoham (Pat. Appl. PCT WO93/20192, 1993), who reported that the thermostable α-L-arabinofuranosidase of *Bacillus stearothermophilus* partially delignified softwood kraft pulp alone, but acted synergistically with a thermophilic xylanase resulting in a net release of about 19% of the lignin under alkaline conditions.

The overall effects of the α-L-arabinofuranosidase treatment on the pulp properties measured from the handsheets made after the enzyme treatment were only minor, although a decrease of 8–11% in the number of PFI -beating revolutions was detected as compared to the control. The proposed denser structure of pulp xylan after α-L-arabinofuranosidase treatment was supported by the slight increase observed in the air resistance (Gurley) (Fig. 3/VI).

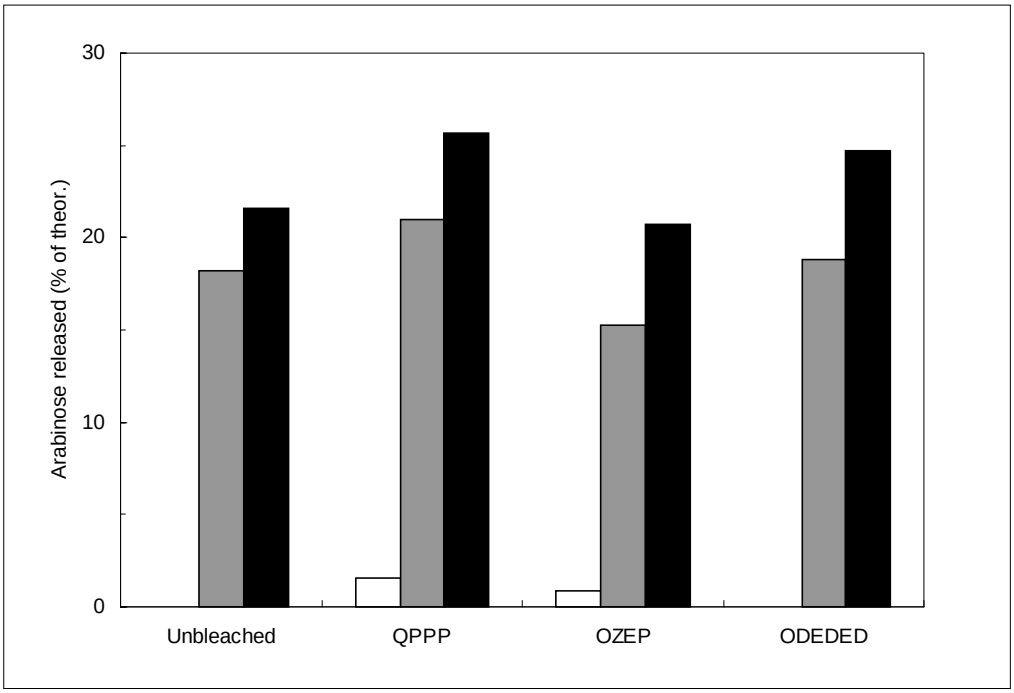

Figure 3. Hydrolysis of arabinose from unbleached and bleached (QPPP, OZEP, ODEDED) softwood kraft pulps by A. terreus α-L-arabino-furanosidase A. □ Reference; ■ 1 000 nkat g^{-1} dry pulp and ■ 5 000 nkat g^{-1} dry pulp. Incubation: pH 4.5, 40°C, 24 h.

3.3.2 Potential of α-L-arabinofuranosidase treatment in wheat bread baking (unpublished results)

The effect of arabinose removal on the extractability of arabinoxylans and β-glucans from wheat and rye flour and bran fractions was studied using α-L-arabinofuranosidase (mixture of *A. terreus* α-L-arabinofuranosidases B1 and B2) and xylanase alone and in combination. α-L-Arabinofuranosidase hydrolysed 36–45 % and 26–44 % of the total arabinose from wheat and rye flours, respectively (Fig. 4), corresponding well to the results obtained with the isolated arabinoxylans. The amounts of arabinose released from the coarser bran fractions were considerably lower: 3.0–4.5 % from wheat and 6.5–12 % from rye (Fig. 4). The addition of xylanase did not significantly affect the amount of arabinose removed. When wheat flour and rye bran were treated with a small α-L-arabinofuranosidase dosage (100 nkat g^{-1} flour), a slight increase in the amount of soluble pentosan was detected. The same was observed in the combined hydrolysis with xylanase, especially in wheat flour and to a lesser extent in rye bran. With the higher α-L-arabinofuranosidase dosage (1 000 nkat g^{-1} flour), the amount of soluble pentosan decreased as compared to the reference and xylanase treated samples in each case, particularly in rye fractions. The extractabilities of β-glucans or proteins were not affected by the arabinose removal (results not shown).

The molecular heterogeneity of the arabinoxylan polymer affects the accessibility of the α-L-arabinofuranosidase. Regions in which a certain type of arabinofuranosyl linkage predominates may be efficiently hydrolysed by a specific enzyme and thus result in specific insolubilization of these regions. In addition, mono- and disubstitution of the xylose residues and the distribution of the substituted residues along the polymer have an effect on the action of α-L-arabinofuranosidase (Andrewartha *et al.*, 1979). According to Izydorczyk and Biliaderis (1995), the bran arabinoxylans are generally more highly substituted both in wheat and in rye, which would make them more resistant to hydrolysis by α-L-arabinofuranosidase as compared to the flour fractions. The bran fractions used in this study had lower arabinose to xylose ratios than the flour fractions, indicating a lower degree of substitution. However, the particle size in the bran fractions was larger, rendering the polysaccharide components less soluble and thus less accessible to the enzyme.

The effect of enzyme supplementation on the softness of wheat doughs was determined by measuring the storage modulus (G') and phase angle (δ) (Table 18). All bonds in dough, the weak van der Waals and hydrogen bonds and the strong S-S bonds, contribute to the magnitude of G'. Soft doughs have low G' and high phase angle values (Amemiya and Menjivar, 1992). α-L-Arabinofuranosidase addition affected dough softness only slightly and the value of G' decreased by 3% as compared to the control.

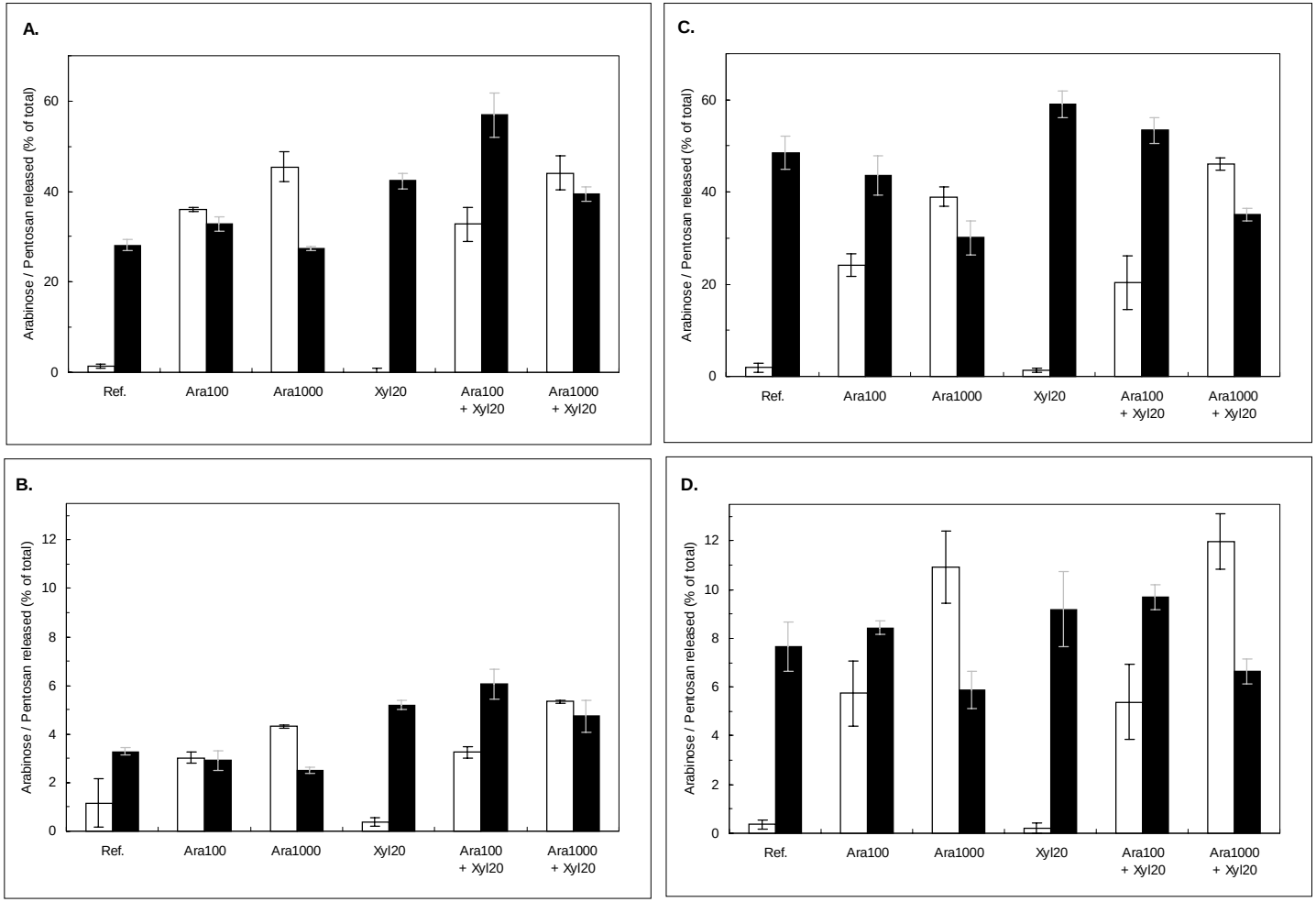

Figure 4. Effect of enzyme treatments on the extractability of arabinoxylans from A. wheat flour; B. wheat bran; C. rye flour and D. rye bran. □ *Free arabinose;* ■ *Soluble arabinoxylans. Enzyme dosages: α-L-Arabinofuranosidase 100 nkat* g^{-1} *(Ara 100) and 1 000 nkat* g^{-1} *(Ara 1000); xylanase 20 nkat* g^{-1} *(Xyl 20). Incubation: pH 4.0, 30°C, 24 h.*

Table 18. Effects of enzyme treatments on the viscoelastic properties of wheat dough.

Sample	Dosage (nkat g^{-1} flour)	Storage modulus G' (kPa)	Phase angle δ
Reference	-	13.8 ± 0.4	23.1 ± 0.8
α-L-Arabinofuranosidase	100	13.4 ± 0.4	23.2 ± 0.6
Xylanase	60	11.9 ± 0.3	24.4 ± 0.6
α-L-Arabinofuranosidase + Xylanase	100 60	11.6 ± 0.3	24.2 ± 0.7

The doughs after xylanase and combined treatments were clearly softer and the values of the storage modulus were about 15% lower than the control value. The loss of elasticity of these doughs was also observed as the increase of phase angle, which was about 5%.

In the wheat bread baking experiments the effects of α-L-arabinofuranosidase treatment alone and in combination with xylanase were studied on the wheat dough and bread properties. Wheat dough is a complex system consisting of gluten network as a continuous phase and air bubbles, starch granules, cellulose fragments and pentosans as dispersed phase. As compared to the isolated substrates or flour-water systems, the amount of water in dough is limited and thus decreases the mobility of enzymes. The addition of α-L-arabinofuranosidase increased the specific volume of the breads by about 5%, whereas after the xylanase and combined treatments the specific volume was increased by 11% as compared to the control without enzymes (Table 19). In an earlier work, *T. reesei* culture filtrate was found to increase the specific volume of wheat and fibre-enriched wheat breads by almost 20%, and the number of greater pores in the crumb increased slightly when enzyme mixtures were used (Laurikainen *et al.*, 1998). In comparison with the results obtained in this study, the purified *T. reesei* xylanase had only a minor effect on loaf properties (Laurikainen *et al.*, 1998). α-L-Arabinofuranosidase was also found to have a positive effect on dough stability and loaf volume when used in combination with xylanase (Mutsaers, 1997). However, this phenomenon was not observed with all the α-L-arabinofuranosidases tested.

α-L-Arabinofuranosidase alone caused only minor changes in crumb porosity (Table 19). Although the specific volumes after the xylanase and combined treatments were the same, the incorporation of α-L-arabinofuranosidase reduced the pore size and produced a more regular pore

Table 19. Effects of enzyme treatments on wheat loaf properties.

Sample	Dosage (nkat g^{-1} flour)	Specific volume (cm^3 g^{-1})	Crumb porosity[a]
Reference	-	2.3 ± 0.3	8.0
α-L-Arabinofuranosidase	100	2.4 ± 0.1	7.0
Xylanase	60	2.6 ± 0.2	6.0
α-L-Arabinofuranosidase + Xylanase	100 60	2.6 ± 0.2	7.5

[a] Scale of diminishing porosity of 1–8.

structure as compared to the xylanase-treated breads. Coarser pore structure was also observed in rye baking when a high dosage of purified *T. reesei* xylanase was added (Autio *et al.*, 1996). On the other hand, lipase supplementation improved the crumb structure of wheat bread as a consequence of the gas-liquid interface stabilization (Poulsen and Borch Søe, 1997). The more even pore size distribution in the breads treated with both α-L-arabinofuranosidase and xylanase might also be due to the formation of compounds stabilizing air bubbles during baking.

The addition of enzymes had a tendency to soften the bread crumb structure during storage (Fig. 5). In the case of the fresh breads, only minor differences in firmness were observed. After one day, the firmness of the enzyme-treated breads was 4–17% lower and after 3 days of storage 9–16% lower as compared to the control. The staling rate, which was calculated from the firmness values, was retarded by 9–10% when α-L-arabinofuranosidase was used alone or in combination with xylanase and by 14% when xylanase was applied alone. Laurikainen *et al.* (1998) observed significant decrease in the firmness of wheat and fibre-enriched wheat breads when enzyme mixtures were applied, whereas with the pure xylanase only minor changes were detected. However, all enzymes, including the xylanase, retarded the staling rate.

According to Bushuk (1966), approximately 45% of the total water in dough is associated with starch, 31% with protein and 23% with pentosans. During the initial mixing of flour with water and other dough ingredients, the water-unextractable pentosans (WUP) of flour absorb high amounts of water. As the xylanases degrade the WUP, part of the water becomes freely available and is redistributed from the pentosan to the gluten phase. This redistribution of water might explain the beneficial effects of xylanases on the volume and appearence of bread. However, during extensive hydrolysis of pentosans more water is released and can no longer be incorporated into

the dough, thus resulting in an increased amount of free water and poor dough properties such as stickiness (Maat *et al.*, 1992; Rouau *et al.*, 1994). Arabinofuranosyl residues affect the solubility of arabinoxylans, but they do not contribute to the water-binding properties of these polymers (Andrewartha *et al.*, 1979). Even so, they could be important for the non-specifically bound water, which may remain unaffected if only part of the arabinosyl residues are removed. According to Delcour *et al.* (1991), the action of the water-soluble pentosans in baking is probably related to an increase in dough viscosity, because the addition of oat β-glucan, corn bran hemicellulose or xanthan gum to gluten-starch doughs gave similar baking results, i.e. increase in loaf volume.

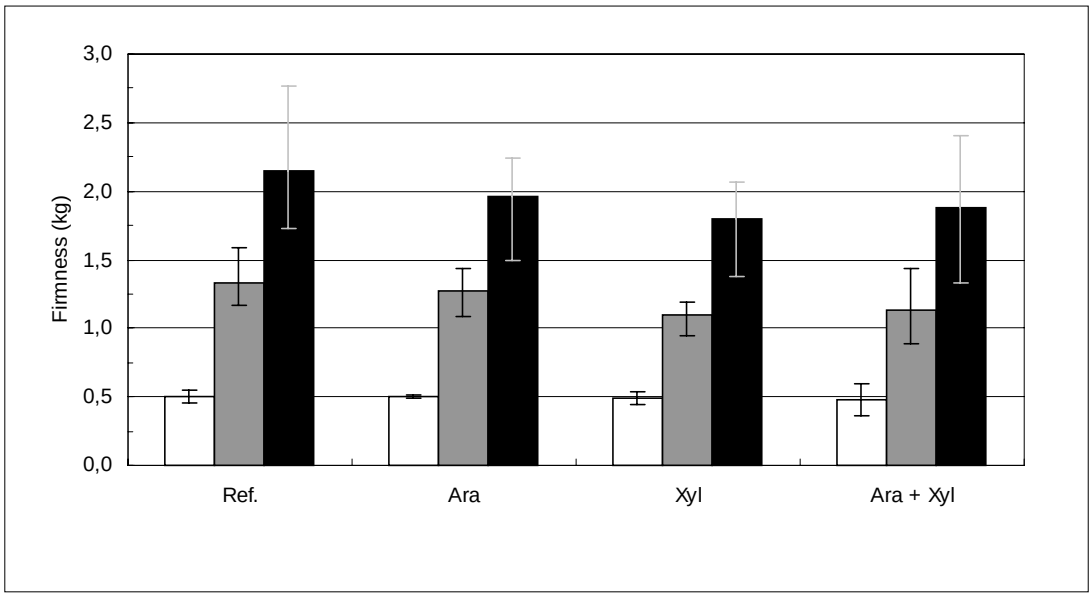

Figure 5. Effects of enzyme treatments on the firmness of wheat bread. Enzyme dosages: α-L-Arabinofuranosidase 100 nkat g^{-1} (Ara), xylanase 60 nkat g^{-1} (Xyl). Storage time: □ 2 h; ■ 24 h and ■ 72 h.

In the present experiments, α-L-arabinofuranosidase addition did not significantly affect the dough properties or the specific volume of the baked loafs. The xylanase and combined treatments caused softer and stickier doughs, but beneficial changes, such as improved loaf volume, were observed in the bread appearence. The major effect obtained by α-L-arabinofuranosidase addition was the more homogeneous pore structure and distribution without loss of the volume increase caused by xylanase.

4 Conclusions

Several natural polymers contain arabinose and galactose residues. Arabinose is commonly found as either a backbone or side-chain component of arabinans and arabinogalactans present e.g. in various fruit, seeds and vegetables, and as a side-group of arabino(glucurono)xylans in annual plants and softwoods. Galactose is the main component of galactans and arabinogalactans, and it is present as a side-group in galactomannans, found especially in legumes, and in galactoglucomannans present e.g. in gymnosperms. These side-groups affect the properties of the polymers, such as their form, solubility and interaction with other components. These properties can be modified by removing the side groups enzymatically.

The properties of different α-L-arabinofuranosidases produced by *Aspergillus terreus* and α-galactosidases produced by *Penicillium simplicissimum* were studied in this work. The enzymes active towards polymeric substrates were of major interest, but the properties of the other enzymes were also characterized. Furthermore, the abilities of the enzymes to act on different matrices were evaluated.

The three *A. terreus* α-L-arabinofuranosidases had rather similar molecular properties and modes of action. In addition, they clearly enhanced the action of xylanase. As they all possessed broad specificity towards polymeric substrates, including arabinans, arabinogalactans and arabino-(glucurono)xylans isolated from various sources (Section 3.2.2 and Table 20), and as they were only able to remove arabinose residues attached to the non-reducing terminal xylose residues of arabinoxylo-oligosaccharides, they were classified as belonging the class of arabinofuranosidase B-type enzymes (Beldman *et al.*, 1997). The further classification of these enzymes into a glycosyl hydrolase family would require knowledge of their complete amino acid sequences.

Most of the isolated arabinose-releasing enzymes have been classified to be of unspecific arabinofuranosidase B-type. Furthermore, it is rather common for a microorganism to produce several α-L-arabinofuranosidases with rather similar properties. It appears that if enzymes with more specific hydrolytic properties, such as the ability to release arabinose residues from double-substituted xylose residues or ferulic acid substituted arabinose residues, are desired, their production should be induced with an appropriate substrate possessing the wanted relevant characteristics. Furthermore, the substrate used for the detection of these enzyme activities has an important role, as the enzymes may lack activity towards the commonly used model substrates, e.g. pNPA.

Table 20. Substrate specificities of Aspergillus terreus α-L-arabinofuranosidases.

Enzyme	Linkages hydrolysed	pNPA	Arabinan		Arabino-galactan		Arabino-xylan	
			o[a]	p	o	p	o	p
αAra A	1→3 1→5	++[b]	++	++	n.d.	++	+/-	+(+)
αAra B1	1→3 1→5	++	++	++(+)	n.d.	+++	+/-	++
αAra B2	1→3 1→5	++	++	++(+)	n.d.	+++	+/-	++

[a] o oligomer; p polymer

[b] +++ high activity; ++ intermediate activity; + low activity; - no activity; n.d. not determined

In comparison to α-L-arabinofuranosidase production, microorganisms appear to produce multiple α-galactosidases with highly divergent molecular and hydrolytic properties. *Penicillium simplicissimum* produces at least four α-galactosidases, of which three were characterized in this work. α-Galactosidase production by this fungus did not appear to depend on the presence of high amounts of galactose in the cultivation medium, as the highest activities were obtained on a carbon source containing only minor amounts of galactose. α-Galactosidases AGLI and AGLIII had rather similar molecular but different hydrolytic properties, whereas the third enzyme, AGLII, varied from the other two proteins in both respects. This differentiation appears very appropriate, since all the enzymes involved have a specific role in the hydrolysis of different substrates (section 3.2.4 and Table 21). AGLI was very efficient in the removal of side groups from polymeric galacto(gluco)mannans even in the absence of the backbone-degrading enzymes, whereas the action of AGLII was limited to the liberation of galactose residues from the non-reducing terminal of various oligosaccharides. The role of AGLIII remained equivocal as its activity towards all the substrates tested was inferior compared to the other two enzymes. Thus it is possible that AGLIII possesses high activity towards some substrate not tested in this study.

Table 21. Substrate specificities of Penicillium simplicissimum α-galactosidases.

Enzyme	pNPG	Raffinose-family oligosaccharides	Polymeric galactomannans	Polymeric galactoglucomannans
AGLI	+[a]	++	+++	+++
AGLII	+++	+++	+/-	+/-
AGLIII	+	+	+	+

[a] +++ high activity; ++ intermediate activity; + low activity; - no activity

On the basis of the classification of glycosyl hydrolases, α-galactosidase AGLI belongs to the family 27. The N-terminal aa sequnece of AGLIII had similarities with AGLI and other enzymes belonging to this family, although there were clear differences in their substrate specificities. Interestingly, the N-terminal aa sequence of AGLII was completely different from those of the other characterized glycosyl hydrolases. However, a complete amino acid sequence and tertiary structure of the protein would be required to allow comparisons between the structure and the mode of action of the enzyme.

The ability of the purified enzymes to release monosaccharides was not restricted to isolated polymers, as they were also able to act in different fibre matrices. The hypothesis of using α-L-arabinofuranosidases or α-galactosidases as pulp bleaching aids is based on the structures of LCC compounds, in which arabinose or galactose side groups of xylan or mannan, respectively, are proposed to be linked to lignin. The hydrolysis of the linkage between the side groups and the main chain has been proposed to facilitate the removal of lignin from pulp. Both α-L-arabinofuranosidase and α-galactosidase were found to be active in pulp and to release the corresponding monosaccharides in amounts comparable to those released by the backbone-degrading enzymes. The effects of the side group removal on the pulp properties were only studied using α-L-arabinofuranosidase. Surprisingly, the treatment decreased the extractability of lignin and thus the pulp bleachability was also decreased. This may have been due to the formation of aggregates of the desubstituted xylan chains, preventing lignin extraction. The same phenomenon was observed in the combined treatment with xylanase, known to have beneficial effects on bleaching. As contradictory results about the ability of α-L-arabinofuranosidase to enhance pulp bleachability have recently been obtained, it is probable that the molecular structure of the enzyme, its substrate specificity and mode of

action in the fibrous matrix and the enzyme dosage used all have important roles.

In another application, wheat bread baking, α-L-arabinofuranosidase treatment had only a minor influence on the baking properties. However, in this case the simultaneous application of α-L-arabinofuranosidase and xylanase resulted in improved structural properties of the crumb without loss of the beneficial alterations caused by xylanase treatment. This is proposed to be due to the formation of oligosaccharide structures possibly stabilizing the gas bubbles.

The usefulness of the hemicellulose side group hydrolysing enzymes in various applications appears to depend on the characteristics of the enzymes as well as on the application in which they are to be used. Their specificities in the matrix environment may differ from those determined with isolated substrates, as the accessibility of the substrates in fibrous material is more limited. For the evaluation of the effects of these enzymes, preparations free of interfering activities are required. Such preparations can be obtained either by purification or by using gene technology. Furthermore, these enzymes have an important role in the complete hydrolysis of complex hemicellulosic and pectic materials, as they facilitate the action of other enzymes involved in the degradation. An interesting possibility for the use of this type of enzymes could also be the production of functional oligosaccharides with beneficial health effects.

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