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Studies on β-Glucanases

SOME PROPERTIES OF A BACTERIAL ENDO-β-(1→3)-GLUCANASE SYSTEM

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A commercial enzyme preparation, originally obtained from a *Flavobacterium* (*Cytophaga*), was fractionated by continuous electrophoresis, giving a protein fraction which hydrolysed laminarin, carboxymethylpachyman, barley β-glucan, lichenin and cellodextrin in random fashion. This enzymic activity was not very stable. Ion-exchange chromatography and molecular-sieve chromatography on Bio-Gel P-60 showed that this activity was due to two specific β-glucanases, an endo-β-(1→3)-glucanase and an endo-β-(1→4)-glucanase. The two enzymes occur in both high- and low-molecular-weight forms, the latter endo-β-(1→3)-glucanase having a molecular weight of about 16000.

For some years, we have been interested in enzymic methods for the structural analysis of polysaccharides. As part of these studies, the action of a bacterial 'laminarinase' preparation on yeast β-glucan and on laminarin has been examined (Manners & Patterson, 1966; Fleming *et al.*, 1967). Enzyme action resulted in the selective hydrolysis of some β-(1→3)-glucosidic linkages, but β-(1→6)-glucosidic linkages were not attacked. For this type of work, it is essential that the specificity of the enzyme be defined in an unambiguous manner. We now describe the partial purification and some properties of the laminarinase preparation. A preliminary account of these results has been given elsewhere (Manners & Wilson, 1972).

Methods and Materials

Analytical methods

Reducing sugars were determined by a modified Nelson method (Robyt & Whelan, 1968), the reagents being calibrated against glucose. This method is more satisfactory than other modifications (Somogyi, 1945, 1952) of the original Nelson (1944) method, which are affected by low concentrations of salts or buffers. Protein was determined by a modified Lowry method (Miller, 1959), calibrated against bovine serum albumin. The protein content of column fractions was determined from the extinction at 280nm.

Separation methods

Descending paper chromatography was carried out on Whatman no. 1 paper by using ethyl acetate–pyridine–water (10:4:3, by vol.) as solvent, and an alkaline $AgNO_3$ reagent to detect the sugars.

Protein material was fractionated by molecular-sieve chromatography or by ion-exchange chromatography, usually at 0–4°C. Columns were prepared from the appropriate support materials, by using the manufacturers' instructions, and fractions were collected in a LKB 7000 Ultrorac Fraction Collector (LKB Instruments, Bromma, Sweden). The fractionation of protein solutions was also carried out on the paper curtain of a Beckman Spinco C.P. continuous-electrophoresis cell (Beckman Instruments, Palo Alto, Calif., U.S.A.). Protein solutions were concentrated in Amicon ultrafiltration cells by using Diaflo membranes (Amicon Ltd., High Wycombe, Bucks., U.K.) under a pressure of 380kPa (55lbf/in²).

Enzyme assays

For reductometric assays, appropriate amounts of substrate, buffer (0.1M-sodium acetate buffer, pH5.0, except where otherwise stated) and enzyme were incubated at 37°C for a suitable time. Enzyme action was stopped by the addition of Nelson (1944) copper reagent to samples of the digest, and the reducing power was determined. Enzyme digests were usually prepared in duplicate, and appropriate enzyme and substrate controls were also analysed. Suspensions of insoluble laminarin were warmed to 60°C before use.

Random hydrolysis of a β-(1→3)-glucan yields a mixture of glucose and laminarisaccharides. The results are expressed as total reducing power, in glucose equivalents, and not as μmol of substrate hydrolysed or product formed. For the routine assay of column fractions, results are expressed as the increase in extinction at 600nm compared with the substrate control, i.e. ΔE_{600}.

The following are typical compositions of digests. (a) For the assay of laminarinase activity in continuous-electrophoresis fractions, 0.1ml of 0.5% laminarin, 0.4ml of buffer (0.01M-sodium acetate, pH5.0) and 0.5ml of enzyme solution were incubated for 45min and then analysed. For the assay of ion-exchange- or molecular-sieve-chromatography fractions, and for the determination of units of β-(1→3)-glucanase activity, a standard digest containing 0.5ml of 1% laminarin, 0.3–0.4ml of the acetate buffer and 0.1–0.2ml of enzyme solution was incubated for 1h at 37°C. Under these conditions, 1 unit of activity is defined as the amount of enzyme that will produce 1μmol of reducing sugar (determined as glucose)/min at 37°C and pH5.0. (b) For laminaribiase activity, 0.5ml of 0.1% laminaribiose in the acetate buffer and 0.5ml of enzyme solution were incubated for 2.5h. (c) For assay of molecular-sieve-chromatography fractions for barley β-glucanase activity, 0.5ml of 0.2% barley β-glucan, 0.3ml of the acetate buffer and 0.2ml of enzyme fraction were incubated for 4h and then analysed. (d) For the assay of ion-exchange chromatography fractions for β-(1→4)-glucanase activity, 0.5ml of 1% CM-cellulose, 0.3ml of the acetate buffer and 0.2ml of enzyme fraction were incubated for 1h at 37°C and then analysed.

For viscometric assays, 1ml of 1% substrate [CM-pachyman (carboxymethylpachyman), barley β-glucan or CM-cellulose] and 1ml of the acetate buffer at 37°C were mixed with 0.5ml of enzyme solution, also at 37°C. Then 2ml of the digest was added to a no. 1 B.S.S. Ostwald viscometer in a constant-temperature water bath (37±0.02°C). Water had a flow time of 24.0s. Enzyme activity was expressed as the rate of increase of the reciprocal of specific viscosity with time, $\frac{d}{dt}\left(\frac{1}{\eta_{sp.}}\right)$. This relationship was linear with the concentrations of enzyme used.

For the qualitative survey of carbohydrase activity, digests contained 5mg of substrate and 5mg of enzyme preparation in 1ml of the acetate buffer, and were analysed after 24h at 37°C.

The isoamylase (i.e. debranching-enzyme) activity of certain fractions was measured reductometrically by incubating 2mg of amylopectin β-limit dextrin with 0.5ml of the acetate buffer and 0.5ml of enzyme fraction for 3h at 30°C (see Gunja-Smith *et al.*, 1970*a*).

Materials

The enzyme preparation used was originally obtained from Glaxo Research Ltd., Greenford, Middx., U.K. The preparation is described in British Patent no. 1048887, and was isolated from the culture medium of a micro-organism, which was temporarily designated L1, and deposited in the National Collection of Industrial Bacteria in Aberdeen as N.C.I.B. 9497. Although the organism was originally classified as a species of the genus *Cytophaga*, more recent evidence (Mitchell *et al.*, 1969) suggests that it is in fact a *Flavobacterium*. Since there is some doubt as to the classification of the bacterium, the genus will not be emphasized in the present paper. The enzyme preparation is now commercially available, as BDH L1 lytic enzyme (BDH Chemicals, Poole, Dorset, U.K.). It is a pale-cream powder that is dissolved in water, and any insoluble material is removed by centrifugation. There was no significant difference in the specific activity of two samples provided by Glaxo Research Ltd. (0.096 and 0.099 unit/mg of protein) and a commercial sample (0.093 unit/mg of protein).

The following substrates were laboratory samples available from previous work (e.g. Manners & Marshall, 1969) on β-glucans and β-glucanases: insoluble laminarin, barley β-glucan, lichenin, luteose, pustulan, cellodextrin, laminarisaccharides ranging in degree of polymerization from 2 to 7, 3^2-β-glucosyl-cellobiose, 4^2-β-glucosyl-laminaribiose, pachyman and CM-pachyman. CM-cellulose was a commercial sample used previously. The other substrates were laboratory samples of established purity.

Results

Carbohydrase activity of the bacterial enzyme preparation

Qualitative assays based on paper-chromatographic analysis showed that the following substrates were readily attacked: laminarin, pachyman, yeast glucan, lichenin. Cellodextrin and the disaccharides laminaribiose and cellobiose were slowly hydrolysed. Carbohydrates containing β-(1→6)-glucosidic linkages (gentiobiose, pustulan, luteose) and a wide range of other possible substrates (maltose, starch, *Rhodymenia palmata* xylan, Esparto xylan, raffinose, melizitose, sucrose, salicin, methyl α- and β-D-glucoside and β-(1→4)-linked D-mannose-containing oligosaccharides) were not attacked.

Purification of the bacterial enzyme preparation by continuous-curtain electrophoresis

The enzyme preparation (500mg) was dissolved in 10ml of water and dialysed against running tap water at 2°C for 18h, diluted to 25ml with distilled water, and the solution clarified by centrifugation. The solution was then applied to the paper curtain of the electrophoresis cell, and the proteins were fractionated in 0.01M-Tris–HCl buffer, pH7.6, at 750V and 33mA at room temperature (22±3°C). During electrophoresis the circulating buffer was cooled with ice. The protein content of the fractions was measured from the extinction at 280nm and a typical result is shown in Fig. 1(*a*). The distribution of enzyme

activities is shown in Fig. 1(*b*). In this experiment, all six racks of tubes showed a similar distribution; in fact, these conditions were highly reproducible for a number of electrophoretic fractionations. The β-(1→3)-glucanase activity was separated from a large amount of other protein. These fractions were removed from each rack, combined and freeze-dried, giving 214mg of freeze-dried enzyme preparation designated CE1. The initial dialysed solution had a specific activity [determined viscometrically against CM-pachyman and expressed as $\frac{d}{dt}\left(\frac{1}{\eta_{sp.}}\right)\Big/$ mg of protein] of 0.022. Preparation CE1, which contained only 4% of the total protein applied to the paper curtain, had a specific activity of 0.333, representing a 15-fold purification. Continuous electrophoresis in 0.01M-sodium citrate buffer, pH5.2, did not separate the β-(1→3)-glucanase activity from the bulk of the protein.

Properties of preparation CE1

The effect of pH on enzyme activity towards laminarin was measured reductometrically. The enzyme digests contained 0.1ml of 0.5% laminarin, 0.4ml of 0.1M-Tris–acetate buffer (pH range 3.3–9.0) and 0.5ml of a 0.2% solution of preparation CE1 in water (previously dialysed against water at 2°C for 18h), and were incubated at 37°C for 40min. The results (Fig. 2) show a fairly broad pH–activity curve with a maximum at pH5.0, and were generally similar to those obtained with a 0.1% solution of the original enzyme preparation. Further enzyme digests were therefore carried out in 0.1M-sodium acetate buffer, pH5.0.

The specificity of preparation CE1 was examined in digests containing 5mg of substrate and 1mg of preparation CE1 in 1ml of the buffer which were incubated at 37°C for 24h. The enzyme was then inactivated by heating (5min at 100°C), and the solutions were cooled, deionized with Biodeminrolit (The Permutit Co. Ltd., London W.4, U.K.), evaporated to dryness, and then dissolved in 0.1ml of distilled water for paper-chromatographic analysis. Laminaribiose gave a trace of glucose; CM-pachyman and laminarin gave a homologous series of laminarisaccharides ranging in degree of polymerization from 1 to 7; both lichenin and barley glucan gave cellobiose (major product), a trisaccharide, tentatively identified as 3^2-β-glucosylcellobiose and a trace of glucose; the xylan from *R. palmata* was not hydrolysed. With laminarin, further experiments showed that a mixture of laminaritriose to laminariheptaose was produced initially (after 3 and 6h) and that glucose and laminaribiose appeared later (after 24h). The results indicate random hydrolysis of β-(1→3)-glucans and of β-glucans containing both (1→3)- and (1→4)-linkages.

The relative rates of hydrolysis of 0.25% solutions of laminarin, lichenin and barley glucan by a 0.1% solution of preparation CE1 are shown in Fig. 3.

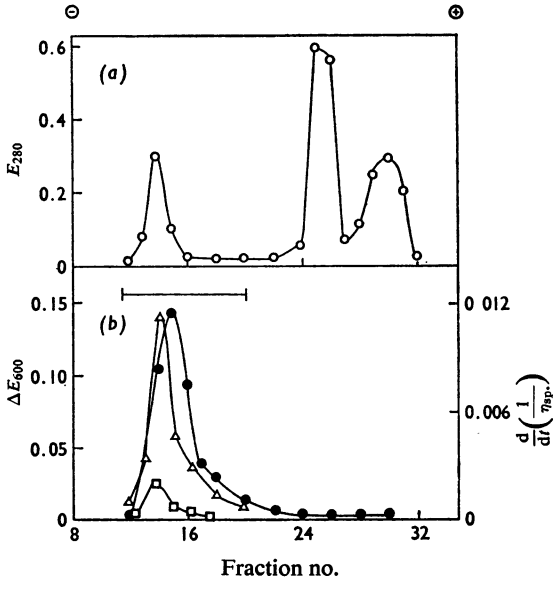

Fig. 1. *Continuous electrophoresis of bacterial enzyme preparation*

(*a*) Distribution of protein (○); (*b*) distribution of enzymic activities. For experimental details see the Methods and Materials section. □, Laminaribiase; ●, laminarinase; △, activity towards CM-pachyman. The fractions under the horizontal bar were pooled to give preparation CE1.

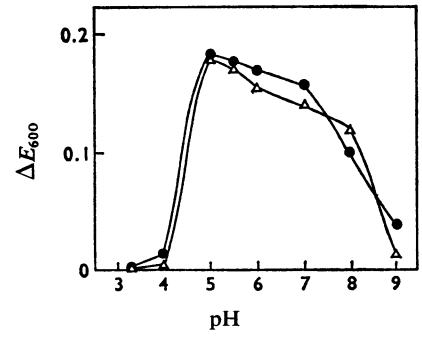

Fig. 2. *Effect of pH on laminarinase activity*

Experimental details are given in the text. ●, Original bacterial enzyme preparation; △, fraction CE1 from continuous electrophoresis.

The molecular weight of preparation CE1 was determined by molecular-sieve chromatography (Andrews, 1965) by using a column of Sephadex G-100, which had been calibrated with urease (mol.wt. 400000), bovine serum albumin (mol.wt. 67000) peroxidase (mol.wt. 40200), pepsin (mol.wt 35000), α-chymotrypsinogen and cytochrome *c* as reference proteins [supplied by Sigma (London) Chemical Co., London S.W.6, U.K.]. The void volume of the column was 102ml, and the elution volumes of α-chymotrypsinogen, laminarinase activity of preparation CE1 and cytochrome *c* were 189, 200 and 213ml respectively. As the two reference proteins have molecular weights of 25000 and 12400 respectively, the results indicate that the β-(1→3)-glucanase of preparation CE1 has a molecular weight of about 16600.

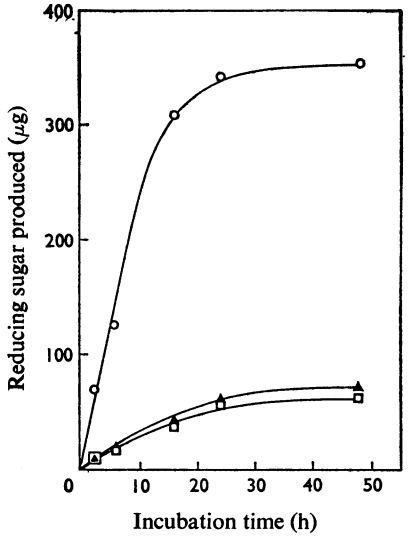

Fig. 3. *Rate of hydrolysis of various β-glucans by enzyme purified by continuous electrophoresis*

For details see the text. ○, Laminarin; ▲, barley β-glucan; □, lichenin.

Studies on the ultrafiltration, dialysis and stability of the enzyme preparation

In view of the above molecular-weight determination, the ultrafiltration of the original enzyme preparation was examined, by using a Diaflo PM-10 membrane. The enzyme preparation (1.0g) was dissolved in 200ml of 0.01M-Tris–HCl buffer, pH7.6, and the solution clarified by centrifugation. A sample (1ml) of the clear supernatant solution was retained for analysis, and the remainder concentrated to 50ml by ultrafiltration at an operating pressure of 380kPa (55lbf/in²). The retentate R1 (i.e. material retained by the membrane; volume 50ml) and the ultrafiltrate (i.e. material passed through the membrane; volume 150ml) were analysed for protein content and β-(1→3)-glucanase activity towards laminarin. The results (Table 1) show that about 50% of the protein passed through the membrane, but that all the enzymic activity was retained.

The results from several experiments have shown that the yield of protein and enzyme obtained from 1g of the original enzyme preparation varied between 200 and 280mg of protein and 15 and 21 units of enzyme activity, and that ultrafiltration resulted in a two- to three-fold increase in specific activity.

Solutions of the enzyme prepared by this method were not very stable on dilution and dialysis. During dialysis against distilled water at 2°C for 18h, about two-thirds of the activity was lost. Although 10mM-Ca^{2+} increased the activity of an undialysed solution by 32%, this concentration of metal ions only partly restored the activity of the dialysed solution. On incubation at 37°C, the enzyme preparation was partly stabilized by the addition of bovine serum albumin (500μg/ml) for periods of up to 3h, but with longer periods (24–48h), the protective action was lessened. Further details of these experiments are given elsewhere (Wilson, 1972).

Table 1. *Protein and endo-β-(1→3)-glucanase activity in enzyme fractions from ultrafiltration with a PM-10 membrane*

For details see the text. Both the endo-β-(1→3)- and endo-β-(1→4)-glucanase activities were retained by a PM-30 membrane.

Fraction	Total protein (mg)	Total endo-β-(1→3)-glucanase activity (units)	Specific activity
Initial extract	200	15.3	0.076
Retentate, R1	84	14.8	0.177
Ultrafiltrate	75	0	0

Fractionation of the bacterial enzyme preparation by ion-exchange chromatography

A solution of the enzyme (1.0g in 200ml of 0.01 M-Tris–HCl buffer, pH 7.6) was clarified and then concentrated to 25ml by ultrafiltration on a PM-10 membrane. The solution contained 3.6mg of protein and 0.84 unit of activity/ml. Part of the solution (5ml) was applied to a column (30cm×2.5cm) of DEAE-cellulose, which had been equilibrated with the same buffer. Fractions (3.2ml) were collected during elution with a linear gradient of NaCl (0–0.5M) in the Tris–HCl buffer. The distribution of protein and enzymic activity towards laminarin and amylopectin β-limit dextrin are shown in Fig. 4. Assayed against laminarin, fraction 42 had a specific activity of 0.49 unit/mg of protein compared with 0.08 and 0.23 unit/mg of protein for the initial enzyme solution before and after ultrafiltration, indicating a twofold increase in endo-β-(1→3)-glucanase activity by DEAE-cellulose chromatography. A second endo-β-glucanase with activity towards CM-cellulose and barley β-glucan was strongly bound to the anion-exchange resin and was eluted at higher concentrations of NaCl (0.5–1.0M). Fig. 5 shows the elution of endo-β-(1→3)-glucanase and endo-β-(1→4)-glucanase activities by a gradient of NaCl (0–1M) in Tris–HCl buffer, followed by further elution with 1M-NaCl.

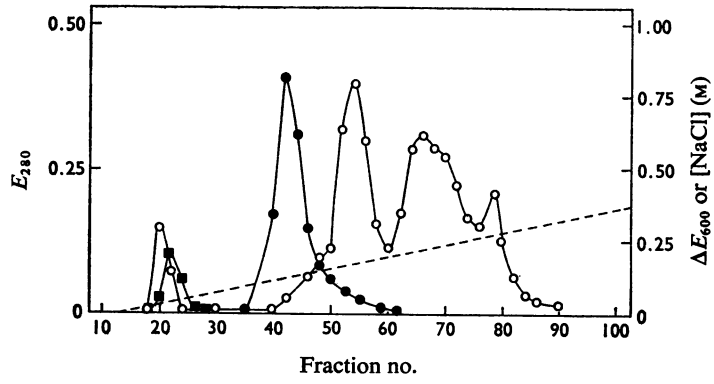

Fig. 4. *DEAE-cellulose chromatography of bacterial enzyme preparation at low salt concentrations*

Fractions of 3.2ml were collected. For other details see the text. ○, Protein; ●, laminarinase activity; ■, isoamylase activity; ----, [NaCl].

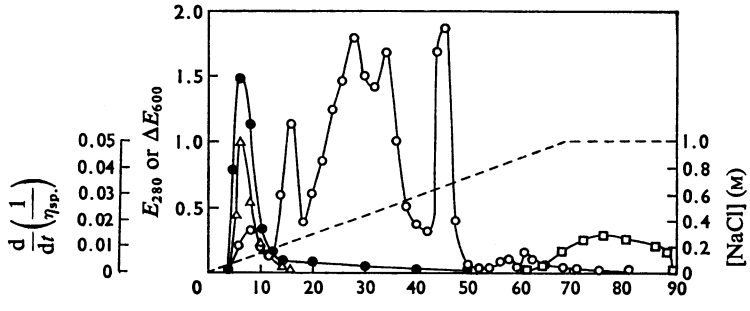

Fig. 5. *DEAE-cellulose chromatography of bacterial enzyme preparation at high salt concentrations*

Fractions of 3.5ml were collected. For other details see the text. ○, Protein; ●, laminarinase activity; △, activity towards CM-pachyman; □, activity towards CM-cellulose; ----, [NaCl].

Fractions from a DEAE-cellulose column, containing endo-β-(1→3)-glucanase activity, were concentrated by ultrafiltration and applied to the calibrated column of Sephadex G-100. The endo-β-(1→3)-glucanase activity was eluted after 204ml, equivalent to a molecular weight of about 15000. The endo-β-(1→4)-glucanase activity was not retained by a PM-10 membrane (suggesting a molecular weight of less than 10000), but was concentrated on a UM-2 membrane. It is noteworthy that the isoamylase activity, which has a molecular weight of about 120000 (Gunja-Smith *et al.*, 1970*a*), was not firmly bound to the DEAE-cellulose at pH7.6.

The specificity of the two β-glucanases was examined by incubating 0.1ml of the concentrated enzyme solutions with 2mg of substrate in 0.1ml of the acetate buffer for 24h at 37°C. Paper-chromatographic analysis showed that the endo-β-(1→3)-glucanase hydrolysed laminarin and CM-pachyman, yielding glucose and oligomers, but did not attack barley β-glucan or cellodextrin. The endo-β-(1→4)-glucanase attacked cellodextrin to produce cellobiose, cellotriose and higher oligosaccharides. This enzyme also hydrolysed barley β-glucan and lichenin, yielding a trisaccharide as the major product together with oligosaccharides of higher degree of polymerization. The trisaccharide was tentatively identified as 3^2-β-glucosylcellobiose by its paper-chromatographic mobility and its paper-electrophoretic mobility in 0.05M-sodium borate buffer, pH10.0, at a potential of 10V/cm for 1h. The mobilities were different from those of 4^2-β-glucosyllaminaribiose.

Fractionation of the bacterial enzyme preparation by molecular-sieve chromatography

A solution containing 12.0mg of protein and 2.30 units of endo-β-(1→3)-glucanase activity/ml was prepared by ultrafiltration on a PM-10 membrane. A 3ml sample was applied to a column (60cm × 2.5cm) of Bio-Gel P-60, which had been equilibrated with 0.01M-acetate buffer, pH5.0. The column was eluted at 2±2°C with the buffer, at a flow rate of 5ml/h, fractions of 3.5ml being collected. The distribution of protein in the fractions and enzymic activity towards laminarin, barley β-glucan and CM-cellulose is shown in Fig. 6. The results show two peaks of β-glucanase activity that are partially separated from each other. Since these peaks are eluted near the void volume of the column, this suggests that their molecular weights approach 60000, which is the exclusion limit of Bio-Gel P-60. Assayed against laminarin, fraction 25 had a specific activity of 0.435 unit/mg of protein compared with 0.07 and 0.20 for the initial enzyme solution before and after ultrafiltration. When incubated with 12.5mM-Ca^{2+} or 2.0mM-EDTA, the activity of fraction 25 was increased by 32% or decreased by 16% respectively.

Fractionation of L1 enzyme solutions on Bio-Gel P-60 under these conditions gave highly reproducible results. It was observed, however, that different experimental conditions produced elution patterns that differed from those shown in Fig. 6. For example, when molecular-sieve chromatography was performed at room temperature (22±3°C), low-molecular-weight forms of β-(1→4)-glucanase were obtained. Fig. 7 shows the results from such an experiment in which all the β-(1→4)-glucanase activity was obtained in a low-molecular-weight form, resulting in a complete separation of the activities towards laminarin and CM-cellulose. Fractions containing enzyme activity were combined and concentrated by ultrafiltration. A portion (0.1ml) of each enzyme solution was incubated at 37°C for 24h with 0.1ml of a 1% solution of laminarin or a 0.5% solution of barley

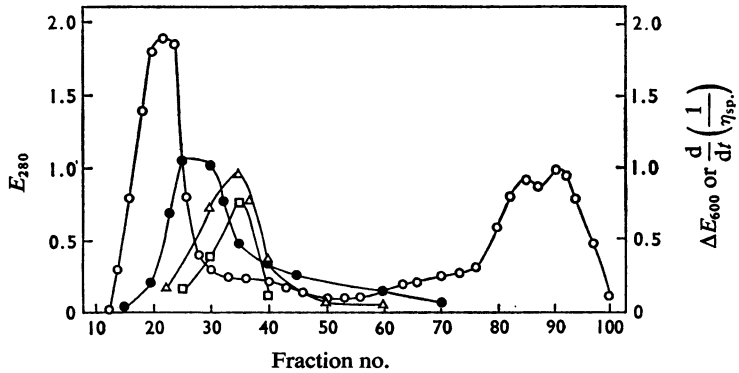

Fig. 6. *Fractionation of bacterial enzyme preparation on Bio-Gel P-60 at 2°C*

Fractions of 3.5ml were collected. For other details see the text. ○, Protein; ●, laminarinase; □, activity towards CM-cellulose; △, activity towards barley β-glucan.

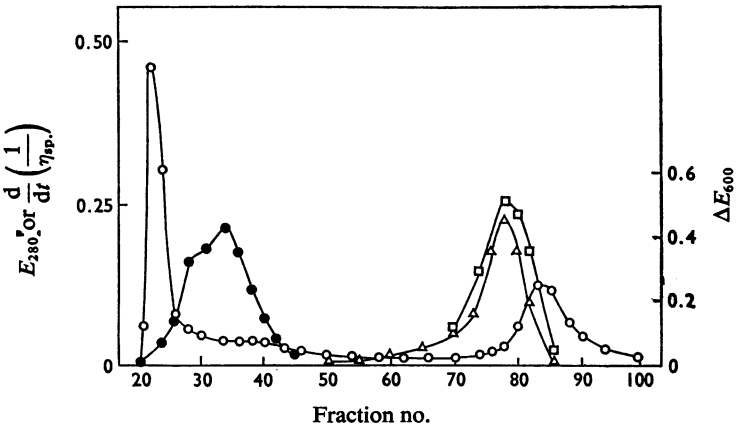

Fig. 7. *Fractionation of bacterial enzyme preparation on Bio-Gel P-60 at 25°C*

Fractions of 3.5ml were collected. For other details see the text. ○, Protein; ●, laminarinase; □, activity towards CM-cellulose; △, activity towards barley β-glucan.

β-glucan. Paper-chromatographic analysis showed that the β-(1→3)-glucanase fraction had no activity towards cellodextrin or barley β-glucan, but caused random hydrolysis of laminarin. It also caused a significant decrease in the viscosity of solutions of CM-pachyman. By contrast, the β-(1→4)-glucanase had no action on laminarin, but hydrolysed barley β-glucan giving 3^2-β-glucosylcellobiose as the major product together with traces of higher oligosaccharides. With cellodextrin it produced cellobiose, cellotriose and higher oligosaccharides.

Molecular-sieve chromatography was also carried out at 22±3°C on Sephadex G-200 as the chromatographic support. Fractionation of enzyme solutions that had been prepared in 0.01M-Tris–HCl buffer, pH7.6 (the same enzyme solution was used for ion-exchange chromatography), yielded both high- and low-molecular-weight forms of β-(1→3)-glucanase and β-(1→4)-glucanase activity.

The stability of the purified β-(1→4)-glucanase at 37°C was examined. After 0, 30, 60 and 180min at 37°C the relative activity of the enzyme towards barley β-glucan was 45, 26, 20 and 0 respectively (expressed as μg of reducing sugar per digest). This activity was therefore unstable at 37°C in the absence of substrate, and there was a complete loss of activity within 3h.

Discussion

The original patent description of the bacterial enzyme preparation mentioned a number of hydrolytic activities, including laminarinase, chitinase and keratinase. The preparation also contains a powerful starch-debranching enzyme, isoamylase (Gunja-Smith *et al.*, 1970*a*), which is proving to be a useful enzyme for the structural analysis of starch and glycogen-type polysaccharides (see, e.g., Gunja-Smith *et al.*, 1970*b*; R. Gordon, D. J. Manners, J. R. Stark & D. Yellowlees, unpublished work).

The present investigation has shown that the bacterial enzyme preparation contained a mixture of β-glucanases, accompanied by weak laminaribiase activity. The results from molecular-sieve and ion-exchange chromatography indicate that laminarin and barley β-glucan or cellodextrin were hydrolysed by specific enzymes, an endo-β-(1→3)-glucanase and an endo-β-(1→4)-glucanase. Continuous electrophoresis at pH7.6 failed to separate these activities. However, they were separated on columns of Bio-Gel P-60 (Fig. 7) and on DEAE-cellulose (Fig. 5). Endo-β-(1→4)-glucanase activity in a BDH bacterial enzyme preparation has been reported independently by Marshall (1972), who also used DEAE-cellulose chromatography to fractionate the preparation.

Both of the enzymes appeared to exist in high- and low-molecular-weight forms. In unfractionated solutions both activities were retained by Amicon PM-30 membranes and therefore presumably had molecular weights in excess of 30000. By contrast, during molecular-sieve chromatography at 25°C on Bio-Gel P-60 the endo-β-(1→3)-glucanase activity was eluted near the void volume (molecular weight approaching 60000), and the endo-β-(1→4)-glucanase activity was observed as a low-molecular-weight form (Fig. 7). Both the endo-β-(1→3)-glucanase and endo-β-(1→4)-glucanase were obtained as low-molecular-weight enzymes from DEAE-cellulose or continuous electrophoresis. The endo-β-(1→3)-glucanase had a

molecular weight of about 16000 (from DEAE-cellulose and continuous electrophoresis).

It would be premature at the present time to consider these changes in terms of monomer⇌trimer or tetramer interconversions, although this clearly represents one possibility. However, the original patent for the enzyme preparation reports the presence of proteolytic activity (pH optimum 8 and temperature optimum 37°C), and in further experiments on ion-exchange chromatography, we have found that those fractions containing endo-β-(1→3)-glucanase activity also show hydrolytic activity towards casein (D. J. Manners & G. Wilson, unpublished work). This latter activity is only partially decreased by protease inhibitors such as methylphenylsulphonyl fluoride. It is therefore possible that proteolytic digestion may play some part in the conversion of high-molecular-weight forms into smaller molecules. This type of digestion has been observed with pullulanase from *Aerobacter aerogenes*, where partial proteolytic degradation may occur during isolation and storage of the debranching enzyme (Eisele *et al.*, 1972).

The major purpose of this work was to examine the suitability of the bacterial enzyme preparation for structural studies on β-glucans. Without purification, it may be used for the degradation of simple β-(1→3)-glucans, such as callose. Since the preparation has no action on β-(1→6)-glucosidic linkages in oligosaccharides, e.g. gentiobiose and 6^2-β-glucosyllaminaribiose (Fleming *et al.*, 1967), or in polysaccharides, e.g. luteose and pustulan, it may be used with caution for the structural analysis of glucans containing both β-(1→3)- and β-(1→6)-linkages. This use may be illustrated by studies on yeast glucan (Manners & Patterson, 1966) and in the preparation of protoplasts from yeasts (Baird & Cunningham, 1971; San Blas, 1972). In the present work, linear β-glucans containing both (1→3)- and (1→4)-linkages, e.g. lichenin and barley β-glucan, were hydrolysed by the endo-β-(1→4)-glucanase, and not by the endo-β-(1→3)-glucanase. For this latter enzyme, long sequences of adjacent β-(1→3)-linked glucose residues appear to be required in the substrate.

In general terms, the β-glucanases are not very stable, and there is loss of activity during continuous electrophoresis and chromatography. This may be partly owing to proteolysis, and partly to a requirement for Ca^{2+} or other metal ions, although the present results do not lead to the conclusion that the endo-β-(1→3)-glucanase is a calcium metalloprotein, resembling α-amylase (see Robyt & Whelan, 1968). Since enzymic activity is diminished during incubation at 37°C (that of the low-molecular-weight form of the β-(1→4)-glucanase being decreased to 44 and 0% after 1 and 3h respectively), by dilution and by dialysis, any experimental conditions must ensure that an excess of active enzyme is always present. However, since the original enzyme preparation contains a significant amount of non-protein material, including galactomannan (D. J. Manners & J. C. Patterson, unpublished work), there is a danger that the products of β-glucan degradation could be contaminated with other carbohydrate material, unless some method of purification is used. The instability of the β-glucanases is similar to that reported for the isoamylase activity of this bacterial preparation (Gunja-Smith *et al.*, 1970*a*); in the absence of substrate, almost all this activity was lost within 1h at 40°C. Nevertheless, in spite of these limitations, the bacterial β-glucanase system represents a convenient source of enzyme for the structural analysis of β-glucans.

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