Purification and properties of three (1 → 3)- β -D-glucanase isoenzymes from young leaves of barley (*Hordeum vulgare*)

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Three (1 → 3)- β -D-glucan glucanohydrolase (EC 3.2.1.39) isoenzymes GI, GII and GIII were purified from young leaves of barley (*Hordeum vulgare*) using $(\text{NH}_4)_2\text{SO}_4$ fractional precipitation, ion-exchange chromatography, chromatofocusing and gel-filtration chromatography. The three (1 → 3)- β -D-glucanases are monomeric proteins of apparent M_r 32 000 with pI values in the range 8.8–10.3. N-terminal amino-acid-sequence analyses confirmed that the three isoenzymes represent the products of separate genes. Isoenzymes GI and GII are less stable at elevated temperatures and are active over a narrower pH range than is isoenzyme GIII, which is a glycoprotein containing 20–30 mol of hexose equivalents/mol of enzyme. The preferred substrate for the enzymes is laminarin from the brown alga *Laminaria digitata*, an essentially linear (1 → 3)- β -D-glucan with a low degree of glucosyl substitution at 0–6 and a degree of

polymerization of approx. 25. The three enzymes are classified as endohydrolases, because they yield (1 → 3)- β -D-oligo-glucosides with degrees of polymerization of 3–8 in the initial stages of hydrolysis of laminarin. Kinetic analyses indicate apparent K_m values in the range 172–208 μM , k_{cat} constants of 36–155 s^{-1} and pH optima of 4.8. Substrate specificity studies show that the three isoenzymes hydrolyse substituted (1 → 3)- β -D-glucans with degrees of polymerization of 25–31 and various high- M_r , substituted and side-branched fungal (1 → 3;1 → 6)- β -D-glucans. However, the isoenzymes differ in their rates of hydrolysis of a (1 → 3;1 → 6)- β -D-glucan from baker's yeast and their specific activities against laminarin vary significantly. The enzymes do not hydrolyse (1 → 3;1 → 4)- β -D-glucans, (1 → 6)- β -D-glucan, CM-cellulose, insoluble (1 → 3)- β -D-glucans or aryl β -D-glycosides.

INTRODUCTION

The (1 → 3)- β -D-glucan endohydrolases (EC 3.2.1.39) of barley (*Hordeum vulgare*) are encoded by a family of at least six genes (Xu et al., 1992). Several (1 → 3)- β -D-glucanases have been purified from germinated barley grain (Manners and Wilson, 1974; Ballance and Svendsen, 1988; Høj et al., 1988, 1989; Skriver et al., 1991), in which relatively high levels of the enzymes develop as germination proceeds (Manners and Marshall, 1969; Ballance et al., 1976). The enzymes are also detected in young vegetative tissues of barley (Xu et al., 1992) and in embryogenic cell cultures (Kragh et al., 1991). Despite the high constitutive levels of (1 → 3)- β -D-glucanases in germinated grain, their functional significance is not clear. One possible role might be in the hydrolysis of (1 → 3)- β -D-glucans that are found in isolated deposits in the sub-aleurone regions of the starchy endosperm (Fulcher et al., 1977; MacGregor et al., 1989), but the amounts of these deposits appear to be too low to explain the high (1 → 3)- β -D-glucanase activities (Fincher, 1992). It has also been suggested that the (1 → 3)- β -D-glucanases might partially hydrolyse the (1 → 3;1 → 4)- β -D-glucans that constitute the major cell-wall polysaccharide of the starchy endosperm (Ballance and Manners, 1978).

Another possible function could be in the protection of the germinating grain against pathogen attack. Plant (1 → 3)- β -D-glucanases are classified among the 'pathogenesis-related' proteins that are expressed in response to microbial invasion (Boller, 1987). The (1 → 3)- β -D-glucanases may function synergistically with other hydrolases, such as chitinases and proteinases, to disrupt the structural integrity of the hyphal tip, thereby causing lysis of the fungal cells (Mauch et al., 1988; Skriver et al., 1991). Alternatively, the (1 → 3)- β -D-glucanases might release oligosaccharides from (1 → 3)- β -D-glucan substrates of either plant or pathogen origin. Oligosaccharides of this type may serve as

chemical signals leading to the activation of other defence responses (Lamb et al., 1989; Ham et al., 1991; Ryan and Farmer, 1991). Although the (1 → 3)- β -D-glucanases in the germinating barley grain are expressed at high levels in the absence of microbial attack (Høj et al., 1988, 1989), they may nevertheless play a pre-emptive role in the protection of the grain at a time when high concentrations of sugars, amino acids and other low- M_r metabolites in the starchy endosperm render the grain particularly susceptible to pathogen invasion (Fincher, 1989). Indeed, the (1 → 3)- β -D-glucanases may represent just one aspect of a much wider strategy to protect the grain against microbial attack (Fincher, 1992).

The detection of at least six (1 → 3)- β -D-glucanase genes in barley (Xu et al., 1992) also raises questions regarding the functional significance for multiple isoenzymes. It could be argued that the occurrence of multiple genes would permit separate genes to be independently regulated in different tissues, both during normal development and in times of stress or pathogen attack, or it could allow different isoenzymes to be targeted to different cellular compartments. Additionally, the multiple (1 → 3)- β -D-glucanase isoforms might exhibit subtle differences in their substrate specificities and action patterns, in particular with respect to their ability to degrade the various (1 → 3;1 → 6)- β -D-glucans which are major cell-wall components of many fungal pathogens (Wessels and Sietsma, 1981). Thus multiple (1 → 3)- β -D-glucanase isoforms capable of hydrolysing a variety of substituted or branched (1 → 3)- β -D-glucans could provide protection against a broad spectrum of potentially pathogenic fungi. To investigate this possibility, we have purified three of the barley (1 → 3)- β -D-glucanases from young leaves and have compared their substrate specificities, kinetic properties and action patterns using a variety of low- and high- M_r linear, substituted and side-branched (1 → 3)- and (1 → 3;1 → 6)- β -D-glucans.

Abbreviations used: DP, degree of polymerization; DS, degree of substitution; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol.

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EXPERIMENTAL

Materials

Laminarin (from *Laminaria digitata*), glucan (from baker's yeast), lichenin (from *Usnea barbata* and *Cetraria islandica*), BSA, dithiothreitol (DTT), phenylmethanesulphonyl fluoride (PMSF), orcinol, cytochrome *c*, Coomassie Brilliant Blue R-250, chloramphenicol, neomycin, nystatin, penicillin G, 4-nitrophenyl β -D-glucoside, 4-nitrophenyl β -D-cellobioside and 4-nitrophenyl β -D-lactoside were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CM-Sephacrose CL-6B, PBE 118 resin, Pharmalyte 8-10.5 and M_r and pI marker proteins were from Pharmacia-LKB (Uppsala, Sweden). DEAE-Cellulose (DE 52) was from Whatman (Maidstone, Kent, U.K.) and (1 $\rightarrow$ 3)- β -D-linked oligoglucoside of DP 2-7 were from Seikagaku Kogyo Co. (Tokyo, Japan). Laminarin (from *Laminaria hyperborea* and *Eisenia bicyclis*) and curdlan (from *Alcaligenes faecalis*) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan), pachyman (from *Poria cocos*) and Miracloth were from Calbiochem (San Diego, CA, U.S.A.), barley (1 $\rightarrow$ 3; 1 $\rightarrow$ 4)- β -D-glucan (from *Hordeum vulgare*) was from Biocon Biochemicals (Kilnagleary, Ireland), CM-cellulose (DS 0.54) from Imperial Chemical Industries (Dingley, Australia), Kieselgel 60 thin-layer plates from Merck (Darmstadt, Germany), YM-5 membrane from Amicon Corporation (Danvers, MA, U.S.A.) and Bio-Gel P-60 (100–200 mesh) from Bio-Rad (Richmond, CA, U.S.A.). Reduced pneumococcal RSIII glucan (from *Streptococcus pneumoniae*), pustulan (from *Umbilicaria popullosa*) and CM-pachyman (DS 0.29) were generously provided by Professor B. A. Stone of this University. Insoluble yeast glucan (from *Saccharomyces cerevisiae*) and soluble CM-yeast glucan (DS 0.42) were provided by Dr. J. Sandula (Institute of Chemistry, Bratislava, Czechoslovakia) and schizophyllan M-2 was from Dr. S. Kitamura (Kyoto Prefectural University, Kyoto, Japan).

Enzyme extraction

Barley (*Hordeum vulgare*, L. cv. Clipper) was surface-sterilized in 0.2% (w/v) AgNO₃ for 30 min, washed successively with sterile distilled water, 0.5 M NaCl and sterile distilled water and steeped for 24 h in sterile distilled water containing chloramphenicol and neomycin (100 μ g/ml) and penicillin G and nystatin (100 units/ml) (Hoy et al., 1980). Grains (3 kg dry weight) maintained at approx. 40% (v/v) moisture content in the antibiotic solution were germinated in the dark at 15 °C for up to 14 days. No bacterial or fungal contamination was observed during this period. The shoots (8–12 cm long) and grains, which contained attached rootlets, were homogenized separately in a Waring Blendor for 10 min (shoots) and 5 min (grain) in 1.5 vol. of 0.1 M sodium acetate buffer, pH 5, containing 10 mM EDTA, 10 mM NaN₃, 3 mM 2-mercaptoethanol and 3 mM PMSF, at 4 °C. All subsequent operations were performed at 4 °C. The homogenates were held at 4 °C for 45 min to extract enzymes, and insoluble material was removed by centrifugation (4400 g, 20 min), followed by filtration through Miracloth. The supernatants were fractionated by precipitation with (NH₄)₂SO₄. Precipitates were collected by centrifugation (4400 g, 20 min), redissolved in the extraction buffer and the crude extracts stored at –70 °C prior to further purification.

Enzyme assays

(1 $\rightarrow$ 3)- β -D-Glucanase activity was determined reductometrically by monitoring the increase in reducing sugars (Nelson, 1944; Somogyi, 1952; Paleg et al., 1959) released in a 0.2% (w/v)

solution of *Laminaria digitata* laminarin in 0.1 M sodium acetate buffer, pH 5, at 37 °C. (1 $\rightarrow$ 3; 1 $\rightarrow$ 4)- β -D-Glucanase activity was measured reductometrically or viscometrically, using barley (1 $\rightarrow$ 3; 1 $\rightarrow$ 4)- β -D-glucan as a substrate (Woodward and Fincher, 1982). One unit of enzyme activity is defined as 1 μ mol of glucose equivalents released/min. One unit corresponds to 16.67 nkat.

Protein and carbohydrate determination

Protein content was measured by the Coomassie Brilliant Blue reagent (Bradford, 1976), using BSA as a standard. Protein in column eluates was estimated by absorbance at 280 nm. Carbohydrate content of the purified enzyme preparations was determined by the phenol/H₂SO₄ acid method (Dubois et al., 1956) using D-mannose or D-glucose standards which contained BSA at concentrations comparable with the protein concentration in the test solutions.

Enzymic properties

The pH optima of the purified (1 $\rightarrow$ 3)- β -D-glucanases, designated isoenzymes GI, GII and GIII, were determined over the pH range 3.5–6 using *L. digitata* laminarin in 0.1 M sodium acetate buffers containing 160 μ g/ml BSA and 0.2, 0.25 and 0.73 pkat of the purified isoenzymes GI, GII and GIII respectively. Heat-stabilities were determined by measuring residual activity after incubation of 0.2, 0.25 and 0.73 pkat of the purified isoenzymes GI, GII and GIII respectively in 0.1 M sodium acetate buffer, pH 4.8, with or without 160 μ g/ml BSA at various temperatures for 15 min.

Substrate specificities and action patterns

Soluble and insoluble β -D-glucans, differing in their linkage types and in the ratio of their linkage types (Table 1), were prepared at a final concentration of 0.1% (w/v) in 0.1 M sodium acetate buffer, pH 4.8, containing 160 μ g/ml BSA. The substrates were incubated with 0.2, 0.25 and 0.73 pkat of the purified isoenzymes GI, GII and GIII respectively for activity measurements.

Action patterns were determined by t.l.c. of the products released after 2.4, 2 and 5.8 pkat of the purified isoenzymes GI, GII and GIII respectively were incubated with *L. digitata* laminarin in 10 mM sodium acetate buffer, pH 4.8, for 1, 30 and 120 min. Final hydrolysis products were examined in the same way, except that the same amounts of fresh enzymes were added after 24 h and the reactions were allowed to proceed for a total of 48 h. The hydrolysates were applied to Kieselgel 60 thin-layer plates, developed in ethyl acetate/acetic acid/water (2:1:1, by vol.) and the reducing sugar products were detected with the orcinol reagent (Farkas and Maclachlan, 1988).

Kinetic parameters

Kinetic parameters were determined at 37 °C by incubating 0.2, 0.25 and 0.73 pkat of the purified isoenzymes GI, GII and GIII respectively in 0.1 M sodium acetate buffer, pH 4.8, containing 160 μ g/ml BSA and *L. digitata* laminarin over the concentration range 0.2–1 mg/ml. Kinetic data were processed with a non-linear-regression-analysis program based on Michaelis–Menten kinetics (Leatherbarrow, 1987).

Gel electrophoresis and isoelectric focusing

Approx. 1 μ g of purified enzymes were analysed by SDS/PAGE (Laemmli, 1970) on 12.5% gels (total acrylamide

Table 1 Structures of β-D-glucan substrates

References: ¹Harada et al. (1968); ²Warsi and Whelan (1957); ³Bull and Chesters (1963); ⁴Usui et al. (1979); ⁵Kogan and Alföldi (1988); ⁶Tabata et al. (1981); ⁷Lindberg and McPherson (1954); ⁸Anderson and Stone (1975); ⁹Perlin and Suzuki (1962); ¹⁰Woodward et al. (1983).

β-D-Glucan (source)	Main type linkages and ratio of linkage type	Structure	Reference
Curdian (<i>Alcaligenes faecalis</i>) and pachyman (<i>Poria cocos</i>)	(1→3)-β	[Glc1→3Glc] _n	1 2
Laminarin (<i>Laminaria digitata</i> , <i>L. hyperborea</i>)	(1→3; 1→6)-β 7:1	$\begin{array}{c} \text{Glc} \\ \\ \text{1} \rightarrow 6 \\ \text{[-(Glc1} \rightarrow 3\text{Glc-)}_3\text{1} \rightarrow 3\text{Glc-]}_n \end{array}$	3
Laminarin (<i>Eisenia bicyclis</i>)	(1→3; 1→6)-β 3:2	$\begin{array}{c} \text{Glc} \\ \\ \text{1} \rightarrow 6 \\ \text{[-Glc1} \rightarrow 3\text{Glc} \\ \rightarrow 6 \\ \text{Glc1} \rightarrow 3\text{Glc1} \rightarrow 3\text{Glc-]}_n \end{array}$	4
Yeast glucan (<i>Saccharomyces cerevisiae</i>)	(1→3; 1→6)-β 4:1	$\begin{array}{c} \text{Glc1} \rightarrow 3\text{Glc)}_{1,2} \\ \rightarrow 6 \\ \text{[-(Glc1} \rightarrow 3\text{Glc-)}_2\text{]}_n \end{array}$	5
Schizophyllan (<i>Schizophyllum commune</i>)	(1→3; 1→6)-β 3:1	$\begin{array}{c} \text{Glc} \\ \\ \text{1} \rightarrow 6 \\ \text{[-Glc1} \rightarrow 3\text{Glc1} \rightarrow 3\text{Glc-]}_n \end{array}$	6
Pustulan (<i>Umbilicaria pustulata</i>)	(1→6)-β	[Glc1→6Glc] _n	7
Reduced pneumococcal glucan RSIII (<i>Streptococcus pneumoniae</i>)	(1→3; 1→4)-β 1:1	[Glc1→3Glc1→4Glc] _n	8
Lichenin (<i>Usnea barbata</i> , <i>Cetraria islandica</i>)	(1→4; 1→3)-β 2:1	[Glc1→4Glc1→4Glc1→3Glc] _n	9
Barley glucan (<i>Hordeum vulgare</i>)	(1→4; 1→3)-β 2.3–2.7:1		10

12.17%; cross-linker 0.33%). M_r marker proteins were phosphorylase *b* (M_r 94000), BSA (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20000) and α-lactalbumin (14400). The gels were stained with Coomassie Brilliant Blue R-250. Analytical isoelectric focusing was performed on 3.75% (w/v) polyacrylamide gels containing 2% (w/v) Pharmalyte over the pH range 8–10.5, using a Pharmacia FBE-3000 flat-bed apparatus. Gels containing approx. 2 μg of purified enzyme were fixed and stained with Coomassie Brilliant Blue R-250 (Vesterberg, 1972). The apparent pI values were calculated by reference to standard marker proteins with pI values in the range 3.5–9.3 (Pharmacia-LKB). Because of the high pI values of the barley (1 → 3)-β-D-glucanases, cytochrome *c* (pI 10.25) was also included with the reference proteins during isoelectric focusing.

Amino-acid-sequence analysis

Automated amino acid sequence analysis was performed in an Applied Biosystems 470A gas-liquid phase sequencer using trifluoroacetic acid chemistry (Hewick et al., 1981). Phenylthiohydantoin derivatives were identified by h.p.l.c. on a Zorbax

C₈ reverse-phase column (du Pont, Wilmington, DE, U.S.A.) eluted with a discontinuous acetonitrile gradient (Zimmerman et al., 1977).

RESULTS

Purification of (1 → 3)-β-D-glucanases from young barley leaves

Leaves and residual grain (with attached rootlets) were excised from 14-day-old seedlings, separately homogenized and precipitated with (NH₄)₂SO₄. Yields and activities of (1 → 3)-β-D-glucanases and (1 → 3; 1 → 4)-β-D-glucanases in the (NH₄)₂SO₄ fractions are compared in Table 2. The majority of both enzymes (63–78%) was precipitated by 20–40%-satd. (NH₄)₂SO₄. The total yield of (1 → 3)-β-D-glucanases found in the leaf and grain/root extracts exceeded that of the (1 → 3; 1 → 4)-β-D-glucanases by 8.5- and 4.4-fold respectively (Table 2).

Although levels of (1 → 3)-β-D-glucanase were highest in the grain/root extract (Table 2), it was subsequently found that the three (1 → 3)-β-D-glucanase isoenzymes GI, GII and GIII could be purified from the leaf extract. Only isoenzymes GI and GII were present in the extracts of grains and roots. The leaf extract was therefore used for the purification procedure shown in

Table 2 Yields of (1 → 3)- β -D-glucanase and (1 → 3;1 → 4)- β -D-glucanase activities in $(\text{NH}_4)_2\text{SO}_4$ fractions of barley extracts

The barley grain (3 kg dry weight) was germinated for 14 days at 15 °C in the dark; grains with roots were separated from leaves, homogenized, and soluble extracts were fractionally precipitated with $(\text{NH}_4)_2\text{SO}_4$.

Fraction [% saturation of $(\text{NH}_4)_2\text{SO}_4$]	Enzyme yields (Units)			
	(1 → 3)- β -D-Glucanase		(1 → 3;1 → 4)- β -D-Glucanase	
	Grain/roots	Leaves	Grain/roots	Leaves
0–20	141	6	28	0
20–40	8075	1089	1785	106
40–80	2400	306	211	32
80–100	1920	0	800	26
Total	12536	1401	2824	164

Scheme 1 Summary of procedures used to purify (1 → 3)- β -D-glucanase isoenzymes GI, GII and GIII from young leaves of barley

Scheme 1. Yields, specific activities, recoveries and purification factors of the three (1 → 3)- β -D-glucanase isoenzymes are shown in Table 3.

DEAE-cellulose chromatography

The desalted 20–40% $(\text{NH}_4)_2\text{SO}_4$ fraction from the leaf extract was applied to a column (4.5 cm × 18 cm) of DEAE-cellulose equilibrated in 20 mM Tris/20 mM HCl, pH 8. Approx. 68% of the total protein in the fraction was retained on the column, but 96% of the (1 → 3)- β -D-glucanase activity was eluted with the buffer (Table 3).

CM-Sepharose chromatography

The unbound fractions from the DEAE-cellulose column were

Table 3 Enzyme yields during purification of (1 → 3)- β -D-glucanase isoenzymes GI, GII and GIII from young barley leaves

Purification step	Yield		Specific activity (units/mg of protein)	Recovery† (%)	Purification factor‡
	Protein (mg)	Activity* (units)			
Crude homogenate	1406	1401	1	100	
20–40% Satd. $(\text{NH}_4)_2\text{SO}_4$	748	1089	1.5	77.7	
DEAE-cellulose	236	1048	4.4	74.8	
Isoenzyme GI					
CM-Sepharose (peak 2)	73.7	752	10.2	53.7	
PBE 118 (peak B)	1.3	185.8	142.9	13.3	
Bio-Gel P-60	0.56	108.4	193.6	7.7	
Bio-Gel P-60	0.17	47.8	281.2	3.4	281
Isoenzyme GII					
CM-Sepharose	73.7	752	10.2	53.7	
PBE 118 (peak A)	3.1	434	140	31	
Bio-Gel P-60	1.3	283.6	218.2	20.2	
Bio-Gel P-60	0.21	50.5	240.5	3.6	241
Isoenzyme GIII					
CM-Sepharose	15.5	158.4	10.2	11.3	
PBE 118	6.1	100.8	16.5	7.2	
Bio-Gel P-60	1.53	60.8	40.5	4.3	41

* As enzyme units recovered.

† Expressed in % of enzyme units.

‡ Calculated on the basis of specific activity (units/mg of protein).

Figure 1 Ion-exchange chromatography on CM-Sepharose

The fraction of leaf extracts unabsorbed on DEAE-cellulose (215 ml, 370 units) was applied to CM-Sepharose and, after washing the column to remove unbound proteins, bound proteins were eluted with a 2 litre linear gradient of 0–0.5 M NaCl (—). Fractions (10 ml) were assayed for (1 → 3)- β -D-glucanase activity (O) and protein (□).

applied to a column (3.2 cm × 30 cm) of CM-Sepharose equilibrated in 0.1 M sodium acetate buffer, pH 5. After unabsorbed proteins were eluted, a linear gradient of 0–0.5 M NaCl in the same buffer (2 litres) was applied to the column. Two peaks of activity, designated 1 and 2, were eluted at approx. 0.075 and 0.125 M NaCl respectively (Figure 1). At this stage the fractions were essentially free of (1 → 3;1 → 4)- β -D-glucanase. The frac-

Figure 2 Chromatofocusing

Pooled fractions from the CM-Sepharose column were adjusted to pH 11, applied to the PBE 118 column and eluted with a linear gradient of Pharmalyte 8–10.5 adjusted to pH 8. Fractions (2 ml) were assayed for (1 → 3)- β -D-glucanase activity (○) and pH (□). (a) Peak 1 from CM-Sepharose (Figure 1), which contained (1 → 3)- β -D-glucanase isoenzyme GIII. (b) Peak 2 from CM-Sepharose (Figure 1) was separated into peaks which contained (1 → 3)- β -D-glucanase isoenzymes GII and GI.

tions containing (1 → 3)- β -D-glucanase were pooled and concentrated by ultrafiltration on a YM-5 membrane.

Chromatofocusing and gel-filtration chromatography

During ultrafiltration, the desalted and concentrated CM-Sepharose fractions (Figure 1) were equilibrated in 25 mM triethylamine buffer, adjusted to pH 11 with HCl, and applied to a column (0.9 cm × 28 cm) of PBE 118 resin equilibrated in the same buffer. The column was eluted at a flow rate of 40 cm · h⁻¹ with Pharmalyte 8–10.5, which had been diluted 45-fold with water and adjusted to pH 8. The chromatofocusing was monitored by measuring the pH of the eluted fractions. In the case of peak 1 from CM-Sepharose chromatography, a single peak of (1 → 3)- β -D-glucanase was eluted at pH 9.9 (Figure 2a), but contaminating proteins were still present. The active fractions from the PBE 118 column, designated (1 → 3)- β -D-glucanase isoenzyme GIII, were therefore concentrated by ultrafiltration and applied to a column (1.6 cm × 100 cm) of Bio-Gel P-60 equilibrated in 50 mM sodium acetate buffer, pH 5, containing 0.2 M NaCl. Typically, size-exclusion chromatography of isoenzyme GIII resulted in the elution of a single protein peak which co-chromatographed with (1 → 3)- β -D-glucanase activity (results not shown). The active fractions were concentrated by ultrafiltration to yield 1.53 mg of isoenzyme GIII. The final purification factor was 41 (Table 3).

Chromatofocusing of peak 2 from the CM-Sepharose column (Figure 1) resolved two components which were eluted at pH 10.3 and 9.8 (Figure 2b). These components corresponded to (1 → 3)- β -D-glucanase isoenzymes GII and GI respectively. The fractions were further purified by gel-filtration chromatography on Bio-Gel P-60 (Table 3), using the protocol described for isoenzyme

Figure 3 SDS/PAGE of purified (1 → 3)- β -D-glucanase isoenzymes GI (0.5 μ g), GII (0.5 μ g) and GIII (1 μ g)

M_r protein standards are indicated.

GIII, except that the column was equilibrated and eluted with 50 mM sodium acetate buffer, pH 5, containing 0.2 M NaCl and 25 mM DTT. The high salt concentration and DTT were found to be necessary for the separation of contaminating proteins which appeared to be bound to both isoenzymes GII and GI. Amino-acid-sequence analysis of the purified isoenzymes confirmed that the component eluted at pH 10.3 contained isoenzyme GII (Høj et al., 1989) and the peak eluted at pH 9.8 contained predominantly (1 → 3)- β -D-glucanase isoenzyme GI (Høj et al., 1988). The final yields of isoenzymes GII and GI were 0.21 and 0.17 mg of protein, and the purification factors were 241- and 281-fold respectively (Table 3).

Each of the purified (1 → 3)- β -D-glucanase isoenzymes could be stored at -15 °C or -70 °C in 50 mM sodium acetate buffer, pH 5.0, for at least 12 months without detectable loss of activity.

Purity of the enzyme preparations

SDS/PAGE under reducing conditions (Figure 3) showed that the purified isoenzyme GI, GII and GIII preparations contained single protein bands with apparent M_r values of about 33 kDa. The N-terminal amino acid sequences of the three isoenzymes are shown below:

GI	T	I	G	V	C	Y	G	V	V	A	N	N	L
GII	I	G	V	C	Y	G	G	I	G	N	N	L	
GIII	I	G	V	C	N	G	V	L	G	N	N	L	

The absence of secondary sequences in the preparations of isoenzymes GII and GIII confirmed their purity. The isoenzyme GI preparation contained a secondary sequence corresponding to isoenzyme GII, which accounted for approx. 10% of the preparation.

Properties of (1 → 3)- β -D-glucanase isoenzymes GI, GII and GIII

pH optima

The purified (1 → 3)- β -D-glucanases GI, GII and GIII each had a pH optimum of pH 4.8 (Figure 4). However, isoenzyme GIII maintained up to 66% of its maximum activity at pH 6, whereas isoenzymes GI and GII retained only between 17–27% of their maximum activity at this pH value. Isoenzyme GI retained less activity than the other two isoenzymes at pH values below 5.0 (Figure 4).

Figure 4 Effect of pH on the activity of purified (1 → 3)- β -D-glucanase isoenzymes GI (○), GII (□) and GIII (△), measured in 0.1 M sodium acetate buffer (containing 160 μ g/ml BSA) at the indicated pH

Heat-stability

Isoenzymes GI and GII were inactivated quite rapidly at temperatures above 10 °C in the absence of BSA, whereas isoenzyme GIII retained full activity after 15 min incubation at 40 °C (Figure 5a). The addition of 160 μ g/ml BSA stabilized all three isoforms. Thus at 50 °C they retained 80–100% of their maximum activities, although isoenzyme GIII was again slightly more stable than the other isoenzymes (Figure 5b).

Figure 5 Temperature-stability of purified (1 → 3)- β -D-glucanases

Residual activities of isoenzymes GI (○), GII (□) and GIII (△) were determined in the absence (a) or presence (b) of 160 μ g/ml BSA in 0.1 M sodium acetate buffer, pH 4.8, after 15 min incubation at the indicated temperatures.

pI values

Partially purified isoenzymes GI, GII and GIII were eluted from the chromatofocusing column at pH values 9.8, 10.3 and 9.9 respectively (Figure 2, Table 4). These values correspond closely to the pI values of the final purified isoenzymes GII and GIII determined by isoelectric focusing (Table 4). However, in the case of isoenzyme GI a difference of about 1 unit was observed between the values determined by chromatofocusing and isoelectric focusing (Table 4). This presumably results from a protein contaminant that was present at the chromatofocusing step, but was subsequently removed from the final preparation by gel-filtration chromatography.

Carbohydrate content

No carbohydrate was detected in isoenzymes GI and GII (Table 4). However, isoenzyme GIII contained 12–17% (w/v) carbohydrate, depending on whether D-mannose or D-glucose was used as a standard in the phenol/ H_2SO_4 assays. On this basis, it can be calculated that isoenzyme GIII contains 20–30 mol of hexose equivalents/mol enzyme (Table 4). Although the structure of the carbohydrate has not yet been studied in detail, the number of steps in the purification procedure and the difference between isoenzymes would suggest that the carbohydrate is covalently associated with the enzyme rather than a contaminant that co-purifies only with isoenzyme GIII.

Kinetic properties

The purified (1 → 3)- β -D-glucanase isoenzymes GI, GII and GIII obey linear Michaelis-Menten kinetics at *L. digitata* laminarin concentrations of 0.2–1.0 mg/ml. Kinetic analysis revealed apparent K_m values of 0.7–0.8 mg/ml (Table 4) which, based on a degree of polymerization (DP) of 25, correspond to substrate concentrations of 170–210 μ M. The catalytic rate constants k_{cat} of isoenzymes GI and GII were similar (155 and 130 s^{-1}), but the k_{cat} of (1 → 3)- β -D-glucanase isoenzyme GIII (36 s^{-1}) was substantially lower, in agreement with the 6–7-fold lower specific activity of this isoform (Table 4, Table 3). The specificity factor k_{cat}/K_m reflected a 3–4.5-fold lower efficiency of laminarin hydrolysis by isoenzyme GIII compared with the other two isoforms.

Table 4 Kinetic parameters, pI values and carbohydrate contents of purified (1 → 3)- β -D-glucanase isoenzymes GI, GII and GIII

Property	Isoenzyme ...	GI	II	III
K_m (mg/ml)*		0.7	0.8	0.7
K_m (μ M)*		172	208	180
k_{cat} (s^{-1})†		155	130	36
k_{cat}/K_m ($10^{-5} \times s^{-1} \cdot M^{-1}$)‡		9	6	2
pI				
Chromatofocusing		9.8	10.3	9.9
Isoelectric focusing		8.8	≥ 10.3	9.8
Carbohydrate content§		0	0	20 or 30†

* Using *L. digitata* laminarin.

† Catalytic rate constant or catalytic-centre activity.

‡ Calculations based on the molar glucose content.

§ As mol of D-mannose or D-glucose/mol of enzyme.

Table 5 Relative rates of hydrolysis of (1 → 3;1 → 6)- β -D-glucans by purified (1 → 3)- β -D-glucanase isoenzymes GI, GII and GIII

No activity was detected towards curdlan, pachyman, CM-pachyman, schizophyllan, pustulan, reduced pneumococcal RSIII glucan, lichenins, barley glucan, CM-cellulose, and aryl β -D-glycosides. The relative rates of hydrolysis of (1 → 3)- β -D-glucanase isoenzymes GI, GII and GIII on *L. digitata* laminarin were arbitrarily set at 100% and correspond to 201, 245 and 59 units/mg of protein respectively.

Substrate	Relative rate (%)		
	GI	II	GIII
Laminarin			
<i>Laminaria digitata</i>	100	100	100
<i>L. hyperborea</i>	44.4	31.6	48.1
<i>Eisenia bicyclis</i>	0.4	0.2	0.5
(1 → 3; 1 → 6)- β -D-Glucan (<i>Saccharomyces cerevisiae</i>)	0.6	0.2	0.5
CM-(1 → 3; 1 → 6)- β -D-glucan (<i>S. cerevisiae</i>)	2.9	0.2	2.2
Glucan (baker's yeast)	18.5	12.2	43.7

Substrate specificities

Six (1 → 3;1 → 6)- β -D-glucans which differ in structural organization (Table 1) were hydrolysed by isoenzymes GI, GII and GIII (Table 5). Laminarin from *L. digitata* was hydrolysed at the highest rate, but *L. hyperborea* laminarin and a β -D-glucan from baker's yeast were also hydrolysed at significant rates (Table 5). In contrast, the insoluble (1 → 3;1 → 6)- β -D-glucan from *Saccharomyces cerevisiae* was degraded slowly by the three isoenzymes (Table 5). The enzymes had no activity against curdlan, pachyman, CM-pachyman, schizophyllan, pustulan, reduced pneumococcal RSIII glucan, lichenin, barley (1 → 3;1 → 4)- β -D-glucan, CM-cellulose, 4-nitrophenyl β -D-glucoside, 4-nitrophenyl β -D-cellobioside or 4-nitrophenyl β -D-lactoside.

Action patterns

The products released from *L. digitata* laminarin by the three isoenzymes during the first 2 h of hydrolysis are shown in Figure 6(a). The initial products were (1 → 3)- β -D-oligoglucosides of DP 3–8, which were seen after 1 min incubation with isoenzymes GII and GIII, and after 30 min with isoenzyme GI. Laminaribiose was detected after 30 min in each of the hydrolysates, and at 2 h, traces of glucose were also observed in the case of isoenzyme GIII. The major hydrolysis products after 48 h (Figure 6b) were laminaribiose and laminaritriose for isoenzymes GII and GIII, although higher oligosaccharides could be detected, whereas isoenzyme GI yielded predominantly (1 → 3)- β -D-oligoglucosides of DP greater than 4.

DISCUSSION

Three (1 → 3)- β -D-glucan endohydrolases, designated isoenzymes GI, GII and GIII, have been purified from extracts of young barley leaves by $(\text{NH}_4)_2\text{SO}_4$ precipitation, ion-exchange chromatography, chromatofocusing and gel-filtration chromatography (Scheme 1). Thus three individual (1 → 3)- β -D-glucanases can be isolated from a single tissue using essentially the same purification procedure. Although the proportion of inactive enzyme in any preparation cannot easily be determined, the similar time periods and chromatographic methods used in the purification, together with the use of identical starting tissue, suggest that comparisons of specific activities and other enzymic

Figure 6 T.L.C. of the hydrolysis products of laminarin by purified barley (1 → 3)- β -D-glucanases in the first 2 h of hydrolysis (a) and after 48 h (b)

Standards are glucose (G) and (1 → 3)- β -D-oligoglucosides of DP 2–7 (L2–L7).

properties of the three isoenzymes will be valid. Purification factors for the three isoenzymes were 281, 241 and 41 respectively. Examination of the purified enzymes by SDS/PAGE showed single protein bands of apparent M_r 33000 for each (Figure 3). Comparison of this value with an apparent M_r 31000 obtained for the three isoenzymes during size-exclusion chromatography (results not shown), indicates that they are monomeric proteins. Sequence analysis of N-terminal amino acids showed that isoenzymes GII and GIII were free of contaminating protein, but that the isoenzyme GI preparation also contained some isoenzyme GII.

The three barley (1 → 3)- β -D-glucanases are basic proteins with pI values of 8.8–10.3 (Table 4). The differences in their pI values allowed their resolution by ion-exchange chromatography and chromatofocusing (Figures 1 and 2). Relatively high levels of up to 20–30 mol of hexose equivalents/mol of enzyme are associated with isoenzyme GIII. In contrast, isoenzymes GI and GII carry little, if any, carbohydrate (Table 4). The isoenzymes also differ with respect to their stability at elevated temperatures; isoenzyme GIII is significantly more stable than isoenzymes GI and GII (Figure 5). Whether the higher thermostability of isoenzyme GIII is related to its higher degree of glycosylation is not yet clear.

Some of the properties of the three isoenzymes can be reconciled with those predicted from the corresponding cDNAs and genomic clones. Thus the N-terminal amino acid sequences determined here for the purified (1 → 3)- β -D-glucanases correspond exactly with the sequences deduced from the nucleotide sequences of cDNAs and genes for isoenzymes GI, GII and GIII (Høj et al., 1989; Wang et al., 1992; Xu et al., 1992). The overall positional identities for the complete primary structures of these isoenzymes are 60–78% (Xu et al., 1992). Similarly, the pI values deduced from the amino acid sequences (Xu et al., 1992) are close

to the values measured here by isoelectric focusing (Table 4), and the relatively high levels of carbohydrate associated with isoenzyme GIII (Table 4) are consistent with the detection of five potential N-glycosylation sites (Asn-Xaa-Ser/Thr) in the complete amino acid sequence of that isoenzyme; isoenzymes GI and GII have 1 and 0 N-glycosylation sites respectively (Xu et al., 1992). In view of the similarity in length of the three polypeptides (310 amino acids in isoenzyme GI, 306 amino acids in isoenzyme GII and 305 amino acids in isoenzyme GIII) (Xu et al., 1992) it is of interest that the 12–17% (w/w) carbohydrate on isoenzyme GIII has little apparent effect on its electrophoretic mobility (Figure 3). In contrast, approx. 4% (w/w) carbohydrate on barley (1 → 3;1 → 4)- β -D-glucanase isoenzyme EII causes a marked decrease in its mobility compared with the non-glycosylated isoenzyme EI (Woodward and Fincher, 1982; Doan and Fincher, 1992).

The barley (1 → 3)- β -D-glucanases share similar properties with those from *Nicotiana* (tobacco) species (Moore and Stone, 1972; Felix and Meins, 1986; Kauffman et al., 1987; Shinshi et al., 1988; Van den Bulcke et al., 1989), from *Phaseolus vulgaris* (French bean) (Edington et al., 1991) and from *Glycine max* (soybean) (Takeuchi et al., 1990). Sequence-identity values of 45–50% are usually observed between barley (1 → 3)- β -D-glucanases and those from dicotyledonous plants, although much higher values are obtained in several localized blocks of highly conserved amino acids, particularly near the C-termini of the enzymes (Xu et al., 1992). However, no correspondence between individual barley isoenzymes and individual isoenzymes from other plants has been detected (Xu et al., 1992). Other plant (1 → 3)- β -D-glucanases are similar in length to the barley enzymes, but both acidic and basic isoforms are found. Thus the relatively large *Nicotiana* family of (1 → 3)- β -D-glucanases was initially classified into acidic and basic isoenzymes, but more recently up to five distinct subfamilies have been distinguished (Ward et al., 1991). Based on the amino acid compositions deduced from cDNA and genomic clones, only one of the six putative barley (1 → 3)- β -D-glucanase isoenzymes would have an acidic pI (Xu et al., 1992), and because this isoenzyme has not yet been purified, its unequivocal identification as a (1 → 3)- β -D-glucanase remains to be established.

The substrate specificities and action patterns of the barley (1 → 3)- β -D-glucanase isoenzymes GI, GII and GIII have been compared using a range of linear, substituted and side-branched β -D-glucans (Tables 1 and 5). The preferred substrate for the three isoenzymes is *L. digitata* laminarin, an essentially linear, soluble, (1 → 3)- β -D-glucan with a low degree of glucosyl substitution at 0–6 (Bull and Chesters, 1963). The *L. hyperborea* laminarin is also hydrolysed by the three isoenzymes, but at a lower rate (Table 5). Similarly, the enzymes hydrolyse baker's-yeast glucan, which contains a mixture of an insoluble, high- M_r , side-branched (1 → 3;1 → 6)- β -D-glucan and a low- M_r (1 → 6)- β -D-glucan that also contains (1 → 3)-linked β -D-glucose residues (Manners et al., 1973a,b), at a significant rate (Table 5).

The barley (1 → 3)- β -D-glucanases do not hydrolyse highly substituted or highly side-branched (1 → 3)- β -D-glucans such as the *E. bicyclis* laminarin or the *S. cerevisiae* β -D-glucan to any great extent. They have no activity on insoluble (1 → 3)- β -D-glucans such as curdlan and pachyman, on (1 → 3;1 → 4)- β -D-glucans, on (1 → 6)- β -D-glucan, on CM-cellulose or on aryl β -D-glucosides, even after prolonged incubation (Table 5). Thus the three (1 → 3)- β -D-glucanases require soluble substrates with relatively long runs of contiguous (1 → 3)- β -D-glucosidic linkages where glucosyl residues are neither substituted nor branched. On this basis, the inability of the enzymes to hydrolyse CM-pachyman was somewhat surprising (cf. Moore and Stone,

1972), but we conclude that the DS of the polysaccharide preparation (0.29) was too high to permit a significant degree of hydrolysis. In the case of the baker's-yeast glucan preparation, the inability of the enzymes to hydrolyse the *S. cerevisiae* β -D-glucan, which consists mostly of the insoluble, high- M_r , side-branched (1 → 3;1 → 6)- β -D-glucan (Manners et al., 1973a), suggests that the barley enzymes are hydrolysing the low- M_r , soluble, component. Although the precise linkage disposition in this polysaccharide is not clear (Manners et al., 1973b), the result indicates that blocks of contiguous (1 → 3)- β -D-glucosyl residues are present. A potentially important difference between the isoenzymes is the apparent ability of isoenzyme GIII to hydrolyse the baker's-yeast glucan at a higher rate than isoenzyme GI and GII (Table 5). This suggests that isoenzyme GIII can tolerate a higher degree of side-branching than the other isoenzymes.

During the early stages of hydrolysis of *L. digitata* laminarin, (1 → 3)- β -D-oligoglucosides of DP 2–9 were released (Figure 6a). Visual examination of the hydrolysis products in Figure 6(a) suggests that there may be relatively less laminaritetraose in the hydrolysate of isoenzyme GII than in those of isoenzymes GI and GIII. After extended hydrolysis, laminaribiose and laminaritriose were the most abundant products (Figure 6b), although the presence of oligosaccharides of higher DP at this stage indicated that the enzymes hydrolyse the low-DP products slowly (Figure 6b). Preliminary experiments indicate that isoenzymes GI and GII also catalyse transglycosylation reactions in the presence of high concentrations of (1 → 3)- β -D-oligoglucosides (M. Hrmova and G. B. Fincher, unpublished work), and this characteristic requires further investigation. In any case, the action patterns of the three isoenzymes are similar and justify their classification as (1 → 3)- β -D-glucan glucanohydrolases (EC 3.2.1.39).

The pH optima (Figure 4) and kinetic properties of the three isoenzymes are also similar, although the k_{cat} of isoenzyme GIII is much lower than the values of isoenzymes GI and GII (Table 4). This is consistent with the lower specific activity of isoenzyme GIII (Table 3) and is also reflected in a lower specificity factor (k_{cat}/K_m) for isoenzyme GIII (Table 4). It should be noted that k_{cat}/K_m ratios of polysaccharide hydrolases are generally low, and because this parameter is a measure of the rate at which an enzyme encounters its substrate, the low values probably reflect low rates of diffusion of polysaccharides in aqueous solutions. It is also important to stress that kinetic parameters such as K_m and k_{cat} of polysaccharide hydrolases should be treated as approximate values only, because of the heterogeneity in size and fine structure inherent in many polysaccharide substrates and because hydrolysis products will often act as additional substrates.

In summary, barley (1 → 3)- β -D-glucanase isoenzyme GIII differs from isoenzymes GI and GII with respect to its lower specific activity, higher carbohydrate content, greater thermostability, its retention of activity over a broader range of pH, and its ability to hydrolyse (1 → 3)- β -D-glucans with a higher DS or side-branching. Whether these differences are of any functional significance in the hydrolysis of fungal-cell-wall β -D-glucans or (1 → 3)- β -D-glucans of plant origin remains to be demonstrated.

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