BIOCHEMISTRY AND MOLECULAR BIOLOGY OF ARABINOXYLAN METABOLISM IN BARLEY

Submitted by

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A thesis submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy

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STATEMENT OF AUTHORSHIP

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Robert C. Lee

April 2002

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PUBLICATIONS

The following papers relate directly to the work described in this thesis:

Lee, R.C., Burton, R.A., Hrmova, M. and Fincher, G.B. (2001). Barley arabinoxylan arabinofuranohydrolases: purification, characterization and determination of primary structures from cDNA clones. *Biochemical Journal*, **356**, 181-189.

Lee, R.C., Hrmova, M., Burton, R.A., Lahnstein, J. and Fincher, G.B. (2002). An α-L-arabinofuranosidase and a β-D-xylosidase from barley: purification, characterization and primary structures. *Manuscript in preparation.*

The cDNA sequences documented in this thesis have been submitted to the GenBank nucleotide sequence database and have accession numbers as follows:

AY029259 *Hordeum vulgare* α-L-arabinofuranosidase/β-D-xylosidase ARA-I

AY029260 *Hordeum vulgare* β-D-xylosidase XYL

AF320324 *Hordeum vulgare* arabinoxylan arabinofuranohydrolase AXAH-I

AF320325 *Hordeum vulgare* arabinoxylan arabinofuranohydrolase AXAH-II

ABBREVIATIONS

A	Absorbance
AU	Absorbance units
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
cm	Centimetre
CM	Carboxymethyl
cM	CentiMorgan
Da	Dalton
kDa	Kilo-Dalton
dCTP	2'-Deoxycytidine 5'-triphosphate
DEAE	Diaminoethyl
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxynucleotide 5'-triphosphate
DP	Degree of polymerisation
EDTA	Ethylene diamine tetra-acetic acid
g	Gram
g	Units of relative centrifugal force
GA_3	Gibberellic acid
h	Hours
HCA	Hydrophobic cluster analysis
IPTG	Isopropylthiogalactoside
kb	Kilo base
LB	Luria-Bertani
M	Molar
min	Minutes
M_r	Relative molecular mass
mRNA	Messenger RNA
4NP-	4-Nitrophenyl-
4NP	4-Nitrophenol
4NPA	4-Nitophenyl α-L-arabinofuranoside
4NPX	4-Nitophenyl β-D-xyloside
PCR	Polymerase chain reaction
pI	Isoelectric point
PMSF	Phenylmethylsulphonyl fluoride
RACE	Randomly amplified cDNA ends
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RP-HPLC	Reversed-phase high-performance liquid chromatography
rpm	Revolutions per minute
RT-PCR	Reverse transcription PCR
sec	Seconds
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSC	Standard saline citrate
Tris	Tris[hydroxymethyl]aminomethane
UV	Ultraviolet
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

NOTE:

Système Internationale (SI) units and prefixes are used throughout this thesis.
IUPAC-IUBMB abbreviations for nucleotides and for unspecified nucleotides are used.
IUPAC-IUBMB conventions for nomenclature and abbreviation of carbohydrates and amino acids are used throughout this thesis.

ABSTRACT

Arabinoxylans are abundant cell wall polysaccharides in the grasses and constitute a significant proportion of the cell walls of vegetative barley tissues and also starchy endosperm, scutellum and aleurone tissues of the barley grain. Arabinoxylans consist of a (1→4)-β-xylan backbone with C(O)2 and C(O)3-α-linked arabinofuranosyl residues. Plant cell walls are varied and dynamic structures and degradation and modification of individual wall components accompanies normal processes of plant growth and development. α-L-Arabinofuranosidases (EC 3.2.1.55) and β-D-xylosidases (EC 3.2.1.37), extensively characterised in microorganisms, participate in arabinoxylan metabolism in plants. The purification and characterization of these enzymes from barley, and the isolation of corresponding cDNAs are described in this thesis.

An α-L-arabinofuranosidase/β-D-xylosidase and a β-D-xylosidase, designated ARA-I and XYL, respectively, have been purified from 5-day-old barley seedlings by fractional precipitation with $(NH_4)_2SO_4$ and column chromatography. Apparent molecular masses of ARA-I and XYL were 65 and 67 kDa, respectively, and their repective isoelectric points were 5.5 and 6.7. Peptide sequences were obtained for each of the purified proteins and BLAST searches revealed that ARA-I and XYL were similar to the microbial β-D-xylosidases of glycoside hydrolase family 3. A second α-L-arabinofuranosidase isoenzyme, designated ARA-II, was partially purified from a barley seedling extract and peptide sequences suggested that this enzyme could also be classified in glycoside hydrolase family 3. An arabinoxylan arabinofuranohydrolase with an apparent molecular mass of 65 kDa was purified from 7-day-old barley seedlings using similar procedures, and was designated AXAH-I. BLAST searches of AXAH-I peptide sequences indicated that AXAH-I was similar to the α-L-arabinofuranosidases from family 51.

ARA-I and XYL hydrolysed aryl glycosides, 4NPA and 4NPX, and removed terminal D-xylosyl residues from arabinoxylan and oligoarabinoxylosides. ARA-I had a limited capacity to remove L-arabinosyl residues from both arabinoxylan and from oligoarabinoxylosides. The arabinoxylan arabinofuranohydrolase, AXAH-I, hydrolysed 4NPA and liberated L-arabinosyl residues from cereal cell wall arabinoxylans. Kinetic constants were determined

for each of the purified enzymes using both aryl glycosides and, for AXAH-I, wheat arabinoxylan. The substrate specificities of purified enzymes were investigated using various polysaccharides and oligosaccharides such as (1→4)-β-linked xylopentaose and (1→5)-α-linked arabinohexaose.

The full-length nucleotide sequences that encode ARA-I and XYL, and a near full-length sequence encoding AXAH-I, were determined from series of partial cDNA clones isolated from several barley cDNA libraries and also using RT-PCR and 3'-end RACE PCR. A full-length cDNA clone encoding a second arabinofuranohydrolase isoenzyme, designated AXAH-II, was isolated. The sequence of the AXAH-II cDNA was 81% identical to that of AXAH-I. The complete primary structures of the four arabinoxylan-hydrolysing enzymes were deduced from cDNA sequences. Surprisingly, the calculated molecular masses of the encoded ARA-I and XYL polypeptides are approximately 80 kDa, therefore implying that a significant portion of the protein has been removed post-translationally. COOH-Terminal amino acid sequence could not be obtained to confirm the location of the processing site. Finally, the expression patterns of genes encoding ARA-I, XYL, AXAH-I and AXAH-II were examined using RT-PCR, and chromosomal locations of the respective genes were determined. Genes encoding XYL, AXAH-I and AXAH-II were expressed in vegetative seedling tissues, such as rootlets, coleoptiles and leaves, in developing grain and in the aleurone and scutellum of germinated barley grain. The AXAH-I gene appeared to be expressed at higher rates in aleurone and scutellum of germinated barley grain than in other tissues. ARA-I expression was not detected in the germinated aleurone and scutellum but was detected at low levels in vegetative seedling tissues and in developing grain.

The two distinct enzyme types described in this thesis, characterized by substrate preference and amino acid sequence, are classified in glycoside hydrolase families 3 and 51. These enzymes are likely to have key roles in cell wall metabolism in barley, such as in modification of arabinoxylans during cell wall deposition or in the hydrolysis of arabinoxylan in germinated grain.

CHAPTER 1.

General Introduction and Research Objectives

1.1 INTRODUCTION

The overall objective of the work described in this thesis was to characterize enzymes, together with their corresponding genes, which mediate in the hydrolysis of cell wall arabinoxylans in barley (*Hordeum vulgare*). Arabinoxylans are major constituents of cell walls in the Poaceae, and attention was particularly focused on the roles of α-L-arabinofuranosidases, β-D-xylosidases and arabinoxylan arabinofuranohydrolases in cell wall metabolism. The purpose of this review is to place these enzymes in the morphological, structural and chemical context of the barley cell wall and to outline where enzyme-mediated changes in the wall accompany key stages of the plant's life cycle, including grain development, grain germination and vegetative growth in young seedlings. The biochemical features of α-L-arabinofuranosidases and β-D-xylosidases from bacteria and fungi are described for comparative reasons, and potential roles for similar enzymes in barley are surveyed in detail.

1.2 BARLEY ANATOMY AND CELL WALL MORPHOLOGY AND COMPOSITION

The major morphological features of flowering plants include the vegetative plant parts such as roots, stems and leaves, and the flowering parts where caryopses develop following fertilization. Each of these plant parts and tissues serve specialised functions that combine to produce viable seeds that ensure the survival of the species through successive generations. *Figure 1.1* shows the tissues of a germinated barley grain in longitudinal section with a detailed view of the major cell types.

Development of the barley grain embryo after sexual reproduction follows fusion of one of two haploid male nuclei with the female egg nucleus of the ovule. Early divisions of the diploid zygote lead to the differentiation of embryonic tissues such as the cotyledon, coleoptile and coleorhiza, first leaf and root initials (Bewley and Black, 1994). The cotyledon forms the scutellum and scutellum epithelium, and lies adjacent to the endosperm. The scutellum participates in the distribution of sugars and other nutrients to the developing seedling following grain germination (Bewley and Black, 1994). The cell walls of the scutellum tissues in barley are 0.125-0.25 μm in thickness and at the scutellum epithelium, consist of a double layer of cell wall material (Niewdorp, 1963; Swift and O'Brien, 1972).

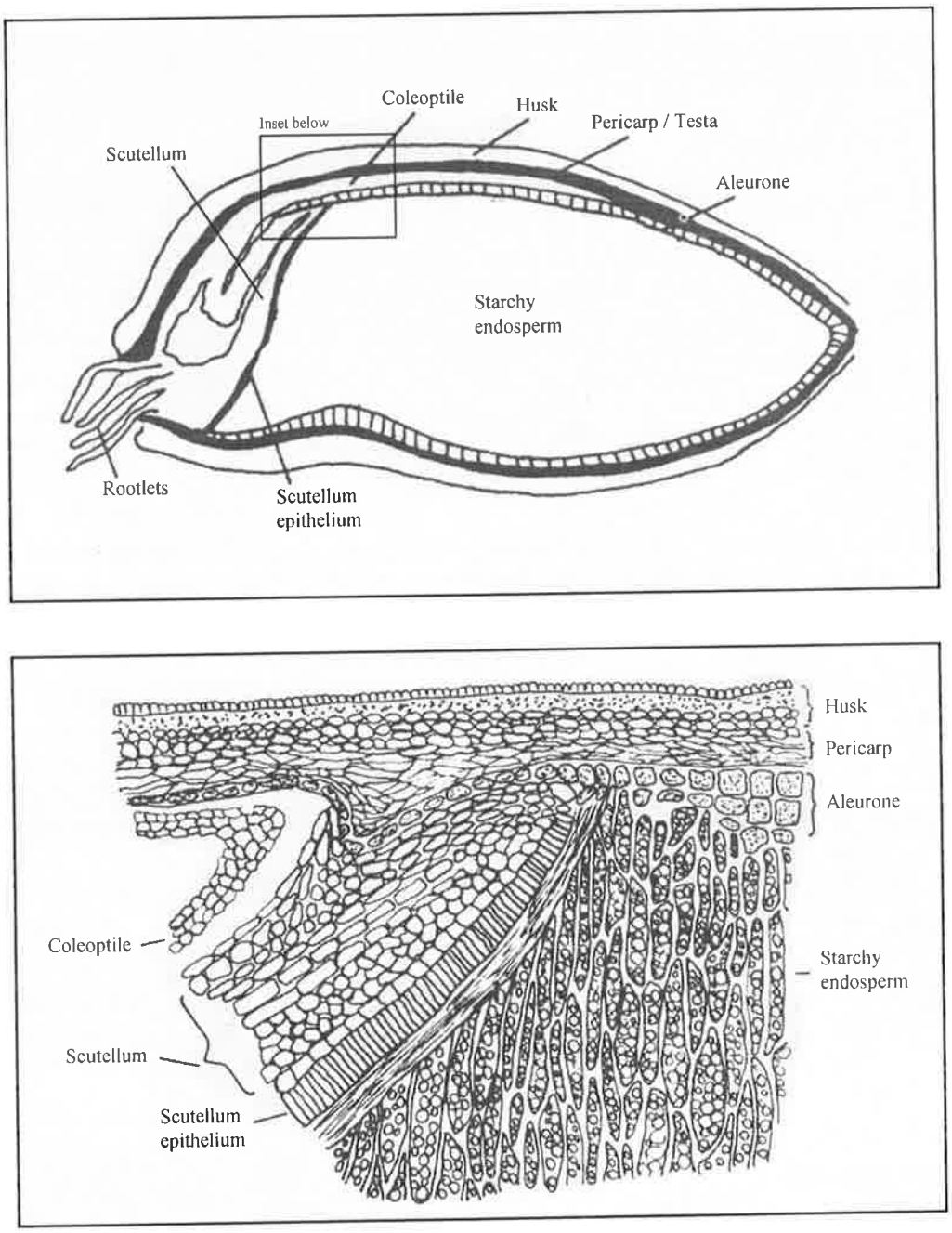

Figure 1.1 **Major tissues of the germinated barley grain shown in longitudinal section.** Inset: detail showing important cell types. Adapted from Bewley and Black (1994).

The constituent polysaccharides of the scutellum cell wall have not been identified unequivocally, although arabinoxylan is probably a major component (Fincher and Stone, 1986).

The endosperm is derived from a triploid nucleus formed by fusion of the second male nucleus with two polar nuclei within the embryo, followed by free nuclear division, without the formation of walls between individual cells. Individual endosperm cells are subsequently formed by compartmentalization of the endosperm with deposited cell walls and further cell division (Olsen, 2001). During the period of grain filling that follows, carbon and nitrogen reserves such as sucrose, fructans and amino acids stored in vegetative plant parts are translocated to the endosperm, where starch and protein are synthesized. During this period the endosperm differentiates into two distinct cell types. An outer layer of cells forms around the periphery of the larger starchy endosperm (Olsen, 2001). Starchy endosperm cell walls vary in thickness, with an average of approximately 2.3 μm, and are composed of around 70% (1→3,1→4)-β-glucan, 20% arabinoxylan, 5% protein and 2-4% glucomannan and cellulose (Fincher, 1975; Ballance and Manners, 1978; Fincher and Stone, 1986).

The aleurone layer surrounds the starchy endosperm in mature barley grain and is derived from the outermost cells of the endosperm, which differentiate into living aleurone cells (Olsen, 2001). These cells are rich in lipids and protein but are essentially devoid of starch (Jones, 1969). The walls of aleurone cells are similar to those of the scutellum epithelium, consisting of a thick outer layer (2.0-3.0 μm) and a thin inner layer (0.3-0.6 μm) (Bacic and Stone, 1981*a*). Arabinoxylan and (1→3,1→4)-β-glucan are the predominant polysaccharides present in aleurone cell walls of barley and account for 65-67% and 26-29% of cell wall dry weight, respectively. Glucomannans, cellulose and protein are present in lesser amounts (Bacic and Stone, 1981*b*; Fincher and Stone, 1986). Aleurone cells become metabolically active after germination of the mature grain and are the major source of hydrolytic enzymes that degrade cell walls, starch and protein for the mobilization of carbon and nitrogen reserves to support seedling growth (Fincher, 1989; Fincher and Stone, 1993; Bewley and Black, 1994).

The outer layers of the grain arise from maternal tissues of the flower and include the testa, the pericarp and husk. The testa and pericarp are derived from the ovule integuments and

ovary wall, respectively, and consist of cuticular layers and compacted cells (Bewley and Black, 1994). In barley, the parental palea and lemma remain fused to the pericarp to form the husk. The cell wall remnants of these outer parental tissues are composed of lignin, cellulose and arabinoxylans and provide protection against entry of moisture and also against attack by pathogens and pests (Fincher and Stone, 1986).

Compositional and structural details of the cell walls of the vegetative tissues of barley plants are less complete than for the various grain tissues. However, these tissues contain a large proportion of cellulose, small amounts of lignin and also arabinoxylans and (1→3,1→4)-β-glucans (Sakurai and Masuda, 1978; Kokubo, 1989).

1.3 BARLEY CELL WALL POLYSACCHARIDES

Chemical analysis of plant polysaccharides, including those with specialised roles in the plant cell wall, has occupied carbohydrate chemists throughout most of the twentieth century. With the development of modern analytical techniques, such as gas chromatography-mass spectroscopy (GC-MS) and ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy, the monosaccharide compositions and glycosidic linkages of most of the cell wall polymers of flowering plants have been described. The use of endoglycanases that hydrolyse polysaccharides only at specific sites, followed by complete structural determination of the resultant oligosaccharides, has provided sufficient information to generate accurate models of the more abundant wall polymers (Bacic *et al.*, 1988; Darvill *et al.*, 1988; Carpita and Gibeaut, 1993, Carpita, 1996). Each of the various polysaccharides in the walls of plants have differences in physical and chemical properties that inherently influence the overall properties of the wall in a particular plant or tissue type. Chemical interactions within the cell wall influence the solubility of wall polymers and therefore, the methods used in their isolation from plant material. It is also worth noting that cell wall polysaccharides are seldom, if ever, defined by a single structure. Rather, they consist of a population of molecules with different degrees of polymerization (DP) and different fine structural features. Furthermore, the fine structure of cell wall polysaccharides varies during cell development to meet the varying functional requirements of the wall during that developmental period.

Nevertheless, generalised structural models for the primary cell walls of flowering plants have been proposed, and envisage cellulose microfibrils interlocked within a matrix of non-cellulosic polysaccharides such as xyloglucan or glucuronoarabinoxylan. The cellulose and matrix polysaccharide network is suspended within an interconnected gel-like domain composed of the pectic polysaccharides, polygalacturonic acid and rhamnogalacturonan (Darvill, *et al.* 1988; Carpita and Gibeaut, 1993; Carpita, 1996). A major division in cell wall constitution exists between the Poaceae and most other flowering plants. Apart from the normal variations in wall composition and wall polysaccharide fine structure, the cell walls of grasses and other commelinoid monocotyledonae are distinct from those of other species. In particular, the grasses have an abundance of glucuronoarabinoxylans and (1→3,1→4)-β-glucans as the major matrix polysaccharides (Dahlgren *et al.*, 1985; Bacic *et al.*,1988; Carpita, 1996). Replacement of xyloglucan, the major wall matrix polymer of dicots and the non-commelinoid monocots, with glucuronoarabinoxylan, is accompanied with reduced pectic polysaccharide content. The charged glucuronyl units of glucuronoarabinoxylans of the Poaceae may be functionally equivalent to the charged pectins in other types of cell walls (Carpita, 1996). Perhaps the most distinctive feature of the cell walls of grasses is the presence of (1→3,1→4)-β-glucan, which exists exclusively in the Poales (Stinard and Nevins, 1980; Carpita, 1996).

Arabinoxylan and (1→3,1→4)-β-glucan are the principal components of the cell walls of cereal grains (Preece and MacKenzie, 1952; Wilkie, 1979). In the mature barley grain, where rapid removal of the wall is an essential process in post-germination endosperm mobilisation, these two polymers account for the major proportion of cell wall dry weight and cellulose content is correspondingly low (Fincher, 1975; Fincher, 1989). In vegetative tissues, such as stems, leaves, coleoptiles and roots, where strength and resilience are required, cellulose is the primary constituent of the wall (Bacic *et al.*, 1988; Delmer and Amor, 1995). The study of cell wall function and structure relies upon a clear understanding of the composition, properties and interactions of the various cell wall components. The structural features of major cell wall components are summarised below.

1.3.1 Cellulose

Cellulose microfibrils provide the "strength" component of primary plant cell walls and are formed by hydrogen bonding between individual cellulose polymers composed of (1→4)-β-

linked glucosyl residues. The glucosyl residues of cellulose form a rigid twofold helical conformation, which is stabilised by intramolecular hydrogen bonding between adjacent glucosyl residues (Delmer and Amor, 1995). Characterization of cellulose in the cell walls of cotton seed hairs suggests that the DP of cellulose in primary walls is relatively low (2000-6000), but in secondary cell walls the DP is much higher (14,000) (Marx-Figini, 1966; Marx-Figini and Schulz, 1966; Bacic *et al.*, 1988).

1.3.2 (1→3,1→4)-β-Glucans

Unique to the grasses, (1→3,1→4)-β-glucans are typically characterized as linear polymers of β-glucosyl residues with both (1→3)- and (1→4)-linkages (Staudte *et al.*, 1983). The presence of (1→3)-linkages along the entire length of (1→3,1→4)-β-glucan disrupts the inflexible conformation of "cellulosic" units and introduces a degree of flexibility that enables partial solubility in aqueous solutions (Woodward *et al.*, 1983*a*; Woodward *et al.*, 1988). In barley (1→3,1→4)-β-glucans, groups of three or four (1→4)-linked β-glucosyl residues are joined by single (1→3)-linkages (Staudte, *et al.*, 1983). Contiguous sequences of (1→3)-linked β-glucosyl residues are not evident in barley (1→3,1→4)-β-glucans but have been reported for other cereals (Kato and Nevins, 1986). Barley (1→3,1→4)-β-glucans are a heterogeneous group of polysaccharides where properties such as DP, linkage distribution and solubility are variable, and influence relationships with other wall components and therefore, extractability from the cell wall. Separation on the basis of water solubility has led to the determination of the physical and chemical characteristics of two broadly defined groups of barley (1→3,1→4)-β-glucans (Woodward *et al.*, 1983*a*; Woodward *et al.*, 1988). (1→3,1→4)-β-Glucans soluble in water at 40°C share the same general linkage arrangement with those soluble in water at 65°C, where cellotriosyl and cellotetraosyl units are separated by single (1→3)-linkages. The 40°C water-soluble (1→3,1→4)-β-glucans contain regions, which account for approximately 10% by weight, where up to 14 contiguous β-glucosyl residues are joined by (1→4)-linkages (Woodward *et al.*, 1983*b*). The 40-60°C water-soluble (1→3,1→4)-β-glucans contain similar relative proportions of (1→4)- and (1→3)-linkages (70% and 30%, respectively), although fewer long-chain "cellulosic" sequences are present (Woodward *et al.*, 1988).

1.3.3 (Glucurono)arabinoxylans

Glucuronoarabinoxylans are abundant cell wall polysaccharides in the grasses and in cereals such as wheat and barley. These polymers are composed of a backbone of (1→4)-β-linked xylopyranosyl residues with α-L-arabinofuranosyl substituents at C(O)2, C(O)3 and C(O)2,3 as shown in *Figure 1.2*. The substitution patterns of arabinoxylans vary between species, between different tissues and also during growth and development of the plant (Reid and Wilkie, 1969; Mares and Stone, 1973; Wilkie, 1979; Bacic and Stone, 1981*b*; Carpita, 1983). The distinctive physicochemical features of arabinoxylans contribute to their biological form and function. These include the arrangement of arabinofuranosyl substituents on backbone xylopyranosyl residues, the presence of contiguous unsubstituted xylopyranosyl residues and also the presence of glucuronyl residues and phenolic groups (Fincher and Stone, 1986; Darvill *et al.*, 1988). The degree of arabinofuranosyl substitution influences the solubility of arabinoxylans. Where substituents are absent, intermolecular associations with other wall components, such as cellulose and (1→3,1→4)-β-glucans, may occur through hydrogen bonding and hydrophobic interactions (Carpita and Gibeaut, 1993; Carpita, 1996). Conversely, heavily substituted regions of the (1→4)-β-D-xylan backbone will not form extended, closely aligned associations with cellulose and related molecules because of the steric hindrance to such associations imposed by the bulky substituent groups.

Many studies have been aimed at characterizing the fine structures of arabinoxylans from wheat and barley. Despite the difficulties associated with molecular mass determination of heterogeneous, asymmetric polymers such as arabinoxylans, molecular masses for water- and alkali-extracted arabinoxylans from starchy endosperm cell walls of wheat (Mares and Stone, 1973) and barley (Forrest and Wainwright, 1977) have been estimated. Using gel-filtration, wheat and barley starchy endosperm walls contain arabinoxylans with apparent molecular mass between 8×10^4 and 5×10^6 Da (DP 600 - 38,000) (Mares and Stone, 1973), and 1×10^6 and 5×10^6 Da (DP 7,500 - 38,000) (Forrest and Wainwright, 1977), respectively. The formation of arabinoxylan aggregates or aggregates with other wall components, together with the extended conformation of this polymer may lead to erroneously high values for polysaccharide molecular mass using gel-filtration chromatography. More reliable methods such as ultracentrifugation and osmometry give estimates of molecular mass for cereal arabinoxylans within the range of 2×10^4 and 1.7×10^6 Da (DP 150 – 12,750) (Andrewartha *et al.*, 1979).

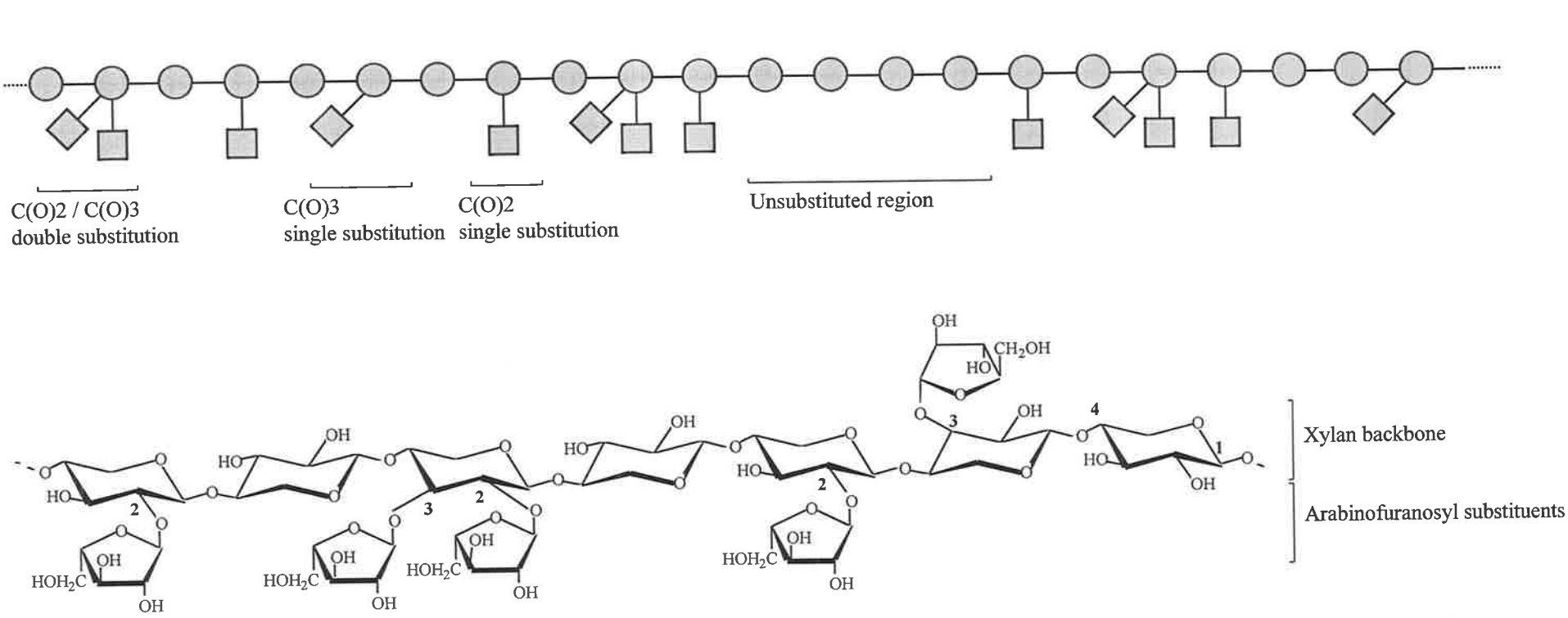

Figure 1.2 **Arabinoxylan structural model (top) and chemical structure (bottom) showing the arrangement of L-arabinofuranosyl substituents on the xylan backbone.** Arabinofuranosyl units are attached to the (1→4)-*β*-D-xylan chain (⊙—⊙) by both C(O)2 (⊙) and C(O)3 (⊙) linkages as indicated and substituted residues usually separated by a single unsubstituted residue. Xylopyranosyl residues may be singly or doubly substituted and regions where two or more xylopyranosyl residues are unsubstituted are present. Adapted from Viëtor *et al.* (1994*a*).

Arabinoxylans are extended polysaccharides with an axial ratio of approximately 140, and therefore produce aqueous solutions of high viscosity. Substitution by single arabinofuranosyl residues affects the shape, flexibility and the solubility of the molecule (Andrewartha *et al.*, 1979). An unsubstituted (1→4)-β-D-xylan polymer forms a threefold left-handed helix, where single intra-chain hydrogen bonds between O(5) and H-O(3') on pairs of contiguous xylopyranosyl residues stabilise the conformation of the polymer (Settineri and Marchessault, 1965; Atkins, 1992). Compared with the twofold helical structure of (1→4)-β-glucan in cellulose chains, where two hydrogen bonds exist between each adjacent pair of glucosyl residues, xylans have a greater degree of flexibility (Atkins, 1992). Arabinofuranosyl substitution in arabinoxylans encourages the extended nature of the polymer yet restricts interaction between polymers, and thereby maintains solubility in aqueous solutions (Andrewartha *et al.*, 1979). Substitution of the xylan chain also influences solubility, and interaction with other components in the plant cell wall. Selective removal of arabinofuranosyl substituents from arabinoxylans in the wall may, in fact, represent a mechanism by which the interactions of arabinoxylans and other wall components can be modulated during growth and development of the plant.

The degree of arabinofuranosyl substitution of arabinoxylans is commonly expressed as the xylose:arabinose ratio and is determined by analysis of constituent sugars of wall preparations. This parameter varies between arabinoxylans extracted from different tissues of cereal grains, such that in wheat and barley, starchy endosperm cell wall arabinoxylans are more highly substituted than those of aleurone tissue (Fincher and Stone, 1986). Xylose:arabinose ratios of barley arabinoxylans range from 1-1.3 in starchy endosperm cell walls (Fincher, 1975; Ballance and Manners, 1978) to 1.5-2.3 in the aleurone (Bacic and Stone, 1981*b*). In barley arabinoxylans, both single C(O)2 and C(O)3 arabinofuranosyl substitution and double C(O)2,3 substitutions are found (McNeil *et al.*, 1975; Fincher and Stone, 1986). In barley husks, only one in six xylopyranosyl residues is substituted by arabinofuranosyl groups and only C(O)3 substitution is found (Aspinall and Ferrier, 1957). In contrast, the degree of substitution of arabinoxylans in wheat is generally lower than that for barley and single C(O)2 substitution is infrequent (Perlin, 1951*a*; Perlin, 1951*b*; Fincher and Stone, 1986).

Early investigations of arabinoxylan fine structure have employed selective degradation of wheat arabinoxylan by periodate oxidation (Ewald and Perlin, 1959), and later, a sequence-specific (1→4)-β-D-xylan endohydrolase from *Streptomyces* (Goldschmid and Perlin, 1963). These studies have shown that the polysaccharide is composed of highly substituted regions, approximately 20-25 xylopyranosyl units in length, separated by open regions of two to five unsubstituted xylopyranosyl residues. Highly substituted regions of wheat arabinoxylan consist of single and paired substituted xylopyranosyl residues that are separated by single unsubstituted xylopyranosyl residues. Isolated substituted xylopyranosyl residues where at least two contiguous unsubstituted residues are located to each side, although uncommon, were also observed. Approximately 60% of the arabinoxylan sample remained as a polymeric fraction that was resistant to hydrolysis by the *Streptomyces* endoxylanase. In this fraction, the extent of substitution was exceptionally high and sites for hydrolysis consisting of at least two unsubstituted xylopyranosyl residues were presumably absent (Goldschmid and Perlin, 1963).

In more recent studies of arabinoxylan structure, monosaccharide and methylation analysis by GC-MS and ^{1}H and ^{13}C NMR spectroscopy have been used to determine the precise arrangement of C(O)2- and C(O)3-linked arabinofuranosyl substituents along the xylan backbone. The structures of oligosaccharides, released from wheat starchy endosperm arabinoxylan by an arabinoxylan-specific (1→4)-β-D-xylan endohydrolase from *Aspergillus*, have been determined using these analytical techniques (Hoffmann *et al.*, 1991; Hoffmann *et al.*, 1992*b*). Together with molecular mass and glycosidic linkage data, ^{1}H resonances for known substitution patterns in simple oligoarabinoxylosides were used to predict the structures of the hydrolysis products. As in earlier studies of wheat starchy endosperm arabinoxylan structure (Goldschmid and Perlin, 1963), one or two contiguous C(O)3 and C(O)2,3 substituted xylopyranosyl residues, were separated by one or two unsubstituted residues and substituted regions were separated by extended un-substituted xylan sequences. Characterization of arabinoxylans from barley cell wall material using similar approaches to those used in the analysis of wheat arabinoxylans, suggest an equivalent distribution of substituents with the added feature of single C(O)2 arabinofuranosyl-substituted xylopyranosyl residues (Viëtor *et al.*, 1994*a*; Viëtor *et al.*, 1994*b*).

Phenolic compounds, such as ferulic acid, are commonly found in the primary cell walls of the grasses, where they are associated with the arabinoxylan component of the wall. Ferulic acid has been observed by fluorescence microscopy in barley starchy endosperm (Fincher, 1976) and aleurone (Bacic and Stone, 1981*a*; 1981*b*) cell walls where it accounts for 0.05% (*w/w*) and 1.2% (*w/w*), respectively. In the absence of any correlation between the pattern of arabinofuranosyl substitution in wheat and barley and ease of extraction from the cell wall, it has been proposed that covalent cross-linking between arabinoxylans may influence their insolubility in the wall matrix (Mares and Stone, 1973; Ahluwalia and Fry, 1986). Ahluwalia and Fry (1986) found that ferulic acid was exclusively associated with the water-insoluble arabinoxylan component of barley starchy endosperm cell walls. Smith and Hartley (1983) isolated a feruloylated disaccharide from a cell wall preparation from wheat bran and found similar compounds in preparations from barley straw, leaves of wheat and Italian ryegrass, and wheat starchy endosperm. Gubler *et al.* (1985) characterized feruloylated tri- and tetrasaccharides among hydrolysis products released from barley aleurone tissue by gibberellic acid (GA_3)-induced enzymes, where the ferulic acid group was linked to O(5) of C(O)3-linked arabinofuranosyl residues (Gubler *et al.*, 1985).

1.4 Biosynthesis and Assembly of Plant Cell Walls

Many of our ideas and concepts of plant cell wall structure have been inferred from our knowledge of the chemical properties and structures of the primary wall components, notably polysaccharides such as cellulose, (1→3,1→4)-β-glucan and glucuronoarabinoxylan. In the rapidly advancing field of plant genomics, new tools for the development of our understanding of cell wall form and function have become available. Plant cell wall researchers are beginning to describe in detail, the enzymes and associated proteins that participate in the synthesis of wall polysaccharides and subsequently, in the deposition and assembly of the extracellular matrix components that constitute the cell wall.

Three stages have been identified in the development of plant cells; these include cytokinesis, elongation and maturation (Gibeaut and Carpita, 1994; Carpita, 1996). During cytokinesis, formation of the cell plate begins by fusion of Golgi-derived vesicles and deposition of non-cellulosic polysaccharides to form the pectin-rich middle lamella. Microtubules direct Golgi vesicles to the cell plate, which grows outward and fuses with

existing cell wall material (Staehelin and Hepler, 1996). Callose synthesis and deposition at the newly formed membrane of the cell plate is followed by further production of cellulose microfibrils and other non-cellulose matrix polysaccharides around the newly formed cell (Carpita, 1996). The elongation phase of plant cell development involves turgor-driven cell expansion and is mediated by the combined processes of enzymic wall loosening, and synthesis and deposition of new cell wall material. Secondary cell wall thickening characterizes the final, maturation phase of cell growth and involves further deposition and cross-linking of cellulosic material and lignin. Formation of secondary walls is a feature of cell growth in woody plants and also in the vascular and epidermal tissues of herbaceous plants (Carpita, 1996).

1.4.1 Biosynthesis of Cell Wall Polysaccharides

Trans-membrane synthase complexes mediate the biosynthesis of linear polymers of glycosyl residues in plants. Cellulose is synthesized exclusively at the plasma membrane (Delmer and Amor, 1995; Delmer, 1999), whereas other matrix polysaccharides, such as (1→3,1→4)-β-glucan, glucuronoarabinoxylan, and in dicotyledons, xyloglucan, are generally believed to be produced within the Golgi apparatus (Driouich *et al.*, 1993; Carpita, 1996). Polysaccharides produced in the Golgi are delivered to the extracellular site of cell wall deposition *via* the Golgi apparatus secretory pathway (Staehelin and Moore, 1995). Addition of glycosyl residues to backbone polysaccharides in substituted cell wall polymers, such as glucuronoarabinoxylans, is accomplished by glycosyl transferases within the Golgi lumen (Gibeaut and Carpita, 1994; Gibeaut, 2000).

1.4.2 Molecular Organisation of Plant Cell Wall Components

The physical and chemical interactions of cell wall components that ensure that the basic structural and functional requirements of the plant cell wall are satisfied, have been described in several proposed models (Keegstra *et al.*, 1973; Cleland, 1981). Models of plant cell wall structure must accommodate properties such as mechanical strength and permeability, and also account for the flexibility required to permit cell enlargement by elongation growth. Helical coils of cellulose microfibrils provide strength in the transverse plane of the cell and allow cell elongation in the longitudinal direction of the cell by pressure forces exerted by cell turgor. Carpita and Gibeaut (1993) describe the cellulosic portion of the plant cell wall as similar to a loose spring that can expand easily in the longitudinal

direction yet has lateral rigidity and strength. Resistance to expansion in the direction of cell growth is provided by the cell wall matrix polysaccharides, such as xyloglucan in dicots and (1→3,1→4)-β-glucan and glucuronoarabinoxylan in the grasses. These associate with cellulose microfibrils by hydrogen bonding and essentially limit the ability of the cellulose "coils" to move apart. Thus, elongation growth may require disruption of hydrogen bonding interactions between matrix polysaccharides, or enzymic hydrolysis of matrix polysaccharides themselves. Hydrolysis of wall polysaccharides is balanced by further synthesis and deposition of matrix polysaccharides, and by the formation of new layers of cellulose microfibrils (Carpita and Gibeaut, 1993).

In the primary cell walls of the Poaceae, deposition of glucuronoarabinoxylan in the wall is accomplished by *in situ* removal of a proportion of the arabinofuranosyl substituents (Carpita, 1983; Gibeaut and Carpita, 1991). Using radiolabelled L-arabinose and D-glucose precursors, Gibeaut and Carpita (1991) found that the degree of substitution of glucuronoarabinoxylans in maize coleoptiles decreased after the cessation of cell elongation and that D-glucosyl units of cell wall (1→3,1→4)-β-glucans exhibit turnover in the wall during growth. (1→3,1→4)-β-Glucans appear only during the growth and elongation phase of coleoptiles in maize (Carpita, 1984; Luttenegger and Nevins, 1985) and hydrolysis, turnover and synthesis of (1→3,1→4)-β-glucans proceed concurrently during cell growth (Carpita and Gibeaut, 1993). At the end of the cell growth stage, the cell wall becomes relatively fixed in structure through the accumulation of structural proteins. In some plant cell types, particularly those that serve as elements of vascular tissues or provide physical support to the plant, a secondary wall consisting of cellulose and lignin may be deposited (Carpita and Gibeaut, 1993). In barley aleurone and starchy endosperm tissues, where strength properties in the cell wall are less important than the need to be rapidly degraded after germination, the cell wall is composed almost entirely of matrix polymers, and cellulose content is low. Considering that these cell types are non-vascular and do not provide structural support to the plant, the low cellulose content of their cell walls is in keeping with their specific functional roles.

1.5 CELL WALL MODIFICATION IN BARLEY GROWTH AND DEVELOPMENT

1.5.1 Barley Germination and Endosperm Mobilisation

During the germination of the barley grain, walls of the aleurone are extensively modified to allow the secretion of hydrolytic enzymes, and walls of the starchy endosperm are completely degraded by glycoside hydrolases. These processes ensure that access of protein- and starch-degrading enzymes to their substrates is not impeded.

Absorption of water by the barley grain initiates a series of molecular and cellular processes that are central to the mobilisation of starchy endosperm reserves and seedling development. Release of gibberellic acid (GA_3) from the imbibed embryo and subsequent diffusion through the aleurone and scutellum is generally accepted as the hormonal signal for expression of genes encoding hydrolases that are to be directed to the starchy endosperm (Fincher, 1989). Although the exact location of GA_3 production is unclear, it has been firmly established that the embryo essentially activates the germination process and that gibberellins induce genes encoding starch (Paleg, 1960; Jones and Jacobsen, 1991), protein and cell wall (Wolf, 1991) hydrolysing enzymes (Fincher, 1989; Fincher and Stone, 1993). After the initiation of barley germination, the cells within the aleurone and scutellum become metabolically active and a range of physiological changes are directed towards synthesis and secretion of endosperm-degrading enzymes (Fincher, 1989). Thus, changes in the aleurone and scutellum epithelium cells are marked by elevation of the level of metabolic activity, as evidenced by morphological changes in mitochondria (Gram, 1982) and development of endoplasmic reticulum (Jones, 1980) and Golgi complexes (Gram, 1982; Fernandez and Staehelin, 1985). Additionally, changes at the scutellum epithelium that serve to increase the absorptive surface area adjacent to the endosperm, indicate the role of the scutellum in absorption and distribution of metabolites to the developing seedling (Niewdorp and Buys, 1964).

Cell walls of the aleurone layer consist of a thick outer layer that is degraded by aleurone-derived enzymes to facilitate enzyme diffusion, and a thin, permeable inner layer that remains to maintain the structural integrity of aleurone cells (Jones, 1969; Bacic and Stone, 1981*a*). Hydrolysis of (1→3,1→4)-β-glucan is detected 6-9 hours after the initiation of germination, preceding amylolytic activity that is detected at 15-18 hours (MacGregor *et al.*,

1994). (1→4)-β-D-Xylan endohydrolase is similarly produced in the barley aleurone (Taiz and Honigman, 1976; Benjavongkulchai and Spencer, 1986; Banik *et al.*, 1997), although it appears several days after the initiation of germination. An indispensable role for (1→4)-β-D-xylan endohydrolase in wall degradation in the germinated grain has not been unequivocally established.

1.5.2 Cell Elongation

The principal mechanisms of cell growth in plants involve dissociation, hydrolysis and transglycosylation of polysaccharides of the cell wall matrix. Several proteins that mediate such processes in plants have been isolated and characterized, and include expansins (Cosgrove, 1999; 2000), xyloglucan endotransglycosylases (Fry *et al.*, 1992; Nishitani and Tominaga, 1992) and glycoside hydrolases (Kim *et al.*, 2000; Harvey *et al.*, 2001). Expansins, first identified in cucumber hypocotyls and oat coleoptiles (McQueen-Mason *et al.*, 1992; Cosgrove and Li, 1993), have been identified as non-enzymic agents of acid-dependent plant cell growth that are presumed to act by disruption of hydrogen bonding between cellulose microfibrils and matrix polysaccharides. Cell walls can be "loosened" by hydrolysis of wall polysaccharides by glycoside hydrolases. Enzymic transglycosylation, leads to the formation of new glycosidic linkages between wall polysaccharides following hydrolysis and accompanied turgor-driven cell expansion.

Auxin-induced elongation growth has been studied extensively in coleoptiles of *Avena* (Loescher and Nevins, 1972), barley (Sakurai and Masuda, 1978) and maize (Carpita, 1984; Luttenegger and Nevins, 1985; Labrador and Nevins, 1989; Inouhe and Nevins, 1991*a*). Hydrolysis of (1→3,1→4)-β-glucan has been suggested to be an important mechanism of auxin-induced cell elongation in maize coleoptiles, based on experiments with antibodies that disrupt the activities of (1→3,1→4)-β-glucanases (Hoson and Nevins, 1989; Inouhe and Nevins, 1991*b*). Increased levels of both (1→3,1→4)-β-glucan endohydrolases and β-D-glucan glucohydrolases have been correlated with elongation growth in developing maize coleoptiles (Inouhe and Nevins, 1998). In barley, transcription of genes encoding (1→3,1→4)-β-glucan endohydrolases and β-D-glucan glucohydrolases has been detected in young leaves and elongating coleoptiles (Slakeski and Fincher, 1992*a*; 1992*b*; Kotake *et al.*, 1997; Harvey *et al.*, 2001).

1.6 WALL MODIFYING ENZYMES IN BARLEY

1.6.1 Hydrolysis of (1→3,1→4)-β-Glucan

The complete depolymerization of (1→3,1→4)-β-glucan to glucose requires hydrolytic activities of (1→3,1→4)-β-glucan endohydrolases (EC 3.2.1.73), β-D-glucan glucohydrolases and possibly β-glucosidases (EC 3.2.1.21). A rapid increase of (1→3,1→4)-β-glucan endohydrolase activity in the aleurone and scutellum is observed within 12 h of germination in barley and this activity is correlated with the removal of (1→3,1→4)-β-glucan in the grain during the course of endosperm modification (Stuart *et al.*, 1986). Two (1→3,1→4)-β-glucan endohydrolase isoenzymes, designated isoenzymes EI and EII, have been purified from germinated barley grains (Woodward and Fincher, 1982*a*). Primary structures for these enzymes have been deduced from gene and cDNA sequences for isoenzymes EI (Slakeski *et al.*, 1990) and EII (Fincher *et al.*, 1986). (1→3,1→4)-β-Glucan endohydrolases specifically hydrolyse (1→4)-β-linkages where there is an adjacent (1→3)-β-linked glucosyl residue towards the non-reducing terminus of the substrate (Parrish *et al.*, 1960; Woodward *et al.*, 1982*b*). (1→3,1→4)-β-Glucan endohydrolase isoenzymes EI and EII have molecular masses of 28 kDa and 33 kDa, respectively. Isoenzyme EII contains 3.6% carbohydrate (Woodward *et al.*, 1982*a*), which enhances the thermostability of this isoform (Doan and Fincher, 1992). Immunological detection of isoenzymes EI and EII by Western blotting of barley aleurone and scutellar preparations showed that both isoforms are synthesized and secreted from GA_3-induced aleurone cells supplemented with calcium ions. In contrast, only isoenzyme EI was detected in scutellum preparations (Stuart *et al.*, 1986). Studies of (1→3,1→4)-β-glucan endohydrolase expression using gene-specific nucleotide probes for *in situ* hybridization and Northern analyses confirm these results and also revealed that the isoenzyme EI is expressed in young leaves and roots (McFadden *et al.*, 1988; Slakeski and Fincher, 1992*a*; 1992*b*). Isoenzyme EI expression in young leaves was enhanced by application of GA_3 or auxin, *in vitro* (Slakeski and Fincher, 1992*a*). (1→3,1→4)-β-Glucan endohydrolase mRNA was not detected in barley coleoptiles, despite reports of (1→3,1→4)-β-glucan turnover during cell elongation processes in both young barley leaves and coleoptiles (Sakurai and Masuda, 1978; Inouhe *et al.*, 1997).

β-D-Glucan glucohydrolases and β-glucosidases hydrolyse terminal (1→3)-β- and (1→4)-β-linked glucosyl residues of (1→3,1→4)-β-glucans and β-oligoglucosides (Leah *et al.*, 1995; Hrmova *et al.*, 1996). Two barley β-D-glucan glucohydrolase isoenzymes, designated ExoI and ExoII, have been purified and characterized (Hrmova *et al.*, 1996; Hrmova and Fincher, 1998) and expression of their genes has been investigated using corresponding cDNAs (Harvey *et al.*, 2001). The β-D-glucan glucohydrolases are produced in the scutellum of germinated barley grain, in elongating coleoptiles and, at low levels, in young leaves and roots (Harvey *et al.*, 2001). The role of β-D-glucan glucohydrolases in endosperm modification and in vegetative tissues has not been investigated to any great extent, but it seems likely that oligoglucosides resulting from (1→3,1→4)-β-glucan endohydrolase action would be target substrates. β-Glucosidases purified from barley (Leah *et al.*, 1995; Hrmova *et al.*, 1996) have similar activities as the β-D-glucan glucohydrolases against oligoglucosides and may participate in (1→3,1→4)-β-glucan degradation in the starchy endosperm where they are produced during late grain development (Simos *et al.*, 1994; Leah *et al.*, 1995).

1.6.2 Hydrolysis of Arabinoxylan

Hydrolysis of arabinoxylan by a range of enzymes in extracts of germinated barley (Preece and MacDougall, 1958) and in GA_3-induced barley aleurone layers (Taiz and Honigman, 1976; Dashek and Chrispeels, 1977) is characterized by the appearance of free L-arabinose and D-xylose. Release of these monosaccharides is clearly the result of the concerted activities of α-L-arabinofuranosidases (EC 3.2.1.55), (1→4)-β-D-xylan endohydrolases (EC 3.2.1.8) and β-D-xylosidases (EC 3.2.1.37). A proposed scheme of arabinoxylan degradation by these enzymes is shown in *Figure 1.3*. Taiz and Honigman (1976) reported that each of the GA_3-induced arabinoxylan degrading activities shared similar expression profiles with α-amylase over the first seven days of germination in intact barley seeds. In isolated barley aleurone layers, a significant proportion of the cell wall material is removed after GA_3 induction and it is this selective process of wall degradation that facilitates release of both α-amylase and (1→4)-β-D-xylan endohydrolase. Although synthesis of (1→4)-β-D-xylan endohydrolase in the aleurone begins to decline at approximately 48 hours after induction, levels of the enzyme in the incubation medium increase dramatically at this stage for, at least, a further 48 hours *in vitro* (Benjavongkulchai and Spencer, 1986). A barley aleurone

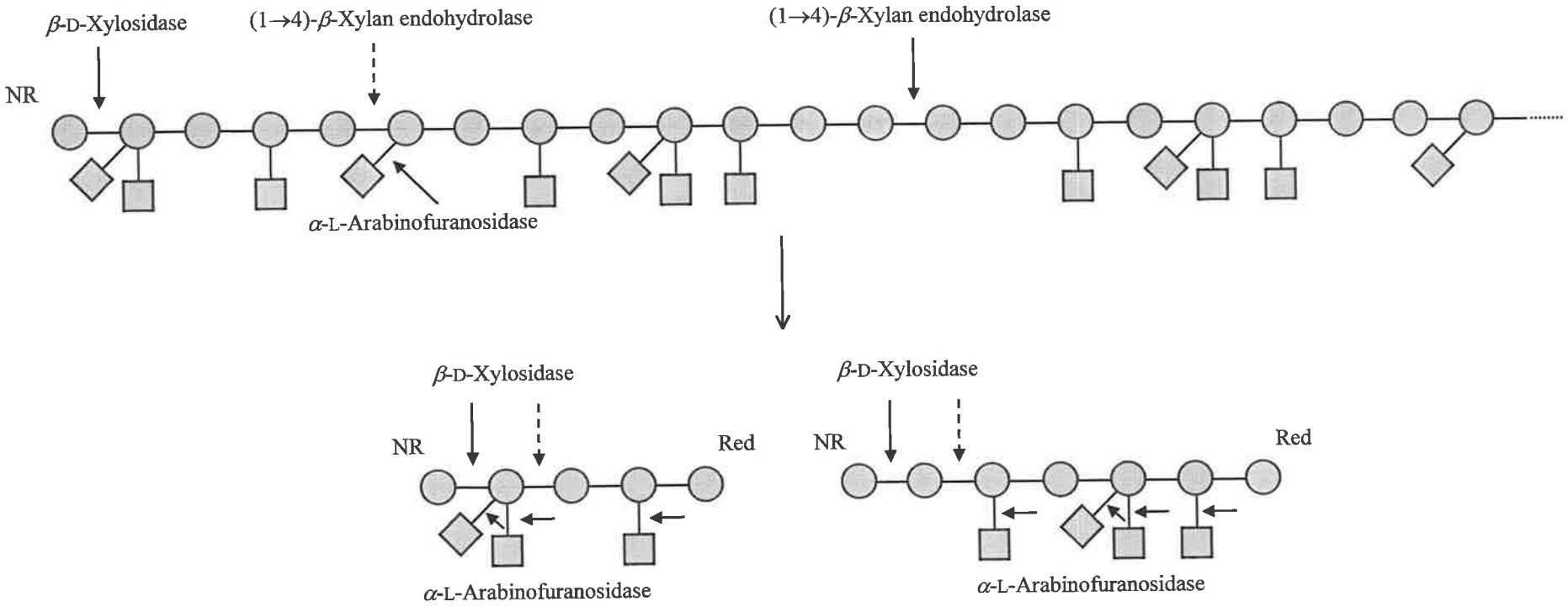

Figure 1.3 **Arabinoxylan structural model with proposed sites of action of major arabinoxylan-hydrolysing enzymes and sequential stages of complete depolymerisation.** The non-reducing end (NR) of arabinoxylan and both reducing (Red) and non-reducing ends of oligosaccharides, generated by partial enzymic degradation, are indicated. *Solid arrows* indicate bonds susceptible to enzymic hydrolysis and *broken arrows* indicate bonds that may be hydrolysed after prior removal of other arabinofuranosyl and/or xylosyl residues. Adapted from Viëtor *et al.* (1994*a*).

(1→4)-β-D-xylan endohydrolase with molecular mass of 34 kDa and pI 4.6 was purified 29-fold from the GA_3-treated aleurone preparation (Benjavongkulchai and Spencer, 1986).

Three (1→4)-β-D-xylan endohydrolase isoforms have been purified from germinated barley grain and each of these has a molecular mass of 41 kDa and an isoelectric point of 5.2. The enzymes share identical NH_2-terminal amino (Slade *et al.*, 1989) acid sequences but exhibited quite different properties than those reported by Benjavongkulchai and Spencer (1986). The germinated barley grain (1→4)-β-D-xylan endohydrolases differ only in separation properties observed during chromatographic purification procedures and may result from a single gene product with varied post-translational modifications (Slade *et al.*, 1989). Two cDNAs encoding closely related (1→4)-β-D-xylan endohydrolase isoenzymes (Banik *et al.*, 1996) have been isolated, and possibly, a further one or two genes may encode (1→4)-β-D-xylan endohydrolases in barley (Banik *et al.*, 1996).

Slade *et al.* (1989) also reported that (1→4)-β-D-xylan endohydrolase activity appeared very late after the initiation of germination. This observation raised doubts as to how important the (1→4)-β-D-xylan endohydrolase really is in wall degradation in the starchy endosperm of germinated grain (Fincher and Stone, 1993). Northern analysis of various barley tissues revealed that the (1→4)-β-D-xylan endohydrolase genes are transcribed only in the aleurone of germinated barley grain (Banik *et al.*, 1996) and in isolated barley aleurone layers (Banik *et al.*, 1997). GA_3 induces (1→4)-β-D-xylan endohydrolase gene transcription in isolated barley aleurone layers from 12 to 48 hours after hormone treatment (Banik *et al.*, 1997).

Recent work by Caspers *et al.* (2001) provides answers to many questions concerning disparities in molecular mass and isoelectric point determinations, and also in the delayed release of (1→4)-β-D-xylan endohydrolase from the barley aleurone observed by Benjavongkulchai and Spencer (1986) and Banik *et al.* (1997). Caspers *et al.* (2001) report that the enzyme is initially translated as a 61.5 kDa proenzyme, which is processed at both the NH_2- and COOH- termini to yield intermediate and mature proteins of 40 kDa and 34 kDa, respectively. These intermediate and mature forms correspond in size to enzyme preparations reported by Slade *et al.* (1989) and Benjavongkulchai and Spencer (1986), respectively. Processing and subsequent release of the barley (1→4)-β-D-xylan

endohydrolase coincides with programmed cell death of cultured aleurone cells treated with GA_3 (Caspers *et al.*, 2001).

Barley α-L-arabinofuranosidase and β-D-xylosidase activities have been identified in germinated grain and in secretions from isolated aleurone layers (Taiz and Honigman, 1976; Dashek and Chrispeels; 1977; Banik *et al.*, 1996), and have been partially purified from wheat bran and germinated wheat grain (Beldman *et al.*, 1996; Cleemput *et al.*, 1997). However, these enzymes are yet to be purified to homogeneity from barley, and the biochemical properties and matching DNA sequences of plant α-L-arabinofuranosidases and β-D-xylosidases remain to be determined.

1.7 α-L-ARABINOFURANOSIDASES AND β-D-XYLOSIDASES IN NATURE

α-L-Arabinofuranosidases and β-D-xylosidases are critical enzymes in the degradation and utilization of arabinose- and xylose-containing polymers, such as arabinans, arabinogalactans, xyloglucans and arabinoxylans (Coughlan and Hazelwood, 1993). Arabinoxylans, consisting of L-arabinofuranosyl and D-xylopyranosyl residues, require the activities of both enzymes, usually in conjunction with (1→4)-β-D-xylan endohydrolase, for complete depolymerization. A wide range of rumen and saprophytic microorganisms utilizes arabinose and xylose derived from these substrates, and α-L-arabinofuranosidases and β-D-xylosidases from many of these sources have been investigated (Coughlan and Hazelwood, 1993). The physical and chemical properties of the enzymes vary widely in accord with the range of environmental conditions and native substrates encountered. Linkage specificity represents the most important property of any of the glycoside hydrolases and given the diversity of glycosidic linkages among the non-cellulosic cell wall polysaccharides of flowering plants, there is a commensurate level of diversity in the microbial α-L-arabinofuranosidases and β-D-xylosidases.

Enzymes have traditionally been classified by their biochemical specificity and catalytic mechanism. Enzyme Commission numbers for glycoside hydrolases, EC 3.2.1.*x*, denote enzymes that hydrolyse *O*-glycosidic linkages. The first three digits are common for all *O*-glycoside hydrolases, and the fourth digit indicates the particular substrate hydrolysed

(IUBMB, 1997). In many cases enzymes are not absolutely specific for a particular substrate and enzymes with broad substrate specificities are therefore particularly difficult to assign to EC classifications (Hrmova *et al.*, 1996). To overcome these difficulties, Henrissat and his colleagues have devised a classification based on primary sequences and, through hydrophobic cluster analysis, secondary structural characteristics (Henrissat, 1991; Henrissat and Davies, 1997; Henrissat, 1998) This sequence-based classification of glycoside hydrolases more accurately reflects structural features and also evolutionary relationships between enzymes, and a rapidly growing list of sequence entries has been collated in a database format (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html) (Coutinho and Henrissat, 1999*a*; 1999*b*). Conservation of amino acid sequence between related proteins implies similarities in protein folds (Chothia and Lesk, 1986) and also catalytic mechanism (Gebler *et al.*, 1992). Sequences within the database have been grouped into 86 glycoside hydrolase families (Henrissat, 1991; Henrissat and Bairoch, 1993; 1996). The families contain enzymes with common genetic origins yet may contain members with different substrate specificity. The sequence-based classification of such a large and diverse group of enzymes as the glycoside hydrolases, therefore permits a more objective analysis of the evolution and function of these enzymes.

Selected examples of α-L-arabinofuranosidases and β-D-xylosidases, for which both biochemical and sequence data have been determined, are compiled in *Table 1.1* where it can be seen these enzymes can be assigned into families 3, 39, 43, 51, 52, 54 and 62. General properties of the enzymes classified in families containing α-L-arabinofuranosidases and β-D-xylosidases are summarised in *Table 1.2*. Sequence-based classification allows direct comparison between enzymes with similar biochemical activities in a particular organism. For example, the α-L-arabinofuranosidases and β-D-xylosidases of *Aspergillus niger* have been extensively characterized at both the genetic and biochemical level (de Vries and Visser, 2001). These represent a diverse array of enzymes with related functional roles within a single species (Kaji, 1984; Gielkens *et al.*, 1997). Classification of α-L-arabinofuranosidases and β-D-xylosidases in glycoside hydrolase families will assist the identification of additional genes encoding arabinoxylan-degrading enzymes arising from current genomics and genome sequencing projects. In the following sections, key characteristics of these microbial enzymes will be discussed with a view to highlighting the

Table 1.1 **Selected α-L-arabinofuranosidases and β-D-xylosidases from bacteria and fungi, for which DNA sequences and biochemical properties have been determined.** Enzymes are grouped within glycoside hydrolase families as listed on the CAZY website (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html) (Coutinho and Henrissat, 1999*a*). Plant genomic DNA sequences have been classified, however, corresponding biochemical data have not been reported.

Organism	Enzyme	GenBank	Summary	References
Family 3: Primarily β-glucosidase (EC 3.2.1.21) and β-xylosidase (EC 3.2.1.37)				
Aspergillus nidulans	β-xylosidase *xlnD* gene	Y13568	*xlnD* gene encodes a protein of 85 kDa that has β-xylosidase activity	Kumar and Ramón (1996) Pérez-González *et al.* (1998)
Aspergillus niger	β-xylosidase *xlnD* gene	Z84377	two β-xylosidases were purified, both of 110 kDa (85 kDa after de-glycosylation) enzymes are active against 4NPX, 4NPA (±5%), oligoxylosides and exo-acting on xylan *xlnD* gene encodes both of the 85 kDa β-xylosidases of *A. niger*	van Peij *et al.* (1997)
Aspergillus oryzae	β-xylosidase A *xylA* gene	AB013851	a β-xylosidase of 110 kDa was purified active against 4NPX, 4NP-α-L-Ara*f* (12%) and 4NP-α-L-Ara*p*(28%) *xylA* gene encodes a polypeptide of 86 kDa	Kitamoto *et al.* (1999)
Thermoanaerobacter brockii	β-glucosidase/xylosidase *xglS* gene	Z56279	81 kDa protein predicted from *xglS* gene recombinant *xglS* product in *E. coli* is active on 4NP-β-D-Glc and 4NP-β-D-Xyl other substrates not tested	Breves *et al.* (1997)
Thermoanaerobacter ethanolicus	β-xylosidase/arabinosidase *xarB* gene	AF135015	88 kDa protein predicted from *xarB* gene recombinant *xarB* product in *E. coli* is active on 4NP-α-L-Ara*p* 4NP-β-D-Xyl*p* and 4NP-β-D-Glc*p* no hydrolysis of oligoarabinosides - liberates L-arabinose from oligoarabinoxylosides	Shao and Weigel (1992) Mai *et al.* (2000)
Trichoderma reesei	β-xylosidase *bxl1* gene	Z69257	80 kDa protein predicted from *bxl1* gene - recombinant *bxl1* product in *S. cerevisiae* is active on 4NP-β-D-Xyl*p*, 4NP-β-D-Glc*p* (34%), 4NP-α-L-Ara*p* (25%)and 4NP-α-L-Ara*f* (15%) - also hydrolyses xylobiose and releases D-xylose from xylan	Poutanen and Puls (1988) Margolles-Clark *et al.* (1996) Herman *et al.* (1997)
Family 39: β-Xylosidase (EC 3.2.1.37)				
Thermoanaerobacterium saccharolyticum	β-xylosidase *xynB* gene	M97883	55 kDa protein predicted from *xynB* gene and by SDS-PAGE of recombinant XynB in *E. coli* hydrolytic activity against xylopentose, xylotriose, xylobiose and 4NPX - no activity on xylan.	Lee and Zeikus (1993)

Abbreviations: 4NP = 4-nitrophenyl; Ara = arabinoside; Xyl = xyloside; Glc = glucoside; Gal = galactoside; *f* = furanoside; *p* = pyranoside

Table 1.1 continued

Organism	Enzyme	GenBank	Summary	References
Family 43: β-Xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), arabinanase (EC 3.2.1.99), xylanase (EC 3.2.1.8)				
Aspergillus niger	endo-1,5-α-L-arabinase *abnA*	L23430	ABN A is a 43 kDa glycosylated protein on SDS-PAGE - 33 kDa deduced from *abnA* gene hydrolyses linear 1,5-α-L-arabinan	Rombouts *et al.* (1988) van der Veen *et al.* (1991) Flipphi *et al.* (1993*b*)
Bacillus pumilus	β-xylosidase *xynB* gene	X05793	62 kDa protein predicted from *xynB* gene sequence - subunit mass 56 kDa from SDS-PAGE hydrolyses 4NP-β-D-Xyl*p* and xylobiose	Panbangred *et al.* (1984) Xu *et al.* (1991)
Bacillus subtilus	endo-1,5-α-L-arabinase *ppc* (protopectinase C)	D85132	30 kDa protein releases soluble pectin from protopectins	Sakai and Sakamoto (1990) Sakamoto *et al.* (1997)
Butyrivibrio fibrisolvens	bifunctional β-D-xylosidase/ α-L-arabinosidase *xylB* gene	M55537	62 kDa protein predicted from *xylB* gene sequence recombinant *xylB* product in *E. coli* hydrolyses 4NP-α-L-Ara*p* and 4NP-β-D-Xyl*p* (57%) - also hydrolyses oligoxylosides (X_2-X_5) - does not hydrolyse xylan	Sewell *et al.* (1989) Utt *et al.* (1991)
Clostridium stercorarium	bifunctional β-D-xylosidase/ α-L-arabinosidase *xylA* gene	D13268	53 kDa protein predicted from *xylB* gene sequence and by SDS-PAGE of recombinant XylA recombinant XylA in *E. coli* – hydrolyses xylobiose, 4NP-α-L-Ara*p* and 4NP-β-D-Xyl*p* (35%) does not hydrolyse xylan	Sakka *et al.* (1993)
Cochliobolus carbonum	β-xylosidase *xyp1* gene	AF095243	37 kDa polypeptide predicted from *xyp1* gene – purified, glycosylated protein was 42 kDa Xyp was active against 4NPX and liberates D-xylose from oat spelt xylan Xyp was slightly active on 4NPA but had no activity on arabinan	Ransom and Walton (1997) Wegener *et al.* (1999)
Prevotella ruminicola	β-xylosidase *xynB* gene	Z49241	36 kDa polypeptide predicted from *xynB* gene recombinant XynB in *E. coli* was active against 4NPX, 4NPA XynB has exo-xylanase activity on beech wood and oat spelt xylan and oligoxylosides	Gasparic *et al.* (1995)
Pseudomonas fluorescens	arabinanase A *arbA* gene	Y10458	recombinant ArbA in *E. coli* 34,000 Da hydrolyses CM arabinan, linear 1,5-α-L-arabinan producing arabinotriose - hydrolysis of A_3 proceeds slowly - does not hydrolyse branched arabinan, 4NPA or 4NPX	McKie *et al.* (1997)
Streptomyces chartreusis	exo-α-L-arabinofuranosidase α-L-arabinofuranosidase II *af ii* gene	AB023626	37 kDa protein, pI 7.5, purified from *S. chartreusis* using 4NPA as substrate hydrolyses 4NPA, linear 1,5-α-L-arabinan - arabinoxylan and arabinogalactan at lower rates does not hydrolyse 4NPX, 4NP-α-L-Ara*p*, 4NP-β-D-Gal*p* or 1,2- and 1,3- arabinosyl linkages	Matsuo *et al.* (2000)

Table 1.1 continued

Organism	Enzyme	GenBank	Summary	References
Family 51: α-L-Arabinofuranosidase (EC 3.2.1.55)				
Aspergillus awamori	α-L-arabinofuranosidase I	AB046702	purified protein was 81 kDa on SDS-PAGE - pI 3.3 - hydrolyses 4NPA, arabinan, arabinoxylan, arabinogalactan - hydrolyses arabinofuranosyl linkages in the order (1→5)>(1→3)>(1→2)	Kaneko *et al.* (1998)
Aspergillus niger	α-L-arabinofuranosidase A *abfA* gene	L29005	purified ABFA was 83 kDa - cDNA isolated using an ABFA specific antibody to probe an expression library - recombinant ABFA in *A. niger* and *A. nidulans* was active against 4NPA (K_m 0.68 mM) and (1→5)-linear arabinan - not active on AX, arabinogalactan or branched arabinan	Rombouts *et al.* (1988) van der Veen *et al.* (1991) Flipphi *et al.* (1993*c*)
Bacillus stearothermophilus	α-L-arabinofuranosidase *abfA* gene	AF159625	purified protein was a homotetramer with 64 kDa subunits - pI 6.5 - hydrolyses 4NPA (K_m 0.42 mM), beet arabinan and oat spelt xylan – no activity on 4NPX or arabinogalactan	Gilead and Shoham (1995)
Clostridium stercorarium	α-L-arabinofuranosidase *arfB* gene	AF002664	molecular mass of 56 kDa deduced from *arfB* gene active against arabinoxylan	Schwarz *et al.* (1995) Zverlov *et al.* (1998)
Cytophaga xylanolytica	α-L-arabinofuranosidase *arfI* and *arfII* genes	AF028018 AF028019	ArfI and ArfII were 58 kDa and 59 kDa deduced from gene sequences, respectively recombinant ArfI and ArfII in *E. coli* hydrolysed 4NPA and liberated L-arabinose from beet arabinan, rye and wheat arabinoxylan	Kim *et al.* (1998)
Pseudomonas cellulosa	Arabinofuranosidase *Abf51A* gene	AY043167	Abf 51A is approx. 55 kDa, 57 kDa predicted from *abf51A* gene removed α-1,2-, α-1,3-linked arabinofuranosyl side chains of arabinans and arabinoxylan hydrolysed 1,5-α-L-linked oligoarabinosides at lower rates	Beylot *et al.* (2001*a*) Beylot *et al.* (2001*b*)
Streptomyces chartreusis	α-L-arabinofuranosidase I *af i* gene	AB023625	80 kDa protein, pI 6.6, purified from *S. chartreusis* using 4NPA as substrate hydrolyses 4NPA, 1,2-, 1,3- and 1,5-α-L-arabinosyl linkages of arabinans and arabinoxylan and arabinogalactan at lower rates - does not hydrolyse 4NPX, 4NP-α-L-Ara*p*, 4NP-β-D-Gal*p*	Matsuo *et al.* (2000)
Streptomyces lividans	α-L-arabinofuranosidase *abfA* gene	U04630	72 kDa polypeptide deduced from the *abfA* gene – purified AbfA was 69 kDa on SDS-PAGE AbfA hydrolyses 4NPA (K_m 0.6 mM), linear arabinan, wheat and rye arabinoxylans and oligoarabinoxylosides - does not hydrolyse 4NPX (or other aryl glycosides) or arabinogalactan	Manin *et al.* (1994)
Thermobacillus xylanolyticus	α-L-arabinofuranosidase *abfD3* gene	Y16849	AbfD3 was 56 kDa by deduction from the *abfD3* gene and by mass spectroscopy recombinant AbfD3 in *E. coli* hydrolysed 4NPA (K_m 0.72 mM at 60°C) - AbfD3 was active on wheat arabinoxylan, larchwood and oat spelt xylan - hydrolyses both C(O)2 and C(O)3 linkages	Debeche *et al.* (2000)
Family 52: β-Xylosidase (EC 3.2.1.37)				
Bacillus stearothermophilus	β-xylosidase *xylA* gene	D28121	75 kDa deduced from gene - 75 kDa on SDS-PAGE. hydrolyses 4NPX and oat spelt xylan	Nanmori *et al.* (1990) Baba *et al.* (1994)

Table 1.1 continued

Organism	Enzyme	GenBank	Summary	References
Family 54: α-L-Arabinofuranosidase (EC 3.2.1.55)				
Aspergillus awamori	α-L-arabinofuranosidase II	AB046701	purified protein was 62 kDa on SDS-PAGE – pI 3.6 - hydrolyses 4NPA, arabinan, arabinoxylan, arabinogalactan - hydrolyses arabinofuranosyl linkages in the order (1→5)>(1→2)>(1→3)	Kaneko *et al.* (1998)
Aspergillus nidulans	α-L-arabinofuranosidase *abfB* gene	Y13759	calculated molecular mass 50.6 kDa, pI 3.9 hydrolyses 4NPA - other substrates not tested	Gielkens *et al.* (1999)
Aspergillus niger	α-L-arabinofuranosidase *abfB* gene	L23502	50.7 kDa polypeptide deduced from *abfB* gene - 67 kDa protein on SDS-PAGE ABFB hydrolyses 4NPA (K_m 0.5 mM), beet arabinan (K_m 0.26 g/l), (1→5)-α-L-arabinan, (1→5)-linked oligoarabinosides and arabinoxylan.	Rombouts *et al.* (1988) van der Veen *et al.* (1991) Flipphi *et al.* (1993*a*)
Trichoderma reesei	α-L-arabinofuranosidase *abf1* gene	Z69252	49.1 kDa deduced from gene sequence - 53 kDa by SDS-PAGE - pI 7.5 hydrolyses 4NPA (K_m 1.2 mM), 4NPX, beet arabinan and arabinoxylan	Poutanen (1988) Margolles-Clark *et al.* (1996)
Family 62: α-L-Arabinofuranosidase (EC 3.2.1.55)				
Aspergillus niger	arabinoxylan arabinofuranohydrolase (AXHA) *axhA* gene	Z78011	33.1 kDa polypeptide deduced from *axhA* gene, pI 4.1	Gielkens *et al.* (1997)
Aspergillus tubingensis	arabinoxylan arabinofuranohydrolase (AXHA) *axhA* gene	Z78010	33.3 kDa polypeptide deduced from *axhA* gene, pI 4.2	Gielkens *et al.* (1997)
Pseudomonas fluorescens	α-L-arabinofuranosidase *xynC* gene	X54523	59 kDa polypeptide deduced from *xynC* releases L-arabinose from oat spelt xylan - does not hydrolyse 4NPA contains a cellulose binding domain	Kellett *et al.* (1990)
Streptomyces lividans	α-L-arabinofuranosidase *abfB* gene	M64551	47 kDa polypeptide predicted from gene and from SDS-PAGE preferred substrates were cereal arabinoxylans (for wheat AX - K_m 1.17 mg/ml) did not hydrolyse arabinogalactan or (1→5)-arabinan contains a xylan binding domain	Vincent *et al.* (1997)

Table 1.2 **Summary of physical and biochemical properties of microbial α-L-arabinofuranosidases and β-D-xylosidases classified in glycoside hydrolase families.**

Family	Enzymes	Substrate specificity	General features
3	β-D-xylosidase β-D-glucosidase exo-glucanase	broad specificity for 4NPX and oligoxylosides minor activities also on 4NPAra*f*, 4NPAra*p*, 4NPGlc*p*, 4NPGal*p*	glycosylated proteins of up to 110 kDa deduced molecular mass 80-88 kDa
39	β-D-xylosidase*	narrow specificity for 4NPX and oligoxylosides only	55 kDa
43	β-D-xylosidase bifunctional arabinosidase/xylosidase arabinanase exo-arabinanase	refer to *Table 1.2* for individual examples	
51	α-L-arabinofuranosidase	narrow specificity for 4NPA and arabinofuranosyl substituents of arabinoxylans and arabinans no activity on 4NPX	56-83 kDa
52	β-D-xylosidase*	narrow specificity for 4NPX xylan	75 kDa
54	α-L-arabinofuranosidase	moderately broad specificity for 4NPA, arabinofuranosyl substituents of arabinoxylans and arabinans low level activity on 4NPX	49-62 kDa
62	α-L-arabinofuranosidase	narrow specificity for arabinosyl substituents of arabinoxylan no activity on 4NPA	some contain a cellulose-binding domain 47-59 kDa 33 kDa without CBD

Abbreviations: 4NP = 4-nitrophenyl; Ara = arabinoside; Xyl = xyloside; Glc = glucoside; Gal = galactoside; *f* = furanoside; *p* = pyranoside
* only one enzyme has been fully characterised in families 39 and 52

relationships between sequence, structure and specificity, and to foreshadow the types of enzymes that are likely to be found in higher plants.

1.7.1 α-L-Arabinofuranosidases

The α-L-arabinofuranosidases are classified in families 43, 51, 54 and 62 of the glycoside hydrolases (Coutinho and Henrissat, 1999*a*), as shown in *Table 1.1*. While the enzymes are grouped according to similarities in amino acid sequence, common features in biochemical and physical properties are evident within each family. The α-L-arabinofuranosidases hydrolyse α-L-arabinofuranosyl linkages of synthetic substrates such as 4-nitrophenyl- and methylumbelliferyl α-L-arabinofuranosides, and terminal α-L-arabinofuranosyl residues of arabinose-containing polymers. Kaji (1984) classified the α-L-arabinofuranosidases in terms of substrate specificity, and two distinct enzyme types distinguished by specificity for arabinofuranosyl residues of polysaccharide or oligosaccharide substrates, became evident (Kaji and Tagawa, 1970; Tagawa, 1970). A third type of arabinofuranosidase, first identified in *Aspergillus awamori*, hydrolyses only α-L-arabinofuranosyl substituents of cereal arabinoxylans (Kormelink *et al.*, 1991; Kormelink *et al.*, 1993). The two arabinosidase types identified by Kaji (1984) are represented in families 51 and 54 and the α-L-arabino-furanosidase that specifically hydrolyses cereal arabinoxylans is classified in family 62.

Family 51 α-L-arabinofuranosidases are 56-83 kDa, and have activity primarily against 4NPA, arabinoxylans and both branched and linear arabinans. The family 51 enzymes generally do not hydrolyse 4NPX or arabinogalactan. Family 54 proteins exhibit similar variations in molecular mass to those of family 51, and hydrolyse 4NPA, arabinans and arabinoxylans, with low-level activity on 4NPX. Family 62 α-L-arabinofuranosidases do not hydrolyse the aryl glycosides, 4NPA or 4NPX, and only have activity against arabinofuranosyl substituents of arabinoxylans. The family 62 α-L-arabinofuranosidases of *Pseudomonas fluorescens* and *Streptomyces lividans* consist of a catalytic domain and an additional NH_2-terminal carbohydrate-binding domain (Kellett *et al.*, 1990; Vincent *et al.*, 1997). Carbohydrate-binding domains are common among the cellulases and β-D-xylan endohydrolases, where they act to increase the efficiency of catalysis by binding to, and effectively concentrating, the substrate (Bolam *et al.*, 1998). In nearly all cases, the mechanism of carbohydrate binding in these domains is through hydrophobic stacking

interactions between glycosyl residues of the xylan or cellulose, and surface tryptophan or tyrosine residues of the binding domain (Nagy *et al.*, 1998; Simpson *et al.*, 2000).

Family 43 contains several enzyme types with varied substrate specificity for aryl glycosides, 4NPA and 4NPX, polysaccharides such as arabinan and arabinoxylans, and oligoxylosides. Individual members of the family 43 group, as listed in *Table 1.1*, hydrolyse their substrate by either exo- or endo- action and some are described as bifunctional. Glycoside hydrolases usually have preferred substrates for which their particular specificity has evolved. Broad specificities against synthetic substrates, such as 4NPA and 4NPX, documented for bifunctional β-D-xylosidase/α-L-arabinosidase enzymes in family 43 may not necessarily predict the true biochemical roles of these enzymes. The best way to characterize and name these enzymes is therefore to determine hydrolytic activities against naturally occurring substrates.

1.7.2 β-D-Xylosidases

Microbial β-D-xylosidases can be classified in glycoside hydrolase families 3, 39, 43 and 52 (*Table 1.1*; *1.2*). The family 3 glycosidases are primarily *exo*-hydrolases with β-D-glucosidase or β-D-xylosidase activity. The β-D-glucan glucohydrolases of barley (Hrmova *et al.*, 1996), for example, hydrolyse non-reducing terminal residues of (1→3)-, (1→4)- and (1→3,1→4)-β-glucans, and also hydrolyse 4-nitrophenyl β-D-glucoside (4NPG). Other family 3 plant glucosidases have been investigated in *Zea mays* (Kim *et al.*, 2000) and *Tropaeolum majus* (Crombie *et al.*, 1998), and at least 13 *Arabidopsis thaliana* sequences that may encode either β-D-glucosidases or β-D-xylosidases, are classified in family 3 (Coutinho and Henrissat, 1999*a*). The family 3 xylosidases are generally glycosylated proteins of up to 110 kDa, with the polypeptide moieties having deduced molecular masses of 80-88 kDa. These enzymes hydrolyse 4NPX, oligoxylosides and xylans, but also exhibit activities on other aryl glycosides such as 4-nitrophenyl-β-D-glucopyranoside, 4NP-α-L-arabino*furano*side and 4NP-α-L-arabino*pyrano*side.

The family 3 β-D-glucan glucohydrolase isoenzyme ExoI from barley has recently been crystallized and the 3-dimensional structure of the protein has been determined (Hrmova *et al.*, 1998; Varghese *et al.*, 1999). The structural coordinates for the β-D-glucan

glucohydrolase isoenzyme ExoI were subsequently used to generate structural models of other family 3 glycoside hydrolases by comparative homology modelling (Harvey *et al.*, 2000). Phylogenetic analysis of around 100 family 3 sequences reveals that the β-D-xylosidases are clustered in two closely related groups and therefore have a substantial level of divergence in amino acid sequence from the β-D-glucosidases that predominate in family 3 (Harvey *et al.*, 2000). Nevertheless, in line with the principle of conserved molecular structure within families of related glycoside hydrolases (Chothia and Lesk, 1986), the microbial family 3 xylosidases will most likely share similarities in structure with the two-domain barley β-D-glucan glucohydrolases (Varghese *et al.*, 1999).

Families 39 and 52 consist of small groups of β-D-xylosidase sequences, few of which have been fully characterized in terms of biochemical properties or amino acid sequence (*Table 1.1*). The family 39 and 52 β-D-xylosidases hydrolyse 4NPX and oligoxylosides and the family 52 β-D-xylosidase from *Bacillus stearothermophilus* also releases D-xylose from polymeric β-D-xylan. Family 43 contains several β-D-xylosidases that similarly hydrolyse 4NPX and (1→4)-β-linked oligoxylosides.

1.7.3 α-L-Arabinofuranosidases and β-D-Xylosidases from Plants

Studies of α-L-arabinofuranosidase and β-D-xylosidase activities in plants have not been as extensive as for bacteria and fungi. Nevertheless, a number of enzymes have been purified and characterized and these are summarised in *Table 1.3*. Of particular relevance to the study of these enzymes in barley is an arabinofuranosidase from wheat, which has activity against 4NPA, but is not active on arabinoxylans (Beldman *et al.*, 1996). An arabinoxylan arabinofuranohydrolase, similar to that purified from *Aspergillus awamori* (Kormelink *et al.*, 1991), with specificity for arabinofuranosyl substituents of arabinoxylan, was also identified in wheat extracts (Beldman *et al.*, 1996). The α-L-arabinofuranosidases so far investigated in plants have molecular masses in the range of 42-94 kDa by SDS-PAGE and K_m values for hydrolysis of 4NPA from 0.22 mM to 34 mM. β-D-Xylosidases from several sources, including wheat (Beldman *et al.*, 1996; Cleemput *et al.*, 1997), hydrolyse only 4NPX and oligoxylosides. α-D-Xylosidases, with xylosidase activity exclusively against oligoxyloglucosides derived from the dicot wall polysaccharide xyloglucan, were purified from pea epicotyls (O'Neill *et al.*, 1989) and also from germinated nasturtium seeds (Fanutti

Table 1.3 **α-L-Arabinofuranosidases and β-D-xylosidases from plants.** Enzymes have been purified and characterised. Corresponding cDNA or gene sequences have not been isolated and therefore, the plant α-L-arabinofuranosidases and β-D-xylosidases cannot be classified in glycoside hydrolase families. An α-D-xylosidase cDNA has been recently isolated from nasturtium (Crombie *et al.*, 2002) and is classified in family 31.

Organism (source)	Summary	References
α-L-Arabinosidases (EC 3.2.1.55)		
Lupinus luteus (lupin cotyledons)	isoenzyme I M_r 70,000, K_m (4NPA) 46.6 mM, pH optimum 4.4 isoenzyme II M_r 120,000, K_m (4NPA) 1.6 mM, pH optimum 3.5 both active on arabinogalactan	Matheson and Saini (1977)
Scopolia japonica (calluses)	M_r 62,000, K_m (4NPA) 6.7 mM	Tanaka and Uchida (1978)
Daucus carota (carrot suspension cultures)	intracellular enzyme, M_r 94,000, K_m (4NPA) 1.33 mM, pH optimum 4.2 hydrolysed beet arabinan and carrot arabinogalactan and pectins	Konno *et al.* (1987)
Daucus carota (carrot suspension cultures)	secreted enzyme, M_r 80,000, K_m (4NPA) 0.22 mM, pH optimum 3.8 hydrolysed beet arabinan and carrot pectin	Konno *et al.* (1994)
Glycine max (soybean seedlings)	M_r 87,000, K_m (4NPA) 0.53 mM, pH optimum 4.8 active on beet arabinan and (1→5)-arabinan	Hatanaka *et al.* (1991)
Raphanus sativus (radish seeds)	M_r 64,000, K_m (4NPA) 9.8 mM released L-arabinose from beet arabinan, soybean arabinogalactan and radish arabinogalactan proteins	Hata *et al.* (1992)
Malus domestica (softening apples)	participates in degradation of arabinogalactans and pectins	Yoshioka *et al.* (1995)
Pyrus serotina (Japanese pear fruit)	cell wall bound, M_r 42,000, K_m (4NPA) 34 mM, pH optimum 5	Tateishi *et al.* (1996)
Triticum aestivum (wheat bran / germinated wheat)	M_r 49,000, active on 4NPA, 4NPX released only D-xylose from oligoarabinoxylosides no activity on high M_r arabinoxylan - also active on α-(1→5)-linked oligoarabinosides	Beldman *et al.* (1996)
β-D-Xylosidases (EC 3.2.1.37)		
Saccharum officinarum (sugar cane immature stalks)	M_r 62,000, pH optimum 4.85, K_m (4NPX) 2.05 mM hydrolysed only 4NPX and low M_r oligoxylosides	Chinen *et al.* (1982)
Cucumis sativus (cucumber seeds)	isoenzyme A - M_r 68,000, pH optimum 4.5, K_m (4NPX) 1.5 mM hydrolysed 4NPX and oligoxylosides isoenzyme B - M_r 68,000, pH optimum 4.5, K_m (4NPX) 5.4 mM	Mujer and Miller (1991)
Triticum aestivum (wheat bran / germinated wheat)	Mr 47,000 released D-xylose only from 4NPX and oligo(arabino)xylosides	Beldman *et al.* (1996)
Triticum aestivum (wheat flour)	M_r 64,000 enzyme released D-xylose from arabinoxylan and oligoxylosides	Cleemput *et al.* (1997)
α-D-Xylosidases		
Pisum sativum (pea epicotyls)	M_r 85,000, released α-linked xylosyl residues from oligoxyloglucosides	O'Neill *et al.* (1989)
Tropaeolum majus (nasturtium seeds)	M_r 85,000, released α-linked xylosyl residues from oligoxyloglucosides cDNA recently isolated - encodes 105 kDa polypeptide (family 31)	Fanutti *et al.* (1991) Crombie *et al.* (2002)
Arabinoxylan arabinofuranohydrolase		
Triticum aestivum (wheat bran / germinated wheat)	M_r 40,000, partially purified AXH released L-arabinose from polymeric arabinoxylan	Beldman *et al.* (1996)

et al., 1991). The recently isolated nasturtium α-D-xylosidase cDNA (Crombie, 2002) encodes a 105 kDa polypeptide that is evidently post-translationally modified to produce the corresponding protein with an apparent molecular mass of 85 kDa (Fanutti *et al.*, 1991). The nasturtium α-D-xylosidase is classified in glycoside hydrolase family 31, along with a number of α-glucosidases (Coutinho and Henrissat, 1999*a*). From these examples, it is clear that the β-D-xylosidases and α-L-arabinofuranosidases of higher plants are highly specific for the various glycosidic linkages that exist in endogenous cell wall polysaccharides. There are similarities in substrate specificity among the plant α-L-arabinofuranosidases and β-D-xylosidases with their counterpart enzymes in bacteria and fungi. Their presence in seeds, germinated seeds and in cotyledons suggest roles in mobilization of stored carbohydrate reserves, as occurs for (1→3,1→4)-β-glucan endohydrolases and (1→4)-β-D-xylan endohydrolases in barley. Occurrence of these enzymes in cultured cells, vegetative tissues and ripening fruits reveals further possibilities in modification of cell wall polysaccharides.

1.8 Summary and Research Objectives

The primary plant cell wall is a varied and dynamic structure and many structural and functional aspects of the different cell types that comprise the plant body are determined by discrete physical and chemical properties of their cell walls. The cell walls of barley grains are essentially composed of two types of carbohydrate polymer; arabinoxylan and (1→3,1→4)-β-glucan. (1→3,1→4)-β-Glucan endohydrolases, and associated enzymes that participate in (1→3,1→4)-β-glucan degradation have been extensively characterized and their mechanisms of action and expression patterns have been established. With its highly substituted molecular structure, arabinoxylan is perhaps a more complex carbohydrate polymer than (1→3,1→4)-β-glucan with respect to degradation by hydrolytic enzymes. Although little is known about the roles that α-L-arabinofuranosidases and β-D-xylosidases play in barley cell wall metabolism, degradation of the arabinoxylan component of barley cell walls probably requires these accessory enzymes acting in conjunction with (1→4)-β-D-xylan endohydrolases. These enzyme activities have been detected in germinated barley but they have not yet been investigated thoroughly in plants. Furthermore, growth and differentiation of plant cells in vegetative tissues is partly governed by changes in the cell wall, many of which are mediated by regulated processes of wall deposition and selective

degradation and modification. Such processes may also require the activities of α-L arabinofuranosidases and β-D-xylosidases to effect polysaccharide deposition and turnover in the wall.

This research project was designed to investigate α-L-arabinofuranosidases and β-D-xylosidases from barley seedlings. The purification of an α-L-arabinofuranosidase and a β-D-xylosidase, designated ARA-I and XYL, respectively, and the partial purification of a second, highly related α-L-arabinofuranosidase, designated ARA-II, is described in *Chapter 2* of this thesis. Amino acid sequences for each of the purified proteins were determined for later use in the isolation of corresponding cDNAs, described in *Chapter 5*. Partial amino acid sequences suggest that the ARA-I, XYL and ARA-II are family 3 glycoside hydrolases. In *Chapter 3*, the purification and identification of an arabinoxylan arabinofuranohydrolase, capable of specifically removing L-arabinofuranosyl substituents of arabinoxylan, is described. On the basis of partial amino acid sequence, this enzyme, designated AXAH-I, was classified in family 51 of the glycoside hydrolases. The biochemical properties of each of the purified enzymes were determined, and these are discussed and compared in *Chapter 4*. *Chapter 5* covers the isolation, sequencing and comparison of four cDNAs, which encode the two family 3 proteins, ARA-I and XYL, the family 51 AXAH-I, and a second, closely related family 51 protein, designated AXAH-II. Finally, the cDNAs were used to design gene-specific oligonucleotide primers for semi-quantitative RT-PCR, and to map respective genes to the barley genome by analysis of RFLPs in doubled haploid mapping populations; this work is described in *Chapter 6*. The overall findings are summarised, and possibilities for further work in this area are discussed, in *Chapter 7*.

CHAPTER 2.

Purification of α-L-Arabinofuranosidase and β-D-Xylosidase Isoenzymes from Barley

2.1 INTRODUCTION

Glucuronoarabinoxylans are important cell wall components in higher plants and are particularly abundant in the cell walls of the Poaceae. In barley, arabinoxylans constitute approximately 70% (*w/w*) of aleurone cell walls (Bacic and Stone, 1981*b*) and approximately 20% (*w/w*) of endosperm cell walls (Fincher, 1975; Ballance and Manners, 1978). The cell walls of vegetative tissues in barley are mainly composed of cellulose embedded in a matrix of other polysaccharides of which glucuronoarabinoxylan, which accounts for 20-30% (*w/w*), is a major component (Sakurai and Masuda, 1978; Kokubo *et al.*, 1989). Barley arabinoxylans are composed of a (1→4)-β-D-xylan backbone with C(O)2-, C(O)3- and C(O)2,3-substituted α-L-arabinofuranosyl residues. Generally, one or two unsubstituted D-xylopyranosyl residues separate one or two contiguous arabinofuranosyl-substituted residues. Highly substituted regions of up to 25 D-xylopyranosyl residues are separated by short sections of around five unsubstituted residues (Viëtor *et al.*, 1994*a*; Viëtor *et al.*, 1994*b*). Arabinoxylans interact with other wall components, such as (1→3,1→4)-β-glucans and cellulose microfibrils, by hydrogen bonding through unsubstituted linear sections of the β-D-xylan backbone (Darvill *et al.*, 1988; Fincher and Stone, 1986).

The substitution pattern of barley cell wall arabinoxylan varies between different plant tissues and also during growth and development of the plant (Carpita, 1983). Differences in the degree of substitution and arrangement of substituents in cell wall arabinoxylans influence the physicochemical properties of individual wall components and also the overall structural and functional properties of the cell wall. Formation of the primary cell wall during vegetative plant growth is accompanied by the production of soluble, highly substituted glucuronoarabinoxylans in the Golgi and their delivery to the exterior of the cell *via* Golgi-derived vesicles (Gibeaut and Carpita, 1994). Deposition of these polysaccharides in the cell wall coincides with a progressive reduction in the degree of arabinofuranosyl substitution, a process that may result from the enzymic removal of substituents by specific hydrolases (Gibeaut and Carpita, 1991). Thus, removal of L-arabinofuranosyl residues reduces arabinoxylan solubility (Andrewartha *et al.*, 1979) and generates unsubstituted regions that could lock the polysaccharides in the wall through extensive intermolecular hydrogen bonding with other wall polysaccharides. Such processes may require α-L-arabinofuranosidases with specificity for arabinoxylans. Cell wall (1→3,1→4)-β-glucans are

subject to degradation and turnover during plant cell growth, and production of (1→3,1→4)-β-glucan endohydrolases and β-glucan glucohydrolases may be associated with elongation processes in young barley leaves and coleoptiles (Slakeski and Fincher, 1992*a*; Harvey *et al.*, 2001). Hydrolysis and turnover of arabinoxylans, a process not yet investigated in barley, may similarly occur during growth of barley tissues and may require both α-L-arabinofuranosidase and β-D-xylosidase activities to act in conjunction with (1→4)-β-D-xylan endohydrolases during cell wall loosening.

α-L-Arabinofuranosidases (EC 3.2.1.55) are widespread among bacteria and fungi where they are produced as secreted enzymes in order to degrade arabinose-containing polymers from plants (Coughlan and Hazelwood, 1993). The ability to hydrolyse arabinofuranosyl linkages has evolved in a diverse array of enzymes that are classified in glycoside hydrolase families 43, 51, 54 and 62 (Coutinho and Henrissat, 1999*a*). β-D-Xylosidases (EC 3.2.1.37) that hydrolyse (1→4)-β-linkages of β-D-xylans and oligoxylosides have also been purified from a variety of microbial sources and are classified in glycoside hydrolase families, 3, 39, 43, and 52 (Coutinho and Henrissat, 1999*a*). α-L-Arabinofuranosidase and β-D-xylosidase activities have been detected in barley malt extracts (Preece and MacDougall, 1958) and also from gibberellic acid (GA_3)-treated barley aleurone layers (Taiz and Honigman, 1976; Dashek and Chrispeels, 1977; Banik *et al.*, 1997). They are therefore suggested to participate with the (1→4)-β-D-xylan endohydrolase (Slade, *et al.*, 1989; Banik *et al.*, 1997; Caspers *et al.*, 2001) in the overall degradation of endosperm arabinoxylans after grain germination.

Glycoside hydrolases commonly have substantial activity against aryl glycosides, and synthetic substrates such as 4-nitrophenyl α-L-arabinofuranoside (4NPA) and 4-nitrophenyl β-D-xylopyranoside (4NPA) are suitable for simple, quantitative assays for α-L-arabinofuranosidase and β-D-xylosidase activity, respectively (Coughlan and Hazelwood, 1993). Here, the purification from barley seedlings, of enzymes that hydrolyse 4NPA and 4NPX is reported. NH_2-terminal and tryptic amino acid sequences of purified proteins have been determined.

2.2 Materials and Methods

2.2.1 *Barley Germination and Extract Preparation*

Barley (*Hordeum vulgare* L. cv. Clipper) grain (3 kg dry weight) was surface sterilised in 0.2% (*w/v*) $AgNO_3$ for 20 min and washed extensively with sterile distilled water, 0.5 M NaCl and sterile distilled water again, prior to steeping for 24 h in water containing chloramphenicol and neomycin (100 mg/ml), and penicillin G and nystatin (100 U/ml) (Hoy *et al.*, 1980). Grains were germinated on autoclaved 3MM Chr paper (Whatman, Maidstone, England) were maintained at approximately 40-45% (*w/w*) moisture content by regular application of freshly-prepared antibiotic solution, for 5 or 7 days in the dark at 22°C. Microbial contamination of the grains was not evident at any stage during this period. Barley seedlings at 5 or 7 days' growth were stored overnight at -20°C prior to homogenisation at 4°C in approximately 1.5 volumes of 0.1 M sodium acetate buffer, pH 5, containing 10 mM EDTA, 10 mM NaN_3, 3 mM 2-mercaptoethanol and 3 mM phenylmethylsulphonyl fluoride (PMSF). Plant material was homogenised using a Waring blender (10 sec low speed, 10 sec medium speed, 20 sec high speed, 20 sec medium speed, 10 sec low speed). The homogenate was allowed to stand for 1 h, after which insoluble material was removed by centrifugation at approximately 6,000g for 20 min at 4°C using a Hitatchi SCR20B centrifuge with a RPR9-2 fixed angle rotor. In the preparation of extract from 7-day-old seedlings, crude homogenate was forced through nylon mesh prior to centrifugation to remove the large volume of leaf and coleoptile debris.

After centrifugation, the supernatant was filtered through Miracloth (Calbiochem-Novabiochem Corporation, La Jolla, CA, U.S.A.) and fractionated by $(NH_4)_2SO_4$ precipitation into 0-20%, 20-40%, 40-60%, 60-80% and 80-100% (*w/v*) fractions. Cold $(NH_4)_2SO_4$ was dissolved at each stage with constant stirring for at least 4 h. Precipitated protein was isolated by centrifugation at approximately 6,000g for 15 min at 4°C, and resuspension in 20 mM Tris-HCl buffer, pH 8.2, containing 4 mM NaN_3 and 3 mM 2-mercaptoethanol. Enzyme activities and protein contents were determined and appropriate fractions were dialysed against 20 mM Tris-HCl buffer, pH 8.2, containing 4 mM sodium azide and 3 mM 2-mercaptoethanol, to remove residual $(NH_4)_2SO_4$. Dialysis tubing (Membracel MD44-14 x 100) was from Selby Biolab (Notting Hill, Australia).

2.2.2 *Ion-Exchange Chromatography*

All protein purification procedures were performed at 4°C. Chromatography media were packed at constant flow rates in siliconized glass columns (Bio Rad, Hercules, CA, U.S.A.) using an ISCO Wiz (ISCO, Lincoln, NE, U.S.A.) or an Ismatec Reglo Digital MS4/8 (Ismatec, Switzerland) low-pressure peristaltic pump. UV absorbance (280 nm) of column eluates was measured using an ISCO UA-6 detector fitted with a 0.5 cm flow-through cell.

DEAE-Cellulose (DE52 pre-swollen microgranular) (Whatman, Maidstone, England) was equilibrated in 20 mM Tris-HCl buffer, pH 8.2, containing 4 mM NaN_3 and 3 mM 2-mercaptoethanol, in a 30 x 5 cm glass column. Bed volume was 400 cm^3. Resuspended, dialysed protein was loaded onto the column at a linear flow rate of 3 $cm.h^{-1}$. Unbound proteins were collected and enzyme activities and protein content were determined. The DEAE-cellulose column with bound proteins was washed with the Tris-HCl column buffer until A_{280} of the column eluate returned to baseline levels (approximately seven column volumes). Bound proteins were eluted with a 3 L linear 0-350 mM NaCl gradient of the Tris-HCl column buffer. Fractions (20 ml) were pooled on the basis of their respective enzyme activities and the examination of contaminating proteins by SDS-PAGE. Pooled fractions were dialysed against 50 mM sodium acetate buffer, pH 5.0, containing 4 mM NaN_3 and 3 mM 2-mercaptoethanol. Dialysis and CM-Sepharose column buffer pH was 5.0 for the purification of β-D-xylosidase and 4.2 for the purification of α-L-arabinofuranosidase isoenzymes ARA-I and ARA-II.

CM-Sepharose CL-6B (Pharmacia Biotech, Uppsala, Sweden) was equilibrated in 50 mM sodium acetate buffer containing 4 mM NaN_3 and 3 mM 2-mercaptoethanol in glass columns; 20 x 5 cm for β-D-xylosidase (bed volume 300 cm^3) and 20 x 2.5 cm for ARA-I and ARA-II (bed volume 85 cm^3). Dialysed protein fractions were loaded onto columns at 3 $cm.h^{-1}$ for β-D-xylosidase and 40 $cm.h^{-1}$ for α-L-arabinofuranosidase isoenzymes. Unbound proteins were eluted with 50 mM sodium acetate column buffer as described for the DEAE-cellulose step above. Bound proteins were eluted with linear NaCl gradients and fractions were collected and assessed as above.

2.2.3 Chromatofocusing

PBE 94 chromatofocusing media (Pharmacia Biotech, Uppsala, Sweden) was pre-equilibrated in 25 mM Tris-acetate buffer, pH 7.8 (for β-D-xylosidase), or 25 mM histidine-HCl buffer, pH 6.2 (for α-L-arabinofuranosidase isoenzymes ARA-I and ARA-II), in 30 x 1.0 cm glass columns (bed volume 22 cm^3) at a linear flow rate of 23 $cm.h^{-1}$. Pooled fractions from CM-Sepharose were concentrated by ultrafiltration using Amicon apparatus fitted with Amicon membranes (Amicon, Beverly, MA, U.S.A.). For large volumes or fractions with high protein concentrations, 200 ml and 50 ml concentrators were used with YM10 membranes and for final preparation of samples for column loading, a 10 ml capacity concentrator with a YM10 membrane was used. Samples were prepared by three rounds of concentration and dilution in the appropriate chromatofocusing starting buffer and sample volumes were reduced to approximately 20% of column volume. Samples were applied to columns and proteins eluted in a descending pH gradient with approximately 12 column volumes of diluted Polybuffer (Pharmacia Biotech, Uppsala, Sweden). For the elution of α-L-arabinofuranosidase isoenzymes ARA-I and ARA-II from PBE 94, Polybuffer 74 was diluted 1:8 in H_2O and pH 3.8 adjusted with HCl. Elution of β-D-xylosidase was achieved with a mixture of Polybuffer 74/Polybuffer 96 (30/70) diluted 1:10 and adjusted to pH 5.5 with acetic acid. Fractions (5 ml) were collected, assessed and pooled as described above.

2.2.4 Size-Exclusion Chromatography

Fractions from PBE 94 chromatography were equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing 200 mM NaCl, 4 mM NaN_3 and 1 mM dithiothreitol, by ultrafiltration using a 10 ml capacity Amicon concentrator with a YM10 membrane. Samples were chromatographed on a 100 x 1.5 cm Bio-Gel P-100 column by elution with the same sodium acetate buffer at a linear flow rate of 1.7 $cm.h^{-1}$.

2.2.5 Enzyme Assays

α-L-Arabinofuranosidase and β-D-xylosidase activities were determined spectrophotometrically using 4NPA and 4NPX, respectively (Sigma Chemical Co., St Louis, MO, U.S.A.). 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 or 4NPX. Reactions were terminated by addition of 2 volumes of saturated di-sodium tetraborate solution. One unit (U) of activity is defined as the amount of enzyme required to produce 1 μmole of 4-nitrophenol in the above

reaction, per min, as determined by measurement of absorbance at 410 nm. Pure 4-nitrophenol (Sigma Chemical Co.) was used as a standard.

2.2.6 *Protein Determination and Polyacrylamide Gel Electrophoresis*

Protein concentration of column eluate was monitored by UV absorbance at 280 nm in a 0.5 cm flow-through cell, calibrated with standard bovine serum albumin (Sigma Chemical Co.). Protein content of pooled column fractions and purified proteins was measured using Coomassie Brilliant Blue reagent from Pierce (Rockford, IL, U.S.A.) where 0.5 ml of reagent was added to 0.5 ml of diluted sample, and protein estimated by measurement of absorbance at 595 nm (Bradford, 1976). Bovine serum albumin was used as standard.

Purity of fractions at each stage of purification was assessed by SDS-PAGE (Laemmli, 1970) with 5% (*w/v*) polyacrylamide (Sigma Chemical Co.) stacking gel, pH 6.8, and 12.5% (*w/v*) polyacrylamide resolving gel, pH 8.8. Proteins were separated at 35-50 mA in electrophoresis buffer, 25 mM Tris-HCl, pH 8.3, containing 250 mM glycine and 0.1% (*w/v*) SDS using a Hoefer SE 250 protein electrophoresis system (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Gels were stained with Coomassie Brilliant Blue R (Sigma Chemical Co.) in 25% (*v/v*) methanol and 7% (*v/v*) acetic acid, and destained in the same solvent. Molecular mass markers (Pharmacia Biotech, Uppsala, Sweden) were phosphorylase *b* (M_r 94,000), albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 20,100) and α-lactalbumin (M_r 14,000).

2.2.7 *Amino Acid Sequence Analysis*

NH_2-Terminal sequence analysis of proteins and RP-HPLC-purified peptides generated by proteolysis with trypsin, were performed on a Hewlett-Packard G1005A automated protein sequencer, using Edman degradation chemistry. Phenylthiohydantoin (PTH) derivatives were identified by reversed-phase HPLC on a PTH-AA 250 x 2.1 mm column using a Hewlett-Packard 1090 system. Amino acid sequence analyses were performed by Mr. Jelle Lahnstein (Department of Plant Science, University of Adelaide).

For digestion with trypsin, purified proteins were concentrated using a Microcon-3 concentrator (Amicon) and resuspended in 50 µl 0.6% SDS (*w/v*) with 2 mM dithiothreitol. Proteins were denatured by incubation in 50 µl 100 mM Tris-HCl, pH 7.2, at 60°C for 2 h.

After denaturation, 50 µl 100 mM Tris-HCl, pH 7.2, containing 8 mM NaCl, 50 µl 80% (*w/v*) dimethylformamide and 2.5 mg TPCK-treated trypsin (Worthington, Lakewood, NJ, U.S.A.) were added and proteins were digested for 24 h at 37°C. The substrate to trypsin ratio was in excess of 20:1 in the hydrolysis of each enzyme. Samples were dried under vacuum and resuspended in 100 µl 0.1% (*v/v*) trifluoroacetic acid (Pierce) and 100 µl 6 M guanidine-HCl (Sigma Chemical Co.).

Tryptic peptides were separated by reversed-phase HPLC on a 250 x 2.5 mm, 5 micron, W-Porex C18 column (Phenomenex, Torrance, CA, U.S.A.) with a C18 guard column using a Hewlett-Packard 1090 HPLC system. Peptides were eluted in 0.05% (*v/v*) trifluoroacetic acid (Pierce) at 0.6 $ml.min^{-1}$ with a linear gradient of 0-70% (*v/v*) acetonitrile (BDH, Poole, England). UV absorbance at 214 nm and 280 nm were monitored and fractions were manually collected according to peak height and shape. Peptide fractions were selected for sequence analysis on the basis of late elution on RP-HPLC, which is often an indication of longer peptides.

BLAST (Altschul *et al.*, 1990) analyses with partial amino acid sequences were performed using the University of Wisconsin, Genetics Computer Group software (Devereux *et al.*, 1984) in the ANGIS suite of programs at the Australian National Genomic Information Service (http://www.angis.org.au).

2.3 RESULTS

2.3.1 *Purification of Barley α-L-Arabinofuranosidase (ARA-I) and β-D-Xylosidase (XYL)*

Preliminary experiments to determine the timing of synthesis of α-L-arabinofuranosidase and β-D-xylosidase isoenzymes in germinated barley revealed that activities of these enzymes reached a peak 4-5 days after germination. From 3 kg barley grain, an extract of soluble proteins from 5-day-old barley seedlings yielded 45 U and 46 U of 4NPA and 4NPX hydrolysing activity, respectively (*Table 2.1*). The majority of α-L-arabinofuranosidase and β-D-xylosidase activity was precipitated in the 40-60%-saturated $(NH_4)_2SO_4$ fraction. This fraction was selected for further purification of the enzymes. Ammonium sulphate fractionation increased the apparent level of β-D-xylosidase above that found in the seedling extract (*Table 2.1*). The sequence of purification steps used for the purification of α-L-arabinofuranosidase and β-D-xylosidase isoenzymes is summarised in *Figure 2.1*.

Large-scale fractionation of the 40-60%-saturated $(NH_4)_2SO_4$ fraction was achieved by anion-exchange chromatography on DEAE-cellulose. The DEAE-cellulose elution profile is shown in *Figure 2.2*. Approximately 2 L of resuspended material was loaded onto the column and about half of the protein was bound to the 400 cm^3 volume of DEAE-cellulose. A large proportion (28 U) of the β-D-xylosidase activity was recovered in the DEAE-cellulose unbound fraction. β-D-Xylosidase was also found in fractions eluted from the DEAE-cellulose column by a 2 L linear gradient of 0-350 mM NaCl, along with the majority of the α-L-arabinofuranosidase activity that was loaded onto the column.

Two sharp peaks of α-L-arabinofuranosidase activity were resolved at approximately 90 mM NaCl and 140 mM NaCl (*Figure 2.2*). Fractions 21-29 (Peak 1) and fractions 37-44 (Peak 2) were pooled in order to collect fractions with the highest specific activities and also to effectively separate the two presumed α-L-arabinofuranosidase isoenzymes. Pooled fractions from peak 1 contained 16.9 U of α-L-arabinofuranosidase activity, representing 38% of the total α-L-arabinofuranosidase activity from the starting material. The peak 2 pool contained 10.5 U of α-L-arabinofuranosidase activity, representing 24% of the starting activity. The two α-L-arabinofuranosidase isoenzymes resolved in peaks 1 and 2 on DEAE-cellulose, were named ARA-I and ARA-II, respectively.

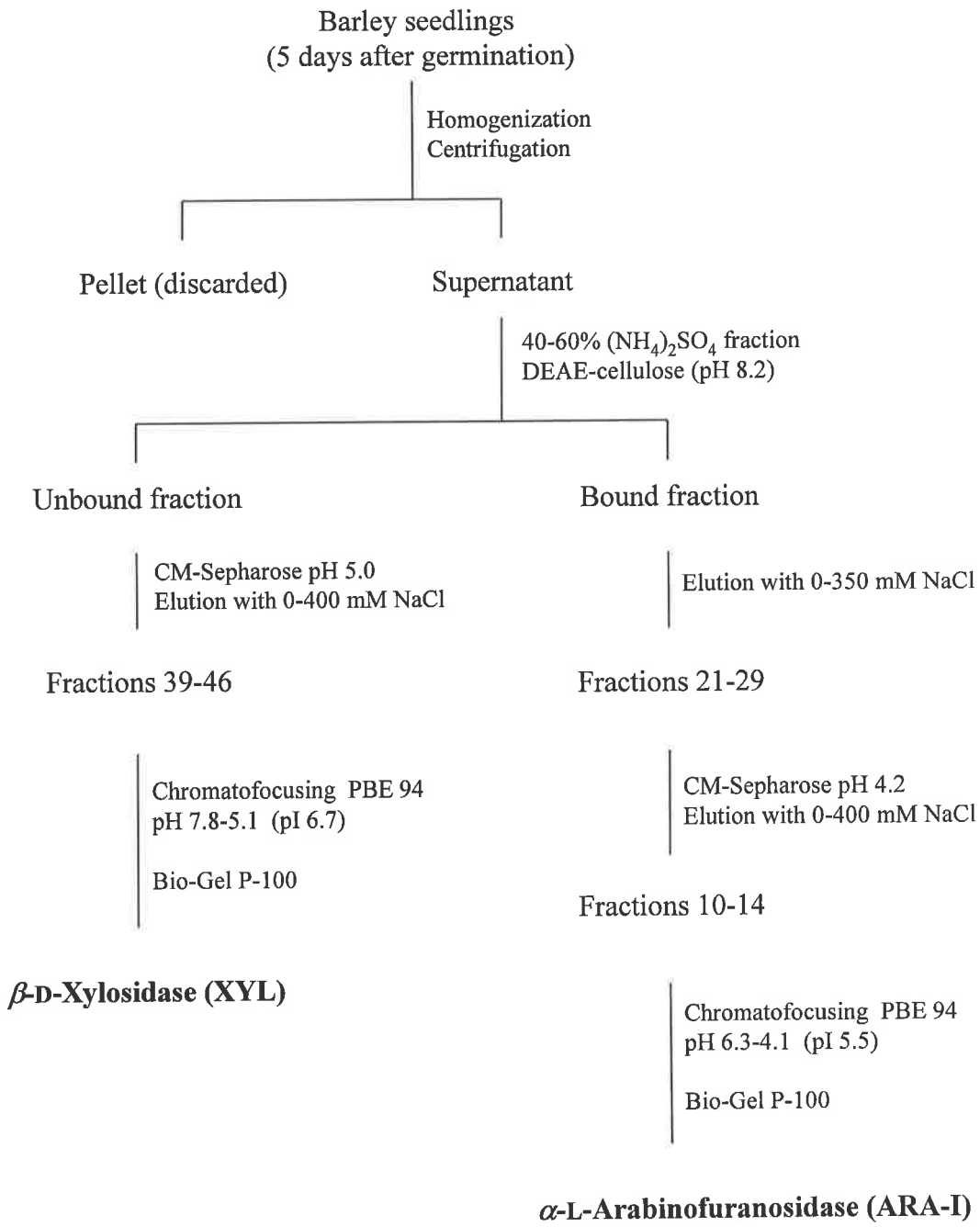

Figure 2.1 **Summary of procedures used in the purification of XYL and ARA-I.**

Table 2.1 **Enzyme yields and purification factors at each stage of the purification of α-L-arabinofuranosidase (ARA-I) and β-D-xylosidase (XYL).** Activities were assayed with 4NPA (ARA-I) and 4NPX (XYL). Recoveries are expressed as percentage of initial activity units and purification factors were calculated on the basis of specific activities.

	Yield				
	Protein *(mg)*	Activity *(mU)*	Specific activity *($mU.mg^{-1}$)*	Recovery *(%)*	Purification factor *(fold)*
α-L-Arabinofuranosidase (ARA-I)					
Crude homogenate	7300	44700	6.1	100	1.0
40-60% $(NH_4)_2SO_4$	1480	40700	28	91	4.5
DEAE-cellulose	62	16900	270	38	45
CM-Sepharose	9.7	12300	1270	28	210
PBE 94	2.0	8500	4200	19	690
Bio-Gel P-100	0.14	950	6610	2	1080
β-D-Xylosidase (XYL)					
Crude homogenate	7300	46000	6.3	100	1.0
40-60% $(NH_4)_2SO_4$	1480	57600	39	125	6.2
DEAE-Cellulose	620	28200	46	61	7.3
CM-Sepharose	320	13200	41	29	6.5
PBE 94	0.58	2180	3770	5	600
Bio-Gel P-100	0.43	2580	6060	6	960

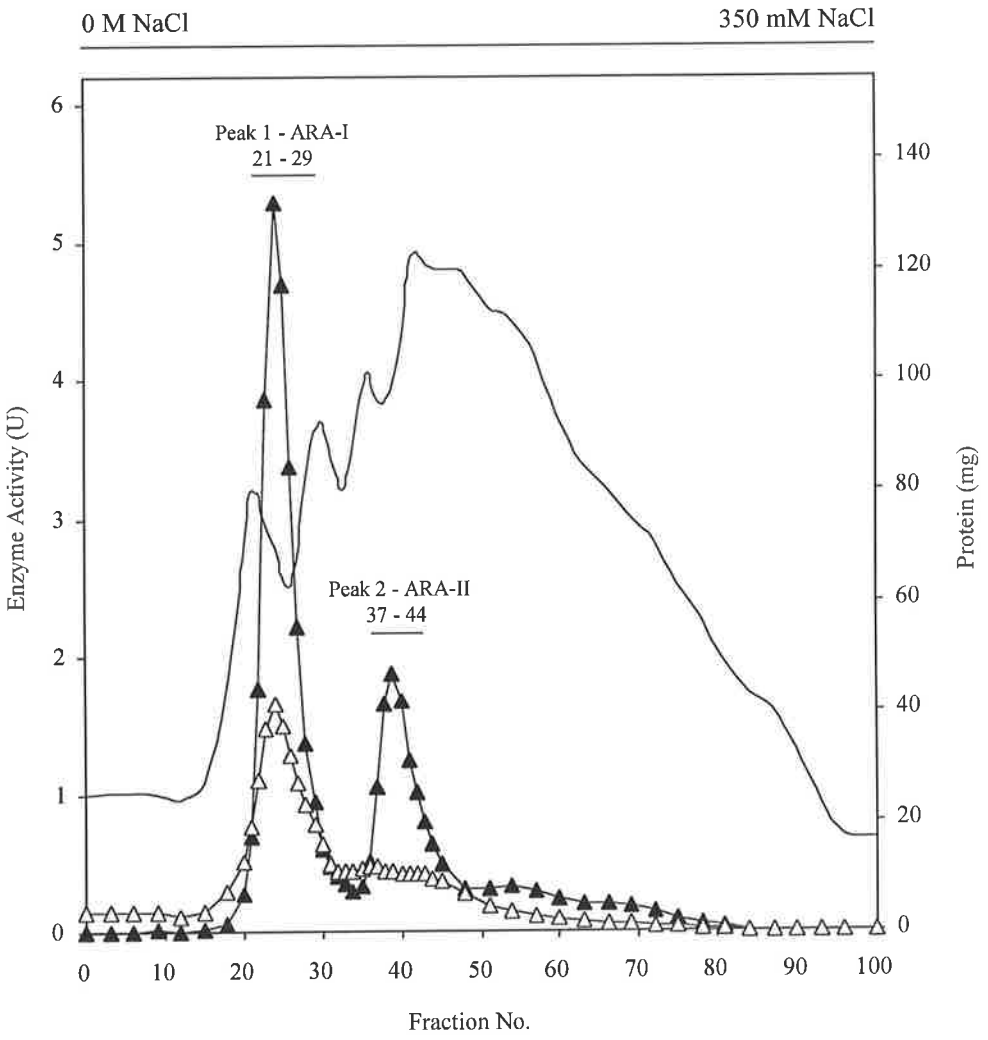

Figure 2.2 **Ion-exchange chromatography of 40-60% $(NH_4)_2SO_4$ fraction on DEAE-cellulose.** Material bound to DEAE-cellulose at pH 8.2 was eluted with a 2 L linear gradient of 0-350 mM NaCl. Fractions (20 ml) were assayed against 4NPA (▲) and 4NPX (△). Protein (—) was measured by absorbance at 280 nm.

DEAE-Cellulose α-L-arabinofuranosidase peak 1 fractions, containing ARA-I, were pooled and equilibrated in 50 mM sodium acetate buffer, pH 4.2, and loaded onto CM-Sepharose. Of the 16.9 U of α-L-arabinofuranosidase activity loaded onto the column, 12.3 U were recovered by elution of bound proteins with a linear gradient of 0-400 mM NaCl (*Figure 2.3*). The ARA-I-containing fractions also contained β-D-xylosidase activity and this was observed to be approximately 15% of the α-L-arabinofuranosidase activity in each fraction. This observation was consistent throughout the purification of ARA-I and in the final purified enzyme preparation, thereby suggesting that although ARA-I has a preference for 4NPA, it can also hydrolyse 4NPX. A broad peak of β-D-xylosidase activity was resolved from the ARA-I fraction by the cation-exchange step (fractions 18-33, *Figure 2.3*). However, because this represented no more than 10% of the starting β-D-xylosidase activity and was of low specific activity, this fraction was not purified further. Fractions 10-14 containing 12.3 U of α-L-arabinofuranosidase activity were pooled and subjected to chromatofocusing on PBE 94 (*Figure 2.4*). A sharp α-L-arabinofuranosidase peak consisting of three 5 ml fractions was eluted from the column at pH 5.5, corresponding with the major protein peak, as indicated by A_{280}. Fractions 14-16 were pooled and applied to a Bio-Gel P-100 column, which was successful in removing small quantities of contaminating proteins with molecular masses of around 30-50 kDa shown on SDS-PAGE (*Figure 2.5*). Bio-Gel P100 UV absorbance profiles were of relatively poor quality, due to the low signal to noise ratio when used at high sensitivities required to detect protein at low concentrations. ARA-I was purified by greater than 1000-fold and 2% of the α-L-arabinofuranosidase activity in the crude extract was recovered as the ARA-I isoenzyme (*Table 2.1*). SDS-PAGE (*Figure 2.5*) shows that the two ion-exchange steps were probably the most useful for the separation of the enzyme from contaminating proteins. ARA-I has a molecular mass of approximately 65 kDa as shown by SDS-PAGE (*Figure 2.5*), and has a pI of 5.5 indicated by chromatofocusing.

The second DEAE-cellulose peak of α-L-arabinofuranosidase activity, containing the ARA-II isoenzyme, was further purified but without success. It was later found that ARA-II was in greater abundance in seedlings harvested 7 days after germination and purification of ARA-II was therefore attempted from a 7-day seedling extract (see *Section 2.3.2*).

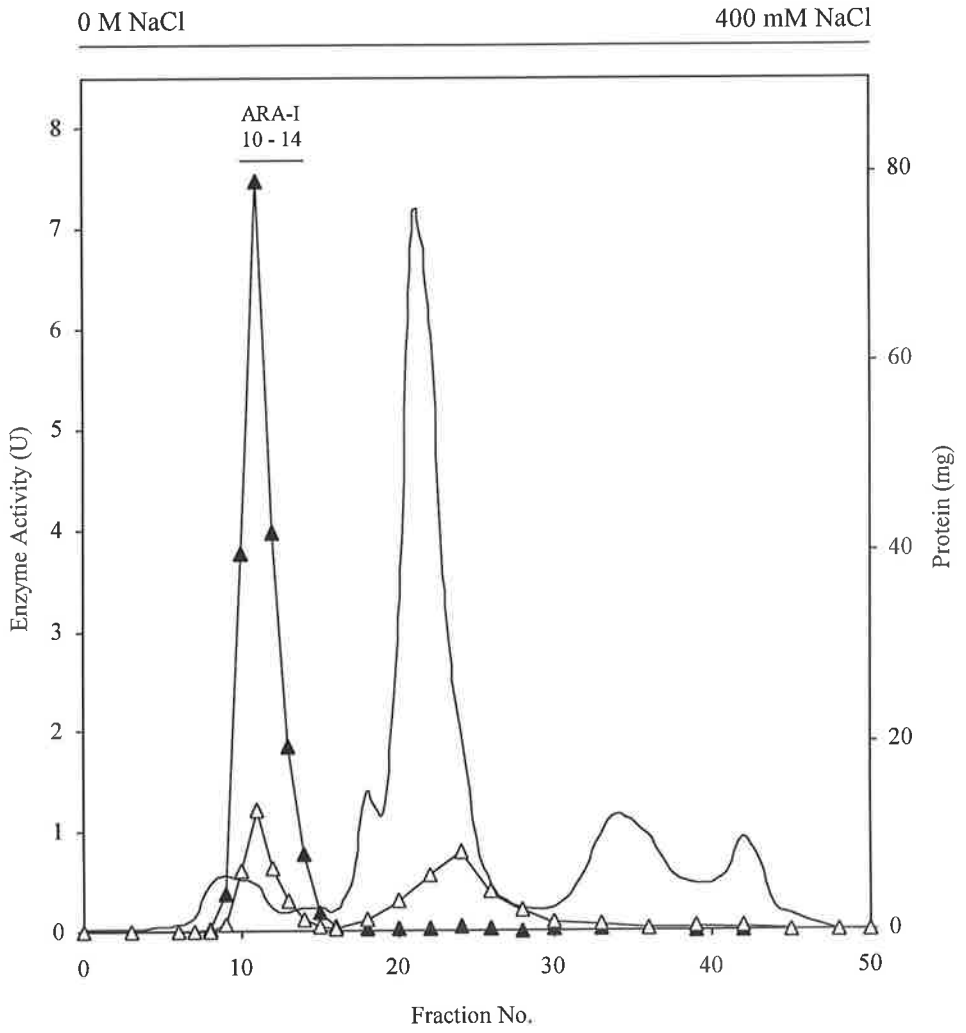

Figure 2.3 **Ion-exchange chromatography of DEAE-cellulose peak 1, containing α-L-arabinofuranosidase (ARA-I) activity, on CM-Sepharose.** Fractions 21-29 from DEAE-cellulose (*Figure 2.2*) were pooled and applied to CM-Sepharose at pH 4.2. Bound proteins were eluted with an 800 ml linear gradient of 0-400 mM NaCl and fractions (16 ml) were assayed against 4NPA (▲) and 4NPX (▵). Protein (—) was measured by absorbance at 280 nm.

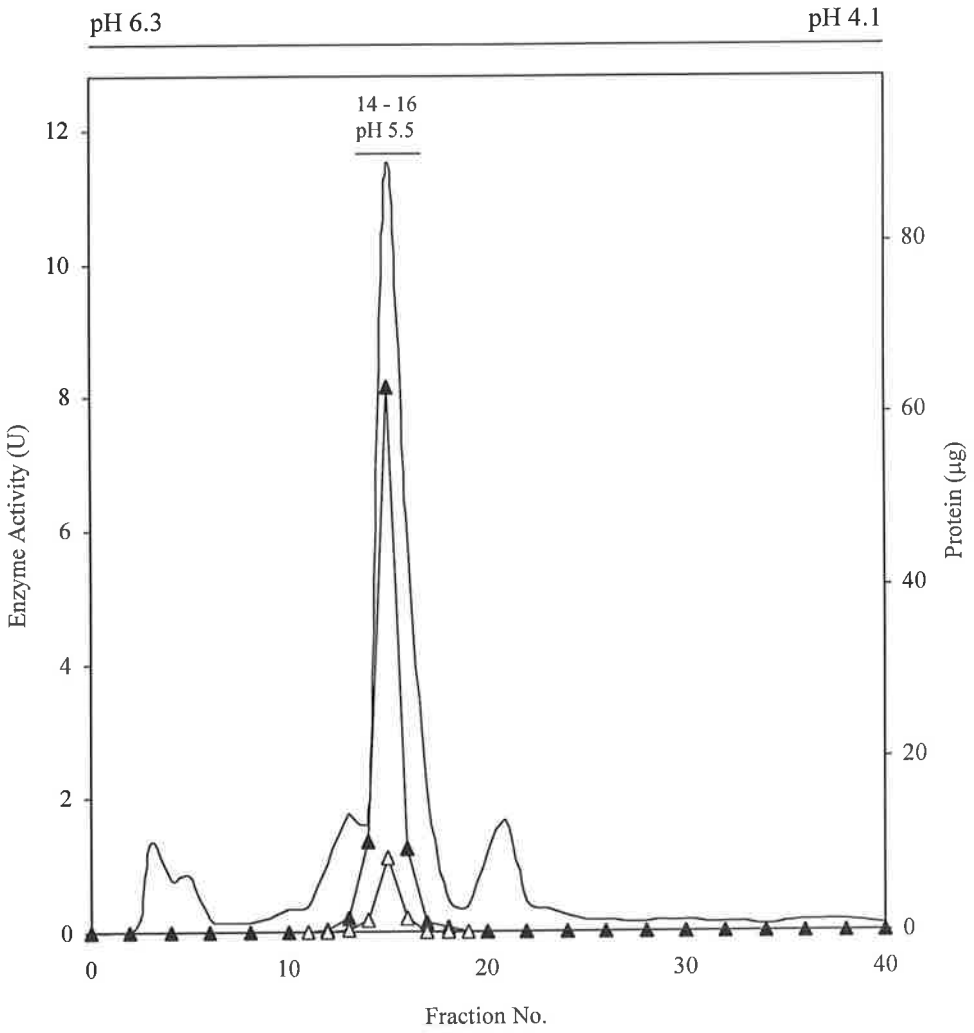

Figure 2.4 **Chromatofocusing of ARA-I on PBE 94.** Pooled fractions (10-14) from CM-Sepharose were concentrated and equilibrated in Polybuffer 74 adjusted to pH 3.8, and applied to PBE 94 equilibrated at pH 6.2 with 25 mM histidine-HCl buffer. Proteins were eluted from the column with Polybuffer 74, pH 3.8. Fractions (5 ml) were assayed against 4NPA (▲) and 4NPX (△) and protein (—) was measured by absorbance at 280 nm.

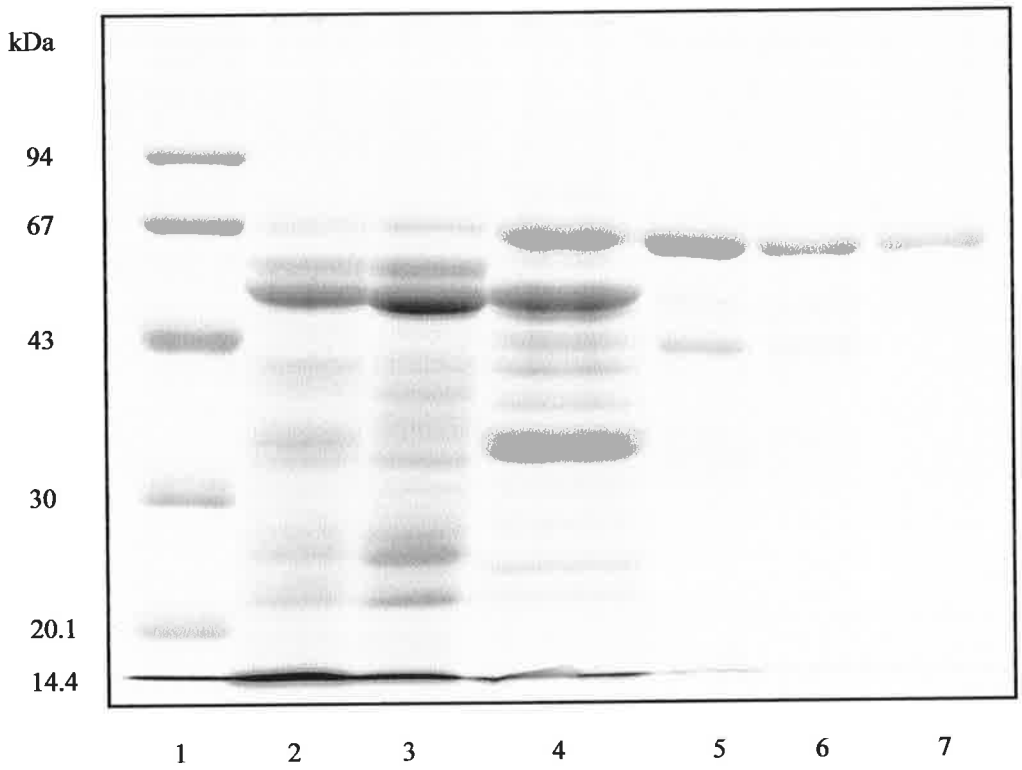

Figure 2.5 **SDS-PAGE of samples taken at each stage of the purification of α-L-arabinofuranosidase (ARA-I) from an extract of 5-day old barley seedlings.** *Lane 1*, molecular mass markers; *lane 2*, crude extract; *lane 3*, 40-60% $(NH_4)_2SO_4$ fraction; *lane 4*, DEAE-cellulose peak 1; *lane 5*, CM-Sepharose pooled fractions 10-14; *lane 6*, PBE 94 fractions; *lane 7*, purified ARA-I after Bio-Gel P-100.

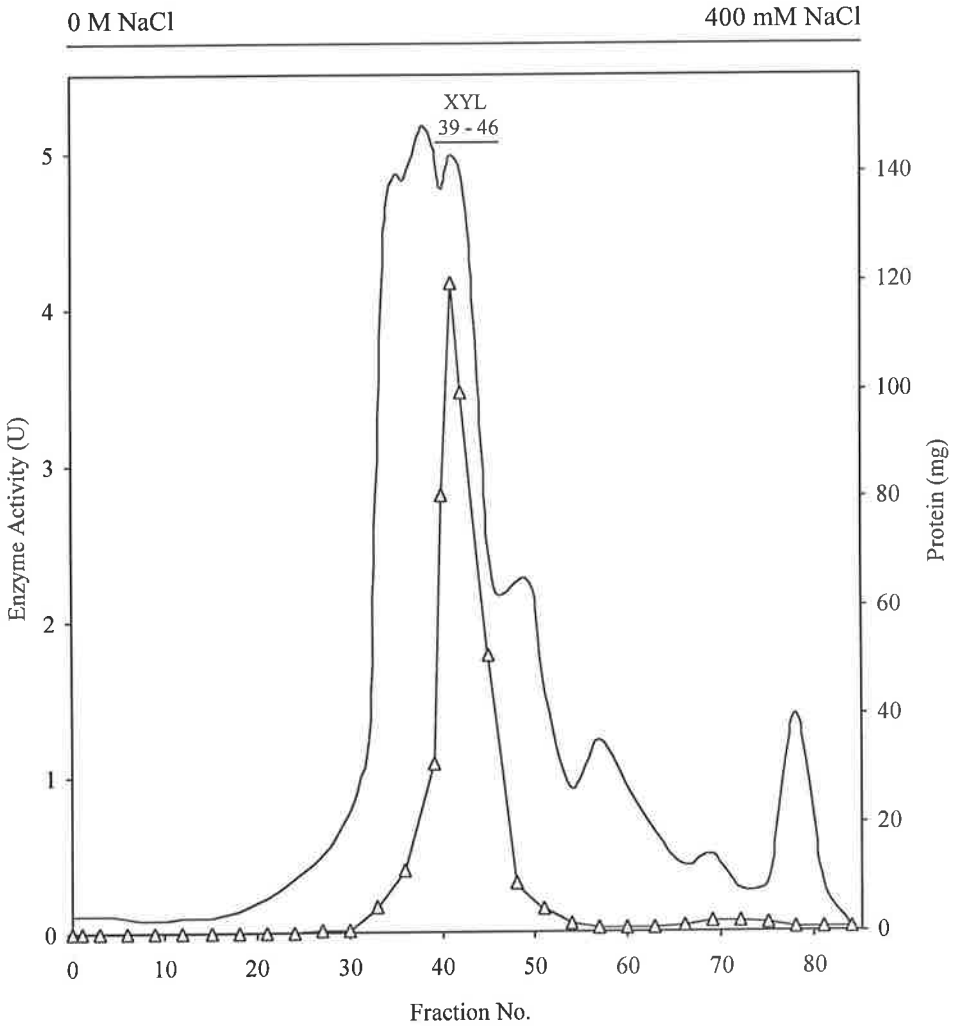

Figure 2.6 **Ion-exchange chromatography of DEAE-cellulose unbound fraction, containing β-D-xylosidase activity, on CM-Sepharose.** Material bound to CM-Sepharose at pH 5.0 was eluted with a 1.6 L linear gradient of 0-400 mM NaCl. Fractions (20 ml) were assayed against 4NPX (▵) and protein (—) was measured by absorbance at 280 nm.

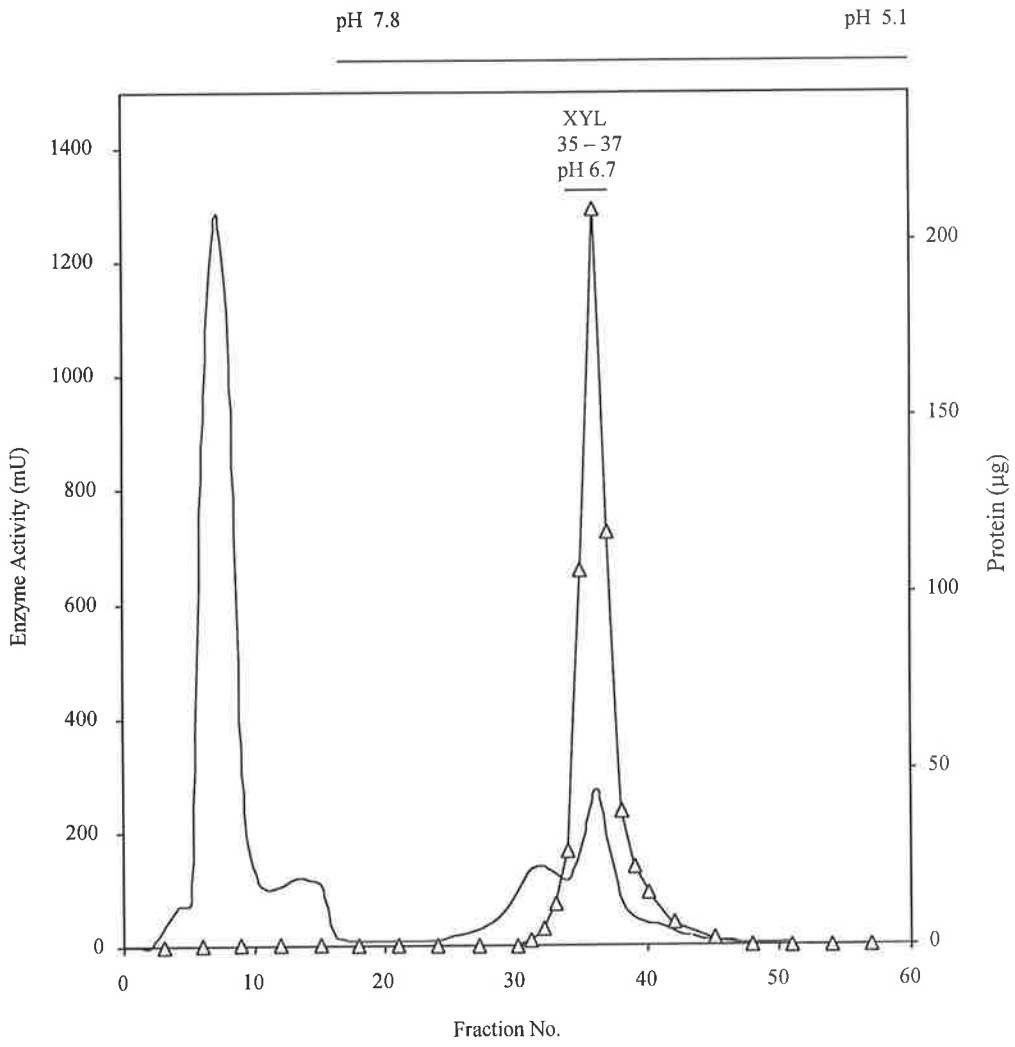

Figure 2.7 **Chromatofocusing of XYL on PBE 94.** Pooled fractions (39-46) from CM-Sepharose were concentrated and equilibrated in 25 mM Tris-acetate buffer, pH 7.8 and applied to PBE 94 equilibrated at pH 7.8. Proteins were eluted from the column, firstly with 25 mM Tris-acetate buffer, pH 7.8, followed by a descending pH gradient established by Polybuffer 74 / Polybuffer 96, pH 5.5. Fractions (5 ml) were assayed against 4NPX (▵) and protein (—) was measured by absorbance at 280 nm.

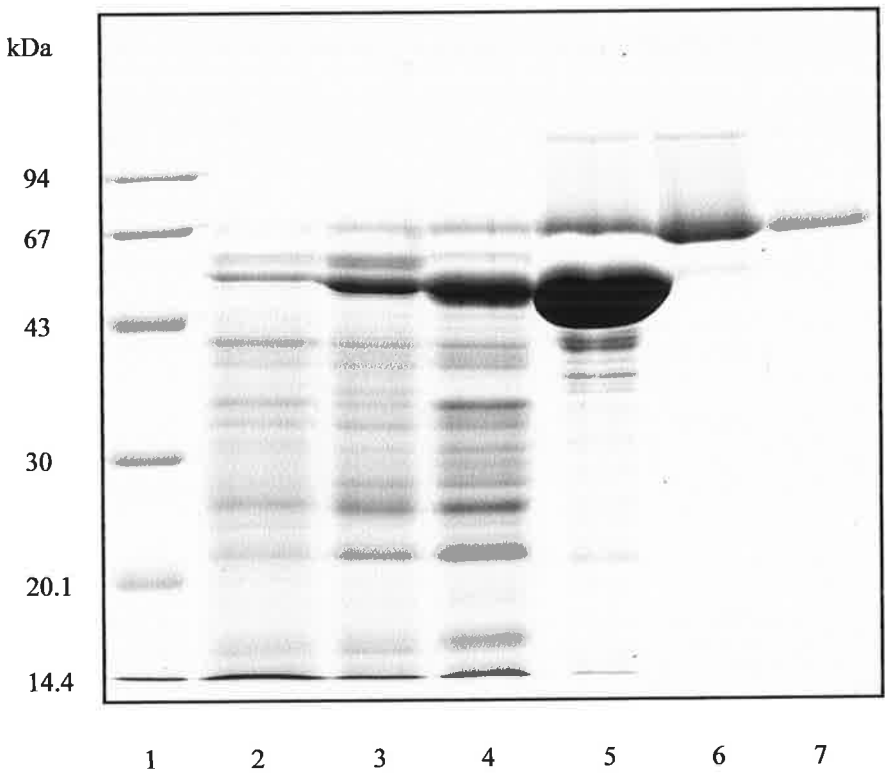

Figure 2.8 **SDS-PAGE of samples taken at each stage of the purification of β-D-xylosidase (XYL) from an extract of 5-day old barley seedlings.** *Lane 1*, molecular mass markers; *lane 2*, crude extract; *lane 3*, 40-60% $(NH_4)_2SO_4$ fraction; *lane 4*, DEAE-cellulose unbound fraction; *lane 5*, CM-Sepharose pooled fractions 39-46; *lane 6*, PBE 94 fractions; *lane 7*, purified XYL after Bio-Gel P-100.

The DEAE-cellulose unbound fraction, containing the major proportion of the β-D-xylosidase activity, was equilibrated in 50 mM sodium acetate buffer, pH 5, and loaded onto a CM-Sepharose column. Almost all of the β-D-xylosidase bound to the CM-Sepharose column. This activity was eluted from the column mid-way through a 1.6 L linear gradient of 0-400 mM NaCl, as a single peak (*Figure 2.6*). This major β-D-xylosidase isoenzyme was designated XYL. Half of the 28.2 U of β-D-xylosidase activity was recovered after CM-Sepharose chromatography and fractions 39-46 were pooled, concentrated and equilibrated in preparation for chromatofocusing. The CM-Sepharose-purified XYL preparation (13.2 U) was applied to a column of PBE 94 at pH 7.8 and the column was eluted with 25 mM Tris-acetate buffer, pH 7.8, to remove major contaminants, prior to elution with a descending pH gradient (*Figure 2.7*). XYL activity eluted at approximately pH 6.7. Three fractions with the highest specific activities (fractions 35-37) were pooled and subjected to a final purification step on a Bio-Gel P-100 column.

Ion-exchange chromatography steps were not particularly effective in the overall purification of XYL; however, minor contaminating proteins were clearly removed by these procedures as shown by SDS-PAGE (*Figure 2.8*). Chromatofocusing removed a highly abundant contaminating protein of approximately 55 kDa (*Figure 2.8*), resulting in a purification factor of greater than 90-fold for this step (*Table 2.1*). The final purified XYL preparation contained a small amount of a high molecular mass contaminant. This could not be removed by further chromatography. XYL, purified 960-fold from the 5-day-old seedling extract and with an overall yield of 6% (*Table 2.1*), has a molecular mass of approximately 67 kDa as determined by SDS-PAGE (*Figure 2.8*) and an isoelectric point of 6.7.

2.3.2 *Partial Purification of Barley α-L-Arabinofuranosidase Isoenzyme ARA-II*

ARA-II was partially purified from 7-day-old barley seedlings using the procedures outlined in *Figure 2.9*. The crude seedling homogenate yielded 24 g soluble protein (*Table 2.2*), 8 g of which was precipitated at 40-60%-saturated $(NH_4)_2SO_4$. α-L-Arabinofuranosidase activity was contained mostly in the 40-60% and 60-80%-saturated $(NH_4)_2SO_4$ fractions. The 40-60% fraction was resuspended in 2 L 20 mM Tris-HCl DEAE-cellulose column buffer at pH 8.0, and applied to DEAE-cellulose. The DEAE-cellulose elution profile (*Figure 2.10*) resembled that for the purification of ARA-I and XYL from the 5-day seedling extract (*Figure 2.2*), except that ARA-II was more abundant. Furthermore, the specific activities of

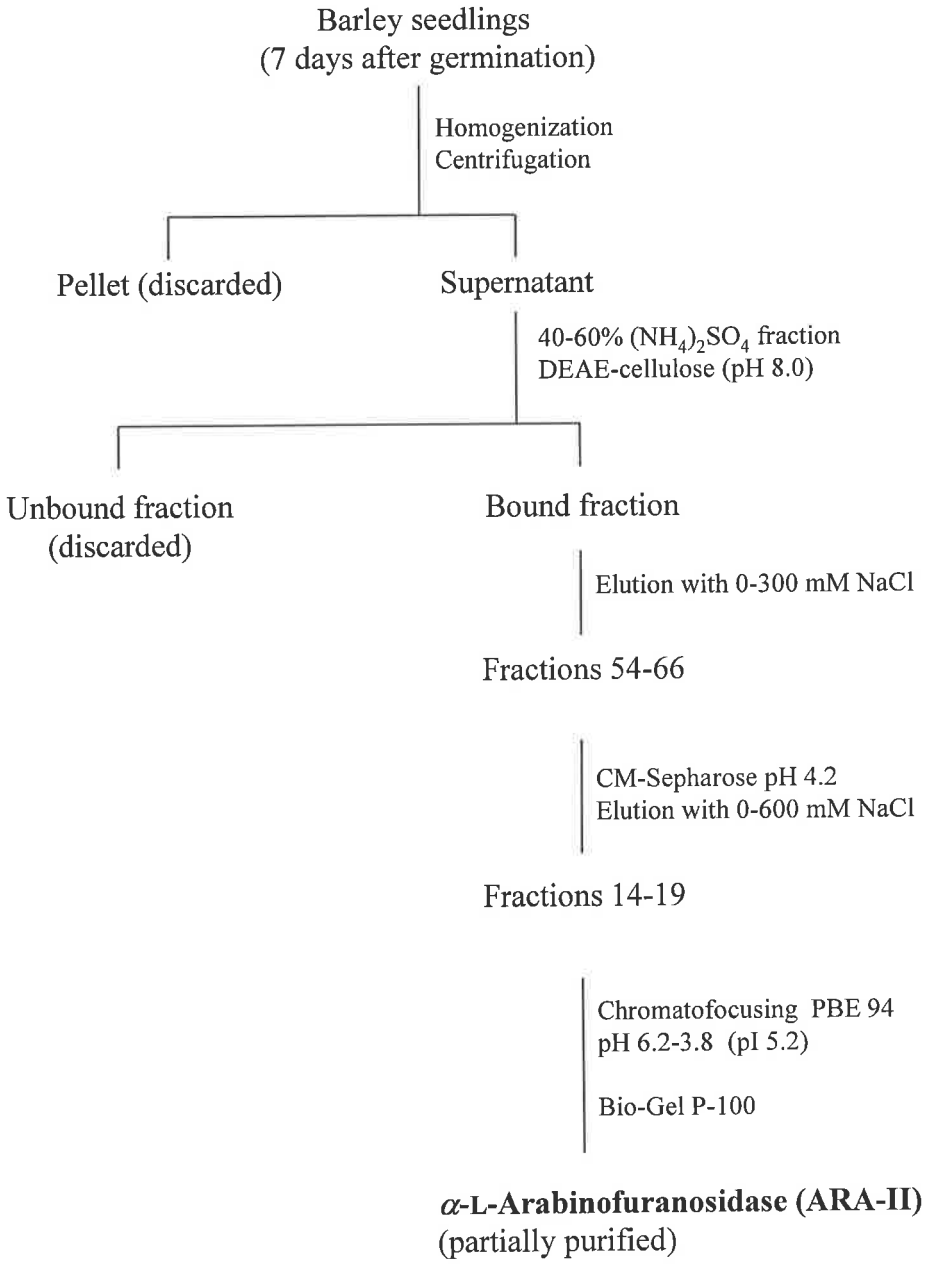

Figure 2.9 **Summary of procedures used in the partial purification of α-L-arabinofuranosidase isoform ARA-II.** The enzyme was partially purified from an extract of 7-day-old barley seedlings. ARA-II was in greater abundance at 7 days after germination than in 5-day-old seedlings used for the purification of XYL and ARA-I.

Table 2.2 **Enzyme yields and purification factors at each stage of the purification of α-L-arabinofuranosidase (ARA-II).** Recovered activity units were assayed with 4NPA. Recoveries are expressed as percentage of initial activity units and purification factors were calculated on the basis of specific activities.

	Yield				
	Protein *(mg)*	Activity *(U)*	Specific activity *($mU.mg^{-1}$)*	Recovery *(%)*	Purification factor *(fold)*
α-L-Arabinofuranosidase (ARA-II)					
Crude homogenate	24000	835	34	100	1.0
40-60% $(NH_4)_2SO_4$	8000	200	25	24	0.7
DEAE-cellulose	322	95.6	300	11	9.0
CM-Sepharose	1.3	68.4	52200	8.2	1500
PBE 94	0.5	28.4	61100	3.4	1800
Bio-Gel P-100	0.004	0.70	192000	0.1	5600

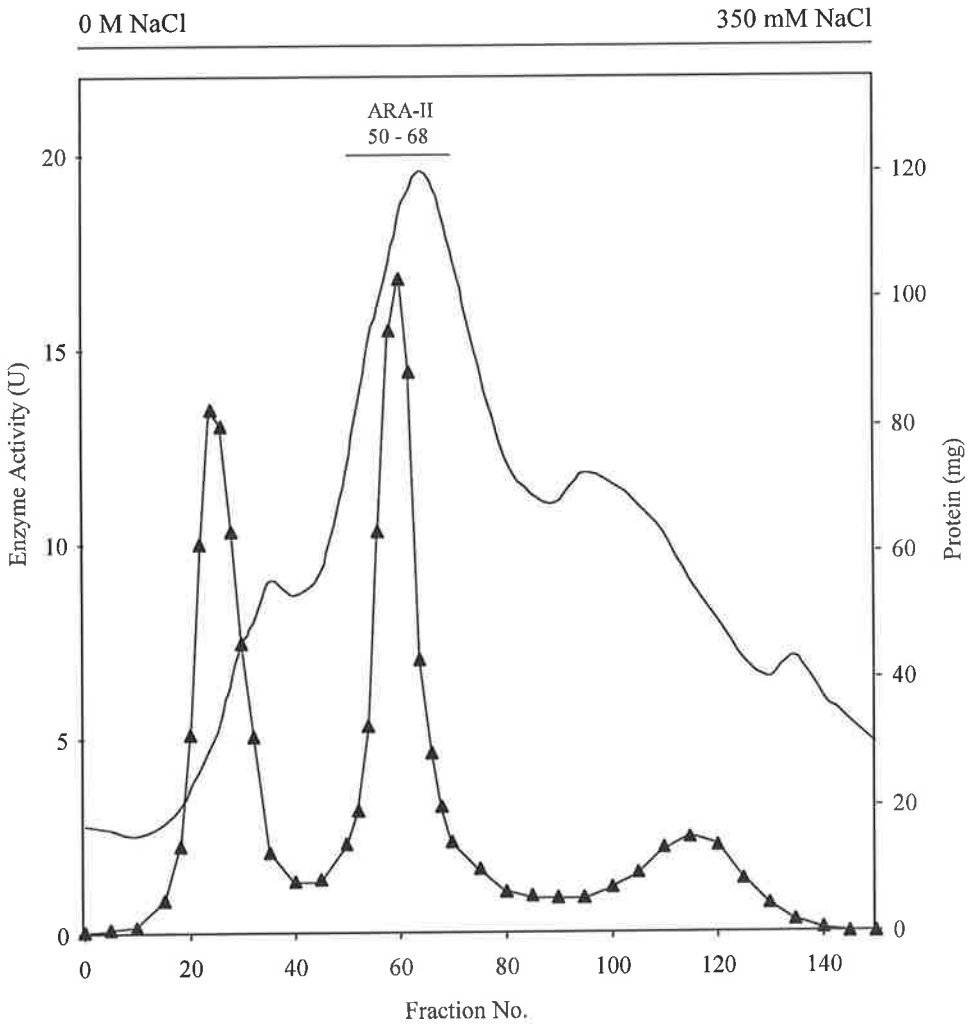

Figure 2.10 **Ion-exchange chromatography of 40-60% $(NH_4)_2SO_4$ fraction on DEAE-cellulose (7-day seedling extract).** Material bound to DEAE-cellulose at pH 8.0 was eluted with a 3 L linear gradient of 0-350 mM NaCl. Fractions (20 ml) were assayed against 4NPA (▲) and protein (—) was measured by absorbance at 280 nm.

fractions in the activity peak corresponding to ARA-II were higher and the amount of enzyme that was available for further purification was approximately 5 times higher than before. A third peak of α-L-arabinofuranosidase activity was observed on this column, eluting at around 270 mM NaCl; this was later purified as an arabinoxylan arabinofuranohydrolase (see *Chapter 3*).

Fractions 50-68 from DEAE-cellulose, containing ARA-II activity, were pooled and equilibrated in 50 mM sodium acetate, pH 4.2, and loaded onto a column of CM-Sepharose. Bound proteins were eluted from the column in a 1 L linear gradient of 0-600 mM NaCl and ARA-II was eluted as a single peak of activity at approximately 190 mM NaCl (*Figure 2.11*). The CM-Sepharose step resulted in a 170-fold purification factor (*Table 2.2*). Fractions 14-19 were pooled and subjected to chromatofocusing on PBE 94 (*Figure 2.12*). A pH gradient from 6.2 to 3.8 was applied to the column and elution of ARA-II at pH 5.2 indicated that the protein has an isoelectric point of approximately 5.2, compared with pI 5.5 for ARA-I. The PBE 94 elution profile shows that a number of contaminating species were successfully eliminated using this procedure. However, only a small increase in specific activity was achieved using this method. Nevertheless, SDS-PAGE of column fractions across the activity peak (*Figure 2.13*) suggested that ARA-II was possibly a protein of about 34 kDa, because the intensity of this band corresponded with the level of ARA-II activity in each successive fraction. As a final step in the purification of ARA-II, fractions 22 and 23 from the chromatofocusing step were applied to a Bio-Gel P-100 column. Further material was removed by this step and the degree of purity of the ARA-II preparation was improved 3-fold. However, very low protein yield meant that further purification of active enzyme was not possible. The ARA-II preparation was separated on SDS-PAGE (*Figure 2.14*) for gel purification of the 34 kDa band and subsequently, identification of the putative ARA-II isoenzyme by amino acid sequencing of tryptic peptides. The *in gel* tryptic digest of the putative ARA-II and subsequent fractionation of peptides was performed by Mr. Jelle Lahnstein (Department of Plant Science, University of Adelaide). If we assume that ARA-II is, in fact, the 34 kDa protein shown on SDS-PAGE (*Figure 2.14*), then we can estimate that the final preparation was of approximately 30% purity. The final ARA-II preparation had a specific activity of 192 $U.mg^{-1}$ and was purified 5600-fold (*Table 2.2*). *Figure 2.15* shows SDS-PAGE of each stage in the partial purification of the putative ARA-II.

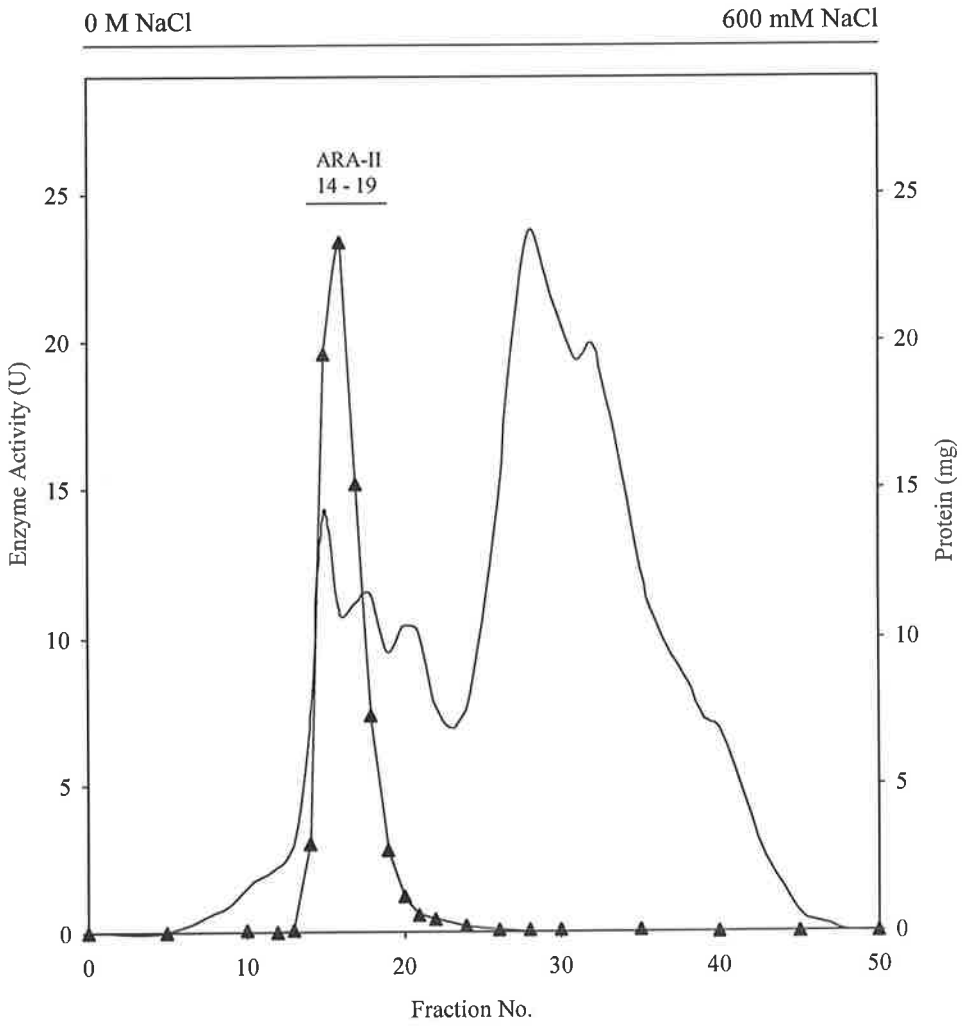

Figure 2.11 **Ion-exchange chromatography of DEAE-cellulose fractions containing α-L-arabinofuranosidase isoform ARA-II on CM-Sepharose.** Material bound to CM-Sepharose at pH 4.2 was eluted with a 1 L gradient of 0-600 mM NaCl. Fractions (20 ml) were assayed against 4NPA (▲) and protein (—) was measured by absorbance at 280 nm.

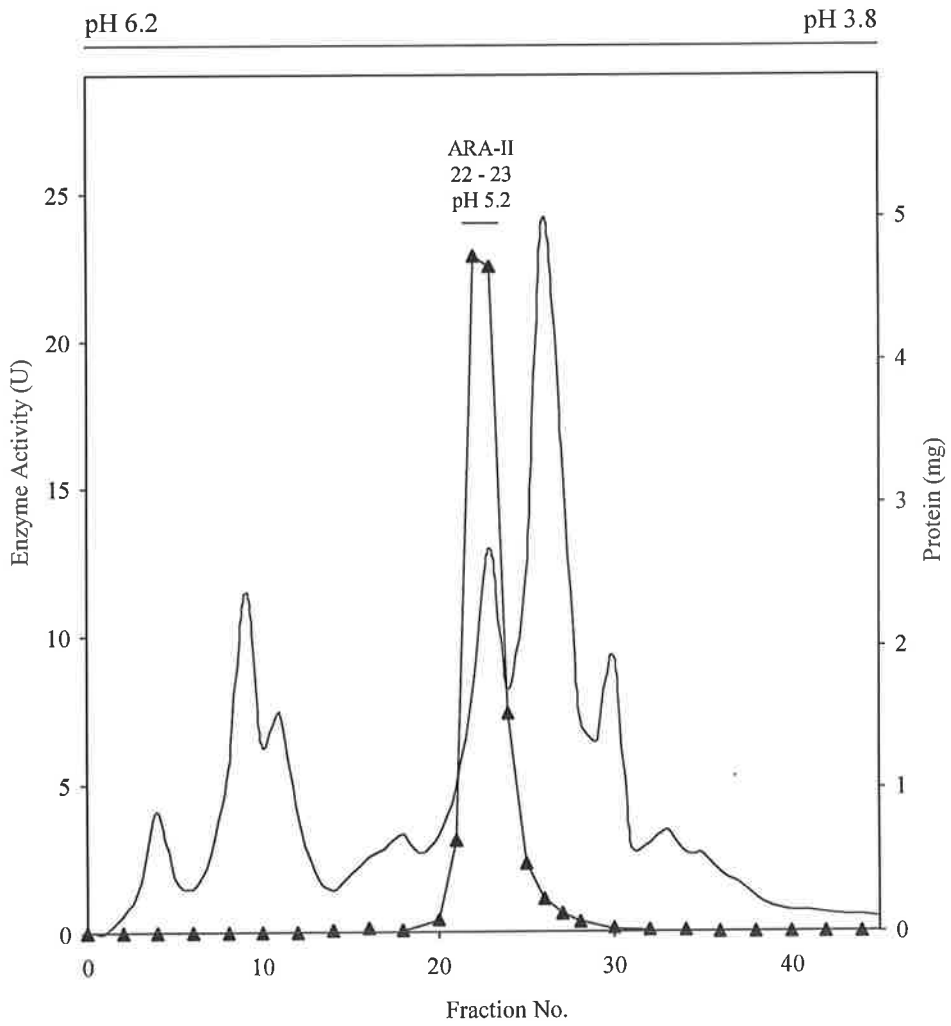

Figure 2.12 **Chromatofocusing of ARA-II on PBE 94.** Pooled fractions (14-19) from CM-Sepharose were concentrated and equilibrated in Polybuffer 74 adjusted to pH 3.8, and applied to PBE 94 equilibrated at pH 6.2 with 25 mM Histidine-HCl buffer. Proteins were eluted from the column with Polybuffer 74, pH 3.8. Fractions (5 ml) were assayed against 4NPA (▲) and protein (—) was measured by absorbance at 280 nm.

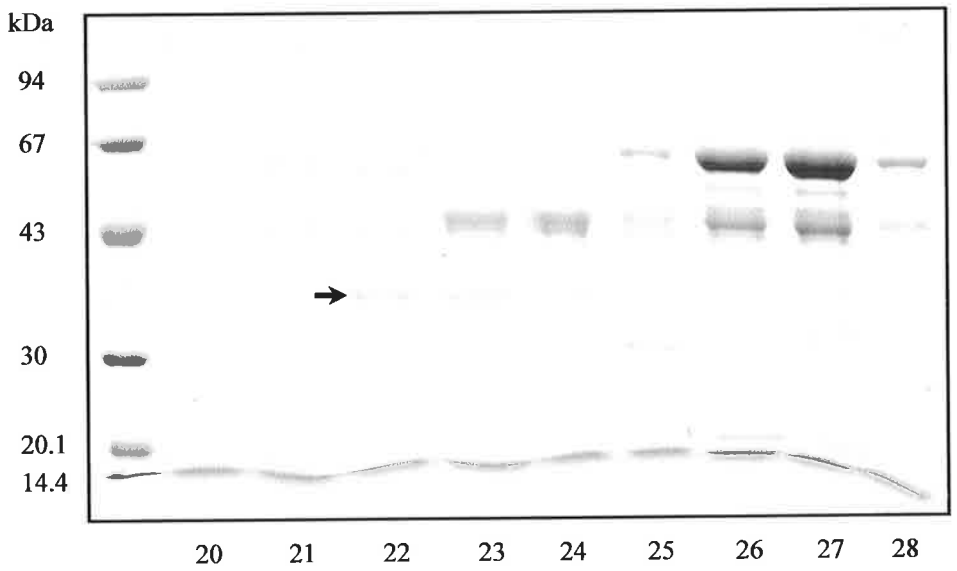

Figure 2.13 **SDS-PAGE of Fractions 20-28 after chromatofocusing of ARA-II on PBE 94.** Lanes contain 20 µl of each 5 ml column fraction and fractions 22 and 23 contain 100 mU α-L-arabinofuranosidase activity. The staining intensity of a 34 kDa band (*arrow*) in fractions 22 and 23 corresponds with the level of α-L-arabinofuranosidase activity in these fractions.

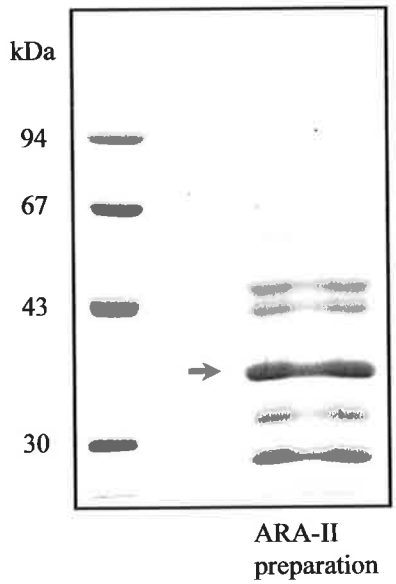

Figure 2.14 **SDS-PAGE of partially purified ARA-II.** The 34 kDa band was excised from the gel for digestion with trypsin and subsequent RP-HPLC purification and NH_2-terminal sequencing of peptides.

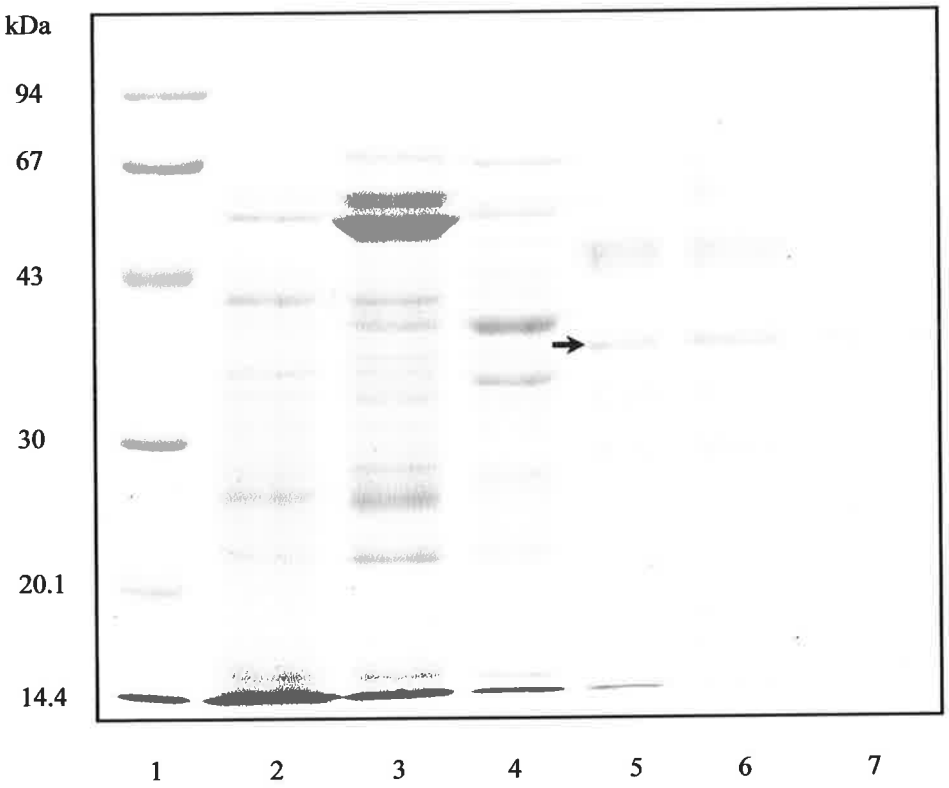

Figure 2.15 **SDS-PAGE of samples taken at each stage of the partial purification of α-L-arabinofuranosidase isoform ARA-II.** *Lane 1*, molecular mass markers; *lane 2*, crude extract; *lane 3*, 40-60% $(NH_4)_2SO_4$ fraction; *lane 4*, DEAE-cellulose fractions 54-66; *lane 5*, CM-Sepharose pooled fractions 14-19; *lane 6*, PBE 94 fractions; *lane 7*, partially purified ARA-II preparation after Bio-Gel P-100. The 34 kDa ARA-II band is indicated (*arrow*).

2.3.3 Amino Acid Sequence Analysis of ARA-I, XYL and ARA-II

Amino acid sequences of ARA-I and XYL, and also the polyacrylamide-gel-purified ARA-II preparation, were determined by Edman degradation sequencing of NH_2-terminal residues of the purified proteins, and of peptides generated by proteolysis with trypsin and purified by RP-HPLC. NH_2-Terminal sequencing of the purified ARA-I protein was unsuccessful, presumably as a result of blockage of the NH_2-terminal residue to Edman degradation. ARA-I tryptic peptides were resolved by RP-HPLC and eight of these, indicated in *Figure 2.16*, were subjected to NH_2-terminal sequence analysis. Their sequences were as follows:

1. WGRGQETPGEDPLLASK
2. LGIPAYEWWSEALHGVSYVGPGTR
3. TPEEAAAITIK
4*a*. FSPLVPGATSFPQPILTAASFFAYLFR
4*b*. LGIPAYEWWSEALHGVSYVGPGTR
5*a*. AGLDLNEGNFLAQHTVAYVQALEDILFG
5*b*. LSDKAYRILS$^{V}/_{E}$GYPGE-G-APG$^{A}/_{T}{}^{L}/_{R}$FE
6*a*. VSQQDLDDTFQPPF
6*b*. HYTAYDV-NWKGVE
7. LGFFDGDPR
8. GEVVSTEAR

Fractions 1, 2 and 3, produced clear sequences and yields of PTH derivatives were in accord with the amount of protein digested and anticipated recovery of RP-HPLC-purified peptides. Two sequences were resolved in RP-HPLC fraction 4, one of which (4*b*) was identical to that determined for neighboring fraction 2. Fractions 5 and 6 produced multiple sequences that were identified by relative abundance of PTH derivatives in each Edman sequencing cycle and were therefore less reliable. Nevertheless, peptide sequences 5*a*, 5*b*, 6*a* and 6*b*, were recorded for later use in matching and aligning of cDNA sequences. Peptide sequences from peaks 7 and 8 were short, consistent with their early elution from RP-HPLC, and of reasonable quality.

The NH_2-terminal sequence of the purified XYL preparation was obtained, the first 38 residues of which were ADPPFS-GAASDAAY-DRALPAERRAADLVS-LSLEEK. Eight of the RP-HPLC purified peptides (*Figure 2.17*), derived by proteolysis of XYL with trypsin were subjected to NH_2-terminal sequencing. Useful amino acid sequences were obtained from six of these purified fractions, with multiple sequences resolved in fractions 1 and 8.

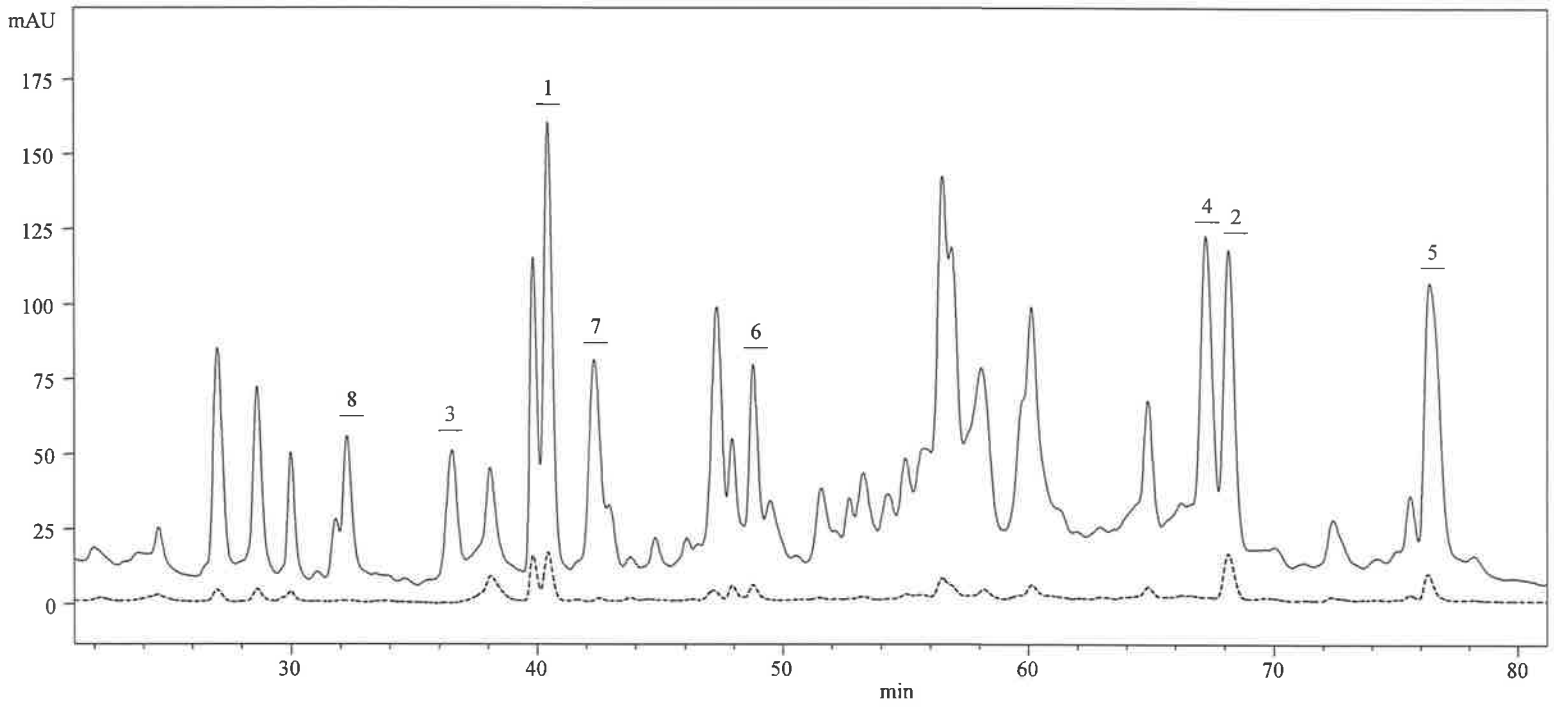

Figure 2.16 **RP-HPLC of peptide fragments generated by digestion of barley α-L-arabinofuranosidase (ARA-I) with trypsin.** A_{280} (—) and A_{214} (---) are shown. Fractions were collected at 1 min intervals and NH_2-terminal amino acid sequences of purified peptides, 1 to 8 as indicated, were determined.

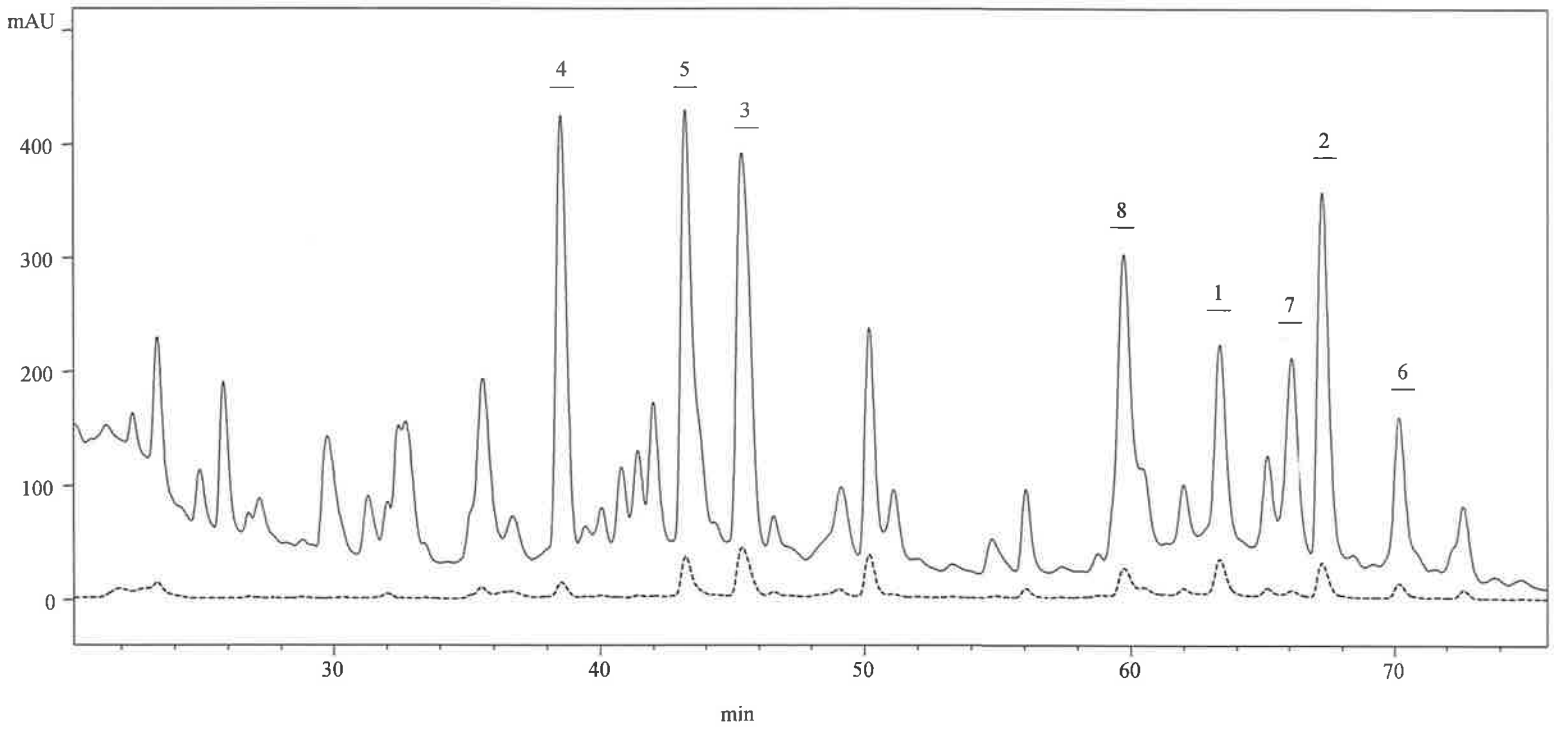

Figure 2.17 **RP-HPLC of peptide fragments generated by digestion of barley β-D-xylosidase (XYL) with trypsin.** A_{280} (—) and A_{214} (---) are shown. Fractions were collected at 1 min intervals and NH_2-terminal amino acid sequences of purified peptides, 1 to 6 as indicated, were determined.

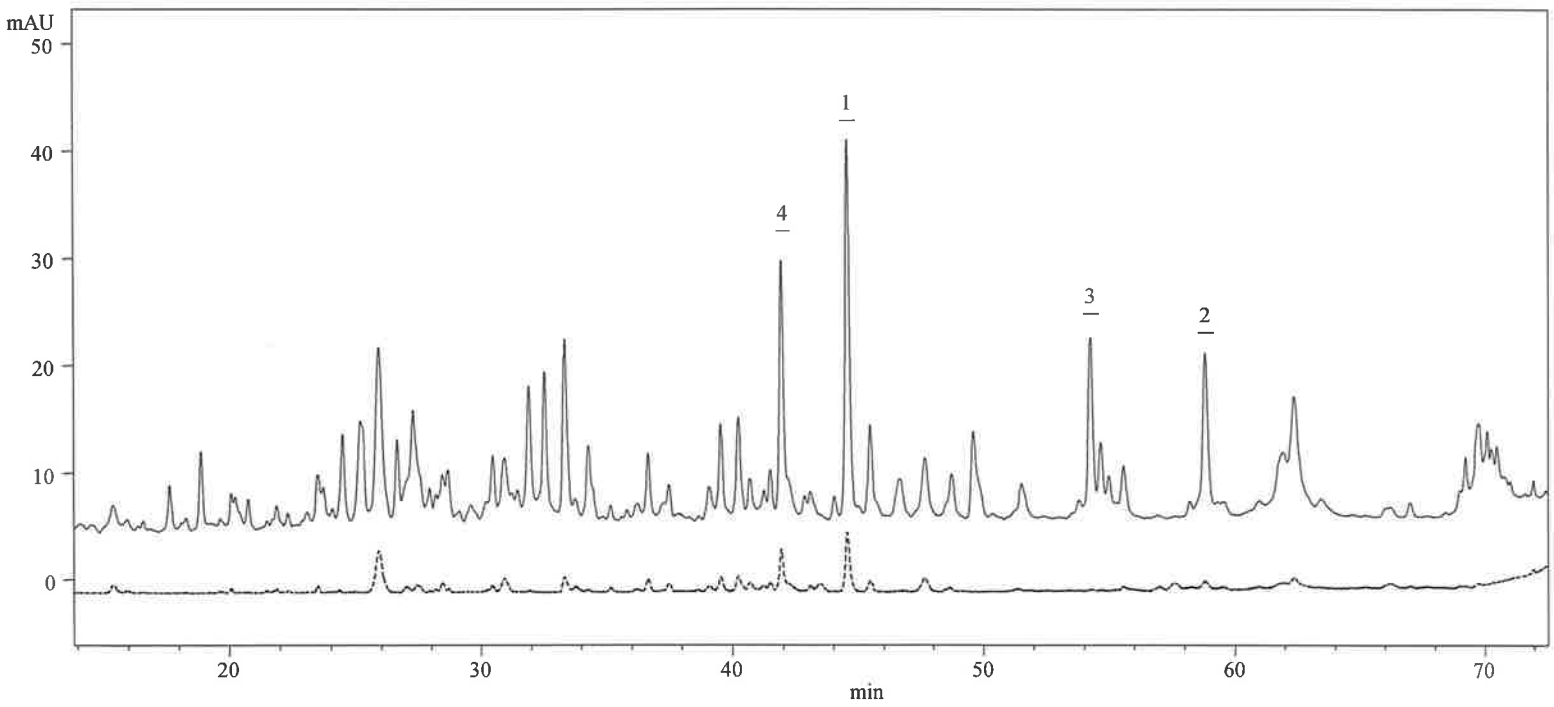

Figure 2.18 **RP-HPLC of peptide fragments generated by digestion of barley α-L-arabinofuranosidase (ARA-II) with trypsin.** Approximately 4 μg ARA-II was separated on SDS-PAGE and peptides were eluted from the gel after proteolysis with trypsin. A_{280} (—) and A_{214} (---) are shown. Fractions were collected and NH_2-terminal amino acid sequences of purified peptides, 1 to 4 as indicated, were determined. This work was performed by Mr. Jelle Lahnstein.

Tryptic peptide sequences for XYL were:

1*a*. LGVPAYKWWSEALHGVA
1*b*. FDWSFNGYIT
2. AATSFPQVILTAASFNPHLWYR
3. LGEDNINVV *(trypsin)*
4. no sequence
5. no sequence
6. IGAIVWAGYPGQAG-IAIAQVLFGDHNPGGR
7. FVQG-NAAV--VSNIGEAVHAAGSADYVVLFMGLDQNQER
8*a*. GVYNNGQAEG
8*b*. EEVDRLELGL

Multiple PTH derivatives in sequencing cycles were common for the RP-HPLC fractions and in peaks 1 and 8, two amino acid sequences for each were distinguished on the basis of abundance of PTH derivatives. From peaks 2 and 3, only single peptide sequences from the multiple PTH derivatives could be interpreted. The peak 3 sequence corresponded with that for trypsin. From peaks 4 and 5, no sequences could be derived from the data. Only peaks 6 and 7 produced single, unequivocal amino acid sequences. Peptide sequences of ARA-I and XYL were similar to β-D-xylosidase sequences from *Aspergillus niger* and *Trichoderma reesei* (GenBank/EMBL accessions Z84377 and Z69257, respectively) as shown by non-redundant BLAST searches of sequence databases. These fungal β-xylosidase sequences are classified in glycoside hydrolase family 3 (Coutinho and Henrissat, 1999*a*).

Four amino acid sequences were obtained from the putative ARA-II tryptic peptides, which were eluted from the trypsin-treated, SDS-PAGE-separated 34 kDa protein. The tryptic peptides were resolved on RP-HPLC (*Figure 2.18*) and the following sequences were obtained:

1. HYAAYDVDAWLTADR
2. VNSLSLVGLLQHI-ATDVMLGDYR
3. LGFFDGIPELESLGANDV-TEEH
4. WLGYNGVEATA

The amino acid sequences of peptides 1 and 3 were remarkably similar to the HYTAYDV and LGFFDGDP sequences of ARA-I peptides 6*b* and 7, respectively. Based on sequence homology, the amino acid sequences suggest that ARA-I, XYL and ARA-II are classified in family 3 of glycoside hydrolase families described by Henrissat (1998).

2.4 DISCUSSION

2.4.1 *Purification of Barley α-L-Arabinofuranosidases and a β-D-Xylosidase*

Two enzymes with hydrolytic activity against 4NPA and 4NPX have been purified from 5-day-old barley seedling extracts and have been designated ARA-I and XYL, respectively. The procedures used for the purification of ARA-I and XYL are presented in *Figure 2.1*. A third enzyme, designated ARA-II, was identified in the extract from 5-day-old barley seedlings and was partially purified from a 7-day-old barley seedling extract in which it was present in greater abundance.

Chromatography on DEAE-cellulose (*Figure 2.2*) resolved the two α-L-arabinofuranosidase isoenzymes, probably because of differences in their isoelectric points, revealed later by chromatofocusing. Other factors that determine binding interactions with DEAE-cellulose include the arrangement of surface charges, the degree and nature of surface glycosylation, and the cellulose-binding capacity of particular proteins (Scopes, 1987). Near-baseline separation of ARA-I and ARA-II (*Figure 2.2*) and careful pooling of column fractions permitted complete separation of the two α-L-arabinofuranosidase isoenzymes. It is noteworthy that a significant level of β-D-xylosidase activity was associated with both α-L-arabinofuranosidase peaks. Further purification revealed that ARA-I had the capacity to hydrolyse both 4NPA and 4NPX (specific activity on 4NPX was typically 15% of that on 4NPA) and throughout the purification of ARA-I, activity peaks for both α-L-arabinofuranosidase and β-D-xylosidase were associated. β-D-Xylosidase activity was also observed with ARA-II. In this case specific activity on 4NPX was approximately 3% of that on 4NPA.

The overall yield and purification factor for ARA-I were 2% and 1080-fold, respectively (*Table 2.1*). The molecular mass of ARA-I, and the isoelectric point determined by chromatofocusing, were 65 kDa and 5.5, respectively. It should be noted that the purification factor for ARA-I was probably underestimated, because at least one third of the initial α-L-arabinofuranosidase activity in the crude seedling extract was contributed by the ARA-II isoform.

The β-D-xylosidase, XYL, did not bind to the DEAE-cellulose column; the β-D-xylosidase activity profile shown in *Figure 2.2* was due primarily to the dual specificity of ARA-I, as discussed above. Of the 57.6 U loaded to the column, 28.2 U was recovered in the unbound fraction. It would be expected that a protein such as XYL, with an apparent isoelectric point of 6.7, would bind to DEAE-cellulose at pH 8.2 (Scopes, 1987). With the large amount of protein and other soluble materials from the barley seedling extract loaded onto the column, the chemical environment within the column may not have been uniform and binding properties of the anion exchange matrix may have been affected by glycosylation or other factors influencing surface charge. Following further chromatography steps, XYL was purified by a factor of 960-fold with a 6% recovery of initial activity. The purified XYL preparation contained a major band of approximately 67 kDa and a minor high molecular mass band of about 120 kDa. It is unclear whether the minor band represented a contaminating protein or an alternative isoform of the XYL isoenzyme, because amino acid sequencing produced only a single, unambiguous sequence. Further analysis of the final XYL preparation using reversed-phase HPLC (results not shown) revealed a major UV absorbance peak, and a second minor peak that may have corresponded to the high M_r band. Further RP-HPLC purification and amino acid sequencing of both protein peaks produced identical NH_2-terminal amino acid sequences, thereby suggesting that the high M_r protein might be a different form of the protein.

Figure 2.10 shows a DEAE-cellulose elution profile for the 40-60%-saturated $(NH_4)_2SO_4$ fraction from a 7-day old barley seedling homogenate. The α-L-arabinofuranosidase profile, was similar to the DEAE-cellulose-fractionated 5-day seedling homogenate (*Figure 2.2*), insofar as two major peaks of enzyme activity were present. However, a third, late-eluting peak of α-L-arabinofuranosidase activity was evident and represented an arabinoxylan arabinofuranohydrolase (AXAH) that was later purified (*Chapter 3*). The 7-day-old seedling homogenate yielded 835 U of α-L-arabinofuranosidase activity, compared with 44.7 U from the 5-day seedling extract. Large increases in α-L-arabinofuranosidase activities in barley seedlings after 7 days of growth were not observed during preliminary trials to determine the most suitable growth stage from which to purify α-L-arabinofuranosidases and β-D-xylosidases. Elevated levels of overall α-L-arabinofuranosidase activity, and of the ARA-II isoenzyme, revealed by fractionation of ARA-I and ARA-II on DEAE-cellulose (*Figure 2.10*), indicated that the 7-day barley seedling extract might prove to be a more suitable

source for the purification of ARA-II. Pooling of active DEAE-cellulose fractions yielded 95.6 U of ARA-II. Despite further purification steps, the final ARA-II preparation remained contaminated and its overall yield was very low. The specific activity of the final preparation, purified several thousand-fold from 7-day old barley seedlings, was 192 $U.mg^{-1}$. This may be compared with a value of 6.6 $U.mg^{-1}$ for ARA-I. The putative molecular mass of 34 kDa for ARA-II is also of interest considering the similarities of its amino acid sequences with those of ARA-I and XYL, which have molecular masses of 65 kDa and 67 kDa, respectively.

2.4.2 Determination of Amino Acid Sequences from Purified Enzymes

To compare amino acid sequences with those in the databases, and to obtain sequence information to facilitate the isolation of cDNAs encoding ARA-I and XYL, Edman degradation amino acid sequencing of purified proteins and of tryptic peptides were performed. Tryptic digestion of ARA-I produced at least 40 peptides, which could be resolved by RP-HPLC (*Figure 2.16*); amino acid sequences of 10 ARA-I peptides were determined. The NH_2-terminal sequence of 38 amino acid residues was obtained for the XYL enzyme, along with sequences of eight tryptic peptides (*Figure 2.17*). Amino acid sequences of peptides 1 and 2 from XYL exhibited similarities to ARA-I tryptic peptides 2 and 4*a*, respectively, and BLAST searches revealed similarities with sequences of family 3 β-D-xylosidases from *Aspergillus niger* (Z84377) and *Trichoderma reesei* (Z69257). These amino acid sequence similarities suggest that ARA-I and XYL are classified in family 3 of glycoside hydrolase families (Coutinho and Henrissat, 1999*a*).

After the putative ARA-II protein was subjected to preparative SDS-PAGE (*Figure 2.14*) and *in situ* tryptic digestion (*Figure 2.18*) four tryptic peptides were obtained. The NH_2-terminal amino acid sequences of three of the tryptic peptides were homologous to *A. niger* and *T. reesei* β-D-xylosidases. Furthermore, the HYAAY and LGFFD amino acid sequences of tryptic peptides 1 and 3 resembled peptides 6*b* and 7 from ARA-I, respectively.

With amino acid sequences similar to the 65 kDa ARA-I and 67 kDa XYL, it is surprising that the putative ARA-II protein appears on SDS-PAGE as a 34 kDa protein band. Family 3 enzymes generally consist of two domains and have a molecular mass of 60-80 kDa. There are two possibilities to explain these observations: ARA-II might be encoded by a truncated

version of a family 3 glycoside hydrolase gene, or alternatively, the native ARA-II polypeptide of approximately 67 kDa might be post-translationally processed to produce a heterodimeric protein with subunits of 34 kDa. The latter suggestion is more likely because analysis of over 100 family 3 sequences revealed none that were this small (Harvey *et al.*, 2000). The further possibility that ARA-II is a different protein altogether, not visible on SDS-PAGE, cannot be dismissed with the current data. If a gene or cDNA could be obtained, these possibilities could be further investigated, or clarified.

2.4.3 Summary

Clearly, ARA-I, XYL and the putative ARA-II are closely related enzymes. The partial amino acid sequences obtained for the three enzymes will provide the basis for the isolation of cDNA sequences and for the determination of complete primary protein structures (*Chapter 5*). The partial amino acid sequences from these barley enzymes, which are active against aryl α-L-arabinofuranoside or aryl β-D-xyloside, suggest that they are family 3 glycoside hydrolases similar to the β-D-xylosidases from *A. niger* and *T. reesei*, and other enzymes from *Aspergillus nidulans* (Y13568) (Pérez-González *et al.*, 1998), *Aspergillus oryzae* (AB013851) (Kitamoto *et al.*, 1999), *Thermoanaerobacter brockii* (Z56279) (Breves *et al.*, 1997) and *Thermoanaerobacter ethanolicus* (AF135015) (Mai *et al.*, 2000) (see *Table 1.1*, *Chapter 1*). The microbial β-D-xylosidases are active primarily on 4NPX, xylan and (1→4)-β-linked oligoxylosides, and have some capacity to hydrolyse 4NPA. The family 3 β-D-xylosidases are not generally active against α-L-arabinofuranosyl residues of cereal arabinoxylans. Preliminary analyses of the substrate specificities of ARA-I and XYL suggested that high molecular mass arabinoxylans were not hydrolysed at a significant rate and that oligosaccharides resulting from the action of (1→4)-β-D-xylan endohydrolases are the most likely substrates for these enzymes, *in vivo*. An investigation of the biochemical properties of ARA-I and XYL is presented in *Chapter 4*.

CHAPTER 3.

Purification of Arabinoxylan Arabinofuranohydrolase from Barley

3.1 INTRODUCTION

The barley α-L-arabinofuranosidase isoenzymes ARA-I and ARA-II, and the β-D-xylosidase XYL, described in *Chapter 2*, are family 3 glycoside hydrolases with no significant activity against arabinoxylans. Preliminary examination of substrate specificities of these purified enzymes suggested that their most likely substrates, *in vivo*, are oligoarabinoxylosides that are released from arabinoxylans by (1→4)-β-D-xylan endohydrolases. Their substrate specificities are described in more detail in *Chapter 4*. However, the removal of arabinofuranosyl substituents of wall arabinoxylans in vegetative tissues during the deposition and modification of cell wall components (Gibeaut and Carpita, 1991) suggests a role in growing plant tissues for α-L-arabinofuranosidases with specificity for the arabinofuranosyl substituents of polymeric arabinoxylan. Moreover, removal of arabinofuranosyl substituents from arabinoxylans of the aleurone or starchy endosperm would complement the activity of (1→4)-β-D-xylan endohydrolases during the degradation of cell walls in post-germination endosperm mobilization (Taiz and Honigman, 1976; Dashek and Chrispeels, 1977). The germinated grain and developing seedling tissues are therefore potential sites for the detection of arabinoxylan-specific α-L-arabinofuranosidases, which are referred to here as arabinoxylan arabinofuranohydrolases, in barley. The objectives of the work described in this Chapter were to detect and subsequently purify arabinoxylan arabinofuranohydrolases from young barley seedlings. When this component of the overall project was initiated, the work of Ferré *et al.* (2000) had not been published.

Arabinoxylan arabinofuranohydrolases (AXAHs), which are active only on high M_r arabinoxylans, have been purified from *Aspergillus awamori* (Kormelink *et al.*, 1991) and *Aspergillus tubingensis* (Gielkens *et al.*, 1997). Cloned cDNA and gene sequences place these enzymes in family 62, along with arabinoxylan arabinofuranohydrolases from *Pseudomonas fluorescens* (Kellett *et al.*, 1990) and *Streptomyces lividans* (Vincent *et al.*, 1997). Microbial α-L-arabinofuranosidases from glycoside hydrolase families 51 and 54 have broader substrate specificities, with activity against 4NPA and various arabinose-containing polymers including arabinoxylans. An α-L-arabinofuranosidase with the ability to liberate L-arabinose from arabinoxylan has been detected in extracts from wheat (*Triticum aestivum*) although no sequence information was available (Beldman *et al.*, 1996), and an

homologous arabinoxylan arabinofuranohydrolase was likely to be produced in barley tissues.

The synthetic substrate, 4NPA, is suitable for the measurement and detection of α-L-arabinofuranosidase activities, as demonstrated with the successful purification of ARA-I and the partial purification ARA-II. However, for the purification and characterization of α-L-arabinofuranosidases that have little, or no activity on synthetic substrates, biochemical assays using naturally occurring substrates such as arabinoxylan must be used. The purification of an arabinoxylan arabinofuranohydrolase is described here. The enzyme was purified from a barley seedling extract using both the 4NPA assay and a simple, semi-quantitative assay based on the release of L-arabinose from wheat flour arabinoxylan. Amino acid sequences of the purified protein were also determined.

3.2 MATERIALS AND METHODS

3.2.1 *Barley Germination and Extract Preparation*

Barley (3 kg dry weight) was surface sterilised and germinated as described in *Chapter 2*, and seedlings, after 7 days of growth in the dark at 22°C, were homogenised in 1.5 volumes of cold extraction buffer (0.1 M sodium acetate buffer, pH 5, 10 mM EDTA, 10 mM NaN_3, 3 mM 2-mercaptoethanol, 3 mM PMSF) as described in *Chapter 2*. The crude seedling homogenate was allowed to stand for 1 h. The soluble fraction was separated from particulate material by filtration through nylon mesh, and centrifugation at 6,000 g for 20 min at 4°C. The soluble seedling extract fraction was filtered through Miracloth and subjected to $(NH_4)_2SO_4$ fractionation as described in *Chapter 2*. Material precipitated at 20-40%-saturated $(NH_4)_2SO_4$ was resuspended in approximately 1 L 20 mM Tris-HCl buffer, pH 8.0, containing 4 mM NaN_3 and 3 mM 2-mercaptoethanol.

3.2.2 *Ion-Exchange Chromatography*

Chromatography was performed at 4°C using the equipment described in *Chapter 2*. The resuspended 20-40% $(NH_4)_2SO_4$ fraction was dialysed against 20 mM Tris-HCl buffer, pH 8.0, containing 4 mM NaN_3 and 3 mM 2-mercaptoethanol, and applied to a 30 x 5 cm column of DEAE-cellulose (Whatman, Maidstone, Kent, U.K.) pre-equilibrated in the same buffer at a linear flow rate of 3 $cm.h^{-1}$. Unbound proteins were eluted with the Tris-HCl column buffer and bound proteins were eluted by application of a 3 L linear gradient of 0-350 mM NaCl in the same Tris-HCl buffer, as previously described. Collected fractions (20 ml) were assayed for α-L-arabinofuranosidase activity on 4NPA and for the capacity to liberate L-arabinose from arabinoxylan, and pooled where fractions contained both activities.

Pooled fractions were dialysed against 50 mM sodium formate buffer, pH 3.8, containing 4 mM NaN_3, and applied to an 18 x 2.5 cm column of SP-Sepharose (Amersham-Pharmacia Biotech, Uppsala, Sweden), pre-equilibrated in 50 mM sodium formate buffer, pH 3.8, containing 4 mM NaN_3, at a linear flow rate of 40 $cm.h^{-1}$. Following the elution of unbound proteins, bound proteins were eluted with a 1 L linear gradient of 0-500 mM NaCl in the sodium formate column buffer. Fractions (20 ml) were collected and assayed as above, and those containing AXAH activity were pooled.

Pooled fractions from SP-Sepharose containing AXAH activity were dialysed against, 50 mM sodium formate buffer containing 4 mM NaN_3, and applied to a 8 x 1.5 cm column of Q-Sepharose (Amersham-Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated in 20 mM histidine-HCl buffer, pH 5.5. Bound proteins were eluted at a linear flow rate of 100 $cm.h^{-1}$ with a 300 ml linear gradient of 0-500 mM NaCl in 50 mM sodium formate buffer. Fractions (10 ml) were collected and assayed for AXAH activity. The final, purified AXAH preparation was concentrated into 10 mM sodium acetate buffer, pH 5.0, containing 4 mM NaN_3 by ultrafiltration using a 10 ml capacity Amicon concentrator fitted with a YM10 membrane (Amicon, Beverly, MA, U.S.A.).

3.2.3 Enzyme Assays

α-L-Arabinofuranosidase activity was measured spectrophotometrically using 4NPA as described in *Chapter 2*. AXAH activities of $(NH_4)_2SO_4$ precipitated fractions and column fractions were determined semi-quantitatively by analysis of released L-arabinose after incubation for up to 30 min with 0.5% (*w/v*) wheat flour arabinoxylan (Megazyme, Bray, Ireland) at 37°C in 50 mM sodium acetate buffer, pH 5.0, containing 4 mM NaN_3. Hydrolysis products were separated by thin-layer chromatography on silica-gel thin-layer plates (Merck, Darmstadt, Germany) developed in ethyl acetate: acetic acid: water (3:2:1 by volume) and sugars were detected using the orcinol reagent, containing 0.5% (*w/v*) orcinol (Sigma Chemical Co.) in 5% (*v/v*) H_2SO_4 in ethanol (Farkas and Maclachlan, 1988). Standard L-arabinose and D-xylose were obtained from Sigma.

3.2.4 Protein Determination and Polyacrylamide Gel Electrophoresis

Protein concentrations of column fractions were estimated by absorbance at 280 nm in a 0.5 cm flow-through cell. Protein content of pooled column fractions and purified proteins was measured using Coomassie Brilliant Blue reagent (Pierce, Rockford, Illinois) (Bradford, 1976). Bovine serum albumin (Sigma Chemical Co.) was used as a standard in all protein estimations. Purity of column fractions and purified proteins was assessed by SDS-PAGE (Laemmli, 1970) on 12.5% polyacrylamide gels stained with Coomassie Brilliant Blue R-250 as described in *Chapter 2*. Molecular mass marker proteins from Pharmacia Biotech (Uppsala, Sweden) were as previously described.

3.2.5 *Amino Acid Sequence Analysis*

NH_2-Terminal sequence analyses of the purified AXAH and RP-HPLC-purified tryptic peptides were performed on a Hewlett-Packard G1005A automated protein sequencer (Palo Alto, CA), as described in *Chapter 2*. Analyses were performed by Dr Neil Shirley and Mr. Jelle Lahnstein (Department of Plant Science, University of Adelaide). Tryptic digestion and RP-HPLC protocols were also as described in *Chapter 2*. BLAST (Altschul *et al.*, 1990) analyses were performed using the University of Wisconsin, Genetics Computer Group software (Devereux *et al.*, 1984) in the ANGIS suite of programs at the Australian National Genomic Information Service (http://www.angis.org.au).

3.3 RESULTS

3.3.1 Purification of Barley Arabinoxylan Arabinofuranohydrolase (AXAH-I)

During DEAE-cellulose chromatography of the 40-60% $(NH_4)_2SO_2$ fraction of a 7-day seedling extract, a broad peak of 4NPA hydrolysing activity eluted from the column at 270 mM NaCl (*Figure 2.10*, *Chapter 2*). This peak of activity, which was not evident in the 5-day seedling extract (*Figure 2.2*, *Chapter 2*), contained approximately 50 U of α-L-arabinofuranosidase activity as measured on 4NPA. When fractions from this column were assayed for their capacity to liberate L-arabinose from wheat arabinoxylan, only fractions corresponding with this third α-L-arabinofuranosidase peak demonstrated this ability (results not shown). It was presumed that the ability to hydrolyse 4NPA and also to hydrolyse the α-L-arabinofuranosyl linkages of arabinoxylan at a relatively high rate were the biochemical properties of an arabinoxylan arabinofuranohydrolase, which was designated AXAH-I. AXAH-I was so-named to distinguish it from a second barley AXAH isoenzyme, designated AXAH-II, later revealed by cDNA cloning (*Chapter 5*).

The major proportion of AXAH activity from the 7-day barley seedling extract was precipitated by 20-40%-saturated $(NH_4)_2SO_2$. DEAE-Cellulose chromatography of the 20-40% $(NH_4)_2SO_2$ fraction resolved the third α-L-arabinofuranosidase peak, eluting at 250 mM NaCl, from minor quantities of ARA-I and ARA-II isoenzymes (*Figure 3.2*). Of the 384 U of α-L-arabinofuranosidase activity in the 20-40% $(NH_4)_2SO_2$ fraction, 270 U were recovered in DEAE-cellulose fractions 90-102 as AXAH-I (*Table 3.2*). AXAH activity of each fraction was confirmed by hydrolysis of wheat arabinoxylan and fractions with the highest specific activity, quantitated using 4NPA, were selected for further purification. The purification of AXAH-I was completed by two further ion-exchange chromatography steps, outlined in *Figure 3.1*. SP-Sepharose (*Figure 3.3*) proved to be particularly effective for the removal of low molecular mass contaminants, including an abundant protein of approximately 26 kDa (*Figure 3.5*). Fractions from SP-Sepharose were pooled, and fractionated on Q-Sepharose (*Figure 3.4*). The elution of bound proteins from Q-Sepharose produced a major protein peak at 320 mM NaCl that was coincident with AXAH activity. The two 10 ml fractions at the height of the activity peak (*Figure 3.4*, fractions 11 and 12) were pooled and concentrated to a minimal volume for further biochemical characterization and analysis (*Chapter 4*).

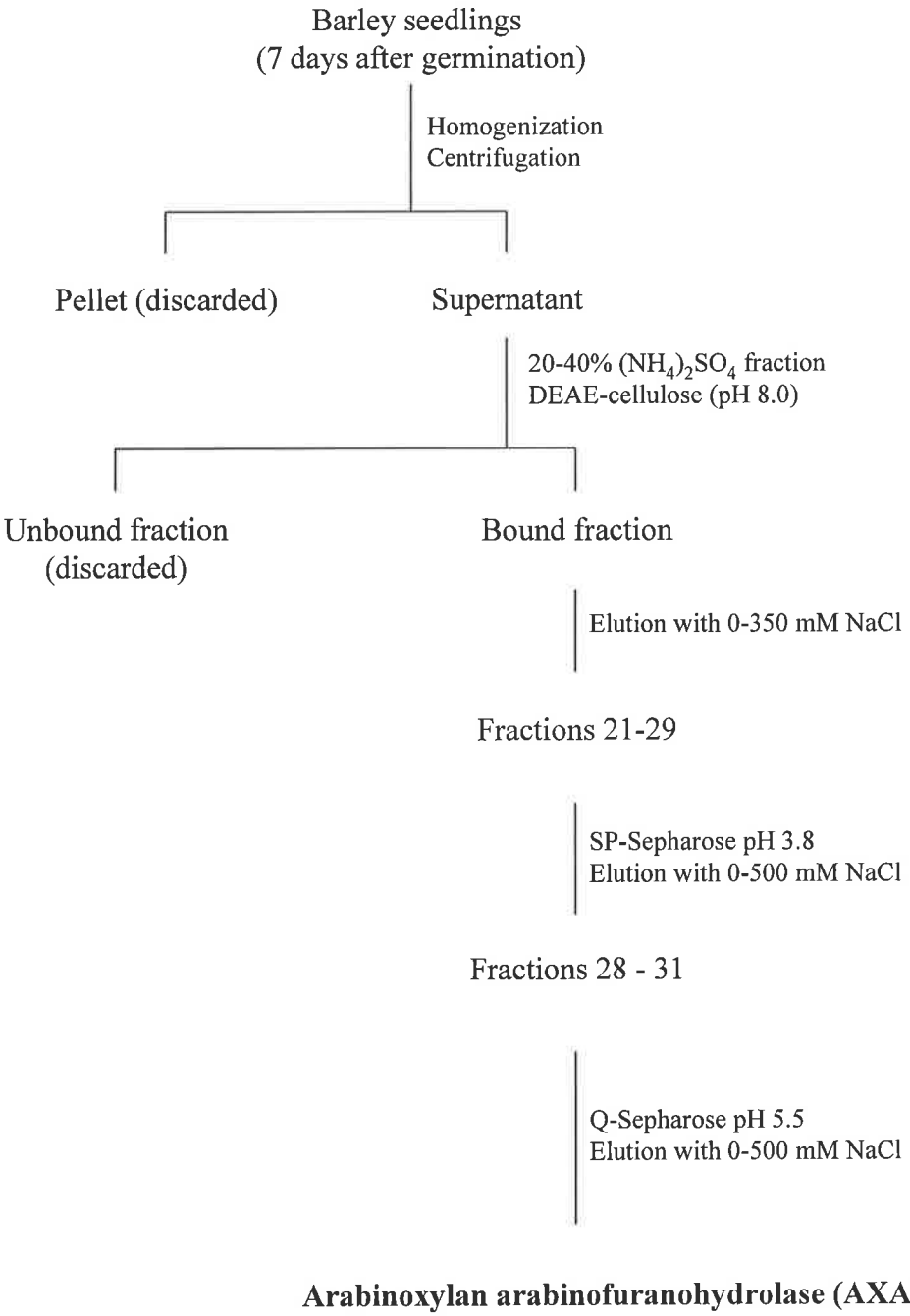

Figure 3.1 **Summary of procedures used in the purification of arabinoxylan arabinofuranohydrolase (AXAH-I).**

Table 3.1 **Enzyme yields and purification factors at each stage of the purification of arabinoxylan arabinofuranohydrolase (AXAH-I).** Activities were assayed with 4NPA. Recoveries are expressed as percentage of initial activity units and purification factors were calculated on the basis of specific activities.

	Yield				
	Protein *(mg)*	Activity *(U)*	Specific activity *($mU.mg^{-1}$)*	Recovery *(%)*	Purification factor *(fold)*
Arabinoxylan arabinofuranohydrolase (AXAH-I)					
Crude homogenate	24000	835	34	100	1.0
20-40% $(NH_4)_2SO_4$	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

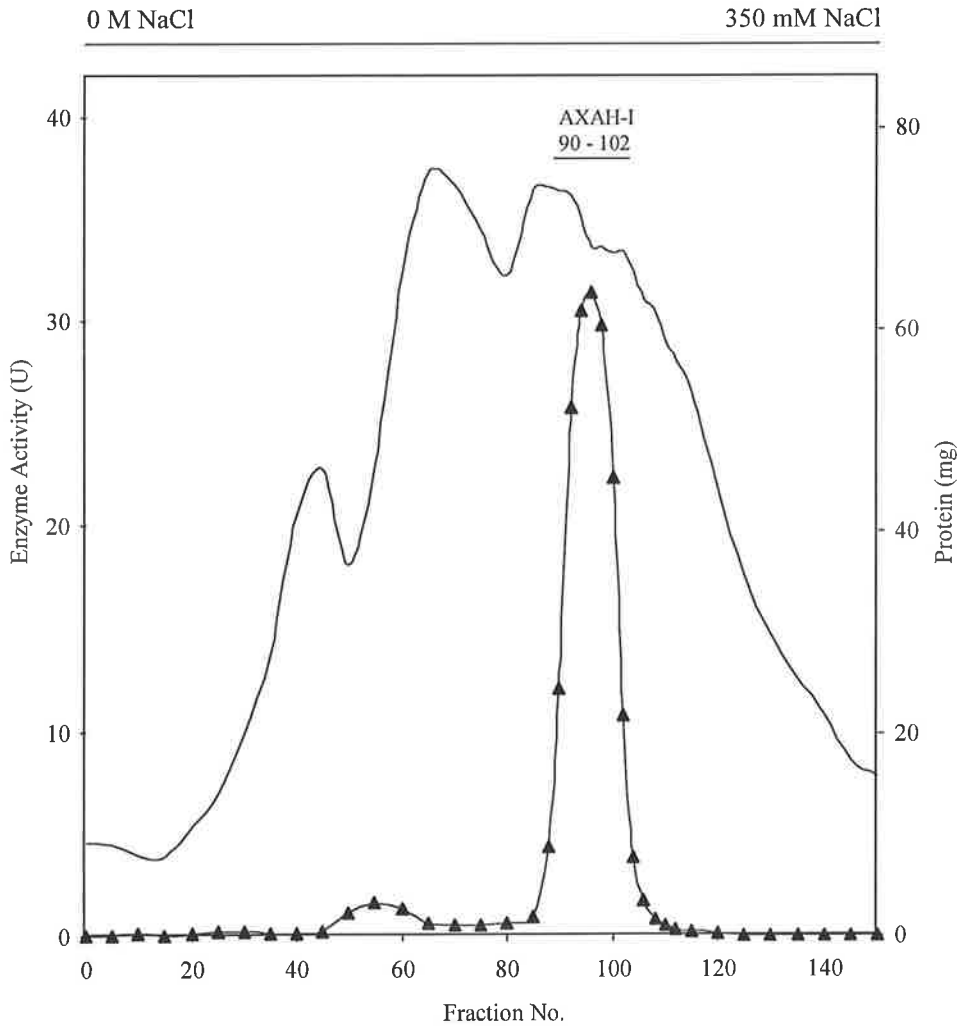

Figure 3.2 **Ion-exchange chromatography of 20-40% $(NH_4)_2SO_4$ fraction on DEAE-cellulose.** Material bound to DEAE-cellulose at pH 8.0 was eluted with a 3 L linear gradient of 0-350 mM NaCl. Fractions (20 ml) were assayed against 4NPA (▲) and protein (—) was measured by absorbance at 280 nm. AXAH-I activity was also measured semi-quantitatively by analysis of hydrolysis products after incubation of column fractions samples with arabinoxylan, using thin-layer chromatography. Liberation of L-arabinose from arabinoxylan was observed for fractions 90-102.

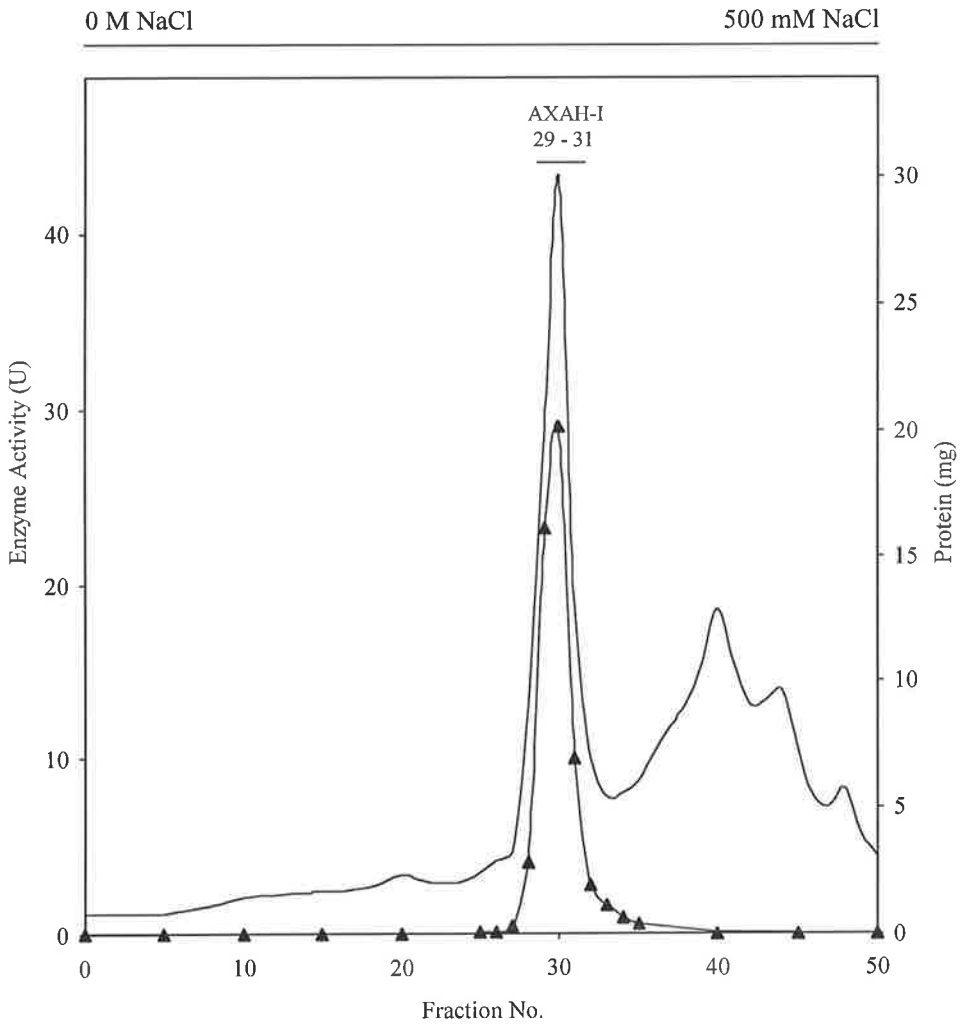

Figure 3.3 **Ion-exchange chromatography of DEAE-cellulose fractions containing arabinoxylan arabinofuranohydrolase activity on SP-Sepharose.** Fractions 90-102 from DEAE-cellulose (*Figure 3.2*) were pooled and applied to SP-Sepharose at pH 3.8. Bound proteins were eluted with a 1 L linear gradient of 0-500 mM NaCl and fractions (20 ml) were assayed against 4NPA (▲). Protein (—) was measured by absorbance at 280 nm.

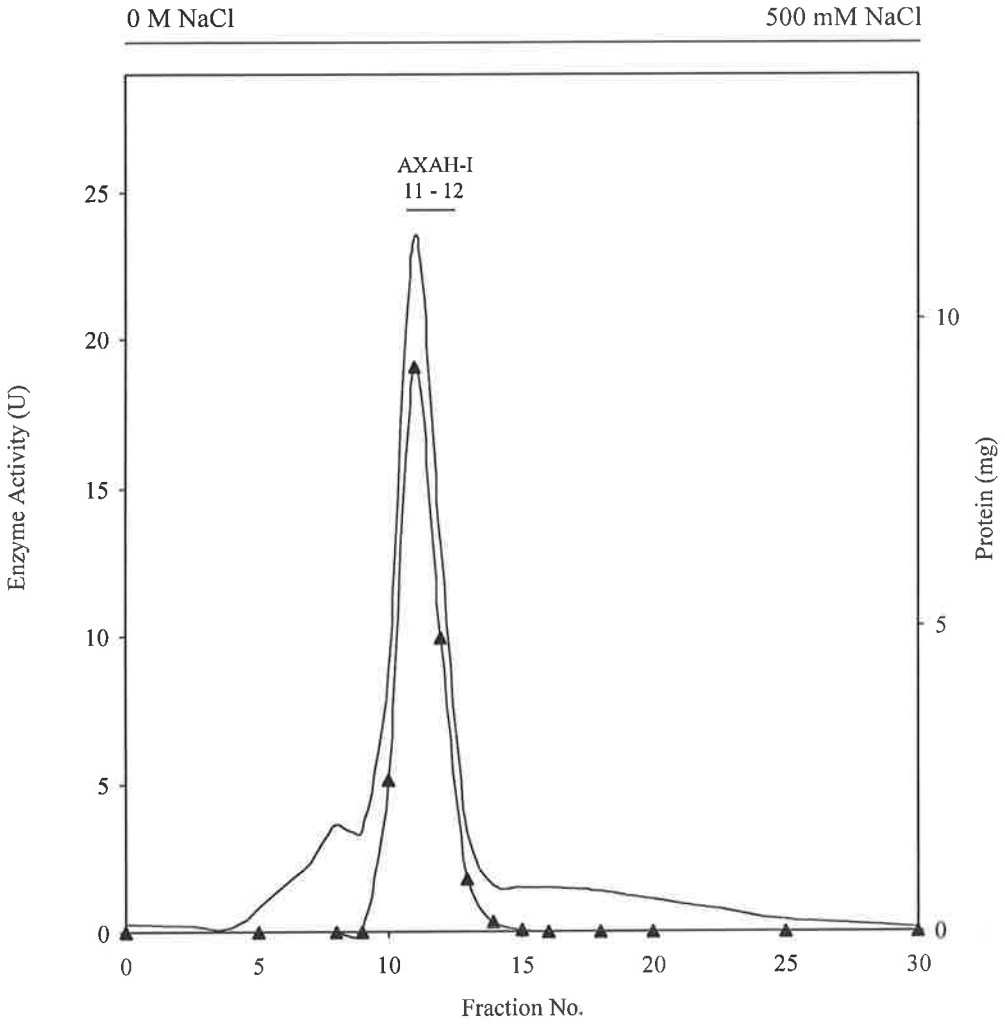

Figure 3.4 **Ion-exchange chromatography of SP-Sepharose fractions containing arabinoxylan arabinofuranohydrolase activity on Q-Sepharose.** Fractions 29-31 from SP-Sepharose (figure 3.3) were pooled and applied to Q-Sepharose at pH 5.5. Bound proteins were eluted with a 300 ml linear gradient of 0-500 mM NaCl and fractions (10 ml) were assayed against 4NPA (▲). Protein (—) was measured by absorbance at 280 nm. Fractions 11 and 12 were pooled and further purified on Q-Sepharose.

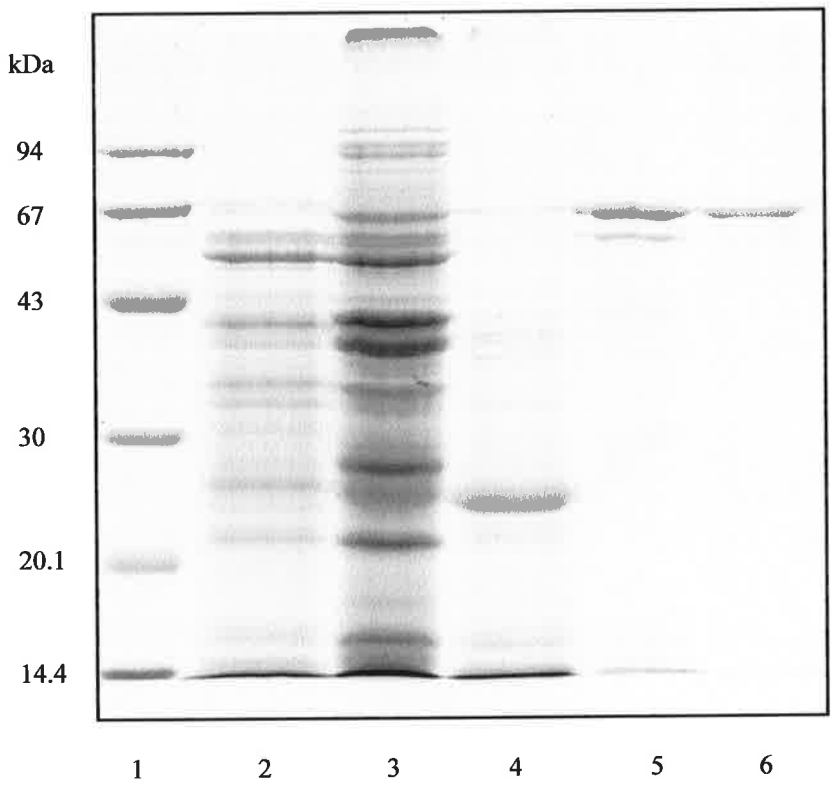

***Figure 3.5* SDS-PAGE of samples taken at each stage of the purification of AXAH-I from an extract of 7-day-old barley seedlings.** *Lane 1*, molecular mass markers; *lane 2*, crude extract; *lane 3*, 20-40% $(NH_4)_2SO_4$ fraction; *lane 4*, DEAE-cellulose fractions 66-87; *lane 5*, SP-Sepharose pooled fractions 28-31; *lane 6*, Q-Sepharose pooled fractions.

The purity of the final enzyme preparation was examined by SDS-PAGE (*Figure 3.5*). A single major band of 65 kDa was present with one minor contaminating band of approximately 60 kDa.

3.3.2 Amino Acid Sequence Analysis

NH_2-Terminal and tryptic amino acid sequences of AXAH-I were determined. The NH_2-terminal sequence was found to be free of secondary sequences and PTH derivatives were recovered with expected yields. The 38 NH_2-terminal residues of AXAH-I were ITQVASLGVDSSPHLARKIPDTLFGIFFEEINHAGAGG.

Tryptic digestion of the AXAH-I preparation produced at least 25 peptide fractions that were resolved by RP-HPLC (*Figure 3.6*). Three of these were selected for NH_2-terminal sequencing. Fraction 1 yielded two amino acid sequences that could be clearly resolved on the basis of abundance of PTH derivatives. The AXAH-I tryptic peptide sequences were:

1*a*. GFEAGGPHTPSNINPWSIIG
1*b*. EAYPDIQMISN-DGSSTPLD
2. IVNFGPDAVGLTISATGLQGSINAFGSTATVLTSGGVMDENSFANPN-VVPV
3. NSDVVQMASYAPLFINDNDRTWNPDAIVFNSWQQYGTPSY

Results for non-redundant BLAST searches revealed similarities between AXAH-I sequences and an arabinosidase from *Bacteroides ovatus* (GenBank/EMBL accession U15178), α-arabinofuranosidase I from *Streptomyces chartreusis* (AB023625) and two *Arabidopsis thaliana* genomic DNA sequences (AF149413 and AC011708). These enzymes are classified in family 51 of glycoside hydrolase families (Coutinho and Henrissat, 1999*a*).

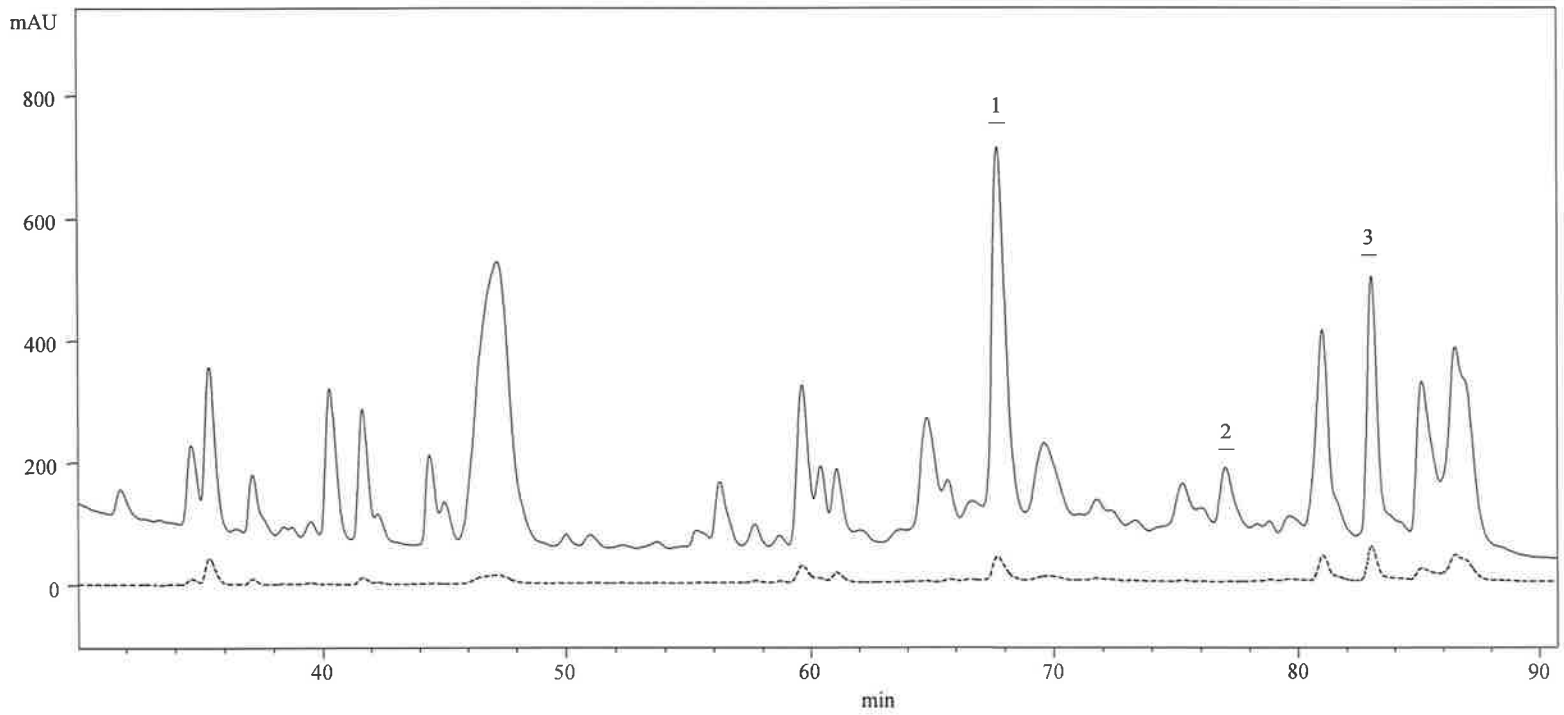

Figure 3.6 **RP-HPLC of peptide fragments generated by digestion of barley arabinoxylan arabinofuranohydrolase (AXAH-I) with trypsin.** A_{280} (—) and A_{214} (---) are shown. Fractions corresponding with peaks shown above were collected and NH_2-terminal amino acid sequences of purified peptides, 1 to 3 as indicated, were determined.

3.4 DISCUSSION

3.4.1 Purification of Barley Arabinoxylan Arabinofuranohydrolase

An enzyme capable of removing L-arabinose from cereal arabinoxylans (AXAH) was first observed by analysis of DEAE-cellulose column fractions from chromatography of the 40-60%-saturated $(NH_4)_2SO_4$ fraction during the partial purification of the α-L-arabinofuranosidase isoenzyme, ARA-II (described in *Chapter 2*). Comparison of column fractions for their capacity to liberate L-arabinose from wheat arabinoxylan identified AXAH-containing fractions corresponding with the third peak of α-L-arabinofuranosidase activity (*Figure 2.10*, *Chapter 2*). As discussed in *Chapter 2*, this late-eluting third α-L-arabinofuranosidase peak was also observed, albeit at a very low level, in DEAE-cellulose fractions of the 40-60%-saturated $(NH_4)_2SO_4$ fraction from a 5-day barley seedling extract (*Figure 2.2*, *Chapter 2*). The analysis of DEAE-cellulose column fractions using both 4NPA and arabinoxylan as substrates for the estimation of α-L-arabinofuranosidase and AXAH activities, respectively, established a number of key observations. Firstly, the enzyme responsible for this late-eluting activity was able to hydrolyse both substrates, therefore suggesting that it might be a family 51- or 54-type enzyme with more broad specificity, rather than the highly specific family 62-type enzyme. Secondly, the differences in substrate specificity between the three "α-L-arabinofuranosidases" were revealed by the use of the two different substrates. The third peak of α-L-arabinofuranosidase activity from DEAE-cellulose chromatography of the barley seedling extracts clearly contained a different class of barley enzyme. DEAE-Cellulose chromatography of the 20-40%-saturated $(NH_4)_2SO_4$ fraction, shown in *Figure 3.2*, indicated that the major component in this fraction with activity against 4NPA was an AXAH.

Here, AXAH was purified from an extract of 7-day-old barley seedlings. The enzyme, designated AXAH-I, was assayed against wheat flour arabinoxylan and released L-arabinose was measured semi-quantitatively by thin-layer chromatography. The scheme in *Figure 3.1* shows that following DEAE-cellulose chromatography, AXAH-I was purified by two further ion-exchange steps on SP-Sepharose and Q-Sepharose. SDS-PAGE of purified fractions after each stage of the purification of AXAH-I (*Figure 3.5*) shows that an abundant contaminant of approximately 25 kDa was removed by the SP-Sepharose step. Further removal of contaminating proteins was achieved by chromatography on Q-Sepharose

(*Figure 3.4*). The two Q-Sepharose fractions (fractions 11 and 12, *Figure 3.4*) from the activity peak were pooled to exclude contaminants from an earlier eluting peak that may have been present in fraction 10. The final AXAH-I preparation contained a single major protein of approximately 65 kDa as shown by SDS-PAGE (*Figure 3.5*). Only a small level of contamination, contributed by a protein of around 60 kDa, was evident in the final purified AXAH-I preparation. AXAH-I was purified by a factor of 184-fold from the 7-day barley seedling extract with an approximate overall recovery of 1% (*Table 3.1*). These values probably underestimate the efficiency of the AXAH-I purification because a large proportion of the α-L-arabinofuranosidase activity in the seedling extract was contributed by the family 3 α-L-arabinofuranosidases ARA-I and ARA-II, and the β-D-xylosidase XYL. Almost half of the α-L-arabinofuranosidase activity from barley seedlings harvested at seven days after germination can be attributed to AXAH-I (*Table 3.1*), as revealed by the almost complete separation of AXAH-I from other α-L-arabinofuranosidases by $(NH_4)_2SO_4$ fractionation.

3.4.2 Determination of Amino Acid Sequences

The NH_2-terminal, 38 amino acid residues of the purified AXAH-I and the NH_2-terminal amino acid sequences of four AXAH-I tryptic peptides were determined by Edman degradation. The four tryptic sequences were derived from three RP-HPLC-purified fractions, with two peptide sequences resolved from differential yields of PTH derivatives for RP-HPLC fraction 1. Sequences from peptides 2 and 3 were 57 and 40 amino acid residues in length, respectively. BLAST search results indicated that AXAH-I is a family 51 glycoside hydrolase (Coutinho and Henrissat, 1999*a*) with sequence similarities to arabinosidases from *Bacteroides ovatus* (U15178) and *Streptomyces chartreusis* (AB023625). An arabinoxylan arabinofuranohydrolase of the type classified in family 62 of the glycoside hydrolases was not found in barley seedling extracts during the course of this work.

At the time the current work was in progress, a description of a barley AXAH was published by Ferré *et al.* (2000). The enzyme described by Ferré *et al.* (2000) had an apparent molecular mass of 66 kDa and a pI of 4.55, and the NH_2-terminal amino acid sequence of GITQVASLGVDSSPHLA$^{R}_{H}$KIP almost exactly matched that obtained for AXAH-I in this study. The enzyme clearly corresponded to the AXAH-I enzyme purified here.

3.4.3 Summary

Chromatography of each of the major α-L-arabinofuranosidase and β-D-xylosidase peaks from two $(NH_4)_2SO_4$ fractions has resolved four glycoside hydrolases with activity on arabinoxylan, or on aryl glycosides. These four enzymes, three of which have been highly purified, probably combine with (1→4)-β-D-xylan endohydrolases into the full complement of glycoside hydrolases necessary for complete hydrolysis of arabinoxylan during normal physiological processes in barley. The possibility remains that other enzymes with similar activities may be produced in different tissues, or at different developmental stages. Nevertheless, the four enzymes investigated here are the major enzymes of their type in barley seedlings and/or germinated grain, and their purification and amino acid sequences provide the foundation for further biochemical characterization (*Chapter 4*) and for the isolation of corresponding cDNA sequences (*Chapter 5*).

CHAPTER 4.

Biochemical Characterization of Purified Enzymes

4.1 INTRODUCTION

Three enzymes with activity against arabinoxylan or the synthetic aryl glycosides 4NPA and 4NPX, have been purified from barley seedling extracts. Two of these enzymes, α-L-arabinofuranosidase isoenzyme ARA-I and a β-D-xylosidase, XYL, are family 3 glycoside hydrolases with sequence similarities to microbial β-D-xylosidases, β-D-glucosidases and β-D-glucan glucohydrolases (Coutinho and Henrissat, 1999*a*). Preliminary experiments (results not shown) had shown that ARA-I and XYL have broad specificity for the aryl glycosides, 4NPA and 4NPX, but their ability to hydrolyse arabinoxylans appeared to be limited. The third purified barley enzyme, an arabinoxylan arabinofuranohydrolase designated AXAH-I, is active on 4NPA and has a relatively high level of activity against arabinoxylans. Based on partial amino acid sequences, AXAH-I is classified as a family 51 glycoside hydrolase.

The detailed characterization of substrate specificity, action pattern and kinetic properties of purified enzymes provides the basis for further studies of their functional roles in barley cell wall metabolism. The activities of enzymes against arabinoxylans are often restricted by the presence of arabinofuranosyl substituents. For example, a (1→4)-β-D-xylan endohydrolase from *Aspergillus niger* requires a series of contiguous unsubstituted β-D-xylopyranosyl residues for hydrolysis of the β-D-xylan backbone (Hoffmann *et al.*, 1992*a*). L-Arabinofuranosyl substituents similarly limit β-D-xylosidase action against arabinoxylans and oligoarabinoxylosides (Tenkanen *et al.*, 1996; Hermann *et al.*, 1997). Furthermore, the capacity for α-L-arabinofuranosidases to remove L-arabinosyl substituents from substituted D-xylopyranosyl residues of arabinoxylan is probably influenced by steric hindrance imposed by neighboring L-arabinofuranosyl substituents.

To more fully understand the enzymic processes of arabinoxylan metabolism in barley, the biochemical properties of purified barley enzymes ARA-I, XYL and AXAH-I have been examined. A comprehensive evaluation of pH optimum, substrate specificity, kinetic parameters and action pattern is reported here.

4.2 MATERIALS AND METHODS

4.2.1 Determination of pH optimum

The pH optimum for each of the purified enzymes was determined by measurement of specific activity, using the preferred substrate in each case, over a range of pH values. The pH optimum for ARA-I and XYL were assayed using 4NPA and 4NPX, respectively, and AXAH-I was assayed against 4NPA. Spectrophotometric determinations were performed, essentially as described in *Chapter 2*, at substrate concentrations of 0.04% (*w/v*) in 0.1 M sodium citrate-sodium phosphate buffers (McIlvaine buffer) over the pH range 3.5-7.5. Activities were expressed as a percentage of the maximum activity observed for each enzyme.

4.2.2 Substrate Specificity

Aryl glycosides were used as substrates with purified ARA-I, XYL and AXAH-I in spectrophotometric assays as described in *Chapter 2*, at substrate concentrations of 2.5 mM. Activities against 4-nitrophenyl-α-L-arabinofuranoside (4NPA), 4-nitrophenyl-α-L-arabinopyranoside, 4-nitrophenyl-β-L-arabinofuranoside, 4-nitrophenyl-β-D-xylopyranoside (4NPX), 4-nitrophenyl-α-D-xylopyranoside, 4-nitrophenyl-α-D-mannopyranoside, 4-nitrophenyl-β-D-mannopyranoside, 4-nitrophenyl-α-D-glucopyranoside, 4-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl-α-D-galactopyranoside and 4-nitrophenyl-β-D-galactopyranoside were determined. Relative activities were calculated as percentage of the specific activity against the preferred glycoside; 4NPX for XYL and 4NPA for ARA-I and AXAH-I. Aryl glycosides were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.)

Hydrolysis of polysaccharide and oligosaccharide substrates by ARA-I, XYL and AXAH-I was assessed by incubation of enzyme with substrate in 50 mM sodium acetate buffer at the respective pH optimum, at 37°C. Increases in reducing sugars were estimated using the method of Nelson (1944) and Somogyi (1952) or by analysis of hydrolysis products by thin-layer chromatography, as described in *Chapter 3*. Polysaccharides were assayed at a substrate concentration of 0.5% (*w/v*) and oligosaccharides, xylopentaose and arabinohexaose, were at 10 mM. Wheat arabinoxylan, branched arabinan and barley (1→ 3,1→ 4)-β-D-glucan were from Megazyme (Bray, Ireland). Laminarin and arabinogalactan were from Sigma. Carboxymethyl-(1→ 4)-β-D-xylan was from Dr Peter

Biely (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak Republic). (1→ 4)-β-D-xylopentaose and (1→ 5)-α-L-arabinohexaose were from Megazyme.

4.2.3 Enzyme Kinetics

Kinetic parameters of ARA-I and XYL were determined for both 4NPA and 4NPX, at 37°C in 50 mM sodium acetate buffer, pH 4.3, containing 4 mM NaN_3 and 160 $\mu g.ml^{-1}$ BSA (Sigma). A fixed amount of enzyme was incubated with substrate, at concentrations ranging from 0.04-0.6, 0.3-6.3, 0.01-0.16 and 0.6-4.6 times the K_m value, for ARA-I with 4NPA and 4NPX, and XYL with 4NPA and 4NPX, respectively. Spectrophotometric assays were performed in triplicate as previously described.

Kinetic parameters of AXAH-I were determined for 4NPA at concentrations in the range of 0.3-6.5 times the K_m value, and for wheat arabinoxylan, up to 5.0 $mg.ml^{-1}$. Assays were performed in triplicate, in 50 mM sodium acetate buffer, pH 4.3, containing 4 mM NaN_3 and 160 $\mu g.ml^{-1}$ BSA. Rates of hydrolysis were determined spectrophotometrically, for each substrate by estimation of released 4-nitrophenol or reducing sugars, respectively, as described above.

Kinetic data were processed by a proportional weighted fit using a non-linear regression analysis program based on Michaelis-Menten enzyme kinetics (Leatherbarrow, 1998).

4.2.4 Thin-Layer Chromatography

Hydrolysis products released from arabinoxylan, xylopentaose and arabinohexaose after incubation with purified ARA-I, XYL and AXAH-I, were examined by thin-layer chromatography on silica-gel thin-layer plates (Merck, Darmstadt, Germany). Purified enzymes, at concentrations specified in the *Results* section, were incubated with substrates at 37°C. Hydrolysates (5 µl), were loaded onto silica-gel plates and plates were subsequently developed in ethyl acetate:acetic acid:water (3:2:1 by volume). Sugars were detected using the orcinol reagent, containing 0.5% (*w/v*) orcinol (Sigma), in 5% (*v/v*) H_2SO_4 in ethanol (Farkas and Maclachlan, 1988). Analysis of arabinoxylan hydrolysis was conducted in this manner for each enzyme, both with and without the addition of *Thermomyces* sp. (1→4)-β-D-xylan endohydrolase. *Thermomyces* sp. (1→4)-β-D-xylan endohydrolase was from Dr Peter Biely (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak

Republic). Standard L-arabinose and D-xylose were obtained from Sigma, and β-D-oligoxylosides (X_2-X_6) were from Megazyme.

4.2.5 Polysaccharide Linkage Analysis

Wheat arabinoxylan, at a concentration of 0.5% (*w/v*), both with and without the addition of AXAH-I, was incubated in 50 mM sodium acetate buffer, pH 4.3, containing 4 mM NaN_3 at 37°C for 16 h. Enzyme-digested and non-digested arabinoxylan solutions were dialysed against Milli-Q H_2O at 4°C and lyophilised. Arabinoxylans were permethylated using the $NaOH/CH_3I$ method (Ciucanu and Kenek, 1984) as described by Nunan *et al.* (1997). Methylated polysaccharides were hydrolysed with trifluoracetic acid, reduced with $NaBD_4$ and acetylated with $HClO_4$ as a catalyst (Harris *et al.*, 1988). 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, Victoria, Australia).

4.3 RESULTS

4.3.1 *Determination of pH optimum*

The pH optimum for ARA-I and XYL was 4.7 and for AXAH-I, pH 4.3 (*Figure 4.1*). Consequently, assays for the determination of substrate specificity and kinetic parameters were conducted in 50 mM sodium acetate buffers at the respective pH optimum.

4.3.2 *Substrate Specificity*

The activities of ARA-I, XYL and AXAH-I against a range of aryl glycosides and several polysaccharides were determined. At 2.5 mM substrate concentration, the preferred aryl glycoside substrates for ARA-I and XYL were 4NPA and 4NPX, respectively. ARA-I was also capable of hydrolysing 4NP-β-D-Xyl*p*, 4NP-β-D-Gal*p* and 4NP-α-L-Ara*p* with 20%, 16% and 11% of the specific activity for 4NPA, respectively (*Table 4.1*). The preference of XYL for 4NPX was more stringent, with specific activities for other aryl glycosides no greater than 3% in each case (*Table 4.1*). In contrast, of the aryl glycosides tested, only 4NPA could be hydrolysed by AXAH-I.

The hydrolysis of various polysaccharide substrates was measured using both a reductometric assay and by detection of hydrolysis products using thin-layer chromatography (TLC). ARA-I and XYL hydrolysed only arabinoxylan; this was at a very low rate that could only be detected after prolonged incubation. AXAH-I hydrolysed arabinoxylan more rapidly and also had activity against larch arabinogalactan and branched arabinan (*Section 4.3.4*). AXAH-I was unable to hydrolyse (1→3,1→4)-β-glucan, laminarin or CM-xylan.

4.3.3 *Enzyme Kinetics*

The kinetic parameters for ARA-I and XYL, using both 4NPA and 4NPX, were determined (*Figure 4.2* and *Figure 4.3*) and for AXAH-I, kinetic analyses for hydrolysis of wheat flour arabinoxylan and 4NPA were performed (*Figure 4.4*). Standard errors for assays in triplicate were less than 5% for aryl glycosides and approximately 17% for AXAH-I using wheat arabinoxylan. The high standard errors for measurement of enzyme activity using wheat arabinoxylan were presumably because the substrate concentrations used to measure kinetic properties of AXAH-I were near the upper limit of solubility for wheat arabinoxylan.

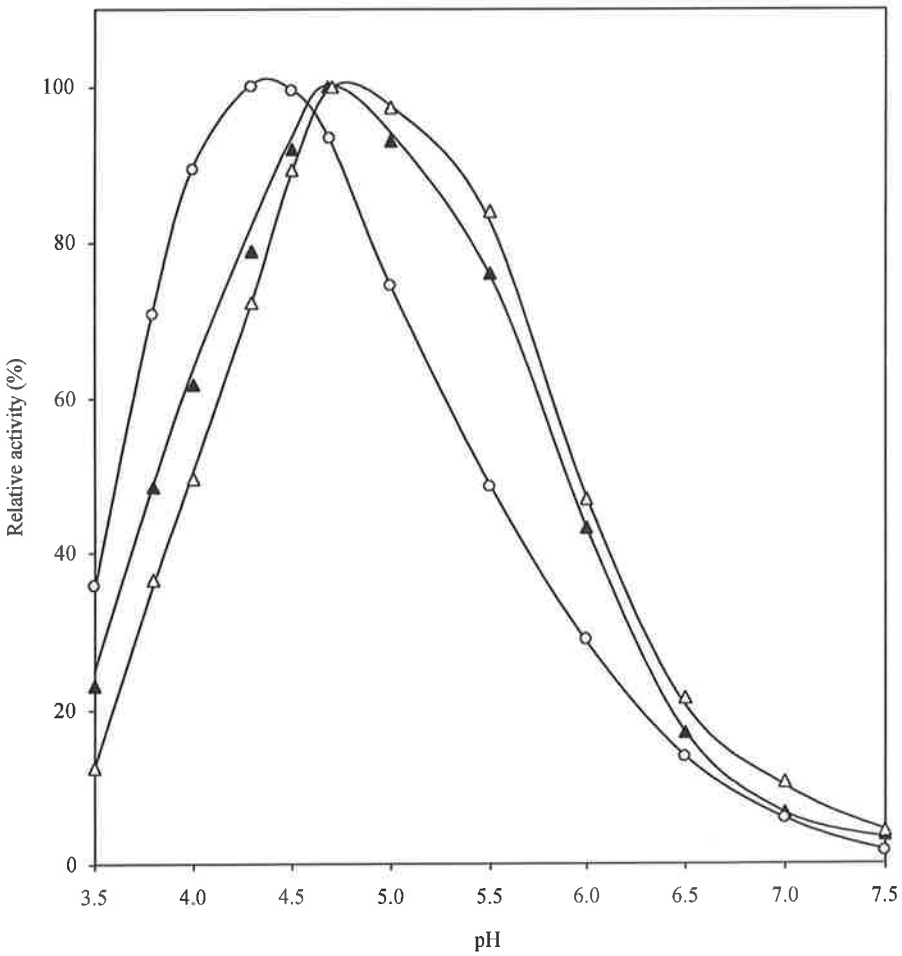

***Figure 4.1* pH Optimum for hydrolytic activity of XYL (△), ARA-I (▲) and AXAH-I (○).** Enzyme activities were determined over a range of pH values between 3.5 and 7.5 in McIlvaine Buffer and were expressed as a percentage of the activity at the pH optimum. The pH optimum for ARA-I and XYL was approximately 4.7 and for AXAH-I, pH 4.3.

Table 4.1 **Relative activities of ARA-I, XYL and AXAH-I against a range of aryl glycosides at 2.5 mM final substrate concentration.** Specific activities of each enzyme were determined using a range of 4-nitrophenyl-glycosides at a substrate concentration of 2.5 mM at pH 4.7. Specific activities for each substrate are expressed as a percentage of that of the preferred substrate for each enzyme.

	Relative activity (%)		
	ARA-I [a]	XYL [b]	AXAH-I [a]
4NP-α-L-Arabinofuranoside (4NPA)	100	3	100
4NP-α-L-Arabinopyranoside	11	2	0
4NP-β-L-Arabinopyranoside	0	0	0
4NP-β-D-Xylopyranoside (4NPX)	20	100	0
4NP-α-D-Xylopyranoside	0	0	0
4NP-α-D-Mannopyranoside	0	0	0
4NP-β-D-Mannopyranoside	0	0	0
4NP-α-D-Glucopyranoside	0	0	0
4NP-β-D-Glucopyranoside	0	1	0
4NP-α-D-Galactopyranoside	0	0	0
4NP-β-D-Galactopyranoside	16	3	0

[a] Activity expressed as % of specific activity against 4NPA
[b] Activity expressed as % of specific activity against 4NPX

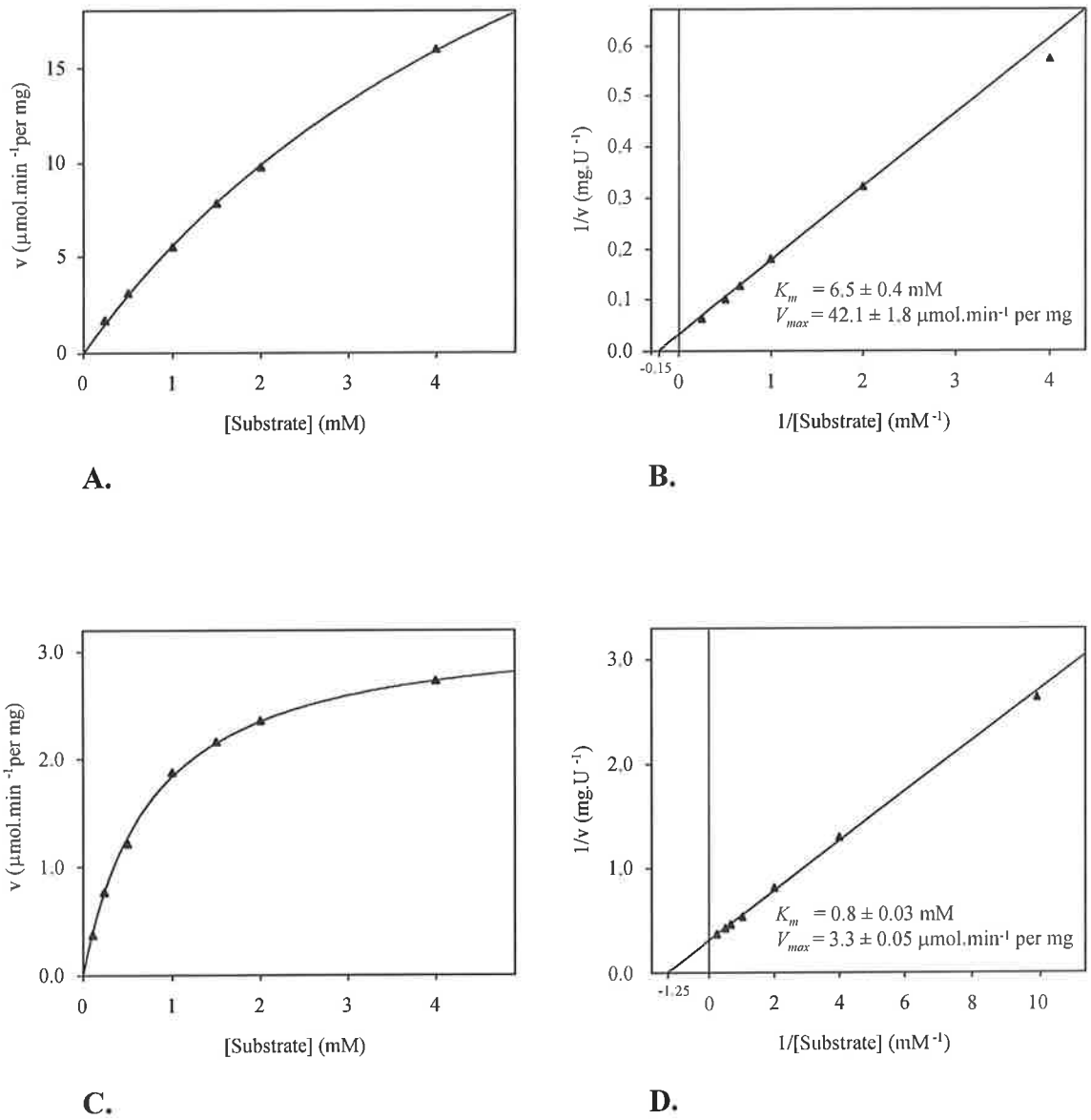

Figure 4.2 **Kinetic analysis of α-L-arabinofuranosidase isoenzyme ARA-I against 4NPA (A. and B.) and 4NPX (C. and D.).** The activity of ARA I was determined over a range of substrate concentrations and expressed as µmol.min^{-1} per mg. Standard errors for assays in triplicate were less than 5%. These data are are represented above as Michaelis-Menten (A. and C.) and Lineweaver-Burk (B. and D.) plots. Kinetic data were processed by a proportional weighted fit with a non-linear regression analysis program based on Michaelis-Menten enzyme kinetics (Leatherbarrow, 1998).

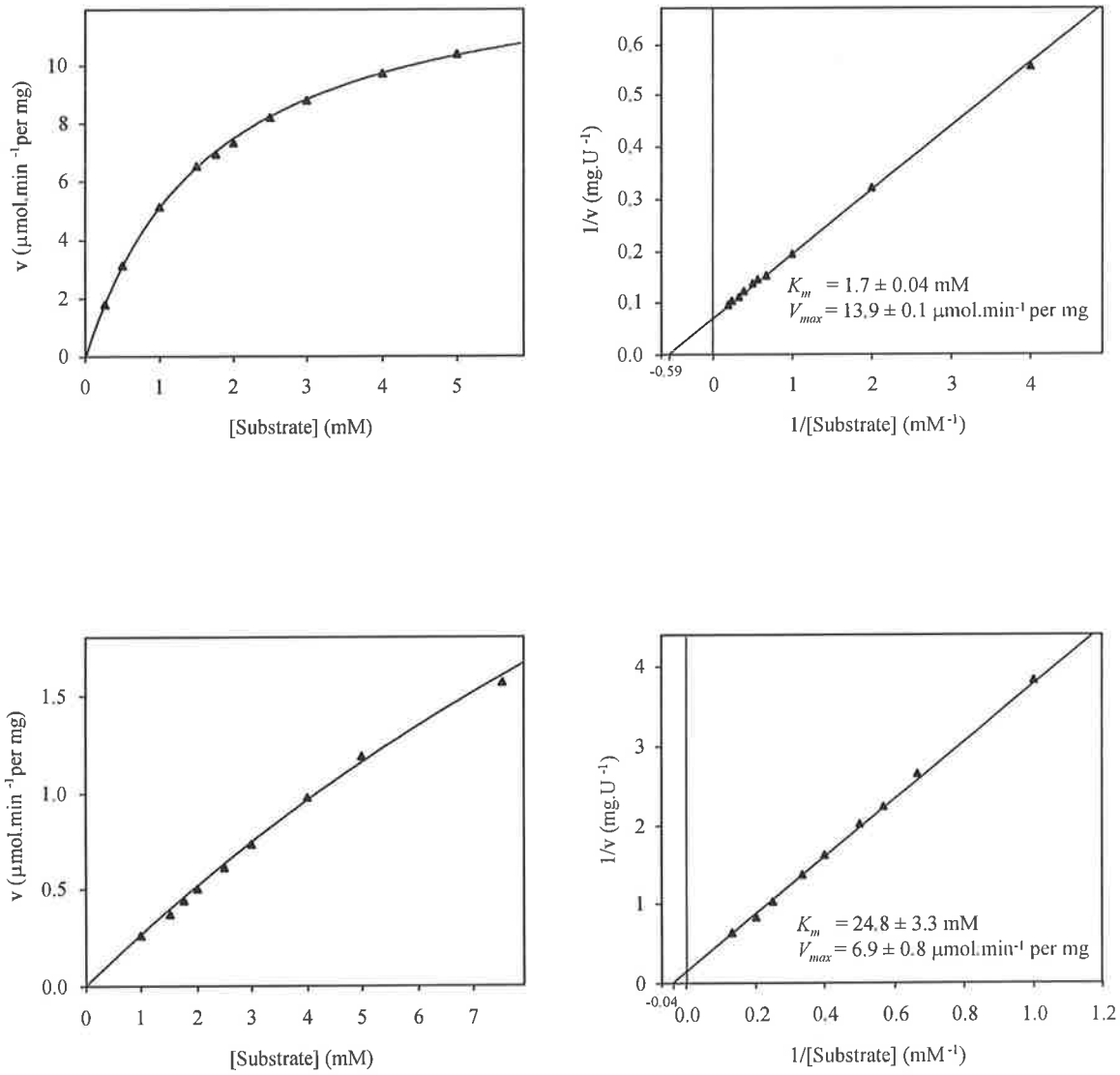

Figure 4.3 **Kinetic analysis of *β*-D-xylosidase (XYL) against 4NPX (A. and B.) and 4NPA (C. and D.).** Enzyme activity was determined over a range of substrate concentrations and expressed as µmol.min^{-1} per mg. Standard errors for assays in triplicate were less than 5%. These data are are represented above as Michaelis-Menten (A. and C.) and Lineweaver-Burk (B. and D.) plots. Kinetic data were processed by a proportional weighted fit with a non-linear regression analysis program based on Michaelis-Menten enzyme kinetics (Leatherbarrow, 1998).

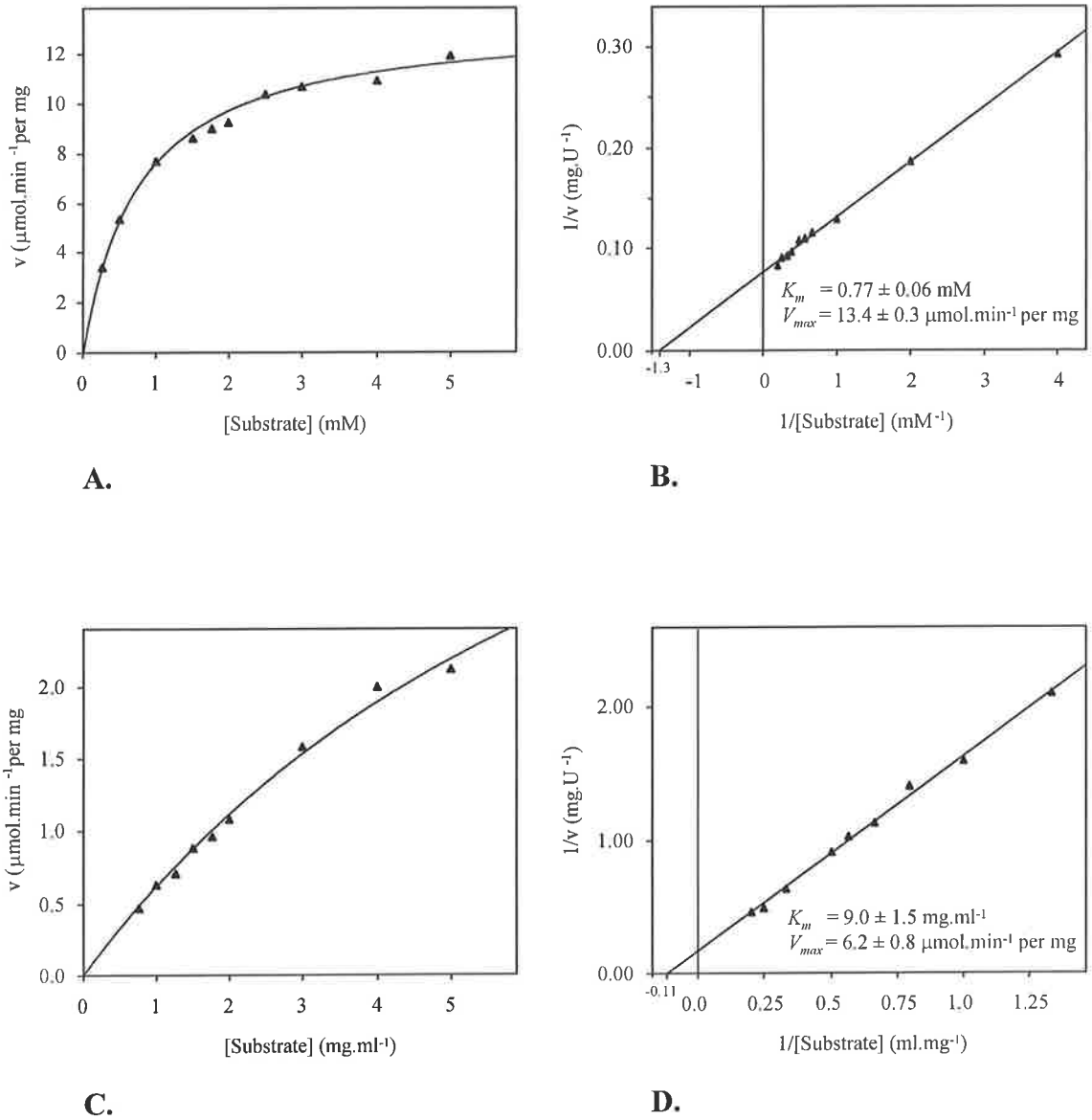

Figure 4.4 **Kinetic analysis of arabinoxylan arabinofuranohydrolase (AXAH-I) against 4NP-α-L-arabinofuranoside (A. and B.) and wheat arabinoxylan (C. and D.).** The activity of AXAH-I was determined over a range of substrate concentrations and expressed as µmol.min^{-1} per mg. Standard errors for assays in triplicate were less than 5%. These data are are represented above as Michaelis-Menten (A. and C.) and Lineweaver-Burk (B. and D.) plots. Kinetic data were processed by a proportional weighted fit with a non-linear regression analysis program based on Michaelis-Menten enzyme kinetics (Leatherbarrow, 1998).

Table 4.2 **Kinetic parameters of ARA-I and XYL against 4NPA and 4NPX.** k_{cat} is the catalytic rate constant and k_{cat}/Km is the catalytic efficiency factor.

Parameter	ARA-I		XYL	
	4NPA	4NPX	4NPA	4NPX
K_m (mM)	6.5	0.8	24.8	1.7
k_{cat} (sec^{-1})	45.6	3.6	7.7	15.5
k_{cat} / K_m ($M^{-1}.sec^{-1}$)	7.02×10^3	3.57×10^3	0.31×10^3	9.1×10^3

Table 4.3 **Kinetic parameters of AXAH-I with 4NPA and wheat arabinoxylan.** k_{cat} is the catalytic rate constant and k_{cat}/Km is the catalytic efficiency factor.

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

K_m values for ARA-I against aryl glycosides revealed that this enzyme has a relatively low K_m for 4NPX (0.8 mM) compared with that for 4NPA (6.5 mM) (*Figure 4.2*; *Table 4.2*). ARA-I achieves a higher rate of catalysis on 4NPA because the catalytic rate constant (K_{cat}), of 45.6 sec^{-1} is almost 13-times that for 4NPX. The catalytic efficiency factor (K_{cat}/K_m) for 4NPA was twice the value determined for 4NPX. Comparison of kinetic parameters for XYL using the substrates 4NPA and 4NPX demonstrated that XYL has a definite preference for 4NPX (*Figure 4.3*; *Table 4.2*). XYL has a K_m value for 4NPX of 1.7 mM compared with a K_m value for 4NPA of 24.8 mM. The catalytic rate constant for 4NPX was twice that for 4NPA and the catalytic efficiency factor for 4NPX was 30 times higher than that measured for 4NPA (*Table 4.2*).

Kinetic parameters determined using 4NPA indicate that AXAH-I hydrolyses 4NPA with a high level of efficiency (*Figure 4.4*; *Table 4.3*). The K_m value 0.77 mM suggests that the enzyme has a relatively high affinity for arabinofuranosides. However, the high K_m value for AXAH-I measured against wheat flour arabinoxylan (9.0 mg/ml) suggests a low affinity for the arabinofuranosyl residues of wheat arabinoxylan. The arrangement of C(O)2- and C(O)3-linked arabinofuranosyl substituents may affect the ability of AXAH-I to remove L-arabinofuranosyl substituents from the polysaccharide. It should be considered that the estimation of kinetic parameters, using polysaccharide substrates such as arabinoxylans, is inherently difficult because released products become new substrates for the enzyme, potentially with different binding affinities and catalytic efficiencies. Furthermore, the diffusion rate of the polymeric substrate will be lower than for that of 4NPA. The catalytic rate constant, K_{cat}, for AXAH-I using wheat arabinoxylan was 6.9 sec^{-1}, which is similar to values for AXAH-I, ARA-I and XYL against 4NPA and 4NPX, and confirms that the arabinofuranosyl substituents of arabinoxylan are hydrolysed relatively efficiently.

4.3.4 Examination of Hydrolytic Activities Using Thin-Layer Chromatography

The action patterns of ARA-I, XYL and AXAH-I were investigated by analysis of hydrolysis products after incubation with arabinoxylan (*Figure 4.5*), (1→5)-α-L-arabinohexaose (*Figure 4.6*), (1→4)-β-D-xylopentaose (*Figure 4.7*), sugar beet arabinan and larch wood arabinogalactan (*Figure 4.8*). Incubation of ARA-I and XYL (5 $U.ml^{-1}$ as measured on 4NPA and 4NPX, respectively) with wheat arabinoxylan for 1 h at 37°C produced similar amounts of D-xylose (*Figure 4.5 A*). ARA-I was also capable of limited removal of α-L-

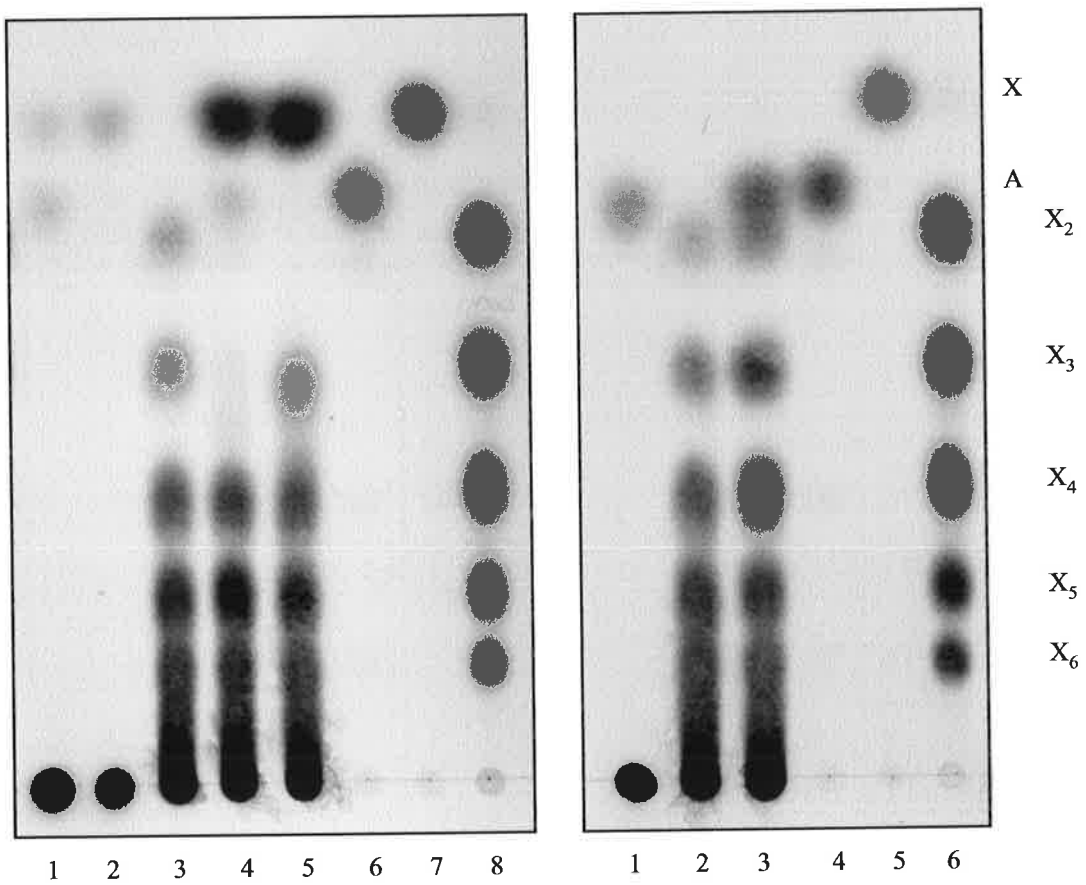

Figure 4.5 **Investigation of substrate specificities for ARA-I and XYL (A) and AXAH-I (B) by hydrolysis of wheat arabinoxylan and analysis of products by thin-layer chromatography.** (A) ARA-I (5 $U.ml^{-1}$, 4NPA) and XYL (5 $U.ml^{-1}$, 4NPX) (*lanes 1* and *2*, respectively) were incubated with wheat arabinoxylan (0.5 %) for 1 h at 37°C. XYL removes xylopyranosyl residues, presumably from the non-reducing end of the polysaccharide, whereas ARA-I liberates terminal xylopyranosyl residues and also has a limited ability to remove arabinofuranosyl substituents. With the addition of *Thermomyces* endoxylanase (*lane 3*), release of D-xylose was increased for both ARA-I (500 $mU.ml^{-1}$, 4-NPA) (*lane 4*) and XYL (500 $mU.ml^{-1}$, 4NPX) (*lane 5*) at reduced levels. Standards were L-arabinose (10 *n*mol), D-xylose (10 *n*mol) and oligoxylosides X_2-X_6 (*lanes 6*, *7* and *8*). (B) AXAH-I (500 $mU.ml^{-1}$, 4NPA) was incubated with wheat arabinoxylan as described for ARA-I and XYL. L-Arabinose was the sole product released by this enzyme (*lane 1*). AXAH-I (500 $mU.ml^{-1}$, 4NPA) and endoxylanase (*lane 3*) appear to act synergistically with an slight increases in the levels of released L-arabinose and low M_r oligosides. Standards were as described for (A) above (*lanes 4*, *5* and *6*).

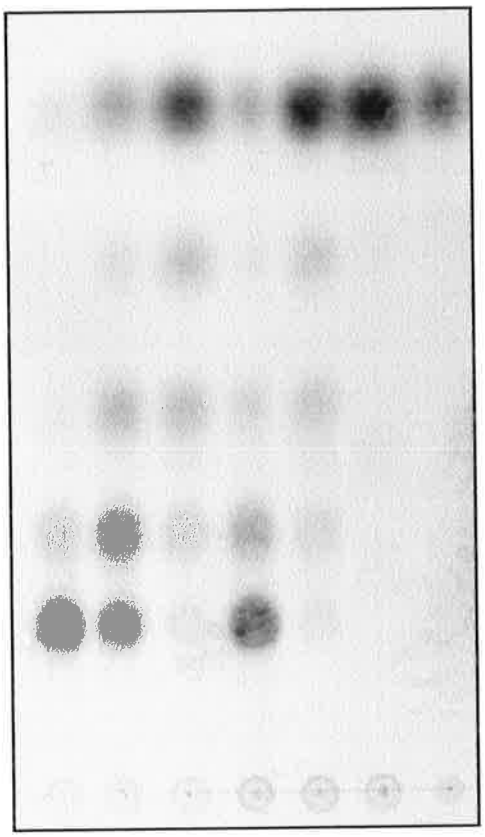

Figure 4.6 **Analysis of hydrolytic activities of ARA-I and XYL on (1→4)-xylopentaose by thin-layer chromatography.** ARA-I (100 $mU.ml^{-1}$, 4NPA) and XYL (100 $mU.ml^{-1}$, 4NPX) were incubated with xylopentaose (10 mM) at 37°C. Progress of hydrolysis of the substrate was analysed by chromatography on silica-gel thin-layer plates, immediately after addition of the enzyme, after 10 min and 60 min. Both ARA-I and XYL can remove xylopyranosyl residues, presumably from the non-reducing end of the oligosaccharide, and the rate of hydrolysis for XYL was higher than for ARA-I. D-Xylose (10 *n*mol) was used as a standard.

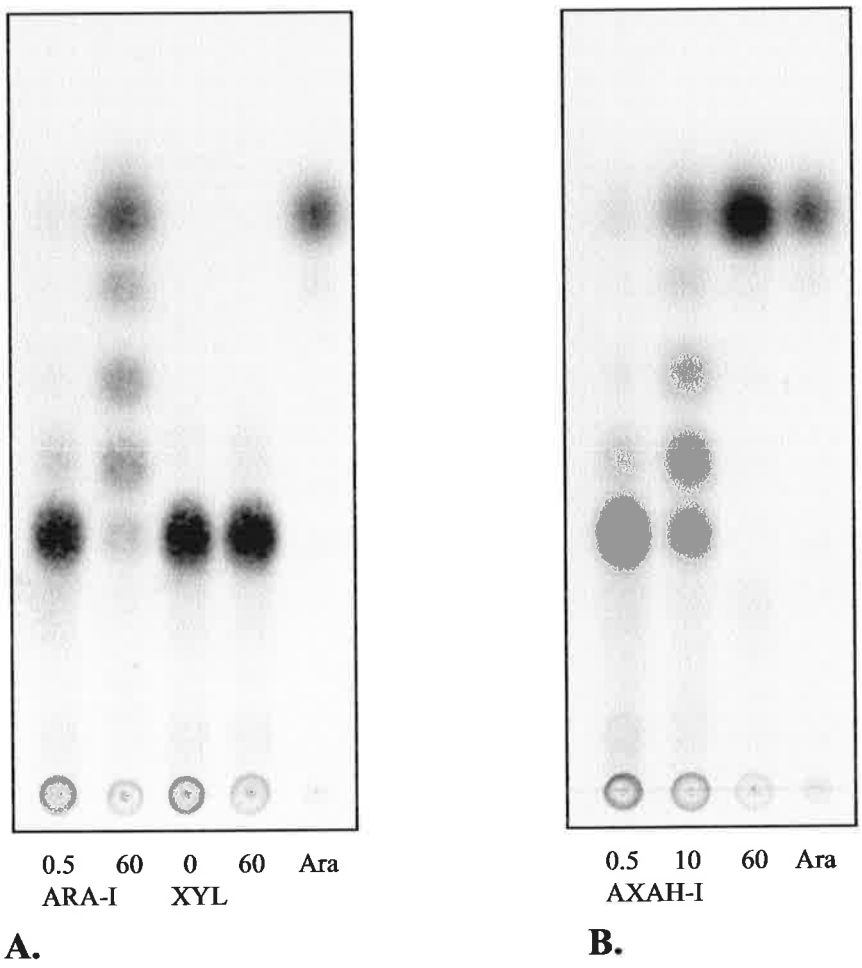

Figure 4.7 **Analysis of hydrolytic activities of ARA-I and XYL (A) and AXAH-I (B) on (1→5)-arabinohexaose by thin-layer chromatography.** (A) ARA-I (100 $mU.ml^{-1}$, 4NPA) and XYL (100 $mU.ml^{-1}$, 4NPX) were incubated with (1→5)-linked arabinohexaose (10 mM) at 37°C. Progress of substrate degradation was analysed by chromatography on silica-gel thin-layer plates, immediately after addition of the enzyme and after 60 min. L-Arabinose (10 *n*mol) was used as a standard. Of these two family 3 enzymes, only ARA-I can hydrolyse arabinohexaose. (B) Hydrolysis of arabinohexaose by AXAH-I (100 $mU.ml^{-1}$, 4NPA) immediately after addition of the enzyme, at 10 min and at 60 min. Assay conditions were as described for (A) above and the standard was L-arabinofuranose (10 *n*mol). AXAH-I is capable of hydrolysing the (1→5)-linkages of oligoarabinosides.

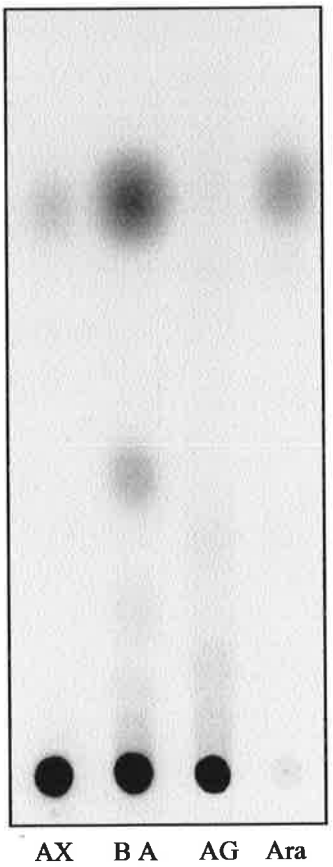

Figure 4.8 **Analysis of hydrolytic activities of AXAH-I on wheat arabinoxylan (AX), beet arabinan (BA) and larch arabinogalactan (AG).** AXAH-I (200 $mU.ml^{-1}$, 4NPA) was incubated with 0.5 % (*w/v*) wheat arabinoxylan, beet arabinan and larch arabinogalactan, for 1 h at 37°C. L-Arabinose was released from each of the polysaccharides by AXAH-I at different rates. Secondary products were evident in the hydrolysis products released from arabinan and arabinogalactan.

arabinofuranosyl residues. *Thermomyces* sp. (1→4)-β-D-xylan endohydrolase hydrolysed the polysaccharide to produce oligoarabinoxylosides; L-arabinose and D-xylose were not released by incubation with this (1→4)-β-D-xylan endohydrolase (*Figure 4.5 A*). Release of D-xylose from arabinoxylan by both ARA-I and XYL was greatly increased in the presence of *Thermomyces* sp. (1→4)-β-D-xylan endohydrolase. As expected, the β-D-xylosidase activities of ARA-I and XYL appear to operate synergistically with (1→4)-β-D-xylan endohydrolase to increase the production of D-xylose.

In contrast, AXAH-I (500 $mU.ml^{-1}$ as measured on 4NPA) more efficiently removes arabinofuranosyl substituents from arabinoxylan compared with ARA-I. AXAH-I was unable to remove D-xylopyranosyl residues, as was achieved by both ARA-I and XYL (*Figure 4.5 B*). The addition of *Thermomyces* sp. (1→4)-β-D-xylan endohydrolase increased the level of L-arabinose released by AXAH-I.

TLC analysis was used to monitor the hydrolysis of (1→4)-β-D-xylopentaose by ARA-I and XYL (*Figure 4.6*). Both enzymes, at concentrations of 100 $mU.ml^{-1}$ determined using the respective preferred substrate, were incubated with xylopentaose. Each was capable of hydrolysing the substrate and XYL completely hydrolysed xylopentaose to D-xylose within 1 h under the conditions used. ARA-I was less efficient in hydrolysing xylopentaose and after 1 h had degraded the substrate to approximately the same degree as XYL after 10 min. AXAH-I was unable to hydrolyse xylopentaose.

Hydrolysis of (1→5)-α-L-arabinohexaose by ARA-I, XYL and AXAH-I was investigated by incubation of 100 $mU.ml^{-1}$ of enzyme, measured using the preferred substrate in each case, with 10 mM arabinohexaose at 37°C for up to 1 h (*Figure 4.7*). Arabinohexaose was only partially degraded by ARA-I in 1 h and XYL was incapable of hydrolysing the α-L-arabinofuranosyl linkages of arabinohexaose (*Figure 4.7 A*). In contrast, AXAH-I completely degraded arabinohexaose within 1h (*Figure 4.7 B*). In comparison to the hydrolysis of wheat flour arabinoxylan, AXAH-I released L-arabinose from sugar beet arabinoxylan at a relatively rapid rate. Low activity was also detected towards larch wood arabinogalactan (*Figure 4.8*).

Table 4.4 **Changes in linkage composition of wheat arabinoxylan after incubation with AXAH-I.** Results are means for two sets of duplicated analyses and the difference column shows the change in amount of linkage type where significant.

Deduced linkage	Mol %		
	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
1,2,3,4-Xyl*p*	8	5	-3

4.3.5 *Investigation of AXAH-I Activity Using Polysaccharide Linkage Analysis*

Linkage composition of hydrolysis products after incubation of AXAH-I with arabinoxylan for 16 h at 37°C, were determined, and compared with that of undigested arabinoxylan (*Table 4.4*). Approximately 60% of the arabinofuranosyl substituents were removed and AXAH-I appears to preferentially hydrolyse (1→3)-α-linked arabinofuranosyl linkages. AXAH-I also removes (1→2)-α-linked arabinofuranosyl substituents and α-L-arabinofuranosyl substituents were released from doubly substituted xylopyranosyl residues. The results presented in *Table 4.4* also show that not all arabinofuranosyl substituents were removed after incubation with AXAH-I. One might expect some precipitation of de-arabinosylated arabinoxylan if a large proportion of arabinofuranosyl residues were removed (Andrewartha *et al.*, 1979), but under the conditions used here no precipitation of residual, modified substrate was observed.

4.4 DISCUSSION

4.4.1 *pH Dependence of Activity*

The pH at which an enzyme is most active is determined primarily by the pK_a values of catalytic amino acids (Segel, 1975). Each of the enzymes had bell-shaped activity curves over the pH range used, indicating that two ionizable amino acid residues are involved in catalysis at the active site. For ARA-I and XYL, the pH optimum was 4.7 and for AXAH-I, the pH optimum was 4.3 (*Figure 4.1*). The pH optima for ARA-I and XYL are close to those found for other hydrolytic enzymes involved in mobilisation of the starchy endosperm (Fincher, 1989). However, the pH optimum of AXAH-I is significantly lower, and this could indicate that AXAH-I functions in a different tissue, at a different time, or at a different subcellular site.

The curves for ARA-I and XYL suggest pK_a values for the catalytic acid/base and catalytic nucleophile of about 5.7 and 3.7, respectively (*Figure 4.1*). Amino acid residues suggested to participate in catalysis in the ARA-I, XYL and AXAH-I were identified in the complete amino acid sequences derived from cDNA clones, and are discussed in *Chapter 5*.

4.4.2 *Substrate Specificity*

The hydrolytic capacities of ARA-I, XYL and AXAH-I were examined using both synthetic glycosides and naturally occurring polysaccharide substrates. In general, the family 3 enzymes ARA-I and XYL, exhibited relatively broad specificity against aryl glycosides and very low levels of activity against polysaccharides. On the other hand, the family 51 enzyme AXAH-I was highly specific for the α-L-arabinofuranosyl moiety of substrates, with hydrolytic activity only against 4NPA and the α-L-arabinofuranosyl substituents of arabinoxylans, arabinogalactan and arabinan.

The relative values for the specific activities of ARA-I and XYL against various aryl glycoside substrates are shown in *Table 4.1*. Both enzymes were capable of hydrolysing 4NPA and 4NPX, substrates that represent the α-L-arabinofuranosyl and β-D-xylopyranosyl residues present in the arabinoxylans of plant cell walls. At a substrate concentration of 2.5 mM and at pH 4.7, the specific activity of ARA-I on 4NPX was 20% of the specific activity determined using 4NPA. Conversely, XYL hydrolysed 4NPA with 3% of the specific

activity determined for XYL using 4NPX (*Table 4.1*). The designations of ARA-I and XYL were made on the basis of these substrate preferences, although it is acknowledged that ARA-I might be better described as an α-L-arabinofuranosidase/β-D-xylosidase.

ARA-I and XYL were also active on 4NP-α-L-arabino*pyran*oside (11% and 2%, respectively) and 4NP-β-D-galactopyranoside (16% and 3%, respectively). Both furanosyl and pyranosyl ring forms of L-arabinosides, and the D-galactoside and D-xylopyranoside have identical conformations of hydroxyl groups on C2 and C3. Hydrolytic activity against each of these glycosides by ARA-I and XYL indicated that enzyme-substrate binding might be more stringent towards the C2-C3 side of glycoside substrates. While the specific activities against aryl glycosides by no means indicate the true biological functions of these enzymes, their broad specificities with respect to the synthetic aryl glycosides are noteworthy properties. Broad specificities of the family 3 β-D-xylosidases have been widely reported. β-Xylosidases from *Aspergillus niger* (van Peij *et al.*, 1997) and *Aspergillus oryzae* (Kitamoto *et al.*, 1999) show highest activity against 4NPX, but also have varying degrees of proportional activity on 4NPA (5% and 12%, respectively). Similarly, the *Trichoderma reesei* β-D-xylosidase hydrolyses 4NP-β-D-glucopyranoside, 4NP-α-L-arabino*pyran*oside and 4NP-α-L-arabino*furan*oside (4NPA) with 34%, 25% and 15% of the activity observed for 4NPX, respectively (Margolles-Clark *et al.*, 1996). A family 3 enzyme from *Thermoanaerobacter brockii* has been characterized as a β-glucosidase/xylosidase with activity against both 4NPG and 4NPX (Breves *et al.*, 1997) and the *Thermoanaerobacter brockii* β-xylosidase/arabinosidase has major activities on 4NP-α-L-arabino*pyran*oside and 4NPX (Shao and Weigel, 1992). The primary purpose of the family 3 β-D-xylosidases in the degradation of β-D-xylans is revealed by their activity against (1→4)-β-linked oligoxylosides. The *T. ethanolicus* bifunctional enzyme is the only enzyme characterized in family 3 that removes L-arabinofuranosyl substituents from oligoarabinoxylosides (Shao and Weigel, 1992). Characterization of specific activities using aryl glycosides has shown that ARA-I and XYL have many of the hallmarks of the microbial family 3 enzymes. Kinetic analyses using both 4NPA and 4NPX for ARA-I and XYL have provided added perspectives to the question of their substrate preferences.

4.4.3 Kinetic Properties

Differences between the enzymes' kinetic properties are reflected in the shapes of Michaelis-Menten and Lineweaver-Burk curves; K_m values for hydrolysis of 4NPA by both enzymes were relatively high in comparison with the K_m values for 4NPX. The K_m values indicate that both ARA-I and XYL have high affinities for the aryl β-D-xyloside. Catalytic rate constants (k_{cat}) shown in *Table 4.2* reveal that ARA-I catalyses the hydrolysis of 4NPA a rate greater than 10-times that for 4NPX, despite having a low K_m for 4NPA. In effect, a molecule of ARA-I can hydrolyse 46 4NPA molecules every second under the conditions used. Under the same conditions, an ARA-I molecule can hydrolyse only 3.6 molecules of 4NPX each second.

The catalytic rate constants for XYL indicate that 4NPX is the preferred substrate for the enzyme but that 4NPA is also hydrolysed at a significant rate. The catalytic efficiency factor (k_{cat}/K_m) combines the substrate affinity and catalytic rate parameters of the enzyme and emphasises the differences between the hydrolytic activities of ARA-I and XYL with respect to the substrates, 4NPA and 4NPX. The β-D-xylosidase, XYL, with a catalytic efficiency factor of 9.1×10^3 $M^{-1}.sec^{-1}$, hydrolyses 4NPX with 30-times the efficiency of the hydrolysis of 4NPA. In contrast, the catalytic efficiency factor for ARA-I on 4NPA was approximately twice the value for the hydrolysis of 4NPX. Thus, the kinetic analysis suggests that ARA-I has a preference for the aryl α-L-arabinofuranoside but has a similar level of activity against the β-D-xyloside. The kinetic properties of ARA-I and XYL were similar to those of a β-xylosidase from *Trichoderma reesei* where the K_m was 0.42 mM for 4NPX (Hermann *et al.*, 1997) and also for the barley β-D-glucan glucohydrolase isoenzymes ExoI and ExoII, for which the K_m and k_{cat} values on 4NPG were 1.4 mM and 0.33 mM, and 5 sec^{-1} and 15 sec^{-1}, respectively (Hrmova and Fincher, 1998).

Kinetic analyses for AXAH-I using 4NPA and wheat arabinoxylan as substrates, were performed at the pH optimum of 4.3. The K_m value for barley AXAH-I against 4NPA (0.77 mM) was similar to K_m values determined for other family 51 α-L-arabinofuranosidases. α-L-Arabinofuranosidases from *Aspergillus niger*, *Bacillus stearothermophilus*, *Streptomyces lividans* and *Thermobacillus xylanolyticus* have K_m values of 0.68 mM (van der Veen, 1991), 0.42 (Gilead and Shoham, 1995), 0.6 mM (Manin *et al.*, 1994) and 0.72 mM (Debeche *et al.*, 2000), respectively. For the *Pseudomonas cellulosa* arabinofuranosidase, K_m, k_{cat} and k_{cat}/K_m

values using 4NPA were 0.18 mM, 30 sec^{-1} and 167 $sec^{-1}.mM^{-1}$ (Beylot *et al.*, 2001*b*). The *P. cellulosa* enzyme therefore has approximately 10-fold greater catalytic efficiency than barley AXAH-I.

Using wheat arabinoxylan as a substrate for the kinetic analysis of AXAH-I, a greater degree of experimental error was observed due to problems associated with polysaccharide substrates. Difficulties in the examination of catalytic properties using substrates such as (1→3,1→4)-β-glucan and laminarin have been ascribed to problems of substrate diffusion and the presence of products in the experimental system that can subsequently be used as new substrates (Akiyama *et al.*, 1997; Hrmova and Fincher, 1998). While a similar situation was experienced at the near-saturation conditions of arabinoxylan, an additional degree of ambiguity probably results from the complexity of the arabinofuranosyl substitution pattern. AXAH-I would almost certainly have different capacities to remove arabinofuranosyl substituents from the C(O)2, C(O)3 and C(O)2,3 of β-D-xylopyranosyl residues. These possibilities raise questions concerning the accuracy of the kinetic properties determined for AXAH-I on arabinoxylan. Further insights on the biochemical properties could be gained using a range of chemically defined oligosaccharides such as those described by Viëtor *et al.* (1994*a*). Detailed studies of this kind were not performed because such substrates were not available. Nevertheless, the K_m, k_{cat} and k_{cat}/K_m values for AXAH-I using wheat arabinoxylan were obtained (*Figure 4.4*; *Table 4.3*). The catalytic rate constant (k_{cat}) of 6.9 sec^{-1} was similar to the k_{cat} value obtained for AXAH-I using 4NPA (15.0 sec^{-1}) and indicates that any limitation in catalytic efficiency of AXAH-I in the hydrolysis of arabinoxylans is probably imposed by the low substrate affinity of the enzyme. The k_{cat} value for AXAH-I using wheat arabinoxylan of 6.9 sec^{-1} was similar to the k_{cat} of 5.4 sec^{-1} reported for the *Pseudomonas cellulosa* family 51 arabinofuranosidase (Beylot *et al.*, 2001*b*).

4.4.4 Action Pattern

Products of hydrolysis after incubation of ARA-I, XYL and AXAH-I with wheat arabinoxylan and other arabinose or xylose-containing substrates were analysed by thin-layer chromatography. ARA-I and XYL (*Figure 4.5 A*; *lanes 1* and *2*, respectively) had minimal activity on wheat arabinoxylan with only a small amount of D-xylose being released from the polysaccharide in each case. The dual α-L-arabinosidase/β-D-xylosidase capacity of ARA-I was confirmed by the analysis of products released from arabinoxylan with both L-arabinose

and D-xylose produced after hydrolysis of arabinoxylan by ARA-I (*Figure 4.5 A*; *lane 1*). β-D-Xylosidase action of ARA-I and XYL is expected to only release non-reducing terminal D-xylopyranosyl residues from arabinoxylans where L-arabinofuranosyl substituents do not prevent such action. Without the removal of L-arabinofuranosyl substituents nearest to the non-reducing terminal xylopyranosyl residue, further β-D-xylosidase action might not proceed. With limits imposed on the β-D-xylosidase activity of ARA-I and XYL against arabinoxylan, roughly the same amount of D-xylose was released by both enzymes. The small number of α-L-arabinofuranosyl residues released by the α-L-arabinofuranosidase capability of ARA-I indicates that only a small proportion of arabinofuranosyl substituents on arabinoxylan are susceptible to removal by ARA-I. These might be single arabinofuranosyl groups on either C(O)2 or C(O)3 of xylopyranosyl residues with several unsubstituted xylopyranosyl residues at each side.

The synergistic effects of (1→4)-β-D-xylan endohydrolase with the activities of ARA-I and XYL were studied by analysis of hydrolysis products after incubation with enzyme combinations (*Figure 4.5 A*; *lanes 3* to *5*). Following hydrolysis of backbone (1→4)-β-D-xylopyranosyl linkages of wheat flour arabinoxylan, the effective concentrations of non-reducing termini and oligosaccharide substrates for the β-D-xylosidase activities of ARA-I and XYL were elevated; hence production of D-xylose was dramatically increased. Xylobiose released by (1→4)-β-D-xylan endohydrolase was completely hydrolysed by the β-D-xylosidase activity of both ARA-I and XYL (*Figure 4.5 A*; *lanes 4* and *5*). Complete hydrolysis of a presumed oligoarabinoxyloside, which had a relative mobility similar to that of xylotriose, was achieved by the combined α-L-arabinofuranosidase and β-D-xylosidase activities of ARA-I (*Figure 4.5 A*; *lane 5*). The amount of L-arabinose released from arabinoxylan by ARA-I was unchanged with the addition of (1→4)-β-D-xylan endohydrolase. It is likely that the susceptibility of L-arabinofuranosyl substituents to hydrolysis is unchanged after hydrolysis of arabinoxylan by (1→4)-β-D-xylan endohydrolase.

The complete depolymerization of arabinoxylan requires the activities of several different enzymes. These experiments show that the family 3 enzymes, ARA-I and XYL, from barley have limited activity on arabinoxylan, but significant β-D-xylosidase activity on

oligosaccharides that result from prior hydrolysis of the polysaccharide by (1→4)-β-D-xylan endohydrolase. The biological substrates for both enzymes, *in vivo*, are probably these low M_r arabinoxylan-derived oligosaccharides. The classification of ARA-I as an α-L-arabinofuranosidase/β-D-xylosidase is supported by this examination of action pattern.

The action pattern of AXAH-I on wheat arabinoxylan is markedly different to that of ARA-I and XYL. Hydrolysis of wheat flour arabinoxylan showed that AXAH-I has significant α-L-arabinofuranosidase activity but has no β-D-xylosidase activity (*Figure 4.5 B*). AXAH-I had a greater capacity than ARA-I to remove α-L-arabinofuranosyl substituents from arabinoxylan (*Figure 4.5 B*; *lane 1*). The addition of (1→4)-β-D-xylan endohydrolase enhanced the rate of production of L-arabinose by AXAH-I, presumably through the generation of smaller substrates that diffused more quickly. Furthermore, AXAH-I may have produced additional sites for the action of (1→4)-β-D-xylan endohydrolase, as indicated by the production of slightly higher amounts of intermediate oligosaccharides than those produced by (1→4)-β-D-xylan endohydrolase alone (*Figure 4.5 B*). Ferré *et al.* (2000) showed that barley AXAH can remove both C(O)2-linked and C(O)3-linked arabinofuranosyl substituents from oligoarabinoxylosides. Barley AXAH attacks C(O)3-linked substituents preferentially and successively removes both residues from doubly-substituted xylopyranosyl residues (Ferré *et al.*, 2000).

Analyses of products released by ARA-I, XYL and AXAH-I, from (1→4)-β-D-xylopentaose and (1→5)-α-L-arabinohexaose were performed using thin-layer chromatography. *Figure 4.6* shows that both XYL and ARA-I hydrolyse xylopentaose at significant rates that reflect their activities on 4NPX. ARA-I has the capacity to hydrolyse (1→5)-α-linked arabinohexaose and could potentially participate in pectin depolymerization. In comparison with the activity of ARA-I, an equivalent 100 $mU.ml^{-1}$ of AXAH-I has a significantly greater ability to degrade arabinohexaose. The ability to hydrolyse (1→2)- and (1→3)-α-L-arabinosides was not tested because these substrates were unavailable, although AXAH-I hydrolyses branched beet arabinan at a relatively high rate (*Figure 4.8*). Family 51 α-L-arabinofuranosidases from *Aspergillus awamori* (Kaneko *et al.*, 1998), *Streptomyces chartreusis* (Matsuo *et al.*, 2000) and *Pseudomonas cellulosa* (Beylot *et al.*, 2001*b*) hydrolyse (1→5)-, (1→3)- and (1→2)-α-L-arabinofuranosyl linkages. Since other

arabinosidases from this group are active on linear and branched arabinans, similar activities could be expected for AXAH-I.

The action of AXAH-I was further examined by analysis of the linkage composition after hydrolysis of arabinoxylan. *Table 4.4* shows that AXAH-I releases both C(O)2- and C(O)3-substituted arabinofuranosyl residues and also residues doubly substituted at C(O)2 and C(O)3. These results are consistent with those reported by Ferré *et al.* (2000).

4.4.5 Summary

The biochemical properties of each of the purified enzymes described here are consistent with those of previously characterized family 3 and family 51 enzymes from bacteria and fungi. ARA-I and XYL have similar β-D-xylosidase activities to other family 3 β-D-xylosidases. ARA-I has an additional α-L-arabinofuranosidase capability and has limited hydrolytic activity against α-L-arabinofuranosyl substituents of arabinoxylan and substantial hydrolytic activity against (1→5)-α-L-oligoarabinosides. With considerable amounts of ARA-I, XYL and AXAH-I produced in young barley seedlings, the target substrates for these enzymes are most likely the abundant cell wall polysaccharide, arabinoxylan, and its degradation products. The enzymes have discrete biochemical activities *in vitro* and probably have defined functional roles in physiological processes such as modification of arabinoxylan structure, or cell wall polysaccharide degradation and turnover. The α-L-arabinofuranosidase activities of ARA-I and AXAH-I may have additional roles in the metabolism of other arabinose-containing compounds such as pectin and arabinogalactan protein. The detailed examination of biochemical function of these important arabinoxylan-degrading enzymes will have further relevance to later studies of their biological significance in barley physiology.

CHAPTER 5.

Molecular Cloning of cDNAs Encoding Barley α-L-Arabinofuranosidases and a β-D-Xylosidase

5.1 INTRODUCTION

In the preceding Chapters, the purification and partial amino acid sequences of three barley enzymes with potential roles in the metabolism of arabinoxylans have been described. Accurate amino acid sequences are central to the identification of particular proteins and provide the basis for the isolation of corresponding DNA sequences. The isolation and characterization of cDNA or gene sequences permit the complete amino acid sequence, or primary structure, of a protein to be deduced. Additional information regarding potential post-translational modification sites and cellular targeting sequences can be extracted from cDNA sequences and derived amino acid sequences. Furthermore, DNA sequences that encode specific proteins are commonly used as molecular probes for the investigation of gene expression and in genetic mapping studies.

Recent developments in the study of plant genomes in *Arabidopsis thaliana* and rice, and transcript sequencing in a wide range of plant species including barley, have produced vast catalogues of DNA sequence information (Bevan *et al.*, 2001). In many cases, the functions of the products of plant genes have been inferred from previously characterized homologous proteins that have been studied in microbial or in other plant systems. Local alignment (BLAST) algorithms (Altschul *et al.*, 1990) reveal conserved amino acid sequence motifs that may reflect common structural and functional properties in homologous proteins. Although genomic information pertaining to barley is limited, EST sequences from barley can be useful for the isolation of cDNAs from plants. However, full-length cDNAs can only be obtained using classical methodologies such as cDNA library screening, reverse-transcription-PCR (RT-PCR) and rapid-amplification-of-cDNA-ends-PCR (RACE-PCR) (Frohman *et al.*, 1988).

The isolation of cDNA sequences encoding barley proteins, ARA-I, XYL and AXAH-I is described in this Chapter. A cDNA encoding a second AXAH isoenzyme, designated AXAH-II, was also identified in this work. The isolation of cDNAs corresponding to the partially purified ARA-II protein (*Chapter 2*) was attempted but was unsuccessful and is not discussed further here. Complete DNA sequences for each of the cDNAs and derived amino acid sequences are presented. The primary structures of the family 3 and family 51 enzymes are discussed, in detail, in the context of known features of other members of these two glycoside hydrolase groups.

5.2 Materials and Methods

5.2.1 Plant Material

Barley (*Hordeum vulgare* L. cv. Clipper) grain was sterilised in 0.2% (*w/v*) $AgNO_3$ and washed with sterile distilled water and 0.5 M NaCl as described in the preparation of germinated grain for protein isolation in *Chapter 2*. Grains were steeped for 24 h in antibiotic solution (100 mg/ml chloramphenicol; 100 mg/ml neomycin; 100 U/ml penicillin G and 100 U/ml nystatin) and germinated grains were grown in a sterile environment in the dark, at 22°C for up to 5 days. Moisture content was maintained at approximately 40-45% (*w/w*) by daily application of fresh antibiotic solution.

Seedling tissues were taken at intervals during early growth and immediately frozen in liquid N_2 for longer-term storage at -80°C. Leaf and coleoptile samples were taken at 5 and 4 days after germination, respectively, by excision as near to the grain as possible. Shoot samples represent the young leaves and coleoptiles combined. Rootlets were removed from the young seedlings five days after germination. Aleurone tissue was isolated at three days after germination, by removal of the scutellum and adjoining rootlets and shoots. Scutella were harvested from germinated grains at three days after germination by removal of rootlets and shoots at their base and excision of scutella from the adjacent aleurone layer and endosperm.

5.2.2 Total RNA Isolation and cDNA Synthesis

Barley seedlings and tissue samples (approximately 100 mg) were ground to a fine powder under liquid N_2 using a mortar and pestle, which had been pre-baked at 150°C for 4 h to eliminate ribonuclease contamination. Approximately 50 mg material was added to 1 ml Trizol reagent (GIBCO-BRL, Rockville, MD, U.S.A.) in a 2 ml Eppendorf tube and mixed vigorously to ensure rapid dispersal of the sample. Homogenised samples were allowed to stand at room temperature for 5 min prior to centrifugation at 12,000g for 15 min at 4°C. Supernatants were carefully removed to a 2 ml Eppendorf tube and RNA was isolated by phase separation with the addition of 200 µl chloroform followed by centrifugation at 12,000g for 15 min at 4°C. After centrifugation, the upper aqueous phase containing RNA was removed to a 2 ml Eppendorf tube. RNA was precipitated by addition of 0.5 ml isopropanol and centrifugation at 12,000g for 10 min at 4°C. The RNA pellet was washed with 1 ml 75% (*v/v*) aqueous ethanol. Following centrifugation at 7,500g for 5 min at 4°C,

supernatants were removed and RNA pellets allowed to air dry. RNA samples were resuspended in 30 µl ribonuclease-free water and incubated at 65°C for 5 min with vortex mixing. Starch and other insoluble polysaccharide materials were separated from the soluble RNA by 15 sec centrifugation at 12,000g. Total RNA samples were quantitated by absorbance at 260 nm and used immediately for first strand DNA synthesis.

First strand cDNA from RNA extracted from a range of barley seedling tissue samples was prepared using the Thermoscript RT-PCR system (GIBCO-BRL) as described by Frohman *et al.* (1988). Total RNA (3 µg) and 3 *p*mol $(dT)_{17}$-adaptor primer, ($^{5'}$GACTCGAGTCGACATCGAT$_{17}$ $^{3'}$) were annealed in 10 µl by denaturation at 65°C for 5 min followed by incubation on ice. The cDNA synthesis reagents [4 µl 5x cDNA synthesis buffer (GIBCO-BRL); 1 µl 0.1 M DTT; 2 µl 10 mM dNTPs; 1 µl ribonuclease-free water; 1 µl RNaseOut, ribonuclease H inhibitor; 1 µl Thermoscript RT (GIBCO-BRL)] were added and mixtures were incubated at 55°C for 60 min. At the completion of first strand synthesis, the RNA template was digested by incubation with 2 U RNase H at 37°C for 20 min, and cDNA was diluted to a final volume of 50 µl in deoxyribonuclease-free water.

5.2.3 Polymerase Chain Reaction (RT-PCR and 3'-end RACE PCR)

To isolate cDNAs corresponding to the purified enzymes, ARA-I, XYL and AXAH-I, RT-PCR, 3'-end RACE PCR and cDNA library screening procedures were employed. As a first approach, RT-PCR using total first strand cDNA from 3-day old seedlings as a template and various combinations of gene-specific degenerate primers, was attempted. Primer sequences were based on the amino acid sequences of NH_2-terminal and tryptic peptides for each of the respective proteins. PCR reactions consisted of the following: cDNA (1-3 µl); 5 µl 10x PCR buffer; 3 µl 25 mM $MgCl_2$; 2 µl 5 mM dNTPs; 1 µl each of 1-0.1 $\mu g.\mu l^{-1}$ degenerate primers (Geneworks, Adelaide, South Australia); 5 µl DMSO (Sigma Chemical Co., St. Louis, MO, U.S.A.); 1 µl *Taq* DNA polymerase (GIBCO-BRL, Rockville, MD, U.S.A.); deoxyribonuclease-free water to a final reaction volume of 50 µl. Reactions were performed in a Perkin-Elmer-Cetus thermal cycler as follows: 94°C, 2 min initial denaturation; 35 cycles of [denaturation at 94°C, 40 sec; annealing at primer specific temp (48-53°C), 40 sec; extension at 72°C, 1 min per 1000 bp of expected product size]; followed by 2 min final extension at 72°C. Amplified products were detected by electrophoresis at 100 V on 1%

(*w/v*) agarose (FMC BioProducts, Rockland, Maine, U.S.A.) in 1x TAE buffer (40 mM Tris-acetate buffer, pH 8.0, 1 mM EDTA) containing 0.1 $\mu g.ml^{-1}$ ethidium bromide. DNA bands were observed under ultraviolet light.

The 3'-end RACE PCR technique was employed for the amplification of cDNA fragments extending to the polyadenylate sequence at the 3'-end of mRNA transcripts. PCR was performed as described above with one gene-specific upstream primer and the 3'-end RACE adaptor primer, $^{5'}$GACTCGAGTCGACATCGA$^{3'}$, which anneals to the $(dT)_{17}$-adaptor primer sequence incorporated at the downstream end of cDNAs during first strand synthesis.

5.2.4 Cloning of cDNAs

PCR-amplified cDNA fragments were isolated by 1% (*w/v*) agarose gel electrophoresis, gel excision and purification using the Geneclean system (Geneworks). The DNA was ligated into pBluescript SK(+) (Stratagene, La Jolla, CA, U.S.A.) or pGEM-TEasy (Promega, Madison, WI, U.S.A.) vectors. Ligation mixtures (10 µl) contained insert and vector in a ratio of approximately 3:1, 5 U T4 DNA ligase (New England BioLabs, Beverly, MA, U.S.A.) and 1x ligation buffer (New England BioLabs). Ligation reactions were incubated for 14 h at 16°C. pBluescript SK(+) vector was prepared by prior digestion with restriction endonuclease *Eco*RV (New England BioLabs) and introduction of dTTPs at the cloning site by incubation of linear vector DNA with dTTP (Sigma Chemical Co.) and 5 U *Taq* DNA polymerase (GIBCO-BRL) in 100 µl containing 1x PCR buffer and 1.5 mM $MgCl_2$, at 72°C for 2 h.

Plasmid constructs were introduced into competent XL1-Blue (Stratagene, La Jolla, CA, U.S.A.) and DH5-α (CLONTECH, Palo Alto, CA, U.S.A.) *Escherichia coli* cells by heat-shock for 30 sec at 42°C, or electroporation using the Bio-Rad Gene Pulser apparatus (Bio-Rad, Hercules, CA, U.S.A.). Transformants were identified by growth of white colonies on Luria-Bertani (LB) agar containing ampicillin (200 $\mu g.ml^{-1}$), IPTG (40 $\mu g.ml^{-1}$) and X-Gal (40 $\mu g.ml^{-1}$). IPTG and X-Gal were from Sigma Chemical Co. Further screening of colonies was achieved by colony lift hybridization with labelled cDNA probes, as described below.

5.2.5 Colony Lift Hybridization

Putative *E. coli* transformants were streaked onto nitrocellulose membranes on duplicate LB agar plates containing ampicillin (200 $\mu g.ml^{-1}$), IPTG (40 $\mu g.ml^{-1}$) and X-Gal (40 $\mu g.ml^{-1}$), and incubated for 16 h at 37°C. DNA was fixed to the membrane by treatment on Whatman 3MM filter paper soaked with 10% SDS (10 min), denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 10 min and neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl buffer, pH 7.2) for 10 min. The membrane was washed in 2x SSC to remove bacterial debris and baked under vacuum at 80°C for 2h, prior to pre-hybridization and hybridization with labelled DNA probe as described in *Section 5.2.8*.

5.2.6 Screening of cDNA Libraries

A cDNA library was prepared by Dr Peilin Xu from poly(A)$^+$ RNA of 24-48 h GA_3-treated barley (cv. Clipper) aleurone layers using the ZAP-cDNA synthesis and cloning kit (Stratagene, La Jolla, CA, U.S.A.) according to the instructions supplied. The Uni-ZAP XR library was plated on competent XL1-Blue MRF' *E. coli* lawns at high density on NZY media and screened by performing duplicate plaque lifts onto nitrocellulose membranes (Micron Separations Inc., Westborough, MA, U.S.A.). Plaque replicas were fixed by treatment of membranes with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 10 min followed by neutralisation (1.5 M NaCl, 0.5 M Tris-HCl buffer, pH 7.2) for 10 min, rinsing in 2x SSC and oven baking at 80°C under vacuum for 2 h. Membranes were pre-hybridized and hybridized at 65°C as described *Section 5.2.8*, with [α-^{32}P]-dCTP-labelled ARA-500 probe and a positive clone was identified by autoradiography. The positive clone was excised and eluted in 500 µl SM buffer with 20 µl chloroform by vortex mixing and incubation at room temperature, and subsequently purified by two further rounds of plating and screening. The pBluescript phagemid containing the ARA-500-positive cDNA insert was excised from the Uni-ZAP XR vector using ExAssist helper phage in competent XL1-Blue MRF' *E. coli* cells, and plated in SOLR *E. coli* cells (Stratagene, La Jolla, CA, U.S.A.). A single colony containing the ARA-I-positive cDNA clone was isolated and DNA sequence determined, as described in *Sections 5.2.9* and *5.2.10*, respectively.

An 18 h GA_3-treated barley (cv. Himalaya) aleurone λZAP cDNA library was provided by Drs Frank Gubler and Jake Jacobsen (CSIRO Division of Plant Industry, Canberra, Australia). A λgt11 cDNA library prepared from 12-day old barley (cv. Klages) seedlings

was obtained from CLONTECH Laboratories (Palo Alto, CA, U.S.A.). These libraries were screened according to the procedures described above, with [α-^{32}P]-dCTP-labelled products of RT-PCR and 3'-end RACE, corresponding to ARA-I and XYL amino acid sequences. A barley (cv. Bark) expressed sequence tag (EST HK04K11), with sequence similarity to AXAH-I amino acid sequences, was generously provided by Dr Andreas Graner (IPK, Gatersleben, Germany) and used as a [α-^{32}P]-dCTP-labelled probe for screening of the 12-day old barley seedling cDNA library. Positive clones were identified by hybridization with the labelled EST. These were isolated as monoclonal phage stocks by successive rounds of screening, and cDNA inserts were sub-cloned into pBluescript SK(+) by ligation of agarose gel-purified, *Eco*RI-digested DNA into *Eco*RI digested pBluescript SK(+) DNA. λ DNA was prepared using a Wizard DNA purification kit (Promega, Madison, WI, U.S.A.) and the *Eco*RI digested cloning vector was de-phosphorylated with calf intestinal alkaline phosphatase (New England BioLabs). DNA ligation and transformation of *E. coli* cells were performed as described above.

5.2.7 *Preparation of [α-^{32}P]-dCTP-Labelled DNA probes*

DNA fragments excised from plasmid preparations, and PCR-amplified DNA purified from agarose gels were labelled with deoxycytidine-5'-[α-^{32}P]-triphosphate (Amersham, UK) in reactions primed with random sequence oligonucleotide nonamers using the Megaprime kit (Amersham). DNA was denatured at 100°C for 5 min and annealed with random primers in 33 µl followed by addition of 10 µl labelling buffer (Amersham), 5 µl [α-^{32}P]-dCTP and 2 µl DNA polymerase I Klenow fragment and incubation at 37°C for 60 min. Labelled DNA was separated from unincorporated [α-^{32}P]-dCTP by fractionation on a Sephedex G50 (Pharmacia, Uppsala, Sweden) column prepared in a glass Pasteur pipet and eluted with 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA. The labelled DNA probe was boiled for 5 min and added to hybridization buffer at 55-65°C.

5.2.8 *DNA Hybridization and Autoradiography*

Nitrocellulose membranes from plaque replica lifts (Micron Separations Inc., Westborough, MA, U.S.A.), Hybond-N membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) with bound PCR products transferred in 0.4 M NaOH from agarose gels, and nitrocellulose membranes from colony lifts were pre-hybridized and hybridized at 55-65°C depending on the degree of similarity between the probe sequence and that of the target DNA. Pre-

hybridization was performed for between 4 and 16 h in pre-hybridization buffer: 6 x standard saline citrate (0.9 M NaCl, 90 mM sodium citrate, pH 7.0); 5x Denhardt's solution (1% *w/v* Ficoll 500; Pharmacia, Uppsala, Sweden), 1% (*w/v*) polyvinylpyrrolidone (Sigma Chemical Co., St Louis, MO, U.S.A.), 1% (*w/v*) BSA (Sigma); 0.5% (*w/v*) SDS (BDH, Poole, England); 50 $\mu g.ml^{-1}$ sheared salmon sperm DNA (Worthington, Lakewood, NJ, U.S.A.). Hybridization of labelled DNA probe, pre-heated at 100°C for 5 min, was for 16 h in: 3x SSC (0.45 M NaCl, 45 mM sodium citrate, pH 7.0); 5x Denhardt's solution; 0.5% (*w/v*) SDS; 50 $\mu g.ml^{-1}$ sheared salmon sperm DNA.

Membranes were washed at the temperature of hybridization, 2-3 times in 0.5x SSC, 0.1% SDS; 0.2x SSC, 0.1% SDS; or 0.1x SSC, 0.1% SDS depending on the required stringency of hybridization. Washed membranes were blotted dry and autoradiography was performed with Fuji Super RX x-ray film (Fuji, Tokyo, Japan) and Du Pont (Wilmington, DE, U.S.A.) intensifying screens at -80°C. Autoradiographs were developed using a Curix system (Agfa, Greenville, SC, U.S.A.).

5.2.9 Isolation of Plasmid DNA and Restriction Enzyme Digests

Overnight cultures of transformed *E. coli* strains were grown at 37°C with shaking. Plasmid DNA was isolated by alkaline lysis of cells followed by removal of bacterial genomic DNA by centrifugation, and precipitation of plasmid DNA with 2 vol ethanol. Cells from 2 ml culture were precipitated by centrifugation, resuspended in 100 µl GTE buffer (0.9% *w/v* glucose; 10 mM EDTA; 25 mM Tris-HCl buffer, pH 7.5) and lysed by addition of 200 µl 0.2 M NaOH, 1% (*w/v*) SDS. After 2 min incubation on ice, lysed cells were neutralised with 150 µl 3 M potassium acetate, pH 4.8, and incubated at -20°C for 15 min. Plasmid DNA was isolated in the supernatant after centrifugation for 15 min at 10,000g at 4°C, and precipitated by addition of 1.0 ml ethanol, incubation on ice for 10 min and centrifugation at 10,000g for 10 min at 4°C. Precipitated DNA was washed once with 500 µl 70% (*v/v*) ethanol, dried under vacuum and resuspended in 50 µl TE buffer (10 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA) containing 40 $\mu g.ml^{-1}$ ribonuclease-H (Sigma). RNA was eliminated by incubation at 37°C for 20 min and DNA samples were stored at -20°C.

To 5 µl plasmid DNA preparation, 2 µl 10x restriction enzyme buffer, 2 µl 100 $\mu g.ml^{-1}$ BSA (if required), 1 µl of restriction enzyme and water to a final volume of 20 µl were added, and samples were digested at 37°C for 2 h. Digests were separated on 1% agarose gels in 1x TAE buffer containing 0.1 $\mu g.ml^{-1}$ ethidium bromide and observed using a UV transilluminator. Restriction endonucleases, *Eco*R1, *Eco*RV and *Xho*I were from New England Biolabs.

5.2.10 DNA Sequencing and Sequence Analysis

DNA sequencing was performed using the dideoxynucleotide chain termination procedure (Sanger *et al.*, 1977) using the ABI PRISM dye terminator kit (Applied Biosystems, Foster City, CA, U.S.A.) and an Applied Biosystems 370 DNA sequencer (Applied Biosystems). Plasmid DNA (0.5 µg) containing cloned insert was used in sequencing reactions primed with oligonucleotide primers corresponding to T7 and SP6 RNA polymerase promoter sequences flanking the cloning site of the pGEM-TEasy vector, or T3 and T7 sequences in the pBluescript vector. Sequencing of clones with unknown sequences was performed by priming from vector sequences in this fashion. For sequencing of the ARA-500 RT-PCR product, approximately 100 ng agarose gel-purified DNA and gene-specific degenerate primer were used. Complete DNA sequences of cDNA clones corresponding to purified proteins were obtained by primer 'walking' along both sense and antisense strands.

Data from automated sequencing were compiled and analysed using the Seq-Ed program (Applied Biosystems) and further alignment analyses of DNA sequences, and database searches were performed using the University of Wisconsin, Genetics Computer Group software (Devereux *et al.*, 1984) in the ANGIS suite of programs at the Australian National Genomic Information Service (http://www.angis.org.au). Sequences for phylogenetic analysis were aligned using the ClustalW program (http://www2.ebi.ac.uk/clustalw/) and unrooted phylogenetic trees were constructed using the PAUP program (Swofford, 1986).

5.3 RESULTS

5.3.1 *Isolation of ARA-I cDNAs*

RT-PCR

Degenerate oligonucleotide primers were designed to correspond with ARA-I tryptic amino acid sequences, which were aligned against the complete amino acid sequence of the homologous *Aspergillus niger* β-D-xylosidase (GenBank/EMBL accession Z84377) (*Figure 5.1*). RT-PCR was performed using all combinations of forward and reverse, gene-specific degenerate primers, with reverse transcribed 3-day old barley seedling mRNA. PCR reactions were performed, both with and without DMSO, and with either 2.5 mM $MgCl_2$ or 25 mM $MgCl_2$. A 500 bp cDNA fragment (*Figure 5.2*) was amplified using ARA-I T2F, $^{5'}$GGNATHCCNGCNTAYGARTGGTGG$^{3'}$ (forward primer) and ARA-I T6aR, $^{5'}$AANGGNGGYTGRAANGTRTCRTC$^{3'}$ (reverse primer) corresponding to GIPAYEWW and DDTFQPPF amino acid sequences of tryptic peptides ARA-I T2 and ARA-I T6a, respectively (*Figure 5.1*). The fragment amplified using these primers was of the expected size, based on the spatial arrangement of tryptic peptides against the *A. niger* xylosidase. The 500 bp fragment, designated ARA-500, was excised and purified from agarose gel for further use as a DNA probe in the subsequent screening of cDNA libraries. DNA sequence analysis showed that the PCR amplified fragment corresponded to the ARA-I cDNA, with the amino acid sequence of tryptic peptide ARA-I T1 (*Figure 5.1*) encoded by the translated cDNA (data not shown).

λZAP cDNA library screening

The ARA-500 cDNA fragment was used as a DNA probe for the screening of the 24-48 h GA_3-induced barley aleurone λZAP cDNA library. Approximately 200,000 cDNA clones were screened in XL1-Blue MRF' *E. coli* cells and one positive clone was identified by hybridization and autoradiography. A single ARA-I-positive clone was isolated after two further rounds of plaque purification (*Figure 5.3*) and a monoclonal phage stock was prepared by elution of an isolated plaque in SM buffer. The pBluescript plasmid containing the λZAP ARA-I cDNA clone was isolated by single-clone excision and an XL1-Blue *E. coli* cell-line was prepared for long-term storage in 20% (*v/v*) glycerol at -80°C. The complete sequence of the 1900 bp λZAP cDNA clone was obtained, only part of which corresponded with the predicted ARA-I cDNA sequence. Approximately 1200 bp

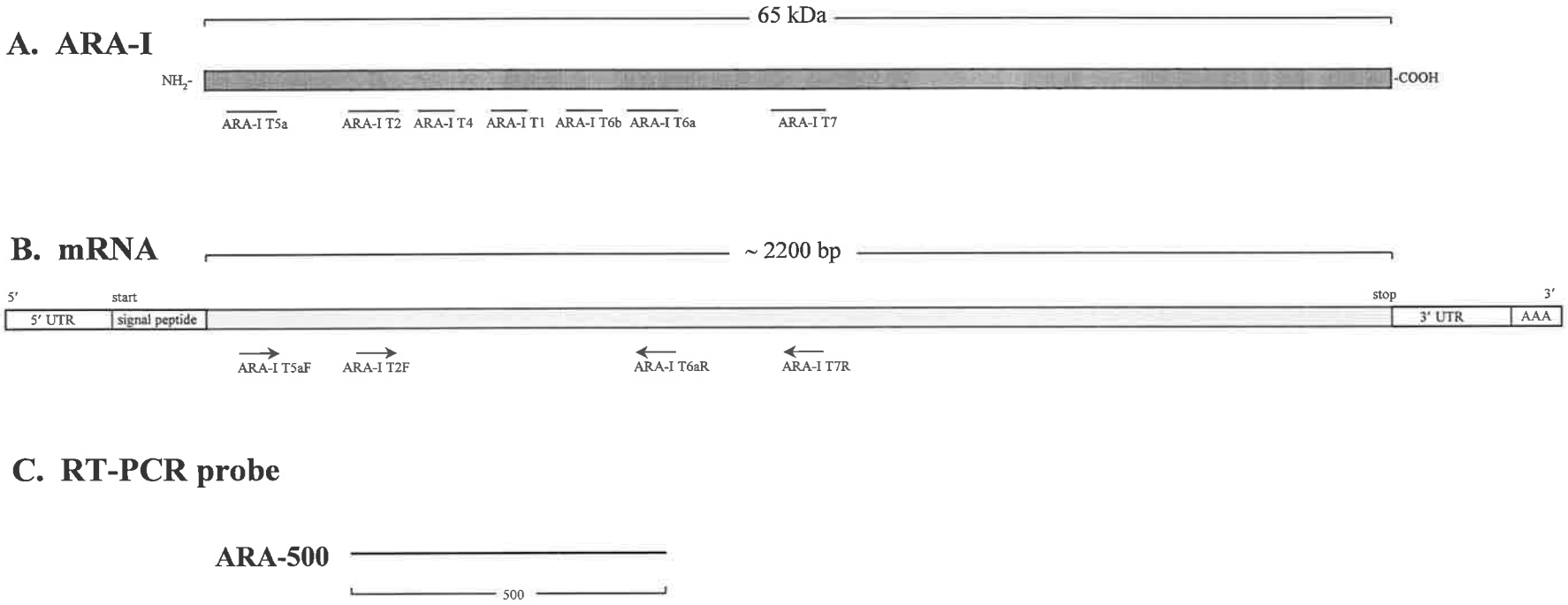

Figure 5.1 **Arrangement of tryptic peptides (A) for α-L-arabinofuranosidase (ARA-I) and corresponding degenerate oligonucleotides (B) against a hypothetical mRNA.** Tryptic peptides were aligned with the *Aspergillus niger* β-D-xylosidase (GenBank/EMBL accession Z84377) amino acid sequence and were used as a guide for oligonucleotide primer combinations for RT-PCR. Approximation of PCR product size is also shown (C).

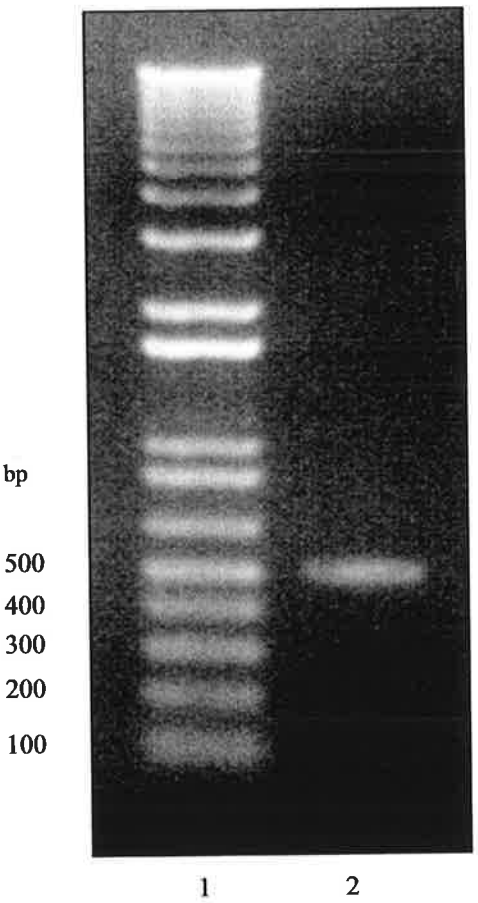

Figure 5.2 **Agarose gel electrophoresis of RT-PCR product from total RNA isolated from 3-day germinated barley.** *Lane 1*, DNA molecular size markers; *lane 2*, RT-PCR product of approximately 500 bp, amplified using ARA-I T2F and ARA-I *T6aR* degenerate oligonucleotide primers.

Figure 5.3 **ARA-I positive clones from a 24-48 h GA_3-induced barley aleurone cDNA library.** Single plaques were isolated after three rounds of screening with the 500 bp ARA-I cDNA fragment. Autoradiography film was exposed for 2 h.

corresponded to the 5' end of ARA-I cDNA but the remainder of the cDNA carried no sequence identifiable with ARA-I. It was therefore likely that the ARA-I clone contained a double insert. The 1200 bp ARA-I sequence was used for construction of the final ARA-I cDNA contiguous sequence. Further cDNA library screening and 3'-end RACE was required for the isolation of other parts of the ARA-I cDNA sequence.

3'-End RACE

Two non-degenerate forward primers, based on the exact DNA sequence of the λZAP cDNA, were used for nested 3'-end RACE PCR amplification of the ARA-I cDNA using root/shoot/scutellum cDNA as a template. ARA-I gene-specific primers, $^{5'}$CGGCGTACGAGTGGTGGTCCGAAG$^{3'}$ and $^{5'}$CGCTGCACGGCGTGTCATACGT$^{3'}$, corresponding to the PAYEWWSEALHGVSYV sequence of tryptic peptide ARA-I T2 (*Figure 5.1*), were used for two successive rounds of PCR in combination with the 3'-end RACE adaptor primer, which anneals to dT_{17}-adaptor-primed cDNAs. Bands were not evident in the first round of PCR, but in the second round, bands with a range of molecular sizes were observed, the largest of which was approximately 2400 bp in length (*Figure 5.4 A*). The 3'-end RACE products were blotted onto Hybond-N membrane and hybridized with the 500 bp ARA-500 cDNA probe. Exposure to autoradiography film for 5 min revealed strongly hybridizing bands that probably corresponded to the ARA-I cDNA (*Figure 5.4 B*). The 2400 bp band, being the largest and presumably the most complete cDNA fragment, was isolated from the agarose gel, purified and subsequently cloned into the pBluescript cloning vector. DNA sequencing revealed that the 2400 bp 3'-end RACE cDNA fragment contained DNA sequence corresponding to ARA-I tryptic peptide sequences and a polyadenylate sequence was present at the 3'-end of the cDNA. Two further independent ARA-I 3'-end RACE clones were isolated and the consensus sequence of these three clones was obtained for the later construction of a contiguous ARA-I cDNA sequence.

ARA-I cDNAs

The λZAP cDNA and the 3'-end RACE cDNA fragment almost covered the complete length of the expected ARA-I cDNA, but a short 5'-end sequence was yet to be isolated. This region was later covered by a λgt11 cDNA clone, one of two corresponding to ARA-I that were found while screening for XYL cDNA clones, described below. *Figure 5.5* shows the four partial cDNAs used to construct the full-length ARA-I cDNA sequence. cDNA inserts were

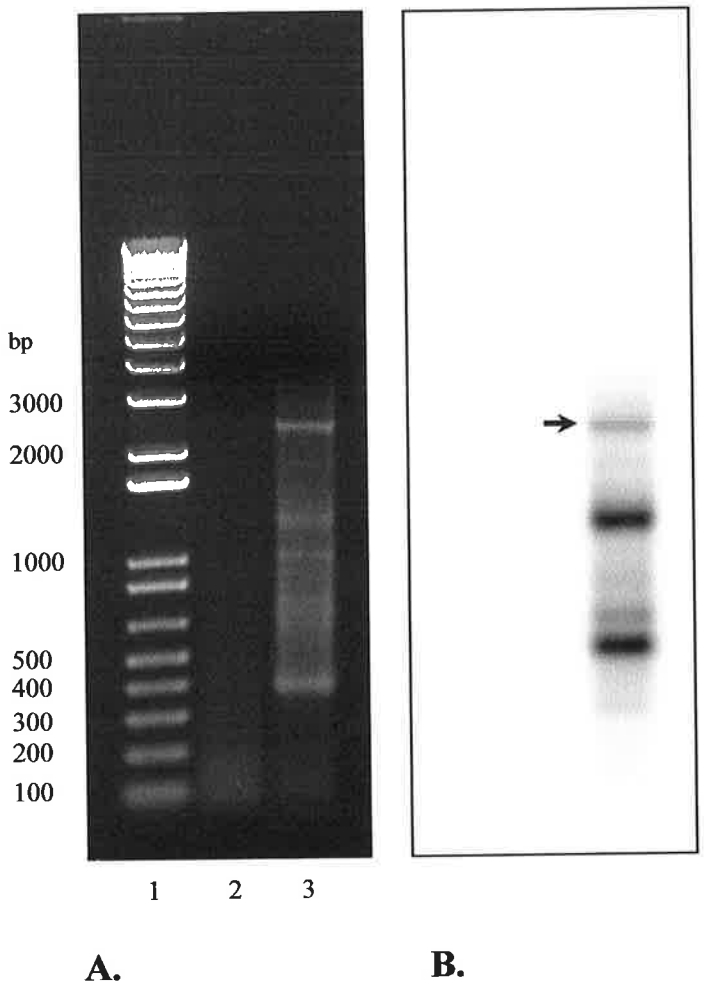

Figure 5.4 **Agarose gel electrophoresis (A) of products from 3'-end RACE-PCR using oligonucleotide primers for ARA-I cDNA and autoradiograph (B) of DNA blot probed with 500 bp ARA-I fragment.** (A) *Lane 1*, DNA molecular size markers; *lane 2*, first round of 3'-end RACE-PCR; *lane 3*, second round of 3'-end RACE-PCR using 1 µl of first round product as template and nested upstream primer with 3′RACE primer. (B) The agarose gel was blotted onto Hybond-N membrane and probed with a [α-^{32}P]-dCTP-labelled 500 bp ARA-I specific probe at 65°C. Autoradiography film was exposed for 5 min. The 2400 bp band (*arrow*) was excised from the gel and cloned.

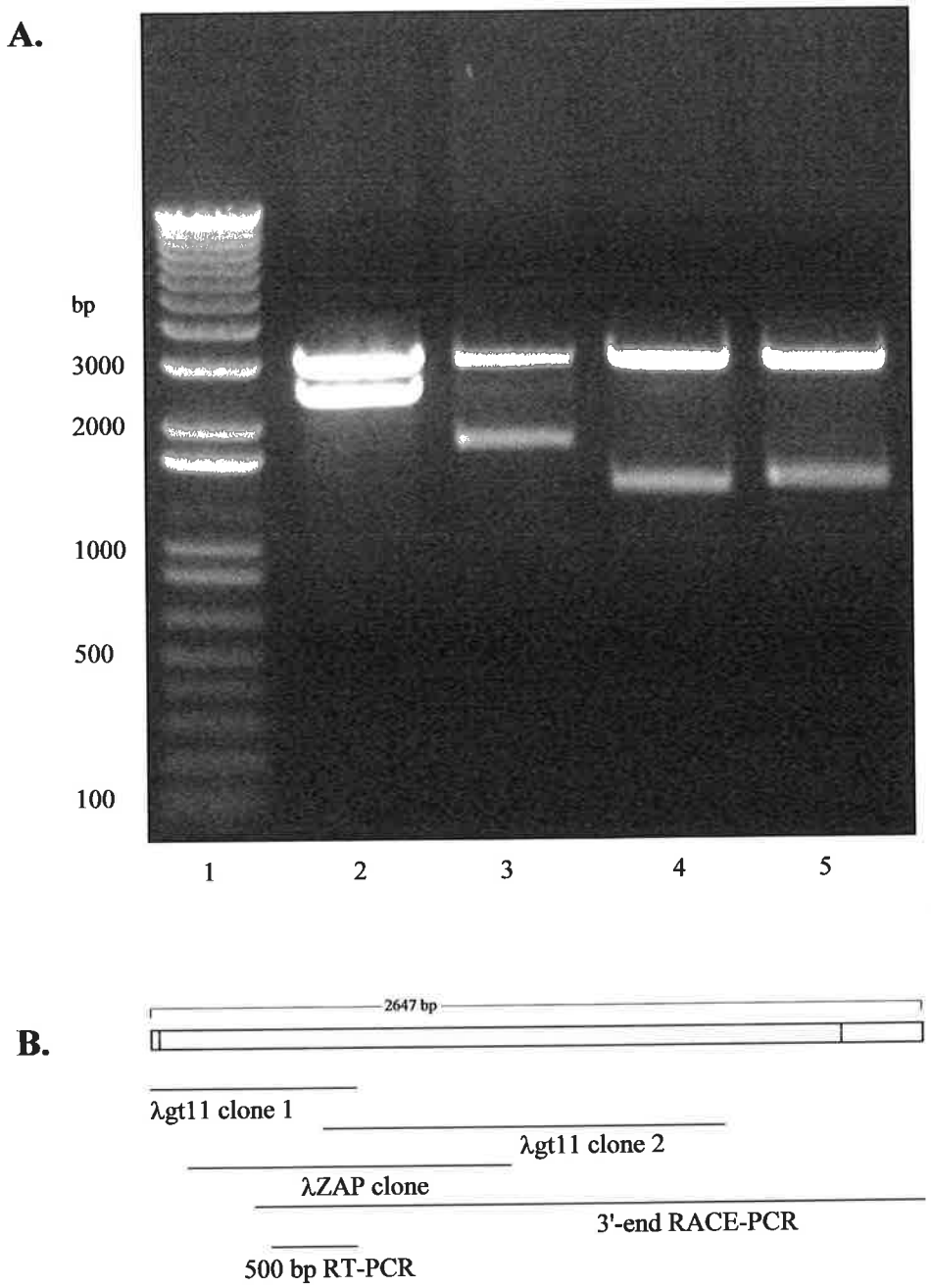

***Figure 5.5* Agarose gel electrophoresis of ARA-I cDNA clones.** (A) cDNA inserts were excised from their respective cloning vectors and electrophoresed on 1% (*w/v*) agarose. *Lane 1*, DNA molecular size markers; *lane 2*, ARA-I 2400 bp RT-PCR fragment (pBluescript SK^+); *lane 3*, ARA-I λZAP clone (pBluescript SK^+); *lane 4*, ARA-I 5'-end λgt11 clone (pBluescript SK^+); *lane 5*, ARA-I 3'-end λgt11 clone (pBluescript SK^+). (B) The full-length cDNA sequence of ARA-I was complied from two overlapping λgt11 clones, one λZAP cDNA clone and a 3'-end RACE-PCR clone. The position of the 500 bp ARA-I RT-PCR fragment is also shown.

excised from respective plasmid cloning vectors (*Figure 5.5 A*). The arrangement of overlapping ARA-I clones is also shown (*Figure 5.5 B*).

5.3.2 Isolation of XYL cDNAs

RT-PCR

RT-PCR was used for the isolation of a cDNA fragment corresponding to the XYL cDNA. Forward and reverse degenerate primers were synthesized to correspond with DNA sequences encoding XYL tryptic peptides (*Figure 5.6*). PCR reactions with various combinations of primers, together with other options of $MgCl_2$ concentration and presence or absence of DMSO, were attempted. Template DNA for RT-PCR was reverse-transcribed root/shoot/scutellum RNA prepared in $(dT)_{17}$-adaptor-primed reverse transcription reactions. A cDNA fragment of approximately 1300 bp was amplified using a combination of primers corresponding to XYL tryptic peptides, XYL T1 and XYL T7 (*Figure 5.7 A*). The sequences of these primers were: XYL T1F, $^{5'}$GTNCCNGCNTAYAAGTGGTGG$^{3'}$, corresponding to amino acid sequence VPAYKWWS, and XYL T7R, $^{5'}$TCYTGRTTYTGRTCNARNCCCAT$^{3'}$, the antisense sequence of that encoding MGLDQNQE. A very faint single band, when blotted onto Hybond-N membrane and hybridized with the [α-^{32}P]-dCTP-labelled 2400 bp ARA-I cDNA probe, was identified as a possible candidate for a cDNA encoding XYL (*Figure 5.7 B*). Hybridization was at low-stringency, and with the apparent homology between ARA-I and XYL amino acid sequences, it was likely that such hybridization conditions might identify a cross-hybridizing XYL cDNA. The 1300 bp band, and one other at approximately 800 bp hybridized the ARA-I probe with a strong hybridization signal. The 1300 bp DNA was purified from agarose gel and cloned into the pGEM TEasy cloning vector. The XYL RT-PCR fragment contained sequences that encoded XYL tryptic peptide amino acid sequences (data not shown). The XYL fragment was subsequently used for further screening of λZAP and λgt11 cDNA libraries for XYL cDNAs.

cDNA library screening

The λZAP 24-48 h GA_3-induced aleurone cDNA library and an 18 h GA_3-treated barley (cv. Himalaya) aleurone cDNA library were screened with the 1300 bp XYL cDNA probe. No positive clones were isolated from these libraries. However, when 200,000 cDNA clones in a 12-day old barley (cv. Klages) seedling λgt11 cDNA library were screened at low stringency with the XYL probe, 27 positive clones were identified. Different hybridization signals

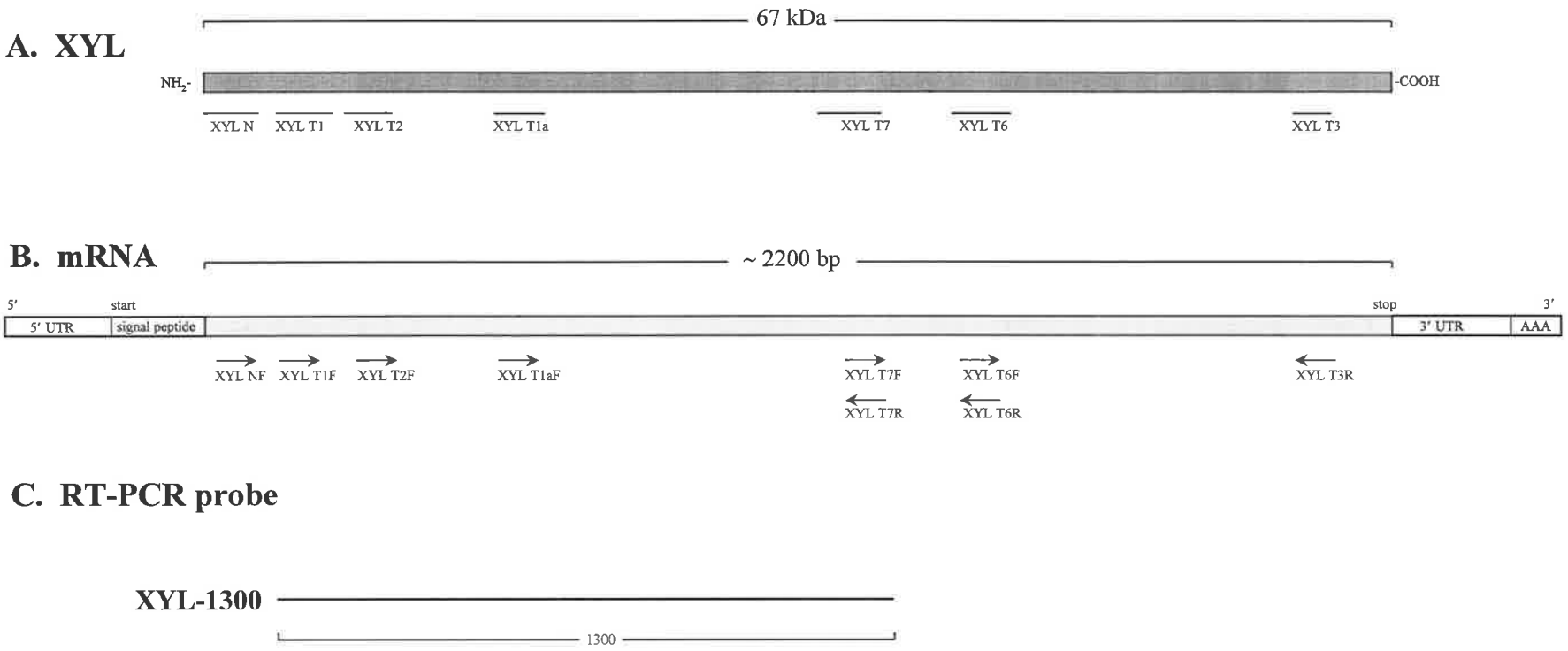

Figure 5.6 **Arrangement of tryptic peptides (A) for β-D-xylosidase (XYL) and corresponding degenerate oligonucleotides (B) against a hypothetical mRNA.** Tryptic peptides were aligned with the *Aspergillus niger* β-D-xylosidase (GenBank/EMBL accession Z84377) amino acid sequence and were used as a guide for oligonucleotide primer combinations for RT-PCR. Approximations of PCR product sizes are also shown (C).

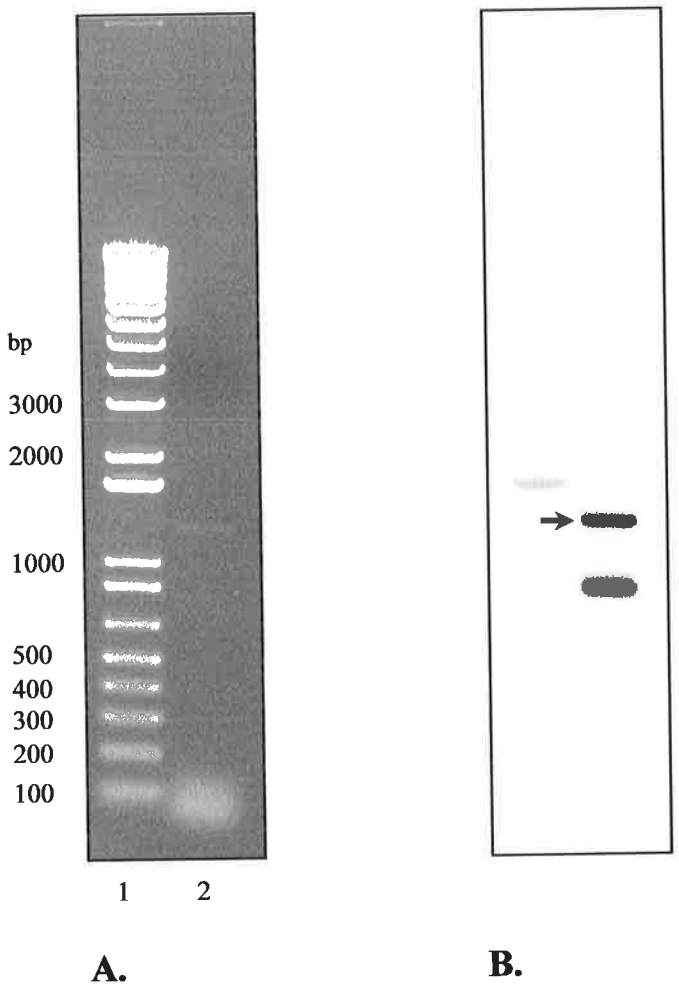

Figure 5.7 **Agarose gel electrophoresis (A) of products from RT-PCR using oligonucleotide primers for XYL cDNA and autoradiograph (B) of DNA blot probed with 2400 bp ARA-I fragment.** (A) *Lane 1*, DNA molecular size markers; *lane 2*, first round of 3'-end RACE-PCR. (B) The agarose gel was blotted onto Hybond-N membrane and probed with [α-^{32}P]-dCTP-labelled 2400 bp ARA-I cDNA. Autoradiography film was exposed for 2 h. The 1300 bp band (*arrow*) was excised from the gel and cloned.

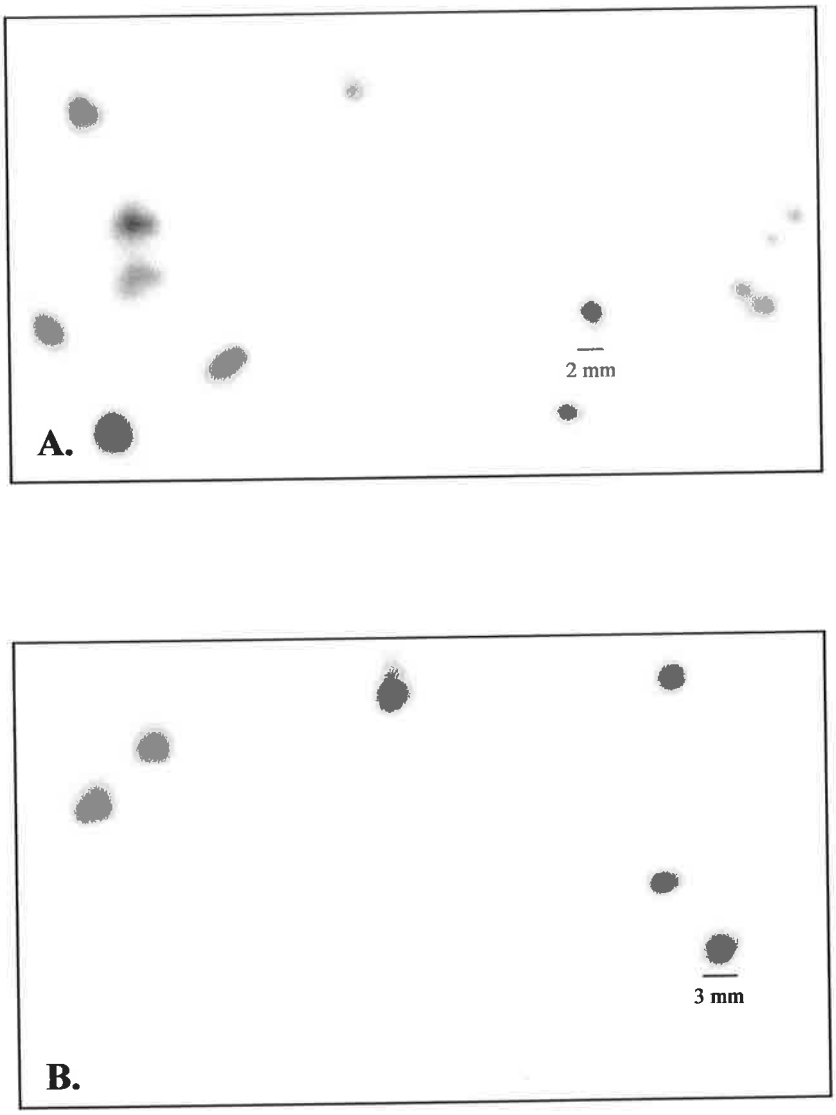

Figure 5.8 **XYL positive clones from a 12-day seedling λgt11 cDNA library hybridized with a 1300 bp RT-PCR fragment.** Single plaques were isolated after three rounds of screening with the 1350 bp XYL fragment. Bacteriophage were grown on *E. coli* Y1090 cells and lifted onto nitrocellulose membrane. After hybridization with [α-^{32}P]-dCTP-labelled probe at 55°C. Two sets of positive clones were identified based on the strength of the hybridization signal. Strong clones were exposed for 3.5 h (A) and weak clones (B) were exposed for 20 h.

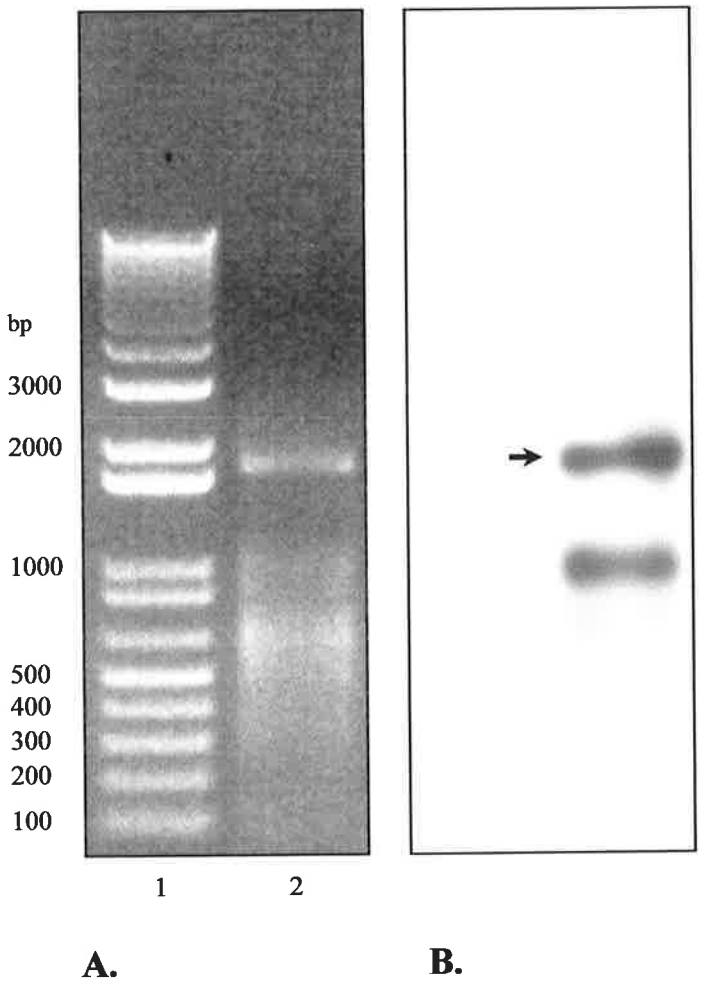

Figure 5.9 **Agarose gel electrophoresis (A) of products from 3'-end RACE-PCR using oligonucleotide primers for XYL and autoradiograph (B) of DNA blot probed with XYL RT-PCR fragment.** (A) *Lane 1*, DNA molecular size markers; *lane 2*, 3'-end RACE-PCR products. (B) The agarose gel was blotted onto Hybond-N membrane and probed with a [α-^{32}P]-dCTP-labelled 1300 bp XYL cDNA fragment at 65°C. Autoradiography film was exposed for 2.5 h. The 1800 bp band (*arrow*) was excised from the gel and cloned.

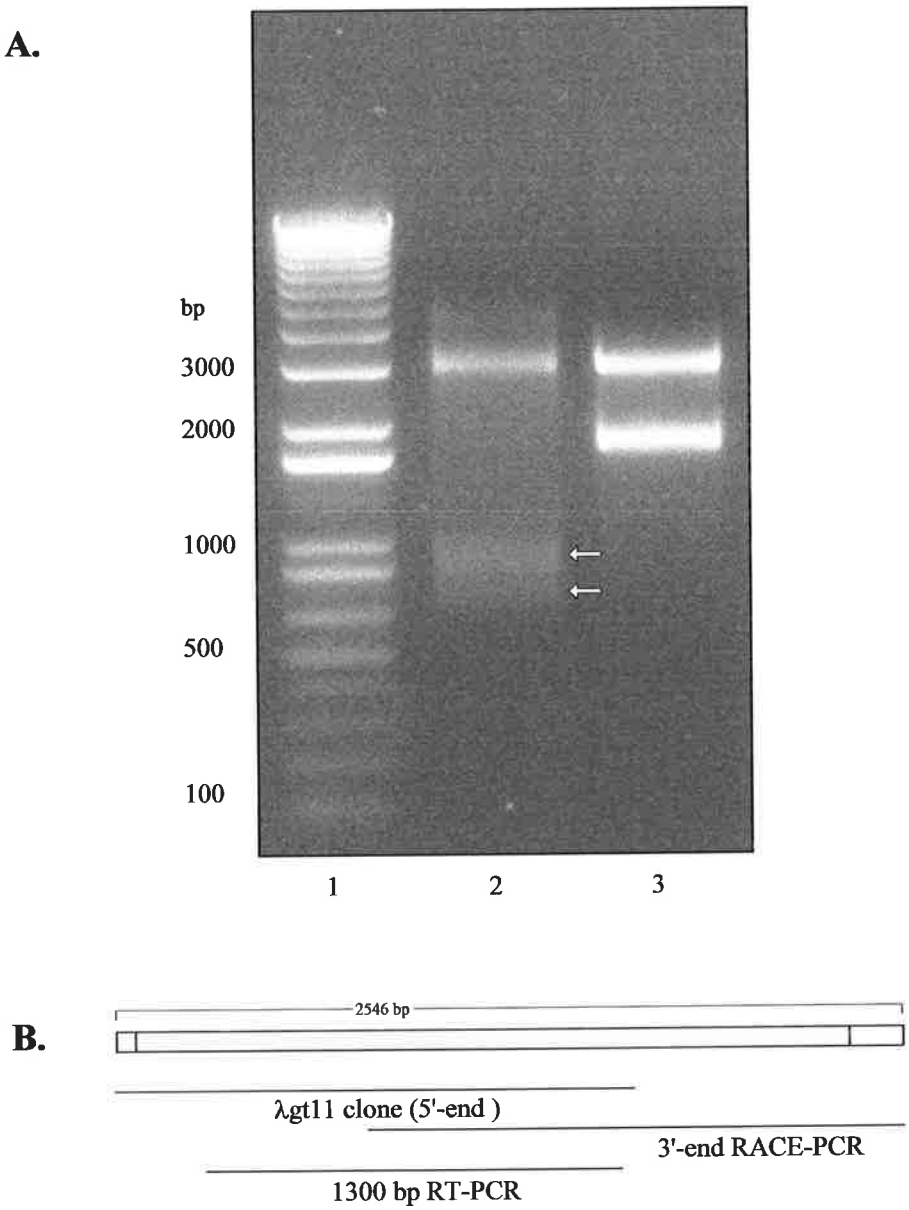

Figure 5.10 **Agarose gel electrophoresis of XYL cDNA clones.** (A) cDNA inserts were excised from their respective cloning vectors and electrophoresed on 1% (*w/v*) agarose. *Lane 1*, DNA molecular size markers; *lane 2*, XYL 5'-end λgt11 clone (pBluescript SK^+); *lane 3*, XYL 3'-end RACE product (pGEM TEasy). Digestion of the XYL 5'-end λgt11 clone with *Eco*RI and *Xho*I produced two bands (*arrows*) due to the presence of a *Xho*I restriction site at 710 bp. (B) The XYL cDNA sequence is a compilation of one λgt11 clone and a 3'-end RACE-PCR clone. The position of a 1300 bp RT-PCR fragment is also shown.

allowed two groups of clones to be distinguished (*Figure 5.8*). Strongly hybridizing plaques exposed to autoradiography film for 3.5 h (*Figure 5.8 A*) produced an equivalent signal to weakly hybridizing clones that were exposed for 20 h (*Figure 5.8 B*). Both types of positive clone were investigated further and 8 were selected for sub-cloning of cDNA inserts into pBluescript (SK+). One cDNA corresponded exactly with the expected XYL cDNA sequence and coincided with the 1300 bp XYL RT-PCR fragment with matching DNA sequence (*Figure 5.10 B*). Two other XYL-positive cDNA clones matched ARA-I clones isolated by RT-PCR, λZAP cDNA library screening and 3'-end RACE, described in *Section 5.3.1*. The overlapping clones corresponding to the ARA-I cDNA were as shown in *Figure 5.5*. Other XYL positive cDNA library clones were truncated versions of the ARA-I or XYL-encoding cDNAs. No obvious reasons for the variation in hybridization signal strength were found, and these observations were not investigated further.

3'-End RACE

The oligonucleotide $^{5'}$GGATACATCACGTCGGAC$^{3'}$ (position 919-936 in the final XYL cDNA sequence; *Figure 5.17*), formerly used for DNA sequencing of the λgt11 XYL cDNA clone, was used for 3'-end RACE, in conjunction with the 3'-end RACE adaptor primer and a root/shoot/scutellum cDNA population. DNA bands from XYL 3'-end RACE were identified by hybridization with a labelled 1300 bp XYL probe (*Figure 5.9 A* and *B*); the larger, 1800 bp band was isolated and subsequently cloned into pGEM TEasy cloning vector. Selection of positive transformants containing the pGEM vector and cloned XYL insert was facilitated by the colony lift hybridization technique. A total of three, independently isolated XYL 3'-end RACE clones were fully sequenced and their consensus sequence, and that of the λgt11 5'-end clone, were used to construct the XYL contiguous cDNA sequence. The Partial XYL cDNA inserts were excised from plasmid cloning vectors to show insert size (*Figure 5.10 A*) and their arrangement against the full-length XYL cDNA is shown (*Figure 5.10 B*).

5.3.3 Isolation of AXAH cDNAs

cDNA library screening using an AXAH EST

Non-redundant BLAST searches of barley EST databases revealed an EST, HK04K11, with a high level of sequence similarity to the expected AXAH-I cDNA sequence, based on the amino acid sequences of AXAH-I tryptic peptides. AXAH-I tryptic peptides are aligned against the full-length amino acid sequence of an *Arabidopsis thaliana* arabinofuranosidase

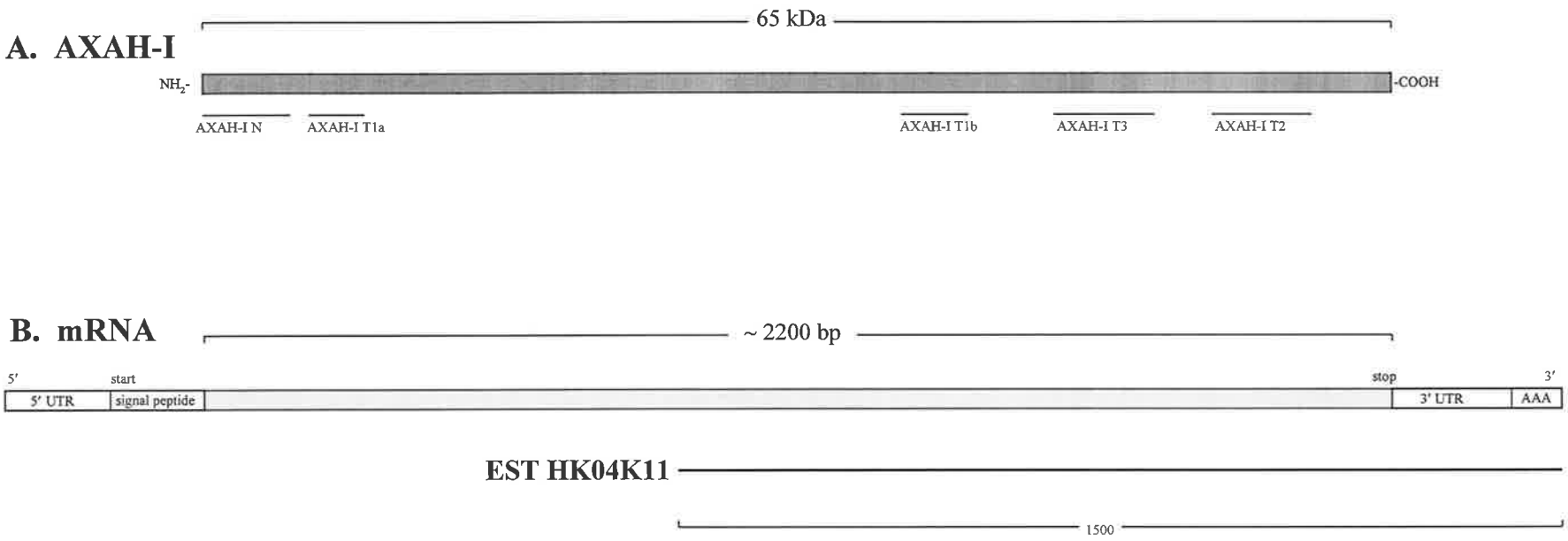

Figure 5.11 **Arrangement of tryptic peptides (A) for arabinoxylan arabinofuranohydrolase (AXAH-I) and the barley EST (HK04K11) (B) against a hypothetical mRNA.** Tryptic peptides were aligned with the *Arabidopsis thaliana* arabinofuranosidase (GenBank/EMBL accession AC011708) amino acid sequence. The barley EST was selected by its high level of homology with the *A.thaliana* DNA sequence.

(GenBank/EMBL accession AC011708) derived from genomic DNA sequence (*Figure 5.11A*). The 1500 bp EST is shown in *Figure 5.11 B*, aligned against a theoretical AXAH-I mRNA. Screening of 200,000 clones with the EST probe at high stringency identified 26 positive clones that were prepared as monoclonal phage stocks by further rounds of plating and screening. Clones that hybridized with the AXAH EST probe at 65°C and washed in 0.2x SSC and 0.1% SDS at 65°C, produced a strong hybridization signal after 20 min exposure to autoradiography film (*Figure 5.12*). The insert sizes of these clones were estimated by PCR amplification using primers based on the flanking sequences of the λgt11 multiple cloning site (results not shown). Six cDNAs were analysed further and found to have identical sequences. The longest of these was subjected to DNA sequence analysis and found to be a near full-length cDNA of approximately 2500 bp encoding a polypeptide with sequence similarity, but not identity, to AXAH-I amino acid sequences (*Figures 5.15 A and 5.15 C*). The encoded AXAH isoenzyme was designated AXAH-II.

AXAH-I cDNA isolation

An AXAH-I λgt11 cDNA clone was found using a differential screening approach. Plaque replicas were first screened with the full-length [α-^{32}P]-dCTP-labelled AXAH-II cDNA at low stringency, to identify all clones that hybridize the AXAH probe. A second, high-stringency wash in 0.1x SSC, 0.1% SDS, indicated clones that were similar, but not identical to the probe DNA, by loss of the hybridization signal, as shown in *Figure 5.13*. A cDNA fragment of approximately 1600 bp corresponding to the 5' region of the AXAH-I cDNA was isolated using this approach (*Figures 5.15 A and 5.15 B*).

3'-End RACE (AXAH-I)

The 3'-end of the AXAH-I cDNA was amplified from root/shoot/scutellum cDNA using the 3'-end RACE protocol, using the $(dT)^{17}$ adaptor primer and an AXAH-I gene-specific primer corresponding to DNA sequence near the 3' end of the λgt11 AXAH-I clone (position 1175-1194 in the final AXAH-I cDNA sequence; *Figure 5.18*). The sequence of this primer corresponded with a position where substantial differences in the nucleotide sequence of AXAH-I and AXAH-II were observed. The AXAH-I-specific 3'-end RACE primer was, $^{5'}$GTGGGAAAGAAAATTACCGT$^{3'}$. The 3'-end RACE cDNA fragment was approximately 750 bp in length and was identified by hybridization with the AXAH-II cDNA at low stringency (*Figure 5.14*). The AXAH-I DNA band was not clearly visible on the agarose gel

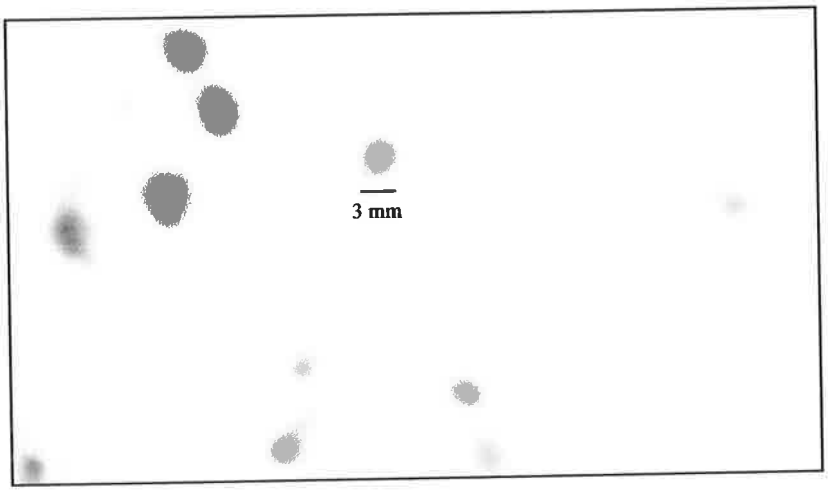

Figure 5.12 **HK04K11 positive clones from a 12-day seedling λgt11 cDNA library.** Single plaques were isolated after three rounds of screening with the 1500 bp HK04K11 EST. Bacteriophage were grown on *E. coli* Y1090 cells and lifted onto nitrocellulose membrane. After hybridization with the [α-^{32}P]-dCTP-labelled EST at 65°C. Autoradiography film was exposed for 20 min.

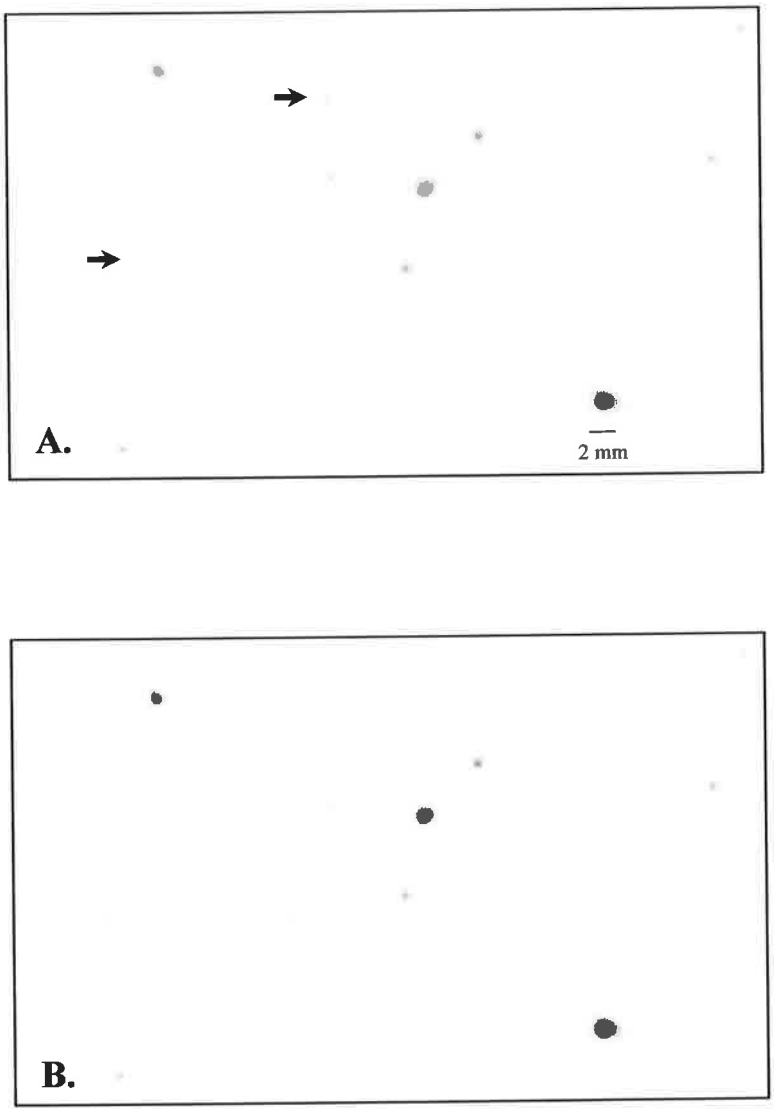

Figure 5.13 **Differential screening of the 12-day seedling λgt11 cDNA library for AXAH-I cDNA.** The cDNA library was plated in *E. coli* Y1090 cells and bacteriophage DNA was lifted onto nitrocellulose membrane. After hybridization with the [α-^{32}P]-dCTP labelled AXAH-II cDNA at 55°C, the membranes were washed two times in 0.5x SSC, 0.1% SDS at 55°C, and exposed to autoradiography film (A). Membranes were subsequently washed in 0.1x SSC, 0.1% SDS at 65°C and again exposed to autoradiography film (B). Plaques (*indicated by arrows*) were selected on the basis of loss of positive hybridization signal after high stringency washing and purified by further rounds of screening.

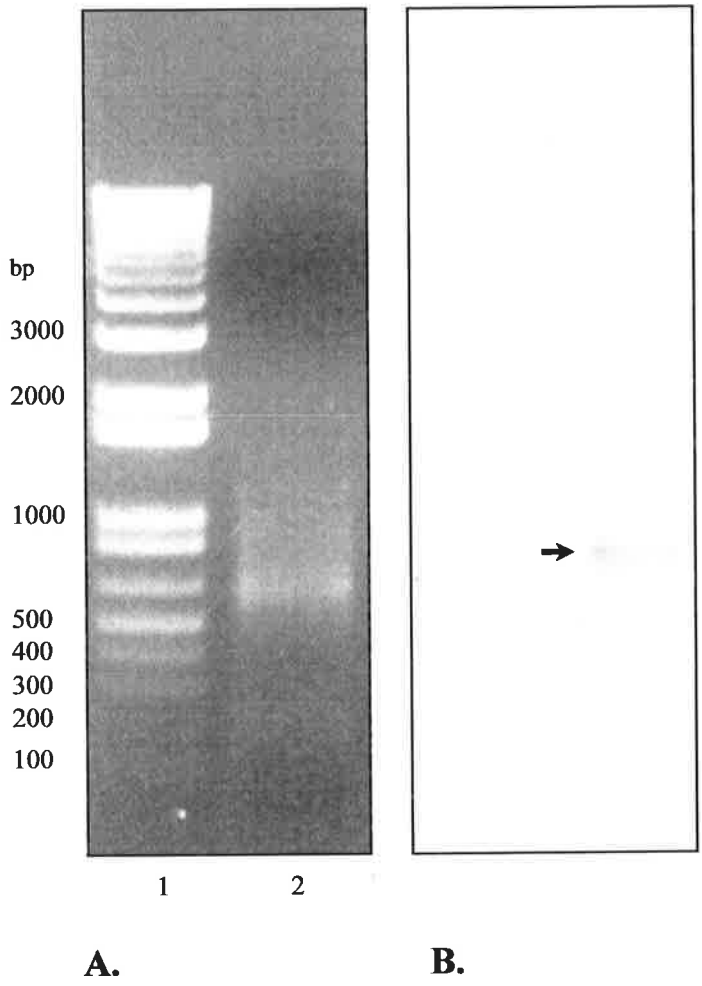

Figure 5.14 **Agarose gel electrophoresis (A) of products from 3'-end RACE-PCR using oligonucleotide primers for AXAH-I and autoradiograph (B) of DNA blot probed with AXAH-II cDNA.** (A) *Lane 1*, DNA molecular size markers; *lane 2*, 3'-end RACE-PCR product. (B) The agarose gel was blotted onto Hybond-N membrane and hybridized with [α-^{32}P]-dCTP-labelled AXAH-II cDNA at 55°C. The membrane was washed 2 times in 0.5x SSC, 0.1% SDS at 55°C, and autoradiography film was exposed for 2.5 h. The 850 bp band (*arrow*) was excised from the gel and cloned.

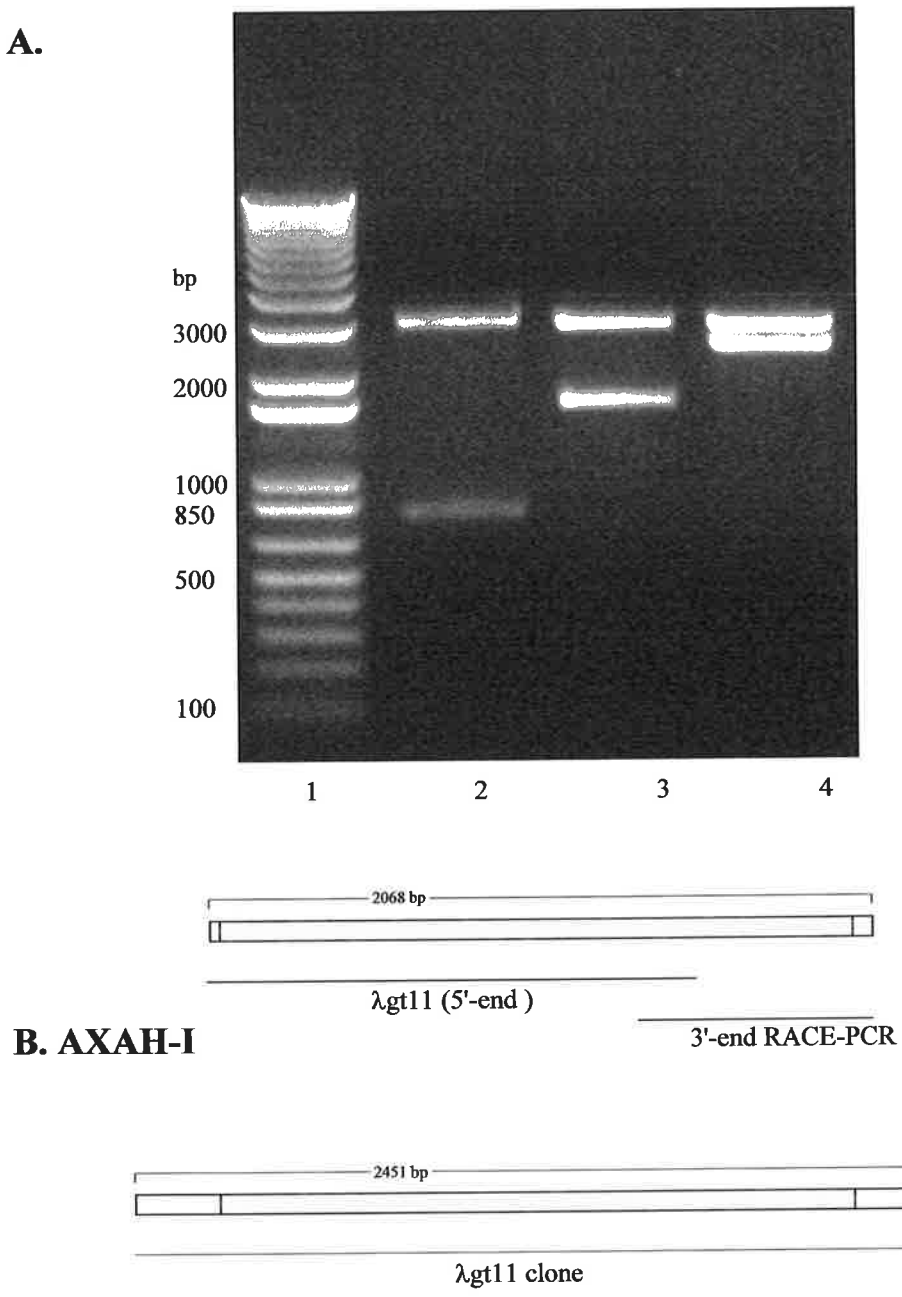

Figure 5.15 **Agarose gel electrophoresis of AXAH-I and AXAH-II cDNA clones.** (A) cDNA inserts were excised from their respective cloning vectors and electrophoresed on 1% (*w/v*) agarose. *Lane 1*, DNA molecular size markers; lane 2, AXAH-I 3'-end RACE product (pGEM TEasy); *lane 3*, AXAH-I 5'-end λgt11 clone (pBluescript SK^+); *lane 4*, AXAH-II full-length λgt11 clone (pBluescript SK^+). (B) The AXAH-I cDNA sequence was constructed from the sequences of a λgt11 clone and an overlapping 3'-end RACE-PCR fragment. (C) The AXAH-II sequence was from a full-length λgt11 cDNA clone.

but was successfully cloned into pGEM TEasy vector (*Figure 5.15 A*). The alignment of the 3'-end RACE AXAH-I cDNA with the 5'-end λgt11 AXAH-I clone is shown in *Figure 5.15 B*.

5.3.4 DNA Sequencing and Amino Acid Sequence Analysis

DNA Sequencing

DNA sequencing was performed using vector sequences as priming sites for initial sequence analyses. Further sequencing was performed with newly synthesized primers based on the 3' ends of preceding sequences. Both sense and antisense strands of each cDNA clone were sequenced and contiguous DNA sequences were constructed. Scale diagrams showing the alignments of contiguous DNA fragments that correspond with the ARA-I, XYL and AXAH cDNAs are presented in *Figures 5.5*, *5.10* and *5.15*, respectively.

Complete cDNA sequences, and derived amino acid sequences of the encoded polypeptides, for ARA-I (*Figure 5.16*), XYL (*Figure 5.17*), AXAH-I (*Figure 5.18*) and AXAH-II (*Figure 5.19*) are presented. The fidelity of derived amino acid sequences for each of the cDNAs was confirmed by comparison with the sequences of tryptic peptides and also with similar, complete amino acid sequences from GenBank/EMBL databases. For each of the purified proteins, the tryptic peptide and NH_2-terminal amino acid sequences corresponded almost exactly to the deduced amino acid sequences from the cDNAs. The exception to this was the NH_2-terminal sequence for XYL, where 7 out of 38 residues were different. The other 168 amino acids of 8 tryptic sequences matched the cDNA-encoded XYL polypeptide exactly. The 861 bp overlapping sequence of the 5'-end λgt11 XYL clone and 3'-end RACE sequence were identical so one can be confident that the contiguous XYL cDNA sequence corresponded to that of a single gene.

The start methionine for each of the sequences was encoded by the first AUG start codon in the same frame as tryptic amino acid sequences and translations were terminated by a TGA stop codon. The NH_2-terminal residues for XYL and AXAH-I were as found by NH_2-terminal sequencing of the final purified protein preparations. For ARA-I and AXAH-II sequences, where the NH_2-terminus was not determined experimentally, the signal peptide-processing site was predicted using the Sigcleave program in the ANGIS suite of genetic sequence analysis programs (*Figure 5.16* and *Figure 5.19*, respectively).

```
CCAGAGCACGCACGCTCGCACGCTAGCCAGCCAGCCAGCAGCAATGGCGACGGCGGCGCGCCCACCGTTCCTGGCCATGGCCGCGGCGGCGCTGCTGGTG  100
                                                M  A  T  A  A  R  P  P  F  L  A  M  A  A  A  A  L  L  V

GCGGCGTGGTGGGGCGGCAATGCGGGGGCGGCGGAGGCGCAGGCGCAGGCGCCGGTGTTCGCGTGCGACGCGTCGAACGCGACGCTGGCGGCCTACGGGT  200
A  A  W  W  G  G  N  A  G  A  A  E  A  Q  A  Q  A  P  V  F  A  C  D  A  S  N  A  T  L  A  A  Y  G  F   24

TCTGCAACCGGAAGGCGACGGCGTCGGCGCGCGCCAGGGACCTAGTGTCGCGGCTGACGCTGGCGGAGAAGGTGGGGTTCCTGGTGAACAAGCAGCCGGC  300
  C  N  R  K  A  T  A  S  A  R  A  R  D  L  V  S  R  L  T  L  A  E  K  V  G  F  L  V  N  K  Q  P  A   57

GCTGGGCCGGCTGGGCATCCCGGCGTACGAGTGGTGGTCCGAAGCGCTGCACGGCGTGTCATACGTCGGCCCCGGCACCCGCTTCTCGCCGCTCGTGCCC  400
  L  G  R  L  G  I  P  A  Y  E  W  W  S  E  A  L  H  G  V  S  Y  V  G  P  G  T  R  F  S  P  L  V  P   90

GGCGCCACCAGCTTCCCGCAGCCCATCCTCACCGCCGCCTCCTTCAACGCCTCCCTCTTCCGCGCCATCGGCGAGGTGGTGTCGACGGAGGCGCGGGCGA  500
G  A  T  S  F  P  Q  P  I  L  T  A  A  S  F  N  A  S  L  F  R  A  I  G  E  V  V  S  T  E  A  R  A  M  124

TGCACAACGTGGGGCTGGCGGGGCTGACGTTCTGGAGCCCCAACATCAACATCTTCAGGGACCCGCGGTGGGGCCGGGGCCAGGAGACGCCCGGCGAGGA  600
  H  N  V  G  L  A  G  L  T  F  W  S  P  N  I  N  I  F  R  D  P  R  W  G  R  G  Q  E  T  P  G  E  D  157

CCCGCTGCTGGCCAGCAAGTACGCCGTGGGATACGTCACGGGGCTCCAGGACGCCGGCGCCGGCGGAGTCACCGACGGCGCGCTCAAGGTCGCCGCCTGC  700
  P  L  L  A  S  K  Y  A  V  G  Y  V  T  G  L  Q  D  A  G  A  G  G  V  T  D  G  A  L  K  V  A  A  C  190

TGCAAGCACTACACCGCCTACGACGTCGACAACTGGAAGGGCGTCGAGCGATACACCTTCGACGCAAAGGTGTCGCAGCAGGACCTGGACGACACGTTCC  800
C  K  H  Y  T  A  Y  D  V  D  N  W  K  G  V  E  R  Y  T  F  D  A  K  V  S  Q  Q  D  L  D  D  T  F  Q  224

AGCCGCCGTTCAAGAGCTGCGTGCTGGACGGCAATGTGGCCAGCGTCATGTGCTCCTACAACAAGGTGAACGGGAAGCCCACCTGCGCCGACAAGGACCT  900
  P  P  F  K  S  C  V  L  D  G  N  V  A  S  V  M  C  S  Y  N  K  V  N  G  K  P  T  C  A  D  K  D  L  257

CCTGGAGGGCGTCATCAGGGGGGACTGGAAGCTCAATGGATACATCGTGTCGGACTGCGACTCCGTCGACGTGCTCTACACCCAGCAGCACTACACCAAG  1000
  L  E  G  V  I  R  G  D  W  K  L  N  G  Y  I  V  S  D  C  D  S  V  D  V  L  Y  T  Q  Q  H  Y  T  K  290

ACGCCCGAGGAGGCCGCCGCTATCACCATCAAATCAGGGGTGGACCTCAACTGCGGCAACTTCCTGGCGCAGCACACGGTGGCGGCGGTGCAGGCCGGCG  1100
T  P  E  E  A  A  A  I  T  I  K  S  G  V  D  L  N  C  G  N  F  L  A  Q  H  T  V  A  A  V  Q  A  G  E  324

AGCTCTCGGAGGAGGATGTCGACCGGGCCATCACCAACAACTTCATCATGCTCATGCGCCTGGGCTTCTTTGACGGCGACCCCCGGCAGCTCGCCTTCGG  1200
  L  S  E  E  D  V  D  R  A  I  T  N  N  F  I  M  L  M  R  L  G  F  F  D  G  D  P  R  Q  L  A  F  G  357

CAGCCTCGGCCCCAAGGACGTGTGCACGTCTTCCAACCGGGAGCTGGCACGCGAGACGGCGCGCCAGGGCATCGTGCTCCTCAAGAACAGCGGCGCGCTG  1300
  S  L  G  P  K  D  V  C  T  S  S  N  R  E  L  A  R  E  T  A  R  Q  G  I  V  L  L  K  N  S  G  A  L  390

CCGCTCTCGGCAAAGTCCATCAAGTCCATGGCCGTCATTGGGCCCAACGCCAATGCCAGCTTCACCATGATCGGCAACTACGAAGGCACACCGTGCAAGT  1400
P  L  S  A  K  S  I  K  S  M  A  V  I  G  P  N  A  N  A  S  F  T  M  I  G  N  Y  E  G  T  P  C  K  Y  424

ACACGACGCCGCTCCAGGGCCTGGGCGCCAAGGTCAACACCGTGTACCAGCCGGGGTGCACCAACGTCGGGTGCAGCGGGAACAGCCTACAGCTCAGTAC  1500
  T  T  P  L  Q  G  L  G  A  K  V  N  T  V  Y  Q  P  G  C  T  N  V  G  C  S  G  N  S  L  Q  L  S  T  457

CGCCGTGGCGGCCGCAGCCAGTGCCGACGTGACCGTGCTCGTCGTCGGCGCCGACCAGTCCATTGAGCGCGAGAGCCTGGACAGGACGAGCCTCCTCTTG  1600
A  V  A  A  A  A  A  S  A  D  V  T  V  L  V  V  G  A  D  Q  S  I  E  R  E  S  L  D  R  T  S  L  L  L  490

CCGGGCCAGCAGACCCAGCTCGTGTCGGCGGTCGCCAACGCCTCCAGCGGGCCTGTCATCCTCGTCGTCATGTCGGGTGGACCATTCGACATCTCGTTCG  1700
P  G  Q  Q  T  Q  L  V  S  A  V  A  N  A  S  S  G  P  V  I  L  V  V  M  S  G  G  P  F  D  I  S  F  A  524

CCAAGGCCAGCGACAAGATCGCTGCCACTCTTTGGGTCGGCTACCCCGGCGAAGCCGGCGGCGCCGCCCTCGACGACACCCTCTTCGGCAGCCACAACCC  1800
  K  A  S  D  K  I  A  A  T  L  W  V  G  Y  P  G  E  A  G  G  A  A  L  D  D  T  L  F  G  S  H  N  P  557

AAGTGGGAGGCTGCCGGTGACGTGGTACCCGGCGTCGTACGCCGACACGGTCACCATGACCGACATGCGGATGCGGCCGGACACGTCGACGGGCTACCCG  1900
S  G  R  L  P  V  T  W  Y  P  A  S  Y  A  D  T  V  T  M  T  D  M  R  M  R  P  D  T  S  T  G  Y  P  590

GGCCGGACGTACCGGTTCTACACGGGCGACACGGTGTTCGCCTTCGGGGACGGCCTGAGCTACACCAAGATGTCACACAGCCTCGTGTCGGCGCCGCCGT  2000
G  R  T  Y  R  F  Y  T  G  D  T  V  F  A  F  G  D  G  L  S  Y  T  K  M  S  H  S  L  V  S  A  P  P  S  624

CGTACGTGTCCATGCGGCTGGCCGAGGACCACCTGTGCCGCGCCGAGGAGTGCGCGTCGGTGGAGGCCGCCGGCGACCACTGCGACGACCTGGCGCTCGA  2100
  Y  V  S  M  R  L  A  E  D  H  L  C  R  A  E  E  C  A  S  V  E  A  A  G  D  H  C  D  D  L  A  L  D  657

CGTCAAGCTCCAGGTGCGGAACGCCGGCGAGGTGGCCGGCGCGCACTCGGTGCTGCTGTTCTCGTCGCCGCCGCCGGCCCACAACGCGCCGGCGAAGCAC  2200
V  K  L  Q  V  R  N  A  G  E  V  A  G  A  H  S  V  L  L  F  S  S  P  P  P  A  H  N  A  P  A  K  H  690

CTGGTCGGGTTCGAGAAGGTGTCGCTGGCGCCCGGGGAGGCCGGCACGGTGGCGTTCCGGGTGGACGTGTGCAGGGACCTGAGCGTGGTGGACGAGCTGG  2300
L  V  G  F  E  K  V  S  L  A  P  G  E  A  G  T  V  A  F  R  V  D  V  C  R  D  L  S  V  V  D  E  L  G  724

GCGGCCGCAAGGTGGCGCTCGGCGGCCACACGCTGCATGACGGCGACCTCAAGCACACCGTGGAACTGCGAGTCTGATGATCGACGGTGATCAGAAAAAT  2400
  G  R  K  V  A  L  G  G  H  T  L  H  D  G  D  L  K  H  T  V  E  L  R  V  *                            748

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TGAAATAACCAAATGGGCCTTTCGTTGGGACAAGAAAGAATTGGATCCACGGGAGGCTTTGGGAGTTGTTTTGTTGCGGTTGTTAAATTTGATTTTATTC  2600

GTTTGGAATTGGAGGGACGATTTGCAACTGTAAAAAAAAAAAAAAAA                                                        2647
```

Figure 5.16 **Nucleotide sequence of ARA-I cDNA and derived amino acid sequence.** The cDNA sequence is a compilation of four cDNA clones as described in *Figure 5.5*. The amino acid sequence is the translation of the open reading frame (44-2374) beginning at ATG codon encoding the start methionine and ending with a TGA stop codon. A signal peptide of 29 amino acids is shown in *bold* and the putative NH_2-terminal alanine residue is indicated with an *arrow*. Tryptic peptides are *underlined* and potential N-glycosylation sites are *double-underlined*. The mature polypeptide consists of 748 amino acid residues.

```
CGCAAGTGAGTGACACTTGTGACAGTTCGGACGCACACTAGAGCCATGGGACGTCGCACTCACGTCGTGCTAGCCGCAGCGGTGCCAGCGCTGCTGCTCG  100
                                  M  G  R  R  T  H  V  V  L  A  A  A  A  V  P  A  L  L  L  V

TCCTCCTCCTCCGGCTGCACGCGGCCGTGGCCGCCGACCCGCCCTTCTCCTGCGGCGCGCCGTCGAGCGCGGCGTTCTGCGACCGGAGGCTGCCGATCGA  200
  L  L  L  R  L  H  A  A  V  A  A  D  P  P  F  S  C  G  A  P  S  S  A  A  F  C  D  R  R  L  P  I  E   23
                                  ↑

GCAGCGGGCGGCGGACCTGGTGTCGAAGCTCACTCTGGAGGAGAAGATCTCGCAGCTCGGCGACGAGTCGCCGGCCGTGGACCGGCTGGGCGTGCCGGCG  300
  Q  R  A  A  D  L  V  S  K  L  T  L  E  E  K  I  S  Q  L  G  D  E  S  P  A  V  D  R  L  G  V  P  A   56

TACAAGTGGTGGTCGGAGGCGCTGCACGGCGTGGCCAATGCCGGCCGCGGCGTCCACCTGGACGGCCCGCTCCGCGCCGCCACCAGCTTCCCGCAGGTCA  400
  Y  K  W  W  S  E  A  L  H  G  V  A  N  A  G  R  G  V  H  L  D  G  P  L  R  A  A  T  S  F  P  Q  V  I   90

TCCTCACCGCCGCCTCCTTCAACCCGCACCTCTGGTACCGCATCGGCCAGGTGATCGGCACGGAGGCGCGGGGCGTGTACAACAACGGGCAGGCGGAGGG  500
  L  T  A  A  S  F  N  P  H  L  W  Y  R  I  G  Q  V  I  G  T  E  A  R  G  V  Y  N  N  G  Q  A  E  G   123

GCTGACCTTCTGGGCGCCCAACATCAACGTGTTCCGGGACCCGCGGTGGGGGCGGGGCCAGGAGACGCCCGGCGAGGACCCGACCATGACGGGCAAGTAC  600
  L  T  F  W  A  P  N  I  N  V  F  R  D  P  R  W  G  R  G  Q  E  T  P  G  E  D  P  T  M  T  G  K  Y   156

GCCGCCGTGTTCGTCCGCGGCGTGCAGGGCTACGGCATGTCCGGCGCCATCAACTCCTCCGACCTGGAGGCCTCCGCCTGTTGCAAGCACTTCACCGCCT  700
  A  A  V  F  V  R  G  V  Q  G  Y  G  M  S  G  A  I  N  S  S  D  L  E  A  S  A  C  C  K  H  F  T  A  Y   190

ACGACCTCGAGAACTGGAAGGGCGTCACCCGCTTCGCCTTCGACGCAAAGGTGACGGAGCAGGACCTGGCAGACACGTACAACCCGCCGTTCAAGAGCTG  800
  D  L  E  N  W  K  G  V  T  R  F  A  F  D  A  K  V  T  E  Q  D  L  A  D  T  Y  N  P  P  F  K  S  C   223

CGTGGAGGACGGCGGCGCCAGCGGCATCATGTGCTCCTACAACCGCGTCAATGGCGTGCCCACCTGCGCCGACCACAACCTGCTCTCCAAGACCGCCAGA  900
  V  E  D  G  G  A  S  G  I  M  C  S  Y  N  R  V  N  G  V  P  T  C  A  D  H  N  L  L  S  K  T  A  R   256

GGCGACTGGAGCTTCAACGGATACATCACGTCGGACTGCGACGCCGTGGCCATCATCCACGACGTGCAGGGGTATGCCAAGGCGCCGGAGGACGCGGTGG  1000
  G  D  W  S  F  N  G  Y  I  T  S  D  C  D  A  V  A  I  I  H  D  V  Q  G  Y  A  K  A  P  E  D  A  V  A  290

CAGATGTCCTCAAGGCCGGGATGGACGTGAACTGCGGCGGCTACATACAGACGCACGGCGTGTCGGCGTACCAGCAGGGCAAGATCACGGGGGAGGACAT  1100
  D  V  L  K  A  G  M  D  V  N  C  G  G  Y  I  Q  T  H  G  V  S  A  Y  Q  Q  G  K  I  T  G  E  D  I   323

CGACCGGGCGCTCCGCAACCTCTTCGCCATCAGGATGAGGCTGGGCCTCTTCGACGGCAATCCCAAGTACAACCGGTACGGCAACATCGGCGCCGACCAG  1200
  D  R  A  L  R  N  L  F  A  I  R  M  R  L  G  L  F  D  G  N  P  K  Y  N  R  Y  G  N  I  G  A  D  Q   356

GTGTGCAGCAAGGAGCACCAGGACCTGGCCCTGCAGGCGGCGCGGGACGGCATCGTCCTGCTCAAGAACGACGGCGCCGCGCTGCCCCTCTCCAAGTCCA  1300
  V  C  S  K  E  H  Q  D  L  A  L  Q  A  A  R  D  G  I  V  L  L  K  N  D  G  A  A  L  P  L  S  K  S  K  390

AGGTCTCCTCCCTCGCCGTCATCGGGCCCAACGGCAACAACGCGTCGCTGCTGCTCGGCAACTACTTCGGGCCGCCCTGCATCTCCGTCACCCCGCTCCA  1400
  V  S  S  L  A  V  I  G  P  N  G  N  N  A  S  L  L  L  G  N  Y  F  G  P  P  C  I  S  V  T  P  L  Q   423

GGCGCTGCAGGGGTACGTCAAGGACGCCCGGTTCGTCCAGGGCTGCAACGCCGCCGTCTGCAACGTGTCCAACATCGGCGAGGCCGTGCACGCGGCCGGC  1500
  A  L  Q  G  Y  V  K  D  A  R  F  V  Q  G  C  N  A  A  V  C  N  V  S  N  I  G  E  A  V  H  A  A  G   456

TCGGCGGACTACGTCGTGCTGTTCATGGGGCTGGACCAGAACCAGGAGCGGGAGGAGGTGGACAGGCTCGAGCTGGGGCTCCCCGGGATGCAGGAGAGCC  1600
  S  A  D  Y  V  V  L  F  M  G  L  D  Q  N  Q  E  R  E  E  V  D  R  L  E  L  G  L  P  G  M  Q  E  S  L  490

TCGTCAACAGCGTGGCCGACGCGGCCAAGAAGCCGGTGATCCTGGTGCTGCTTTGCGGTGGCCCGGTCGACGTGACGTTCGCCAAGAACAACCCTAAGAT  1700
  V  N  S  V  A  D  A  A  K  K  P  V  I  L  V  L  L  C  G  G  P  V  D  V  T  F  A  K  N  N  P  K  I   523

CGGCGCCATTGTCTGGGCTGGTTACCCTGGCCAGGCCGGTGGCATCGCCATCGCCCAGGTCCTCTTCGGCGACCACAACCCCGGTGGGAGGCTGCCGGTG  1800
  G  A  I  V  W  A  G  Y  P  G  Q  A  G  G  I  A  I  A  Q  V  L  F  G  D  H  N  P  G  G  R  L  P  V   556

ACGTGGTACCCGAAGGAGTTCACGGCGGTGCCGATGACGGACATGCGGATGCGCGCCGACCCGTCCACTGGCTACCCGGGGCGCACCTACAGGTTCTACA  1900
  T  W  Y  P  K  E  F  T  A  V  P  M  T  D  M  R  M  R  A  D  P  S  T  G  Y  P  G  R  T  Y  R  F  Y  K  590

AGGGCAAGACCGTGTACAACTTCGGCTACGGCCTGAGCTACTCCAAGTACTCCCACCGCTTCGCGTCCAAGGGCACGAAGCCGCCGTCCATGAGCGGCAT  2000
  G  K  T  V  Y  N  F  G  Y  G  L  S  Y  S  K  Y  S  H  R  F  A  S  K  G  T  K  P  P  S  M  S  G  I  623

CGAGGGCCTCAAGGCGACGGCAAGGGCGTCGGCGGCGGGCACGGTGAGCTACGACGTGGAGGAGATGGGCGCGGAGGCGTGCGACAGGCTCCGGTTCCCG  2100
  E  G  L  K  A  T  A  R  A  S  A  A  G  T  V  S  Y  D  V  E  E  M  G  A  E  A  C  D  R  L  R  F  P   656

GCGGTGGTGCGGGTGCAGAACCACGGGCCCATGGACGGGGGGCACCTGGTGCTGCTGTTCCTGCGGTGGCCGAACGCGACGGACGGGCGGCCCGCCAGCC  2200
  A  V  V  R  V  Q  N  H  G  P  M  D  G  G  H  L  V  L  L  F  L  R  W  P  N  A  T  D  G  R  P  A  S  Q  690

AGCTGATCGGGTTCCAGAGCGTGCACCTGCGGGCGGACGAGGCCGCGCACGTGGAGTTCGAGGTGAGCCCCTGCAAGCACTTGAGCCGGGCGGCGGAGGA  2300
  L  I  G  F  Q  S  V  H  L  R  A  D  E  A  A  H  V  E  F  E  V  S  P  C  K  H  L  S  R  A  A  E  D   723

CGGCCGCAAGGTGATCGACCAGGGCTCGCACTTCGTCAGGGTCGGCGACGACGAGTTCGAGTTGAGCTTCATGGCCTGAGCCGGCCGGCCGGCGGCCGGC  2400
  G  R  K  V  I  D  Q  G  S  H  F  V  R  V  G  D  D  E  F  E  L  S  F  M  A  *                          748

GGTTGGATCGATCGAACACGATAAACCTGCTGCTGCTGCTGCTGCTGCTTCGGCAGAGGAGGACTTCGATCATAAATTGTAGCCACGTAACTTATGAGAA  2500

GGAGATAATGATGATAAATTAAAAGCGAAAAAAAAAAAAAAAAAAA                                                          2546
```

Figure 5.17 **Nucleotide sequence of XYL cDNA and derived amino acid sequence.** The cDNA sequence is a compilation of two cDNA clones as described in *Figure 5.10*. The amino acid sequence is the translation of the open reading frame (46-2376) beginning at ATG codon encoding the start methionine and ending with a TGA stop codon. A signal peptide of 29 amino acids is shown in *bold* and the NH_2-terminal alanine residue is indicated with an *arrow*. Tryptic peptides are *underlined* and potential N-glycosylation sites are *double-underlined*. A putative polyadenylation signal is *underlined*. The mature polypeptide consists of 748 amino acid residues.

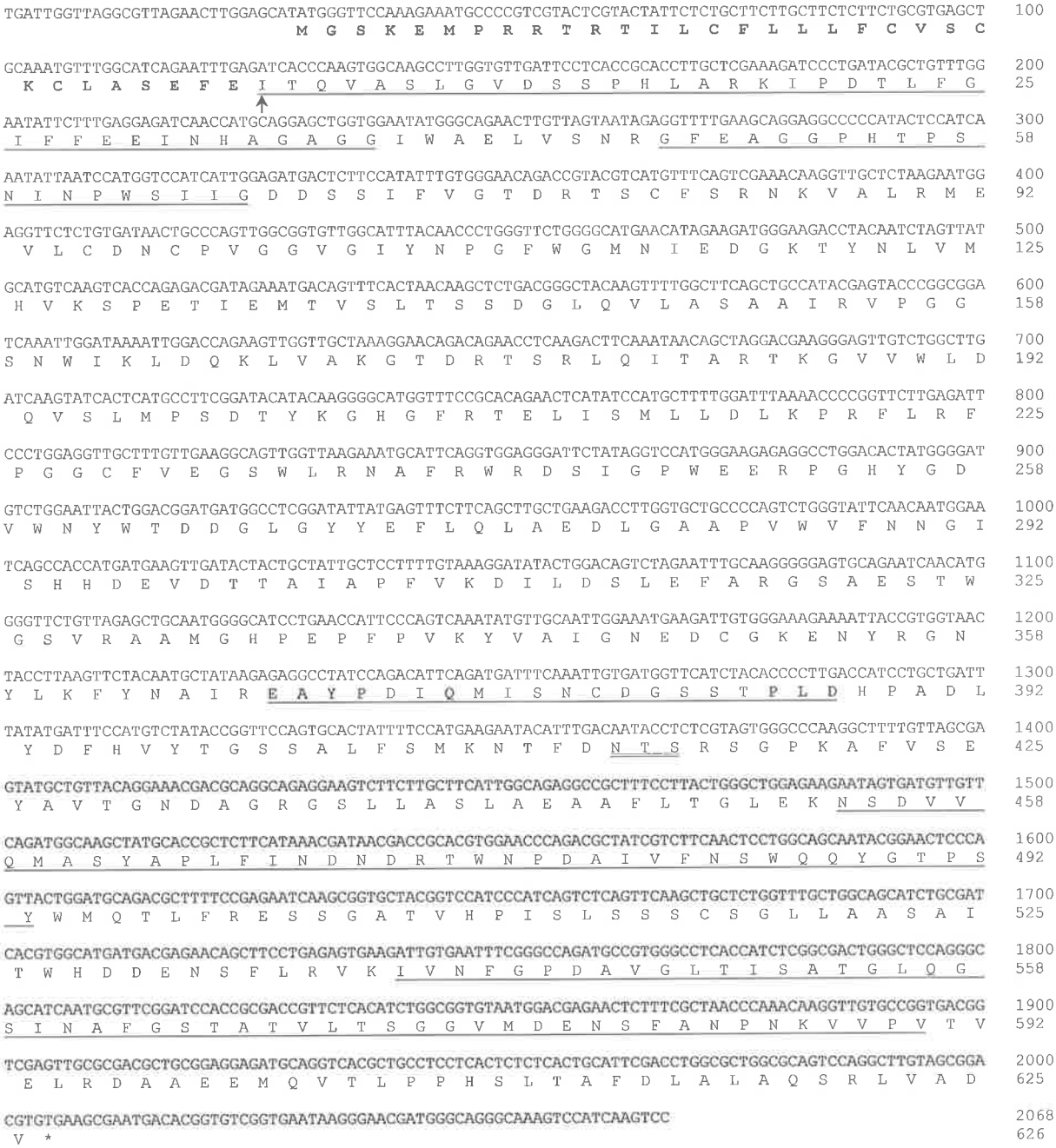

Figure 5.18 **Nucleotide sequence of AXAH-I cDNA and derived amino acid sequence.** The cDNA sequence is a compilation of two cDNA clones as described in *Figure 5.15*. The amino acid sequence is the translation of the open reading frame (31-2004) beginning at ATG codon encoding the start methionine and ending with a TGA stop codon. A signal peptide of 32 amino acids is shown in *bold* and the NH_2-terminal isoleucine residue is indicated with an *arrow*. Tryptic peptides are *underlined* and potential N-glycosylation sites are *double-underlined*. The mature polypeptide consists of 626 amino acid residues.

```
CAACCAACCAACCGCCGAAGCGGCCCACTCCGGCAAGCAACTGGCCGTGGCCTCCGGCGTCCGATCGCCATCCGGAGCCACCCTTCCCTTCAAAGCCACT  100

GCTTCCTCGCCGTGGCGGCTTCGCCTACTTAAGCGCGCGCCTCCGCTGCTCCTGCACTCACTCGCAGGATCCTGACTCCGCCTCCGTCTTCCTCAGCCGC  200

TTGCGCTTGCTGCTGGCTGTGGGTGGGATTCATGACACACGGACTACATTAGTGCTCTTTACAAGATGAAGCGTATGGGTTCCAAACAAGTGTTCGTTCC  300
                                                                  M  K  R  M  G  S  K  Q  V  F  V  P

TACCATCGCATGCGTGCTGCTCCTATTCTGCTTTGGATGCAAATGCATAGCATCGGAATTACAAGCGACACAGACGGCCACCCTCAAGGTTGATGCCTCA  400
 T  I  A  C  V  L  L  L  F  C  F  G  C  K  C  I  A  S  E  L  Q  A  T  Q  T  A  T  L  K  V  D  A  S   12
                                                                  ↑

TCACAGCTTGCCCGAAAGATACCAGACACACTGTTTGGGATGTTTTTTGAGGAGATCAACCATGCAGGAGCCGGTGGCATATGGGCAGAACTTGTTAGTA  500
 S  Q  L  A  R  K  I  P  D  T  L  F  G  M  F  F  E  E  I  N  H  A  G  A  G  G  I  W  A  E  L  V  S  N   46

ATAGAGGTTTTGAAGCTGGAGGACTCCATACGCCATCAAATATTGACCCATGGTCTATAATTGGAGACGACTCTTCCATATTTGTGGCGACTGATCGTAC  600
    R  G  F  E  A  G  G  L  H  T  P  S  N  I  D  P  W  S  I  I  G  D  D  S  S  I  F  V  A  T  D  R  T   79

ATCATGTTTCAGTCGAAACATTATCGCTCTTAGGATGGAGGTTCTCTGTGATGACTGCCCAGCCAGTGGTGTTGGCATTTACAACCCTGGGTTCTGGGGC  700
 S  C  F  S  R  N  I  I  A  L  R  M  E  V  L  C  D  D  C  P  A  S  G  V  G  I  Y  N  P  G  F  W  G  C  112

ATGAACATAGAAGATGGAAAGACCTACAATCTAGTTATGTATGTTAAATCAGCAGAAGCTGCAGAATTGACAGTTTCATTAGCAAGCTCCGATGGGTTGC  800
 M  N  I  E  D  G  K  T  Y  N  L  V  M  Y  V  K  S  A  E  A  A  E  L  T  V  S  L  A  S  S  D  G  L  Q  146

AGAATCTTGCTTCTGTTACTGTACCAGTTGCTGGCACTTCCAACTGGACAAAAGTGGAGCAAAAATTGATTGCTAAAGGGACAAACAGAACCTCAAGACT  900
    N  L  A  S  V  T  V  P  V  A  G  T  S  N  W  T  K  V  E  Q  K  L  I  A  K  G  T  N  R  T  S  R  L  179

TCAGATAACATCTAACAAGAAGGGAGTTGTATGGTTTGATCAAGTATCACTCATGCCTTCAGACACGTTCAAGGGACATGGTTTTCGCACCGAACTGATA  1000
 Q  I  T  S  N  K  K  G  V  V  W  F  D  Q  V  S  L  M  P  S  D  T  F  K  G  H  G  F  R  T  E  L  I  212

TCCATGCTTTTGGATCTAAAACCACGATTCTTGAGATTTCCTGGTGGTTGCTTCGTTGAAGGCGAATGGTTAAGAAATGCATTCAGGTGGAGGGAATCTA  1100
 S  M  L  L  D  L  K  P  R  F  L  R  F  P  G  G  C  F  V  E  G  E  W  L  R  N  A  F  R  W  R  E  S  I  246

TTGGTCCATGGGAAGAGAGGCCTGGACACTTCGGAGATGTTTGGCATTACTGGACTGACGATGGCCTTGGATATTATGAGTTTCTTCAGCTTTCTGAACA  1200
    G  P  W  E  E  R  P  G  H  F  G  D  V  W  H  Y  W  T  D  D  G  L  G  Y  Y  E  F  L  Q  L  S  E  H  279

CCTGGGGGCTGCTCCAATCTGGGTATTCAACAATGGAATCAGCCACAATGATGAAGTTAGTACCGCTGCCATTGCCCCTTTTGTAAAGGATGTATTGGAT  1300
 L  G  A  A  P  I  W  V  F  N  N  G  I  S  H  N  D  E  V  S  T  A  A  I  A  P  F  V  K  D  V  L  D  312

AGTCTAGAATTTGCAAGGGGGAGTGCAAACTCAACATGGGGCTCTGTTAGAGCTGCAATGGGGCATCCTGAACCGTTCCCAGTCAAATATGTTGCAATCG  1400
 S  L  E  F  A  R  G  S  A  N  S  T  W  G  S  V  R  A  A  M  G  H  P  E  P  F  P  V  K  Y  V  A  I  G  346

GAAATGAAGATTGTGGGAAAAAATACTACCTCGGTAATTACCTCAAGTTCTACAATGCTATAAGAGAATCCTATCCAGACATTCAGATGATTTCAAATTG  1500
    N  E  D  C  G  K  K  Y  Y  L  G  N  Y  L  K  F  Y  N  A  I  R  E  S  Y  P  D  I  Q  M  I  S  N  C  379

TGATGGTTCATCTAAACCACTTGACCATCCTGCTGATCTGTATGACTTCCATGTCTACACCGATTCTAAGACATTGTTTAACATGAAGGGTACGTTTGAT  1600
 D  G  S  S  K  P  L  D  H  P  A  D  L  Y  D  F  H  V  Y  T  D  S  K  T  L  F  N  M  K  G  T  F  D  412

AAAACTTCTCGGACTGGTCCCAAGGCCTTTGTCAGTGAGTATGCGGTATGGAGAACTGATGCAGGTAGAGGAAGCCTTCTTGGTTCACTGGCAGAGGCTG  1700
 K  T  S  R  T  G  P  K  A  F  V  S  E  Y  A  V  W  R  T  D  A  G  R  G  S  L  L  G  S  L  A  E  A  A  446

CCTTCCTTACTGGGCTGGAGAAGAACAGTGATATTGTTCATATGGCAAGTTATGCACCGCTCTTTGTAAACGACAACGATCAAACGTGGAATCCAGATGC  1800
    F  L  T  G  L  E  K  N  S  D  I  V  H  M  A  S  Y  A  P  L  F  V  N  D  N  D  Q  T  W  N  P  D  A  479

TATCGTCTTCAACTCCTGGCAACAATACGGAACTCCTAGTTACTGGATGCAGAAGTTTTTCCGCGAATCTAGTGGTGCCATGATTCATCCAATTACAATC  1900
 I  V  F  N  S  W  Q  Q  Y  G  T  P  S  Y  W  M  Q  K  F  F  R  E  S  S  G  A  M  I  H  P  I  T  I  512

AGTTCCAGCTACTCTGGTTCTCTAGCAGCATCTGCTATCACCTGGCAGGATTCCGGGAATAACTTCCTGAGAGTAAAGATTGTGAACTTCGGGTCGGACA  2000
 S  S  S  Y  S  G  S  L  A  A  S  A  I  T  W  Q  D  S  G  N  N  F  L  R  V  K  I  V  N  F  G  S  D  T  546

CCGTGAGCCTCACAATCTCTGTGTCCGGGCTCCAGGCTAGCATCAACGCGCTGGGGTCAAACGCTACTGTTCTCACGTCCAGCAATGTAAAGGATGAAAA  2100
    V  S  L  T  I  S  V  S  G  L  Q  A  S  I  N  A  L  G  S  N  A  T  V  L  T  S  S  N  V  K  D  E  N  579

TTCTTTCAGTAACCCAACCAAGGTCGTGCCCGTGACAAGTCAGTTGCATAACGCCGCGGAACAGATGCAGGTCACGCTCGCTGCCCACTCCTTCTCCTCG  2200
 S  F  S  N  P  T  K  V  V  P  V  T  S  Q  L  H  N  A  A  E  Q  M  Q  V  T  L  A  A  H  S  F  S  S  612

TTCGACCTTGCGCTTGCTCAGTCCGAGCTTGTCGCAGAGATGTGAAGCAGCGACACGGCGTTAGTGACACGTGGAAACAGGATTAATACACGAGGCGTGA  2300
 F  D  L  A  L  A  Q  S  E  L  V  A  E  M  *                                                          626

TATGGAAATGGAGGAGCTTGTCAGTAATTATGCTTATATCTTGATAAAGCACTCACTGTACAGTGTAAGAAAGAGGAGATACTCATACATACAGCAGTAC  2400

TAAATAAAGTCCATATCAGGCCGATTTGCAGTTAAAAAAAAAAAAAAAAAA                                                    2451
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Figure 5.19 **Nucleotide sequence of AXAH-II cDNA and derived amino acid sequence.** The amino acid sequence is the translation of the open reading frame (266-2242) beginning at ATG codon, encoding the start methionine, and ending with a TGA stop codon. A signal peptide of 33 amino acids is shown in *bold* and the putative NH_2-terminal alanine residue is indicated with an *arrow*. Potential N-glycosylation sites are *double-underlined* and a putative polyadenylation signal is *underlined*. The mature polypeptide consists of 626 amino acid residues.

Amino Acid Sequence Analysis

The XYL cDNA had four potential N-glycosylation sites, defined by the consensus sequence, NXS or NXT. In the AXAH-I sequence there was one potential N-glycosylation site and for ARA-I and AXAH-II, there were five sites each. Putative polyadenylation signals were found in the XYL and AXAH-II cDNAs, 4 and 26 bases upstream from the polyadenylation sequences, respectively. A polyadenylation sequence was present for the ARA-I sequence but no polyadenylation signal sequence was evident. The AXAH-I cDNA was truncated at the 3' end and no polyadenylation signal was found.

Theoretical values for molecular mass and isoelectric point of mature proteins were calculated using the ANGIS Pepstats program and were as follows: ARA-I, M_r 79,184, pI 5.7; XYL, M_r 80,500, pI 6.5; AXAH-I, M_r 68,279, pI 4.8; AXAH-II, M_r 68,760, pI 5.2. Codon usage statistics were compiled using the ANGIS Codonfrequency program. No bias in codon usage was observed for AXAH-I and AXAH-II, but both the ARA-I and XYL sequences showed an extreme bias towards the use of G or C in the third base position. Approximate percentages of codons having G or C in the third base position were 95% for ARA-I and XYL, and 45% for AXAH-I and AXAH-II.

The deduced amino acid sequences of ARA-I and XYL are aligned in *Figure 5.20* and positions of sequence identity are highlighted. The sequences were 51% identical. The putative catalytic nucleophilic residues, D275 and D268 in ARA-I and XYL, respectively, were conserved in a 'GXXXSD' sequence that is common in related barley β-D-glucan glucohydrolases (GenBank/EMBL accessions AF102868 and U46003) (Harvey *et al.* 2000). The putative catalytic acid/base residues in ARA-I and XYL were glutamic acid residues E481 and E474, respectively (*Figure 5.20*). *Figure 5.21* shows the phylogenetic relationships between ARA-I, XYL and other family 3 glycoside hydrolases. The barley ARA-I and XYL sequences are grouped with closely related *Arabidopsis thaliana* sequences. These sequences are related to microbial β-D-xylosidases and also to plant β-D-glucan glucohydrolases and β-D-glucosidases.

The deduced amino acid sequence of AXAH-I is aligned with that of AXAH-II in *Figure 5.22*. The mature enzymes have 81% sequence identity. The amino acid sequences surrounding the putative catalytic acid/base (E348) and catalytic nucleophile (E425),

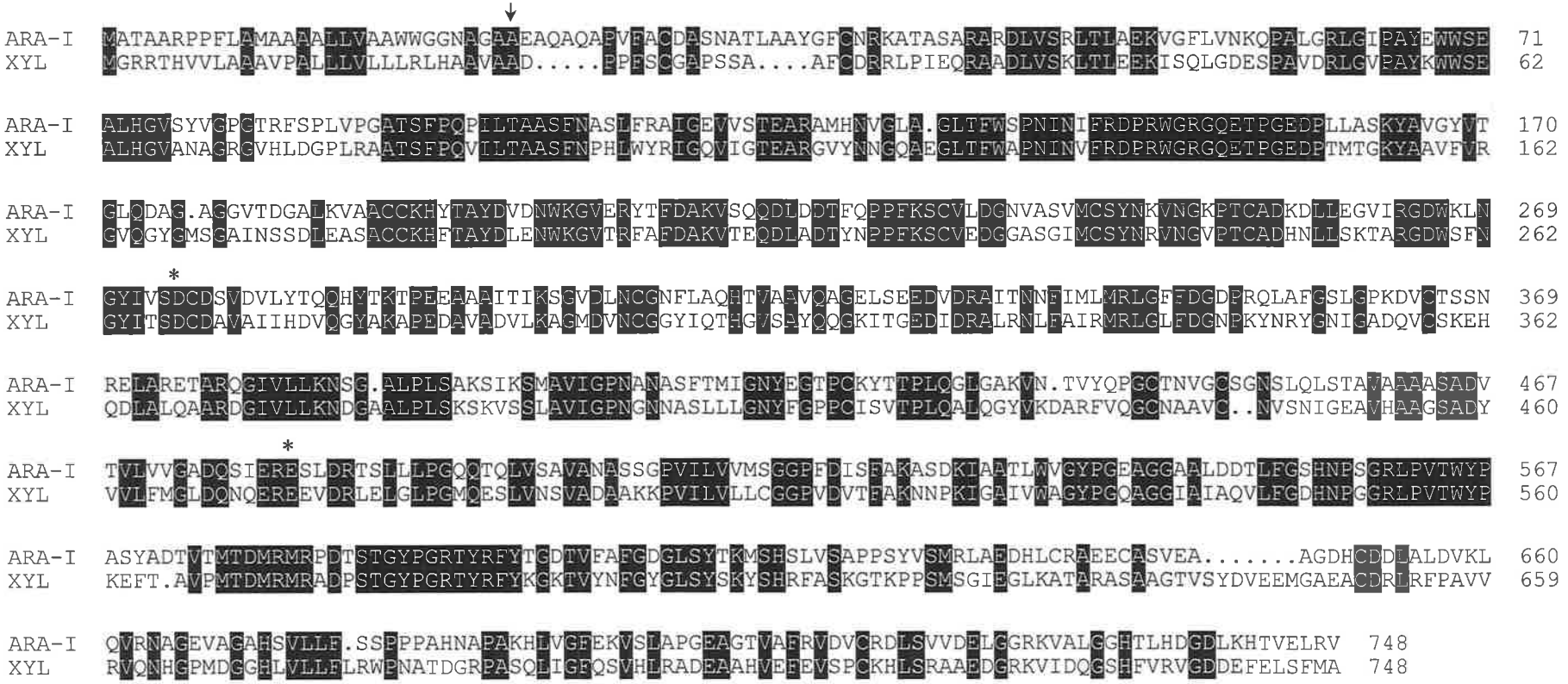


Figure 5.20 **Alignment of ARA-I and XYL amino acid sequences.** The cDNAs were translated from the putative start methionine to the amino acid residue preceding the TGA stop codon. The ARA-I and XYL cDNAs encode mature polypeptides of 748 amino acids with signal peptides of 29 residues. The amino acid sequences above are numbered from the NH_2-termini of the mature enzymes, indicated by an *arrow*. The putative catalytic nucleophile, (D275 for ARA-I and D268 for XYL), and the putative catalytic acid in each sequence (E481 for ARA-I and E474 for XYL), are indicated by *asterisks*.

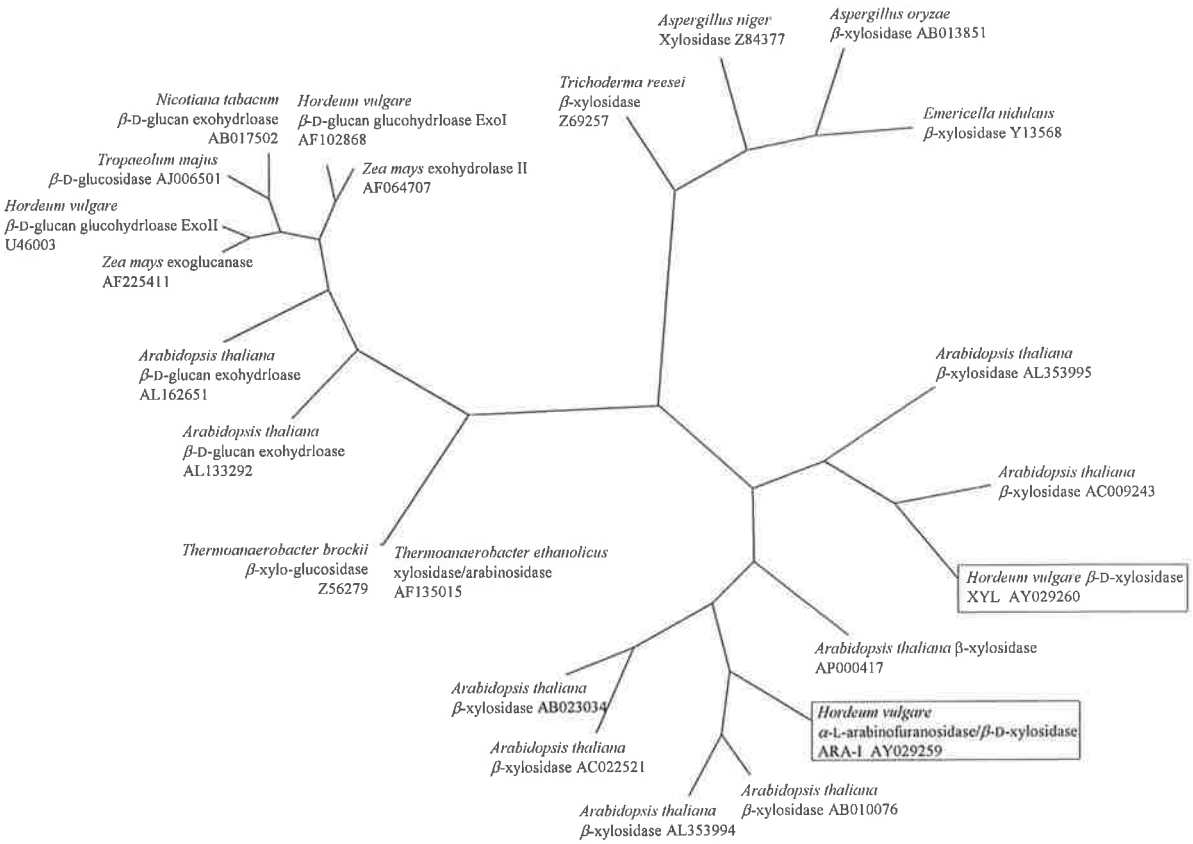


Figure 5.21 **Unrooted radial phylogenetic tree of selected family 3 glycoside hydrolases.** Amino acid sequences were aligned with the ClustalW program and the unrooted phylogenetic tree was constructed with the PAUP program (Swofford, 1988). ARA-I and XYL amino sequences have a high degree of similarity to *Arabidopsis thaliana* genomic sequences encoding β-xylosidase-like proteins (GenBank/EMBL accessions AB010076, AL353994, AP000417, AC022521, AB023034, AL353995, AC009243) ARA-I and XYL are also similar to a cluster of related microbial β-xylosidases from *Aspergillus niger* (Z84377), *Trichoderma reesei* (Z69257), *Emericella nidulans* (Y13568) and *Aspergillus oryzae* (AB013851). Non-specific enzymes from *Thermoanaerobacter brockii* (β-xylo-glucosidase) (Z56279) and *Thermoanaerobacter ethanolicus* (xylosidase/arabinosidase) (AF135015) also have some sequence similarities with ARA-I and XYL. Family 3 also contains β-glucosidases and β-glucan glucohydrolases such as *Zea mays* exoglucanase (AF225411), *Hordeum vulgare* β-D-glucan glucohydrolase isoenzymes ExoI (AF102868) and ExoII (U46003), *Tropaeolum majus* β-D-glucosidase (AJ006501), *Zea mays* exohydrolase II (AF064707), *Nicotiana tabacum* β-D-glucan exohydrolase (AB017502) and *A. thaliana* genomic sequences encoding β-D-glucan glucohydrolase-like proteins (AL133292 and AL162651).

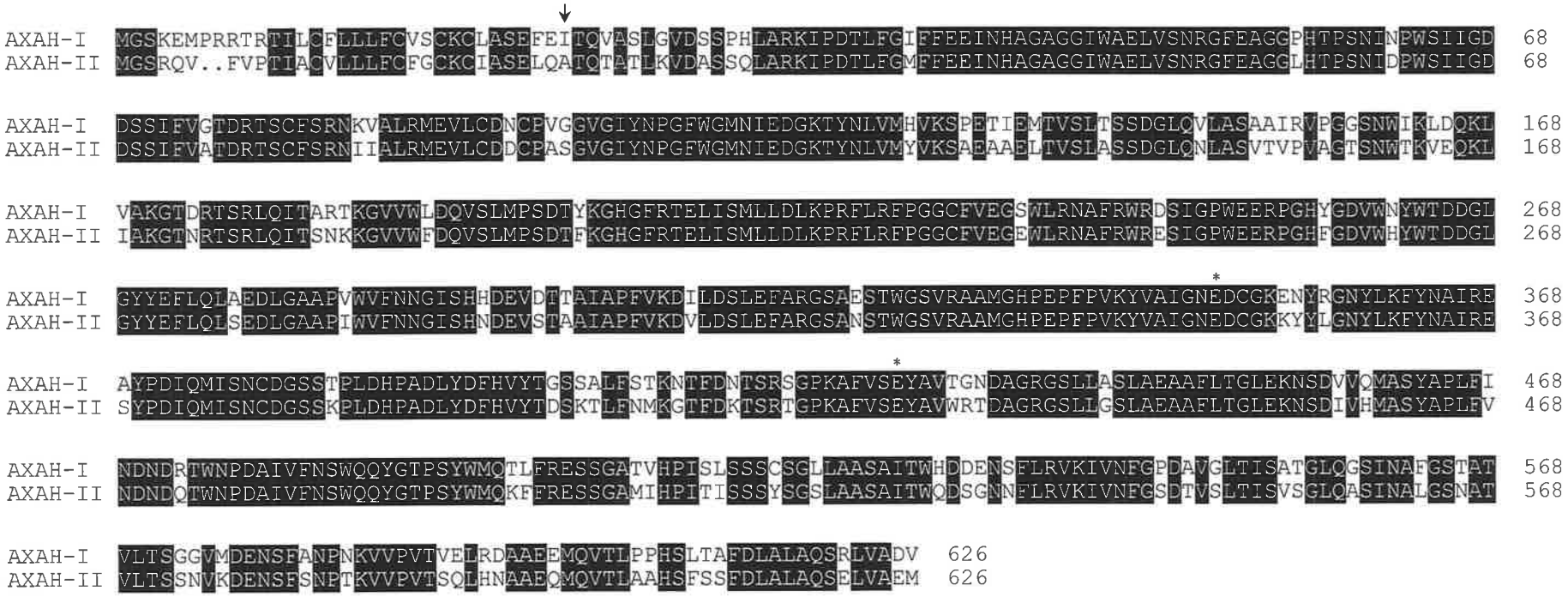


Figure 5.22 **Alignment of AXAH-I and AXAH-II amino acid sequences.** The cDNAs were translated from the putative start methionine to the amino acid residue preceding the TGA stop codon. The ARA-I and XYL cDNAs encode mature polypeptides of 626 amino acids with signal peptides of 32 and 30 residues, respectively. The amino acid sequences above are numbered from the NH_2-termini of the mature enzymes, indicated by an *arrow*. The putative catalytic acid (E348) and catalytic nucleophile (E425) in each sequence are indicated by *asterisks*.

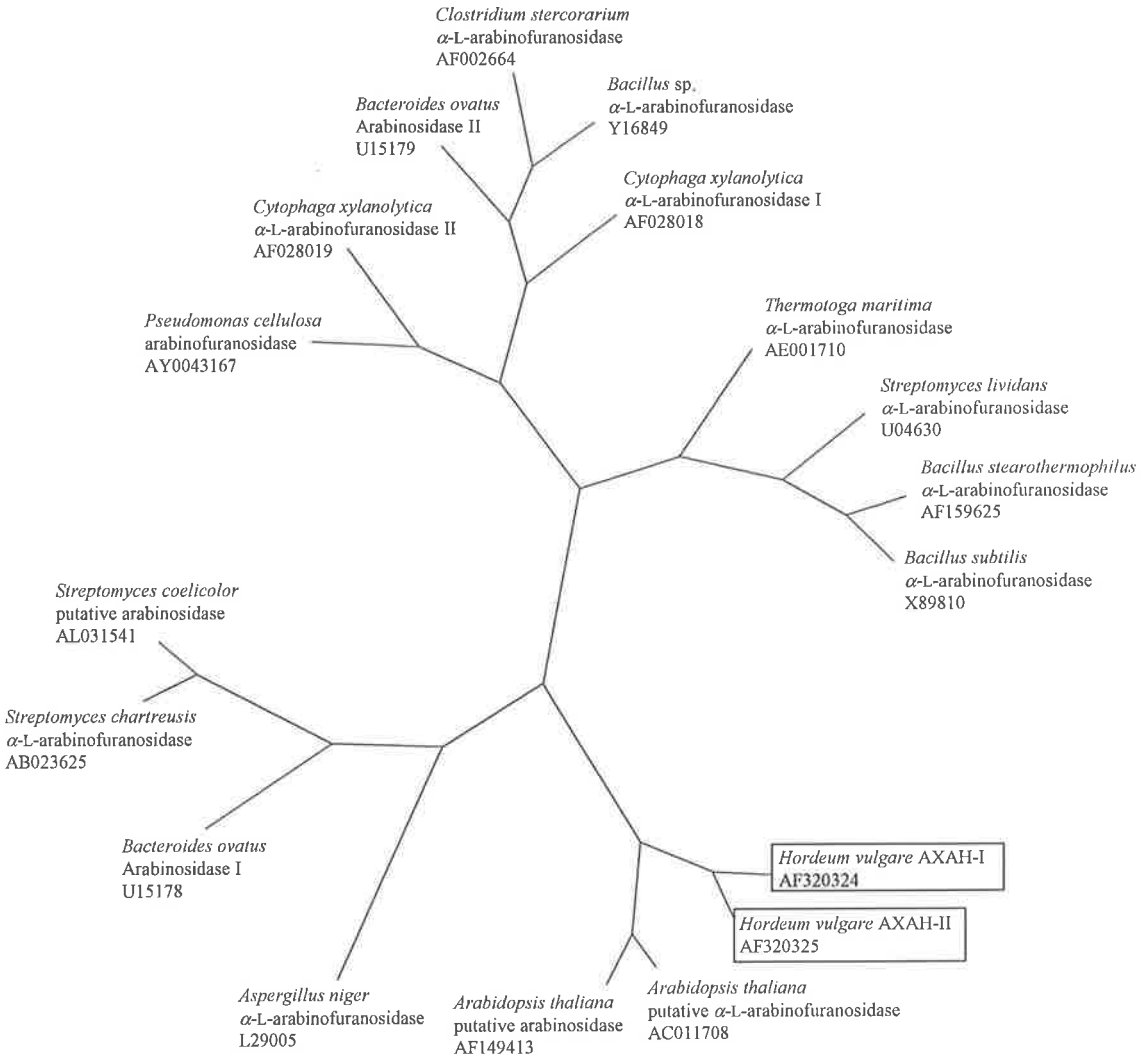


Figure 5.23 **Unrooted radial phylogenetic tree of selected family 51 glycoside hydrolases.** Sequences were aligned using the ClustalW program and the unrooted phylogenetic tree was constructed using the PAUP program (Swofford, 1988). AXAH-I and AXAH-II have a high degree of homology with *Arabidopsis thaliana* genomic sequences encoding arabinosidase-like proteins (GenBank/EMBL accessions AC011708 and AF149413). More distantly related family 51 glycoside hydrolases include arabinosidases asdI and asdII from *Bacteroides ovatus* (U15178 and U15179), α-L-arabinofuranosidases ArfI and ArfII 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), *Pseudomonas cellulosa* (AY043167), *Streptomyces lividans* (U04630), *Streptomyces chartreusis* (AB023625), *Streptomyces coelicolor* (AL031541) and *Thermotoga maritima* (AE001710).

predicted for the family 51 α-L-arabinofuranosidase from *Clostridium stercorarium* (Zverlov *et al.*, 1998), are conserved. The two AXAH sequences were compared with those of other family 51 glycoside hydrolases by phylogenetic analysis (*Figure 5.23*). The barley AXAH-I and AXAH-II isoenzymes are grouped with two putative arabinofuranosidases from *A. thaliana*. Other, more distantly related microbial enzymes share up to 35% sequence identity with the barley AXAH sequences.

5.4 DISCUSSION

5.4.1 *Isolation of cDNAs*

The cDNAs encoding the barley α-L-arabinofuranosidase/β-D-xylosidase, ARA-I, a β-D-xylosidase, XYL and two arabinoxylan arabinofuranohydrolases, AXAH-I and AXAH-II, were isolated using a range of molecular cloning methods. RT-PCR with gene-specific oligonucleotide primers was used for the isolation of cDNA fragments that could be used for cDNA library screening for full-length clones. A 1500 bp EST with sequence homology to AXAH-I was used for cDNA library screening. cDNA Library screening produced many partial cDNA clones corresponding to ARA-I and XYL sequences. The 3'-end regions of the ARA-I and XYL cDNAs were amplified by 3'-end RACE PCR. Overlapping 3'-end RACE sequences were identical to corresponding 5'-end sequences obtained from cDNA libraries. Each of the ARA-I and XYL cDNA fragments was sequenced in both directions and the published cDNAs were thus compiled.

Screening of the 12-day barley seedling λgt11 cDNA library with the AXAH EST identified several identical clones, one of which comprised a full-length cDNA encoding a polypeptide sequence with homology to the purified barley AXAH-I amino acid sequences. The full-length cDNA encoded a second AXAH isoenzyme, designated AXAH-II, which was later found to have 81% sequence identity to AXAH-I. The AXAH-II cDNA sequence exactly matched the sequence of the 1500 bp barley EST used for library screening. A partial AXAH-I cDNA was isolated by heterologous hybridization with the AXAH-II cDNA probe and the AXAH-I 3'-end sequence was isolated using 3'-end RACE.

Thus, complete cDNA sequences encoding ARA-I (AY029259), XYL (AY029260), AXAH-I (AF320324) and AXAH-II (AF320325) were determined and have been published in the GenBank and EMBL databanks with accession numbers given.

5.4.2 *Analysis of ARA-I and XYL cDNA and Amino Acid Sequences*

The amino acid sequences of ARA-I and XYL, derived from cDNA sequences, were aligned to highlight sequence similarity for the two proteins (*Figure 5.20*). Both cDNAs encoded signal peptides of 29 amino acid residues in which charged arginine residues were located near the NH_2-terminus and a polar histidine or asparagine residue was present near the signal

cleavage site. A hydrophobic core of up to 18 residues, consisting of mainly alanine, leucine and valine residues, is consistent with protein translation across the endoplasmic reticulum and secretion from the cell (Watson, 1984; von Heijne, 1986). The AXAA sequence representing the signal peptide cleavage site in XYL, was conserved in the ARA-I amino acid sequence. This consensus sequence satisfies the -3, -1 rule of von Heijne (1986) which states that the amino acid at the -1 position is small, and in the -3 position, only small, polar or aliphatic, non-polar amino acids are permitted and proline must not be present in any of the positions from -3 to +1 (von Heijne, 1986). The ADPPF NH_2-terminal sequence of XYL, determined experimentally as described in *Chapter 2*, was confirmed by the translated XYL cDNA.

The NH_2-terminus of the purified ARA-I protein could not be obtained by Edman degradation because the NH_2-termial residue was blocked. However, the ARA-I NH_2-terminus was predicted by the von Heijne rule, and with consideration of conserved sequence between ARA-I and XYL, mentioned above. An alternative signal-processing site in ARA-I is located 5 amino acid residues downstream, in an AQAQ sequence (*Figure 5.16*). Processing at this alternative site, also in accordance with the -3, -1 principles, would leave a glutamine residue at the NH_2-terminus of the mature ARA-I protein. An NH_2-terminal glutamine residue could be spontaneously cyclized to form a 5-oxoprolyl residue that would be resistant to Edman degradation (Robinson *et al.*, 1973). A further possibility might involve signal peptide processing at the former AXAA site with subsequent aminopeptidase action and cyclization of the resultant glutamine residue. The amino terminus of the family 3 β-D-xylosidase from *Trichoderma reesei* was also blocked (Margolles-Clark *et al.*, 1996). Prediction of the signal cleavage site was similarly based on alanine residues in the -3 and -1 positions and a glutamine residue at the NH_2-terminus (Margolles-Clark *et al.*, 1996; van Peij *et al.*, 1997). Nevertheless, the NH_2-terminal sequence of the mature ARA-I is probably in the vicinity of the sites discussed here. For the purposes of this discussion of signal peptide and mature protein properties, the NH_2-terminal sequence is accepted as the AEAQAQ sequence, indicated in *Figure 5.16*.

Amino acid sequences determined for purified ARA-I and XYL, described in *Chapter 2*, in most cases, exactly matched the ARA-I and XYL amino acid sequences derived from respective cDNAs. A total of 96 amino acid residues from ARA-I tryptic peptides 1, 2, 3, 6*a*,

6*b*, 7 and 8, contained no errors when compared with corresponding regions in the ARA-I sequence. Two out of 27 residues from tryptic peptide 4 were incorrect and the mixed sequences from the ARA-I tryptic peptide fraction corresponding with RP-HPLC peak 5 (*Chapter 2, Figure 2.16*) contained several errors when compared with the deduced ARA-I amino acid sequence. The reliability of the peak 5 sequences was questioned in the discussion of amino acid sequencing results in *Chapter 2* and the amino acid sequence of ARA-I derived from the cDNA remains credible despite these anomalies. Overall, 126 amino acid residues determined by amino acid sequence analysis of the purified protein matched the derived amino acid sequence of ARA-I.

Of the 38 NH_2-terminal amino acid residues determined for the purified β-D-xylosidase, XYL, seven did not match the corresponding sequence derived from the XYL cDNA. Despite these sequence discrepancies in the NH_2-terminal region, all 126 amino acid residues from peptides 1*a*, 2, 6, 7, 8*a* and 8*b* matched the cDNA-derived XYL sequence exactly. A single mismatch in peptide 1*b* and four errors out of nine sequenced residues in peptide 3 were most likely the result of uncertainties in interpreted data for these peptides. The sequences of peptide 1*b* and peptide 3 were determined on the basis of relative abundance of up to six PTH derivatives detected in each sequencing cycle, and mismatches with the deduced sequence were not surprising. A total of 139 tryptic peptide residues were identical to the deduced amino acid sequence of XYL and suggest that the XYL cDNA encodes the protein purified as described in *Chapter 2*. The seven mismatched residues in the NH_2-terminal region conflict with the fidelity of determined amino acid sequences throughout the remainder of the protein. These discrepancies cannot be explained with the current data.

5.4.3 Properties of Mature Enzymes

The ARA-I and XYL cDNAs encode mature polypeptides of 748 amino acids that have 51% positional identity to one another (*Figure 5.20*). The calculated pI values of 5.7 and 6.5 for ARA-I and XYL were similar to pI values determined experimentally during respective protein purifications, described in *Chapter 2*. However, predicted molecular mass values of 79,184 and 80,500 Da for ARA-I and XYL were not consistent with the 65 kDa and 67 kDa molecular masses estimated for these proteins on denaturing electrophoresis gels. COOH-Terminal amino acid sequence analyses of the purified proteins were attempted but were

unsuccessful. Based on the consistent molecular mass determination using denaturing PAGE and extensive analysis of the cDNA sequences, one must conclude that ARA-I and XYL polypeptides have been modified post-translationally. Given the absence of tryptic peptides from the COOH-terminal regions of both ARA-I and XYL (*Figures 5.16* and *5.17*), this post-translational modification is likely to involve removal of a large proportion (perhaps 120 amino acid residues) of the COOH-terminus. Such a process has been recently reported for (1→4)-β-xylan endohydrolase in barley (Caspers *et al.*, 2001). Microbial family 3 β-D-xylosidase cDNAs encode polypeptides of 80-88 kDa, and glycosylation produces proteins of up to 110 kDa. Clearly, the microbial β-D-xylosidases from family 3 are not processed in the same fashion as the homologous family 3 proteins from barley appear to be. Local alignment of *Arabidopsis thaliana* genomic sequences reveals a family of seven genes that encode proteins with sequence similarity to ARA-I and XYL. All seven encoded polypeptides contain approximately 780 amino acids and most probably have calculated molecular mass values in the vicinity of the 80 kDa predicted for ARA-I and XYL. The question of post-translational removal of polypeptide sequence in ARA-I and XYL from barley requires further investigation.

5.4.4 Relationships with other Family 3 Enzymes

The classification of β-D-glucosidases and β-D-xylosidases in glycoside hydrolase family 3 is based on amino acid sequence identity of approximately 30%. However, substrate specificities and consequently biological functions of the two groups of proteins are quite distinct. In barley, the β-glucan glucohydrolases ExoI and ExoII, hydrolyse terminal β-glucosyl linkages of β-glucans (Hrmova *et al.*, 1996). In contrast, the β-D-xylosidase enzymes investigated here, ARA-I and XYL, with no activity against β-glucosides, only target β-D-xylosides. Evolution of substrate specificity in order to generate novel proteins that can accomplish different chemical outcomes, may occur through gene duplication and subsequent accumulation of critical point mutations that alter substrate binding and hydrolytic activity. Evidence for such an evolutionary process is provided by the biochemical and genetic characterization of barley (1→3,1→4)-β-glucan endohydrolases and (1→3)-β-glucan endohydrolases (Høj and Fincher, 1995). These two discrete enzyme-types, with approximately 50% positional identity in amino acid sequence (Xu *et al.*, 1992), have clearly distinct biochemical capacities, and consequently quite separate biological roles in

barley. (1→3)-β-Glucan endohydrolases are implicated in plant defence while (1→3,1→4)-β-glucan endohydrolases participate in metabolism of cell wall (1→3,1→4)-β-glucans (Høj and Fincher, 1995). Furthermore, (1→3,1→4)-β-glucan endohydrolase, isoenzyme EII, and (1→3)-β-glucan endohydrolase, isoenzyme GII, from barley have almost identical three-dimensional structures (Varghese *et al.*, 1994).

Figure 5.21 illustrates relationships between the barley and *Arabidopsis* family 3 β-D-xylosidase sequences with microbial β-D-xylosidases, and with a group of plant β-glucosidase and β-glucan glucohydrolase sequences. The seven *Arabidopsis* β-D-xylosidase sequences are clustered with the barley ARA-I and XYL sequences. It is likely that the diversity of biochemical properties evidenced for the two barley proteins would similarly be found among the *Arabidopsis* proteins. Fungal β-D-xylosidases are grouped on a separate branch from the plant β-D-xylosidase sequences, indicating that substantial divergence has occurred during the evolution of these genes. Two bacterial sequences from *Thermoanaerobacter* sp. encode proteins with broad specificity for β-D-xylosides, β-D-glucosides and α-L-arabinosides (Shao and Weigel, 1992; Breves *et al.*, 1997). These observations are supported by the more comprehensive phylogenetic analysis of the family 3 glycoside hydrolases performed by Harvey *et al.* (2000).

Despite having a greater degree of divergence in both substrate specificity and amino acid sequence, the β-xylosidases and β-glucan glucohydrolases probably have common genetic origins, and analogous to the family 17 β-glucan endohydrolases, probably have similar three-dimensional structures. Homology modelling of ARA-I and XYL primary structures using the three-dimensional structure of barley β-glucan glucohydrolase isoenzyme ExoI (Varghese *et al.*, 1999), as described for the generation of structural models of other family 3 glycoside hydrolases (Harvey *et al.*, 2000), was unsuccessful. With positional identity between the template and target polypeptide sequences no more than 30%, three-dimensional structural models were unreliable and a high proportion of amino acid residues were in unfavourable conformations (results not shown). Nevertheless, hydrophobic cluster analysis (Gaboriaud *et al.*, 1987) showed that elements of secondary structure identified in the β-glucan glucohydrolase isoenzyme ExoI three-dimensional structure (Varghese *et al.*, 1999) were conserved in the ARA-I and XYL sequences (results not shown).

5.4.5 Catalytic Amino Acids and Substrate Binding

Catalytic amino acids identified in barley β-glucan glucohydrolase isoenzymes ExoI and ExoII were conserved in the ARA-I and XYL amino acid sequences. The SDCD sequence, conserved between both ARA-I and XYL and also among all *Arabidopsis* β-xylosidase-like sequences, corresponded with the ExoI and ExoII SDW sequence that contains the aspartic acid catalytic nucleophile residue (Varghese *et al.*, 1999; Harvey *et al.*, 2000). These were D275 and D268 for ARA-I XYL, respectively. Putative catalytic acid/base residues in ARA-I and XYL were glutamic acid residues E481 and E474, respectively. These corresponded with similarly positioned glutamic acid residues in barley β-glucan glucohydrolases ExoI and ExoII (Varghese *et al.*, 1999; Harvey *et al.*, 2000).

Several additional amino acid residues recognised in the β-glucan glucohydrolase ExoI structure as participating in substrate binding, are conserved in ARA-I and XYL, and also in each of the seven β-xylosidase-like sequences from *A. thaliana*. Arginine residue R158, lysine K206, histidine H207 and tyrosine Y253 hydrogen bond with C1 to C4 hydroxyls of the glucosyl residue in subsite +1 of ExoI (Varghese *et al.*, 1999; Hrmova *et al.*, 2002) and each of these residues is conserved in the β-xylosidase enzymes in barley and *A. thaliana*. Furthermore, key catalytic site tryptophan residues, W286 and W434 in the barley β-glucan glucohydrolase ExoI (Varghese *et al.*, 1999; Hrmova *et al.*, 2002), are substituted with other amino acids in ARA-I and XYL, such as cysteine, alanine or proline.

The divergence of substrate specificity and enzyme function among the β-D-xylosidase-like enzymes and closely related β-D-glucan glucohydrolases in family 3 is of considerable interest. Difficulties in homology modelling of the three-dimensional structures of ARA-I and XYL mean that further understanding of these enzymes and their evolution of substrate specificity will depend on the future determination of structure from a crystallised family 3 β-D-xylosidase.

5.4.6 Analysis of AXAH-I and AXAH-II cDNA and Amino Acid Sequences

The deduced amino acid sequences of AXAH-I and AXAH-II were 81% identical to one another as shown in *Figure 5.22*. The similarity between the two AXAH cDNA sequences was also evident from their cross hybridization during cDNA isolation. The 38-residue NH_2-

terminal amino acid sequence of AXAH-I was determined experimentally by Edman sequencing of purified AXAH-I (*Chapter 3*) and corresponded exactly with that deduced from the AXAH-I cDNA. The NH_2-terminal residue of the mature AXAH-I was an isoleucine residue and a 32-residue signal peptide was encoded by the preceding cDNA sequence. The AXAH-II cDNA encodes an homologous amino acid sequence in the NH_2-terminal region and an alanine residue is the predicted NH_2-terminal residue. Glutamic acid residues in position -3 and a glutamic acid or glutamine residue in the -1 position for AXAH-I and AXAH-II, respectively, do not fit the rules of von Heijne (1986). Nevertheless, the signal peptides of both AXAHs do contain a core of hydrophobic residues that suggest that they are secreted proteins. The mature AXAH polypeptides contain 626 amino acid residues and calculated molecular mass values and isoelectric points were similar to experimentally determined values (*Chapter 3*).

In addition to the 38 NH_2-terminal amino acid residues, a further 130 AXAH-I residues were determined by NH_2-terminal sequencing of tryptic peptides and all of these were encoded by the AXAH-I cDNA. Based on the deduced amino acid sequences, AXAH-I and AXAH-II can be classified in glycoside hydrolase family 51. Family 51 contains 13 microbial enzymes for which enzyme properties have been determined and also two genomic sequences from *Arabidopsis thaliana* that presumably encode AXAHs. The higher-plant sequences, with 52-63% sequence identity, are clustered on a common branch, and have 30-35% sequence identity with the microbial sequences.

Hydrophobic cluster analysis (Gaboriaud *et al.*, 1987) and comparative alignment of AXAH-I and AXAH-II sequences allow the prediction of catalytic amino acid residues and also tertiary protein structure. Zverlov *et al.* (1998) identified two conserved regions of $(\beta/\alpha)_8$ barrel proteins in glycoside hydrolase families 1, 2, 5, 10, 39 and 51 that contained putative catalytic residues in the family 51 α-L-arabinofuranosidase from *Clostridium stercorarium*. Beylot *et al.* (2001*b*) have confirmed similarly positioned glutamic acid residues in the *Pseudomonas cellulosa* α-L-arabinofuranosidase as catalytic amino acids using site-directed mutagenesis. Two glutamic acid residues, E348 and E425, were conserved in the barley AXAH-I and AXAH-II sequences, and are the likely catalytic acid and catalytic nucleophile, respectively.

5.4.7 *Summary*

The cDNA sequences of the arabinoxylan-degrading enzymes described in this chapter add to the growing list of glycoside hydrolase sequences. Many of the existing sequences in the glycoside DNA hydrolase family database (Coutinho and Henrissat, 1999*a*) are from microbial sources. The biochemical properties of purified barley proteins, ARA-I, XYL and AXAH-I, described in *Chapter 4*, can now be linked to specific barley genes. Furthermore, a second barley AXAH isoenzyme was identified. Heterologous expression of the AXAH-II cDNA would be required to fully characterize enzymic properties, however, with 81% amino acid sequence identity one can be confident that AXAH-II has similar biochemical properties to those of AXAH-I. cDNAs can now be used as specific molecular probes for genetic mapping and for examination of the expression of respective genes (*Chapter 6*).

CHAPTER 6.

Expression Patterns and Mapping of Barley α-L-Arabinofuranosidase and β-D-Xylosidase Genes

6.1 INTRODUCTION

The purification of barley α-L-arabinofuranosidases and a β-D-xylosidase, and the subsequent isolation of corresponding cDNAs, have enabled the characterization of key enzymes involved in arabinoxylan degradation in barley. The enzymes ARA-I and XYL have substrate specificities suggestive of a biological function in the degradation of oligosaccharide breakdown products of arabinoxylans. In contrast, the activity of AXAH-I against arabinoxylan indicates that the two family 51 AXAH isoenzymes might participate in the de-arabinosylation of polymeric arabinoxylans. Such a process may occur during deposition of arabinoxylans in newly formed cell walls of barley tissues, or alternatively, in the degradation of starchy endosperm, aleurone or scutellum cell walls following germination in barley grain. The next question to address in this investigation of the molecular processes of arabinoxylan metabolism in barley is that of the biological roles of these arabinoxylan-degrading enzymes. Attributing discrete physiological functions to particular endogenous enzymes in organisms as complex as plants can be a difficult exercise. In many cases, we can only infer biological roles of enzymes on the basis of *in vitro* enzymic properties, observed chemical changes within plant tissues themselves, and the location and timing of expression of corresponding genes. Further suggestions on the biological functions of specific genes may be revealed by genetic mapping and correlation of genetic loci with quantitative trait loci (QTLs).

In this final experimental chapter, the expression patterns of ARA-I, XYL, AXAH-I and AXAH-II genes have been examined using semi-quantitative RT-PCR. Genetic mapping of each of the genes is also documented here.

6.2 MATERIALS AND METHODS

6.2.1 *Plant Material*

For the analysis of gene expression in various barley tissues, barley (*Hordeum vulgare* L. cv. Clipper) grain was sterilised, washed and germinated as described for the isolation of seedling tissues for cDNA synthesis in *Chapter 5*. Grains were steeped for 24 h in antibiotic solution and germinated grains were grown on autoclaved 3MM Chr paper (Whatman, Maidstone, England) in the dark, at 22°C for up to 5 days. Moisture content was maintained at approximately 40-45% (*w/w*) and microbial contamination of seedlings was not evident throughout seedling development. Germinated grains were also grown for 5 days in sunlight at 22°C for the isolation of light-grown leaf tissue. Tissues were harvested from the seedlings, immediately immersed in RNALater solution (Ambion Inc., Austin, Texas, U.S.A.) and incubated at room temperature for 30 min. Samples were stored in RNALater at 4°C for subsequent RNA isolation using Trizol reagent (GIBCO-BRL, Rockville, MD, U.S.A.) and cDNA synthesis using Thermoscript RT-PCR system (GIBCO-BRL) as described in *Chapter 5*. Coleoptile and leaf samples were taken 4- and 5-days after germination, respectively. Rootlets were removed from the young seedlings 5-days after germination. Aleurone tissue and scutella were harvested from seedlings after 3-days and developing grains were harvested from barley plants 6-days after anthesis. Procedures for the isolation of each type of sample are described in detail in *Chapter 5*.

Doubled haploid mapping populations of F1 plants from 'Chebec x Harrington', 'Clipper x Sahara' and 'Galleon x Haruna-Nijo' crosses, and wheat aneuploid lines were made available by Professor Peter Langridge (Department of Plant Science, University of Adelaide).

6.2.2 *Semi-Quantitative RT-PCR*

Oligonucleotide primers for gene-specific amplification from cDNAs of developing barley grain and seedling tissues were designed to correspond to DNA sequences within the coding regions of respective cDNAs (forward primer), and from 3'-untranslated regions (reverse primer). The cDNA synthesis reactions, primed with $(dT)_{17}$ primer and containing 3 µg total RNA, were performed as described in *Chapter 5*. PCR reactions contained: 1 µl cDNA; 2.5 µl 10x PCR buffer; 1.5 µl 25 mM $MgCl_2$; 1 µl 5 mM dNTPs; 1 µl each of forward and reverse primers (0.1 $\mu g.\mu l^{-1}$); 2.5 µl DMSO (Sigma Chemical Co., St. Louis, MO, U.S.A.);

0.5 µl *Taq* DNA polymerase (GIBCO-BRL, Rockville, MD, U.S.A.); deoxyribonuclease-free water to a final reaction volume of 25 µl. DNA fragments corresponding to each gene were amplified using a Perkin-Elmer-Cetus thermal cycler, by 30 cycles of PCR consisting of: 94°C, 40 sec (denaturation); 55°C, 40 sec (annealing); 72°C, 30 sec (extension). Amplified products were detected by electrophoresis at 100 V on 1% (*w/v*) agarose (FMC BioProducts, Rockland, Maine, U.S.A.) in 1x TAE buffer (40 mM Tris-acetate buffer, pH 8.0, containing 1 mM EDTA) and 0.1 $\mu g.ml^{-1}$ ethidium bromide. DNA bands were observed under ultraviolet light. Forward and reverse primer pairs for barley *β*-actin and glyceraldehyde phosphate dehydrogenase (GAPDH) were used in control reactions. PCR reactions without cDNA template were included for each primer pair as negative controls. For each set of primers, amplified DNA was excised from the agarose gel and purified using the Geneclean kit (Geneworks, Adelaide, Australia) for subsequent DNA sequence analysis. DNA sequences, in each case, exactly matched the DNA sequence of the respective cDNA clone.

6.2.3 Genomic DNA Isolation and Southern Blot Analysis

Barley genomic DNA from doubled haploid FI plants from three mapping populations, and from wheat aneuploid lines, was prepared by Mrs Margaret Pallotta. Approximately 5 g leaf material was ground to a fine powder under liquid N_2 and extracted in 600 µl 100 mM Tris-HCl buffer, pH 8.5, containing 1% (*w/v*) sarkosyl (Sigma), 100 mM NaCl, 10 mM EDTA, 2% polyvinylpyrrolidone (Sigma). Phenol/chloroform/iso-amylalcohol (25:24:1) (600 µl) was added and leaf homogenates were mixed by inversion for 10 min. Tubes were centrifuged for 10 min at 13,000 rpm and DNA contained in the upper aqueous phase was precipitated by addition of 60 µl 3 M sodium acetate buffer, pH 4.8, and 600 µl isopropanol. After centrifugation at 13,000g and removal of the supernatant, precipitated DNA was washed with 70% (*v/v*) ethanol, air-dried and resuspended in 30 µl TE buffer (10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA) containing 40 $\mu g.ml^{-1}$ ribonuclease-H (Sigma). Genomic DNA from mapping population lines was restricted using restriction endonucleases *Eco*RI, *Eco*RV, *Dra*I, and *Hin*dIII, and electrophoresed on 1% (*w/v*) agarose gels in 1x TAE buffer (40 mM Tris-acetate buffer, pH 8.0, containing 1 mM EDTA) at 33 V. DNA was observed on a UV transilluminator and transferred to Hybond N membranes in 0.4 M NaOH, prior to pre-hybridization and hybridization with [α-^{32}P]-dCTP-labelled probes as described

in *Chapter 5*. DNA probes for ARA-I, XYL, AXAH-I and AXAH-II were used to screen mapping populations where restriction fragment length polymorphisms (RFLPs) were evident. *Hin*dIII-digested wheat aneuploid lines were also screened with each of the probes listed above. Membranes were washed two times in 0.2x SSC, 0.1% SDS and exposed to autoradiography film for 4-5 days at -80°C as previously described.

Genetic linkage analyses were performed using Mapmaker (Lander *et al.*, 1987) and JoinMap (Stam, 1993) software. Recombination values were translated into map distances in centiMorgan (cM) units using the Kosambi function (Kosambi, 1944). Chromosomal locations were also assigned using the wheat aneuploid lines, based on the absence of bands in nullisomic lines.

6.3 RESULTS

6.3.1 *Transcript Analysis of β-D-Xylosidase and α-L-Arabinofuranosidase Genes*

Levels of ARA-I, XYL, AXAH-I and AXAH-II mRNA transcripts in various barley tissues, including developing grain, aleurone, scutellum, rootlets, coleoptiles, and leaves, both etiolated and light grown, were assessed using semi-quantitative RT-PCR. Initially, Northern analyses were attempted for the examination of the transcriptional activities of these genes. However, difficulties were encountered in the isolation of total RNA from plant tissues with high polysaccharide contents and hybridization signals were very low. Problems of probe specificity also arise where multiple isoenzymes with similar mRNA sequences exist and this was particularly relevant in the case of the closely related AXAH-I and AXAH-II. Gene-specific probes corresponding to the 3'-untranslated regions of cDNAs were found to be of insufficient length to produce effective radiolabelled DNA probes for Northern hybridization analyses. Gene-specific primers were therefore synthesised to specifically amplify short DNA fragments from reverse-transcribed total RNA from each plant tissue sample. Sense oligonucleotide primers corresponded to cDNA sequences within the coding region of respective cDNAs and antisense primers were designed for sequences within the unique 3'-untranslated regions of respective cDNAs.

Primer pairs for gene-specific, semi-quantitative RT-PCR were as follows: ARA-I, $^{5'}$GGAGGCTGCCGGTGACGTGG$^{3'}$ (sense), $^{5'}$TGAAATCGATTCTCCCTACG$^{3'}$ (antisense); XYL, $^{5'}$GGAGGAGATGGGCGCGGAGGCGTG$^{3'}$ (sense), $^{5'}$GCAGCAGGTTTATC-GTGTTCG$^{3'}$ (antisense); AXAH-I, $^{5'}$GTTGTTCAGATGGCAAGCTATGC$^{3'}$ (sense), $^{5'}$GTTCCCTTATTCACCGACACC$^{3'}$ (antisense); AXAH-II, $^{5'}$CGGGAATAA-CTTCCTGAGAG$^{3'}$ (sense), $^{5'}$CAAGCTCCTCCATTTCCATATC$^{3'}$ (antisense). Primers were tested through their ability to produce DNA of the expected size from plasmid DNA preparations of appropriate cDNA constructs described in *Chapter 5*. RT-PCR bands were consistent with expected product sizes of 619 bp (ARA-I), 376 bp (XYL), 545 bp (AXAH-I) and 367 bp (AXAH-II), calculated from respective cDNA sequences (results not shown).

Figure 6.1 shows the results for RT-PCR from barley tissue samples using primers specific for each of the ARA-I, XYL, AXAH-I and AXAH-II cDNAs. Control PCR reactions for each of the cDNA preparations were performed using primers specific for barley actin and

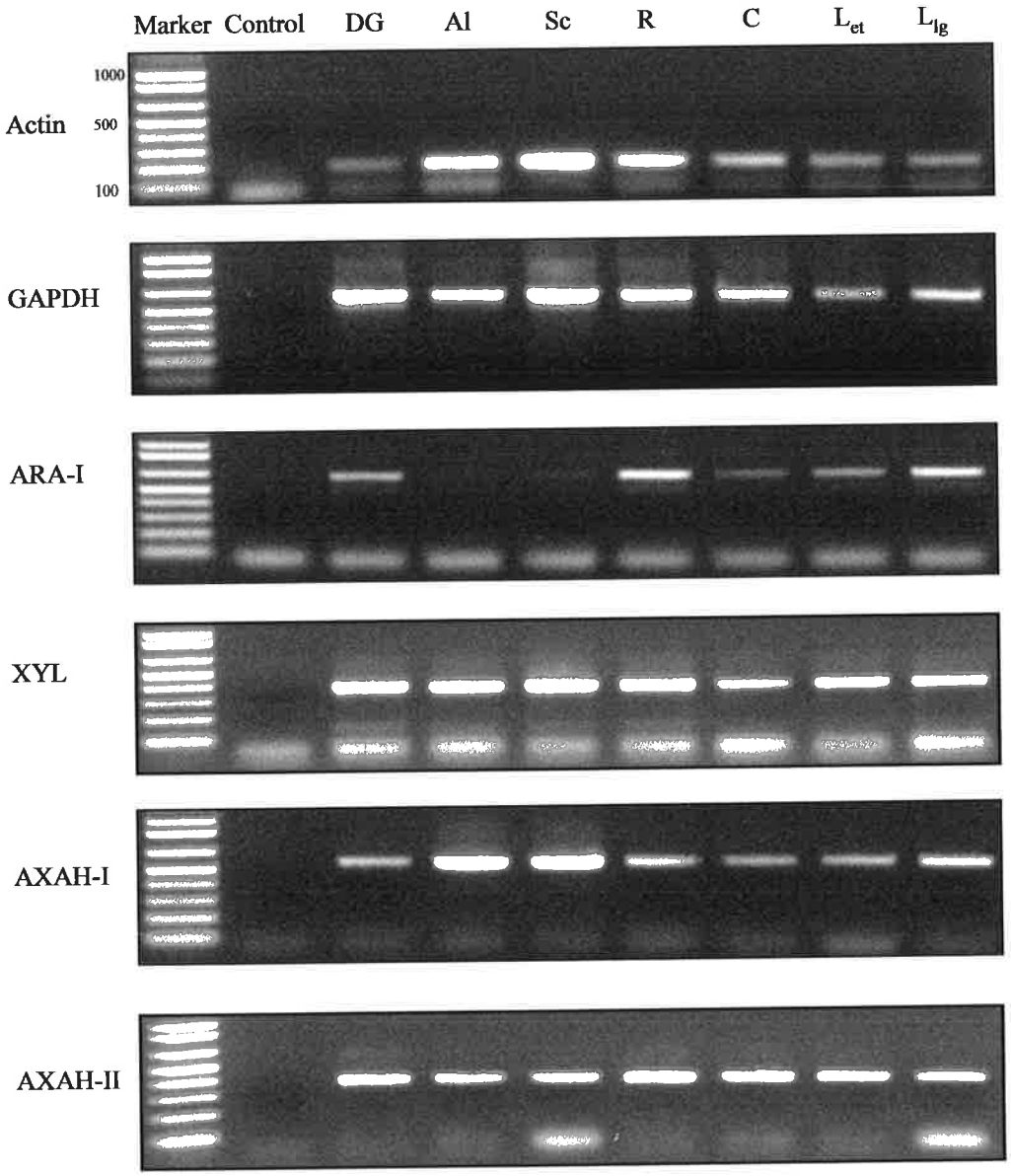


Figure 6.1 **Semi-quantitative RT-PCR for Actin (control), GAPDH (control), ARA-I, XYL, AXAH-I and AXAH-II using cDNA prepared from various barley tissues (developing grain, DG; aleurone, Al; scutellum, Sc; root, R; coleoptile, C; etiolated leaf, L_{et}; light-grown leaf, L_{lg}).** cDNAs were prepared from 3 μg total RNA using Thermoscript reverse transcriptase incubated at 60°C for 1 h. DNA fragments corresponding to each gene were amplified by 30 cycles of PCR (94°C, 40 sec; 55°C, 40 sec; 72°C, 30 sec) using gene-specific primers.

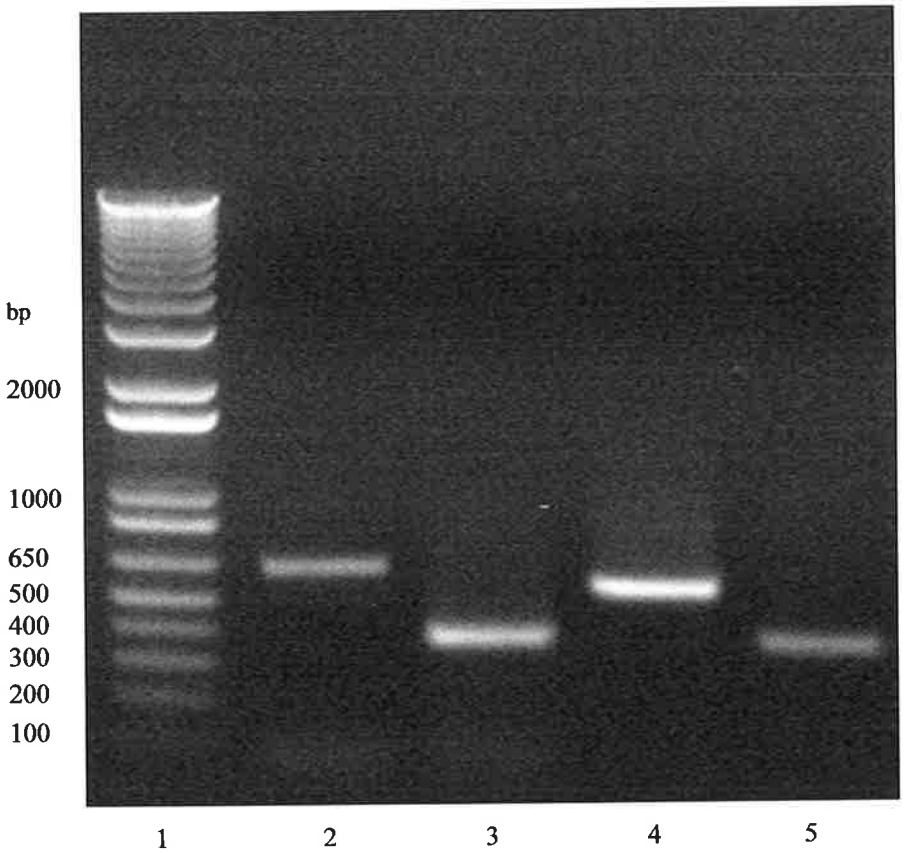


Figure 6.2 **RT-PCR bands excised for sequence analysis and validation.** cDNAs for ARA-I (*lane 2*), XYL (*lane 3*), AXAH-I (*lane 4*) and AXAH-II (*lane 5*), generated by RT-PCR, were purified from agarose gel after electrophoresis and DNA sequences for each were determined. In each case the DNA sequence obtained exactly matched that of the respective cDNA clone. DNA size markers are included (*lane 1*).

GAPDH genes, which should be expressed at reasonable levels in all the barley tissues tested. For each of the cDNA synthesis reactions, a standard amount of total RNA (3 μg) was used for RT-PCR. This was assumed to provide a consistent level of representative mRNAs and therefore, following reverse transcription, a representative population of cDNAs from which PCR amplified bands could be quantitated. Levels of actin and GAPDH varied between samples, thus reflecting differences in the metabolic activities of different tissue types. ARA-I transcripts were detected in developing grains and in vegetative rootlets, coleoptiles and leaves. ARA-I does not appear to be expressed in aleurone or scutellum tissue at 3-days after germination. XYL transcripts were found in each of the barley tissues and at relatively high levels. AXAH-I was transcribed in each of the barley tissues tested, with elevated levels of mRNA in aleurone and scutellum tissue. AXAH-II was transcribed in all tissues tested. Each of the four PCR products (excluding controls) was excised from agarose gel (*Figure 6.2*) and sequence identities with respective cDNAs were confirmed by nucleotide sequence analysis.

6.3.2 *Genetic Mapping of β-D-Xylosidase and α-L-Arabinofuranosidase Genes*

Barley mapping population parental lines were screened at high stringency with DNA probes corresponding to each of the α-L-arabinofuranosidase/β-D-xylosidase, β-D-xylosidase and AXAH cDNAs. DNA probes were as follows: ARA-I, 2400 bp 3'-end RACE fragment; XYL, 1800 bp 3'-end RACE fragment; AXAH-I, both the 5'-end λgt11 cDNA and the 3'-end RACE cDNA fragment; AXAH-II, full-length λgt11 cDNA. Restriction fragment length polymorphisms (RFLPs) for each of the DNA probes were rare, with only one RFLP for the ARA-I probe, between Chebec and Harrington genomic DNA digested with restriction endonuclease *Hin*dIII (*Figure 6.3*). For the XYL probe, RFLPs were present for Clipper and Sahara genomic DNA restricted with *Eco*RI (*Figure 6.4*) and *Dra*I (*Figure 6.5*). For AXAH-I, an RFLP between Galleon and Haruna-Nijo DNA digested with *Dra*I, was used for mapping purposes (*Figure 6.6*). For AXAH-II, there were no RFLPs between the parental lines of available mapping populations, with any of the restriction endonucleases used.

Mapping of RFLPs was accomplished by scoring each of the doubled haploid F1 individuals as having inherited a particular RFLP from either parent A or parent B. Chromosomal locations for the ARA-I, XYL and AXAH-I genes were determined by correlation with genetic markers using the Mapmaker and JoinMap software. The positions of the ARA-I,

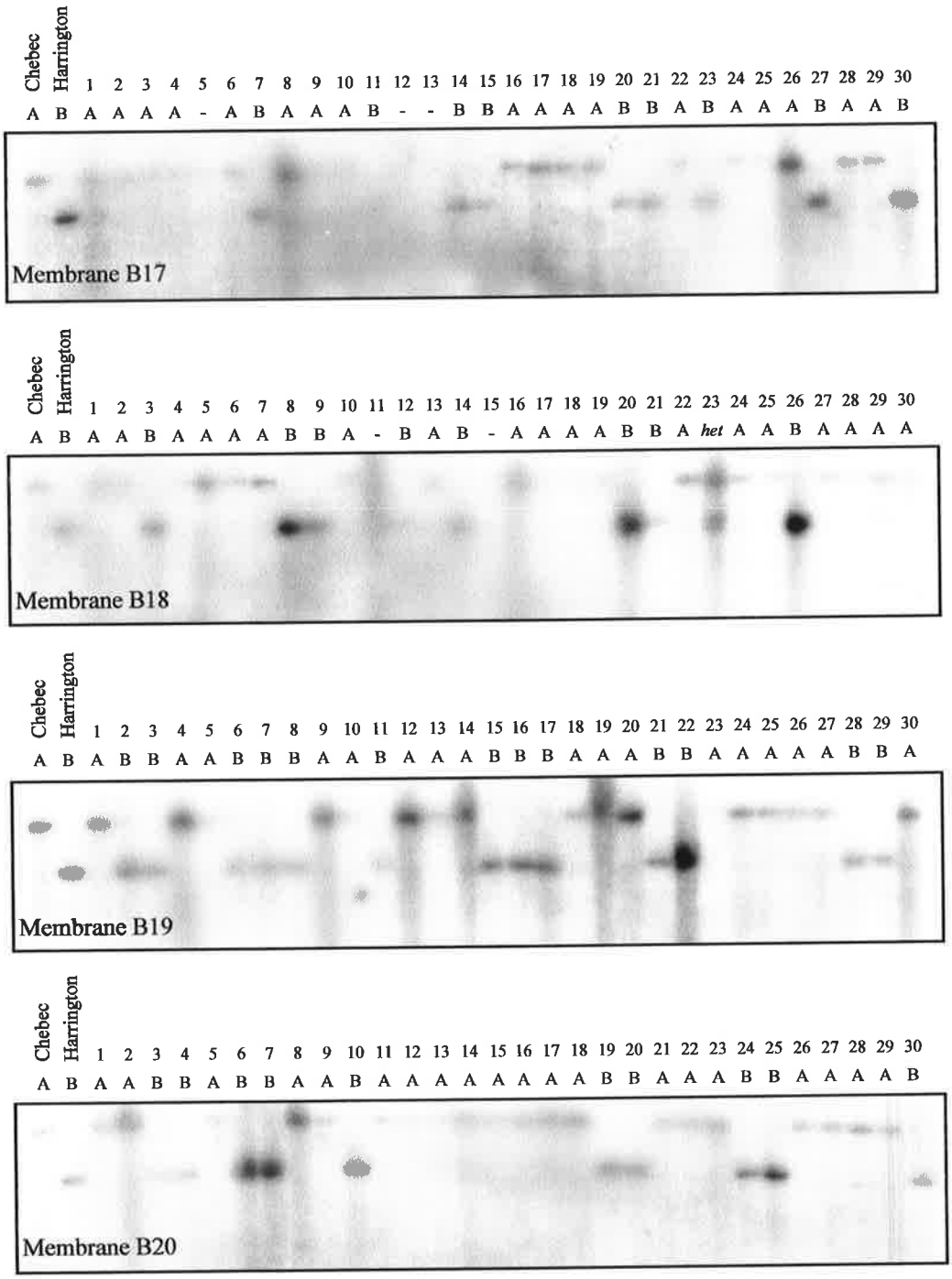


Figure 6.3 **Mapping of the ARA-I gene using 'Chebec x Harrington' doubled haploid mapping population.** Genomic DNA samples from 120 F1 doubled haploid individuals were digested with *Hin*dIII, electrophoresed on four gels and blotted onto nylon membranes. Genomic Southern blots were hybridized at 65°C with a radiolabelled 2400 bp ARA-I cDNA probe and after washing with 0.1 x SSC, 0.1% SDS at 65°C, exposed on autoradiography film for 5 days at -80°C. RFLPs for the Chebec (A) and Harrington (B) parents and for each of the F1 hybrids are shown (*het* = heterologous).

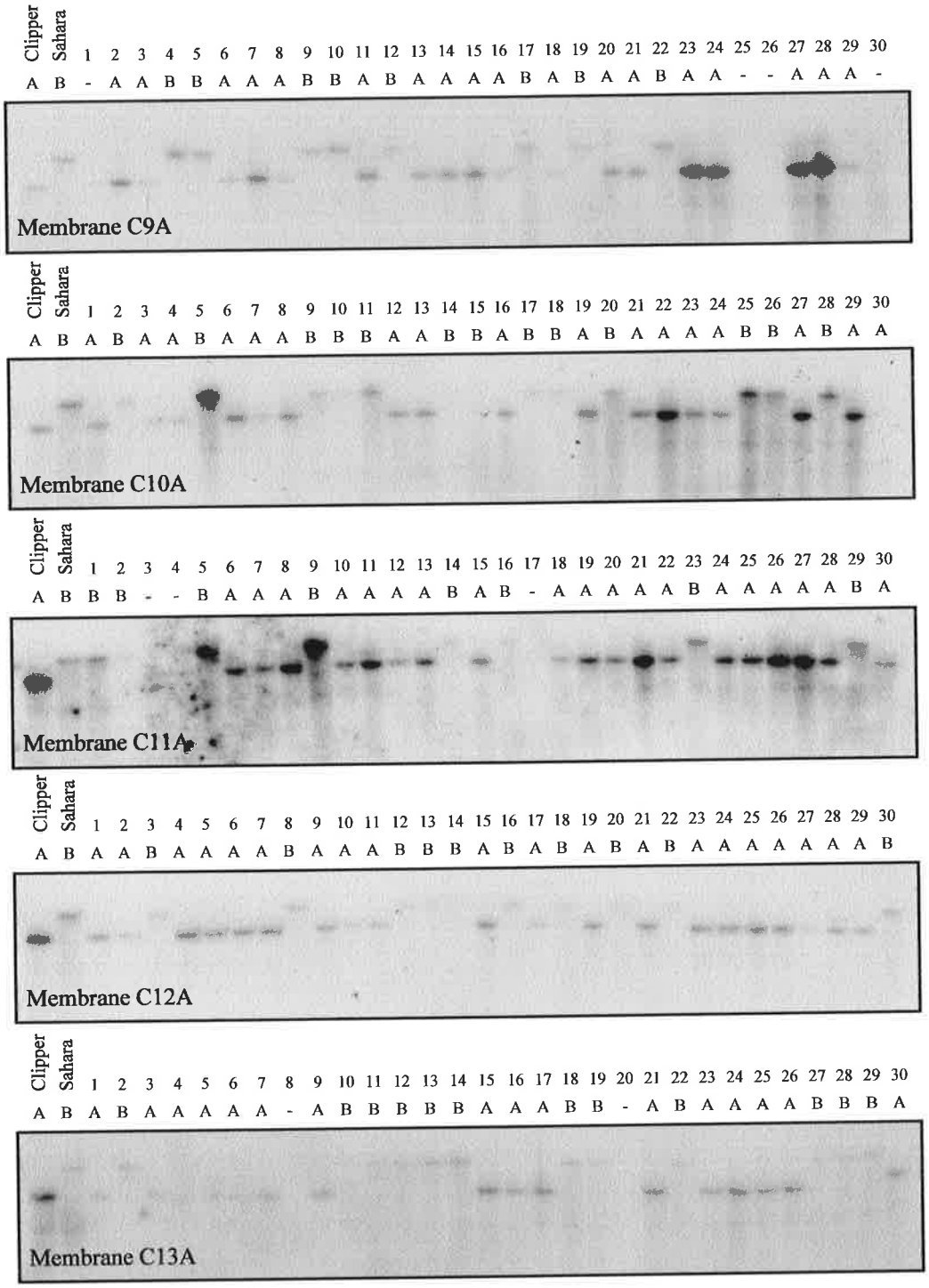
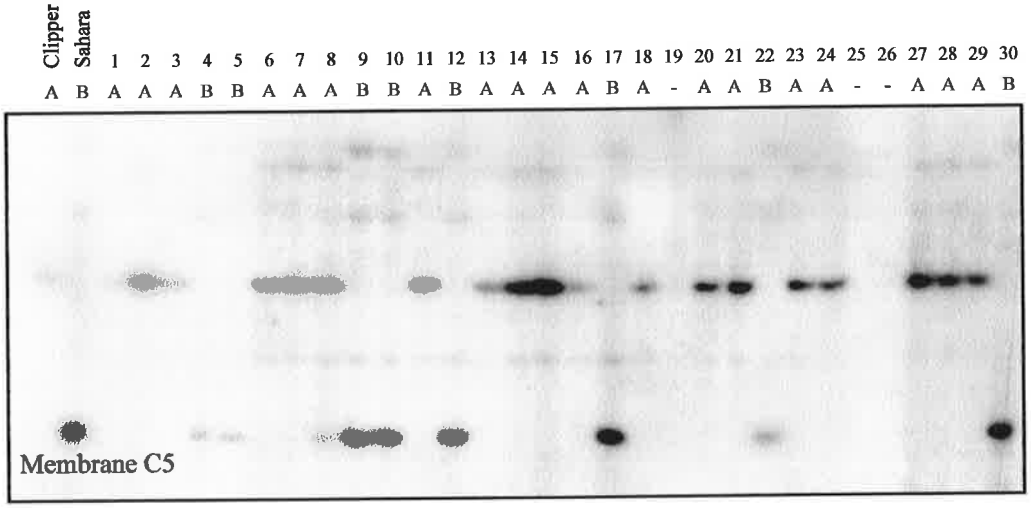


Figure 6.4 **Mapping of the XYL gene using 'Clipper x Sahara' doubled haploid mapping population.** Genomic DNA samples from 150 F1 doubled haploid individuals were digested with *Eco*RI, electrophoresed on five gels and blotted onto nylon membranes. Genomic Southern blots were hybridized at 65°C with a radiolabelled 1800 bp XYL cDNA probe and after washing with 0.1 x SSC, 0.1% SDS at 65°C, exposed on autoradiography film for 5 days at -80°C. RFLPs for the Clipper (A) and Sahara (B) parents and for each of the F1 hybrids are shown.



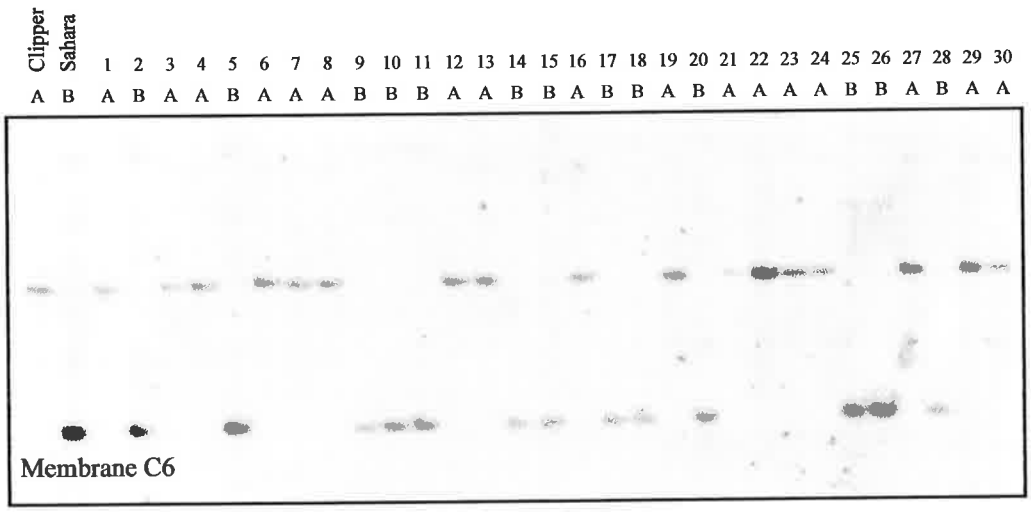


Figure 6.5 **Mapping of XYL using 'Clipper x Sahara' doubled haploid mapping population.** Genomic DNA samples from 150 F1 doubled haploid individuals were digested with *Dra*I, electrophoresed on five gels and blotted onto nylon membranes. Genomic Southern blots were hybridized at 65°C with a radiolabelled 1800 bp XYL cDNA probe and after washing with 0.1 x SSC, 0.1% SDS at 65°C, exposed on autoradiography film for 5 days at -80°C. RFLPs for the Clipper (A) and Sahara (B) parents and for each of the F1 hybrids are shown. *Continued overleaf.*

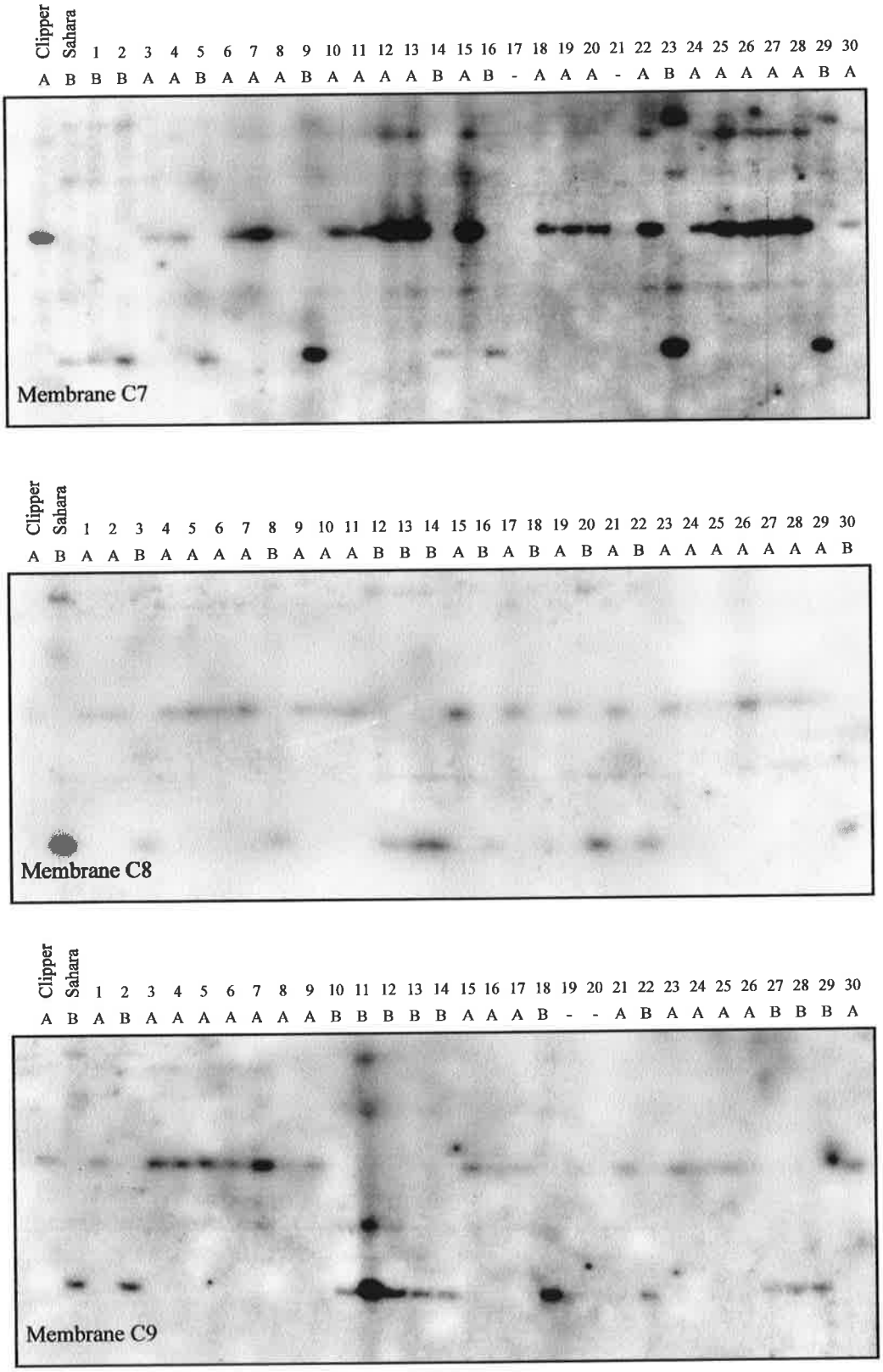


Figure 6.5 *Continued from previous page.*

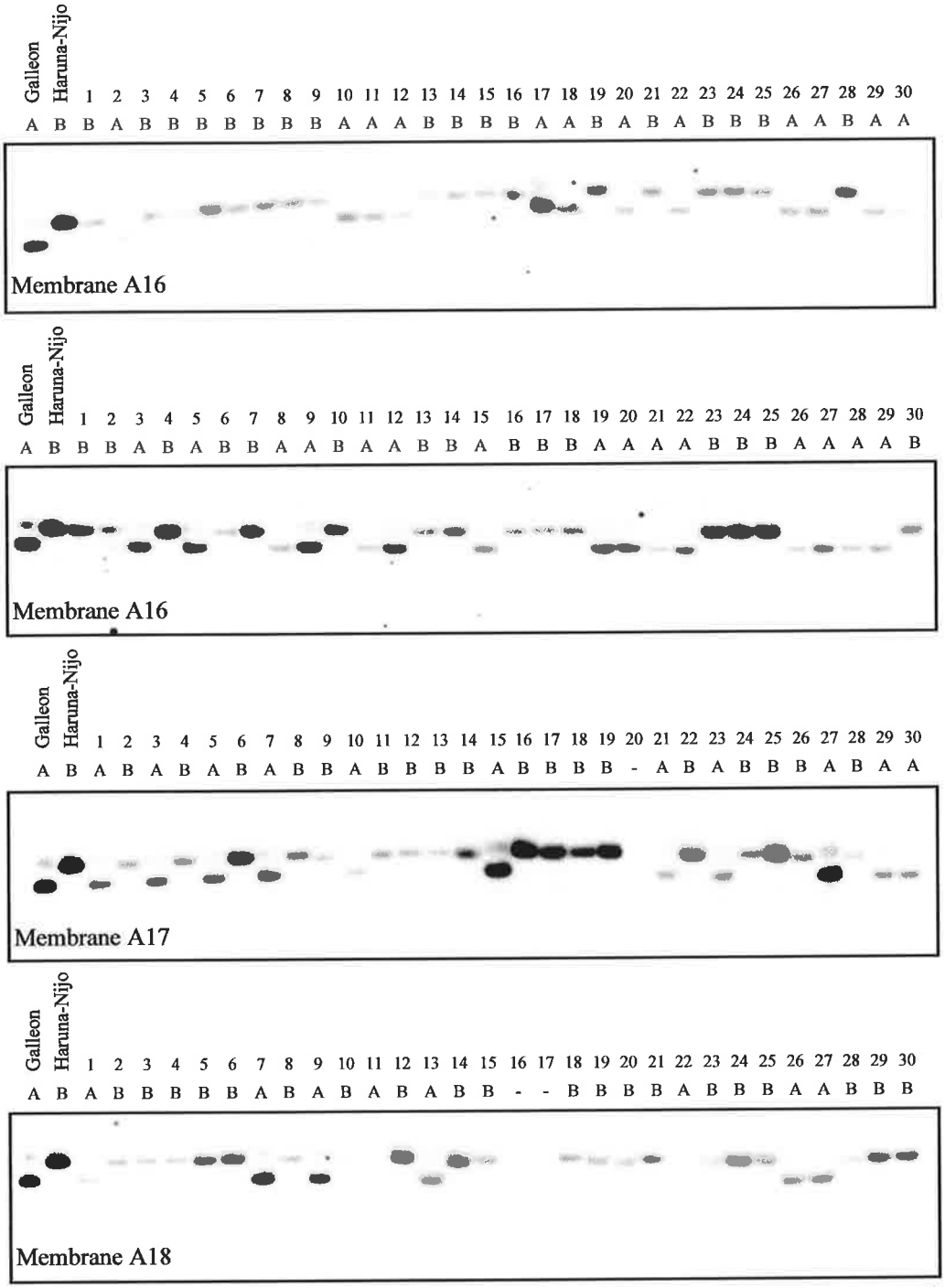


Figure 6.6 **Mapping of AXAH-I using 'Galleon x Haruna-Nijo' doubled haploid mapping population.** Genomic DNA samples from 120 F1 doubled haploid individuals were digested with *Dra*I, electrophoresed on four gels and blotted onto nylon membranes. Genomic Southern blots were hybridized at 65°C with radiolabelled AXAH-I 5'end and AXAH-I 3'-end RACE cDNA probes and after washing with 0.1 x SSC, 0.1% SDS at 65°C, exposed on autoradiography film for 4 days at -80°C. RFLPs for the Galleon (A) and Haruna-Nijo (B) parents and for each of the F1 hybrids are shown.

XYL and AXAH-I genes, together with previously characterized genetic markers, on linkage maps of relevant chromosomes are shown in *Figures 6.7*, *6.8* and *6.9*, respectively. The ARA-I gene is located on the long arm of barley chromosome 2H and the gene encoding XYL is found near the centromere of barley chromosome 6H. The gene encoding AXAH-I is located on the short arm of barley chromosome 5H.

The gene encoding AXAH-II could not be mapped in the available mapping populations because RFLPs were not present with the restriction enzymes used. *Hin*dIII-digested genomic DNA samples from wheat aneuploid lines were screened for the loss of bands hybridizing the AXAH-II DNA probe (*Figure 6.10*). Six major hybridizing bands were evident in the *Hin*dIII-digested wheat genomic DNA; these bands represented AXAH-II restriction fragment lengths for the A, B and D, wheat genomes. Two bands were absent in each of the aneuploid lines that were deficient in either the 4A, 4B or 4D chromosome, thereby indicating that the homoeologous AXAH-II gene in wheat is located on wheat chromosome 4. The likely chromosomal location for the AXAH-II gene in barley is therefore chromosome 4H. Wheat aneuploid lines were screened with DNA probes for ARA-I, XYL and AXAH-I and the chromosomal assignments for each homoeologous gene in wheat matched that determined by mapping of respective RFLPs in barley doubled haploid F1 populations.

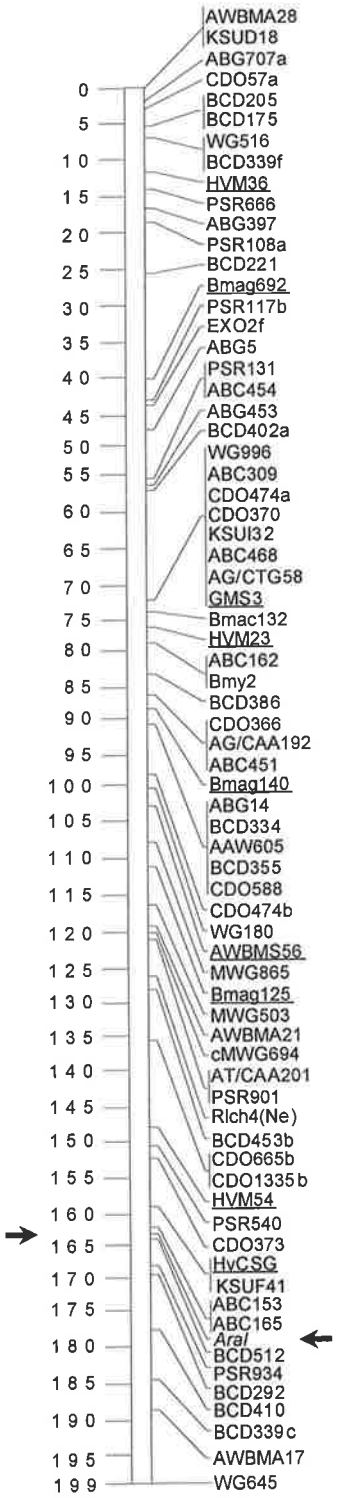


Figure 6.7 **Chromosome map of barley chromosome 2H showing the position of the *AraI* gene that encodes ARA-I.** Molecular markers and *AraI* were mapped in the 'Chebec x Harrington' mapping population. *AraI* is located at 163 cM and is indicated with an *arrow*. This figure was produced by Mr. Angelo Karakousis.

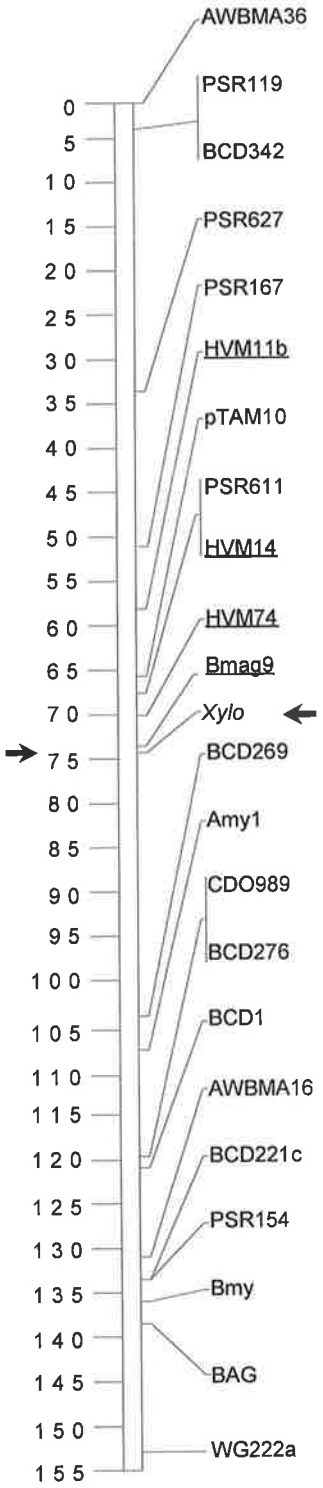


***Figure 6.8* Chromosome map of barley chromosome 6H showing the position of the *Xylo* gene that encodes XYL.** Molecular markers and *Xylo* were mapped in the 'Clipper x Sahara' mapping population. *Xylo* is located at 75 cM and is indicated with an *arrow*. This figure was produced by Mr. Angelo Karakousis.

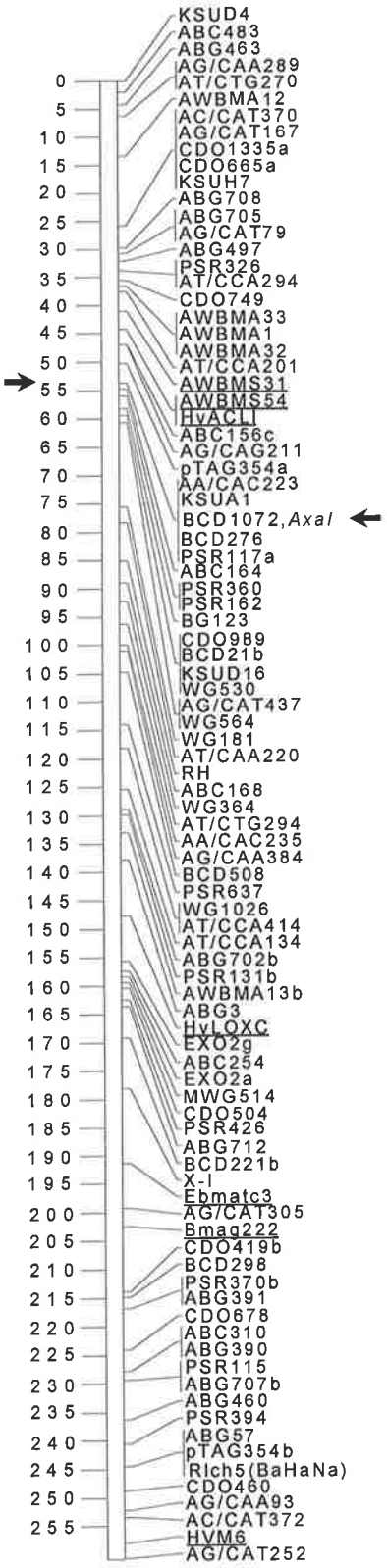


Figure 6.9 **Chromosome map of barley chromosome 5H showing the position of the *AxaI* gene that encodes AXAH-I.** Molecular markers and the *AxaI* gene were mapped in the 'Galleon x Haruna-Nijo' mapping population. *AxaI* is located at 54 cM and is indicated with an *arrow*. This figure was produced by Mr. Angelo Karakousis.

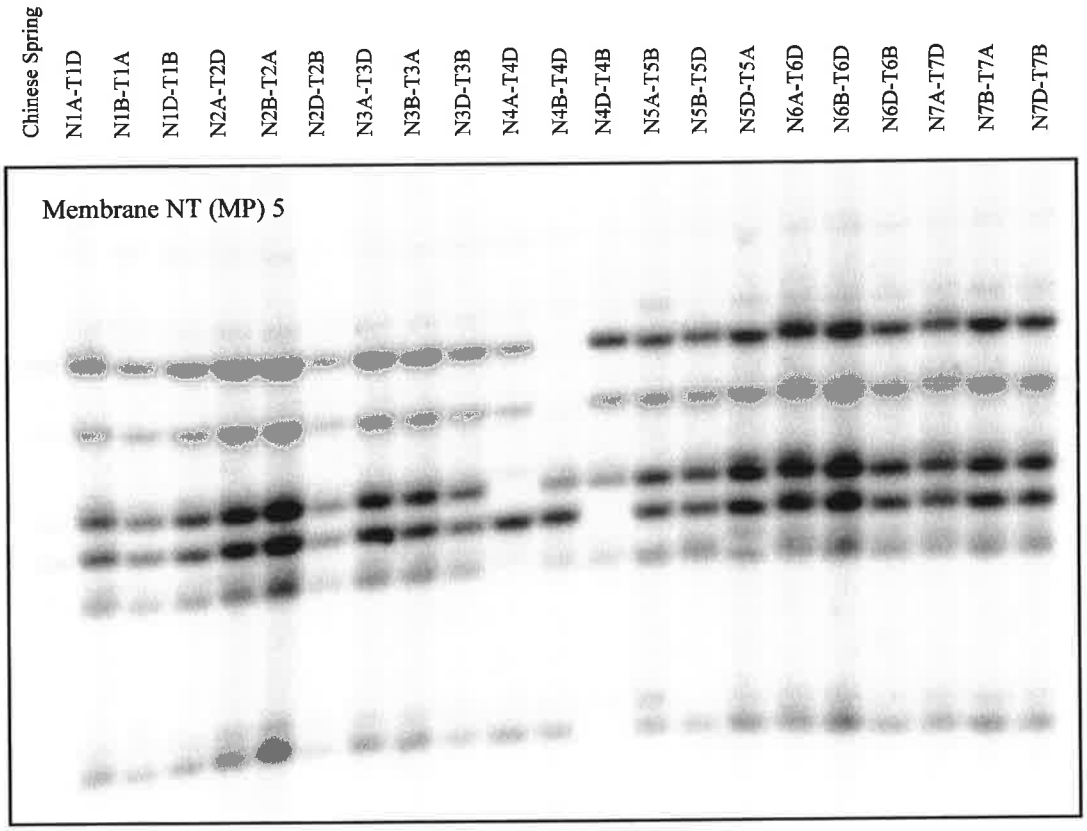


Figure 6.10 **Mapping of AXAH-II using wheat nuli-tetrasomic lines.** Genomic DNA samples from Chinese Spring wheat and 21 nuli-tetrasomic lines were digested with HindIII, electrophoresed and blotted onto nylon membrane. Southern blots were hybridized at 65°C with radiolabelled AXAH-II cDNA probe and after washing with 0.1 x SSC, 0.1% SDS at 65°C, exposed on autoradiography film for 5 days at -80°C. Loss of bands for N4A, N4B and N4D indicate that the homoeologous AXAH-II gene is located on wheat chromosome 4, and suggests that in barley the AXAH-II gene is located on chromosome 4H.

6.4 DISCUSSION

6.4.1 Gene Expression

Transcription of genes encoding ARA-I, XYL, AXAH-I and AXAH-II has been investigated using semi-quantitative RT-PCR. RT-PCR is a simple method that can be used to compare transcript levels in a range of plant tissues using a representative cDNA population generated from a standard level of total RNA. RT-PCR produced clear bands with expected sizes for each of the primer pairs used. The identity and purity DNA amplified using ARA-I, XYL, AXAH-I and AXAH-II primer pairs, was verified by DNA sequencing. Actin and GAPDH primers produced bands with various degrees of intensity (*Figure 6.1*), which suggested that the levels of transcription of the control genes were not uniform between the various barley tissues. Alternatively, the proportion of mRNA in each total RNA sample, or the quality of mRNA may have varied between samples. In the etiolated and light-grown leaf samples, the large amount of rubisco mRNA may lead to lower levels of other mRNA transcripts in the standard 3 μg total RNA sample and therefore an underestimate of particular transcript levels. Conversely, the relatively high level of mRNA in the total RNA isolated from aleurone tissue (results not shown) may exaggerate the estimated level of transcription. Nevertheless, the RT-PCR procedure shows in which tissues each of the genes is transcribed and comparison of levels of gene expression between tissues can be approximated from the intensity of each band.

Expression of the α-L-arabinofuranosidase/β-D-xylosidase (ARA-I) gene was detected at low levels in developing grain, rootlets, coleoptiles and both in etiolated and light-grown leaves. The β-D-xylosidase XYL was expressed in each of the barley seedling tissues sampled and transcripts were detected at relatively high levels. ARA-I was not expressed at high levels in aleurone or scutellum tissue of germinated barley grain, which suggested that ARA-I does not participate in arabinoxylan degradation during post-germination mobilisation of the barley endosperm. In contrast, the gene encoding XYL is expressed at high levels in these tissues and it is possible that the β-D-xylosidase functions cooperatively with (1→4)-β-D-xylan endohydrolase, which is produced exclusively in the aleurone of germinated barley (Banik *et al.*, 1996). Genes encoding ARA-I and XYL are expressed in vegetative rootlets, coleoptiles and leaves, and in the developing grain. In the apparent absence of (1→4)-β-D-xylan endohydrolase in these tissues (Banik *et al.*, 1996), it is unclear exactly what role

ARA-I and XYL might play. In vegetative tissues and in developing barley caryopses, arabinoxylan modification processes associated with cell wall synthesis and deposition are potential sites of action for these enzymes. Chemical analysis of developing maize coleoptiles (Gibeaut and Carpita, 1991) suggests that L-arabinofuranosyl residues are removed from cell wall arabinoxylans at the end of the cell growth phase. Growth-related changes in the amount of D-xylose in vegetative grass tissues have not been examined in detail. However, the level of β-D-xylosidase activity contributed by the expression of both ARA-I and XYL in vegetative tissues of barley and in developing grain might be considerable.

Removal of L-arabinofuranosyl residues from arabinoxylans, *in situ*, requires the arabinoxylan arabinofuranohydrolase activity of AXAH-I and presumably also that of a second isoenzyme, AXAH-II. Genes encoding both AXAH-I and AXAH-II were transcribed in all barley tissue samples tested (*Figure 6.1*). Bands of roughly equal intensity were observed for AXAH-II across developing grain, aleurone, scutellum, rootlet, coleoptile, and etiolated and light-grown leaf. Transcription of the gene encoding AXAH-I appeared to be significantly higher in aleurone and scutellum tissue isolated from germinated barley grain than in vegetative barley seedling tissues or in developing barley grain. It seems highly likely that the two AXAHs function in the removal of L-arabinofuranosyl residues from arabinoxylan in both germinated grain tissues and also in the cell walls of vegetative barley tissues. AXAH-I and AXAH-II activity in the aleurone layer and scutellum might facilitate the action of (1→4)-β-xylan endohydrolases by producing unsubstituted xylan sites on cell wall arabinoxylans. Release of AXAH-I and AXAH-II from the scutellum and aleurone into the barley endosperm would facilitate the action of (1→4)-β-xylan endohydrolase and hence, degradation of endosperm arabinoxylans in germinated grain. The biochemical properties of AXAH-I suggest that removal of L-arabinofuranosyl residues from arabinoxylans of newly synthesised cell walls, as described by Gibeaut and Carpita (1991), could be accomplished by the AXAH isoenzymes. Localisation of transcription of the AXAH genes in vegetative barley tissues and in developing barley grain supports this possibility.

The simple RT-PCR procedure has been useful for the analysis of gene expression of the arabinoxylan degrading enzymes described in this thesis. More specifically, these experiments establish the presence or absence of transcription of particular genes in various

tissues. Localisation of gene expression in barley supports the proposed biological roles for ARA-I, XYL, AXAH-I and AXAH-II in both degradation and turnover of arabinoxylans, and in modification of arabinofuranosyl substitution pattern that might be associated with wall deposition.

6.4.2 Genetic Mapping

The ARA-I, XYL and AXAH-I genes were mapped using RFLPs of doubled haploid F1 mapping populations. In each case, hybridization signals were strong and with low level of background signal. Precise chromosomal locations for ARA-I, XYL and AXAH-I genes, named *AraI*, *Xylo* and *AxaI* respectively, were assigned as described in the *Results* section. None of the three genetic loci were correlated with known quantitative trait loci (QTLs) for the respective mapping populations and clustering of the genes encoding arabinoxylan-degrading enzymes was not evident. For example, the *X*-I gene encoding (1→4)-β-xylan endohydrolase isoenzyme X-I is located on the long arm of barley chromosome 5H (Banik *et al.*, 1997), whereas the *AxaI* gene is located on the short arm of chromosome 5H.

The absence of RFLPs within the AXAH-II gene in available barley mapping populations prevented the assignment of chromosomal location for the AXAH-II gene. However, screening of DNA from wheat nuli-tetrasomic lines indicates that the gene encoding AXAH-II in wheat is on wheat chromosome 4, and that the likely location for the AXAH-II gene in barley is chromosome 4H. This position was not correlated with potential QTLs and does not suggest any clustering with genes for similar enzymes.

CHAPTER 7.

Summary and Concluding Remarks

7.1 SUMMARY OF EXPERIMENTAL RESULTS

Germinated barley has provided the starting point for the characterization of several glycoside hydrolases with activity against cell wall polysaccharides. Enzymes such as (1→3,1→4)-β-glucan endohydrolases (Woodward and Fincher, 1992*a*) and β-glucan glucohydrolases (Hrmova *et al.*, 1996) participate in the degradation of cell wall (1→3,1→4)-β-glucans in barley (Fincher, 1989; Fincher and Stone, 1993). Purification of these proteins has led to further isolation of cDNA and gene sequences (Fincher *et al.*, 1989; Slakeski *et al.*, 1990; Hrmova *et al.*, 1996; Harvey *et al.*, 2001), analysis of expression of corresponding genes (Slakeski and Fincher, 19992*a*; 1992*b*; Harvey *et al.*, 2001) and determination of protein structures (Varghese *et al.*, 1994; Varghese *et al.*, 1999). The biological function of (1→3,1→4)-β-glucan endohydrolase isoenzymes is primarily in the degradation of barley endosperm cell walls after grain germination. However, gene expression studies suggest that (1→3,1→4)-β-glucan endohydrolase is also produced in young barley leaves and roots (Slakeski and Fincher, 1992*a*). Similarly, β-Glucan glucohydrolase isoenzymes are synthesized in young leaves, rootlets and elongating coleoptiles in barley seedlings (Kotake *et al.*, 1997; Harvey *et al.*, 2001). It is suggested that hydrolysis of wall (1→3,1→4)-β-glucans in elongating tissues serves to loosen the wall matrix and to turnover component sugars (Inouhe and Nevins, 1998; Harvey *et al.*, 2001). Clearly, there are roles for (1→3,1→4)-β-glucan-hydrolysing enzymes in both the germinated grain and in developing vegetative tissues of barley seedlings.

Arabinoxylans are abundant polysaccharides in barley and in other cereals and grasses; they are an important component of cell walls from both barley grain and vegetative tissues. In parallel with the metabolism of wall (1→3,1→4)-β-glucans, there is clear evidence that arabinoxylans are hydrolysed by a range of enzymes during normal physiological processes. (1→4)-β-Xylan endohydrolases are produced in the aleurone of germinated barley grain (Slade *et al.*, 1989; Banik *et al.*, 1996) and α-L-arabinofuranosidase and β-D-xylosidase activities have been detected in germinated grain and in isolated aleurone layers (Taiz and Honigman, 1976; Dashek and Chrispeels; 1977; Banik *et al.*, 1996). Furthermore, removal of L-arabinose from cell wall arabinoxylans coincides with cessation of elongation growth in

maize coleoptiles (Gibeaut and Carpita, 1991). It is apparent that degradation and modification of arabinoxylans also occur in vegetative tissues in barley.

Five glycoside hydrolases from barley, with exohydrolytic activity against arabinoxylans, have been investigated during the course of this research. Three of these enzymes, ARA-I, XYL and AXAH-I, were purified to homogeneity, their biochemical and physical properties were determined and corresponding cDNA sequences were isolated. A fourth enzyme, ARA-II, was partially purified from the barley seedling extract and a fifth enzyme designated AXAH-II was revealed by cDNA cloning. The five barley proteins are divided into two distinct groups, based on sequence homology with microbial α-L-arabinofuranosidases and β-D-xylosidases. ARA-I, XYL and ARA-II share similarities in both amino acid sequence and substrate specificity with family 3 β-D-xylosidases from bacteria and fungi. The microbial β-D-xylosidases from family 3 commonly have molecular masses of greater than 100 kDa. Polypeptide sequences encoded by the genes are approximately 85 kDa, and the enzymes can be highly glycosylated. The cDNA sequences encoding ARA-I and XYL from barley also have theoretical molecular masses of approximately 80 kDa, yet SDS-PAGE of purified ARA-I and XYL indicate molecular masses of 65 kDa and 67 kDa, respectively. It seems that the barley family 3 enzymes, ARA-I and XYL, are subject to post-translational processing and that the processing site is located towards the COOH-terminal end of the encoded polypeptide. The exact position of processing could not be determined, presumably because the COOH-terminus was "ragged" rather than discrete.

ARA-I and XYL from barley have similar substrate specificities to other microbial β-D-xylosidases, with primary activity against (1→4)-linked oligoxylosides and also oligoarabinoxylosides, but no significant capacity to hydrolyse arabinoxylans. Family 3 β-D-xylosidases commonly hydrolyse both 4NPA and 4NPX, but have a clear preference for the β-D-xyloside. Kinetic parameters for barley ARA-I show that this isoenzyme has a slight preference for 4NPA. ARA-I is also notable for its ability to hydrolyse (1→5)-linked arabinosides and to liberate some L-arabinose and also D-xylose from arabinoxylan. The partially-purified ARA-II isoenzyme from barley also had a preference for the aryl α-L-arabinofuranoside and apparently hydrolyses 4NPA with high specific activity.

The barley arabinoxylan arabinofuranohydrolases, AXAH-I and AXAH-II, represent a second type of arabinoxylan-hydrolysing enzyme, and are classified in glycoside hydrolase family 51. AXAH-I is a 65 kDa protein, with the capacity to remove L-arabinofuranosyl substituents from arabinoxylan. AXAH-I also hydrolyses (1→5)-α-linked oligoarabinosides but has no activity on either 4-nitrophenyl- or oligo-β-D-xylosides. AXAH-II, with 81% amino acid sequence identity to AXAH-I, probably has biochemical properties similar to AXAH-I.

7.2 FURTHER RESEARCH

The description of arabinoxylan-degrading exohydrolases and their corresponding genes presents opportunities for further investigation of arabinoxylan metabolism in cereals using barley as a model system. Recent developments in the application of gene silencing procedures and plant transformation allow analysis of gene function in a biological context (Wang and Waterhouse, 2002). DNA constructs for barley transformation that produce double stranded RNA intermediates corresponding to a particular plant gene, when transcribed in the plant, cause endogenous gene expression to be silenced (Waterhouse *et al.*, 1998; Smith *et al.*, 2000). Alternatively, insights on gene function can be gained by over-expression of specific genes in experimental plants. Altered phenotypes in the transgenic plant in which gene silencing or over-expression has been effective may indicate the biological role of the gene in question. The family 3 α-L-arabinofuranosidase and β-D-xylosidase activities of ARA-I and XYL, might have secondary roles in oligosaccharide degradation and turnover of sugars, but AXAH-I and AXAH-II potentially have more important, primary roles in modifying arabinoxylan structures. Whether the AXAHs act cooperatively with (1→4)-β-xylan endohydrolase in germinated grains or whether they selectively remove arabinofuranosyl substituents of arabinoxylans to promote interactions between wall polysaccharides in developing tissues is unclear. Silencing of AXAH genes in transformed barley plants may produce altered performance in grain germination or in cell wall strength. Over-expression of AXAH genes might produce opposite effects. These experiments are underway and should further our understanding of the plant cell wall as a whole and of the ways in which individual polysaccharide components contribute to overall wall properties.

7.3 Concluding Remarks

The biochemical approach for the study of a particular aspect of plant physiology follows the logic of isolating pure preparations of specific plant enzymes and evaluating their biochemical properties. Determination of partial amino acid sequences by Edman sequencing of both whole protein and of tryptic peptides leads to the isolation of cDNAs corresponding to the purified proteins and subsequently, the description of complete amino acid sequence or primary structure. These procedures have been successfully applied to the question of arabinoxylan degradation in barley, resulting in the complete description of three arabinoxylan-degrading enzymes in barley; namely ARA-I, XYL and AXAH-I. Two further proteins, ARA-II and AXAH-II, were identified, but only partially characterized. Clearly, the four proteins encountered during protein purifications are the major arabinoxylan hydrolysing exohydrolases in barley seedlings; AXAH-II was not detected in barley seedling extracts. The next important question to address in the study of arabinoxylan metabolism in barley is that of the biological functions of arabinoxylan-degrading enzymes. RT-PCR indicated that both the family 3 type enzymes and the family 51 AXAHs are synthesized in a range of both vegetative and germinated grain tissues. The substrate specificities and expression sites of each of the enzymes suggest important roles in arabinoxylan degradation in germinated grain, and in the modification and turnover in newly-synthesized arabinoxylans in the cell walls of vegetative tissues. No conclusive evidence of biological functions of the arabinoxylan degrading enzymes in barley is available from this work. However, the results of this research provide important information for the further analysis of the biological significance of arabinoxylan metabolism processes in barley.

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