Carbohydrate Research, 37 (1974) 9–22

PURIFICATION AND PROPERTIES OF AN ENDO-(1→3)-β-D-GLUCANASE FROM MALTED BARLEY*†

DAVID J. MANNERS AND GLYNN WILSON

Department of Brewing and Biological Sciences, Heriot-Watt University, Edinburgh EH1 1HX (Great Britain)

(Received January 28th, 1974; accepted March 18th, 1974)

ABSTRACT

An endo-(1→3)-β-D-glucanase has been isolated from malted barley and purified 92-fold. The enzyme preparation appeared to be physically homogeneous and had a molecular weight of about 12,800 daltons. The enzyme is highly specific for (1→3)-β-D-glucosidic linkages, and has an endo action on laminaran. The effect of certain group-specific reagents on the enzyme has been examined.

INTRODUCTION

Previous studies from these and other laboratories[2–4] have shown that extracts of germinated or malted barley contain a complex mixture of β-glucanases and β-glucosidases. In order of decreasing molecular weight, we have provided evidence[2] for two β-glucosidases, an endo-(1→4)-β-D-glucanase, an endo-barley-β-D-glucanase and an endo-(1→3)-β-D-glucanase. We now describe the purification and properties of the endo-(1→3)-β-D-glucanase. A preliminary account of some of the results has been given elsewhere[5].

EXPERIMENTAL

Substrates. — The following substrates were laboratory samples available from previous work[2] on β-glucans and β-glucanases: insoluble laminaran from *Laminaria hyperborea*, and lichenan from *Cetraria icelandica*. Cellodextrin was prepared by the partial acid hydrolysis of cellulose. Barley β-D-glucan was prepared by the method of Preece and MacKenzie[6]. 4-*O*-β-Laminaribiosyl-D-glucose was prepared by Dr D. C. Taylor[7] using laminaribiose phosphorylase from *Astasia ocellata*, and 3-*O*-β-cellobiosyl-D-glucose was prepared from lichenan by use of an enzyme preparation from *Rhizopus arrhizus*[8]. Sodium carboxymethylpachyman (CMP) was prepared from pachyman [a (1→3)-β-D-glucan from the fungus *Poria cocos*] by the method of Clarke and Stone[9]. Carboxymethylcellulose (CMC) was a gift from Imperial Chemical Industries Ltd. Laminarisaccharides (G-G8) were prepared from pachyman by

*Dedicated to the memory of Professor W. Z. Hassid.
†Studies on β-glucanases. Part II. For Part I, see Ref. 1.

partial acid hydrolysis, followed by charcoal–Celite chromatography and preparative paper chromatography. Cellobiose was a commercial sample of established purity.

Enzyme inhibitors. — D-Glucono-(1,5)-lactone was obtained from BDH (Poole, England), 2-hydroxy-5-nitrobenzyl bromide from Eastman Organic Chemicals, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methyl toluene-*p*-sulphonate from Ralph Emanuel (Wembley, England), chloro(*N*-α-tosyl-L-lysyl)methane from Calbiochem (San Diego, Calif., U.S.A.), and *N*-bromosuccinimide, glycine methyl ester hydrochloride and crystalline *N*-acetylimidazole from the Sigma Chemical Company Ltd. (London).

Enzyme source. — Malted barley (Var. Proctor, 1969 harvest) was a gift from Campbell, Hope and King (Edinburgh).

Analytical methods. — Reducing sugars were determined by a modified Nelson method[10], the reagents being calibrated against D-glucose. Protein was determined by a modified Lowry method[11], calibrated against crystalline bovine serum albumin. The protein content of column fractions was estimated from the extinction at 280 nm.

Separation methods. — Descending paper chromatograms on Whatman No. 1 paper were developed in ethyl acetate–pyridine–water (10:4:3) as solvent. Reducing sugars were detected with an alkaline silver nitrate reagent[12]. Protein material was fractionated by molecular-sieve chromatography at 0–4° on columns of Biogel P-60 or P-30 resins (Bio-Rad Laboratories Ltd., Bromley, Kent). Protein solutions were concentrated in Amicon ultrafiltration cells equipped with Diaflo membranes (Amicon Ltd., High Wycombe, Bucks), under a pressure of 4 atm. Polyacrylamide gel electrophoresis was performed with a Shandon Analytical Electrophoresis apparatus (Shandon, London). 7.5% Acrylamide gels were prepared according to the method of Tombs and Akroyd[13].

Enzyme assays. — (*a*) *Reductometric assays.* Activities towards various substrates based on reducing power measurements may be due to exo-β-D-glucanase, β-glucosidase, or endo-β-D-glucanase. Activities towards cellobiose, laminaribiose, cellodextran, laminaran, barley β-D-glucan, and lichenan were determined by incubating the substrate with 0.01M sodium acetate buffer, pH 5.3, and a suitable amount of enzyme solution. The amount of substrate present in the digest varied between 0.25 mg/ml and 1 mg/ml depending upon its reducing power. After incubation for suitable lengths of time at 37°, the reaction was stopped by the addition of Nelson copper reagent[10] to aliquots of that solution. Appropriate controls containing either substrate or enzyme were also analysed. Suspensions of insoluble laminaran were warmed to 60° prior to use. In experiments with purified endo-(1→3)-β-D-glucanase, 0.5 ml of laminaran (1% w/v) was incubated with sodium acetate buffer (0.1M, pH 5.0) and enzyme solution (total digest volume 1 ml) for 1 h at 37°. As the products of enzymic degradation of laminaran by endo-(1→3)-β-D-glucanase are a mixture of sugars, enzyme activities have been expressed in terms of μg of reducing sugar formed as D-glucose equivalents. For the routine assay of column fractions, enzyme activities have been expressed as the increase in extinction of Nelson determinations over that of the substrate blank (ΔE600).

(b) *Viscometric assay of endo-β-D-glucanase activities.* The reduction in viscosity of β-D-glucans was measured in a No. 1 BSS. Ostwald viscometer. For the assay, 1 ml of substrate solution (1%, w/v) and 1 ml of sodium acetate buffer (0.2M, pH 5.0) were mixed with 0.5 ml of enzyme solution at 37°. A sample of the digest (2 ml) was transferred to the viscometer at a constant temperature of 37°, and the flow time measured at intervals after mixing. Water had a flow time of 24.0 sec in the viscometer used. Enzyme activity is expressed as the rate of increase of the reciprocal specific viscosity with time $d(1/\eta_{sp})/dt$. The viscometric method has been used primarily for the assay of column fractions, to distinguish between endo-β-glucanase and β-glucosidase activities, which is not possible with a reductometric method. Carboxymethylpachyman has been used for the viscometric assay of endo-(1→3)-β-D-glucanase activity. Endo-(1→4)-β-D-glucanase activity has been measured with carboxymethylcellulose as substrate, whilst barley β-D-glucan was the substrate for the viscometric assay of enzymes that hydrolyse β-D-glucans containing both β-D-(1→4)- and β-D-(1→3)-glucosidic linkages. Such activity has been termed endo-barley-β-D-glucanase.

Inhibitor assays. — (a) *Standard reductometric procedure.* Purified endo-(1→3)-β-D-glucanase (0.1 ml in sodium acetate–acetic acid buffer pH 5.0) was allowed to react with inhibitor solution (0.1 ml) for 30 min at 25°. Following the addition of laminaran (0.5 ml, 1%, w/v) and 0.05M sodium acetate–acetic acid buffer (0.3 ml, pH 5.0), the enzyme was then assayed by the reductometric method. The activity was compared with that of a control containing water in place of inhibitor solution. Each inhibitor was tested for possible interference with the Nelson reagent[10].

(*b*) *Reaction of enzyme with cyanate and iodoacetate.* A 1:10 dilution of purified enzyme fraction E6 was treated with an equal volume of cyanate (0.4M) or iodoacetate (20mM) solution in 10mM 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris-HCl) buffer (pH 8.0). The mixtures were dialysed against the same buffer for 24 h (at 0–4°) before assay. A control solution was also dialysed.

(*c*) *Reaction of enzyme with* N-*bromosuccinimide.* The enzyme solution (1:2 dilution of Fraction E5 in 0.01M sodium acetate buffer, pH 5.0) was treated with an equal volume of *N*-bromosuccinimide (10mM) for 30 min at 25°. Endo-(1→3)-β-D-glucanase activity in control and test solutions was determined by the viscometric procedure.

(*d*) *Reaction of enzyme with* N-*acetylimidazole.* Purified enzyme Fraction E6 was diluted 1:10 with Tris-HCl buffer (0.01M, pH 7.6). Solid *N*-acetylimidazole was added to an aliquot of the diluted enzyme to a concentration of 23mM. Four separate, equal additions of solid *N*-acetylimidazole were made at 15-min intervals to a second aliquot of diluted enzyme to give a final concentration of 92mM (calculated to give approximately 100 mg reagent/mg protein). A third aliquot of diluted enzyme served as a control. The solutions were kept at 25° and samples were withdrawn at suitable time intervals, cooled on ice, and dialysed against distilled water (18 h, 0–4°). Enzyme activities were determined by the reductometric procedure with the estimation of appropriate controls.

(*e*) *Reaction of enzyme with 2-hydroxy-5-nitrobenzyl bromide.* 2-Hydroxy-5-nitrobenzyl bromide (0.2M, 1 vol.) in acetone was added to 20 vol. of a 1:1 dilution of purified enzyme Fraction E6 in sodium acetate buffer (0.1M, pH 5.0). After 30, 60, and 120 min in the dark (at 25°), samples were withdrawn and assayed with the reductometric procedure. The reagent was replaced by acetone in controls.

(*f*) *Reaction of enzyme with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methyl toluene*-p-*sulphonate.* This was used with glycine methyl ester as the nucleophilic modifying reagent. The reaction mixture contained equal volumes of purified enzyme Fraction E6 and a solution containing 0.2M carbodiimide and 2M glycine methyl ester in 0.5M pyridine–pyridinium chloride buffer (pH 4.8). Control mixtures were prepared with the omission of ester, carbodiimide, or of both. Test and control solutions were kept at 25° and, at suitable time intervals, aliquots were removed and excess reagent was quenched with M acetate buffer (0.5 ml, pH 5.0). After 15 min at 25°, the samples were dialysed (18 h, 0–4°) against acetate buffer (0.01M, pH 5.0). Enzyme activities were determined with the standard reductometric digest.

Purification of endo-(1→3)-β-D-glucanase. — (*a*) *Enzyme extraction.* Malted barley flour was extracted with 0.2M acetate (sodium acetate–acetic acid) buffer, pH 5.0 (3 l/kg of flour), for 3 h at room temperature (18–20°). The extract was dialysed against running tap water (0–5°) for 24 h (Fraction E1).

(*b*) *Ammonium sulphate precipitation.* Protein was precipitated from Fraction E1 by the addition of solid ammonium sulphate to 50% saturation. The precipitate was dissolved in acetate buffer (0.01M, pH 5.0), dialysed, and freeze-dried (Fraction E2). There was no loss of activity during freeze-drying.

(*c*) *Molecular-sieve chromatography.* A portion of Fraction E2 (300 mg) was dissolved in acetate buffer (0.01M, pH 5.0) and applied to a column (2.5 × 70 cm) of Biogel P-60 resin. The flow rate of buffer (0.01M acetate, pH 5.0) through the column was kept constant by a LKB peristaltic pump, fractions of 4 ml being collected. The distributions of protein and enzyme activities throughout the column fractions are shown in Fig. 1. Several fractionations of Fraction E2 on Biogel P-60 resin gave highly reproducible results similar to those shown in Fig. 1. Fractions containing endo-(1→3)-β-D-glucanase but little or no endo-barley-β-D-glucanase (from three separate fractionations of E2 on Biogel P-60 resin) were pooled and freeze-dried to give Fraction E3. This was then applied to a column (2.5 × 40 cm) of Biogel P-30 resin. Fractions containing enzyme activity were pooled and freeze-dried (Fraction E4) and rechromatographed on the column of Biogel P-30 resin (Fraction E5). Fig. 2 shows the distribution of protein and endo-(1→3)-β-D-glucanase in column fractions from the second column of Biogel P-30 resin. The increase in specific activity of endo-(1→3)-β-D-glucanase during the purification of 1 kg of flour is shown in Table I. The purified enzyme (Fraction E5) was concentrated to a final volume of 9 ml by ultrafiltration on a PM-10 membrane and was stored in the frozen state (Fraction E6). All enzyme activity was retained by the membrane. The final yield of purified enzyme was of the order of 100 mg/kg of malted barley.

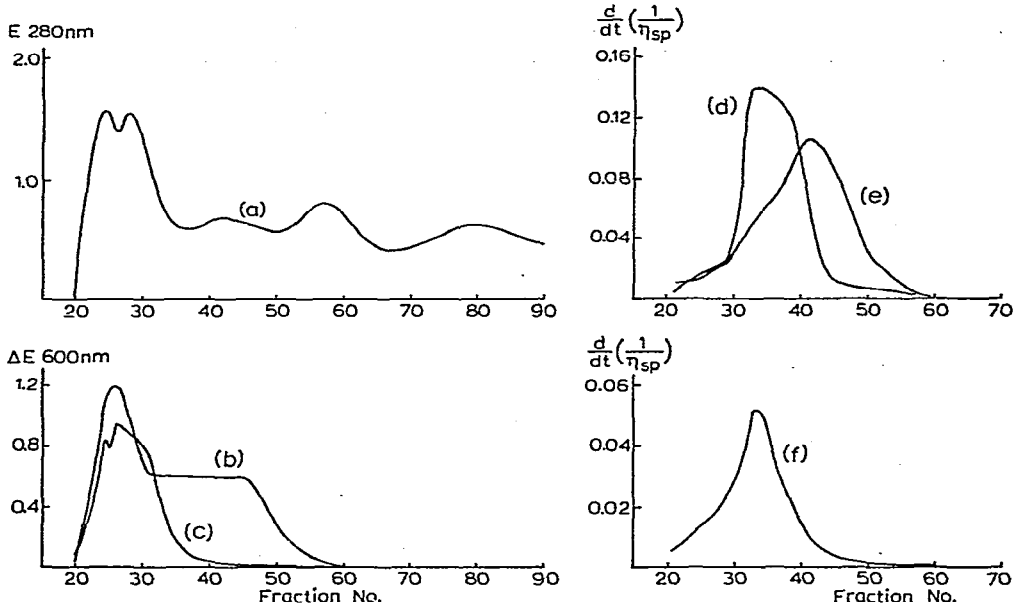

Fig. 1. Fractionation of a malted barley extract on Biogel P-60 resin. Distribution of: (a) protein, (b) laminarinase (reductometric assay), (c) cellodextrinase (reductometric assay), (d) endo-barley-β-D-glucanase (viscometric assay), (e) endo-(1→3)-β-D-glucanase (viscometric assay), and (f) endo-(1→4)-β-D-glucanase (viscometric assay). Reductometric assays towards other substrates were also performed, but for the sake of brevity, are not shown. Activity towards laminaribiose was present in fractions 20–35 with a maximum at 27, towards cellobiose in fractions 20–35 with two maxima at 24 and 28, towards lichenan in fractions 20–40 with a maximum at 26, and towards barley β-D-glucan in fractions 20–40 with a maximum at 23. All these activities are mainly due to the two β-glucosidases present in the extract, supplemented to varying extents by the endo-β-D-glucanases.

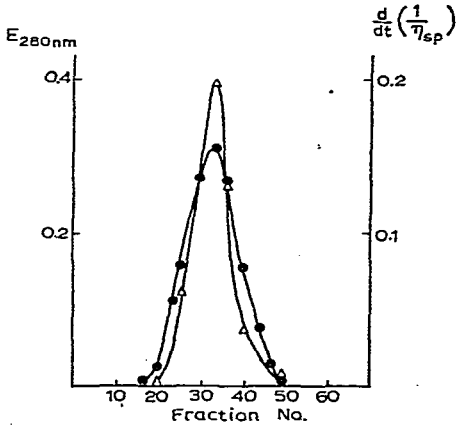

Fig. 2. Elution profile of endo-(1→3)-β-D-glucanase from the second column of Biogel P-30 resin; ●—●, protein; △—△, enzymic activity.

TABLE I

PURIFICATION OF MALTED BARLEY ENDO-(1→3)-β-D-GLUCANASE

Stage	*Fraction*	*Spec. activ.*[a]	*Enrichment*
Dialysed initial extract	E1	0.028	1
Ammonium sulphate 0–50%	E2	0.067	2.3
Biogel P-60 resin	E3	0.800	28
Biogel P-30 resin (1)	E4	1.676	58
Biogel P-30 resin (2)	E5	2.661	92

[a]As d/dt (1/ηsp)/mg protein; determined by viscometric assay with CMP.

Ultracentrifugation of purified endo-(1→3)-β-D-glucanase. — Sedimentation was performed in a Beckman Spinco, Model E ultracentrifuge using analytical rotor A. The sample was run in a synthetic boundary cell filling at 10,000 r.p.m. Photographs taken at 0, 16, 48, and 80 min (58,780 r.p.m.) showed that the enzyme sedimented as a single symmetrical peak.

Polyacrylamide gel electrophoresis. — Electrophoresis of E5 on 7.5% acrylamide gels, in Tris–glycine buffer (0.01M, pH 8.75) showed a single protein band that had migrated towards the anode.

Properties of malted barley endo-(1→3)-β-D-glucanase. — (*a*) *pH optimum.* The effect of pH on enzyme activity was investigated by use of 0.1M citrate–phosphate buffer in the reductometric assay. A typical dumb-bell shaped curve was observed with optimum activity at pH 5.0.

(*b*) *Effect of temperature.* The effect of temperature during incubation of the enzyme and substrate for 1 h was investigated within the range of 0–60° by the use of the reductometric assay with laminaran. The optimum temperature was 37°.

(*c*) *Effect of substrate concentration on reaction velocity.* The reaction velocity (v) was measured by the reductometric procedure at a series of concentrations of laminaran ranging from 0.02–1.2% (w/v) in the final incubation mixture. From a Lineweaver–Burke plot (Fig. 3), the K_M was 92 mg/100 ml. Since the laminaran sample had DP21, the molecular weight was 3420, and the K_M value therefore 2.7×10^{-4}M.

(*d*) *Molecular-weight estimation by molecular-sieve chromatography.* Chromatography was performed on a column of Sephadex G-100 (dimensions 60 × 2.5 cm) which was calibrated with standard proteins (urease, bovine serum albumin, peroxidase, pepsin, and cytochrome C). The molecular weight calculated from a plot of V/V_0 against log molecular weight was approximately 12,800 daltons. The enzyme and cytochrome C (molecular weight 12,400 daltons) had elution volumes of 215 and 216 ml, respectively.

(*e*) *Specificity of malted barley endo-(1→3)-β-D-glucanase.* The action of the purified enzyme on a variety of β-D-linked substrates was investigated by incubating substrate (1 mg) with acetate buffer (0.1 ml, 0.01M, pH 5.0) and enzyme solution (0.1 ml, 1:10 dilution, E6) for 24 h at 37°. With insoluble substrates, digests were shaken during incubation. After incubation, the enzyme was inactivated by boiling

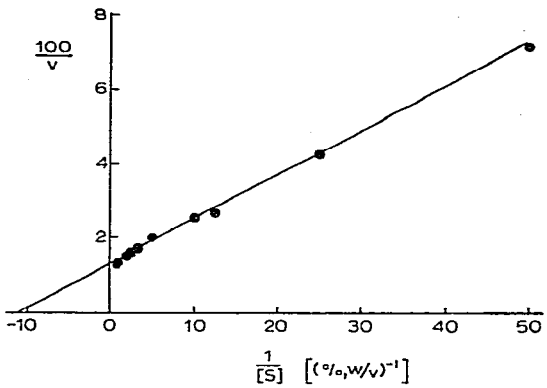

Fig. 3. Lineweaver–Burk plot for the hydrolysis of laminaran by malted barley endo-(1→3)-β-D-glucanase. The enzyme solution was purified enzyme fraction E5.

and the solutions were cooled and deionized with Biodeminrolit (The Permutit Company Ltd., London). The hydrolysis products were detected by descending paper chromatography. The results are shown in Table II.

TABLE II

THE ACTION OF MALTED BARLEY ENDO-(1→3)-β-D-GLUCANASE ON VARIOUS SUBSTRATES

Type of linkage	*Substrate*	*Products of hydrolysis*[a]
(1→3)-β-D-Glucosidic	Insoluble laminaran	G2–G7 G (trace)
	Pachyman	G2–G7 G (trace)
	Laminaripentaose (G5)	G2, G3, G (trace)
	Laminaritriose (G3)	G2 (trace), G (trace)
	Laminaribiose (G2)	Nil
(1→3)- and (1→4)-β-D-Glucosidic	Barley glucan	Nil
	Lichenan	Nil
	4-*O*-β-Laminaribiosyl-D-glucose	Nil
	3-*O*-β-Cellobiosyl-D-glucose	Nil
(1→4)-β-D-Glucosidic	Cellodextran	Nil
	Cellobiose	Nil
(1→3)-β-D-Xylosidic	Xylan from *Caulerpa filiformis*	Nil

[a]Detected by chromatography. G, D-Glucose, G2–G7, laminaribiose–laminariheptaose.

(*f*) *Action pattern of purified enzyme on laminaran.* A diagram of the products formed during the progressive hydrolysis laminaran is shown in Fig. 4.

(*g*) *Comparison of the action pattern of (1→3)-β-D-glucanase from malted barley and Basidiomycete QM806.* A solution of CMP (3 ml, 1%, w/v), acetate buffer (3 ml,

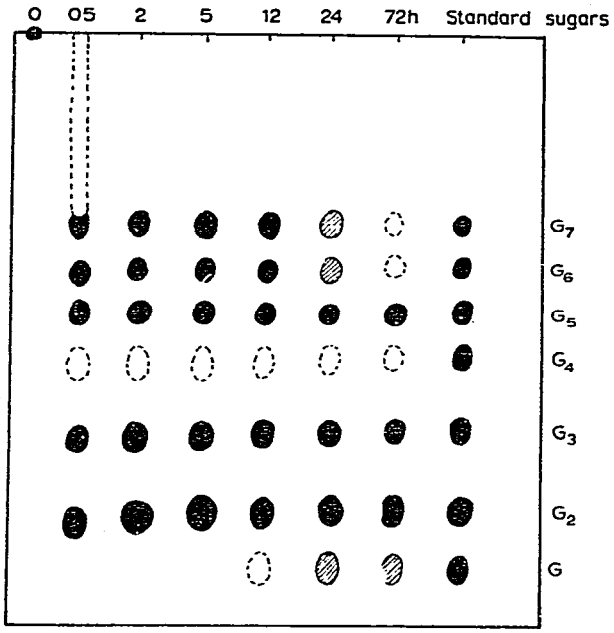

Fig. 4. Diagrammatic representation of the action of the endo-(1→3)-β-D-glucanase on laminaran; G–G7 represent the laminaricsaccharides ranging from D-glucose to laminariheptaose.

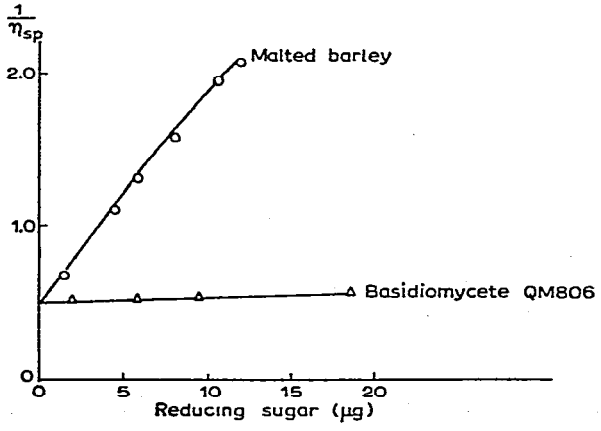

Fig. 5. Relationship between $1/\eta_{sp}$ and reducing sugar formation for the hydrolysis of CM-pachyman by malted barley endo-(1→3)-β-D-glucanase and an exo-(1→3)-β-D-glucanase from *Basidiomycete* QM806.

0.1M, pH 5.0), and enzyme solution (1.5 ml) was incubated at 37°. Immediately after addition of the enzyme solution, 2 ml of the digest was transfered to a viscometer for flow-time measurements. At intervals, 0.5-ml aliquots of the digest were assayed for reducing sugar. The relationship between the increase in reciprocal specific viscosity ($1/\eta_{sp}$) and reducing sugar production is shown in Fig. 5. The slope of the graph for malted barley endo-(1→3)-β-D-glucanase is steep compared with the flattish curve obtained with a freeze-dried culture filtrate (0.1 mg/ml) of *Basidiomycete* QM806 which showed high exo-(1→3)-β-D-glucanase activity[14].

(*h*) *Effect of metal ions on the activity of purified malted barley endo-(1→3)-β-D-glucanase.* The effect of calcium and other metal ions on enzyme activity was tested by mixing equal volumes of enzyme and metal ion solution for 30 min at 25°. Enzyme activity was measured by adding 0.2 ml of the mixture to the reductometric digest containing laminaran as substrate. Each metal ion solution was tested for possible interference with the Nelson reagents. It was noted that calcium chloride affected the viscometric assay procedure. At a final concentration of 50mM in the digest, the flow time of a 1% solution of CMP (at 37°) was reduced by 40%. This effect could be minimized by the addition of sodium chloride[9] to digests, but it could not be completely counteracted. The results for the effect of various metal ions on enzyme activity are shown in Table III.

TABLE III

EFFECT OF METAL IONS AND OF BSA ON ENDO-(1→3)-β-D-GLUCANASE ACTIVITY

Metal ion	*Concentration in mixture with enzyme* (mM)	*Relative activity* (%)	*BSA concentration in digest (μg/ml)*	*Relative activity* (%)
None	—	100	0	100
Ca^{2+}	125	125	10	108
Mg^{2+}	50	100	20	113
Ba^{2+}	5	128	50	122
Co^{2+}	2	118	100	126
Mn^{2+}	2	120	200	135

(*i*) *Effect of bovine albumin serum (BSA) on endo-(1→3)-β-D-glucanase activity.* The effect of different concentrations of BSA in enzyme digests was investigated by the use of the reductometric assay. The enzyme solution was a 1:10 dilution of purified Fraction E6. The results are shown in Table III.

(*j*) *Inhibition of malted barley endo-(1→3)-β-D-glucanase.* The effect of various inhibitors was determined by incubating the enzyme with inhibitor and assaying the resulting activity. Where possible, the standard reductometric method (see inhibition assays) was used. Reaction conditions with cyanate, iodoacetate, *N*-bromosuccinimide, *N*-acetylimidazole, 2-hydroxy-5-nitrobenzyl bromide, and carbodiimide are given in inhibitor assays. The results obtained are shown in Table IV.

TABLE IV

INHIBITION OF MALTED BARLEY ENDO-(1→3)-β-D-GLUCANASE BY VARIOUS COMPOUNDS[a]

Compound	*Conc. in mixture with enzyme* (mM)	*Inhibition* (%)
Cu^{2+}	0.2	11
	2.0	40
	20.0	61
Hg^{2+}	0.2	85
Fe^{2+}	20	24
EDTA	50	12
Mercuri-*p*-carboxyphenyl chloride	0.2	16
Mercuri-phenyl nitrate	0.2	52
	2.0	92
Cyanate ion	200	10
Iodoacetate ion	10	41
N-Bromosuccinimide	5	86
N-Acetylimidazole	23	40[b]
	90	93[b]
		95[c]
2-Hydroxy-5-nitrobenzyl bromide	10	83
		91[b]
		92[c]
1-Cyclohexyl-3-(morpholinoethyl)carbodiimide methyl toluene-*p*-sulphonate	100	64[c]

[a]Preincubation time of 30 min, unless otherwise stated. No inhibition was observed with the following compounds: D-Glucono-(1,5)-lactone (5mM), chloro(*N*-α-tosyl-L-lysyl)methane (2mM), and *N*-ethylmaleimide (1mM). [b]Preincubation time, 1 h. [c]Preincubation time, 2 h.

DISCUSSION

An endo-(1→3)-β-D-glucanase from malted barley has been purified to a state of homogeneity, as judged by ultracentrifugation and gel electrophoresis. The purified enzyme has a sharp pH optimum in the region of pH 5 and, during incubation for 1 h, a temperature optimum of 37°. With laminaran, the K_M was 2.7×10^{-4}M. The only other plant endo-(1→3)-β-D-glucanases that have been purified to a state of homogeneity appear to be those from the leaves of red kidney bean (*Phaseolus vulgaris*)[15] and the leaves of *Nicotiana glutinosa*[16]. Both of these enzymes show optimum activity in the region of pH 5. A partially purified endo-(1→3)-β-D-glucanase from ungerminated rye[17] has a pH optimum of 5.2. The temperature optimum of 37° for the malted barley enzyme is lower than values of 45° and 50° reported for the *Nicotiana*[16] and *Phaseolus*[15] enzymes, respectively.

The molecular weight of the purified enzyme is 12,800 daltons, as determined by molecular-sieve chromatography. This value is higher than a preliminary estimate[2] of 9000 daltons obtained in the study of an unfractionated extract of malted barley. It is likely that the present result is more accurate, since the molecular weight was

determined with the purified enzyme. The malted barley enzyme is thus smaller than that *from Nicotiana glutinosa* (45,000 daltons)[16] but is similar in size to the enzyme from *Phaseolus vulgaris* (about 12,000 daltons)[15].

The malted barley endo-(1→3)-β-D-glucanase is highly specific for (1→3)-β-D-glucans. The (1→3)-β-D-xylan is not a substrate and there is no action on oligosaccharides containing (1→4)-β-D-glucosidic linkages. Similarly, oligosaccharides and polysaccharides containing both (1→3)- and (1→4)-β-D-glucosidic linkages are not attacked. The products of hydrolysis of essentially linear (1→3)-β-D-glucans, such as laminaran, are laminaribiose, laminaritriose, laminaripentaose, and higher oligosaccharides, together with traces of D-glucose. It is apparent that only small amounts of laminaritetraose are formed. Laminaribiose is not a substrate, whilst laminaritriose is slowly attacked. Presumably the D-glucose detected during the later stages of the hydrolysis of laminaran is produced by the limited hydrolysis of laminaritriose. Laminaripentaose is attacked and yields the disaccharide plus the trisaccharide together with traces of D-glucose. These results show that the enzyme has an endo action pattern. This has been illustrated by the fact that it rapidly reduces the viscosity of CMP solutions with a corresponding slow release of reducing sugars (Fig. 5).

Since only small amounts of laminaritetraose were produced during the hydrolysis of laminaran, it is probable that the enzyme requires a significant number of (perhaps five or more) adjacent (1→3)-β-D-glucosidic linkages in its substrate for efficient binding. The malted barley enzyme, thus, has a similar specificity to the endo-(1→3)-β-D-glucanases from *Nicotiana glutinosa*[16] and *Phaseolus vulgaris*[15]. Other highly specific endo-(1→3)-β-D-glucanases have been found in ungerminated rye[17], germinated oats[18], pepper callus[19], and wheat and barley root callus[20]. All these enzymes have endo-action patterns, are highly specific for (1→3)-β-D-glucans, and produce oligosaccharides but little or no D-glucose. These enzymes, therefore, belong to the class EC 3.2.1.39 and can be distinguished from an endo-β-D-glucanase from *Rhizopus arrhizus*[21], which will also hydrolyse (1→4)-β-D-linkages adjacent to (1→3)-β-D-linkages in cereal D-glucans, thus belonging to the class EC 3.2.1.6.

We have shown in the present studies that β-D-glucan, prepared from ungerminated barley by the method of Preece and MacKenzie[6], is not attacked by the malted barley endo-(1→3)-β-D-glucanase. This substrate contains about 70% of (1→4)- and 30% of (1→3) linkages. The (1→3) linkages appear to be randomly distributed along the chain, since Smith-degradation studies[22] show the presence of a significant proportion of two and three adjacent (1→3)-linked D-glucose residues, although appreciable amounts of longer sequences were not detected. Recent work[23] has shown that β-D-glucan extracted from barley after germination for 48 h has an unexpectedly high viscosity, and hence molecular size, and on Smith-degradation there is evidence for sequences of up to five or more adjacent (1→3)-β-D-linkages. This sample of barley β-D-glucan is hydrolysed to a limited extent by malted barley endo (1→3)-β-D-glucanase. Moore and Stone[16] have reported that incubation of their sample of barley β-D-glucan with an endo-(1→3)-β-D-glucanase from *Nicotiana glutinosa* resulted in limited hydrolysis, as shown by a small decrease in the viscosity

of the substrate but without the production of reducing sugars. We consider that these observations show a difference in the structure of the substrates, rather than a difference in enzyme specificity, and emphasise the need for caution in drawing conclusions on the specificity of β-D-glucanases when "barley β-D-glucan" is the substrate.

The observation that malted barley endo-(1→3)-β-D-glucanase is activated by calcium ions[2] has been confirmed; however, the activity of the purified enzyme is also enhanced by low levels of Ba^{2+}, Mn^{2+}, and Co^{2+}. It has been shown[24] that some (1→3)-β-D-glucanases are stimulated by Mn^{2+} and Co^{2+}, and it has been suggested that these enzymes are not specifically ion-activated, since such chelating agents as EDTA have little effect on enzyme activity. In the present work, the malted barley endo-(1→3)-β-D-glucanase is inhibited by EDTA, although only a 12% loss of activity was detected by reducing power assays compared with a 44% loss previously reported[2], based on a viscometric assay, which is less reliable owing to the effect of metal ions on the viscosity of CMP. Although the effect of metal ions and EDTA on the activity of malted barley endo-(1→3)-β-D-glucanase might suggest that a metal ion is essential for activity, endo-(1→3)-β-D-glucanases from *Nicotiana glutinosa*[16] and *Phaseolus vulgaris*[15] are not inhibited by EDTA. Thus, unlike the α-amylases, dependence upon a cation for enzyme activity is not a common feature of the plant endo-(1→3)-β-D-glucanases.

The activity of malted barley endo-(1→3)-β-D-glucanases is higher in the presence of BSA (Table III). A number of workers have shown that the addition of BSA or other proteins to dilute solutions of purified polysaccharide hydrolases results in stabilization or an increase in activity; for example, *Myrothrecium verrucaria* cellulase was stabilized by BSA[25], as was the purified endo-(1→3)-β-D-glucanase from *N. glutinosa*[16].

The results of the inhibitor tests (Table IV) have provided additional information on the enzyme. D-Glucono-(1,5)-lactone, which is a competitive inhibitor of β-D-glucosidases but not of endo-β-D-glucanases[26], does not inhibit the enzyme. Chloro(*N*-α-tosyl-L-lysyl)methane, a substrate analogue of trypsin, which reacts with histidine residues at the active site[27], and also with cysteine residue in papain[28], does not effect enzyme activity. Although thiol and disulphide groups are important for the activity of some enzymes, it is doubtful whether they are essential for the malted barley enzyme. *N*-Ethylmaleimide was not an inhibitor, and mercuri-*p*-carboxyphenyl chloride (PCMB) was only slightly inhibitory (16%) at relatively high concentrations (0.2mM); at 0.01mM, mercuri-*p*-carboxyphenyl chloride has no effect[2]. Inhibition by mercuri-phenyl nitrate may not be due to reaction with thiol groups, since this inhibitor reacts with other groups in α-amylase[29] and catalase[30]. Similarly, inhibition by iodoacetate and Hg^{2+} is not diagnostic of any particular amino acid residue. However, there is preliminary evidence that both tryptophan and tyrosine residues are required for activity. The former is shown by the inhibition by 2-hydroxy-5-nitrobenzyl bromide[31] and *N*-bromosuccinimide[32], and the latter from the results with *N*-acetylimidazole[33]. The inhibition by carbodiimide may

confirm the requirement for tyrosine, but most likely indicates involvement of glutamyl, aspartyl, or a C-terminal amino acid residue[34].

Although there is little information on the inhibition of other β-D-glucanases by group-specific reagents, it has been shown that tryptophan and possibly histidyl residues are important for the activity of a cellulase from *Penicillium notatum*[35]. In lysozyme, tryptophan residues participate in binding and glutamic and aspartic acid residues in catalysis[36]. As our results (Table IV) show a marked similarity to those obtained with the endo-(1→3)-β-D-glucanase from *Nicotiana glutinosa*[16], it is possible that the endo-(1→3)-β-D-glucanases may well show structural homologies analogous to those found in, for example, the esterases[37] and the mammalian serine proteinases[38].

The function of a highly specific endo-(1→3)-β-D-glucanase, which develops during the germination of barley[2], is unclear since the evidence for a (1→3)-β-D-glucan in barley is equivocal. Taiz and Jones[39] have concluded from histochemical evidence that barley aleurone cell-walls contain (1→3)-β-D-glucosidic linkages and have suggested that the (1→3)-β-D-glucanase produced by these cells is responsible for the observed cell-wall degradation. Studies on the aleurone cell-walls of wheat by Fulcher *et al.*[40] do not support this view, but provide evidence for the presence of a ferulic acid–carbohydrate complex in these cell walls. Although these observations cast considerable doubts on the histochemical evidence presented by Taiz and Jones[39], the observations of Pollard[41] support the idea that the aleurone cells contain some (1→3)-β-D-glucan.

The major structural polysaccharides in cereals are pentosans, cellulose, and endospermic β-D-glucan. Since some samples of barley β-D-glucan are susceptible to the action of endo-(1→3)-β-D-glucanases[23], it is possible that the *in vivo* function of the malted-barley enzyme is to assist in the degradation of this glucan during germination.

ACKNOWLEDGEMENT

We are indebted to the Science Research Council for the award of a research studentship (to G. W.).

REFERENCES

1 D. J. MANNERS AND G. WILSON, *Biochem. J.*, 135 (1973) 11–18.
2 D. J. MANNERS AND J. J. MARSHALL, *J. Inst. Brew.*, 75 (1969) 550–561.
3 W. W. LUCHSINGER, *Cereal Sci. Today*, 11 (1966) 69–77.
4 E. J. BASS AND W. O. S. MEREDITH, *Proc. Amer. Soc. Brew. Chem.*, (1960) 38–47.
5 D. J. MANNERS AND G. WILSON, *Abstr. Int. Carbohyd. Conf.*, 6 (1972) 17–18.
6 I. A. PREECE AND K. G. MACKENZIE, *J. Inst. Brew.*, 58 (1952) 353–362.
7 D. J. MANNERS AND D. C. TAYLOR, *Arch. Biochem. Biophys.*, 121 (1967) 443–451.
8 W. L. CUNNINGHAM AND D. J. MANNERS, *Biochem. J.*, 90 (1964) 596–602.
9 A. E. CLARKE AND B. A. STONE, *Phytochemistry*, 1 (1962) 175–188.
10 J. F. ROBYT AND W. J. WHELAN, in J. A. RADLEY (Ed.), *Starch and its Derivatives*, 4th ed., Chapman and Hall, London, 1968, p. 432.

11 G. L. MILLER, *Anal. Chem.*, 31 (1959) 964.
12 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature*, 166 (1950) 444–445.
13 M. P. TOMBS AND P. AKROYD, *Shandon Instrument Applications*, Shandon Scientific Co. Ltd., London, 1967, No. 18.
14 T. E. NELSON, J. JOHNSON, E. JANTZEN, AND S. KIRKWOOD, *J. Biol. Chem.*, 244 (1969) 5972–5980.
15 F. B. ABELES, R. P. BOSHART, L. E. FORRENCE, AND W. H. HABIG, *Plant Physiol.*, 47 (1971) 129–136.
16 A. E. MOORE AND B. A. STONE, *Biochim. Biophys. Acta*, 258 (1972) 238–247; 248–264.
17 D. J. MANNERS AND J. J. MARSHALL, *Phytochemistry*, 12 (1973) 547–553.
18 D. J. MANNERS AND G. WILSON, unpublished work.
19 M. MANDELS, F. W. PARRISH, AND E. T. REESE, *Phytochemistry*, 6 (1967) 1097–1100.
20 O. L. GAMBORG AND D. E. EVELEIGH, *Can. J. Biochem.*, 46 (1968) 417–421.
21 E. T. REESE AND M. MANDELS, *Can. J. Microbiol.*, 5 (1959) 173–185.
22 M. FLEMING AND D. J. MANNERS, *Biochem. J.*, 100 (1966) 4P.
23 G. N. BATHGATE, G. H. PALMER, AND G. WILSON, *J. Inst. Brew.*, 80 (1974) 278–285.
24 C. G. C. CHESTERS AND A. T. BULL, *Biochem. J.*, 86 (1963) 38–46.
25 G. L. MILLER AND R. BIRZGALIS, *Anal. Biochem.*, 2 (1961) 393–395.
26 E. T. REESE, A. H. MAGUIRE, AND F. W. PARRISH, *Can. J. Biochem.*, 46 (1968) 25–34.
27 E. SHAW AND S. SPRINGHORN, *Biochem. Biophys. Res. Commun.*, 27 (1967) 391–397.
28 J. R. WHITAKER AND J. PEREZ-VILLASENOR, *Arch. Biochem. Biophys.*, 124 (1968) 70–78.
29 C. T. GREENWOOD AND E. A. MILNE, *Stärke*, 20 (1968) 101–107.
30 M. R. SOHLER, M. A. SIEBERT, C. W. KREKE, AND E. S. COOK, *J. Biol. Chem.*, 198 (1952) 281–291.
31 H. R. HORTON AND D. E. KOSHLAND, *J. Amer. Chem. Soc.*, 87 (1965) 1126–1132.
32 T. F. SPANDE AND B. WITKOP, *Methods Enzymol.*, 11 (1967) 498–506.
33 T. F. RIORDAN, W. E. C. WALKER, AND B. L. VALLEE, *Biochemistry*, 4 (1965) 1758–1765.
34 D. G. HOARE AND D. E. KOSHLAND, *J. Biol. Chem.*, 242 (1967) 2447–2453.
35 G. PETTERSSON, *Arch. Biochem. Biophys.*, 126 (1968) 776–784.
36 C. C. F. BLAKE, L. N. JOHNSON, G. A. MAIR, A. C. T. NORTH, D. C. PHILLIPS, AND V. R. SARMA, *Proc. Roy. Soc., Ser. B.*, 167 (1967) 378–388.
37 N. K. SCHAFFER, H. O. MICHEL, AND A. F. BRIDGES, *Biochemistry*, 12 (1973) 2946–2950.
38 B. S. HARTLEY AND D. M. SHOTTON, in P. D. BOYER (Ed.), *The Enzymes*, Vol. 3, 3rd ed., Academic Press, New York, 1971, p. 323–373.
39 L. TAIZ AND R. J. JONES, *Planta* (Berlin), 92 (1970) 73–84.
40 R. G. FULCHER, T. P. O'BRIEN, AND J. W. LEE, *Aust. J. Biol. Sci.*, 25 (1972) 23–34.
41 C. J. POLLARD, *Plant Physiol.*, 44 (1969) 1227–1232.