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Barley β-D-glucan exohydrolases. Substrate specificity and kinetic properties

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Abstract

Two β-D-glucan exohydrolases purified from germinated barley (*Hordeum vulgare*) and designated isoenzymes ExoI and ExoII release glucose during the hydrolysis of a range of polymeric β-D-glucans, β-linked oligo-D-glucosides, and aryl β-D-glucosides. Of the polysaccharide substrates examined the enzymes show a preference for (1 → 3)-β-glucans, although (1 → 3;1 → 6)- and (1 → 3;1 → 4)-β-D-glucans are also hydrolysed. Oligosaccharides containing (1 → 2)-, (1 → 3)-, (1 → 4)- and (1 → 6)-β-linked glucosyl residues are hydrolysed by both enzymes, which therefore exhibit a relatively broad specificity with respect to linkage positions in their substrates. During the hydrolysis of laminarabiose at high substrate concentrations (5–20 mM), transglycosylation reactions can be detected. Both isoenzymes have a pH optimum of 5.25 and bell-shaped pH-activity curves. Detailed kinetic analyses using the (1 → 3)-β-glucan, laminaran from *Laminaria digitata*, allow the calculation of apparent K_m values of 98 and 120 μM, catalytic rate constants (k_{cat}) of 73 and 28 sec^{-1}, and catalytic efficiency factors (k_{cat}/K_m) of 7.4×10^5 and 2.3×10^5 sec^{-1} M^{-1} for isoenzymes ExoI and ExoII, respectively. The kinetic analyses also show a positive cooperativity of binding of the enzymes for the barley (1 → 3;1 → 4)-β-D-glucan, which suggests the presence of an allosteric substrate-binding site. Because of important differences between these barley enzymes and previously described (1 → 3)-β-D-glucan exohydrolases (EC 3.2.1.58) from other sources, they can not be readily assigned to existing Enzyme Commission groups. However, amino acid sequence similarities suggest that the enzymes are members of the family 3 group of glycosyl hydrolases. © 1998 Elsevier Science Ltd

Keywords: Action pattern; Barley; β-Glucan exohydrolase; Kinetic analysis; Substrate specificity

Abbreviations: CM, *O*-(carboxymethyl); dp, degree of polymerization; DS, degree of substitution; G4G3G, 3-*O*-β-cellobiosyl-D-glucose; HPAEC, high performance anion-exchange chromatography; 4NP, 4-nitrophenol; 4NPG, 4-nitrophenyl β-D-glucopyranoside; PAD, pulsed amperometric detector; PR, pathogenesis-related; EC 3.2.1.58, (1 → 3)-β-D-glucan glucohydrolase or (1 → 3)-β-glucan exohydrolase; EC 3.2.1.39, (1 → 3)-β-D-glucan glucanohydrolase or (1 → 3)-β-glucan endohydrolase; EC 3.2.1.73, (1 → 3;1 → 4)-β-D-glucan 4-glucanohydrolase or (1 → 3;1 → 4)-β-glucan endohydrolase; EC 3.2.1.21, β-D-glucoside glucohydrolase or β-glucosidase

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1. Introduction

Two β-D-glucan exohydrolases have been purified from eight-day germinated barley grain by Hrmova et al. [1]. Amino acid sequences of the purified enzymes, which have been designated isoenzymes ExoI and ExoII, provide good evidence that they represent the products of separate genes, but that the two genes have probably evolved from a common ancestral gene. Nucleotide sequence analysis of a near full-length cDNA encoding isoenzyme ExoII enabled the primary structure of this enzyme to be deduced. The two isoforms have isoelectric points of 7.8 and 8.0, and apparent M_r values of 69,000 and 71,000, respectively [1]. A β-D-glucan exohydrolase released from the cell walls of barley seedlings has recently been described [2]. This enzyme corresponds to isoenzyme ExoII; no isoenzyme ExoI was detected in the seedling cell walls [2].

The ability of the β-glucan exohydrolases to hydrolyse both (1 → 3)-β-glucans and (1 → 3;1 → 4)-β-glucans raises some doubt as to their correct Enzyme Commission classification. Enzymes with similar specificities have been reported in walls of soybean cell cultures [3], in cultured *Acacia verek* cells [4] and in maize coleoptiles [5], although the activities of these enzyme preparations against a broad range of substrates have not always been examined. The work described here was undertaken to characterize the substrate specificities and action patterns of the two barley β-glucan exohydrolases, and to define their kinetic properties. This detailed characterization of the enzymes' substrate specificities, action patterns and kinetic properties represents a prerequisite for future studies on the location and functional significance of β-glucan exohydrolases in germinated grain or growing barley seedlings, and for a detailed description of the mechanism of catalysis.

2. Experimental

Materials.—Laminaran (from *Laminaria digitata*), lichenans (from *Usnea barbata* and *Cetraria islandica*), 2-nitrophenyl β-D-glucopyranoside and 4-nitrophenyl β-D-glucopyranoside (4NPG), 2-nitrophenyl and 4-nitrophenyl β-D-galactopyranosides, 4-nitrophenyl β-D-xylopyranoside, 4-nitrophenyl β-D-fucopyranoside, 4-nitrophenyl β-cellobioside, 4-nitrophenyl β-lactoside, 4-nitrophenyl β-D-glucosiduronic acid, 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucoside, 4-nitrophenyl β-gentiobioside, 4-nitrophenyl β-D-arabinopyranoside, gentiobiose, orcinol and the glucose diagnostic kit were obtained from Sigma Chemical (St. Louis, MO, USA). Kieselgel 60 thin-layer plates were from Merck (Darmstadt, Germany) and low M_r marker proteins were from AMRAD Pharmacia Biotech (Uppsala, Sweden). Laminarans (from *L. hyperborea* and *Eisenia bicyclis*) and curdlan (from *Alcaligenes faecalis*) were from Tokyo Kasei Kogyo (Tokyo, Japan), (1 → 4)-β-D-xylan (beechwood) was from the Institute of Chemistry (Bratislava, Slovakia), pachyman (from *Poria cocos*) was from Calbiochem (San Diego, CA, USA), barley (1 → 3;1 → 4)-β-D-glucan was from Biocon Biochemicals (Kinagleary, Ireland), CM-cellulose (degree of substitution, DS 0.54) was from Imperial Chemical Industries (Dingley, Australia) and (1 → 3)-β-D-linked oligosaccharides of dp 2–7 and (1 → 4)-β-D-linked oligosaccharides of dp 2–6 were purchased from Seikagaku Kogyo (Tokyo, Japan). Pneumococcal SIII polysaccharide (from *Streptococcus pneumoniae*), pustulan (from *Umbilicaria pustulata*), CM-pachyman (DS 0.29), 3-*O*-β-cellobiosyl-D-glucose (G4G3G) and sophorose were generously provided by Professor B.A. Stone (La Trobe University, Melbourne, Australia). Schizophyllan M-2 (from *Schizophyllum commune*) was obtained from Dr. S. Kitamura (Kyoto Prefectural University, Kyoto, Japan) and insoluble yeast (1 → 3;1 → 6)-β-D-glucan (from *Saccharomyces cerevisiae*) and soluble yeast CM-glucan (DS 0.42) were kindly provided by Dr. J. Sandula (Institute of Chemistry, Bratislava, Slovakia).

Enzyme assays.—Purified β-glucan exohydrolase isoenzymes ExoI and ExoII were prepared from extracts of germinated barley as described by Hrmova et al. [1]. Activity was determined reductometrically by monitoring the increase in reducing sugars [6,7] released in 0.2% (w/v) solutions of *L. digitata* laminaran or barley (1 → 3;1 → 4)-β-glucan and spectrophotometrically by the increase in absorbance at 410 nm in 0.2% (w/v) 4NPG in 50 mM NaOAc buffer, pH 5.25 at 37° C. The reaction with 4NPG was stopped and developed by adding two volumes of 4% (w/v) Na_2CO_3 [1], and absorbance was measured at 410 nm. Glucose released by enzyme action was measured by the glucose oxidase method, using *o*-dianisidine as a diagnostic reagent, according to the manufacturer's (Sigma) instructions.

One unit of activity is defined as the amount of enzyme required to release 1 μmol glucose equivalents from laminaran or barley (1 → 3;1 → 4)-β-glucan per min, or to release 1 μmol 4NP from 4NPG per min. One unit corresponds to 16.67 nanokatals.

Protein determination and electrophoresis.—Protein concentrations during enzyme purification and characterization, and SDS–PAGE were performed as described previously [1].

Action patterns.—The products released from 2.9% (w/v) *L. digitata* laminaran and 1.0% (w/v) (1 → 3;1 → 4)-β-glucan were monitored by TLC after incubating with 132 pkat and 391 pkat of purified isoenzymes ExoI and ExoII, respectively, at 37 °C in 5 mM NaOAc buffer, pH 5.25, for 0, 0.2 and 2 h. Final hydrolysis products were examined in the same way, except that one-third of the initial amount of fresh enzyme was added after 2 h and the reactions were allowed to proceed for a total of 24 h under a toluene vapour. Hydrolysates were applied to Kieselgel 60 thin-layer plates developed in EtOAc/CH_3COOH/water (3:2:1, by volume) and reducing products were detected with the orcinol reagent [8].

Products released from *L. digitata* laminaran and (1 → 3;1 → 4)-β-glucan were also analysed by high performance anion-exchange chromatography (HPAEC), using a CarboPac PA10 analytical separation column (4 × 250 mm) and a CarboPac PA10 guard column (4 × 50 mm), and a pulsed amperometric detector (PAD) fitted with a combination pH-Ag/AgCl reference electrode (Dionex Sunnyvale, CA, USA). Peaks were identified by comparison with standard glucose, gentiobiose, and cello- and laminar-oligosaccharides of dp 2–4. Concentrations of individual components in enzyme hydrolysates were estimated from peak areas [9]; PAD response factors for these components varied by 3–10% between assays.

To investigate whether the β-glucan exohydrolases catalyze glucosyl transfer reactions, 264 pkat isoenzyme ExoI and 782 pkat of isoenzyme ExoII were incubated with 5 and 20 mM laminarabiose in 5 mM NaOAc buffer, pH 5.25 for 0, 0.2, 4 and 18 h. Reaction mixtures were separated by TLC and detected as described above, or by HPAEC-PAD.

Substrate specificities.—Soluble or insoluble β-glucans, differing in the identity, proportion and arrangement of their linkage types (Table 1), were prepared at a final concentration of 0.2% (w/v) in 0.1 M NaOAc buffer, pH 5.25, containing 160 μg/mL BSA. The substrates were incubated with 22 and 40 pkat of the purified β-glucan exohydrolase isoenzymes ExoI and ExoII, respectively, and activities were measured reductometrically.

Hydrolytic rates of 2-nitrophenyl and 4-nitrophenyl β-glycosides were assayed at a final concentration of 0.2% (w/v) in 0.1 M NaOAc buffer, pH 5.25, containing 160 μg/mL BSA. The substrates were incubated with 5 and 2 pkat of the purified isoenzymes ExoI and ExoII, respectively, and activity was measured spectrophotometrically at 410 nm.

Enzymic properties.—The pH optima of the purified β-glucan exohydrolases were determined over the pH range 4–7 in 0.1 M sodium citrate–0.2 M sodium phosphate buffers (McIlvaine buffers). Purified isoenzymes ExoI and ExoII (22 and 40 pkat, respectively) were incubated with 0.2% (w/v) 4NPG, in the presence of 160 μg/mL BSA. Thermostabilities were determined by measuring residual activity with 0.2% (w/v) 4NPG after incubation of 5 and 2 pkat of the purified isoenzymes ExoI and ExoII, respectively, in 0.1 M NaOAc buffer, pH 5.25 with or without BSA at temperatures between 0 and 80 °C for 15 min.

Kinetic parameters.—Kinetic parameters were determined at 37 °C by incubating 66–198 and 773–1030 pkat of the purified β-glucan exohydrolases ExoI and ExoII, respectively, in 0.1 M NaOAc buffer, pH 5.25, containing 160 μg/mL BSA. Rates of hydrolysis were determined at substrate concentrations ranging from 0.4–3, 0.4–4, 0.1–6 and 0.1–10 times the K_m value for laminaran, barley (1 → 3;1 → 4)-β-glucan, laminarabiose and 4NPG, respectively. Enzymic reactions with laminaran and (1 → 3;1 → 4)-β-glucan were stopped by the addition of the alkaline copper reagent in reductometric assays [6,7], by the addition of Na_2CO_3 with 4NPG in spectrophotometric assays [1], or by heating for 2 min to 100 °C in glucose oxidase assays. Kinetic data were processed by a proportional weighted fit using a non-linear-regression analysis program based on Michaelis–Menten and Hill model equations [10]; variations of approximately 10% were usually observed in the kinetic analyses with polysaccharide substrates. Calculations of molecular masses for K_m determinations were based on a dp of 25 for laminaran [11] and on a dp of 1300 for the barley (1 → 3;1 → 4)-β-glucan [12]. Special care was taken to ensure that initial reaction rates were measured [13].

3. Results

Purity of β-glucan exohydrolases.—The purity of β-glucan exohydrolases isolated from barley seedlings according to Hrmova et al. [1] were assessed by SDS–PAGE, where single protein bands were de-

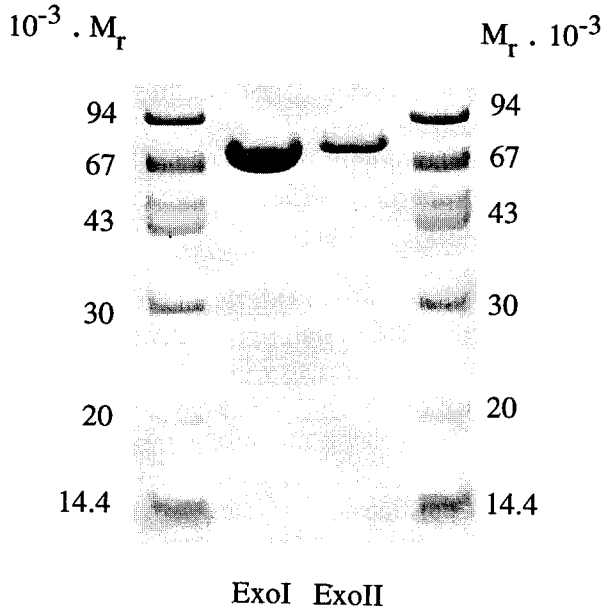

Fig. 1. SDS–PAGE of purified β-glucan exohydrolase isoenzymes ExoI (lane 2, 18 μg loaded) and ExoII (lane 3, 9 μg loaded). Molecular size protein markers are shown in lanes 1 and 4.

tected, even at high protein loadings (Fig. 1). Additional confirmation of purity was obtained from NH_2-terminal amino acid sequence analysis. No secondary sequences were detected in the first 100 amino acid residues from isoenzyme ExoI or in the first 80 residues from isoenzyme ExoII; the sequences were identical to those previously reported [1]. In addition, recoveries of amino acid residues during the sequencing analysis were close to theoretical values; this provided further evidence of enzyme purity.

Action patterns.—Examination of reaction products released by the barley β-glucan exohydrolases during the hydrolysis of laminaran and barley (1 → 3;1 → 4)-β-glucan shows that glucose appears early and continues to accumulate as hydrolysis proceeds (Fig. 2). In the case of the (1 → 3;1 → 4)-β-glucan no other major products can be detected, but a major oligosaccharide with a mobility similar to gentiobiose can be detected in the laminaran hydrolysate, together with several minor oligosaccharides (Fig. 2). Data shown in Fig. 2 are for isoenzyme ExoI, but almost identical results were obtained with isoenzyme ExoII

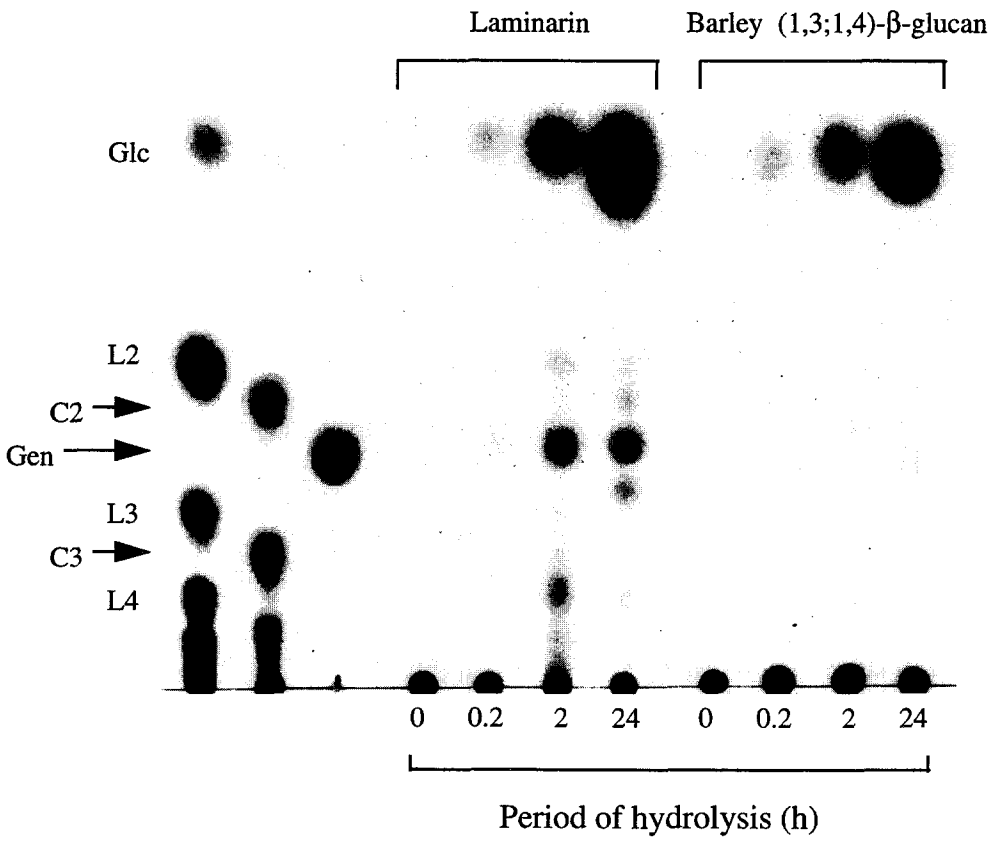

Fig. 2. TLC of hydrolysis products of *L. digitata* laminaran and barley (1 → 3;1 → 4)-β-glucan by β-glucan exohydrolase isoenzyme ExoI after 0, 0.2, 2 and 24 h. Standards are glucose (Glc), gentiobiose (Gen), (1 → 3)-β-linked oligoglucosides of dp 2–4 (L2–L4), and (1 → 4)-β-linked oligoglucosides of dp 2 and 3 (C2 and C3).

Table 1
Relative rates of hydrolysis of β-linked poly- and oligosaccharides and aryl β-D-glucosides by barley β-glucan exohydrolase isoenzymes ExoI and ExoII[a]

Substrate	Relative rate (%)	
	ExoI	ExoII
Polysaccharides		
laminaran		
L. digitata	100	100
L. hyperborea	50	75
E. bicyclis	9	10
CM-pachyman (*P. cocos*)	40	34
CM-(1 → 3;1 → 6)-β-glucan (*S. cerevisiae*)	25	29
(1 → 3;1 → 4)-β-glucan (*Hordeum vulgare*)	10	14
(1 → 3;1 → 4)-β-glucan (lichenin)		
U. barbata	11	27
C. islandica	18	49
SIII polysaccharide (*S. pneumoniae*)	2	5
Oligosaccharides		
sophorose	55	54
laminarabiose	70	75
cellobiose	14	20
gentiobiose	36	20
Aryl β-D-glucopyranosides		
4-nitrophenyl β-D-glucoside	10	56
2-nitrophenyl β-D-glucoside	5	12

[a]The rates on β-linked poly- and oligosaccharides were measured reductometrically and on aryl β-D-glucosides spectrophotometrically. The rates on *L. digitata* laminaran were arbitrarily set at 100% and correspond to 63 and 24 units/mg protein, respectively. Little or no activity was detected against CM-cellulose, xylan, curdlan (*A. faecalis*), pachyman (*P. cocos*), schizophyllan (*Schizophyllum commune*), pustulan (*Umbilicaria pustulata*), (1 → 3;1 → 6)-β-glucan (*S. cerevisiae*), 2-nitrophenyl β-D-galactopyranoside and 4-nitrophenyl β-D-glycopyranosides of D-arabinose, D-glucuronic acid, *N*-acetyl-D-glucosamine, gentiobiose, lactose, D-galactose, D-xylose, D-fucose and cellobiose.

(data not shown). Gentiobiose could be released from the laminaran itself, because the preparation from *L. digitata* that was used in these experiments consists of a linear backbone of 20–25 (1 → 3)-β-linked glucosyl residues in which approximately every seventh residue is substituted at O-6 position with a single glucosyl residue [11]. The accumulation of this (1 → 6)-β-linked disaccharide implies either that the enzymes can by-pass glucosyl residues that are substituted at O-6, and that the released gentiobiose is hydrolysed very slowly, or that it is produced concurrently by transglycosylation, particularly as the concentrations of released glucose and oligosaccharides increase. Proton NMR has been used to identify gentiobiose in laminaran hydrolysates [1]. The results shown in Fig. 2 also indicate that the rate of hydrolysis of the released gentiobiose is sufficiently slow to allow it to accumulate. It is likely that the gentiobiose is eventually hydrolysed to glucose (Table 1).

The exceedingly low amounts of gentiobiose detected by TLC in (1 → 3;1 → 4)-β-glucan hydrolysates (Fig. 2) are not consistent with the relatively higher levels (approximately 15%) found by HPAEC analysis (Fig. 3). The apparent discrepancy is pre-

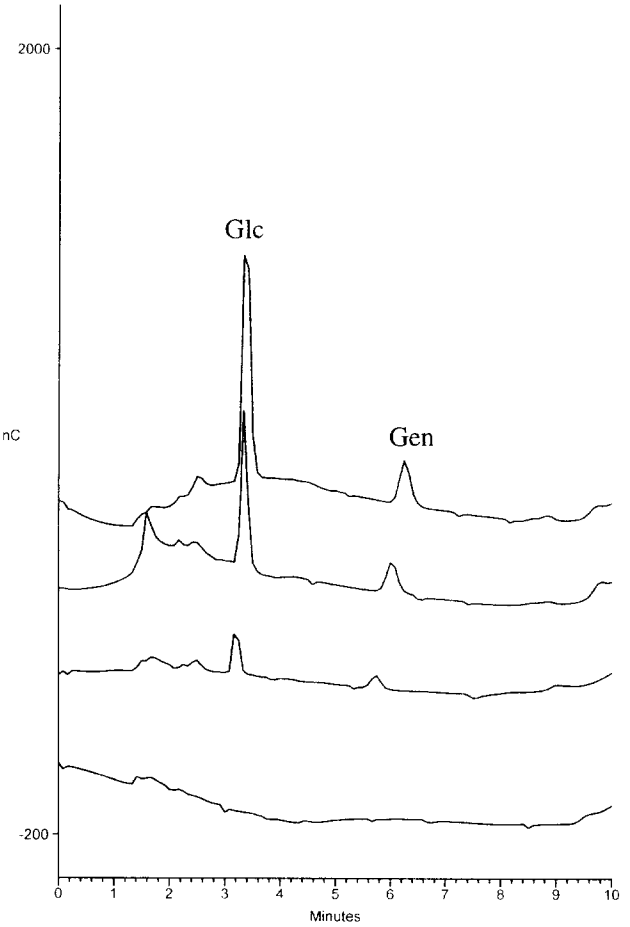

Fig. 3. HPAEC-PAD (Dionex) analysis of products released from barley (1 → 3;1 → 4)-β-glucan by β-glucan exohydrolase isoenzyme ExoI. The four profiles represent time courses of hydrolysis after 0, 0.2, 2 and 24 h. The products were eluted from the column at 23 °C at a flow rate of 1 mL/min, using a gradient of 150 mM NaOH prepared in 18 MΩ cm^{-1} deionised water (solvent A) and 500 mM sodium acetate in 150 mM NaOH (solvent B). The eluent program at 0–2 min was 98% (v/v) solvent A and 2% (v/v) solvent B (isocratic); 2–5 min, 95% A and 5% B (linear); 5–8 min, 82% A and 18% B (linear); 8–15 min, 75% A and 25% B (linear). Retention times of glucose and gentiobiose are indicated.

sumably explained by the lower response of gentiobiose to orcinol detection on thin layer plates (M. Hrmova and G.B. Fincher, unpublished data). The presence of gentiobiose in hydrolysates of barley (1 → 3;1 → 4)-β-glucan (Fig. 3) is also suggestive of transglycosylation reactions, but the peak that has the same chromatographic mobility as gentiobiose in Fig. 3 has not yet been subjected to NMR or mass spectrometric characterization.

To further examine the potential glucosyltransferase or transglycosylation activity of the enzymes, isoenzymes ExoI and ExoII were incubated with laminarabiose of concentrations up to 20 mM and the reaction mixtures separated by TLC. Again, significant levels of a reducing oligosaccharide with a mobility similar to gentiobiose were detected, together with some higher oligosaccharides. Results for isoenzymes ExoII (Fig. 4) and ExoI (data not shown) were essentially the same. This also suggests that the enzymes are capable of catalysing complex transglycosylation reactions, in which two major transglycosylation products are transiently formed; these are eventually hydrolysed to glucose with low levels of gentiobiose (Fig. 4A). Preliminary results, based on elution times during HPAEC-PAD (Dionex) analysis, indicate that the major higher oligosaccharides in the

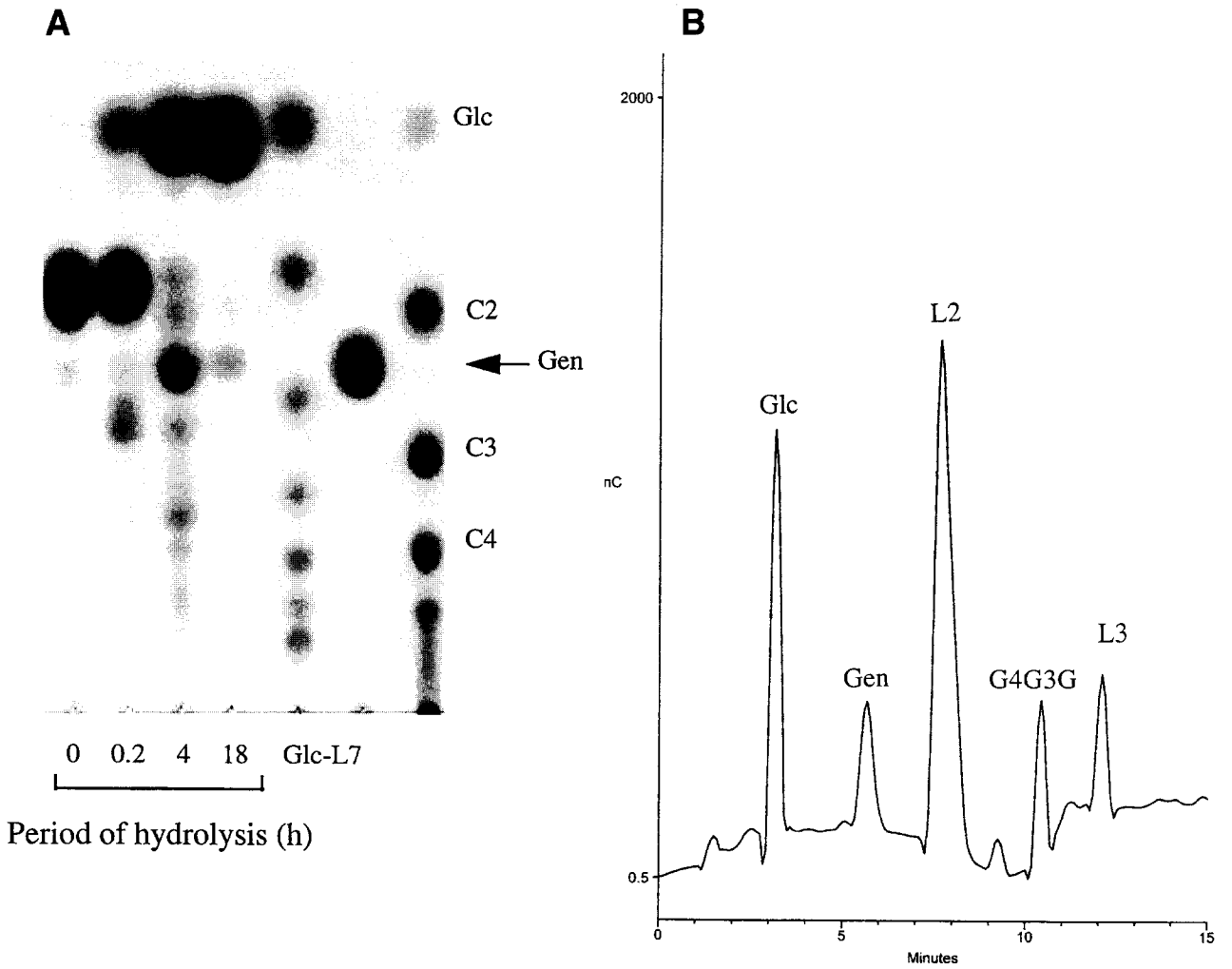

Fig. 4. (A) Thin-layer chromatography of transglycosylation reaction products formed from laminarabiose by ß-glucan exohydrolase isoenzyme ExoII. Laminarabiose at a final concentration of 20 mM was incubated with the enzyme for 0, 0.2, 4 and 18 h at 37 °C. Aliquots were concentrated and chromatographed with glucose (Glc), gentiobiose (Gen), (1 → 3)-β-linked oligoglucosides of dp 2–7 (L2–L7) and (1 → 4)-β-linked oligoglucosides of dp 2–5 (C2–C5) as standards. (B) HPAEC-PAD (Dionex) analysis of products formed from laminarabiose by β-glucan exohydrolase isoenzyme ExoII after 0.2 h at 37 °C; these products correspond to those shown in lane 2 in (A). The oligosaccharides were identified by comparison of retention times with standard glucose (Glc), gentiobiose (Gen), laminarabiose (L2), 3-*O*-β-cellobiosyl-D-glucose (G4G3G) and laminaratriose (L3). Conditions of chromatography were as described in Fig. 3.

0.2 h hydrolysates are laminaratriose and 3-*O*-β-cellobiosyl-D-glucose (Fig. 4B), but a detailed characterization of these products has not yet been undertaken.

Nevertheless, it is clear that both enzymes can hydrolyse polymeric β-glucans at a significant rate and that glucose is the major product released. This warrants their classification as exohydrolases with the systematic name, β-glucan glucohydrolase.

Substrate specificity: Polysaccharides.—Reaction rates on the polysaccharide substrates are compared in Table 1, where the relatively rapid hydolysis of the *L. digitata* laminaran is apparent. The laminaran from *E. bicyclis* is hydrolysed much more slowly by both enzymes and this is probably related to the relatively higher content of (1 → 6)-β-linkages in this (1 → 3;1 → 6)-β-glucan (Table 1). The high molecular weight, linear (1 → 3)-β-glucan, CM-pachyman is also hydrolysed more slowly than the *L. digitata* laminaran, but in this case the carboxymethyl groups on the higher molecular weight substrate might be expected to interfere, to some extent, with hydrolysis (Table 1). Isoenzyme ExoII appears to be affected less by branching and substitution than does isoenzyme ExoI (Table 1).

As observed previously (Fig. 2) [1], both β-glucan exohydrolases can hydrolyse (1 → 3;1 → 4)-β-glucans; under the conditions used here, they hydrolyse lichenans more quickly than the barley (1 → 3;1 → 4)-β-glucan (Table 1). Again, the rate of hydrolysis of (1 → 3;1 → 4)-β-glucans compared with laminaran is higher for isoenzyme ExoII (Table 1).

No hydrolysis of CM-cellulose, xylan, curdlan, native pachyman, schizophyllan or pustulan was observed (Table 1). In some instances the absence of activity might result from the low solubility of the polysaccharides and attendant problems with their reduced accessibility to enzymic action.

Substrate specificity: Oligosaccharides.—Substrate specificities of the β-glucan exohydrolases were further examined using oligomeric substrates, including laminara-oligosaccharides, cello-oliogosaccarides and the (1 → 2)- and (1 → 6)-β-linked disaccharides, sophorose and gentiobiose (Table 1). The substantial degree of hydrolysis of these oligosaccharides indicates that both enzymes are capable of hydrolysing (1 → 2)-, (1 → 3)-, (1 → 4)- and (1 → 6)-β-glucosyl linkages. The enzymes hydrolyse (1 → 3)-linked oligosaccharides faster than (1 → 4)-linked oligosaccharides.

Although the rates of hydrolysis of cellobiose are significant and increase with the dp of the cellodextrins [1], the trends in relative rates of hydrolysis of

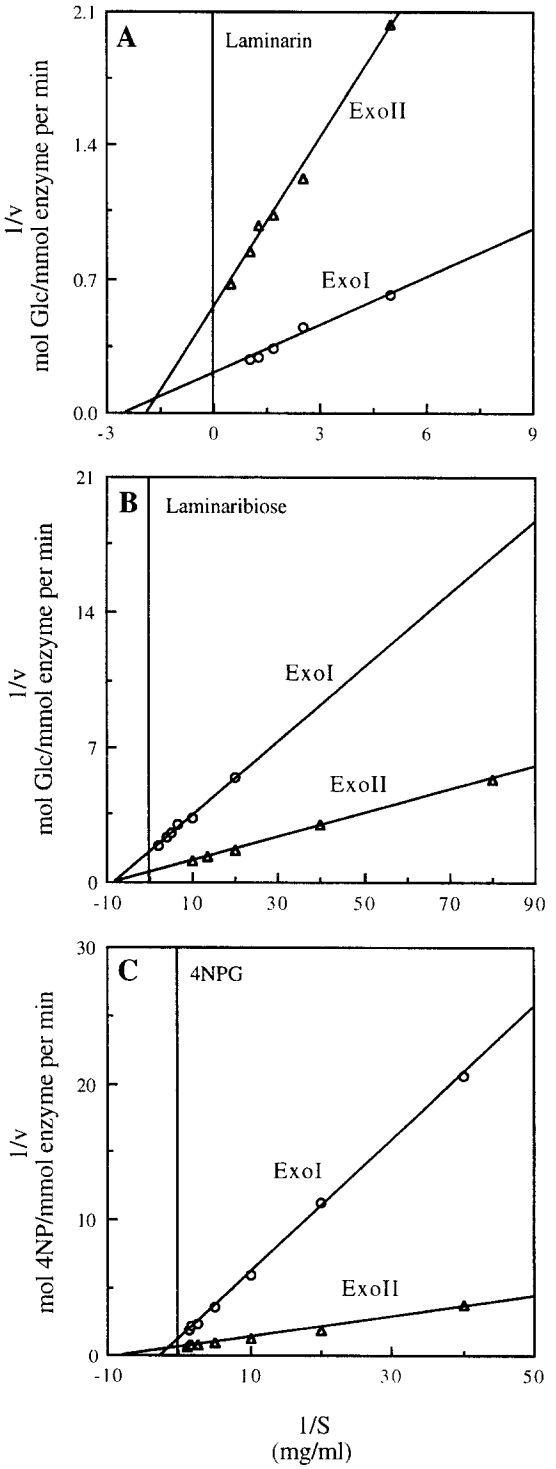

Fig. 5. Double-reciprocal (Lineweaver–Burk) plots of β-glucan exohydrolase isoenzymes ExoI and ExoII. Enzyme activity was measured reductometrically with *L. digitata* laminaran (A), by the glucose oxidase method with laminarabiose (B), or spectrophotometrically with 4NPG (C). Substrate concentrations ranged from 0.1–10 times the value of the K_m.

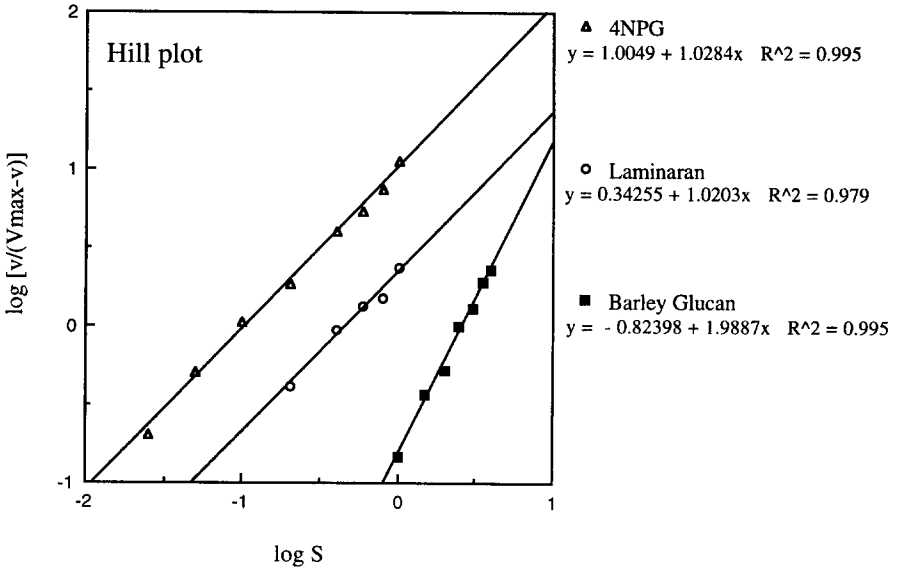

Fig. 6. Hill plot of the kinetic data. The activity of β-glucan exohydrolase isoenzyme ExoII was measured on 4NPG, *L. digitata* laminaran, and barley (1 → 3;1 → 4)-β-glucan.

the laminaradextrin series are somewhat more difficult to reconcile with published work. Previously characterized (1 → 3)-β-glucan exohydrolases hydrolyse laminarabiose slowly, if at all, and rates of hydrolysis generally increase with the dp of the oligosaccharide [14–18]. Both isoforms studied here hydrolyse laminarabiose at relatively high rates and no clear correlation between reaction rate and dp of the substrate is apparent. The products of hydrolysis of laminarabiose and other glucosyl disaccharides following incubation with isoenzymes ExoI and ExoII were analysed. Glucose is the major product in all cases, although residual sophorose and gentiobiose substrates remain.

Substrate specificity: Aryl β-glycosides.—The barley β-glucan exohydrolases can hydrolyse aryl β-glucosides to a significant degree, in particular 4NPG. The 2-nitrophenyl β-D-glucoside substrate is hydrolysed more slowly (Table 1) and activity on two β-galactosides, a β-xyloside and a range of other glycosides is very low (Table 1).

Kinetic properties.—Lineweaver–Burk plots for the hydrolysis of laminaran, laminarabiose and 4NPG by the barley β-glucan exohydrolase isoenzymes ExoI and ExoII are compared in Fig. 5. Linear curves were always obtained and when the data were rearranged according to the Hill plot [19,20], the gradients of the curves are very close to 1.0 (Fig. 6). This indicates

Table 2
Kinetic parameters of barley β-glucan exohydrolase isoenzymes ExoI and ExoII

Isoenzyme	Substrate Laminaran[a]	(1 → 3;1 → 4)-β-glucan	Laminarabiose	4NPG[b]
ExoI				
K_m (μM)	98	12	365	1400
k_{cat} (s^{-1})	73	4	10	5
k_{cat}/K_m (10^{-3} s^{-1} M^{-1})	740	330	27	3
ExoII				
K_m (μM)	120	12	355	330
k_{cat} (s^{-1})	28	11	36	15
k_{cat}/K_m (10^{-3} s^{-1} M^{-1})	230	920	101	46

[a]Using *L. digitata* laminaran.
[b]Data for isoenzyme ExoI taken from [1].

that each enzyme has a single substrate binding site for laminaran and 4NPG [20]. A notable exception was observed during the hydrolysis of barley (1 → 3;1 → 4)-β-glucan with both isoenzymes, where a non-hyperbolic or sigmoidal velocity curve was observed (data not shown). This indicates that the enzyme might have more than one substrate-binding site for this polysaccharide. After fitting the (1 → 3;1 → 4)-β-glucan data to the Hill model equation [10] it was possible to calculate kinetic parameters (Table 2). Kinetic data rearrangement in the form of the Hill plot [19,20] yielded a gradient close to 2.0 (Fig. 6), again suggesting a positive cooperativity between separate substrate-binding sites. Data for isoenzyme ExoII is shown in Fig. 6, but the same effect was observed with isoenzyme ExoI.

The kinetic parameters calculated from the data presented in Fig. 5 are shown in Table 2. The K_m values for the two isoenzymes acting on laminaran, (1 → 3;1 → 4)-β-glucan and laminarabiose are similar, but isoenzyme ExoII exhibits higher catalytic rate constants (k_{cat}) than isoenzyme ExoI during hydrolysis of all substrates examined here, except laminaran. Catalytic efficiency factors (k_{cat}/K_m) are substantially higher for isoenzyme ExoII during hydrolysis of barley (1 → 3;1 → 4)-β-glucan, laminarabiose and 4NPG (Table 2).

In considering and comparing the kinetic parameters in Table 2, it should be emphasized that K_m and k_{cat} values for polysaccharide hydrolases should be treated as approximate values only, because polysaccharide substrates are often heterogeneous with respect to size and structure, and because hydrolysis products can also act as additional substrates [8]. For this reason, it is important to ensure that only initial reaction rates are measured [13].

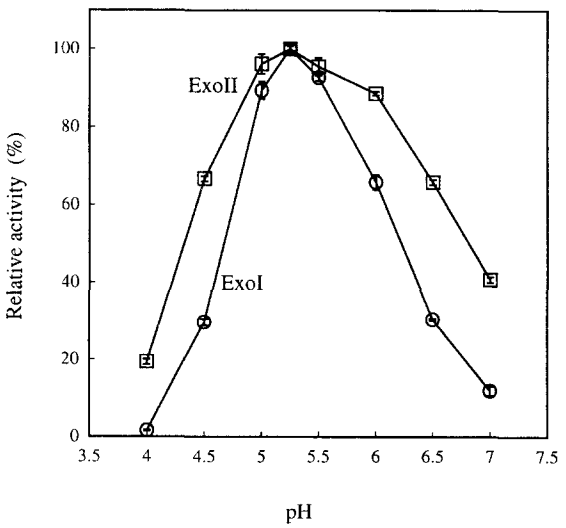

Fig. 7. Effect of pH on the activity of barley β-glucan exohydrolase isoenzymes ExoI (○) and ExoII (□). Enzyme activities were measured in 0.1 M sodium citrate–sodium phosphate buffers (McIlvaine buffers) containing 160 μg/mL BSA, using 4NPG as a substrate.

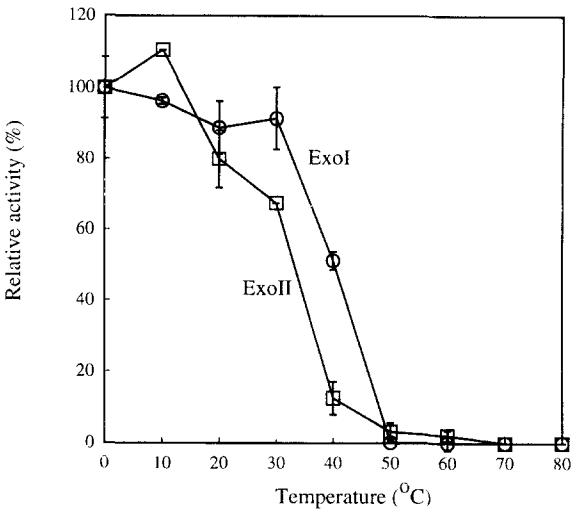

Fig. 8. Temperature stability of β-glucan exohydrolase isoenzymes ExoI (○) and ExoII (□). Residual activities after 15 min incubation at the indicated temperatures were determined using 4NPG.

ph Dependence.—For both isoenzymes, maximal activity is achieved at pH 5.25 during hydrolysis of 4NPG in the McIlvaine citrate–phosphate buffers (Fig. 7). Similar results were obtained in NaOAc buffers. The pH dependence curves are bell-shaped; half maximal activity is retained approximately 1 pH unit either side of the pH optimum and activity is almost completely lost one-and-a-half pH units from the optimum. This suggests that at least two ionizable amino acids participate in the catalytic cleavage of glycosidic linkages in these substrates. When these pH data were re-calculated as Dixon plots (data not shown), ionization constants of the enzyme–substrate complex were 4.7 and 5.9.

Thermostability.—When residual activities of the enzymes are measured after heating for 15 min at temperatures up to 80 °C, it is apparent that the β-glucan exohydrolases rapidly lose activity at temperatures above 30–35 °C (Fig. 8). Isoenzyme ExoI retains 50% of its activity after 15 min at 39 °C, while the 50% point for isoenzyme ExoII is approximately 33 °C. The addition of 160 μg/mL BSA provides limited protection against thermal inactivation.

4. Discussion

The two β-glucan exohydrolases from germinated barley have been assigned the isoenzyme names ExoI and ExoII. Despite their ability to hydrolyse aryl β-glucosides, the enzymes are classified as polysaccharide exohydrolases rather than β-glucosidases because they can also release glucose from polysaccharides. The released glucose originates from the non-reducing terminus of the substrates. In contrast, two β-glucosidases (EC 3.2.1.21) purified simultaneously from germinated barley have no activity on polysaccharides and their NH_2-terminal amino acid sequences show a high degree of similarity with well-characterized β-glucosidases from plants, animals and microorganisms [1].

Detailed analyses of the action patterns of the barley β-glucan exohydrolases have now confirmed that the enzymes are indeed β-glucan exohydrolases that catalyse the hydrolytic cleavage of single glucose units from (1 → 3)-β-glucans, (1 → 3;1 → 4)-β-glucans and (1 → 3;1 → 6)-β-glucans (Fig. 2, Table 1). Of the polysaccharides tested the preferred substrate for both isoforms is the (1 → 3)-β-glucan, laminaran, from *L. digitata*. As the degree of branching or substitution of (1 → 3)-β-glucan substrates increases, the rate of hydrolysis decreases (Table 1). Nevertheless, the enzymes hydrolyse (1 → 3;1 → 6)- and (1 → 3;1 → 4)-β-glucans to a significant extent, but isoenzyme ExoII appears to better tolerate the presence of (1 → 4)-linkages and (1 → 6)-branch points or substituents in the polysaccharide substrates (Table 1).

The relatively broad specificity for linkage type in polysaccharide substrates (Table 1) was confirmed in studies on the hydrolysis of oligosaccharides (Table 1). The (1 → 2)-β-diglucoside, sophorose, together with the corresponding (1 → 3)-β-glucosyl disaccharide, laminarabiose, the (1 → 4)-β-linked disaccharide, cellobiose, and the (1 → 6)-β-linked disaccharide, gentiobiose, are all hydrolysed by the two barley isoenzymes. Specificity of the isoenzymes for the glycosyl moiety of substrates was examined using aryl β-glucosides. The two barley β-glucan exohydrolases exhibit a high, but not an absolute specificity for β-glucosyl residues; aryl β-galactosides, a β-xyloside, a β-cellobioside and a β-fucoside are hydrolysed at very low rates (Table 1).

Of particular interest was the evidence from the kinetic analyses that the enzymes might have more than one substrate-binding site for barley (1 → 3;1 → 4)-β-glucan (Fig. 6). This suggests that the enzymes might have, in addition to the catalytic site, an allosteric site [21] at which substrate or substrate analogues could bind and influence activity. This kinetic behaviour has been defined as positive cooperativity or a positive homotropic response; the binding of one substrate molecule facilitates the binding of another substrate molecule by increasing the affinity of the second binding site [19]. It is also possible that this other substrate-binding site has a function in the binding of the enzyme to insoluble substrates, such as plant or fungal cell walls, in a similar fashion to the starch granule-binding domain of α-amylases [22] and the cellulose-binding domains of cellulases [23].

Bearing in mind the relatively broad substrate specificities of the enzymes, we are unwilling at this stage to assign the barley β-glucan exohydrolases to any existing Enzyme Commission group. However, we note significant similarities to (1 → 3)-β-glucan exohydrolases (EC 3.2.1.58) associated with cell walls in maize coleoptiles [5,24] and in suspension-cultured soybean cells [3]. Polysaccharide exohydrolases can either retain or invert anomeric configuration during hydrolysis, while endohydrolases often retain anomeric configuration [25–27]. Furthermore, glycanases that retain anomeric configuration frequently catalyse transglycosylation reactions, while those that invert configuration do not [27,28]. The retention of anomeric configuration by the barley β-glucan exohydrolases [1] is consistent with the apparent ability of the enzymes to catalyse transglycosylation reactions (Fig. 4) [29,30]. Henrissat and Bairoch [31] have classified glycosyl hydrolases into 57 families, although members of different families seem to have similar three-dimensional conformations and may indeed be classified in the same family [32]. Amino acid sequence alignments and hydrophobic cluster analyses (data not shown) indicate that the two barley β-glucan exohydrolases described here can be classified in family 3, where they share sequence similarities with a bacterial (1 → 4)-β-glucan glucohydrolase [33].

The abundance of the β-glucan exohydrolases in germinated barley and their relatively broad substrate specificities raise important questions regarding their function. The grain used for the purification of the β-glucan exohydrolases had been germinated for 8 days at 19 °C [1] and the enzymes may have originated in one or more tissues or cell types present, including the aleurone layer, the scutellum, the starchy endosperm, young roots, coleoptiles, and possibly young leaves. One role for the β-glucan exohydrolases could be to convert completely oligo-β-gluco-

Table 3
Comparison of barley (1 → 3)-β-glucan endohydrolases with barley β-glucan exohydrolases[a]

	(1 → 3)-β-Glucan endohydrolases[b]			β-Glucan exohydrolases	
	GI	GII	GIII	ExoI	ExoII
Kinetic parameters[c]					
K_m (μM)	12	208	180	98	120
k_{cat} (sec^{-1})	155	130	36	73	28
k_{cat}/K_m (10^{-5} s^{-1} M^{-1})	9	6	2	7	2
Relative rates of hydrolysis (%)					
laminaran					
L. digitata	100	100	100	100	100
L. hyperborea	44	32	48	50	75
E. bicyclis	0.4	0.2	0.5	9	10
CM-(1,3;1,6)-β-glucan					
S. cerevisiae	3	0.2	2	25	29
CM-pachyman (*P. cocos*)	0	0	0	40	34

[a]The relative rates of hydrolysis of substrates by (1 → 3)-β-glucan endohydrolases GI, GII, GIII and β-glucan exohydrolases ExoI and ExoII were arbitrarily set at 100% and correspond to 201, 245, 59 and 63 and 24 units/mg protein, respectively.
[b]Data taken from [8].
[c]Using *L. digitata* laminaran.

sides to glucose (Fig. 2), which would then be available as an energy source for the developing seedling. High levels of (1 → 3;1 → 4)-β-glucan in walls of starchy endosperm cells are hydrolysed to tri- and tetrasaccharides by the action of two (1 → 3;1 → 4)-β-glucanases of the EC 3.2.1.73 group [34,35]. It is highly likely that enzymes capable of salvaging glucose from these oligosaccharides would be present in germinated grain, because glucose present in cell wall (1 → 3;1 → 4)-β-glucan constitutes up to 18% of total grain glucose [36] and this clearly represents a valuable source of energy for the growing seedling. The development of an enzyme system to recover this energy component of endosperm cell walls would clearly represent a competitive advantage to developing seedlings, particularly in the relatively hostile environment of arid areas where cereals evolved. It has also been suggested that the β-glucosidases from germinated barley grain could participate in the complete hydrolysis of (1 → 3;1 → 4)-β-glucan to glucose [37]. Because of the much slower rate of hydrolysis of (1 → 3)-β-glucosyl linkages by the β-glucosidases [1] and the relative abundance of this linkage type in the tri- and tetrasaccharides released from (1 → 3;1 → 4)-β-glucan by (1 → 3;1 → 4)-β-glucan endohydrolases [35], it might appear that the β-glucan exohydrolases would be more efficient in effecting the complete conversion of these cell-wall polysaccharides to glucose in the germinated grain. However, although there is evidence for the expression of β-glucan exohydrolase genes in the scutellum of germinated barley (A.J. Harvey and G.B. Fincher, unpublished data), the locations of these enzymes and their participation in (1 → 3;1 → 4)-β-glucan depolymerization in the starchy endosperm of germinated grain remain to be demonstrated unequivocally.

A second possible function for the β-glucan exohydrolases could be in the auxin-induced elongation of cells in growing coleoptiles, which has been studied in detail in both barley and maize [2,38,39]. In this case the substrate for the exo-β-glucanases would be the (1 → 3;1 → 4)-β-glucan of the coleoptile cell wall, which decreases significantly during coleoptile growth [38,40] in a process that has been linked to wall 'loosening' and auxin-induced cell elongation [5,24]. How a β-glucan exohydrolase might allow this 'loosening' through partial exohydrolysis of cell wall (1 → 3;1 → 4)-β-glucans is not clear. The decrease in (1 → 3;1 → 4)-β-glucans in the walls of barley coleoptiles is unlikely to be mediated by (1 → 3;1 → 4)-β-glucan endohydrolases of the EC 3.2.1.73 group because neither the (1 → 3;1 → 4)-β-glucanases of this group nor their corresponding mRNAs are detectable in coleoptiles, even after auxin treatment [41,42]. However, a β-glucan endohydrolase which only partially hydrolyses polymeric (1 → 3;1 → 4)-β-glucan and has a specificity which is clearly distinguishable from the EC 3.2.1.73 enzymes has been detected in the walls of elongating maize coleoptiles [24]. The 'specialized' (1 → 3;1 → 4)-β-glucan endo-

hydrolase might act in concert with β-glucan exohydrolases to remove (1 → 3;1 → 4)-β-glucans from walls of the elongating cells.

A third possible role for β-glucan exohydrolases in the germinating grain or in young barley seedlings could be to hydrolyse (1 → 3)-β-glucosyl linkages in (1 → 3)- and (1 → 3;1 → 6)-β-glucans of the type commonly found in fungal cell walls (Table 3) [43]. Thus, (1 → 3)-β-glucan endohydrolases are classified with the pathogenesis-related (PR) group of proteins that are induced in plants in response to pathogen attack [44]. The (1 → 3)-β-glucan endohydrolases are believed to offer germinating barley grain some degree of protection against fungal invasion because they can hydrolyse fungal cell wall (1 → 3)- and (1 → 3;1 → 6)-β-glucans [45], and it is possible that β-glucan exohydrolases of the type characterized here could act in synergy with the endohydrolases to degrade walls of invading fungi. Indeed, reductometric assays commonly used to measure (1 → 3)-β-glucan endohydrolases in studies on pathogenesis-related proteins in plants will not discriminate between endo- or exo-hydrolases. With the current focus on (1 → 3)-β-glucan endohydrolases, the exohydrolases could therefore go undetected. Barley (1 → 3)-β-glucan endohydrolases of the PR protein class hydrolyse highly branched or substituted (1 → 3;1 → 6)-β-glucans of fungal cell-wall origin slowly, if at all [8]. The low rate of hydrolysis has been attributed to the relatively long substrate binding site, which accommodates eight glucosyl residues of the substrate and in which there is little room for branching or substitution at the O-6 positions of glucosyl residues [46,47]. This, in turn, has led to some questions as to the effectiveness of (1 → 3)-β-glucan endohydrolases in hydrolysing fungal β-glucans. Comparison of kinetic parameters and relative rates of hydrolysis of various substrates reveals that while the catalytic rate constants (k_{cat}) measured against the laminaran from *L. digitata* are generally higher for the endohydrolases (Table 3), the exohydrolases are relatively more active than the endohydrolases as the degree of substitution or branching of (1 → 3)-β-glucans increases (Tables 1 and 3). Based on these comparisons, it seems quite possible that the β-glucan exohydrolases could participate in the plant's defence strategies against invading fungal pathogens. Furthermore, the enzymes' ability to catalyse transglycosylation reactions (Fig. 4) raises the possibility that they participate in the synthesis of branched (1 → 3;1 → 6)-β-oligoglucosides of the type that are known to elicit responses such as phytoalexin or PR protein production in plants during microbial attack [48]. Alternatively, the β-glucan exohydrolases could trim larger oligosaccharides during the generation of such elicitors or could remove them after elicitation of the defence response. It should now be possible to use gene-specific probes for β-glucan exohydrolase isoenzymes ExoI [1] and ExoII (A.J. Harvey and G.B. Fincher, unpublished data) to locate transcription sites for the genes and to monitor transcriptional activities of the genes, particularly in relation to the expression of the PR (1 → 3)-β-glucan endohydrolases, during pathogen attack.

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