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Substrate specificities of *Aspergillus terreus* α-arabinofuranosidases

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Abstract

The substrate specificities of three purified *Aspergillus terreus* α-arabinofuranosidases, pI 7.5, 8.3 and 8.5, were studied using various isolated arabinose-containing polysaccharides and oligosaccharides as substrates. In addition, their mode of action was compared with those of some other arabinose-releasing enzymes. All three *A. terreus* α-arabinofuranosidases preferred branched pectic polysaccharides, such as sugar beet arabinan and β-1,4-arabinogalactans as substrates, but they were also able to release arabinose from linear arabinan, β-1,3/1,6-arabinogalactans and different arabinoxylans. α-Arabinofuranosidases pI 7.5 and pI 8.5 were able to hydrolyse arabinose from oligosaccharide mixtures of wheat flour and larchwood arabinoxylans, forming arabinose and linear xylo-oligosaccharides. However, isolated arabinoxylo-oligosaccharides (DP 3-5) with arabinose substituted at *O*-3 of an internal xylose residue were only poorly degraded. Arabinose linked to *O*-2 of either internal or non-reducing end xylose residues was not hydrolysed.

1. Introduction

Various arabinose-containing polysaccharides, such as arabinans, arabinogalactans and arabinoxylans, exist in different tissues of plants, e.g. leaves, roots, seeds and flowers. Arabinans are composed of a highly branched α-1,5-linked L-arabinofuranose backbone containing 3- or 2-linked α-L-arabinofuranose side groups. In β-1,4-arabinogalactans, mainly present in grasses and cereals, the galactopyranose backbone is substituted at the *O*-3 and *O*-6 positions by α-L-arabinofuranose side-chains with varying degrees of polymerization. In side-chains the residues are connected via α-1,5-linkages (Aspinall, 1980; van de Vis, 1994). These polymers can further be esterified with feruloyl groups. Arabinans, galactans and arabinogalactans have been isolated from pectin-rich sources, and it is possible that they are associated with the rhamnogalacturonan chains at the hairy regions of pectic substances.

The highly branched β-1,3/1,6-arabinogalactans are most abundant in gymnosperms, especially in larchwood, but they are also found in some flowering plants. The β-1,3-D-galactopyranose backbone is mainly branched by β-D-galactopyranose at *O*-6 and α-L-arabinofuranose residues at *O*-3 with an average of two residues per side-chain. In addition, some β-1,3-L-arabinopyranose and D-glucuronic acid residues may be present in the side-chains (Timell, 1967; Aspinall, 1980; Puls & Schuseil, 1993; van de Vis, 1994).

Arabinoxylans are composed of a linear β-1,4-D-xylopyranose backbone which is variably substituted depending on the origin. Softwood arabinoxylans are substituted by α-L-arabinofuranoside groups at *O*-3 of the xylose residues. In addition, they contain 4-*O*-methyl-α-D-glucuronic acid groups at *O*-2 of the xylose (Timell, 1967). According to Kormelink & Voragen (1993), larchwood arabinoxylan contains not only *O*-3 linked but also single *O*-2 linked arabinofuranoside residues. Cereal arabinoxylans have a more complex structure. They contain less uronic acids and are more highly branched with L-arabinofuranosyl side groups than the softwood arabinoxylans. Arabinofuranose residues can be linked either to *O*-3 or *O*-2 or to both *O*-2 and *O*-3 of xylopyranose. Some of the arabinofuranose residues in cereal arabinoxylans are esterified with ferulic acid and less frequently with *p*-coumaric acid (Mueller-Harvey et al., 1986; Puls & Schuseil, 1993).

Several α-arabinofuranosidases with different substrate specificities have been isolated from various fungi, bacteria and plants, e.g. from *Aspergillus* (Rombouts et al., 1988, Kormelink et al., 1991, Kaneko et al., 1993, Luonteri et al., 1995, Wood & McCrae, 1996), *Trichoderma* (Poutanen, 1988), *Streptomyces* (Kaji et al., 1981, Komae et al., 1982) and *Bacillus* species (Kaneko et al., 1994, Bezalel et al.,

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1993, Gilead & Shoham, 1995) and *Scopolia japonica* (Tanaka & Uchida, 1978). Some of these enzymes have interesting characteristics, being highly specific only towards arabinoxylans (Kormelink et al., 1991), or being able to hydrolyse ferulic acid-substituted arabinose residues (Wood & McCrae, 1996).

In a previous study, purified *Aspergillus terreus* α-arabinofuranosidases pI 7.5, pI 8.3 and pI 8.5 were found to hydrolyse polymeric arabinoxylans (Luonteri et al., 1995). In this work, the specificities of these enzymes were studied further using different isolated poly- and oligosaccharides as substrates. Their action was compared with that of *A. niger* α-arabinofuranosidase B, able to hydrolyse polymeric substrates (Rombouts et al., 1988). Furthermore, *Trichoderma reesei* α-arabinofuranosidase, which has a similar N-terminal amino acid sequence to that of *A. terreus* α-arabinofuranosidases pI 8.3 and pI 8.5 (Luonteri et al., unpublished results), and a highly specific (1,4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) from *A. awamori* (Kormelink et al., 1991) were used as references.

2. Materials and methods

2.1. Enzymes

α-Arabinofuranosidases pI 7.5, pI 8.3 and pI 8.5 from *Aspergillus terreus* VTT-D-82209 were purified to homogeneity as described previously (Luonteri et al., 1995). Other enzymes used were α-arabinofuranosidase B from *Aspergillus niger* (Rombouts et al., 1988) and (1,4)-β-D-arabinoxylan arabinofuranohydrolase (AXH; Kormelink et al., 1991), endoxylanase I and endoxylanase III (EXI and EXIII) from *Aspergillus awamori* (Kormelink et al., 1993c). α-Arabinofuranosidase, β-xylosidase and endoxylanase pI 9 from *Trichoderma reesei* Rut C30 were purified according to Poutanen (1988), Poutanen & Puls (1988) and Tenkanen et al. (1992), respectively. Activities of the *A. terreus* and *T. reesei* α-arabinofuranosidases were determined using 2 mM *p*-nitrophenyl-α-L-arabinofuranoside as substrate as described by Poutanen (1988). Specific activities of the α-arabinofuranosidases used in this study are presented in Table 1.

2.2. Substrates

Branched and linear sugar beet arabinans were from British Sugar (Peterborough, UK). β-1,4-Arabinogalactan from soya was a gift from Novo Nordisk Ferment (Dittingen, Switzerland). Isolated and purified β-1,4-arabinogalactan from onion and β-1,3/1,6-arabinogalactan from green coffee beans were prepared as described by van de Vis (1994). β-1,3/1,6-Arabinogalactan from larchwood (Stractan) was from St Regis Paper Company (Tacoma, WA, USA). Wheat flour arabinoxylan was obtained from Megazyme (Sydney, Australia). Arabinoxylan isolated from wheat bran and two fractions of sorghum arabinoglucuronoxylan were prepared as described by Bergmans et al. (1996) and Verbruggen et al. (1995), respectively. Larchwood arabinoxylan was isolated using the method of Kormelink et al. (1993c). Softwood kraft pulp arabinoxylan was kindly provided by Jan Jansson (Central Laboratory, Espoo, Finland). The monosaccharide compositions of the substrates are presented in Table 2.

Xylo-oligosaccharides substituted with arabinose: 3^2-α-L-arabinofuranosyl-xylotriose (3-Ara^{2}Xyl$_3$); $3^{2/3}$-α-L-arabinofuranosyl-xylotetraose (3-Ara$^{2/3}$Xyl$_4$) and $3^{2/3}$-α-L-arabino-furanosyl-xylopentaose (3-Ara$^{2/3}$Xyl$_5$), were isolated and purified from pine pulp (Teleman et al., 1996, Tenkanen et al., 1996). 2^3-α-L-Arabinofuranosyl-xylotetraose (2-Ara^{3}Xyl$_4$) was obtained from a double-substituted oligomer 2^3, 3^3-α-L-arabinofuranosyl-xylotetraose (2,3-Ara^{3}Xyl$_4$) isolated from wheat flour by hydrolysing the *O*-3-linked arabinofuranoside with *Bifidobacterium adolescentis* DSM 20083 α-arabinofuranohydrolase AXH-d$_3$ as described by van Laere et al. (1997).

2.3. Hydrolysis experiments

The progress of hydroysis was followed using wheat bran and wheat flour arabinoxylans and branched beet arabinan (1 mg ml^{-1}) as substrates. They were hydrolysed by α-arabinofuranosidases and AXH (0.5 μg ml^{-1}) at pH 4.5 (pH 4.0 for *T. reesei* α-arabinofuranosidase) at 30°C for 48 h. Samples were also taken after 2, 5 and 24 h. After the incubation, enzymes were inactivated by heat treatment at 100°C for 10 min. In substrate blanks a corresponding volume of

Table 1
Specific activities of α-arabinofuranosidases (αAra) and AXH

Enzyme	Specific activity (nkat mg^{-1} protein)	Substrate	Reference
Aspergillus terreus αAra pI 7.5	1220	2 mM pNPA[1]	This study
Aspergillus terreus αAra pI 8.3	1700	2 mM pNPA	This study
Aspergillus terreus αAra pI 8.5	1880	2 mM pNPA	This study
Aspergillus niger αAra B	2450	3.7 mM pNPA (0.1% w/v)	Rombouts et al., 1988
Trichoderma reesei αAra	1760	2 mM pNPA	This study
Aspergillus awamori AXH[2]	367	Wheat alkali-soluble arabinoxylan	Kormelink et al., 1991

[1] pNPA p-nitrophenyl-α-L-arabinofuranoside
[2] No activity against pNPA

50 mM sodium acetate, pH 4.5, was used instead of enzyme solution. On the basis of the results obtained from the first incubations, further experiments with polymeric substrates were carried out under the same conditions but with an incubation time of 5 h (2 h for AXH).

Enzyme digests of the polymeric substrates were analysed by HPAEC using a Dionex Bio-LC GMP-II quaternary gradient module (Dionex Co., Sunnyvale, CA, USA) equipped with a Dionex Carbopac PA-100 column (250 × 4 mm) in combination with a CarboPac PA guard column (25 × 3 mm) and a PED detector in the pulsed amperometric detection (PAD) mode according to Gruppen et al. (1992).

Mixtures of different arabinose-containing xylo-oligosaccharides were prepared from wheat flour, larchwood and softwood kraft pulp xylans by endoxylanase hydrolysis. Wheat flour and larchwood arabinoxylans (2 mg ml^{-1}) in 50 mM sodium acetate buffer, pH 4.5, were treated with EXI or EXIII (0.2 μg ml^{-1}) at 30°C for 24 h. After inactivation of the enzymes, the hydrolysates were lyophilized and the dry samples were redissolved in 100 mM sodium acetate, pH 4.0 (wheat flour 25 mg ml^{-1}; larchwood 50 mg ml^{-1}). Softwood kraft pulp arabinoxylan in 100 mM sodium acetate, pH 4.0, was treated with *T. reesei* endoxylanase pI 9 (1.5 μg ml^{-1}) at 40°C for 24 h and then inactivated by heating. Five hydrolysates obtained (final concentration 5.0 mg ml^{-1}) and the isolated arabinoxylo-oligosaccharides (0.5 mg ml^{-1}) were treated with *A. terreus* α-arabinofuranosidases pI 7.5 (0.4 μg ml^{-1}) and pI 8.5 (0.3 μg ml^{-1}) and the latter oligosaccharides also in combination with β-xylosidase from *T. reesei* (0.9 μg ml^{-1}) at pH 4.0 and 40°C for 24 h. Samples were analysed by HPAEC-PAD using a Dionex 4500i series chromatograph according to Hausalo (1995).

3. Results

3.1. Hydrolysis of polymeric substrates

The progress of hydrolysis was followed for 48 h with three different substrates: wheat bran and wheat flour arabinoxylans and branched beet arabinan. Three *Aspergillus terreus* α-arabinofuranosidases released 20%–25% of the total arabinose present in arabinoxylans from wheat bran and wheat flour in 48 h (Fig. 1). *A. niger* α-arabinofuranosidase

Table 2
Monosaccharide compositions of the polysaccharides in mol%

Polysaccharide	Total sugar % (w/w)	Ara[1]	Xyl	Gal	Man	Glc	Rha/Fuc[2]	AUA	Reference
Linear beet arabinan	>90	>90	?[3]	?	?	?	?	?	McCleary et al., 1989
Branched beet arabinan	>80	>80	?	?	?	?	?	?	McCleary et al., 1989
β-1,4-Arabinogalactan; onion	71	7.5	Trace	71	5.2	15	0.2	1.0	van de Vis, 1994
β-1,4-Arabinogalactan; soya	75	38	1.4	57	–[4]	–	1.8	2.4	van de Vis, 1994
β-1,3/1,6-Arabinogalactan; coffee	85	24	–	65	3.9	2.1	0.4	4.1	van de Vis, 1994
β1,3/1,6-Arabinogalactan; larchwood	88	17	–	83	–	–	0.4	1.3	van de Vis, 1994
Wheat flour arabinoxylan	>98	41	59	–	–	–	–	–	Megazyme
Wheat bran arabinoxylan BE.RT 80	83.7	41	50	1.4	–	3.2	?	3.7	Bergmans et al., 1996
Sorghum glucuronoarabinoxylan BE 1.2	>95	44	39	2.3	0.6	3.5	0.3	9.9	Verbruggen et al., 1995
Sorghum glucuronoarabinoxylan 1K4	>95	25	30	2.7	–	32	0.7	11	Verbruggen et al., 1995
Larchwood arabinoxylan (St. Regis)	?	11	56	3.1	–	26	–	4.2	Kormelink & Voragen, 1993
Softwood kraft-pulp arabinoxylan	97.5	10	79	Trace	Trace	Trace	–	11	Jan Jansson, personal communication

[1] Ara, arabinose; Xyl, xylose; Gal, galactose; Man, mannose; Glc, glucose; Rha, rhamnose; Fuc, fucose; AUA, uronic acids (= 4-*O*-methylglucuronic and hexenuronic acids)
[2] Rha and/or Fuc
[3] ? unknown
[4] – not detected

B had a slightly lower activity against these substrates and hydrolysed about 16% of the arabinose linkages. *Trichoderma reesei* α-arabinofuranosidase was tested only on wheat flour arabinoxylan, which was poorly degraded. The amount of arabinose hydrolysed from branched arabinan in 48 h was about 18% with all the α-arabinofuranosidases studied. The reaction was fastest during the first 5 h of incubation, and therefore 5 h was chosen as the incubation time for the rest of the experiments with polymeric substrates.

Aspergillus awamori AXH liberated 20%–30% of the arabinose in wheat flour and bran arabinoxylan in 48 h (Fig. 1). Because it did not show any activity towards branched arabinan, only arabinoxylans were used as substrates in the subsequent experiments. The maximal degree of hydrolysis was reached already in 2 h, and therefore an incubation time of 2 h was chosen for AXH.

In the next experiments, the aim was to compare the ability of different α-arabinofuranosidases to release arabinose from various polysaccharides, rather than to reach maximal hydrolysis. On the basis of the molar amount of arabinose released, the highly branched beet arabinan appeared to be the best substrate for all the α-arabinofuranosidases studied (1490–1870 μmol arabinose mg^{-1} protein released; Table 3). All these enzymes released 12%–16% of the arabinose present in the substrate (Table 3). The linear

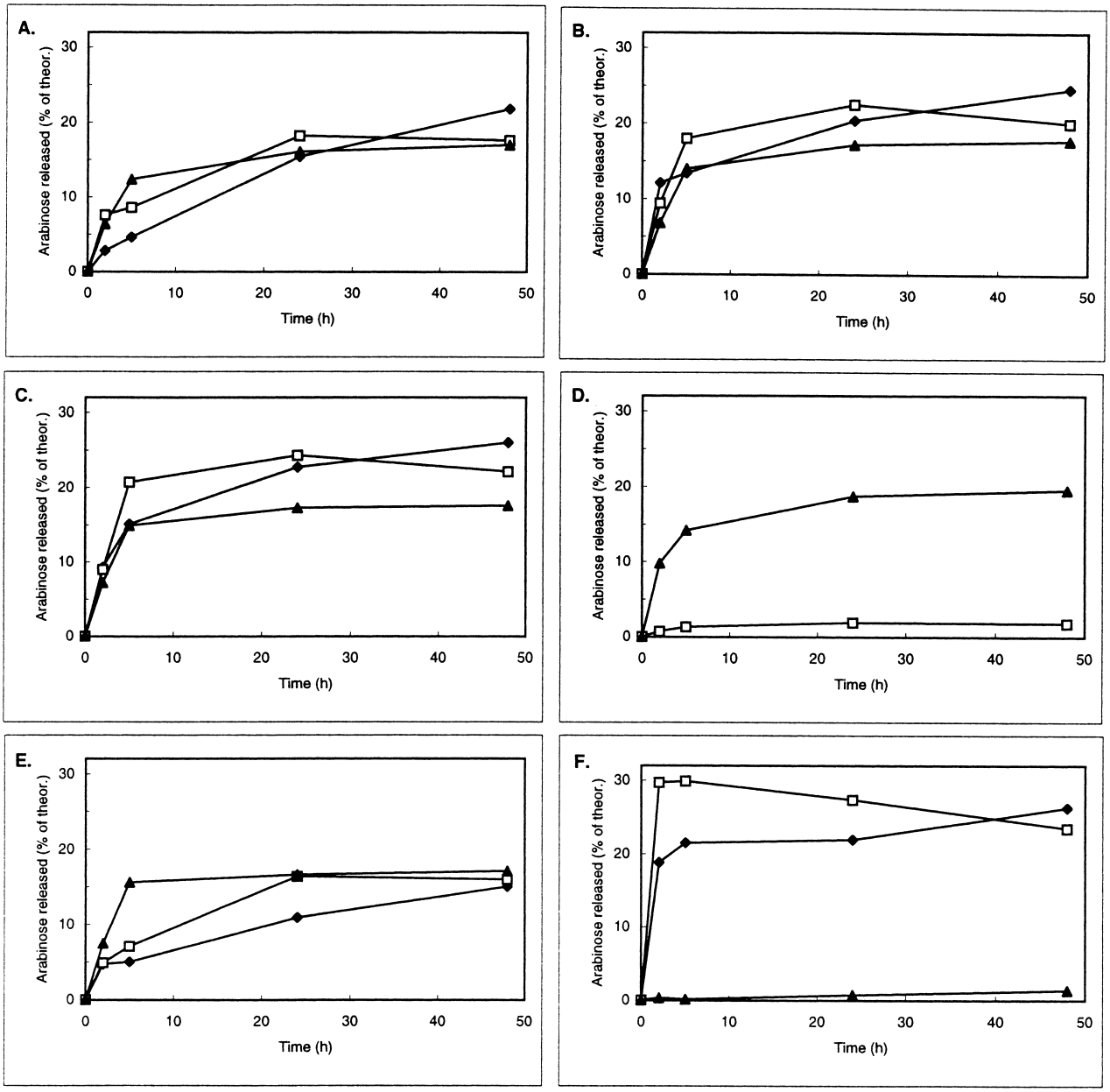

Fig. 1. Degradation of branched arabinan (▲), wheat bran arabinoxylan (♦) and wheat flour arabinoxylan (□) by (A), *A. terreus* α-arabinofuranosidase pI 7.5; (B), *A. terreus* α-arabinofuranosidase pI 8.3; (C), *A. terreus* α-arabinofuranosidase pI 8.5; (D), *T. reesei* α-arabinofuranosidase; (E), *A. niger* α-arabinofuranosidase B and (F), *A. awamori* AXH.

α-1,5-L-arabinan appeared to be a more difficult substrate and only about 5% of the total arabinose was liberated, corresponding to 440–870 μmol mg^{-1} protein. The slow rate of degradation of the linear α-1,5-L-arabinan was probably due to the lower concentration of end-groups susceptible to hydrolysis rather than to inability of the α-arabinofuranosidases to cleave the α-1,5-linked arabinose residues.

The β-1,4-arabinogalactans were the best polymeric substrates for the α-arabinofuranosidases when expressed as the percentage of arabinose liberated from the polymer (Table 3). On the basis of the amount of arabinose released per mg of protein, the soya β-1,4-arabinogalactan was degraded almost as efficiently as the branched arabinan. α-Arabinofuranosidase pI 7.5 released about 20%, whereas the other α-arabinofuranosidases liberated 45%–55% of the total arabinose in soya β-1,4-arabinogalactan. The soya β-1,4-arabinogalactan used was slightly branched at *O*-6 with arabinose and/or branched arabinan side-chains. However, it is also possible that the preparation contained some arabinan (van de Vis, 1994). On the other hand, only 14%–39% of the arabinose units in onion β-1,4-arabinogalactan was released (100–280 μmol mg^{-1} protein; Table 3).

The β-1,3/1,6-arabinogalactans from coffee beans and larchwood were more complex substrates. α-Arabinofuranosidases pI 8.3 and pI 8.5 both released 21% and 10% of the arabinose from coffee and larchwood β-1,3/1,6-arabinogalactans, respectively (160–440 μmol mg^{-1} protein; Table 3). Other α-arabinofuranosidases liberated only 1–7% of the arabinose from these substrates. The less efficient hydrolysis of the β-1,3/1,6-arabinogalactans compared to that of β-1,4-arabinogalactans may have been due to the higher degree of branching of the backbone by galactose side-chains. The arabinose residues may also have been further substituted with other sugar residues (van de Vis, 1994).

Surprisingly, the more branched arabinoxylans isolated from cereals were better substrates for all α-arabinofuranosidases and AXH compared to the arabinoxylans isolated from larchwood or softwood pulp. α-Arabinofuranosidases pI 8.3 and pI 8.5 released 11%–20% of the arabinose present in the cereal-derived and about 10% of that present in the wood-derived arabinoxylans (Table 4). The amount of arabinose released from the cereal arabinoxylans was 600–1130 μmol mg^{-1} protein, whereas only 130–170 μmol mg^{-1} protein was released from the wood arabinoxylans (Table 4). *Aspergillus* α-arabinofuranosidases pI 7.5 and B and *T. reesei* α-arabinofuranosidase released 3%–10% of arabinose from arabinoxylans. AXH hydrolysed the different arabinoxylans most efficiently, releasing higher amounts of arabinose in a shorter time (2 h vs. 5 h) than any of the α-arabinofuranosidases. It hydrolysed 20%–55% of the arabinose from these polysaccharides (Table 4). As well as releasing the highest amount of arabinose from larchwood arabinoxylan, AXH also had the greatest efficiency towards wheat flour arabinoxylan (1620 μmol mg^{-1} protein; Table 4). The AXH contained a minor amount of xylanase activity, which may have slightly decreased the degree of hydrolysis of arabinose, since it is known that the polymer is a much better substrate for AXH than the oligomers (van Laere and co-workers, unpublished results). According to Kormelink et al. (1991), AXH is highly specific for arabinoxylans but is unable to hydrolyse other arabinose-containing substrates.

3.2. Action of α-arabinofuranosidases towards oligomeric substrates

The action of *A. terreus* α-arabinofuranosidases pI 7.5 and pI 8.5 was further studied using different arabinoxylo-oligosaccharides. As the efficiencies of α-arabinofuranosidases

Table 3
Release of arabinose (μmol mg^{-1} protein and % of theoretical) from arabinans and arabinogalactans (0.1% w/v) by different α-arabinofuranosidases (αAra; 0.5 μg protein ml^{-1}). Incubation: pH 4.5 (*T. reesei*, pH 4.0), 30°C, 5 h

Substrate	αAra pI 7.5 *A. terreus*		αAra pI 8.3 *A. terreus*		αAra pI 8.5 *A. terreus*		αAra B *A. niger*		αAra *T. reesei*	
	(μmol mg^{-1})	(%)	(μmol mg^{-1})	(%)	(μmol mg^{-1})	(%)	(μmol mg^{-1})	(%)	(μmol mg^{-1})	(%)
Linear α-1,5-L-arabinan; sugar beet	440	3	750	6	870	7	n.d.	n.d.	640	5
Branched arabinan; sugar beet	1490	12	1680	14	1790	15	1870	16	1700	14
β-1,4-Arabinogalactan; onion	100	14	250	35	280	39	190	27	n.d.	n.d.
β-1,4-Arabinogalactan; soya	750	22	1900	55	1640	48	1700	50	1460	43
β-1,3/6-Arabinogalactan; coffee bean	150	7	440	21	440	21	62	3	n.d.	n.d.
β-1,3/6-Arabinogalactan; larchwood	19	1	170	10	160	10	84	5	67	4

[1] n.d., not determined

Table 4
Release of arabinose (μmol mg^{-1} protein and % of theoretical) from different arabino(glucurono)xylans (0.1% w/v) by different α-arabinofuranosidases (αAra) and AXH (0.5 μg protein ml^{-1}). Incubation: pH 4.5 (*T. reesei*, pH 4.0), 30°C, 5 h (AXH, 2 h)

Substrate	αAra pI 7.5 *A. terreus*		αAra pI 8.3 *A. terreus*		αAra pI 8.5 *A. terreus*		αAra B *A. niger*		αAra *T. reesei*		AXH *A. awamori*	
	(μmol mg^{-1})	(%)	(μmol mg^{-1})	(%)	(μmol mg^{-1})	(%)	(μmol mg^{-1})	(%)	(μmol mg^{-1})	(%)	(μmol mg^{-1})	(%)
Wheat flour arabinoxylan	470	9	980	18	1130	21	390	7	69	0.8	1620	30
Wheat bran arabinoxylan BE.RT 80	230	5	660	13	740	15	250	5	n.d.[1]	n.d.	920	19
Sorghum arabinoxylan BE 1.2	360	6	810	14	980	17	380	7	480	8	1000	18
Sorghum arabinoxylan 1K4	230	7	n.d.	n.d.	600	11	260	9	340	10	n.d.	n.d.
Larchwood arabinoxylan	67	5	130	9	170	12	39	3	71	5	790	55
Softwood kraft-pulp arabinoxylan	85	6	n.d.	n.d.	140	11	37	3	37	3	290	23

[1] n.d., not determined

pI 8.3 and pI 8.5 towards polymeric substrates were almost identical, only the pI 8.5 enzyme was used in these studies. The modes of action of *A. awamori* endoxylanases I and III on arabinoxylans are known to be different (Kormelink et al., 1993a). In the EXI digest, the proportion of short DP oligosaccharides is higher than in the EXIII digest. Kormelink et al. (1993a) analysed most of the structures of the wheat arabinoxylan oligomers formed. Endoxylanase pI 9 from *T. reesei* hydrolysed the softwood kraft pulp arabinoxylan to oligosaccharides, as reported by Teleman et al. (1996).

Both α-arabinofuranosidases appeared to act in a similar way against the oligosaccharide mixtures, although the hydrolysis was not very efficient. After the α-arabinofuranosidase treatments, the peaks corresponding to the substituted oligosaccharides (retention times 35.0–46.4 for wheat (not shown); 35.0–42.6 for larchwood (Fig. 2); 35.6–49.2 for kraft pulp (not shown)) diminished or disappeared and the amounts of linear xylobiose and -triose (Xyl_2, Xyl_3) increased and/or xylotetraose and -pentaose (Xyl_4, Xyl_5) were formed (Fig. 2, Table 5). The amount of longer branched oligosaccharides in the EXIII digests was higher than in the EXI digests, and thus more Xyl_4 and Xyl_5 could be formed from them in α-arabinofuranosidase hydrolyses. According to Kormelink et al. (1993a), EXI requires one unsubstituted xylopyranosyl residue adjacent to single-substituted and two unsubstituted residues adjacent to double-substituted xylopyranosyl residues at the reducing end, whereas EXIII requires two unsubstituted residues in both cases. In addition, EXIII leaves at least one unsubstituted xylopyranosyl residue at the non-reducing end of the formed oligomer, whereas EXI is also able to hydrolyse the linkage from the non-reducing end of single- and even double-substituted xylopyranosyl residues. Hence, not only the DP of the oligosaccharide, but also the site of arabinose substitution on the backbone appeared to affect the action of the α-arabinofuranosidases.

Further confirmation of the preference of α-arabinofuranosidases pI 7.5 and pI 8.5 for longer DP substrates with arabinose attached to the non-reducing end xylose residue was provided by the fact that the isolated arabinoxylo-oligomers of DP 3-5, in which arabinose was linked to xylose residues in the middle of the oligomer, were only poorly hydrolysed (Table 6). However, arabinoxylobiose with arabinose attached to the *O*-3 of the non-reducing end xylose was efficiently degraded (Luonteri et al., unpublished). Arabinoxylotriose (3-Ara^2Xyl_3) and arabinoxylotetraose (3-$Ara^{2/3}Xyl_4$) were not degraded by the α-arabinofuranosidases alone or in combination with β-xylosidase. α-Arabinofuranosidase pI 7.5 could liberate only a minor amount of arabinose from arabinoxylopentaose (3-$Ara^{2/3}Xyl_5$). Recently it was reported that an L-arabinofuranosyl group linked to the *O*-3 of a xylopyranosyl group protects the β-1,4-linkage before the substituted xylose unit from being cleaved by *T. reesei* β-xylosidase (Tenkanen et al., 1996). Thus β-xylosidase treatment never produced

oligomers with a substituted non-reducing end xylose unit. Arabinoxylotetraose (2-Ara^{3}Xyl$_4$), in which arabinose is *O*-2-linked to the xylose, was not degraded by α-arabinofuranosidase pI 7.5 or pI 8.5 alone. In the combined hydrolysis with β-xylosidase, both α-arabinofuranosidases were also inactive but the β-xylosidase was able to release the xylose residue from the non-reducing end of the oligomer despite the arabinose substitution in the adjacent xylose residue.

4. Discussion

α-Arabinofuranosidases have been classified into two groups. *Aspergillus niger*-type enzymes release arabinose not only from arabinan, arabinoxylan and arabinogalactan but also from simple synthetic substrates and oligosaccharides, whereas the *Streptomyces purpurascens*-type enzymes act only on low moleculer weight arabinosides (Kaji, 1984). The classification proposed by Beldman et al. (1997) also takes into account the ability of enzymes to cleave α-1,2-, α-1,3- and/or α-1,5-linkages and to cleave off arabinose substituents from single- or double-substituted xylose residues. Previously, the specificities of several α-arabinofuranosidases purified from various organisms towards arabinans and different arabinoxylans have been studied, whereas the hydrolysis of arabinogalactans has attracted less attention. In this work, the substrate specificities of *Aspergillus terreus* α-arabinofuranosidases towards several polymeric and oligomeric substrates were studied and compared with those of *A. niger* and *Trichoderma reesei* α-arabinofuranosidases and *A. awamori* arabinoxylan arabinofuranohydrolase (AXH).

Three *A. terreus* α-arabinosisdases were able to hydrolyse various polymeric substrates with different degrees of substitution. All the enzymes preferred branched pectic polymers, such as sugar beet arabinan and β-1,4-arabinogalactans as substrates. However, they also possessed activity against β-1,3/1,6-arabinogalactans and arabinoxylans. *A. niger* α-arabinofuranosidase B and *T. reesei* α-arabinofuranosidase had similar hydrolytic properties to those of the *A. terreus* enzymes. *A. awamori* AXH was

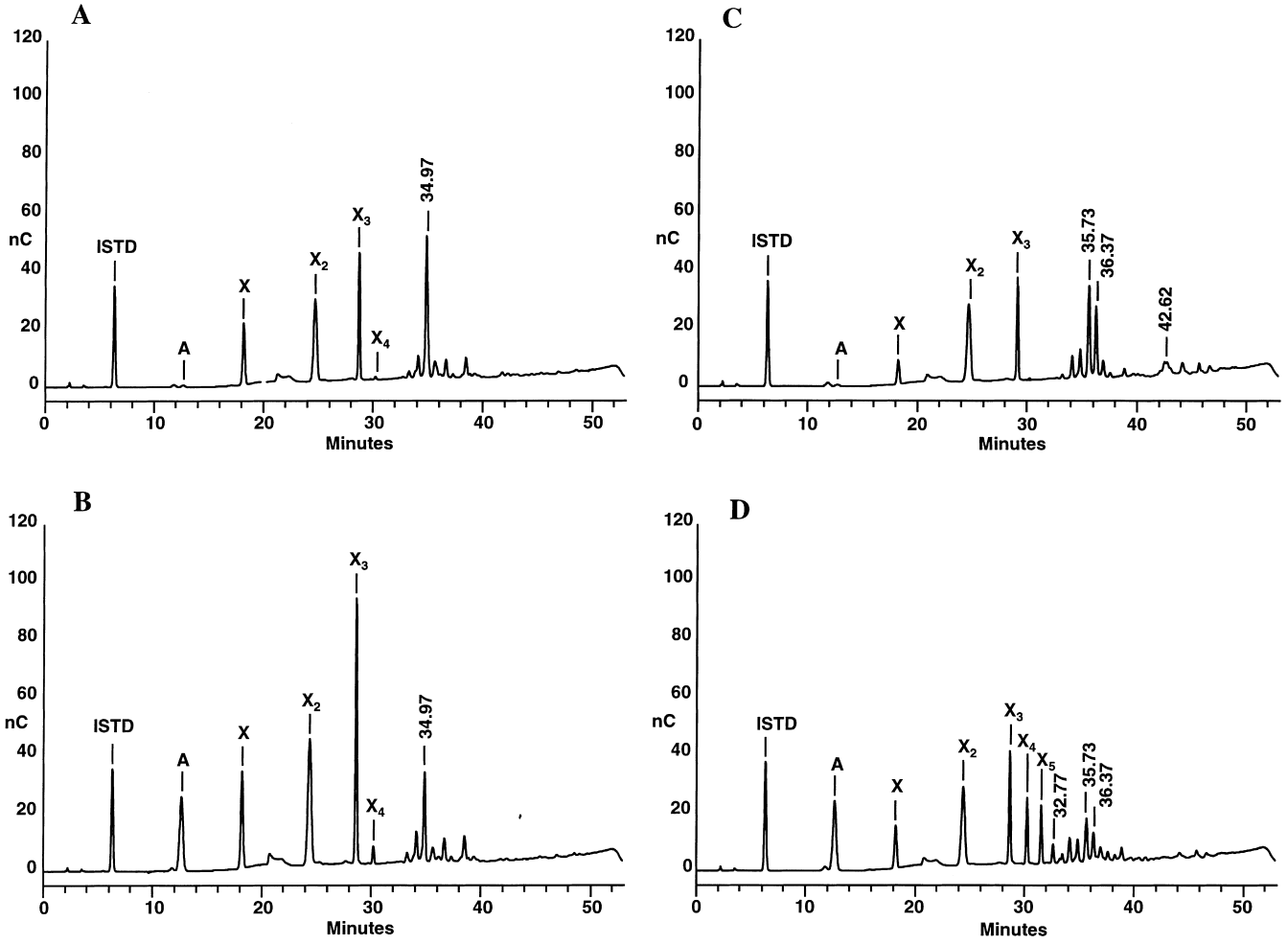

Fig. 2. HPLC chromatograms of arabinose and oligosaccharides produced from larchwood arabinoxylan by different enzyme treatments. (A), EX I hydrolysate; (B), EX I hydrolysate treated with αAra pI 8.5; (C), EX III hydrolysate; (D), EX III hydrolysate treated with αAra pI 8.5. Abbreviations: ISTD, internal standard; A, arabinose; X, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose.

unable to hydrolyse polysaccharides other than arabinoxylans, against which it had clearly higher activity than any of the α-arabinofuranosidases.

Although the *A. terreus* and *A. niger* α-arabinofuranosidases had similarities in their activities against polymeric substrates, clear differences in their mode of action against short arabinoxylo-oligosaccharides were observed. According to Kormelink et al. (1993b), *A. niger* α-arabinofuranosidase A is only active against low molecular weight arabinoxylans (DP 1-4), and is able to release all *O*-3-linked arabinofuranosyl substituents from single-substituted xylopyranosyl residues. On the other hand, α-arabinofuranosidase B could only split off the *O*-3-linked arabinofuranosyl substituents from the single-substituted, non-reducing end xylopyranosyl residues. The *A. terreus* α-arabinofuranosidases were able to degrade only some of the longer oligosaccharides in the endoxylanase digests of various arabinoxylans. Moreover, they had very limited activity towards the isolated arabinoxylo-oligosaccharides of DP 3-5, in which arabinose is attached to the internal xylose residues. However, they were able to release arabinose linked to the non-reducing end xylopyranosyl in arabinoxylobiose (Luonteri et al., unpublished). Similarly, α-arabinofuranosidases of *A. niger* 15-6 and *Bacillus subtilis* have been reported to be able to degrade only short arabinoxylo-oligosaccharides with the arabinofuranosyl attached to xylopyranosyl in the non-reducing end (Kaneko et al., 1993; Kaneko et al., 1994). The *A. awamori* AXH is able to release all *O*-3-linked arabinofurosyl residues from single-substituted xylopyranoses, regardless of whether the xylose residue is in a terminal or in a non-terminal position (Kormelink et al., 1993b). In addition, it is able to release the *O*-2-linked arabinofuranosyl residue from 2-Ara^{3}Xyl$_3$. This might explain the more efficient hydrolysis of arabinosyl linkages in larchwood arabinoxylan, in which arabinose is mainly *O*-2-linked to xylose (Kormelink & Voragen, 1993), compared to the α-arabinofuranosidases which were inactive towards this linkage. *Bifidobacterium adolescentis* has been reported to produce a unique enzyme, AXH-d3, specific for arabinofuranosyl groups linked at *O*-3 of double-substituted xylopyranosyl residues (van Laere et al., 1997). Similarly to *A. awamori* AXH, this enzyme is specific for arabinoxylans and is not able to hydrolyse pNPA, arabinan, soy arabinogalactan or oligosaccharides derived from them.

The substrate specificities of α-arabinofuranosidases isolated from several other *Aspergillus* strains have also been studied. α-Arabinofuranosidase of *A. niger* 5-16 released arabinose from small synthetic substrates and arabinan, but was unable to degrade gum arabic or arabinoxylan (Kaneko et al., 1993). *A. niger* KI α-arabinofuranosidase, which was slightly smaller than the enzyme isolated from the strain 5-16 but had a similar isolelectric point, hydrolysed not only the low molecular-weight substrates but also polymeric beet arabinan, arabinoxylan and gum arabic (Kaji & Tagawa, 1970, Tagawa & Kaji, 1969). Similarly, *A. sojae* α-arabinofuranosidase exhibited activity not only against low- but also against high molecular-weight substrates such as beet arabinan, rice-straw arabinoxylan and soybean

Table 5
Formation of arabinose (Ara) and linear xylo-oligosaccharides (Xyl–Xyl$_5$) from arabinoxylan hydrolysates (5 mg ml^{-1}) by *A. terreus* α-arabinofuranosides (αAra) pI 7.5 (0.4 μg ml^{-1}) and pI 8.5 (0.3 μg ml^{-1}) after pretreatment with *A. awamori* (EXI, EXIII) or *T. reesei* (EX pI 9) endoxylanases. Incubation: pH 4.0, 40°C, 24 h

Substrate	Enzyme	Concentration (μg ml^{-1})					
		Ara	Xyl	Xyl$_2$	Xyl$_3$	Xyl$_4$	Xyl$_5$
Wheat flour arabinoxylan	EXI	< 5	85	170	110	< 5	< 5
	EXI → αAra pI 7.5[1]	160	93	210	160	< 5	< 5
	EXI → αAra pI 8.5	260	95	210	210	39	210
	EXIII	< 5	20	130	< 5	< 5	< 5
	EXIII → αAra pI 7.5	190	25	130	79	42	53
	EXIII → αAra pI 8.5	350	30	130	86	86	120
Larchwood arabinoxylan	EXI	< 5	62	190	140	< 5	< 5
	EXI → αAra pI 7.5	80	81	260	230	13	< 5
	EXI → αAra pI 8.5	110	97	280	290	20	< 5
	EXIII	< 5	23	160	110	< 5	< 5
	EXIII → αAra pI 7.5	74	34	170	120	50	62
	EXIII → αAra pI 8.5	100	40	160	120	73	89
Softwood kraft-pulp arabinoxylan	EX pI 9	< 5	81	800	180	9	< 5
	EX pI 9 → αAra pI 8.5	300	170	1070	250	230	110

[1] → Sequential hydrolysis. First enzyme was inactivated by heat treatment before the additions of the second enzyme

arabinogalactan (Kimura et al., 1995). The *A. terreus* enzymes and the *A. niger* α-arabinofuranosidase B resemble the two latter enzymes in their hydrolytic properties, despite their divergent molecular properties (Luonteri et al., 1995; Rombouts et al., 1988). *A. awamori* α-arabinofuranosidase was also able to release arabinose from polymeric cereal and larchwood arabinoxylans, arabinogalactan and arabinan, but arabinose-substituted oligosaccharides were better substrates for the enzyme (Wood & McCrae, 1996). Furthermore, this α-arabinosidase possessed a novel property, as it was able to release feruloylated arabinose residues from steam-extracted wheat-straw arabinoxylan.

In previous studies, *T. reesei* α-arabinofuranosidase has been found to release arabinose from beet arabinan, wheat-straw arabinoxylan and different isolated cereal arabinoxylans (Poutanen, 1988; Margolles-Clark et al., 1996). The low activity of the *T. reesei* α-arabinofuranosidase towards different cereal arabinoxylans observed in this study might be due to the lower incubation temperature, the lower enzyme dosage or even partial inactivation of the enzyme.

α-Arabinofuranosidases of several *Streptomyces* species have also been studied thoroughly. *Streptomyces lividans* α-arabinofuranosidase, which contained a specific xylan-binding domain, resembled the *A. awamori* AXH as it preferred cereal arabinoxylans (oat, rye and wheat) as substrates (Vincent et al., 1997). It did not hydrolyse larchwood or birchwood xylan, arabinogalactan or linear α-1,5-arabinan. On the other hand, α-arabinofuranosidases isolated from *Streptomyces* sp. No. 17-1 (Kaji et al., 1981) and *S. diastaticus* (Tajana et al., 1992) also released arabinose from arabinogalactan, beet α-1,5-arabinan and debranched arabinan, respectively, whereas *S. purpurascens* IFO 3389 α-arabinofuranosidase was only active against low molecular-weight substrates (Komae et al., 1982). Both the *S. purpurascens* IFO 3389 enzyme (Komae et al., 1982) and the *A. niger* α-arabinofuranosidase A (Rombouts et al., 1988) are large proteins, which might explain their poor activity towards polymeric substrates. The substrate specificities of the *T. reesei*- and the other *Streptomyces* α-arabinofuranosidases resembled those of the *Aspergillus* enzymes used in this study.

Only a few amino acid sequences of α-arabinofuranosidases have been published and classified hitherto. The N-terminal amino acid sequences (20 aa) of the *A. terreus* α-arabinofuranosidases pI 8.3 and pI 8.5 were found to be identical with that of *T. reesei* α-arabinofuranosidase, which is similar to the amino acid sequence of *A. niger* α-arabinofuranosidase B (Luonteri et al., unpublished results; Margolles-Clark et al., 1996, Flipphi et al., 1993a). Furthermore, the N-terminus of *A. terreus* α-arabinofuranosidase pI 7.5 had four different amino acids in the sequence of 20 amino acids when compared with these enzymes (Luonteri et al., unpublished results). All these α-arabinofuranosidases possessed a broad substrate specificity. On the other hand, the N-terminal amino acid sequences of e.g. *A. niger* α-arabinofuranosidase A (Flipphi et al., 1993b), which hydrolyses only low molecular weight substrates, and *S. lividans* α-arabinofuranosidase (Vincent et al., 1997), which was highly specific towards arabinoxylans, were unlike the sequences of the other α-arabinofuranosidases mentioned above. However, complete amino acid sequences are required before further conclusions concerning the structure of the protein and its function can be drawn.

α-Arabinofuranosidases pI 7.5, pI 8.3 and pI 8.5 from *A. terreus*, α-arabinofuranosidase B from *A. niger* and *T. reesei* α-arabinofuranosidase had very similar activities against the polymeric substrates tested. Unlike *A. awamori* AXH, which was only active on different arabinoxylans, all three *A. terreus* α-arabinofuranosidases were able to release at

Table 6
Degradation of arabinoxylo-oligosaccharides (0.5 mg ml^{-1}) by *Aspergillus terreus* α-arabinofuranosides pI 7.5 (0.4 μg ml^{-1}) and pI 8.5 (0.3 μg ml^{-1}) alone and in combination with *T. reesei* β-xylosidase (βX, 0.9 μg ml^{-1}). Incubation: pH 4.0, 40°C, 24 h

Substrate Abbreviation[1]	Structure[2]	Enzyme	Degradation		Xylose formation[3]
			Alone	With βX	
3-Ara^{2}Xyl$_3$	Ara$_1$ \ Xyl–Xyl^3–Xyl	Ara pI 7.5	Not degraded	Not degraded	–
		Ara pI 8.5	Not degraded	Not degraded	–
3-Ara^{2}Xyl$_4$ (2/3)	Ara$_1$ \ Xyl–Xyl–Xyl^3–Xyl	Ara pI 7.5	Not degraded	Not degraded	+ /-
3-Ara^3/Xyl$_4$ (1/3)	Ara$_1$ \ Xyl–Xyl^{3}Xyl–Xyl	Ara pI 8.5	Not degraded	Not degraded	+ /-
3-Ara^{3}Xyl$_5$ (major)	Ara$_1$ \ Xyl–Xyl–Xyl^3–Xyl–Xyl	Ara pI 7.5	Slightly degraded	Not degraded	+
3-Ara^{2}Xyl$_5$ (minor)	Ara$_1$ \ Xyl–Xyl–Xyl–Xyl^3–Xyl	Ara pI 8.5	Slightly degraded	Not degraded	+
2-Ara^{3}Xyl$_4$	Ara$_1$ | Xyl–Xyl^2–Xyl–Xyl	Ara pI 7.5	Not degraded	Not degraded	+
		Ara pI 8.5	Not degraded	Not degraded	+

[1] See text.
[2] –, β-1,4-linkage; \, α-1,3-linkage; |, α-1,2-linkage; Ara, arabinose; Xyl, xylose; Xyl, xylose unit in the reducing end
[3] +, xylose formed; – no xylose formed

least part of the arabinose from arabinans, both types of arabinogalactans as well as from several arabinoxylans. Thus they could be classified as *Aspergillus niger*- or arafur B-type α-arabinofuranosidases as proposed by Kaji (1984) and Beldman et al. (1997), respectively. The type of linkage by which arabinose was attached to the other monosaccharide residues in the polymer clearly had an effect on the action of the enzymes studied. Furthermore, the degree of substitution of the polymer affected the extent of hydrolysis.

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