Barley β-D-Glucan Exohydrolases with β-D-Glucosidase Activity

PURIFICATION, CHARACTERIZATION, AND DETERMINATION OF PRIMARY STRUCTURE FROM A cDNA CLONE*

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Two β-glucan exohydrolases of apparent molecular masses 69,000 and 71,000 Da have been purified from extracts of 8-day germinated barley grains and are designated isoenzymes Exol and ExolI, respectively. The sequences of their first 52 NH₂-terminal amino acids show 64% positional identity. Both enzymes hydrolyze the (1,3)-β-glucan, laminarin, but also hydrolyze (1,3;1,4)-β-glucan and 4-nitrophenyl β-glucoside. The complete sequence of 602 amino acid residues of the mature β-glucan exohydrolase isoenzyme Exol has been deduced by nucleotide sequence analysis of a near full-length cDNA. Two other enzymes of apparent molecular mass 62,000 Da, designated βII and βIII, were also purified from the extracts. Their amino acid sequences are similar to enzymes classified as β-glucosidases and although they hydrolyze 4-nitrophenyl β-glucoside, their substrate specificities and action patterns are more typical of polysaccharide exohydrolases of the (1,4)-β-glucan glycohydrodolase type. Both the β-glucan exohydrolase isoenzyme Exol and the β-glucosidase isoenzyme βII release single glucosyl residues from the nonreducing ends of substrates and proton-NMR shows that anomer configurations are retained during hydrolysis by both classes of enzyme. These results raise general questions regarding the distinction between polysaccharide exohydrolases and glucosidases, together with more specific questions regarding the functional roles of the two classes of enzyme in germinating barley grain.

Cell walls in the starchy endosperm of barley and other agriculturally important cereal grains are composed predominantly of (1,3;1,4)-β-glucans and arabinoxylans (reviewed by Fincher (1992)). In the case of barley, these two polysaccharides account for more than 90% by weight of walls of the starchy endosperm, with (1,3;1,4)-β-glucan levels of approximately 70% and arabinoxylan levels of about 20% (Fincher, 1975; Ballance and Manners, 1978). In the germinating grain the cell walls represent a physical barrier between hydrolytic enzymes, such as α-amylases and endopeptidases which are secreted from the aleurone cells surrounding the starchy endosperm, and their starch and storage protein substrates which are packaged inside the starchy endosperm cells. Following germination, walls of starchy endosperm cells are extensively degraded and eventually disappear (Selvig et al., 1986). The removal of the walls is mediated by two (1,3;1,4)-β-glucanases (EC 3.2.1.73), designated isoenzymes EI and EII (Woodward and Fincher, 1982a), and by the combined action of xylan endo- and exohydrolases, and α-arabinofuranosidases (Preece and MacDougall, 1958; Slade et al., 1989).

Because of the quantitative importance of (1,3;1,4)-β-glucans in barley endosperm cell walls and difficulties encountered with the extreme instability of the endo-xylanases, most attention has been focussed on the (1,3;1,4)-β-glucanases in studies of cell wall degradation in germinating grain. The genes encoding the two isoforms are subject to independent regulation. The isoenzyme EI gene is expressed mainly in the scutellum of germinating grain, but also in young leaves and roots of barley seedlings, whereas expression of the isoenzyme EII gene appears to be restricted to the aleurone layer of germinating grain (Slakeski and Fincher, 1992a, 1992b). Both isoenzymes release the characteristic tri- and tetrasaccharides 3-O-β-cellobiosyl-α-glucose (G4G3Gred) and 3-O-β-cellobiosyl-α-glucose (G4G4G3Gred) as the major oligomeric products from (1,3,1,4)-β-glucans of the starchy endosperm walls (Parrish et al., 1960; Woodward and Fincher, 1982b). Given that cell wall (1,3,1,4)-β-glucans may represent up to 18% of the total glucosyl residues stored in the starchy endosperm of barley (Morall and Briggs, 1978), it is highly likely that glucosyl residues bound in the tri- and tetrasaccharide products of (1,3,1,4)-β-glucan exohydrolase action would eventually be released as free glucose and translocated as an energy source to the developing seedling. Prime candidates for the release of glucose from oligosaccharides derived from (1,3,1,4)-β-glucans would be β-glucosidases or β-glucan exohydrolases, both of which have been detected in crude extracts of germinating barley grain (Manners and Marshall, 1969; Simos et al., 1994; Leah et al., 1995).

In the work described here we have purified two β-glucan exohydrolases, which can hydrolyze both (1,3,1,4)-β-glucans and (1,3)-β-glucans, and two β-glucosidases. The chemical and enzymatic properties of the enzymes have been defined and a cDNA encoding one β-glucan exohydrolase isoform has been characterized. Comparisons of the substrate specificities and action patterns of the two classes of enzyme suggest that both classes release glucose from the nonreducing ends of (1,3,1,4)-β-oligoglucosides, but that the β-glucan exohydrolases more rapidly catalyze the complete hydrolysis of these oligosaccharides.
**Barley β-Glucan Exohydrolases and β-Glucosidases**

**Materials—** Laminarin from Laminaria digitata, dithiothreitol, 4-nitrophenyl β-o-glucoside (4-NPG), 1-β-D-glucan-1,5-lactone, ornithine, and bovine serum albumin (BSA) were from Sigma. CM-Sepharose CL-6B, PBE 94 and 118 resins, Pharmalyte 8–10.5, Polybuffer 96, and low molecular mass protein standards were from AMRAD Pharmacia Biotech (Uppsala, Sweden). DEAE-Cellulose (DE52) and CM-cellulose (CM52) were from Whatman (Maidstone, Kent, United Kingdom), and Bio-Gel A-5m (100–200 mesh) was from Bio-Rad (Hercules, CA). The 1(3,4)-(1,4)-β-p-glucanase was from Merck (Darmstadt, Germany), and YM-10 membranes were from Amicon Corporation (Beverly, MA). D-[1-14C]glucosamine hydrochloride (Cambridge Isotope Laboratories, Andover, MA).

**Enzyme Extraction—** Barley (Hordeum vulgare L., cv. Clipper) grain was surface-sterilized as described by Hoy et al. (1980). Grains (3.5 kg), maintained at approximately 40% (w/v) moisture content in the antibiotic solution, were germinated in the dark at 19 ± 2°C for 8 days. The homogenization of grain and ammonium sulfate precipitation were as described previously (Hrmova and Fincher, 1993).

**Enzyme Assays—** Enzyme activity was determined reductometrically by monitoring the increase in reducing sugars (Nelson, 1944; Somogyi, 1952) released from solutions of L. digitata laminarin (or barley (1,3,1,4)-β-glucan (Woodward and Fincher, 1982a), or spectrophotometrically at 410 nm on 0.04% (w/v) 4-NPG in 50 mM sodium acetate buffer, pH 5, at 37°C. The reaction with 4-NPG was stopped by adding 2 volumes of 4% (w/v) Na2CO3 (Biely et al., 1980). One unit of activity is defined as the amount of enzyme required to release 1 μmol of glucose from laminarin and barley (1,3,1,4)-β-glucan, or to release 1 μmol of 4-nitrophenol from 4-NPG per min. One unit corresponds to 16.67 nkat.

**Substrate Specificities—Hydrolysis of L. digitata laminarin, barley (1,3,1,4)-β-glucan, and 4-NPG was assayed at a final substrate concentration of 0.2% (w/v) in 0.1 M sodium acetate buffer, pH 5.25, containing 160 μg/ml BSA. The substrates were incubated with 22.1 and 4.0 pkat (based on glucose released from laminarin) of purified exo-β-glucanase isoenzymes Exo I and Exo II, respectively, and with 16.7 and 14.5 pkat (based on 4-nitrophenol released from 4-NPG) of the purified β-glucosidase isozymes βI and βII, respectively.

The oligosaccharides G3G4Gemp, G3G4G4G4Gemp, and G3G4G4G4G4G4Gemp were purified from hydrolysates of barley (1,3,4)-β-glucan after treatment with the Streptomyces sp. cellulase (EC 3.2.1.40), Cellobiase (EC 3.2.1.21), and Cellulase Exo I (Novozymes). The sequence of the oligonucleotide probe was determined at 37°C by incubating 2.8 and 30.4 pkat (based on 4-nitrophenol released from 4-NPG) of the purified β-glucosidase isozymes βI and βII, respectively, and hydrolysis rates were determined reductometrically.

**Kinetic Analyses and Inhibition Studies—** Kinetic parameters were determined at 37°C by incubating 2.8 and 30.4 pkat (based on 4-nitrophenol released from 4-NPG) of purified isoenzymes Exo I and βII, respectively, in 0.1 M sodium acetate buffer, pH 5.25 (isoenzyme Exo I) or pH 5.0 (isoenzyme βII), containing 160 μg/ml BSA. Rates of hydrolysis were determined spectrophotometrically at 4-NPG concentrations in the range 0.1–4 (isoenzyme Exo I) and 0.2–6 (isoenzyme βII) times the K_m value.

The inhibition of the β-glucan exohydrolase isozyme Exo I and the β-glucosidase isozyme βII by n-β-glucan-1,5-lactone and glucose was monitored by incubating 35.1 and 45.6 pkat isoenzymes Exo I and βII, respectively, in 0.1 M sodium acetate buffer, pH 5.25 (isoenzyme Exo I) or pH 5.0 (isoenzyme βII), containing 160 μg/ml BSA. Rates of hydrolysis were determined after the first 10 min of hydrolysis (Leget, 1990) after the addition of a fixed amount of enzyme to incubation mixtures containing different concentrations of 4-NPG and inhibitors. Kinetic data (K_m and V_max) and the inhibition constants (K_i) of enzyme-inhibitor complexes were processed by a non-linear regression analysis program based on Michaelis-Menten kinetics (Perrella, 1988).

**Protein Determination and Polyacrylamide Gel Electrophoresis—** Protein content and SDS-PAGE were performed as described previously (Hrmova and Fincher, 1993).

**Tryptic Digestions and Amino Acid Sequencing—** Tryptic fragments of iso-β-glucanase isozyme Exo I were generated and analyzed essentially as described by Chen et al. (1993). Selected peptides and enzyme isoenzymes were sequenced with the Hewlett-Packard 1100A protein sequencer (Palo Alto, CA) using the Hewlett-Packard 3.0 sequencing routine, based on Edman degradation chemistry.

**Materials—** Laminaria digitata or barley (1,3,1,4)-β-glucan was from Biocon Biochemicals (Kilnagley, Ireland), Kieselgel 60 thin-layer plates and molecular mass protein standards were from AMRAD Pharmacia Biotech (Uppsala, Sweden). DEAE-Cellulose (DE52) and CM-cellulose (CM52) were from Whatman (Maidstone, Kent, United Kingdom), and Bio-Gel A-5m (100–200 mesh) was from Bio-Rad (Hercules, CA). D-[1-14C]glucosamine hydrochloride (Cambridge Isotope Laboratories, Andover, MA).

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**RESULTS**

**Purification of Exo-β-glucanases—** The procedures developed to purify the exo-β-glucanases from extracts of germinated barley are shown in Scheme 1. Enzyme yields, specific activities, and purification factors are presented in Table I.

Unpurified grain extract and fractions precipitated with ammonium sulfate were assayed for (1,3)-β-glucanase, (1,3,1,4)-β-glucanase, and β-glucosidase using the substrates laminarin,
Germinated grain (8 days, 19±2°C)

1. Homogenization
2. Centrifugation (4,400g; 30 min)

Pellet (discarded)  
Supernatant

1. 40-80% (NH₄)₂SO₄ saturation
2. DEAE-cellulose (pH 7.6)

Unbound fraction
CM-Sepharose (pH 4.9)  Elution with 0-0.6 M NaCl
Peak I (0.1 M NaCl)
Chromatofocusing (pH 9.4)
Peak 1
Phenyl-Sepharose (2 M NaCl)
Peak 1
Bio-Gel P-100

Isoenzyme ExoI

Bound fraction (discarded)

Isoenzyme βI

Isoenzyme βII

Barley β-Glucan Exohydrolases and β-Glucosidases

Barley (1,3;1,4)-β-glucan and 4-NPG, respectively. For each assay, most activity was detected in the material precipitated by 40–80% saturated ammonium sulfate, which was used for subsequent purification steps. Exploratory experiments at this stage showed that the exo-β-glucanases to be purified could hydrolyze both laminarin and 4-NPG, but that the (1,3)-β-glucan endohydrolases, which were present at very high levels (Hrmova and Fincher, 1993) did not hydrolyze 4-NPG. Because the major objective of the work was to purify exo-β-glucanases rather than the (1,3)-β-glucan endohydrolases, 4-NPG was used to assay exo-β-glucanases in all subsequent purification procedures, despite the fact that it was not the preferred substrate and that it would also detect β-glucosidases.

Following dialysis against 20 mM Tris-HCl buffer, pH 7.6, containing 10 mM sodium azide and 3 mM 2-mercaptoethanol, the 40–80% ammonium sulfate fraction was applied to a 4.5 cm × 20-cm column of DEAE-cellulose equilibrated in 20 mM Tris-HCl buffer, pH 7.6. Most activity passed through the column (Table I) and was immediately transferred to a 3.2 cm × 24-cm column of CM-Sepharose equilibrated in 50 mM sodium acetate buffer, pH 4.9. After unbound proteins were eluted, a linear gradient (3.8 liters) of 0 to 0.6 M NaCl in the same buffer was used to assay the eluted β-glucanases.
applied to the column at a linear flow rate of 76 cm h\(^{-1}\). Five peaks of activity against 4-NPG were detected (Fig. 1). Of these, peaks I and IV were active against laminarin, (1,3;1,4)-β-glucan, and 4-NPG, and proved to contain exo-β-glucanases. Peak III was most active against 4-NPG and was subsequently shown to contain the β-glucosidases (Scheme 1, Fig. 1). Peak II was subjected to further purification by chromatofocusing, but no β-glucan exohydrolase activity could be detected (data not shown). Peak V contained a third isof orm of β-glucosidase, which had an isoelectric point of 9.3, but the fractions were contaminated with α-amylase and have not been purified further.

Fractions associated with peaks I (fractions 42–50, Fig. 1), III (fractions 63–72), and IV (fractions 127–137) were pooled and concentrated by ultrafiltration. During ultrafiltration, fractions from peaks I and IV were equilibrated in 25 mM ethanolamine-HCl buffer, pH 9.4, while fractions from peak III were equilibrated in 25 mM triethylamine-HCl buffer, pH 11. Fractions (13 ml) were assayed for activity against 4-NPG (▲) and protein (- - -).

In the same buffer. However, SDS-PAGE showed that the active enzyme fractions from the phenyl-Sepharose column still contained minor contaminating proteins, which were subsequently removed by size exclusion chromatography on a 1.5 cm × 96-cm Bio-Gel P-100 column equilibrated in 50 mM sodium acetate buffer, pH 5.0, containing 200 mM NaCl and 1 mM dithiothreitol, at a flow rate of 0.5 cm h\(^{-1}\).

After size exclusion chromatography of the Exo I and Exo B preparations, the active fractions were concentrated by ultrafiltration. Amino acid sequence data showed that their NH\(_2\)-terminal sequences were identical for the first 52 residues. While it is formally possible that the isoenzyme Exo I and Exo I preparations represent separate isof orms, we believe that these more probably correspond to a single protein and that the resolution of two peaks during chromatofocusing (Fig. 2) may result from partial deamination of glutamine or asparagine residues, or from different levels of post-translational modification. These enzyme fractions are designated β-glucan exohydrolase isoenzyme Exo I.

When the pooled fractions of CM-Sepharose peak IV (Fig. 1) were subjected to chromatofocusing, two peaks of 4-NPG activity were eluted at pH values of 8.0 and 7.7 and although SDS-PAGE showed that the major proteins had apparent molecular mass values of 70–71 kDa, some contaminating proteins were still present (data not shown). The contaminating proteins were removed by size exclusion chromatography on the Bio-Gel P-100 column, as described above. Following the separate purification of the chromatofocusing peaks on Bio-Gel P-100, active fractions were concentrated by ultrafiltration and their NH\(_2\)-terminal amino acid sequence determined. Both proteins had identical sequences for 52 NH\(_2\)-terminal amino acids and we conclude that they too probably represent a single protein in which deamination or other post-translational modifications result in slightly different mobilities on the chromatofocusing column (data not shown). These enzymes are designated β-glucan exohydrolase isoenzyme Exo I. The NH\(_2\)-terminal amino acid sequences of the β-glucan exohydrolase isoenzymes Exo I and Exo II are compared in Table II.

Purification of β-Glucosidases—The pooled fractions of peak III from the CM-Sepharose column (Fig. 1) were also resolved into two peaks of 4-NPG activity after chromatofocusing (Fig. 3), but in this case the fractions under the peak exhibited no activity against laminarin or (1,3;1,4)-β-glucan. It seemed likely, therefore, that these were β-glucosidases. Amino acid
sequence analyses revealed a single amino acid difference in the sequence of their 50 NH2-terminal amino acids (Table II) and the enzymes are designated \( \beta \)-glucosidase isoenzyme \( \beta I \) (pl 8.9) and isoenzyme \( \beta I I \) (pl 9.0) (Fig. 3). However, after pooling the fractions SDS-PAGE revealed the presence of low levels of contaminating proteins in each; these contaminating proteins were removed by size exclusion chromatography on Bio-Gel P-100, as described above. The amino acid sequence of the \( \beta \)-glucosidase isoenzymes corresponds with those already published for barley isoenzymes \( \beta I \) and \( \beta I I \) (Simos et al., 1994; Leah et al., 1995). and is similar to other \( \beta \)-glucosidase sequences in the data bases (Table II).

Purity and Yields—The final enzyme preparations were examined by SDS-PAGE, which revealed single protein bands at the loadings used (Fig. 4). For the \( \beta \)-glucan exohydrolases, isoenzyme Exol was represented by a single band of apparent Mr 69,000, while the value for isoenzyme ExolI was 71,000 (Fig. 4). The \( \beta \)-glucosidases also exhibited single bands following SDS-PAGE analysis, but in this case their apparent Mr values were 62,000 (Fig. 4). The purities of all four enzyme preparations were confirmed by NH2-terminal amino acid sequence analysis, in which no secondary sequences could be detected. The high degree of purity of the enzymes enabled the sequence of 50 or more amino acids to be determined (Table II).

The final recoveries of the enzymes were generally low when expressed on a percentage basis (Table I), primarily because of the highly selective pooling of column fractions that was necessary to separate the enzymes from contaminating proteins, particularly after CM-Sepharose ion exchange chromatography and size exclusion chromatography. Furthermore, we believe the high pH values (pH 11) used during chromatofocusing (Fig. 3) caused considerable losses in enzyme activity. The yields are also lower than those reported by Leah et al. (1995), although the enzymes were purified from extracts of different tissues, using different procedures. Nevertheless, milligram quantities of each enzyme were obtained from extracts of 3.5 kg of germinated barley. The final purification factors were also relatively low (Table I) but this is because of the presence, in the crude extract, of a battery of enzymes capable of hydrolyzing the 4-NPG substrate. Thus, the initial specific activity was much higher than that attributable to any single enzyme and the final purification achieved therefore appears correspondingly lower.

Table II

|   | Exol | Exol I | Exol II
|---|------|-------|-------|
|   | DYVL KADTK PVEDR VADLL GRMTL AEKIG QMTQI ERLVA TDV LRDNPI | **LK** **PKQ** **LG** IK**** **** **** **** **E**** **AEAM** SRY**** | 60 70 80 90 100 |
|   | GSSL GGGSV PRKGA TAKEN QDMVD GFQK -MSTR LGIPM IYGID AVHQ | GSSL GGGSV PRKGA TAKEN QDMVD GFQK -MSTR LGIPM IYGID AVHQ | 60 70 80 90 100 |

**Fig. 3.** Chromatofocusing of \( \beta \)-glucosidase isoenzymes \( \beta I \) and \( \beta I I \) on PBE 118. Pooled fractions 63–72 from the CM-Sepharose column (Fig. 1) were concentrated, equilibrated in 25 mM triethylamine-HCl buffer, pH 11, applied to the PBE 118 column and eluted by generating a linear pH gradient with Pharmalyte 8–10.5 which had been adjusted to pH 8. Fractions (1.5 ml) were assayed for protein (---), pH and activity against 4-NPG (△).

**Fig. 4.** SDS-PAGE of samples taken at various stages during the purification (Scheme 1) of \( \beta \)-glucan exohydrolase isoenzymes Exol and ExolI, and \( \beta \)-glucosidase isoenzymes \( \beta I \) and \( \beta I I \). Lane 1, crude extract; lane 2, fraction not bound to DEAE-cellulose; lane 3, Peak I from CM-Sepharose (Fig. 1); lane 4, Peak IV from CM-Sepharose (Fig. 1); lanes 5 and 6, purified \( \beta \)-glucan exohydrolase isoenzymes Exol and ExolI, respectively; lanes 7 and 8, purified \( \beta \)-glucosidase isoenzymes \( \beta I \) and \( \beta I I \), respectively; lane 9, molecular size standards.
ifivities of the enzymes purified here are summarized and compared in Table III. The two \( \beta \)-glucan exohydrolases can hydrolyze the polymeric substrates laminarin and (1,3,1,4)-\( \beta \)-glucan, in addition to cellobiose and 4-NPG (Table III). Thin-layer chromatography showed that glucose is the sole product released from each of these substrates (data not shown). When hydrolysis rates of isoenzyme Exol against a variety of oligosaccharides with (1,3)-\( \beta \)-, (1,4)-\( \beta \)-, or (1,3,1,4)-\( \beta \)-linkages were compared, no decrease in activity was observed as the degree of polymerization (DP) of the oligosaccharides increased (Fig. 5A). The specific activities of \( \beta \)-glucan exohydrolases on the (1,3)-\( \beta \)-glucan, laminarin, are higher than those measured with (1,3;1,4)-\( \beta \)-glucan, and isoenzyme Exol has a 3-fold higher specific activity than isoenzyme ExoII during hydrolysis of laminarin (Table III). Both \( \beta \)-glucan exohydrolases have higher specific activities against 4-NPG than the \( \beta \)-glucosidases (Table III).

In contrast, the \( \beta \)-glucosidases hydrolyze 4-NPG but have no detectable activity on laminarin or (1,3,1,4)-\( \beta \)-glucan (Table III). However, isoenzyme \( \beta II \) had a relatively high activity against cellobiose and the rate of hydrolysis increased markedly with DP in the (1,4)-\( \beta \)-oligoglucoside series (Fig. 5B). These enzymes preference for (1,4)-\( \beta \)-glucosyl residues at the nonreducing terminus of oligomeric substrates was further demonstrated by their ability to hydrolyze G4G3Gred at much higher rates than G3G4Gred (Figs. 5 and 6). It should also be noted that isoenzyme \( \beta II \) hydrolyzed cellobiose more rapidly than laminaribiose (Fig. 5B), but that the preparation of Leah et al. (1995) hydrolyzed laminaribiose more rapidly than cellobiose. We are unable to explain this difference at this stage.

The availability of the two (1,3,1,4)-\( \beta \)-oligoglucosides G4G3Gred and G3G4Gred, in which the arrangements of (1,3)- and (1,4)-linkages differ, enabled us to investigate whether glucose units were released from the reducing or nonreducing ends of the substrates. For both types of enzyme initial reaction products on G4G3Gred were glucose and laminaribiose (G3G4Gred), while for G3G4Gred the initial products were glucose and cellobiose (G4G3Gred) (Fig. 6). Thus, both enzyme types remove glucose from the nonreducing termini of these substrates. The faster rate of hydrolysis of G4G3Gred by the \( \beta \)-glucosidase, compared with its rate against G3G4Gred, also confirmed the marked preference of this enzyme for (1,4)-\( \beta \)-linkages (Fig. 6), as already observed in its relatively high activity against (1,4)-\( \beta \)-oligoglucosides (Fig. 5B).

Inhibition by \( \alpha \)-gluco-1,5-lactone and \( \alpha \)-glucosidases have often been distinguished on the basis of differential inhibition with \( \alpha \)-glucosyl-1,5-lactone; \( \beta \)-glucosidases are generally more susceptible to inhibition (Reese et al., 1968). Kinetic parameters calculated from inhibition data are shown in Table IV, where it is apparent that \( K_i \) values for both \( \alpha \)-gluco-1,5-lactone and glucose inhibition of \( \beta \)-glucosidase isoenzyme Exol are significantly lower than for the isoenzyme \( \beta II \) (Table IV). Thus, the \( \alpha \)-gluco-1,5-lactone shows a greater affinity for the \( \beta \)-glucan exohydrolase.

In the case of the barley \( \beta \)-glucan exohydrolase isoenzyme Exol, values for \( K_i(\text{glucose})/K_i(\text{4-NPG}) \) of 6.3 and for \( K_i(\text{glucose})/K_i(\text{lactone}) \) of 4,190 are exceptionally low and high, respectively. Previously recorded values are in the 20–100 range and approximately 1000, respectively (Legler et al., 1990). This indicates that the nitrophenyl group in 4-NPG makes a large contribution to binding (K/\( K_m = 6.3 \)) (Legler et al., 1990).

Anomeric Configuration of Hydrolysis Products—\( ^1H \) NMR was used to investigate the anomeric configuration of oligosaccharides released during hydrolysis of substrates by the \( \beta \)-glucan exohydrolase isoenzyme Exol and the \( \beta \)-glucosidase \( \beta II \) (Fig. 7). During the hydrolysis of laminarin by \( \alpha \)-glucanase, which include G3G4Gred, G3G4G3G4Gred, and G3G4G4G3G4Gred, are labeled S3-S7; the (1,3,1,4)-\( \beta \)-oligoglucosides of barley (1,3,1,4)-\( \beta \)-glucan with the Streptomyces cellulase, which include G3G4Gred, G3G4G3G4Gred, and G3G4G4G3G4Gred, are labeled B3-B6; the relative rate of hydrolysis of 4-NPG (+) is included for comparative purposes. Hydrolysis rates are expressed as a percentage of the rate against (A) laminaribiose (L2) or (B) cellobiose (C2).
from overlapping doublets of inter-residue anomic protons of both intrachain and nonreducing terminal glycosidic linkages of the cellopentaose substrate. As hydrolysis proceeds this triplet decreases in intensity (Fig. 7B). The signal corresponding to the α-anomeric protons of glucose at δ = 5.23, J = 3.7 Hz increased in intensity during hydrolysis and reflects mutarotation of the β-anomeric protons of the glucose initially released from the substrate (Fig. 7B).

Based on these data, it is clear that both the barley β-glucan exohydrolase and the β-glucosidase catalyze hydrolysis of their substrates with retention of anomic configuration in the glucose product released (Fig. 7).

Characterization of a cDNA Encoding β-Glucan Exohydrolase Isoenzyme ExoI—In the initial screening of the cDNA library a single 600-base pair cDNA clone was identified among 6 × 10⁴ plaques and nucleotide sequence analysis showed that it encoded the NH₂-terminal region of the barley β-glucan exohydrolase isoenzyme ExoI. The cDNA was subsequently labeled and used to re-screen 10⁵ plaques, two of which hybridized strongly with the probe. One was 1.6 kilobases in length and a 1-kilobase fragment from its 3’-end was used to screen another 3 × 10⁴ plaques. This resulted in the isolation of a near full-length cDNA encoding the β-glucan exohydrolase isoenzyme ExoI; the sequences of the overlapping regions of the three CDNAs were identical.

Both strands of the 2,105-base pair cDNA were sequenced (Fig. 8). To assist with the nucleotide sequencing, purified β-glucan exohydrolase isoenzyme ExoI was hydrolyzed with trypsin and, after separation of tryptic peptides by high performance liquid chromatography, nine fragments were subjected to amino acid sequence analysis (data not shown). This isoform was chosen because initial purification protocols resulted in much lower yields of isoenzyme ExoI, which was therefore not available at the time for the generation of tryptic peptides for amino acid sequence analysis. However, subsequent minor modifications to the procedure led to much higher relative yields of isoenzyme ExoI (Table I). Nevertheless, the peptides from isoenzyme ExoI were similar to blocks of amino acid sequences deduced from the cDNA and were therefore useful in ensuring no errors were made with the reading frame during nucleotide sequencing.

The cDNA has an open reading frame which extends from nucleotides 26 to 1897 and the amino acid sequence deduced from nucleotides 92–247 corresponds exactly with that determined directly from the 52 NH₂-terminal amino acids of β-glucan exohydrolase isoenzyme ExoI (Table II). On this basis, we conclude that the cDNA encodes β-glucan exohydrolase isoenzyme ExoI.

The region from the codon specifying the NH₂-terminal aspartic acid residue at nucleotide 92 to the stop codon at nucleotide 1898 encodes a polypeptide of 602 amino acid residues, with a calculated Mₙ of 65,102 and an estimated pl of 7.9. Four potential N-glycosylation sites are present, and are encoded by sequences beginning at nucleotides 206, 749, 983, and 1580 (Fig. 8). Immediately 5’ to the codon which specifies the NH₂-terminal aspartic acid residue is a sequence corresponding to a putative signal peptide of 22 residues (Fig. 8). The coding region of the mature polypeptide is followed by a TGA stop codon, which becomes part of a 208-nucleotide pair 3’-untranslated region, and is followed by a polyadenylic acid tail of 30 residues (Fig. 8). A potential polyadenylation signal AATAAA begins 80 nucleotides upstream from the start of the polyadenylyl tail. The coding region of the β-glucan exohydrolase cDNA has an overall (G + C) content of 54% and some preference for the use of G or C in the wobble base position of codons (65%).

### Table IV

| Isoenzyme ExoI | Isoenzyme βII |
|----------------|--------------|
| Kₘ (4-NPG) (m) | 1.4 × 10⁻³ | 0.5 × 10⁻³ |
| Kₘ (4-NPG) (s⁻¹) | 4.7 | 0.5 |
| Kₘ/Kₘ⁺ (m⁻¹) | 3.3 × 10³ | 1.0 × 10³ |
| Kₙ (lactone) (m) | 2.1 × 10⁻⁶ | 1.6 × 10⁻⁵ |
| Kₙ (glucose) (m) | 8.8 × 10⁻³ | 2.1 × 10⁻² |
| Kₙ/Kₙ⁺ (4-NPG) | 6.3 | 42.0 |
| Kₙ (glucose/Kₙ⁺) | 4,190 | 1,313 |

*a* Catalytic rate constant or catalytic center activity.
*b* Catalytic efficiency factor.
*c* Dissociation constant of the enzyme-inhibitor complex.

2 M. Hřmova and G. B. Fincher, unpublished data.
DISCUSSION

Two basic β-glucan exohydrolases of apparent Mr, 69,000 and 71,000 have been purified from extracts of 8-day germinated barley grain by fractional precipitation with ammonium sulfate, ion exchange chromatography, chromatofocusing, hydrophobic interaction chromatography, and gel filtration chromatography (Scheme 1). The purification procedure allowed the separation of β-glucan exohydrolases from (1,3)- and (1,3;1,4)-β-glucan endohydrolases, both of which were present at high levels in the initial grain extracts. The exohydrolases have been designated isoenzymes ExoI and ExoII in order to distinguish them from the (1,3)-β-glucan endohydrolase isoenzymes GI-GVII and the (1,3;1,4)-β-glucan endohydrolase isoenzymes EI and EII (Woodward and Fincher, 1982a; Xu et al., 1992; Høj and Fincher, 1995). The purified enzymes hydrolyze 4-NPG, but have been identified with polysaccharide exohydrolases rather than β-glucosidases because they also hydrolyze polymeric substrates such as laminarin and (1,3;1,4)-β-glucan (Table III), releasing glucose as the primary degradation product. Furthermore, the use of (1,3;1,4)-β-oligoglucosides shows clearly that the glucose units are released from the nonreducing termini of the oligomeric substrates (Fig. 6). The β-glucan exohydrolases can be readily distinguished from two β-glucosidases that were also purified from the grain extracts (Scheme 1), not only on the basis of differences in substrate specificity (Table III), but also because of their different isoelectric points, molecular weights, and NH₂-terminal amino acid sequences (Figs. 2–4 and Table II). No linkage specificity has been assigned to the β-glucan exohydrolases, because they are able to release glucose from (1,3)-β-glucans, (1,3;1,4)-β-glucans, and (1,2)-, (1,3)-, (1,4)-, (1,6)-, and 1,3;1,4-β-oligoglucosides (Table III).²

Two isoforms of the barley β-glucan exohydrolase isoenzyme ExoII were resolved during the purification procedure. The sequences of their NH₂-terminal amino acids show considerable divergence; only 33 of the first 52 residues are identical (Table II). The complete primary structure of the β-glucan exohydrolase isoenzyme ExoI has been deduced from a near full-length cDNA clone (Fig. 8). The nucleotide sequence reveals the presence of a putative signal peptide of 22 residues, suggesting that the enzyme is secreted from cells in which it is expressed. The putative signal peptide region is followed by a region that encodes a protein with 602 residues, a calculated molecular mass of 65,102 Da, and a deduced pl of 7.9. These values may be compared with an apparent Mr of 71,000 estimated from SDS-PAGE (Fig. 4) and an isoelectric point of 8.0 estimated by chromatofocusing (data not shown). The difference between the molecular weight calculated from the deduced amino acid sequence and that measured by SDS-PAGE may result from glycosylation of the mature enzyme, which carries four potential N-glycosylation sites (Fig. 8).

When the barley β-glucan exohydrolase isoenzyme ExoI sequence (Fig. 8) was compared with sequences in the DNA and protein data bases, a sequence tagged site (clone ABC254; accession number L43939) from barley was found to have a nucleotide sequence identical with nucleotides 1853–2016 of the cDNA isolated here (Fig. 8). The ABC254 sequence is also in the GrainGenes data base and has been mapped to the long arm of barley chromosome 1 (7H), close to the centromere (Langridge et al., 1995). It is therefore possible to positively identify the ABC254 probe as part of the gene encoding the barley β-glucan exohydrolase isoenzyme ExoI, and to incorporate this gene into high density genetic maps.

An important question raised in the work described here...
relates to the differences between \( \beta \)-glucan exohydrolases and \( \beta \)-glucosidases, and how they should be classified within current Enzyme Commission groups. Jermyn (1961) and later Fry (1995) have pointed out that the commonality between these enzymes lies in their ability to cleave nonreducing terminal glucosyl residues from their substrates. Their specificity for the "aglycone" portion of the substrate and the type of linkage hydrolyzed is quite variable and their classification according to the two subtypes as recognized by the Enzyme Commission is somewhat arbitrary. The barley \( \beta \)-glucan exohydrolase isoenzymes ExoI and ExoII release glucose from the nonreducing end of substrates, with retention of anomeric configuration (Figs. 6 and 7). In some respects they are similar in specificity and action pattern to plant (1,3)-\( \beta \)-glucan exohydrolases that have been classified in the EC3.2.1.58 group (Cline and Albertsheim, 1981; Labrador and Nevins, 1989), but they are capable of hydrolyzing substrates with linkages other than those in the (1,3)-position. While they are clearly polysaccharide exohydrolases, rates of hydrolysis of oligomeric substrates do not increase dramatically with increasing DP (Fig. 5A), but this may simply reflect subsite binding energies and the organization of the substrate-binding site (Varghese et al., 1994; Hrmova et al., 1995).

Classification of the barley \( \beta \)-glucosidases within existing EC groups (Webb, 1994) is also difficult. Although their amino acid sequences show high degrees of similarity to a wide range of \( \beta \)-glucosidases (EC 3.2.1.21) in the protein and DNA data bases, they hydrolyze cellodextrins at significantly higher rates than the aryl-glucoside 4-NPG and, more importantly, the rate of hydrolysis increases with DP of the cellodextrin substrate (Table III; Fig. 8B). The \( \beta \)-glucosidases release glucose from the nonreducing ends of substrates (Fig. 6), with retention of anomeric configuration and the organization of the \( \beta \)-glucosidases is somewhat arbitrary. The final consideration here relates to the functions of the barley \( \beta \)-glucan exohydrolases and \( \beta \)-glucosidases in germinating barley grain or in young seedlings. It should be noted that the enzymes could be derived from the aleurone, scutellum, starchy endosperm, coleoptile, young leaves, or young roots, all of which are likely to be present in the 8-day germinated barley grain used for enzyme isolation. The availability of cDNA clones for both classes of enzyme (Fig. 8C; Leah et al. (1995))

**Fig. 8.** Nucleotide sequence and derived amino acid sequence of the cDNA encoding barley \( \beta \)-glucan exohydrolase isoenzyme ExoI. The putative signal peptide is shaded and the arrow indicates the NH\(_2\)-terminal amino acid residue of the mature enzyme. The four potential N-glycosylation sites are underlined, and the potential polyadenylation signal is shown by double underlining.
opens the way for defining their precise tissue location. β-Glucosidases are likely to have many functions in cellular processes during normal growth and development of plants, depending on their substrate specificities (Leah et al., 1995). Maize β-glucan exohydrolases have been implicated in cell wall loosening in elongating coleoptiles (Hoson and Nevin, 1989), in which high levels of this class of enzyme are correlated with significant decreases in cell wall (1,3;1,4)-β-glucan (Sakurai and Masuda, 1978). Although it is difficult to envisage how exohydrolases might mediate in wall loosening (Fry, 1995), there is certainly little or no (1,3;1,4)-β-glucan endohydrolase of the EC 3.2.1.73 group in elongating barley coleoptiles (Slakesi and Fincher, 1992a).

A second possible function for the enzymes is to participate in the complete depolymerization of endosperm cell wall (1,3;1,4)-β-glucan to glucose. The major oligosaccharides released from this polysaccharide by (1,3;1,4)-β-glucan endohydrolases are G4G3Gβred and G4G4G3Gβred (Woodward and Fincher, 1982b). Both the β-glucan exohydrolases and the β-glucosidases can hydrolyze these oligosaccharides (Figs. 5 and 6; Leah et al., 1995), although the rate of hydrolysis of (1,3)-β-glucosyl linkages by the β-glucosidases is relatively slow. The third possible role for the enzymes, in particular the β-glucan exohydrolases, could be related to the protection of germinating grain or young seedlings from pathogen attack (Høj and Fincher, 1995), (1,3)-β-Glucan endohydrolases (EC 3.2.1.39) are classified among the pathogenesis-related proteins and could protect the young plant from microbial infection through their ability to hydrolyze the (1,3,6)-β-glucons found in fungal cell walls. The β-glucan exohydrolases purified here are also able to hydrolyze (1,3)- and (1,3,6)-β-glucons (Table III).2 In any case, the availability of cDNAs for the exo-β-glucanase will enable the time course and regulation of its gene expression to be examined, both in normal growth and development, and after pathogen attack, and this will undoubtedly provide clues as to the functions of the β-glucan exohydrolases and the β-glucosidases in germinated grain and young barley seedlings.

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Barley -D-Glucan Exohydrolases with -D-Glucosidase Activity: PURIFICATION, CHARACTERIZATION, AND DETERMINATION OF PRIMARY STRUCTURE FROM A cDNA CLONE

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