Barley arabinoxylan arabinofuranohydrolases: purification, characterization and determination of primary structures from cDNA clones

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A family 51 arabinoxylan arabinofuranohydrolase, designated AXAH-I, has been purified from extracts of 7-day-old barley (*Hordeum vulgare* L.) seedlings by fractional precipitation with $(NH_4)_2SO_4$ and ion-exchange chromatography. The enzyme has an apparent molecular mass of 65 kDa and releases L-arabinose from cereal cell wall arabinoxylans with a pH optimum of 4.3, a catalytic rate constant (k_{cat}) of 6.9 s^{-1} and a catalytic efficiency factor (k_{cat}/K_m) of 0.76 ($ml \cdot s^{-1} \cdot mg^{-1}$). Whereas the hydrolysis of α-L-arabinofuranosyl residues linked to C(O)3 of backbone (1 → 4)-β-xylosyl residues proceeds at the fastest rate, α-L-arabinofuranosyl residues on doubly substituted xylosyl residues are also hydrolysed, at lower rates. A near full-length cDNA encoding barley AXAH-I indicates that the mature enzyme consists of 626 amino acid residues and has a calculated pI of 4.8. A second cDNA, which is 81% identical with that encoding AXAH-I, encodes another barley AXAH, which has been designated AXAH-II. The barley AXAHs are likely to have key roles in wall metabolism in cereals and other members of the Poaceae. Thus the enzymes could participate in the modification of the fine structure of arabinoxylan during wall deposition, maturation or expansion, or in wall turnover and the hydrolysis of arabinoxylans in germinated grain.

Key words: arabinofuranosidase, cell walls, enzymic hydrolysis, heteroxylans, wall metabolism.

INTRODUCTION

Glucuronoarabinoxylans are widely distributed in cell walls of higher plants and are particularly abundant in walls of the Poaceae. In barley, arabinoxylans constitute approx. 70% of aleurone walls [1], approx. 20% of walls from the starchy endosperm [2,3] and 20–30% of walls from young vegetative tissues [4,5]. The barley arabinoxylans consist of (1 → 4)-β-D-xylopyranosyl residues that form a molecular backbone carrying single α-L-arabinofuranosyl residues. Most of the arabinosyl substituents are attached to C(O)3 of the backbone xylosyl residues but they are also linked to C(O)2 or to both C(O)3 and C(O)2 [6]. Other substituents, such as glucuronosyl residues, and short oligosaccharide side chains can also be present but are usually found in low abundance [7,8].

The fine chemical structures of wall arabinoxylans are subject to modification during normal plant growth and development; many of the changes that occur involve the removal of arabinofuranosyl substituents from the (1 → 4)-β-xylan backbone. Thus when arabinoxylans are initially deposited into walls of elongating maize coleoptiles, the (1 → 4)-β-xylan backbone is heavily substituted with arabinofuranosyl residues [9]. Subsequently, arabinosyl residues are removed from this highly soluble form of the polysaccharide in a process that presumably leads to decreased solubility and an enhanced ability of unsubstituted regions of the (1 → 4)-β-xylan backbone to 'lock' into the wall through hydrogen-bonding interactions with unsubstituted regions of other (1 → 4)-β-xylan chains, with cellulose or with other wall polysaccharides such as (1 → 3,1 → 4)-β-glucans [7,9,10]. Similarly, arabinofuranosyl residues will be removed from arabinoxylans during the turnover of wall polysaccharides in elongating coleoptiles [11–13] or during the depolymerization of walls in germinated grain [7,14].

Removal of the arabinofuranosyl residues from arabinoxylans is generally attributed to the α-L-arabinofuranoside arabinofuranohydrolases (EC 3.2.1.55) that are most commonly found in rumen or saprophytic micro-organisms; however, these enzymes are also found in higher plants. For example, α-L-arabinofuranosidases are secreted from isolated barley aleurone layers [15,16]. The α-L-arabinofuranosidases are most commonly assayed with the synthetic aryl glycoside 4-nitrophenyl α-L-arabinofuranoside (4NPA) as substrate in convenient spectrophotometric assays that are quick and easy to perform. However, these enzymes have a broad range of specificities for the various (1 → 2)-, (1 → 3)- and (1 → 5)-α-L-arabinofuranosyl linkages of arabinans and arabinoxylans. Thus we have purified α-L-arabinofuranosidases from young barley seedlings that rapidly hydrolyse 4NPA but have little or no activity on polymeric arabinoxylans (R. C. Lee, M. Hrmova and G. B. Fincher, unpublished work). Nevertheless, the well-documented changes in arabinoxylan structure that occur in normal growth and development indicate that AXAHs must exist; this activity has been reported in extracts of germinated cereal grain [17,18].

Here we describe the purification of an arabinoxylan-specific α-L-arabinoside arabinofuranohydrolase, designated arabinoxylan arabinofuranohydrolase (AXAH-I), from barley seedlings, and defined its action pattern, substrate specificity and kinetic properties. The complete primary structure of the enzyme and

Abbreviations used: AXAH, arabinoxylan arabinofuranohydrolase; EST, expressed sequence tag; 4NPA, 4-nitrophenyl α-L-arabinofuranoside; PTH, phenylthiohydantoin; RACE, rapid amplification of cDNA ends.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession numbers AF320324 and AF320325.

that of a second isoform have been deduced from cDNA clones. Although the cDNA sequences are very similar to expressed sequence tag (EST) sequences in the barley and *Arabidopsis* databases, these are the first sequences reported for genes encoding higher-plant AXAHs.

EXPERIMENTAL

Barley germination and enzyme purification

Barley (*Hordeum vulgare* L. cv. Clipper) grain (3 kg dry weight) was surface-sterilized with 0.2 % (w/v) $AgNO_3$, washed successively with sterile distilled water, 0.5 M NaCl and sterile distilled water, and immersed for 24 h in sterile distilled water containing chloramphenicol (100 mg/ml), neomycin (100 mg/ml), penicillin G (100 i.u./ml) and nystatin (100 i.u./ml) [19]. Germinated grain was maintained at approx. 40–45 % (w/w) moisture content for 7 days in the dark at 22 °C by the regular application of fresh antibiotic solution. No visible evidence of bacterial or fungal contamination of the grains was detected at any stage during this period. The germinated grain was stored overnight at −20 °C before homogenization at 4 °C in 1.5 vol. of 0.1 M sodium acetate buffer, pH 5, containing 10 mM EDTA, 10 mM NaN_3, 3 mM 2-mercaptoethanol and 3 mM PMSF (Sigma Chemical Co, St Louis, MO, U.S.A.), followed by $(NH_4)_2SO_4$ fractional precipitation [20]. The purification procedure was undertaken at 0–4 °C and is summarized below.

Step 1

Material precipitated with 20–40 %-satd $(NH_4)_2SO_4$ was resuspended in approx. 1 litre of 20 mM Tris/HCl buffer, pH 8.0, containing 4 mM NaN_3 and 3 mM 2-mercaptoethanol. Resuspended material was dialysed against the same buffer and applied to a 30 cm × 5 cm column of DEAE-cellulose (Whatman, Maidstone, Kent, U.K.) equilibrated in 20 mM Tris/HCl buffer, pH 8.0, containing 4 mM NaN_3 and 3 mM 2-mercaptoethanol, and eluted with the same buffer at a linear flow rate of 3 cm/h. After the elution of unbound proteins, bound proteins were eluted by application of a 3-litre linear gradient of 0–350 mM NaCl in the same buffer.

Step 2

Fractions with AXAH activity were pooled and dialysed against 50 mM sodium formate buffer, pH 3.8, containing 4 mM NaN_3, and applied to an 18 cm × 2.5 cm column of SP-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated in 50 mM sodium formate buffer, pH 3.8. After unbound proteins had been eluted, a linear gradient of 0–500 mM NaCl was applied at a flow rate of 40 cm/h.

Step 3

Pooled fractions from SP-Sepharose containing the AXAH-I were dialysed against 20 mM histidine/HCl buffer, pH 5.5, containing 4 mM NaN_3, and applied to a 8 cm × 1.5 cm column of Q-Sepharose (Amersham Pharmacia Biotech) equilibrated in 20 mM histidine/HCl buffer, pH 5.5. Bound proteins were eluted at a flow rate of 100 cm/h with a linear gradient of 0–500 mM NaCl.

Enzyme assays

α-L-Arabinofuranosidase activity was determined spectrophotometrically with 4NPA (Sigma). Assays were performed at 37 °C in 50 mM sodium acetate buffer, pH 5, containing 4 mM NaN_3 and 0.04 % (w/v) 4NPA. The reaction was terminated by the addition of 2 vol. of satd di-sodium tetraborate solution. One unit of activity is defined as the amount of enzyme required to produce 1 μmol of 4-nitrophenol/min, as determined by measurement of A_{410}. AXAH activities of column fractions were assessed semiquantitatively by analysis of released arabinose after incubation for up to 30 min with 0.5 % (w/v) wheat arabinoxylan (Megazyme, Bray, Ireland) at 37 °C in 50 mM sodium acetate buffer, pH 5, containing 4 mM NaN_3. Products were separated by TLC on silica-gel plates (Merck, Darmstadt, Germany) in ethyl acetate/acetic acid/water (3:2:1, by vol.). Sugars were detected with the orcinol reagent [20].

Protein determination and PAGE

Protein concentrations in column fractions were estimated from A_{280}. Protein contents of pooled fractions were determined with Coomassie Brilliant Blue (Pierce, Rockford, IL, U.S.A.) [21]. The purity of column fractions and purified proteins was assessed by SDS/PAGE [22] on 12.5 % (w/v) polyacrylamide gels stained with Coomassie Brilliant Blue R-250. Molecular mass marker proteins were from Amersham Pharmacia Biotech.

Substrate specificity

Aryl glycosides (Sigma) were used as substrates with purified AXAH as described above, at a substrate concentration of 2.5 mM. The ability to hydrolyse polysaccharide and oligosaccharide substrates was assessed by the separation and detection of hydrolysis products by TLC as described above. Wheat arabinoxylan, (1 → 4)-β-linked xylopentaose, (1 → 5)-α-linked arabinofuranohexaose and branched (1 → 3)- and (1 → 5)-linked α-arabinan from sugar beet and barley (1 → 3, 1 → 4)-β-glucan were from Megazyme (Bray, Ireland). Laminarin and larch wood arabinogalactan were from Sigma. Carboxymethyl-(1 → 4)-β-xylan and a (1 → 4)-β-xylan endohydrolase from *Thermomyces* sp. were from Dr Peter Biely (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak Republic); gum arabic was a gift from Professor B. A. Stone (La Trobe University, Melbourne, Australia). Hydrolysis products were analysed by TLC as described above.

Kinetic studies

Kinetic parameters of AXAH-I were determined for 4NPA at concentrations in the range of 0.3–6.5-fold the K_m, and for wheat arabinoxylan at substrate concentrations up to 5.0 mg/ml. The rate of hydrolysis of wheat arabinoxylan was estimated from the increase in reducing sugars [23,24]. Assays were performed in triplicate, in 50 mM sodium acetate buffer, pH 4.3, containing 4 mM NaN_3 and 160 μg/ml BSA (fraction V; Sigma Chemical Co.). A fixed amount of enzyme was incubated with various amounts of substrate; rates of hydrolysis were determined for each substrate as described above. Kinetic data were processed by a proportional weighted fit with a non-linear regression analysis program based on Michaelis–Menten enzyme kinetics [25].

Polysaccharide linkage analysis

Arabinoxylans were permethylated by using the $NaOH/CH_3I$ method [26] as described by Nunan et al. [27]. Methylated polysaccharides were hydrolysed with trifluoroacetic acid, reduced with $NaBD_4$ and acetylated with $HClO_4$ as a catalyst

[28]. Partly methylated alditol acetates were separated on a BPX70 capillary column (SGE, Melbourne, Australia) in a MAT 1010B GC-MS (Finnigan, San José, CA, U.S.A.). All methylation analyses were performed in quadruplicate in the laboratory of Professor Tony Bacic (University of Melbourne, Melbourne, Victoria, Australia).

Tryptic digestions and amino acid sequence analysis

N-terminal sequence analyses of AXAH-I and of tryptic peptides were performed on a Hewlett-Packard G1005A automated protein sequencer (Palo Alto, CA, U.S.A.), with Edman degradation chemistry. Phenylthiohydantoin (PTH) derivatives were identified with a Hewlett-Packard 1090 HPLC system. The tryptic peptides were generated and purified as described previously [29].

cDNA isolation

A λgt11 cDNA library prepared from 12-day-old barley (cv. Klages) seedlings (Clontech Laboratories, Palo Alto, CA, U.S.A.) was screened by performing duplicate plaque lifts on to nitrocellulose membranes (Micron Separations, Westborough, MA, U.S.A.). Plaque replicas were hybridized with [α-^{32}P]dCTP-labelled barley EST HK04K11 as described by Sambrook et al. [30] in 3 $\times$ SSC (SSC being 0.15 M NaCl/0.015 M sodium citrate) at 58 °C and washed in 0.2 $\times$ SSC containing 0.1 % SDS. The barley EST was selected on the basis of nucleotide sequence similarity to corresponding amino acid sequences generated from the purified AXAH-I enzyme. The EST was provided by Dr Andreas Graner (IPK, Gatersleben, Germany). Positive clones were identified by autoradiography and purified by further rounds of screening. The cDNA inserts were excised from purified λDNA, prepared with a Wizard λDNA kit (Promega, Madison, WI, U.S.A.), with restriction endonuclease *Eco*R1 (New England Biolabs, Beverly, MA, U.S.A.) and ligated into pBluescript SK(+) (Stratagene, La Jolla, CA, U.S.A.) with T4 DNA ligase (New England Biolabs). Ligation mixes were transformed into competent DH5-α *Escherichia coli* cells by electroporation with the Bio-Rad (Hercules, CA, U.S.A.) Gene Pulser apparatus. The 3′ rapid amplification of cDNA ends (RACE) protocol was performed as described by Frohman et al. [31] with cDNA prepared from 5-day-old barley (cv. Clipper) shoots with Trizol™ reagent (Gibco-BRL, Rockville, MD, U.S.A.) for RNA isolation and the Thermoscript™ reverse-transcriptase-mediated PCR system (Gibco-BRL) for reverse transcription.

Nucleotide sequencing and sequence analysis

DNA sequencing was performed with the dideoxyribonucleotide chain termination procedure [32] with the ABI Prism dye terminator kit on an Applied Biosystems 370 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Data from automated sequencing were compiled and analysed with the Seq-Ed program (Applied Biosystems) and further analyses of DNA sequences and database searches were performed with the University of Wisconsin Genetics Computer Group software [33] in the ANGIS suite of programs at the University of Sydney (Sydney, New South Wales, Australia). Sequences were aligned using the ClustalW program (http://www2.ebi.ac.uk/clustalw/) and the unrooted phylogenetic tree was constructed with the PAUP program [34].

RESULTS

Purification of AXAH

Approximately 90 % of AXAH activity was recovered in the material precipitated by 20–40 %-satd $(NH_4)_2SO_4$. However, other α-L-arabinofuranosidases, which were unable to hydrolyse arabinoxylans, were present, predominantly in the 40–60 %-satd $(NH_4)_2SO_4$ fractions. These have also been purified and characterized (R. C. Lee, M. Hrmova and G. B. Fincher, unpublished work). We therefore used the 20–40 %-satd $(NH_4)_2SO_4$ fraction for the purification of barley AXAH-I. After chromatography on DEAE-cellulose, minor peaks of activity, corresponding to the two α-L-arabinofuranosidases observed for the 40–60 %-satd $(NH_4)_2SO_4$ fraction in preliminary experiments, were eluted at approx. 90 and 120 mM NaCl. Approximately 80 units of 4-NPA activity, confirmed as AXAH-I by its ability to hydrolyse arabinoxylan, was eluted at 220 mM NaCl (Figure 1A). These fractions, purified more than 10-fold during the DEAE-cellulose step, were pooled and applied to an SP-Sepharose column: a single peak of AXAH-I activity was eluted at approx. 300 mM NaCl (Figure 1B). The SP-Sepharose step proved to be highly effective for the removal of many low-molecular-mass contaminants, including an abundant protein of approx. 26 kDa (Figure 2). Pooled fractions containing AXAH-I were subsequently fractionated on Q-Sepharose. The elution of bound proteins yielded a major protein peak at 320 mM NaCl that was coincident with AXAH-I activity (Figure 1C). The two 10 ml fractions at the height of the activity peak (Figure 1C, fractions 11 and 12) were pooled and concentrated in 10 mM sodium acetate buffer, pH 5.0, containing 4 mM NaN_3. This sample contained the purified AXAH-I that was used for subsequent biochemical characterization and analysis.

The purity of the final enzyme preparation was examined by SDS/PAGE (Figure 2). A single major band at 65 kDa was present with one minor contaminating band of approx. 60 kDa. The final recovery and fold purification factors of AXAH-I were relatively low (Table 1) but were probably underestimated because more than half of the 4NPA-hydrolysing activity in the crude seedling extract was contributed by other α-L-arabinofuranosidases.

Amino acid sequence analysis

Both the N-terminal amino acid sequence of the purified AXAH-I and the sequences of tryptic peptides derived from it were determined. The N-terminal sequence was free of secondary sequences and PTH derivatives were recovered with expected yields. The first 38 residues were ITQVA SLGVD SSPHL ARKIP DTLFG IFFEE INHAG AGG.

Tryptic digestion of the AXAH-I preparation produced at least 25 peptide fractions that were resolved by reverse-phase HPLC (results not shown); three of these were selected for N-terminal sequencing. One fraction yielded two amino acid sequences that could be clearly resolved on the basis of abundance of PTH derivatives: GFEAG GPHTP SNINP WSIIG and EAYPD IQMIS N–DGS STPLD. The sequences of two other fractions were IVNFG PDAVG LTISA TGLQG SINAF GSTAT VLTSG GVMDE NSFAN – VVPV and NSDVV QMASY APLFI NDNDR TWNPD AIVFN SWQQY GTPSY.

Results of non-redundant BLAST searches at this stage revealed similarities to an arabinosidase from *Bacteroides ovatus* [35], α-arabinofuranosidase I from *Streptomyces chartreusis* [36] and two *Arabidopsis thaliana* genomic DNA sequences (GenBank® accession numbers AF149413 and AC011708).

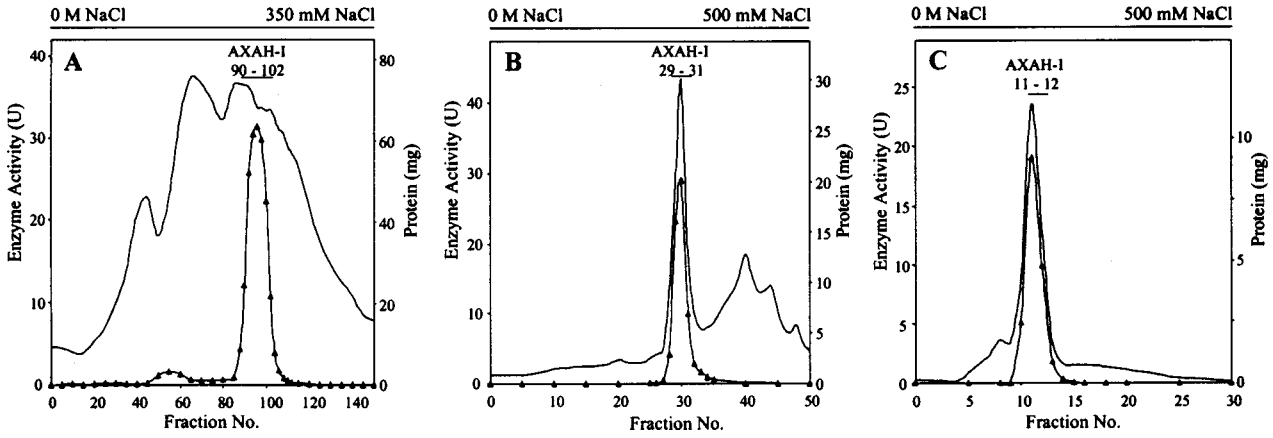

Figure 1 Ion-exchange chromatography steps in the purification of AXAH-I

(**A**) Material precipitated with 20–40%-satd $(NH_4)_2SO_4$ was applied to a DEAE-cellulose column at pH 8.0 and, after the removal of unbound proteins, bound proteins were eluted with a 3-litre linear gradient of 0–350 mM NaCl. Arabinofuranosidase activity of 20 ml fractions was determined against 4NPA (▲) and wheat arabinoxylan (results not shown). Protein (unbroken line) was measured by A_{280}. (**B**) Fractions 90–102 from the DEAE-cellulose column were pooled and applied to SP-Sepharose at pH 3.8. Bound proteins were eluted with a 1-litre linear gradient of 0–500 mM NaCl and fractions (20 ml) were assayed as described for DEAE-cellulose. (**C**) Fractions 29–31 from SP-Sepharose were concentrated and applied to Q-Sepharose at pH 5.5. Bound material was eluted with a 300 ml linear gradient of 0–500 mM NaCl and fractions (10 ml) were assayed as described above.

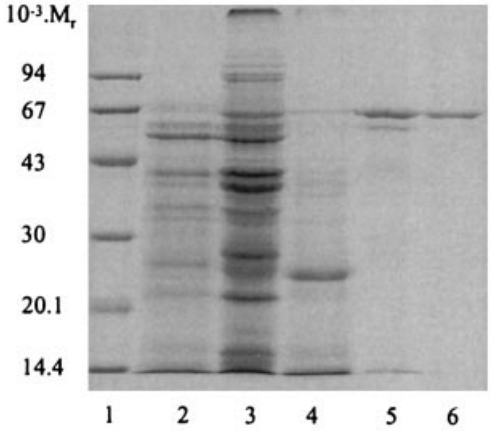

Figure 2 SDS/PAGE of protein fractions during the purification of AXAH-I

Lane 1, molecular mass markers (indicated at the left in kDa); lane 2, crude extract; lane 3, 20–40%-satd $(NH_4)_2SO_4$ fraction; lane 4, DEAE-cellulose pooled fractions 90–102; lane 5, SP-Sepharose pooled fractions 29–31; lane 6, Q-Sepharose pooled fractions.

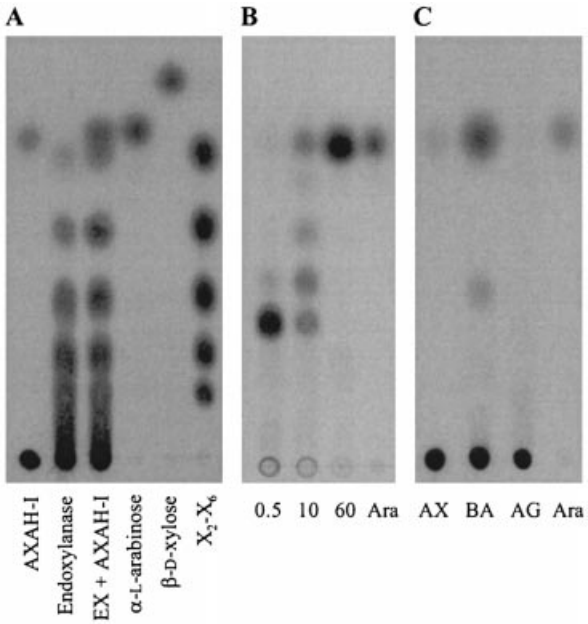

Figure 3 TLC of products of AXAH-I on wheat arabinoxylan (A), (1 → 5)-arabinohexaose (B) and arabinan and arabinogalactan (C)

(**A**) Lane 1, AXAH-I (0.5 unit/ml) incubated with 0.5% (w/v) wheat arabinoxylan for 1 h at 37 °C. L-Arabinose was the sole product released by this enzyme. Lane 2, fungal (1 → 4)-β-xylan endohydrolase incubated with 0.5% (w/v) wheat flour arabinoxylan. Lane 3, AXAH-I (0.5 unit/ml) and endoxylanase incubated with 0.5% (w/v) arabinoxylan. Lanes 4–6, standard L-arabinofuranose, D-xylopyranose and oligoxylobiose–oligoxylohexaose (X_2–X_6). (**B**) AXAH-I (0.1 unit/ml) incubated with 10 mM (1 → 5)-linked arabinofuranohexaose for 0.5, 10 and 60 min, as indicated. (**C**) AXAH-I (0.2 unit/ml) incubated with 0.5% (w/v) wheat arabinoxylan (AX), beet arabinan (BA) and larch arabinogalactan (AG) for 60 min at 37 °C.

Table 1 Enzyme yields and purification factors at each stage of the purification of AXAH-I

Recovered activity units were assayed with 4NPA; recoveries are expressed as percentages of initial activity units; purification factors are calculated on the basis of specific activities.

Step	Yield		Specific activity (m-units/mg)	Recovery (%)	Purification factor (fold)
	Protein (mg)	Activity (units)			
Crude homogenate	24000	835	34	100	1.0
20–40%-satd $(NH_4)_2SO_4$	1530	384	251	46	7.3
DEAE-cellulose	82	270	3300	32	96
SP-Sepharose	9.7	38	3900	4.5	113
Q-Sepharose	1.4	8.7	6300	1.0	184

Specificity, kinetics and action pattern

The activities of AXAH-I on aryl glycosides and several polysaccharides were determined. Of these substrates, 4NPA,

Table 2 Kinetic parameters of AXAH-I with 4NPA and wheat arabinoxylan

k_{cat} is the catalytic rate constant; k_{cat}/K_m is the catalytic efficiency factor.

Parameter	Substrate...	4NPA	Wheat arabinoxylan
K_m		0.77 mM	9.0 mg/ml
k_{cat} (s^{-1})		15.0	6.9
k_{cat}/K_m		1.95×10^4 $M^{-1} \cdot s^{-1}$	0.76 $ml \cdot s^{-1} \cdot mg^{-1}$

Table 3 Changes in linkage composition of wheat arabinoxylan (AX) after incubation with AXAH-I

Results are means for two sets of duplicated analyses. The difference column shows the change in amount of linkage type (where significant).

	Composition (mol%)		
Deduced linkage	AX	AX + AXAH-I	Difference
t-Ara*f*	24	10	−14
t-Xyl*p*	2	3	–
1,2-Ara*f*	1	2	–
1,4-Xyl*p*	53	76	23
1,3,4-Xyl*p*	11	4	−7
1,2,4-Xyl*p*	2	1	–
1,2,3,4-Xyl*p*	8	5	−3

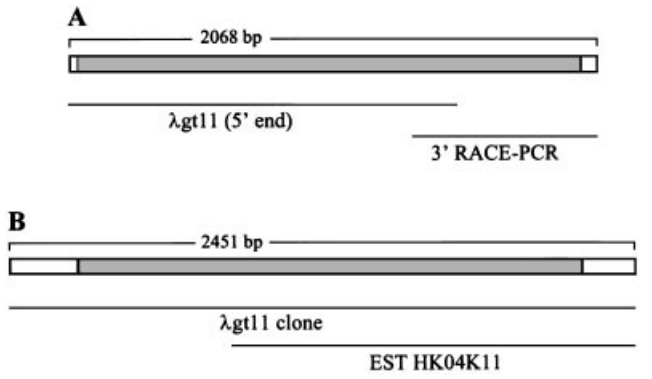

Figure 4 Alignment of cDNA species encoding AXAH-I (A) and AXAH-II (B) sequences

The full-length cDNA sequence of AXAH-I is a compilation of an overlapping λgt11 cDNA clone and a 3′ RACE-PCR product. The cDNA sequence of AXAH-II was derived from a single λgt11 clone. The position of the 1400 bp barley EST (HK04K11) used as a probe for library screening is shown.

(1 → 5)-α-L-arabinofuranohexaose, beet arabinan, larch wood arabinogalactan and wheat flour arabinoxylan could be hydrolysed by AXAH-I; TLC showed that arabinose was the final product released (Figure 3). After hydrolysis of the sugar beet arabinan, a product of intermediate mobility was detected in addition to arabinose (Figure 3C). This is likely to have been a rhamnogalacturonan fragment. The enzyme was unable to hydrolyse 4-nitrophenyl arabinopyranosides, xylopyranosides, mannopyranosides, glucopyranosides or galactopyranosides, whether α- or β-linked (results not shown). The enzyme did not hydrolyse the polysaccharide substrates laminarin, (1 → 3,1 → 4)-β-glucan, gum arabic or carboxymethylxylan (results not shown).

The kinetic parameters for AXAH-1 with 4NPA and wheat flour arabinoxylan are compared in Table 2. The K_m for 4NPA had a standard error of less than 10% in triplicate assays. However, the K_m for AXAH-I on arabinoxylan was less reliable, with a standard error of 17%. This was presumably because the K_m of 9.0 mg/ml was near the upper limit of solubility for the wheat flour arabinoxylan. It must also be noted that the estimation of kinetic parameters for enzymes hydrolysing polysaccharide substrates is inherently difficult because released products can also be new substrates for the enzyme. Care was therefore taken to ensure that only initial reaction rates were measured [29,37].

The action pattern of AXAH-I on arabinoxylan was investigated (Figure 3A) and showed that the enzyme removed single arabinofuranosyl substituents from the polysaccharide. When both AXAH-I and a fungal endoxylanase were added to the substrate, the rate of release of arabinose was enhanced. In addition, AXAH-I completely hydrolysed the (1 → 5)-α-linked arabinofuranohexaose to arabinose (Figure 3B) and released arabinofuranose from sugar beet arabinan at a relatively rapid rate (Figure 3C). Low activity was also detected towards the larch wood arabinogalactan (Figure 3C).

Linkage analyses of arabinoxylan incubated for 16 h, either with or without enzyme, suggested that AXAH-I removes both the (1 → 3)-α- and (1 → 2)-α-linked arabinofuranosyl substituents of arabinoxylan (Table 3). Furthermore, arabinofuranosyl residues were released from doubly substituted xylopyranosyl residues. The results presented in Table 3 also show that not all arabinofuranosyl residues had been removed after incubation for 16 h.

Isolation of cDNA species encoding AXAHs

A 1400 bp barley EST HK04K11, obtained from Dr Andreas Graner, was used to probe the 12-day-old seedling λgt11cDNA library. Of the 200000 clones screened, 26 hybridized with the EST probe. The insert sizes of these clones were estimated by PCR amplification with primers based on λgt11 sequences flanking the multiple cloning site. Six cDNA species were analysed further and, after preparation of monoclonal phage stocks and the subcloning of cDNA inserts into pBluescript, a full-length cDNA of 2451 bp was identified that encoded a polypeptide with similarity to AXAH-I amino acid sequences (Figure 4). The complete DNA sequence of this cDNA clone was determined. The amino acid sequence of the encoded polypeptide derived from the cDNA is shown in Figure 5. Comparison of this amino acid sequence with the known sequences of the purified AXAH-I protein revealed identities of up to 90%. The cDNA sequence therefore did not correspond exactly to the amino acid sequence of the purified AXAH-I enzyme but the sequence similarity suggested that the cDNA was likely to represent a second AXAH isoenzyme, which was designated AXAH-II.

The AXAH-II cDNA had 5′ and 3′ untranslated regions of 273 and 206 bp respectively and included a polyadenylate tail of 18 residues. A polyadenylation signal of AATAAA was present 26 bases upstream of the polyadenylate sequence and five potential N-glycosylation sites were found. On the assumption that the N-terminus of AXAH-II is in a similar position to that in AXAH-I, the N-terminal amino acid residue would be the Ala residue indicated in Figure 5. On this basis the theoretical molecular mass and isoelectric point of AXAH-II would be 68760 Da and 5.2 respectively.

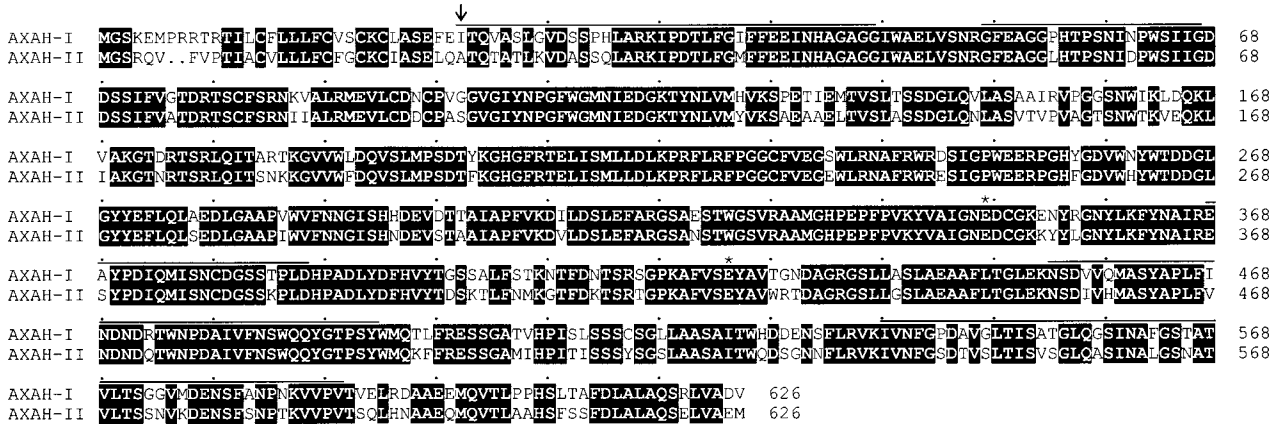

Figure 5 Alignment of AXAH-I and AXAH-II amino acid sequences

The cDNA species were translated from the putative start methionine to the amino acid residue preceding the TGA stop codon. The AXAH-I and AXAH-II cDNA species encode mature polypeptides of 626 residues with signal peptides of 32 and 30 residues respectively. The amino acid sequences are numbered from the N-termini of the mature enzymes, indicated by an arrow, and lines above the AXAH-I sequence indicate tryptic peptide sequences obtained by amino acid sequence analysis. The putative catalytic acid (Glu-348) and catalytic nucleophile (Glu-425) are indicated by asterisks.

The AXAH-II cDNA was subsequently used as a probe to rescreen the λgt11 cDNA library. Plaque replicas that hybridized with the AXAH-II probe were identified by autoradiography and potential AXAH-I clones were selected as those for which the hybridization signal was relatively faint. A 1386 bp cDNA corresponding to the 5′ region of the AXAH-I cDNA was isolated by using this approach. A 741 bp 3′ end fragment of the AXAH-I cDNA was isolated using 3′ RACE-PCR [36] with oligonucleotide primers based on the 5′ sequence of the AXAH-I cDNA, in positions where differences in nucleotide sequences between AXAH-I and AXAH-II were observed. In conjunction with the 3′ end RACE primer (5′-GACTCGAGTCGACATC-GA-3′) [31], the 5′ AXAH-I-specific primers for the primary and secondary rounds of PCR were 5′-GTGGGAAAGAAAATTA-CCGT-3′ and 5′-TGTCTATACCGGTTCCAGTG-3′ respectively. The final AXAH-I cDNA sequence that has been lodged in the databases is therefore a compilation of the sequences from the two cDNA species (Figure 4). The total AXAH-I cDNA was 2068 bp in length and had 5′ and 3′ untranslated regions of 30 and 64 bp respectively. No polyadenylic acid sequence was present. One potential N-glycosylation site was found. The N-terminal amino acid sequence and those of each of the tryptic peptides, as determined by automated amino acid sequence analysis of purified AXAH-I enzyme and its tryptic fragments, corresponded exactly to the deduced amino acid sequence from the cDNA. The theoretical molecular mass and isoelectric point were 68279 Da and 4.8 respectively. The sequences of AXAH-I and AXAH-II have 81% identity at the nucleotide level and 83% at the amino acid level of the mature enzyme. No bias in codon usage was evident. The deduced amino acid sequence of AXAH-I is aligned with that of AXAH-II in Figure 5.

DISCUSSION

An AXAH has been purified from 7-day-old barley seedlings and designated AXAH-I. The purification procedure involved precipitation with $(NH_4)_2SO_4$ and three ion-exchange chromatography steps. It was apparent throughout the purification procedure that the enzyme had a relatively low pI, which undoubtedly contributed to the relative ease of purification. The final fold purification factor of 184 (Table 1) is likely to be a great underestimate of the true purification factor for the enzyme because there are also α-L-arabinofuranosidases with relatively high activities on 4NPA in the initial seedling extract (R. C. Lee, M. Hrmova and G. B. Fincher, unpublished work). These α-L-arabinofuranosidases hydrolyse the 4NPA substrate used to monitor AXAH-I activity during the purification process, but do not hydrolyse arabinofuranosyl substituents of polymeric arabinoxylan. The α-L-arabinofuranosidases were completely removed after the first ion-exchange chromatography step.

The purity of the final AXAH-I preparation was examined by SDS/PAGE (Figure 2) and, in addition, by close examination of N-terminal amino acid sequencing profiles. The purified AXAH-I had an apparent molecular mass of 65 kDa and was observed as a single major band on the SDS/PAGE gels (Figure 2). A very faint band can also be observed at a molecular mass of approx. 60 kDa (Figure 2, lane 6), although during N-terminal amino acid sequence analysis of the purified AXAH-I preparation no secondary sequences were ever detected and PTH derivatives were recovered in yields expected for the major protein band (results not shown). It remains possible that the faint protein band at 60 kDa represents a second AXAH isoenzyme or a truncated version of AXAH-I.

The substrate specificity of the purified barley AXAH-I was tested against a range of aryl glycosides, oligosaccharides and polysaccharides. It was clear that the enzyme exhibits a high degree of specificity for the α-L-arabinofuranosyl moiety of substrates but is relatively tolerant to different aglycone moieties. Most importantly, barley AXAH-I could be distinguished from several α-L-arabinofuranosidases in the seedling extracts because of its ability to hydrolyse arabinoxylan. The α-L-arabinofuranosidases are unable to do this, although both types of enzyme can hydrolyse 4NPA (R. C. Lee, M. Hrmova and G. B. Fincher, unpublished work). Care must therefore be exercised in using 4NPA as a substrate to monitor AXAH activity, especially if it is believed that the 4NPA activity is measuring enzymes that directly remove arabinosyl residues from polymeric arabinoxylan [16]. Preliminary substrate specificity studies on the barley α-L-arabinofuranosidases indicate that these enzymes are most likely to participate in the final hydrolysis of low-molecular-mass

oligosaccharides released from arabinoxylans by the action of endohydrolases (R. C. Lee, M. Hrmova and G. B. Fincher, unpublished work).

Thus the barley AXAH-I enzyme can hydrolyse 4NPA, wheat flour arabinoxylan, sugar beet arabinan, larch wood arabinogalactan and (1 → 5)-linked α-L-oligoarabinosides at significant rates (Table 2, Figure 3). AXAH-I might therefore catalyse the removal of short oligomeric side chains that occur in low abundance on cereal arabinoxylans [38]. Furthermore, (1 → 5)-α-L-oligoarabinosides are attached to rhamnosyl residues of cereal pectins [39], which raises the possibility that the AXAH enzymes might also participate in pectin hydrolysis during cell wall metabolism. The hydrolysis of the sugar beet arabinan (Figure 3) is consistent with this possibility.

A comparison of kinetic parameters calculated from the action of barley AXAH-I on the synthetic substrate, 4NPA, and wheat flour arabinoxylan shows that the catalytic rate for 4NPA is significantly higher than that for the arabinoxylan (Table 2). However, large differences in the rates of substrate diffusion make such comparisons difficult to interpret.

The action pattern of barley AXAH-I was investigated by the examination of products of hydrolysis of 4NPA, wheat flour arabinoxylan, sugar beet arabinan, larch wood arabinogalactan and (1 → 5)-α-L-arabinofuranohexaose, where arabinose was the sole product released in all cases (Figure 3). The addition of a fungal endoxylanase seemed to enhance hydrolytic rates on arabinoxylan (Figure 3A); intermediate (1 → 5)-α-L-oligoarabinosides could be detected in the course of hydrolysis of (1 → 5)-α-L-arabinofuranohexaose (Figure 3B). The action pattern was further investigated through methylation analysis of the wheat flour arabinoxylan before and after hydrolysis. During hydrolysis the molar percentage of terminal arabinofuranosyl residues decreased markedly, as expected; this was accompanied by an increase in unsubstituted (1 → 4)-xylopyranosyl residues (Table 3). In addition, the results show that the enzyme removes the arabinosyl residues not only from C(O)3-substituted xylopyranosyl residues, as indicated by the decrease in the 1,3,4-substituted xylopyranosyl residues, but also from xylopyranosyl residues that are doubly substituted at C(O)3 and C(O)2, as indicated by the decrease in 1,2,3,4-substituted xylopyranosyl derivatives (Table 3). The methylation data also suggest that barley AXAH-I can hydrolyse arabinosyl residues on C(O)2 of the backbone xylosyl residues; however, because of the relatively low abundance of C(O)2-substituted xylopyranosyl residues (Table 3), errors would be relatively large and this result needs to be viewed with caution. Investigations of the substrate specificity of a recently reported AXAH from barley, with the use of NMR techniques and defined arabino-oligoxylosides, support these findings [18]. The enzyme has the capacity to remove both (1 → 2)- and (1 → 3)-linked arabinofuranosyl substituents from the non-reducing terminal xylosyl residue but not from internal xylosyl residues [18].

The ability of barley AXAH-I to hydrolyse both 4NPA and arabinoxylan can be contrasted with an earlier report that an AXAH in extracts of wheat bran and germinated wheat grain could not hydrolyse 4NPA [17]. Similarly, an AXAH from *Aspergillus awamori* is unable to hydrolyse 4NPA [40]. These differences suggest that further attention might be directed to their Enzyme Commission classification in class EC 3.2.1.55 because there seem to be subgroups of α-L-arabinofuranoside arabinofuranohydrolases with different substrate specificities. The difficulty in classifying these enzymes is consistent with the presence of α-L-arabinofuranosidases in several of the families of glycoside hydrolases identified by Henrissat [41], including families 43, 51, 54 and 62. Generally, enzymes in families 51 and 54 can hydrolyse (1 → 2)- and (1 → 3)-linkages, whereas those in family 43 exhibit a preference for (1 → 5)-linkages [40]. The barley enzyme described here is a member of the family 51 group, on the basis of amino acid sequence data, but has an apparently broader substrate specificity than other family 51 enzymes in that it can hydrolyse (1 → 2)-, (1 → 3)- and (1 → 5)-linkages.

The purified barley AXAH-I was subjected to N-terminal amino acid sequence analysis; amino acid sequences of tryptic peptides generated from the enzyme were also determined. The N-terminal sequence was essentially the same as that of the enzyme purified recently by Ferré et al. [18]. On the basis of these sequences, a barley EST that potentially encoded AXAH-I was identified and used to screen a cDNA library from 12-day-old barley seedlings. Through a combination of cDNA library screening and 3′ RACE-PCR [31], two overlapping cDNA species were isolated and sequenced. One corresponded to the mRNA encoding AXAH-I because it could be translated into an amino acid sequence that exactly matched the sequences of 38 N-terminal residues of the purified enzyme and a total of 128 residues determined from four tryptic peptides.

The second cDNA clearly encoded a related AXAH from barley; this isoform has been designated AXAH-II. The AXAH-I and AXAH-II cDNA species were 2068 and 2451 bp in length respectively, encoding mature enzymes with respective molecular masses of 68279 and 68760 Da and with calculated pI values of 4.8 and 5.2. In both cases the mature enzymes contain 626 residues. The calculated molecular masses of the enzymes are somewhat higher than the apparent molecular mass of 65 kDa observed for the purified AXAH-I on SDS/PAGE gels (Figure 2).

The amino acid sequences for the two enzymes are aligned in Figure 5 and exhibit 83 % sequence identity. Although AXAH-II was not detected during the purification of AXAH-I, isolation of the cDNA indicates that its gene is expressed in young barley seedlings, perhaps at relatively low levels or at different times after germination. The presence of at least two AXAH isoenzymes is also consistent with our preliminary Southern hybridization analyses, which indicate that barley AXAHs are encoded by a small family of two or three genes (R. C. Lee, R. A. Burton and G. B. Fincher, unpublished work). In contrast with genes encoding other polysaccharide hydrolases from barley [7,14], there was no apparent bias in codon usage in either of the AXAH genes.

The availability of the complete primary structures for barley AXAH-I and AXAH-II has enabled the enzymes to be placed in the family 51 glycoside hydrolases [41] (http://afmb.cnrs-mrs.fr), in which there are currently 22 microbial enzymes, classified mostly as α-L-arabinofuranosidases, and two BAC (bacterial artificial chromosome) clones from *A. thaliana*. Nucleotide and amino acid sequence identities between the barley AXAH enzymes and the microbial α-L-arabinofuranosidases are generally approx. 30–35 % but rise to 52–63 % between the barley and *Arabidopsis* sequences. An unrooted phylogenetic tree summarizing the relatedness of family 51 glycoside hydrolases is presented in Figure 6 in which, as expected, the higher-plant enzymes are clustered on a common branch.

The sequence alignments shown in Figure 5 and comparative hydrophobic cluster analyses allow the identification of likely catalytic amino acid residues in the enzymes [42]. Thus the catalytic acid in the barley AXAH enzymes is likely be Glu-348, which is found in a highly conserved Gly-Asn-Glu sequence (Figure 5) [42]. The catalytic nucleophile of the barley AXAH enzymes is likely to be Glu-425, although sequence conservation around this residue is not particularly high in other family 51 glycoside hydrolases [42].

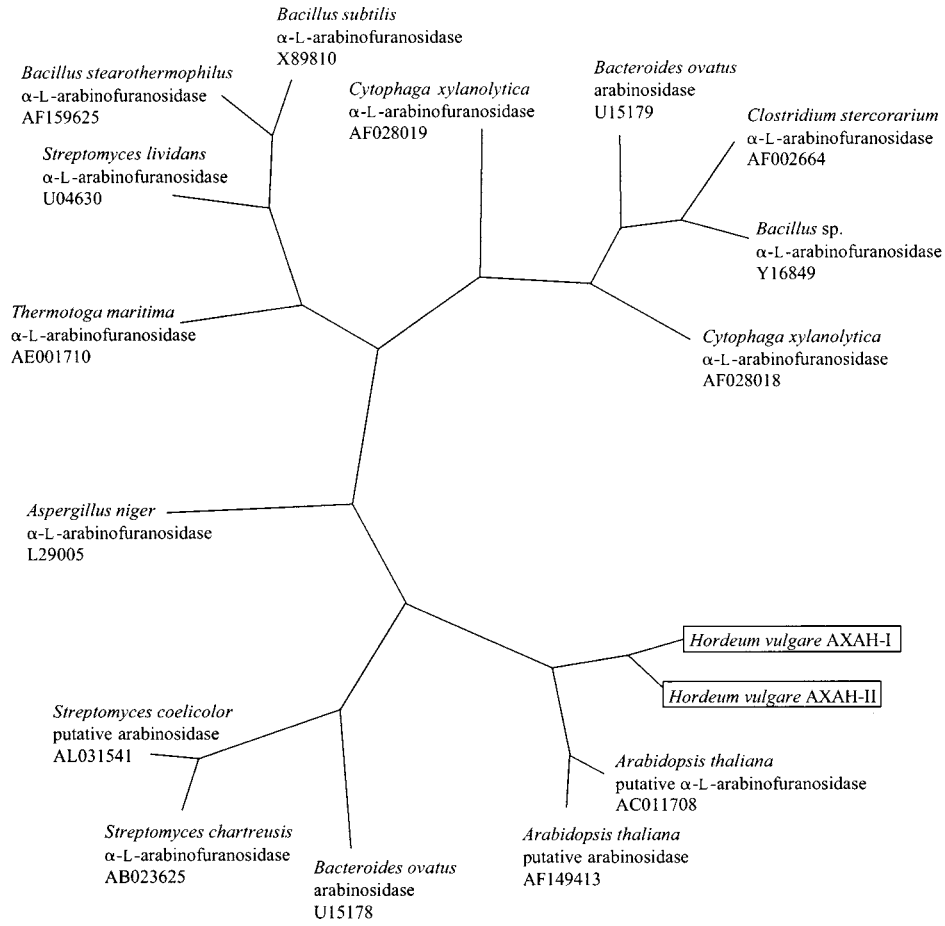

Figure 6 Unrooted radial phylogenetic tree of family 51 glycoside hydrolases

Sequences were aligned with the ClustalW program; the unrooted phylogenetic tree was constructed with the PAUP program [34]. AXAH-I and AXAH-II have a high degree of similarity to *Arabidopsis thaliana* genomic sequences encoding arabinosidase-like proteins (GenBank®/EMBL accession numbers AC011708 and AF149413). More distantly related family 51 glycoside hydrolases include arabinosidases from *Bacteroides ovatus* (U15178 and U15179), α-L-arabinofuranosidases from *Cytophaga xylanolytica* (AF028018 and AF028019), α-L-arabinofuranosidases from *Aspergillus niger* (L29005), *Bacillus* sp. (Y16849), *Bacillus stearothermophilus* (AF159625), *Bacillus subtilis* (X89810), *Clostridium stercorarium* (AF002664), *Streptomyces lividans* (U04630), *Streptomyces chartreusis* (AB023625), *Streptomyces coelicolor* (AL031541) and *Thermotoga maritima* (AE001710).

The final consideration here is the biological functions of the barley AXAHs. In higher plants and particularly in the Poaceae, in which arabinoxylans constitute a major component of cell walls, the enzymes almost certainly have a central role in cell wall metabolism during normal growth and development and might also be involved in the modification of the wall in response to biotic or abiotic stresses. A central role for AXAH-I in arabinoxylan metabolism can be inferred by the abundance of this polysaccharide in barley; however, the hydrolysis of branched arabinans and (1 → 5)-linked arabinosides indicates a broader specificity. A thorough description of the substrate specificity, and hence biological function, of the barley AXAHs will depend on the determination of the three-dimensional structure of the enzyme.

As mentioned above, enzymes that catalyse the hydrolytic removal of α-L-arabinofuranosyl residues from cell wall arabinoxylans can generate changes in arabinoxylan fine structure that will have marked effects on such physicochemical properties of the polysaccharides as solubility [10] and hence on their interactions with other wall polysaccharides. Thus the removal of arabinofuranosyl residues might be expected not only to 'stiffen' the cell wall but also to decrease its permeability to metabolites and its penetrability by pathogenic micro-organisms. The availability of cDNA species encoding two AXAH isoenzymes from barley will now permit the generation of gene-specific probes for use in Northern hybridization analyses. Patterns of individual gene transcription in various tissues of growing seedlings could be compared with precise information on enzyme activities and changes that occur in the fine structure of cell wall arabinoxylans. The combined information will allow more confident conclusions to be drawn regarding the roles of the enzymes during the deposition of newly synthesized arabinoxylans into the wall matrix, during changes and turnover of wall polysaccharides that occur in elongating cells, and during the depolymerization of arabinoxylans in germinated grain.

We thank Dr Andreas Graner for providing the EST clones, Dr Neil Shirley and Jelle Lahnstein for assistance with amino acid analysis, Professor Tony Bacic for performing the methylation analyses, and Professor Bruce Stone and Dr Peter Biely for providing polysaccharides and enzymes that are not commercially available. This work was supported by grants from the Australian Research Council and the Grains Research and Development Corporation of Australia. R. C. L. gratefully acknowledges receipt of a Grains Research and Development Corporation Postgraduate Scholarship.

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Received 13 November 2000/29 January 2001; accepted 27 February 2001