Biochemistry and Molecular Biology of Arabinoxylan Metabolism in Germinated Barley

Robert Lee, Maria Hrmova, Rachel Burton and Geoffrey Fincher

Department of Plant Science, The University of Adelaide, Waite Road, Urrbrae S.A. 5064.

Introduction

Following water uptake and germination of a barley grain, hydrolytic enzymes that are mainly secreted from the scutellar epithelial layer and the aleurone layer, catalyse the depolymerization of starch and protein reserves in the starchy endosperm. Degradation products are subsequently absorbed by the scutellum and translocated to the developing seedling. During commercial malting operations, barley grain is steeped in water and germinated under conditions designed to maximise the modification of the starchy endosperm, but to minimise vegetative growth of the young seedling (Bamforth and Barclay, 1993; Briggs *et al.*, 1981). In both processes enzymic degradation of cell walls in the starchy endosperm is crucial. Because most of the key enzymes are secreted from the aleurone layers, the walls of starchy endosperm cells represent a physical barrier which denies the enzymes free access to their substrates within starchy endosperm cells. As a result, the rapid early removal of the cell wall is of central importance both for the successful germination of the grain (Fincher, 1989) and for the production of a well-modified malt (Bamforth and Barclay, 1993).

The major constituents of walls in the starchy endosperm of barley are the (1→3,1→4)-β-glucans and the arabinoxylans (Fincher, 1975; Ballance and Manners, 1978). The (1→3,1→4)-β-glucans are the most abundant components in the wall, and a good deal of attention has been focussed on their chemical structures, solution properties and enzymic depolymerization (Woodward and Fincher, 1983; Fincher and Stone, 1993). Similarly, the chemical structures and solution properties of cereal arabinoxylans have been described in detail (Perlin, 1951; Andrewartha *et al*., 1979; Voragen *et al*., 1987; Viëtor *et al*., 1994), but there is less information on the enzymes that catalyse their hydrolysis in germinated barley grain.

Both the (1→3,1→4)-β-glucans and the arabinoxylans can be extracted from walls with hot water and both form solutions of high viscosity. The latter property can be attributed to the asymmetrical molecular conformation of both polysaccharides. If cell walls are not adequately degraded during malting, malt extracts can contain high levels of the polysaccharides and attendant difficulties associated with the filtration of viscous extracts can significantly slow the brewing process (Bamforth, 1985).

These problems have usually been related to undegraded (1→3,1→4)-β-glucan, because this is the most abundant polysaccharide in the walls. However, arabinoxylans constitute about 25% of starchy endosperm walls (Fincher, 1975) and about 70% of aleurone walls (Bacic and Stone, 1981). The enzymes that degrade arabinoxylans are often produced late in the germination process (Banik *et al.*, 1997), and high levels of this polysaccharide can survive through the brewing process into the final beer (Coote and Kirsop, 1976; Viëtor *et al*., 1993). The contribution of arabinoxylans to the undesirable effects normally ascribed to (1→3,1→4)-β-glucans has often been underestimated, or completely overlooked.

In the work described here, enzymes that participate in arabinoxylan hydrolysis have been purified from extracts of germinated barley and characterized. Corresponding cDNAs have been isolated and sequenced, and will enable the expression patterns of key genes to be monitored during endosperm modification.

Materials and Methods

Enzyme Purification

Barley grain (*Hordeum vulgare* cv. Clipper) was surface-sterilized (Hoy *et al*., 1980), steeped for 24h in water containing a cocktail of antibiotics, and germinated at approximately 40% (v/v) moisture content in the dark at 22˚C for 5 days (Hrmova and Fincher, 1993). No bacterial or fungal growth could be detected during this period. The germinated grain was homogenised at 4˚C in 100 mM sodium acetate buffer, pH 5.0, containing 10 mM EDTA, 4 mM sodium azide, 3 mM 2-mercaptethanol and 3 mM PMSF. After 45 min insoluble material was removed by centrifugation and the enzymes were purified by fractional precipitation with ammonium sulphate, ion exchange chromatography, chromatofocussing and gel filtration chromatography, using protocols described by Hrmova *et al.* (1996).

Enzyme Assays

Activity in column fractions and in purified enzyme preparations was measured using 0.04% (w/v) 4-nitrophenyl α-L-arabinofuranoside (4NPA) or 4-nitrophenyl β-D-xylopyranoside (4NPX) in 50 mM sodium acetate buffer, pH 5.0 at 37˚C. The reactions were stopped with 2 vol. saturated $Na_2B_4O_7$ (Biely *et al.*, 1980) and absorbance measured at 410 nm. Activity against wheat flour arabinoxylan was measured reductometrically (Banik *et al.*, 1997). Products released from the substrates were identified by thin layer chromatography on Kieselgel 60 plates developed in 3:2:1 (v/v) ethyl acetate:acetic acid:water (Hrmova and Fincher, 1993).

Isolation of cDNA Clones

The NH_2-terminal amino acid sequences of purified enzymes or of tryptic peptides derived from purified enzymes were determined in a Hewlett-Packard (G1005A protein sequencer. Oligonucleotide primers were designed on the basis of amino acid sequences and used in RT-PCR reactions to generate cDNA probes (Burton *et al.*, 1999) from RNA preparations from 4 day-old barley seedlings. The PCR products were sequenced (Sanger *et al.,* 1977) and used as probes to screen cDNA libraries produced from poly(A)$^+$- RNA from young barley seedlings (Hrmova *et al.*, 1996).

Results

Enzyme Purification

Two α-L-arabinofuranosidases, designated isoenzymes ARA I and ARA II, were purified from extracts of young barley seedlings. In addition, a β-xylosidase (designated XYL) was purified and characterized. Although the enzyme preparations were highly purified, some cross-reactivity between substrates was observed. Thus, the α-L-arabinofuranosidases exhibited some activity against 4NPX and the β-xylosidase had low levels of activity against 4NPA. Some properties of the enzymes are compared in Table 1.

Neither the arabinofuranosidases nor the xylosidase were capable of rapidly removing arabinose or xylose from wheat flour arabinoxylan. However, another enzyme which had

minimal activity on 4NPA was found to remove arabinose units from the arabinoxylan substrate at a rate approximately 500 times that observed for the arabinofuranosidase isoenzyme ARA II. This enzyme has been designated arabinoxylan arabinofuranohydrolase, but it has not yet been purified to homogeneity or subjected to detailed analysis.

Table 1. Properties of Arabinofuranosidases and Xylosidases from Germinated Barley.

Enzyme	Molecular Weight (Da)	Isoelectric Point	Specific Activity on 4NPA (mU/mg)	Specific Activity on 4NPX(mU/mg)
ARA I	N.D.[a]	5.3	N.D.	N.D
ARA II	70,000	5.5	12,300	2,500
XYL	70,000	6.7	320	10,500

[a] not determined

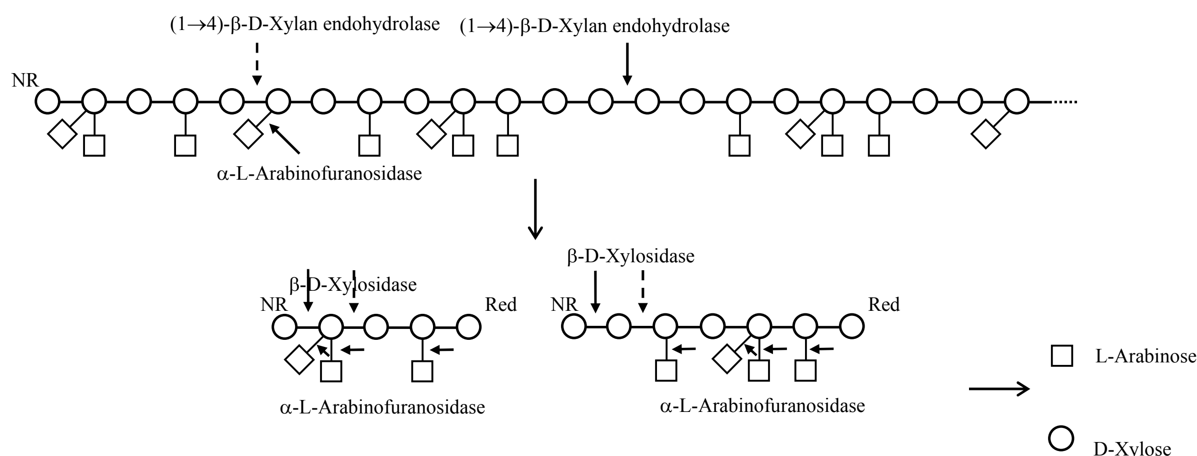

Figure 1. Arabinoxylan structural model with proposed sites of action of major arabinoxylan hydrolysing enzymes and sequential stages of complete depolymerisation. The (1→4)- linked xylan backbone (o-o) is shown with arabinofuranosyl residues linked via C(O)2 ( ), C(O)3 ( ) and C(O)2,3 ( ). The non - reducing end of arabinoxylan and both reducing 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 xylopyranosyl residues.

Isolation of cDNA Clones

Using oligonucleotide primers based on amino acid sequences from tryptic peptides, a PCR product of approximately 500 bp was amplified. Nucleotide sequence analysis showed that the PCR product corresponded to arabinofuranosidase isoenzyme ARA II. The PCR product was used to screen cDNA libraries and to design primers for additional RT-PCR amplifications. At this stage a 2304 bp cDNA encoding barley arabinofuranosidase isoenzyme ARA II has been sequenced. The cDNA has an open reading frame equivalent to 664 amino acids, a 312 bp 3’ untranslated region and a polyadenylic acid tail of 16 residues. Preliminary Northern blot analyses indicate that the mRNA encoding the enzyme is about 2800 nucleotides, and the cDNA is therefore about 500 bp short of full-length at its 5’ end.

The coding region of the cDNA is characterized by a distinct bias in codon usage; most codons end in G or C. Similar biases in codon usage have been observed in many genes encoding barley enzymes that participate in endosperm mobilization (Fincher, 1989; Banik *et al.*, 1997).

Discussion

Two α-L-arabinofuranosidases and a β-D-xylosidase have been identified in extracts of germinated barley grain. The original objective of the work was to investigate the role of those enzymes in arabinoxylan hydrolysis during cell wall degradation in germinated grain. The structural features of the polysaccharide, together with the expected sites of hydrolysis by the α-L-arabinofuranosidases and the β-D-xylosidase, are shown in Figure 1.

Although each of the enzymes was active on the synthetic aryl-glycoside substrates 4NPA and 4NPX, their activity on polymeric arabinoxylan was very low and it would seem unlikely that they play a major role in the removal of arabinosyl or xylosyl residues from polymeric arabinoxylan. However, it remains possible that both classes of enzyme are required for the hydrolysis of low molecular weight arabinoxylosides released from the polysaccharide by (1→4)-β-xylan endohydrolase activity (Figure 1). To demonstrate such a function convincingly, high level activity against well-defined oligosaccharide substrates would need to be demonstrated.

In addition to the α-L-arabinofuranosidases and the β-D-xylosidase studied here, a small amount of a different, previously unknown enzyme with a much greater capacity to remove arabinofuranosyl residues from polymeric arabinoxylan was detected in the grain extract. This enzyme will now be purified for detailed analysis of its substrate specificity and action pattern. It may be concluded at this stage that there is likely to be a group of enzymes involved in the removal of arabinosyl residues from polymeric and oligomeric substrates, and that the group might be larger than was first envisaged. Thus, different enzymes might be required to remove arabinose units from C(O)2 and C(O)3 atoms of xylosyl residues in the backbone, and another might be required for doubly substituted xylosyl residues (Figure 1). Furthermore, different groups of enzymes might be required for the hydrolysis of polysaccharides and oligosaccharides.

Finally, the work here has resulted in the isolation of a near full-length cDNA encoding α-L-arabinofuranosidase isoenzyme ARA II. This can now be used as a probe to define not only the number of genes encoding α-L-arabinofuranosidases, but also the positions of the genes on the barley genome. The cDNAs will also be used in Northern hybridization analyses to monitor expression patterns of the genes in various tissues during normal growth and development of barley.

Acknowledgments

This work is supported by a grant from the Grains Research and Development Corporation and by a GRDC Postgraduate Student Award to Robert C. Lee.

References

Andrewartha, K., Phillips, D.R. and Stone, B.A. (1979) Carbohydr. Res., 77:191-204.
Bacic, A. and Stone, B.A. (1981) Aust. J. Plant Physiol., 8:475-495.

Ballance, G.M. and Manners, D.J. (1978) Carbohydr. Res., 61:107-108.
Bamforth, C.W. (1985) J. Inst. Brew., 91:154-160.
Bamforth, C.W. and Barclay, A.H.P. (1993). Malting technology and the uses of malt. Pages 297-354 in: Barley: Chemistry and Technology. A.W. MacGregor and R.S. Bhatty, Eds. American
Association of Cereal Chemists, St Paul, MN.
Banik, M., Li C.-D., Langridge, P. and Fincher, G.B. (1997) Mol. Gen. Genet., 253:599-608.
Biely, P., Vrsanska, M., and Kratsky, Z. (1980) Eur. J. Biochem., 108:313-321.
Briggs, D.E., Hough, J.S., Stevens, R. and Young, T.W. (1981). Malting and Brewing Science.
Volume 1. Malt and Sweet Wort. Chapman and Hall, London.
Burton, R.A., Zhang, X.Q., Hrmova, M. and Fincher, G.B. (1999) Plant Physiol., 119:859-871.
Coote, N. and Kirsop, B.H. (1976) J. Inst. Brew., 82:34.
Fincher, G.B. (1975) J. Inst. Brew., 81:116-122.
Fincher, G.B. (1989) Ann. Rev. Plant Physiol. Plant Mol. Biol. 40: 305-346.
Perlin, A.S. (1951) Cereal Chem., 28:382-393.
Hoy, J.L., Macauley, B.J. and Fincher, G.B. (1980) J. Inst. Brew., 87:77-80.
Hrmova, M. and Fincher, G.B. (1993) Biochem. J., 289:453-461.
Hrmova, M., Harvey, A.J., Wang, J., Shirley, N.J., Jones, G.P., Stone, B.A., Høj, P.B. and Fincher, G.B. (1996) J. Biol. Chem., 271:5277-5286.
Sanger, F., Nicklen, S. and Coulson, A.K. (1977) Proc. Natl. Acad. Sci. U.S.A., 74:5463-5467.
Viëtor, R.J., Kormelink, F.J.M., Angelino, S.A.G.F. and Voragen, A.G.J. (1994) Carbohydr. Polym., 24:113-118.
Viëtor, R.J., Voragen, A.G.J. and Angelino, S.A.G.F. (1993) J. Inst. Brew., 99:243-248.
Voragen, A.G.J., Scholls, H.A., Marijs, J., Rombouts, F.M. and Angelino, S.A.G.F. (1987) J. Inst. Brew., 93:202-208.
Woodward, J.R. and Fincher, G.B.. (1983) Brewers Digest, 58:28-32.