Carbohydrate Research, 106 (1982) 111–122
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

SUBSTRATE SPECIFICITIES AND KINETIC PROPERTIES OF TWO (1→3),(1→4)-β-D-GLUCAN ENDO-HYDROLASES FROM GERMINATING BARLEY (*Hordeum vulgare*)

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(Received October 14th, 1981; accepted for publication, December 15th, 1981)

ABSTRACT

Two β-D-glucan endo-hydrolases purified from germinating barley (*Hordeum vulgare*) hydrolyse (1→4)-β linkages in (1→3),(1→4)-β-D-glucans where the D-glucosyl residue is substituted at O-3, but will not hydrolyse (1→3)-β-D-glucans or (1→4)-β-D-glucans. Methylation analysis of hydrolytic products released from barley (1→3),(1→4)-β-D-glucan indicates that 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose are the major oligomers formed. The enzymes exhibit characteristic endo-hydrolase action-patterns on this substrate. Both enzymes can therefore be classified as (1→3),(1→4)-β-D-glucan 4-glucanohydrolases (EC 3.2.1.73). The reduced, pneumococcal polysaccharide RS III, which consists of alternating (1→3)- and (1→4)-linked β-D-glucosyl residues, is hydrolysed by the enzymes to release laminaribiose as a major oligomeric product. Although the kinetic parameters of the two enzymes are similar, one hydrolyses barley (1→3),(1→4)-β-D-glucan at a significantly higher rate than the other and is more stable at elevated temperatures.

INTRODUCTION

Two enzymes capable of rapidly reducing the viscosity of (1→3),(1→4)-β-D-glucan solutions have been purified from extracts of germinating barley[1] (*Hordeum vulgare*). However, three distinct classes of β-D-glucan endo-hydrolases present in the germinating grain might hydrolyse this polysaccharide. These include (1→3),(1→4)-β-D-glucan 4-glucanohydrolase (lichenase, EC 3.2.1.73), which requires adjacent (1→3)- and (1→4)-β-D-glucosyl residues for activity[2,3], and (1→4)-β-D-glucan 4-glucanohydrolase (cellulase, EC 3.2.1.4)[3,4], although it has recently been shown that cellulase is derived largely from the fungal population associated with germinating grain[5]. In addition, (1→3),(1→4)-β-D-glucans containing contiguous (1→3)-linked β-D-glucosyl residues[6] may be hydrolysed by (1→3)-β-D-glucan glucanohydrolase[7] [endo-(1→3)-β-D-glucanase, EC 3.2.1.39]. Relatively high concentrations of (1→3)-β-D-glucanase have been reported in germinating barley[8].

In view of these possibilities, the substrate specificities of the two highly purified enzymes from germinating barley[1] have been examined in detail, in order to define

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the class of β-D-glucan endo-hydrolase to which each should be assigned, and their action patterns, kinetic parameters, and relative susceptibility to heat inactivation have been compared.

EXPERIMENTAL

Substrates. — Arabinoxylan from wheat flour, carboxymethylxylan (CM-xylan), rhodymenan, yeast mannan, cellodextrin standards (d.p. 2–5), laminaridextrin standards (d.p. 2–6), carboxymethylpachyman (CM-pachyman), *Phytophthora cinnamomi* β-D-glucan, and the reduced, pneumococcal polysaccharide RS III were generously provided by Professor B. A. Stone (Department of Biochemistry, La Trobe University). Larch arabinogalactan, bovine serum albumin, *p*-nitrophenyl α-D-glucopyranoside, *p*-nitrophenyl β-D-glucopyranoside, pullulan, and gliadin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Laminarin from *Laminaria hyperborea* was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; laminarin from *Eisenia arborea* was from Tokyo Kasei Kogyo Co., Ltd., Tokyo; CM-cellulose (Edifas B 50) was from ICI Australia Pty. Ltd.; soluble starch was from BDH (Australia) Pty. Ltd.; and *N*-benzyloxycarbonyl-glycyl-L-leucine was provided by Dr. L. Sparrow, CSIRO Division of Protein Chemistry, Parkville, Victoria, Australia. Barley (1→3),(1→4)-β-D-glucan was extracted and purified from barley flour as described previously[1], and was shown by methylation analysis to contain 28% of (1→3) and 72% of (1→4) linkages.

Assay procedures. — The activity of (1→3),(1→4)-β-D-glucan endo-hydrolases was routinely determined viscometrically over 15 min at 40° by using 550 μl of barley (1→3),(1→4)-β-D-glucan [0.2% w/v in 50mM sodium acetate buffer (pH 4.7) containing 5mM sodium azide and bovine serum albumin (400 μg/ml)] and 50 μl of enzyme solution.

In substrate specificity studies, 1.1 ml of substrate in 50mM sodium acetate buffer [pH 4.7; containing 5mM sodium azide and bovine serum albumin (400 μg/ml)] was incubated with a final concentration of enzyme [0.40 μg/ml; 1.10 and 1.34 nkat of enzyme I and II, respectively]. Substrate concentrations were 1.9 mg/ml, except for CM-cellulose (2.5 mg/ml), CM-xylan (6 mg/ml), and the two *p*-nitrophenyl D-glucosides (0.9 mg/ml). After 13 h at 40°, reactions were stopped by heating at 100° for 10 min and reducing equivalents were measured[9]. The relatively long period of incubation was necessary to detect hydrolysis of the RS III polysaccharide (see Discussion) and to check for small amounts of contaminating enzymes in the preparations. Control incubations showed that 55% of enzyme I activity and 89% of enzyme II activity were retained over this period. Activities of the enzymes on solutions of arabinoxylan, CM-xylan, and CM-pachyman were also assayed viscometrically, as described for barley (1→3),(1→4)-β-D-glucan.

Enzymic action on *p*-nitrophenyl D-glucosides was measured by mixing 100 μl of the incubation mixture with 1 ml of 0.1M NaOH, diluting to 3.0 ml, and measuring the absorbance at 400 nm. The enzymes (1 μg/ml; containing 2.75 nkat of enzyme I

and 3.35 nkat of enzyme II) were tested for proteolytic activity using gliadin (0.35 mg/ml) or *N*-benzyloxycarbonyl-glycyl-L-leucine (2 mg/ml) in 50mM sodium acetate buffer (pH 4.7).

In each substrate specificity study, substrate and boiled enzyme controls were assayed.

Determination of kinetic parameters. — For the determination of pH optima, 0.2% (w/v) (1→3),(1→4)-β-D-glucan was prepared in McIlvaine's citric acid–Na_2-HPO_4 buffer[10] containing bovine serum albumin (400 μg/ml) in the pH range 2.6–9.0, enzyme was added to a final concentration of 0.4 μg/ml (1.10 nkat of enzyme I, 1.34 nkat of enzyme II), and activity was measured viscometrically. To estimate the kinetic parameters V_{max} and K_m, (1→3),(1→4)-β-D-glucan was dissolved in 2.1 ml of 50mM sodium acetate buffer [pH 4.7; containing 5mM sodium azide, bovine serum albumin (400 μg/ml), and 1.10 nkat of enzyme I or 1.34 nkat of enzyme II] in the concentration range of 0.2–3.0 mg/ml. Concentrations in excess of 3 mg/ml were difficult to attain with the (1→3),(1→4)-β-D-glucan used. After 30 min at 40°, the mixture was heated at 100° for 10 min and reducing equivalents were measured[9]. The reaction rate remained linear during this period. In control incubations, heat-inactivated enzyme was added to the substrate solutions.

Isolation of oligomeric products. — Oligosaccharides released by the action of barley (1→3),(1→4)-β-D-glucanase on barley (1→3),(1→4)-β-D-glucan were purified from hydrolysates by preparative-scale gel-filtration chromatography[11]. Barley (1→3),(1→4)-β-D-glucan (140 mg) in 20 ml of 50mM sodium acetate buffer (pH 4.7) was incubated with enzyme (final concentration, 8 μg/ml; containing 22 nkat of enzyme I or 26.8 nkat of enzyme II) at 35° in the presence of toluene for 24 h, when more enzyme was added to produce a total concentration of 16 μg/ml. After a further 24 h, the hydrolysate was heated at 100° for 10 min, insoluble material was removed by centrifugation, and 4 volumes of ethanol were added. After 16 h at 4°, precipitated material was removed by centrifugation and washed thoroughly with 80% ethanol, and the 80% ethanol-soluble fraction was concentrated *in vacuo* at 40°.

The 80% ethanol-soluble oligosaccharides were fractionated on Bio-Gel P-2 (batch 188434, minus 400 mesh; Bio-Rad Laboratories, Richmond, California). The column (0.9 × 170 cm) was equilibrated in distilled water and operated[11] at 60° and 200 kPa, at a flow rate of ~34 ml/h. The column was calibrated with cellodextrins and laminaridextrins. A solution of ~10 mg of carbohydrate in ~300 μl of water was applied to the column and, after combining the appropriate fractions, the tri- and tetra-saccharides were rechromatographed until single, symmetrical peaks were obtained. Oligosaccharides in the eluate were detected with an Optilab Multiref 902 differential refractometer (Optilab, Vällingby, Sweden).

Methylation analysis. — Linkage positions in oligo- and poly-saccharides were determined by the methylation procedure of Hakomori[12], as described by Björndal *et al.*[13]. To sequence linkages in mixed-linkage oligoglucosides, tri- and tetra-saccharides (1 mg) were dissolved in 1 ml of M NH_4OH and reduced with 10 mg of sodium borodeuteride for 4 h at room temperature and 1 h at 40° prior to methylation[14].

Permethylated alditol acetates were chromatographed on a glass column (1.8 m × 3.2 mm) packed with 3% of OV-225 on Gas Chrom Q (100–120 mesh), using a Jeol JGC-20K gas chromatograph interfaced to a Jeol JMS-D100 double-focusing mass spectrometer. Injection-port and interfacing temperatures were ~230° and the oven temperature was 200°. Helium was used as the carrier gas at a flow rate of ~30 ml/min.

Heat stability of enzymes. — Enzymes were dissolved in 50mM sodium acetate buffer (pH 4.7; containing 5mM sodium azide) at a final concentration of 0.4 μg of enzyme protein/ml (1.10 nkat of enzyme I, 1.34 nkat of enzyme II). After maintaining the solutions at various temperatures for 15 min in the presence or absence of bovine serum albumin (400 μg/ml), residual activity was measured viscometrically.

RESULTS

Substrate specificity. — The substrate specificities of the enzymes are shown in Table I. Both hydrolyse the barley (1→3),(1→4)-β-D-glucan and the reduced, pneumo-

TABLE I

SUBSTRATE SPECIFICITY OF BARLEY (1→3),(1→4)-β-D-GLUCANASES

Substrate	*Preponderant linkage*	*Activity[a] (reducing equivalents released)*	
		Enzyme I	*Enzyme II*
β-D-Glucans			
Barley β-D-glucan	(1→3),(1→4)-β	+	+
RS III	(1→3),(1→4)-β	+	+
Laminarin (*Laminaria hyperborea*)	(1→3)-β	—	—
Laminarin (*Eisenia arborea*)	(1→3),(1→6)-β	—	—
Phytophthora glucan	(1→3),(1→6)-β	—	—
CM-Pachyman	(1→3)-β	Trace*	Trace*
CM-Cellulose	(1→4)-β	—	—
Cellopentaose	(1→4)-β	—	—
α-D-Glucans			
Soluble starch	(1→4),(1→6)-α	—	—
Pullulan	(1→4),(1→6)-α	—	—
β-D-Xylans			
Arabinoxylan (wheat)	(1→4)-β	Trace*	Trace*
CM-Xylan	(1→4)-β	—	—
Rhodymenan	(1→3),(1→4)-β	—	—
Glucosides and other polysaccharides			
Mannan (yeast)	(1→2),(1→3),(1→6)-α	—	—
Arabinogalactan (larch)	(1→3),(1→6)-β	—	—
p-Nitrophenyl α-D-glucopyranoside		—	—
p-Nitrophenyl β-D-glucopyranoside		—	—
Peptides and proteins			
N-Benzyloxycarbonyl-glycyl-L-leucine		—	—
Gliadin		—	—

[a] +, Denotes activity; —, no activity; *detected viscometrically.

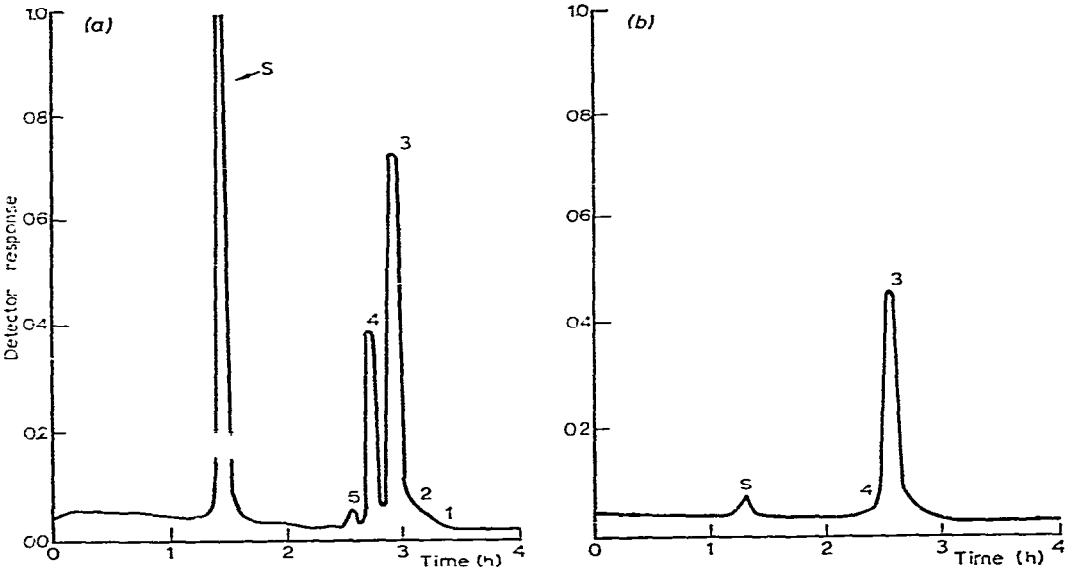

Fig. 1. Bio-Gel P-2 elution profile of the 80% ethanol-soluble material released by enzyme I acting on barley (1→3),(1→4)-β-D-glucan. The numbers refer to the d.p. of the oligosaccharides, and peak S represents buffer salts (which are eluted at the void volume). (*a*) Total 80% ethanol-soluble fraction; (*b*) rechromatography of fractions containing the trisaccharide.

coccal polysaccharide RS III, and very slight activity towards arabinoxylan and CM-pachyman was detected when sensitive viscometric assays were used. However, neither enzyme hydrolyses laminarin, other β-D-glucans, α-D-glucans, CM-xylan, glucosides, or peptides.

Fractionation of the hydrolysis products of barley (1→3),(1→4)-β-D-glucan. — Paper chromatography of the enzymic hydrolysates of (1→3),(1→4)-β-D-glucan (data not shown) demonstrated that two major oligosaccharides were released by the barley enzymes, and that these had mobilities similar to those of the (1→3),(1→4)-β-D-oligoglucosides produced by the *Bacillus subtilis* β-D-glucanase during hydrolysis of barley (1→3),(1→4)-β-D-glucan[11], but were readily distinguishable from the products formed by the *Streptomyces* cellulase[15].

When the 80% ethanol-soluble fraction from the barley enzyme-I hydrolysate was fractionated on Bio-Gel P-2, the preponderant products were a trisaccharide (64%) and a tetrasaccharide (33%), with smaller amounts of penta- (3%) and hexasaccharide material (Fig. 1a). The profile of the enzyme-II hydrolysate was almost identical. After rechromatography of the fractions containing trisaccharide on Bio-Gel P-2, a single, major peak was observed (Fig. 1b). This material, and tetrasaccharide purified in the same way, was used for methylation analysis.

Methylation analysis. — The results of methylation analysis of the oligomeric products released by the enzymes are shown in Table II. Again, the structures of the products formed by the action of the two enzymes are similar. Both trisaccharides

TABLE II

METHYLATION ANALYSIS OF 80% ETHANOL-SOLUBLE OLIGOSACCHARIDES RELEASED FROM BARLEY (1→3), (1→4)-β-D-GLUCAN BY (1→3),(1→4)-β-D-GLUCANASES

Sample	*Pre-reduced with $NaBD_4$*	*Terminal Glc* (R_F *1.00*)	*Molar ratio*		*1,2,4,5,6-Penta-O-methyl-glucitol* (R_F *0.38*)
			(1→3)-Linked Glc (R_F *1.79*)	*(1→4)-Linked Glc* (R_F *2.24*)	
Enzyme I					
Trisaccharide	No	1.0	0.7	0.8	—
	Yes	1.0	trace	0.9	0.5
Tetrasaccharide	No	1.0	0.9	1.9	—
	Yes	1.0	trace	1.5	0.3
Enzyme II					
Trisaccharide	No	1.0	0.9	0.9	—
	Yes	1.0	trace	0.8	0.4
Tetrasaccharide	No	1.0	0.9	1.9	—
	Yes	1.0	trace	1.5	0.3
Standards					
Laminaribiose	No	1.0	0.8	—	—
	Yes	1.0	0.1	—	0.7
Laminaritriose	No	1.0	1.7	—	—
	Yes	1.0	0.9	—	0.7

contain a single 1,3- and a single 1,4-D-glucosyl residue, consistent with their mobility during paper chromatography. The tetrasaccharides contain a single 1,3-D-glucosyl and two 1,4-D-glucosyl residues.

To determine the sequence of (1→3) and (1→4) linkages in the oligosaccharides, samples were reduced with sodium borodeuteride before methylation. The (1→3) linkage was shown to be adjacent to the reducing end of each oligosaccharide, since reduction with sodium borodeuteride prior to methylation analysis resulted in loss of the 1,3-linked D-glucosyl derivative 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol (Table II) and the appearance of a product with mobility 0.38 relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. The identity of this component was established by mass spectrometry to be 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methyl-D-glucitol and the major mass fragments can be rationalised in terms of its molecular structure (Fig. 2). The base peak of the spectrum is *m/z* 90, which corresponds to one of the primary fragments formed by fission between the methoxylated C-2 and the acetoxylated C-3. The absence of its complementary ion *m/z* 205 is consistent with the formation of the carboxonium ion on methoxylated carbon atoms after fission between acetoxylated and methoxylated carbons[16]. Similarly, fission between C-3 and C-4 would be expected to give rise to *m/z* 133, but not *m/z* 162. The abundant ion *m/z* 101 may be derived by β-elimination of methanol from *m/z* 133, while loss of formaldehyde from *m/z* 90 would explain[16] the secondary fragment *m/z* 60. Fission between C-4 and C-5 would result in the appearance of primary fragments *m/z* 206 and 89,

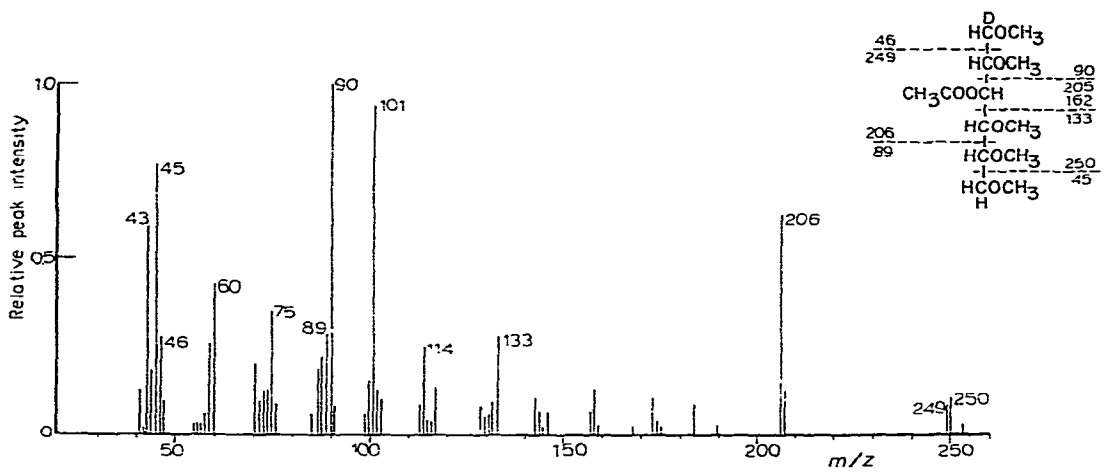

Fig. 2. Mass spectrum of 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methyl-D-glucitol obtained from 3-*O*-β-cellobiosyl-D-glucose pre-reduced with sodium borodeuteride.

and fission between C-1 and C-2, and C-5 and C-6, would lead to primary fragments *m/z* 46 and 250, which contain C-1 and thus the deuterium label, together with their complementary ions *m/z* 249 and 45. The molecular ion *m/z* 295 could not be detected in the spectrum.

The identity of 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methyl-D-glucitol was confirmed by comparison of its retention time and mass spectrum with those of a peak generated by borodeuteride reduction of laminaribiose and laminaritriose followed by methylation analysis. This derivative is much more volatile than those derived from terminal, (1→3)-, or (1→4)-linked D-glucosyl residues and is preferentially lost during the removal of acetic anhydride from the partially methylated alditol acetates (Table II). Björndal *et al.*[13] concluded that, for this and other reasons, quantitative recovery of methylated sugars may not be attained and these losses are evident in Table II.

However, the results demonstrate unequivocally that, for both enzymes, the trisaccharide released from barley (1→3),(1→4)-β-D-glucan is 3-*O*-β-cellobiosyl-D-glucose and that the tetrasaccharide is 3-*O*-β-cellotriosyl-D-glucose.

Products of hydrolysis of RS III. — Both enzymes hydrolyse the RS III polysaccharide, although paper chromatography revealed that hydrolysis was not complete even after the addition of further enzyme (to 3 μg/ml) during incubation for 39 h (data not shown). Based on relative mobilities, the major products were concluded to be laminaribiose and higher oligosaccharides that correspond to those detected amongst the products released by the action of the *Bacillus subtilis* (1→3),(1→4)-β-D-glucanase on the RS III polysaccharide[11]. No cellobiose was released.

Action pattern of the enzymes. — Both (1→3),(1→4)-β-D-glucanases exhibit characteristic endo-hydrolase action-patterns on (1→3),(1→4)-β-D-glucan (Fig. 3). During the rapid, initial decrease in viscosity, reducing equivalents were produced in an essentially linear manner. The degree of hydrolysis after 3 h is ~30% for

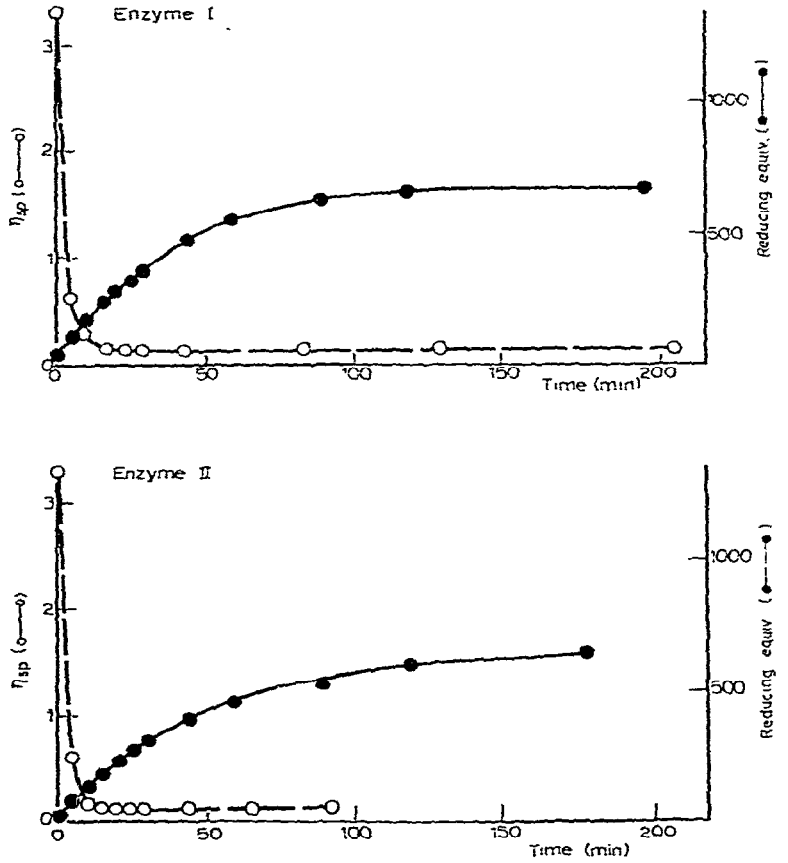

Fig. 3. Action patterns of barley (1→3),(1→4)-β-D-glucan hydrolases on barley (1→3),(1→4)-β-D-glucan: ○---○, specific viscosity η_{sp}; ●——●, reducing equivalents released.

each enzyme, which corresponds to an average chain-length of 3–4 residues for the oligomeric products and is thus consistent with the proportions of the major oligosaccharide products [3-*O*-β-cellobiosyl-D-glucose (64%) and 3-*O*-β-cellotriosyl-D-glucose (33%)] in the hydrolysate (Fig. 1).

TABLE III

KINETIC PROPERTIES OF (1→3),(1→4)-β-D-GLUCANASES FROM GERMINATING BARLEY

	V_{max} (*μmol of Glc equiv./min/μmol of enzyme*)[a]	K_m (*mg/ml*)	*Optimum pH*
Enzyme I	7.06×10^3	3.0	4.7
Enzyme II	11.62×10^3	3.4	4.7

[a]Mol. wts.: enzyme I, 28,000; enzyme II, 33,000 (ref. 1).

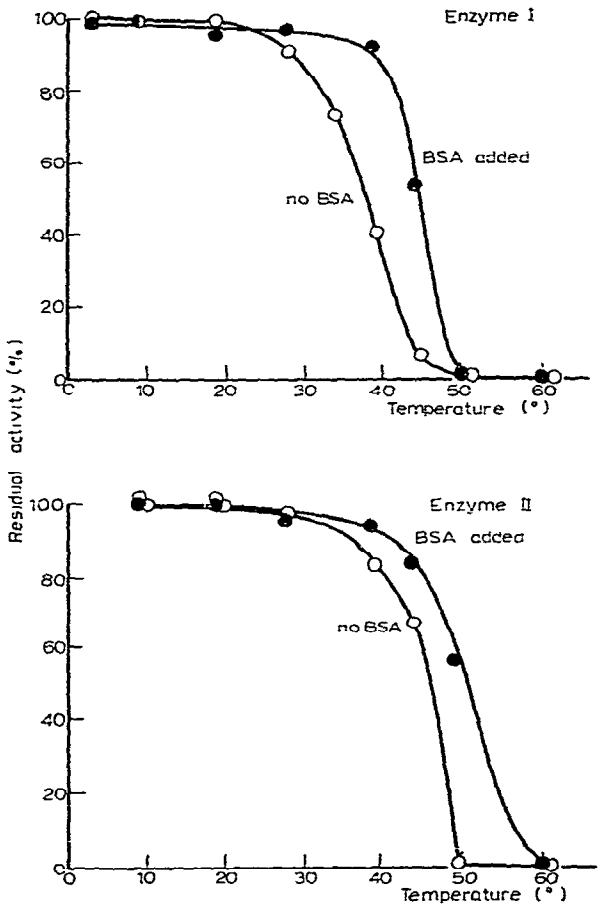

Fig. 4. Temperature stability of barley (1→3),(1→4)-β-D-glucan hydrolases in the presence (●——●) or absence (○——○) of bovine serum albumin (400 μg/ml). Enzyme solutions were maintained at various temperatures for 15 min and the residual activity was measured.

Kinetic properties. — The kinetic parameters of the two enzymes are summarised in Table III. Both exhibit the same pH optimum and both lose ~90% of their activity at 1.5 pH units away from the optimum. The Michaelis–Menten constants for the enzymes acting on barley (1→3),(1→4)-β-D-glucan are similar, although enzyme II appears to hydrolyse the substrate at a significantly higher rate than does enzyme I (Table III). Since the molecular weight of the barley (1→3),(1→4)-β-D-glucan is unknown, K_m values are expressed as mg/ml.

Stability of the enzymes. — Both enzymes lose activity rapidly at temperatures above 40°, but the addition of bovine serum albumin stabilises both considerably (Fig. 4). Enzyme I is appreciably less stable than enzyme II and retained <50% of its initial activity after heating at 40° for 15 min, in the absence of bovine serum albumin. Enzyme II lost ~15% of its activity under similar conditions. In the presence of bovine serum albumin, both enzymes were essentially stable at 40°.

DISCUSSION

The two highly purified enzymes from germinating barley appear to have identical substrate specificities. Both hydrolyse barley (1→3),(1→4)-β-D-glucan and the RS III polysaccharide, but exhibit little or no activity on other substrates tested. The slight hydrolysis of wheat-flour arabinoxylan is probably due to small proportions of contaminating (1→3),(1→4)-β-D-glucan in the arabinoxylan rather than to xylanase activity in the enzyme preparations, since (1→3),(1→4)-β-D-glucan has recently been identified in wheat endosperm cell-walls[17] and re-examination of the preparation has revealed traces of (1→3),(1→4)-β-D-glucan (unpublished data). This view is supported by the absence of any activity on CM-xylan or rhodymenan (Table I). In extended incubations (13 h), the enzymes reduce, very slightly, the viscosity of CM-pachyman solutions, whereas other (1→3)-β-D-glucans are not hydrolysed. The reason for this is not clear.

The algal (1→3),(1→4)-β-D-xylan rhodymenan contains (1→3) and (1→4) linkages in the ratio of ~1:2; although these are thought to be arranged randomly, there are many sequences where (1→3) and (1→4) linkages are juxtaposed[18]. In this respect, the polysaccharide has structural features similar to those of the barley (1→3),(1→4)-β-D-glucan. The inability of the barley enzymes to hydrolyse rhodymenan suggests that the hydroxymethyl groups of glucans, which are absent from xylans, are important determinants of specificity.

An enzyme preparation of undefined purity and substrate specificity isolated from germinating barley releases (1→3),(1→4)-β-D-glucan from barley endosperm cell-walls, and it has been suggested that an acidic carboxypeptidase in the preparation is responsible[19]. Neither of the enzymes purified in the present study had any action on a dipeptide or on a cereal storage protein known to be hydrolysed by extracts of germinating barley[20] (Table I).

It is clear that the two enzymes purified from germinating barley are true (1→3),(1→4)-β-D-glucan hydrolases, requiring both (1→3)- and (1→4)-β-D-linked residues in β-D-glucan substrates. They do not hydrolyse β-D-glucans containing only (1→3) or only (1→4) linkages (Table I). Each enzyme degrades ~90% of the barley (1→3),(1→4)-β-D-glucan to 80% ethanol-soluble oligosaccharides, of which ~64% is the trisaccharide 3-*O*-β-cellobiosyl-D-glucose, ~33% is the tetrasaccharide 3-*O*-β-cellotriosyl-D-glucose, and ~3% is a pentasaccharide of unknown structure. The major products are the same as those reported[21] for oligosaccharides released by a single, partially purified enzyme from germinating barley. Approximately 10% of the (1→3),(1→4)-β-D-glucan remained insoluble in 80% ethanol after exhaustive hydrolysis, and the structure of this material is under investigation.

The release of 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose as the major oligomeric products, the decrease in the ratio of (1→4) to (1→3) linkages (2.6 in the polysaccharide to 1.3 in the oligomeric products), and the presence of a (1→3) linkage at the reducing ends of the oligosaccharides indicate that only (1→4) linkages are hydrolysed and that the glycosyl portion must be substituted at O-3.

Thus, in the barley (1→3),(1→4)-β-D-glucan, linkages are hydrolysed as follows: ...G 3 G ⇞ G 4 G 3 G ⇞ G ...$_{Red}$ or ...G 3 G ⇞ G 4 G 4 G 3 G ⇞ G ...$_{Red}$ (the subscript "Red" indicates the reducing end of the polysaccharide).

The specificity requirement was confirmed by the action of the enzymes on the reduced, pneumococcal polysaccharide RS III, which is a (1→3),(1→4)-β-D-glucan containing alternating (1→3) and (1→4) linkages[11]. The presence of laminaribiose and the absence of cellobiose in hydrolysates indicate that the enzymes hydrolyse (1→4)-β-D linkages only. The RS III polysaccharide is hydrolysed at a significantly lower rate than the barley (1→3),(1→4)-β-D-glucan. Binding of the enzyme to its substrate is thus likely to extend beyond the residues directly involved in hydrolysis, so that the sequence ... G 3 G ⇞ G 4 G ...$_{Red}$ may be hydrolysed more rapidly than ... G 3 G ⇞ G 3 G ...$_{Red}$. Linkage arrangement on the non-reducing side of the linkage hydrolysed may also be important for binding, such that ... G 4 G 4 G 3 G ⇞ G ...$_{Red}$ is hydrolysed more rapidly than ... G 3 G 4 G 3 G ⇞ G ...$_{Red}$.

Anderson and Stone[11] observed that a (1→3),(1→4)-β-D-glucan endo-hydrolase from *Bacillus subtilis*, which resembles the barley (1→3),(1→4)-β-D-glucanases with respect to substrate specificity, also hydrolyses the RS III polysaccharide much more slowly than barley (1→3),(1→4)-β-D-glucan, whereas a β-D-glucanase [(1→3)-(1→3),(1→4)-β-D-glucan 3(4)-glucanohydrolase, EC 3.2.1.6] from *Rhizopus arrhizus* very rapidly hydrolyses the RS III polysaccharide to laminaribiose. Whilst this may reflect similarities in the binding sites of the barley and *Bacillus subtilis* (1→3),(1→4)-β-D-glucan hydrolases, it is apparent that the binding sites of these enzymes differ significantly from those of the *Rhizopus arrhizus* enzyme.

The action pattern of the enzymes during hydrolysis of barley (1→3),(1→4)-β-D-glucan is characteristic of endo-hydrolases, but there have been reports that some exo-hydrolases can hydrolyse internal glycosidic linkages in the main polysaccharide chain. For example, a fungal (1→3)-β-D-glucan exo-hydrolase can initiate endo-cleavage in (1→3),(1→6)-β-D-glucans by hydrolysing internal (1→3)-β-D linkages where these are adjacent to (1→6)-β-D linkages[22]. Similarly a (1→3)-β-D-glucan exo-hydrolase from *Euglena gracilis*[23] can hydrolyse internal (1→3)-β-D linkages in (1→3),(1→6)-β-D-glucans[24]. The possibility that the barley enzymes are (1→4)-β-D-glucan exo-hydrolases that hydrolyse internal (1→4)-β-D linkages in (1→3),(1→4)-β-D-glucans in an analogous manner is ruled out by the absence of activity against cellopentaose.

In summary, the enzymic properties of the two (1→3),(1→4)-β-D-glucanases indicate that both are endo-hydrolases that hydrolyse a (1→4)-β-D linkage in β-D-glucans where the D-glucosyl residue is substituted at O-3. Both enzymes are therefore (1→3),(1→4)-β-D-glucan 4-glucanohydrolases and fall into the enzyme classification EC 3.2.1.73.

The enzymes are remarkably similar with respect to their kinetic properties, although enzyme II hydrolyses the (1→3),(1→4)-β-D-glucan more rapidly than enzyme I. Catalytic-centre activities (k_{cat}) calculated from V_{max} values (Table III) indicate that enzyme I catalyses ~7,100 hydrolytic cleavages in barley (1→3),(1→4)-

β-D-glucan per minute, and that enzyme II hydrolyses ~11,600 linkages per minute.

Enzyme II is significantly more stable at elevated temperatures than enzyme I (Fig. 4). However, in the purified form, neither exhibits a high degree of temperature stability. Although both are essentially stable at 40° in the presence of bovine serum albumin, treatment for 15 min at 60° abolished activity. Whether or not the greater temperature stability of enzyme II is related to its higher content of carbohydrate is unknown.

A functional significance for the presence of two (1→3),(1→4)-β-D-glucan 4-glucanohydrolases of similar specificity in germinating barley has yet to be established. Amino acid sequence analysis has shown that the two enzymes are derived from different genes, but it is not known if both genes are expressed in the endosperm during germination or whether the enzymes are located in different tissues in the grain and are involved in different metabolic processes[25].

ACKNOWLEDGMENTS

We thank Professor B. A. Stone (Department of Biochemistry, La Trobe University) for providing polysaccharides and enzymes, and for helpful advice. The work was supported by grants from the Australian Research Grants Committee.

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