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Purification and properties of a β-1,6-glucanase from *Penicillium brefeldianum*

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An inducible endo-β-1,6-glucanase was purified from *Penicillium brefeldianum* by DEAE-cellulose, Bio-Gel P-150 and high-pressure liquid chromatography. The final preparation was essentially free from β-1,3-glucanase and β-glucosidase activities. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed one protein band with an M_r of 44000. The $V_{max.}$ and K_m values were calculated to be 624 units (μmol/min)/mg and 2.78 mg/ml respectively. The glucanase had lytic activity against mycelial cells of the yeast *Candida albicans*. The yield of purified β-1,6-glucanase from 100 mg dry weight of freeze-dried culture filtrate varied from 60 to 180 units.

The cell wall of the opportunistic pathogenic yeast *Candida albicans* is involved in the host immune response and is a target for antifungal drugs. The cell-wall glucan contains β-1,3 and β-1,6 linkages (Bishop *et al.*, 1960; Gopal *et al.*, 1984). The degradation of glucans by enzymes of known specificity is an important technique for determining the fine structure of polysaccharides such as cell-wall glucans. However, as pointed out by Duffus *et al.* (1982), the lack of specificity of the enzymes available limits the usefulness of this approach. One reason for the paucity of detailed information on β-1,6-glucans is the absence of β-1,6-glucanases purified free of β-1,3-glucanase activity.

In the present paper we report on the purification, properties and specificity of a 1,6-β-D-glucan glucanohydrolase (EC 3.2.1.75) from *Penicillium brefeldianum*.

Experimental

Materials

Pustulan, bovine serum albumin (for protein assays) and Azocoll were purchased from Calbiochem–Behring, La Jolla, CA, U.S.A. PAHBAH, *p*-nitrophenyl β-D-glucopyranoside, gentiobiose, horseradish peroxidase (type I), glucose oxidase (type II), *o*-dianisidine, Coomassie Brilliant Blue R and molecular-mass markers (bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, soya-bean trypsin inhibitor, lactalbumin and myoglobin) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Laminarin was supplied by the U.S. Biochemical Corporation, Cleveland, OH, U.S.A. DEAE-cellulose (DE 52) was from Whatman Separation Ltd., Maidstone, Kent, U.K. and Bio-Gel P-150 was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. The TSK-GEL G3000SW column was purchased from Toya Soda Manufacturing Co., Shin Nan-Yo City, Yamaguichi, Japan. Dowex 50W (X8; H^+ form), Biodeminrolit (mixed-bed cation/anion-exchanger) and SDS (specially pure) were from BDH Chemicals Ltd., Poole, Dorset, U.K. Other chemicals were of analytical-reagent grade.

Penicillium brefeldianum, strain QM 1873, was kindly supplied by Dr. E. T. Reese, U.S. Army Natick Research and Development Laboratories, Natick, MA, U.S.A. Free β-1,6-pachyman [from *Poria coccus* (Wolf)], its carboxymethylated derivative, *Ecklonia radiata* glucan, lactate oxidase (purified from *Mycobacterium smegmatis*) and ornithine transcarbamylase (purified from *Escherichia coli* W) were available in the laboratory.

Growth of organism and enzyme induction

P. brefeldianum QM 1873 was propagated on slopes comprising the following (per litre): yeast extract, 5g; peptone, 10g, glucose, 20g; NaCl, 1.0g; $MgSO_4,7H_2O$, 0.5g; KH_2PO_4, 1.0g; agar, 30g. Submerged cultures were prepared in 2-litre Erlenmeyer flasks containing 1 litre of the following medium (per litre): glucose, 3g; KH_2PO_4, 2g; $(NH_4)_2SO_4$, 1.4g; urea, 0.3g; $MgSO_4,7H_2O$,

Abbreviations used: PAHBAH, *p*-hydroxybenzoic acid hydrazide; SDS, sodium dodecyl sulphate; h.p.l.c., high-pressure liquid chromatography.

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0.3g; $CaCl_2$, 0.3g; trace elements (Fe, 1mg; Mn, 0.5mg; Co, 0.5mg; Zn, 0.8mg) and crude ground pustulan, 0.15% (w/v). The flasks were shaken on a gyratory shaker (200rev./min) at 28°C for approx. 3 weeks. The cultures were filtered (Whatman no. 1 paper) to remove cells and the combined filtrates were concentrated to about 50ml by ultrafiltration (Ulvac membrane G10T; exclusion limit 10kDa). The concentrate was centrifuged (3000*g* for 10min) to remove debris, dialysed against distilled water at 4°C and freeze-dried. The yield of dry material was approx. 50mg per litre of culture filtrate.

Purification of pustulan

Crude pustulan was ground with a mortar and pestle, sieved through two layers of gauze, and purified as follows.

Pustulan (4g) was refluxed at 100°C in 200ml of 0.15M-H_2SO_4 for 30min, cooled, and insoluble material was removed by centrifugation (3000*g* for 10min). After neutralizing with NaOH, Na^+ and SO_4^{2-} ions were removed batchwise with Biodeminrolit. The pustulan was precipitated with ethanol (85%, v/v), recovered by filtration on a sintered-glass funnel, washed with 95% (v/v) ethanol and air-dried. For enzyme assays, the purified pustulan was redissolved in water and reduced with $NaBH_4$ (0.3g/g of pustulan) at 100°C for 10min. The solution was then neutralized with acetic acid, passed through a Dowex 50W (X8; H^+ form) column to remove Na^+ ions, dried by rotary evaporation and washed five times with methanol to remove borate ions. The reduced pustulan was dissolved in water, precipitated with ethanol (85%, v/v) and air-dried.

Assay of reducing sugars and total carbohydrate

Reducing sugars were assayed by a modification of the method of Lever (1973). PAHBAH reagent was prepared immediately before use by mixing, in the given order, 5ml of each of the following: 1M-Na_2SO_3, 0.2M-$CaCl_2$, 0.5M-trisodium citrate and 5M-NaOH. This solution was then diluted to 100ml with water and PAHBAH (1.0g) was added. For the assay of reducing sugars, 1.0ml of this reagent was added to 0.1ml of sample containing 0–30μg of glucose or reducing sugar. Each sample was heated at 100°C for 10min, 3ml of water was added and the absorbance was read at 420nm.

Glucose was assayed by the glucose oxidase method of Lloyd & Whelan (1969), except that glycerol was omitted from the buffer. The assay was valid over the range 0–20μg of glucose.

Total carbohydrate was assayed by the method of Dubois *et al.* (1956), with glucose as the standard.

Determination of protein

Protein was assayed by the method of Bradford (1976), with crystalline bovine serum albumin as the standard. Protein in column eluents was monitored for A_{280}.

Enzyme assays

All enzyme assays were carried out at 50°C, in 25mM-sodium acetate buffer, pH4.2, unless otherwise stated.

β-1,6-Glucanase activity was assayed on reduced pustulan (5mg/ml) in a total volume of 500μl, for 15min. The reaction was terminated by heating each tube at 100°C for 10min. Reducing sugar was then determined by the PAHBAH method. The enzyme assay was linear up to 250μg of reducing sugar/ml. Controls without enzyme gave less than 20% of the values with glucanase present. One unit of β-1,6-glucanase was defined as the amount of enzyme that produces 1μmol of reducing-sugar equivalents per min.

To determine the optimal ionic strength, assays were performed in the following concentrations of sodium acetate buffer (mM): 2.5, 10, 25, 50, 100. The pH optimum of the β-1,6-glucanase was determined by assaying the enzyme in the following buffers (25mM final concn.): glycine/HCl (pH1.5, 2.0, 2.5), sodium citrate (pH3.0, 3.5), sodium acetate (pH4.0, 4.2, 4.5, 5.0, 5.5), imidazole/HCl (pH6.0, 6.5, 7.0). Separate controls, with no enzyme, were included for each pH.

The effect of pH on stability was determined after incubation at −20°C, 4°C and 25°C for 24h in buffers of the following pH values: 3.0, 4.2, 5.0, 6.0, 7.0, 9.0. The buffers used were those described in the previous paragraph, and 25mM-glycine/NaOH, pH9.0. The enzyme was then diluted with assay buffer and its activity was measured as described above.

To determine the temperature optimum, enzyme activity was assayed over the range 20–80°C for 15min. The temperature stability of the enzyme at pH4.2 and at pH7.0 was measured by incubating the enzyme in the absence of substrate for 1h at temperatures in the range 4–60°C. After incubation, tubes were cooled on ice and assayed by the usual method.

β-1,3-Glucanase was assayed in a similar manner to β-1,6-glucanase, with reduced laminarin (4mg/ml) as substrate. The assay was linear up to 300μg of reducing sugar/ml, and activity was defined as for the β-1,6-glucanase.

β-Glucosidase activity was determined using *p*-nitrophenyl β-D-glucopyranoside (6mM) in a total volume of 250μl. After incubation at 50°C for 30min or longer, the reaction was terminated with 0.5ml of 5% (w/v) trichloroacetic acid. NaOH (1M, 0.75ml) was added and the absorbance read at

405nm. One unit of activity was defined as the amount of enzyme that produces 1μmol of *p*-nitrophenol/min.

Proteinase activity was estimated from the release of dye from Azocoll (5mg) in a 1.5ml assay mixture. Microfuge tubes were incubated in a gyratory shaker (260 rev./min), centrifuged (10000*g* for 5min) and the A_{520} of the supernatants read. One unit of proteinase activity was defined as the amount of enzyme that produces an A_{520} of 1.0/h.

Purification of β-1,6-glucanase

Freeze-dried material from the culture filtrate was dissolved in 2–3ml of 25mM-imidazole/HCl buffer, pH7.4, centrifuged (3000*g* for 10min) and applied to a 60ml column (16cm × 2.2cm) of DEAE-cellulose (DE 52) that had been equilibrated with the imidazole buffer at 4°C. The column was washed with 10ml of buffer and then eluted with 400ml of a pH gradient of 50mM-sodium citrate buffer (200ml of pH6.2; 200ml of pH3.0). Fractions (5.6ml) were collected at a rate of 40–50ml/h, and those containing the major peak of β-1,6-glucanase were pooled, dialysed against distilled water at 4°C, and freeze-dried.

The glucanase from the DEAE-cellulose DE 52 column was dissolved in 2ml of 50mM-sodium acetate buffer, pH4.5, and chromatographed on a 260ml column (96cm × 1.85cm) of Bio-Gel P-150 equilibrated with acetate buffer at 4°C. Fractions (2.9ml) were collected at a rate of 3ml/h, and those with the highest β-1,6-glucanase/β-1,3-glucanase activity ratios were pooled, dialysed against distilled water at 4°C and freeze-dried.

The enzyme from the Bio-Gel P-150 column was dissolved in 0.2–0.5ml of 10mM-sodium phosphate buffer, pH6.5 [prepared in deionized water and membrane (0.45μm pore size)-filtered], centrifuged (10000*g* for 10min) and then chromatographed on a G3000SW h.p.l.c. column (1200mm × 7.5mm), equilibrated with the phosphate buffer, at room temperature. The pressure was 2760–3450kPa and the flow rate 1ml/min. Protein in the eluate was monitored at both 214nm and 280nm, and 0.5ml fractions were collected. Again, those fractions with the highest β-1,6-/β-1,3-glucanase activity ratios were pooled, dialysed against distilled water at 4°C, and freeze-dried. The column was calibrated with the following standards: lactate oxidase (molecular mass 344kDa), bovine serum albumin dimer (135kDa), ornithine transcarbamylase (112.5kDa), bovine serum albumin (66kDa), ovalbumin (45kDa), myoglobin (16.7kDa) in (*a*) 100mM-sodium phosphate buffer, pH7.0, containing 200mM-Na_2SO_4, and (*b*) 10mM-sodium phosphate buffer, pH6.5.

Polyacrylamide-gel electrophoresis

The method of Laemmli (1970) for vertical-slab (10cm × 8cm × 0.5cm) SDS/polyacrylamide-gel electrophoresis was performed with the mini-gel apparatus described by Matsudaira & Burgess (1978). Separation was on a gradient of 9–15% (w/v) acrylamide, and the stacking gel was 4% (w/v) acrylamide. Samples were dissolved in the buffer [3% (w/v) SDS/6M-urea/50mM-β-mercaptoethanol/0.005% Bromophenol Blue/62.5mM-Tris/HCl, pH6.7) and heated at 70°C for 4min before loading. Gels were stained with Coomassie Brilliant Blue R, destained, and scanned with an LKB densitometer. A standard curve was constructed with the following molecular-mass markers: bovine serum albumin (66kDa), ovalbumin (45kDa), glyceraldehyde-3-phosphate dehydrogenase (36kDa), carbonic anhydrase (29kDa), soya-bean trypsin inhibitor (20.1kDa) and lactalbumin (14.2kDa).

Paper chromatography

Oligosaccharides were separated by descending paper chromatography for 64h on Whatman no. 1 paper with propan-1-ol/ethyl acetate/water (7:1:3, by vol.) as the solvent system. Spots were detected by silver staining (Trevelyan *et al.*, 1950). Oligomers with β-1,6-linkages were obtained from a partial acid hydrolysate of pustulan.

Results

Induction of β-1,6-glucanase

The β-1,6-glucanase was inducible (Fig. 1) and its activity in the culture filtrate reached a maximum value after 3 weeks incubation at 28°C. Activity varied over the range 450–1450 units/litre with crude pustulan as inducer, but in one experiment it was 5800 units/litre. The yield of enzyme was lower (180 units/litre) with acid-purified pustulan.

Purification of β-1,6-glucanase

Typical results for the purification of the β-1,6-glucanase are given in Table 1. The crude culture filtrate material contained about 9–13% (w/w) of protein and about 40% (w/w) of carbohydrate. After fractionation on DEAE-cellulose, the total protein that could be measured in the column fractions increased such that 40% (w/w) of the initial dry weight could be accounted for as protein. Since this step effected partial separation of protein and carbohydrate, this disparity may be due to masking of protein by carbohydrate in the crude material.

The DEAE-cellulose chromatography separated all of the carbohydrate and proteinase, and most of

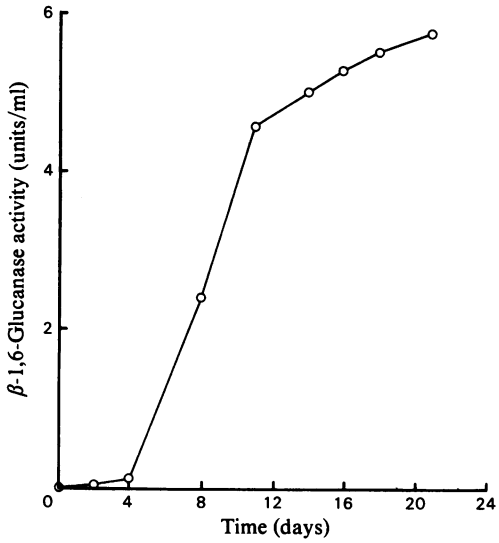

Fig. 1. *Induction of β-1,6-glucanase*
β-1,6-Glucanase activity in the culture filtrate of *P. brefeldianum* was monitored over the 3 weeks after inoculation. Portions of the culture were sampled at the times shown, centrifuged, and the supernatants were assayed for β-1,6-glucanase activity.

ratio of β-1,6-/β-1,3-glucanase activities was thus increased from 2–6-fold to 2500–3000-fold; a higher degree of purity was obtained when two TSK-GEL columns were used in series, as shown in Table 1. The overall degree of purification (Table 1) was 7080-fold with respect to β-1,3-glucanase. In the final preparation, β-1,3-glucanase activity was only just detected by using undiluted enzyme and increasing the incubation time from 15 to 180min; β-1,6-glucanase was measured on 100-fold dilutions for 15min. The yield of purified β-1,6-glucanase from 100mg dry weight of freeze-dried culture filtrate (i.e. from 2 litres of culture) varied from 60 to 180 units.

Analysis of the final purified preparation by SDS/polyacrylamide-gel electrophoresis gave one band with Coomassie Blue staining (Fig. 3). The more sensitive silver stain did reveal other bands (results not shown).

Properties of the purified β-1,6-glucanase

Ionic strength optimum. The optimum concentration of sodium acetate for assay of the enzyme was 25mM. Activity at 2.5mM and at 100mM was approx. 90% of the maximum.

Table 1. *Purification of β-1,6-glucanase from P. brefeldianum*
Abbreviation used: n.d., not determined.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	β-1,6-/β-1,3-Glucanase activity ratio
Culture filtrate	1.9	33.8*	581.4	17.2	0.96
DEAE-cellulose eluate	34	4.9	182.6	37.3	26
Bio-Gel P-150 eluate	20	n.d.	215.1	n.d.	501
TSK-GEL G3000SW eluate†	1.8	0.15	60.8	405	6800

* Obtained from cumulative protein contents of DEAE-cellulose fractions.
† Results shown are for two TSK-GEL columns in series.

the protein, β-1,3-glucanase and β-glucosidase from the β-1,6-glucanase (Fig. 2). This separation resulted in a 10–30-fold increase in the β-1,6-/β-1,3-glucanase activity ratio.

The Bio-Gel P-150 column (V_0 55ml) removed most of the remaining protein and β-1,3-glucanase, with 60–100% total recovery of β-1,6-glucanase and a 25–65-fold increase in the β-1,6-/β-1,3-glucanase activity ratio.

Chromatography on the TSK-GEL column separated essentially all of the remaining β-1,3-glucanase and β-glucosidase from the β-1,6-glucanase, with 50–60% total recovery of the latter. The

Effect of pH. The optimum pH for assay of the enzyme was 4.2, as shown in Fig. 4. At −20°C, the enzyme was stable at pH5.0–6.0 in the absence of substrate. At 4 and 25°C there was no loss of activity after 24h at pH7.0; at pH5.0, 80–85% of the activity was retained. The enzyme was less stable as the pH was increased above 8.0 or decreased below 5.0.

Effect of temperature. The optimum temperature for assay of the enzyme was 50°C (Fig. 5). When the enzyme was incubated for 1h at pH4.2, activity decreased steadily as the temperature of incubation was increased from 4°C (no loss of activity) to

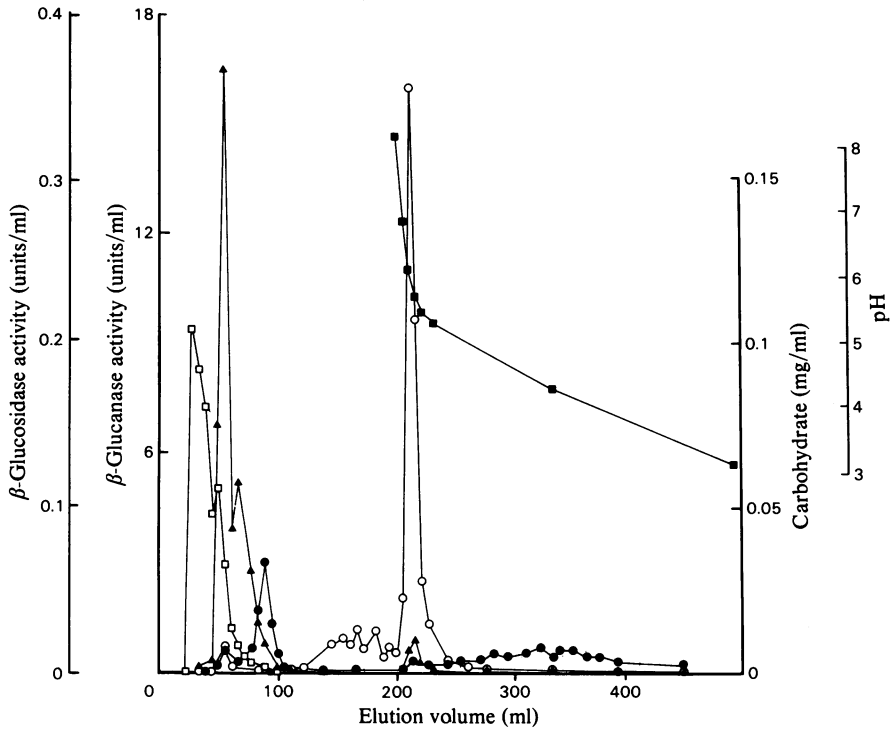

Fig. 2. *DEAE-cellulose chromatography of β-1,6-glucanase*
Crude culture filtrate material (92mg) was chromatographed on a DEAE-cellulose column as described in the text. Fractions (5.6ml) were analysed for β-1,6-glucanase activity (○), β-1,3-glucanase activity (●), β-glucosidase activity (▲), carbohydrate (□) and pH (■). Selected fractions are represented in the graph.

60°C (2% of initial activity). At pH7.0, however, the enzyme was stable at temperatures up to 30°C, above which there was a sharp decrease in activity to 7% at 50°C (Fig. 5).

Stability. The β-1,6-glucanase was stable at −20°C and pH6.0 for at least 2 months. Freezing and thawing resulted in the loss of approx. 10% activity after 12 cycles.

Kinetics. The β-1,6-glucanase exhibited classical Michaelis–Menten kinetics, and a double-reciprocal plot (Fig. 6) gave a $V_{max.}$ of 624 units/mg and a K_m of 2.78mg/ml. On the basis of a subunit molecular mass of 44kDa, the catalytic-centre activity was calculated to be 471 mol of reducing sugar/s per mol of enzyme. A direct linear plot gave a $V_{max.}$ value of 598 units/mg and a K_m of 2.38mg/ml.

Activity towards other substrates. When the purified β-1,6-glucanase was tested for activity towards gentiobiose, pachyman and carboxymethyl-pachyman (each at 5mg/ml in the assay), the reducing sugar produced was less than 2% of that released from pustulan. The corresponding degree of hydrolysis of *Ecklonia radiata* glucan, which contains both β-1,6 and β-1,3 linkages, was 4–5%; of this, less than 10% of the reducing sugar released was glucose. The ratio of activities towards *p*-nitrophenyl β-D-glucoside and pustulan was 7100.

Nature of β-1,6-glucanase action. Under the standard assay conditions, less than 3% of the released reducing sugar was glucose. However, this value increased gradually as the time of enzyme incubation was extended, and after 72h glucose accounted for 8.5% of the reducing sugar, which was 45% of the total carbohydrate.

Paper chromatography of 20min, 24h and 72h hydrolysates supported these results, revealing a gradual accumulation of spots which co-migrated with gentiobiose and β-1,6 oligomers. Glucose spots were visible in the 24h- and 72h-hydrolysate chromatograms, but were much lighter than the spots representing the higher-order sugars. These results indicate that the enzyme is an endoglucanase.

Determination of molecular mass. From SDS/polyacrylamide-gel electrophoresis a minimal molecular mass of 44kDa was calculated for the

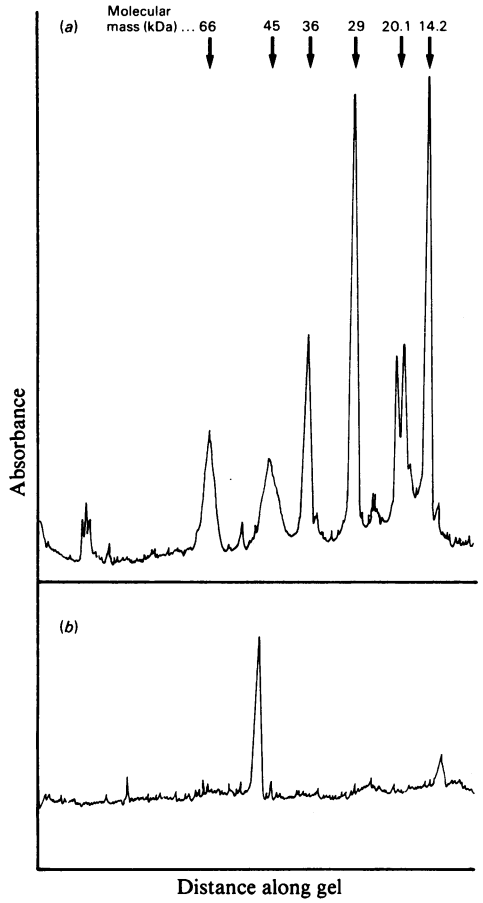

Fig. 3. *Polyacrylamide-gel electrophoresis of purified β-1,6-glucanase*
SDS/polyacrylamide-gel electrophoresis of molecular-mass markers (approx. 0.5μg of each) and purified β-1,6-glucanase (approx. 5μg) was performed as described in the text. The gel was stained with Coomassie Blue, destained, and scanned with an LKB densitometer. The profiles show: (*a*) markers of indicated molecular-mass values (kDa); (*b*) β-1,6-glucanase.

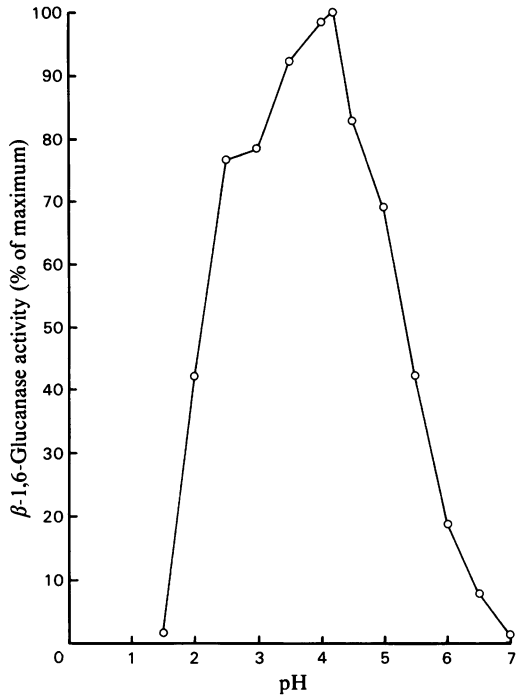

Fig. 4. *Effect of pH on activity of β-1,6-glucanase*
The effect of pH on the activity of the purified β-1,6-glucanase was measured at the pH values shown, using buffers described in the text.

β-1,6-glucanase. The elution volumes on the TSK-GEL column indicated values of 61.5kDa in low-ionic-strength buffer (10mM-phosphate, pH6.5) and 16kDa in 100mM-phosphate buffer, pH7.0, containing 200mM-Na_2SO_4. Furthermore, the elution volume of the β-1,6-glucanase on the Bio-Gel P-150 column was 160ml, which was greater than the V_e for myoglobin, and suggested an apparent molecular mass of less than 17kDa. These data indicate that the enzyme behaves anomalously on both Bio-Gel and TSK gel-permeation columns.

Discussion

In the present study with *P. brefeldianum*, the β-1,6-glucanase was induced by pustulan, as reported by Reese *et al.* (1962). Induction was also found with a number of other fungal and bacterial systems (Shibata & Fukimbara, 1972, 1973; Fleet & Phaff, 1974; Miyazaki & Oikawa, 1976). However, Santos *et al.* (1977) claimed that the β-1,6-glucanase of *Penicillium italicum* was constitutive and subject to catabolite repression by glucose and other carbon sources that support active growth. Stimulation of β-1,6-glucanase activity in the presence of glucose was found with *Trichoderma viride* (del Rey *et al.*, 1979).

The β-1,6-glucanase of *P. brefeldianum* was purified by conventional techniques, which have been used for the purification of the enzyme from other sources (Shibata & Fukimbara, 1973; Yamamoto *et al.*, 1974; Rombouts *et al.*, 1978; Katohda *et al.*, 1979). The final preparation was pure as judged by Coomassie Blue staining of the SDS/polyacrylamide gel, but, most important, the β-1,3-glucanase activity was less than 0.02% of the β-1,6-glucanase activity. A comparison of the properties

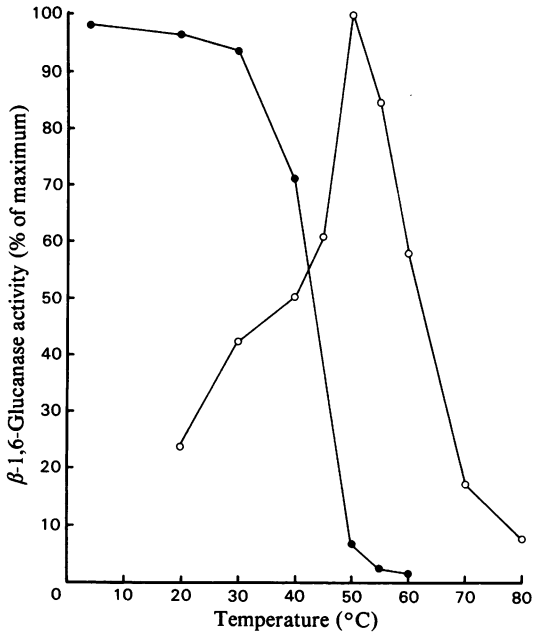

Fig. 5. *Effect of temperature on the activity and stability of β-1,6-glucanase*

The activity of the purified β-1,6-glucanase was measured at the temperatures shown (○). The effect of temperature on the stability of the enzyme was determined by incubating in 25mM-imidazole/HCl buffer, pH 7.0, for 1 h at the temperatures indicated and measuring activity by the standard assay (●).

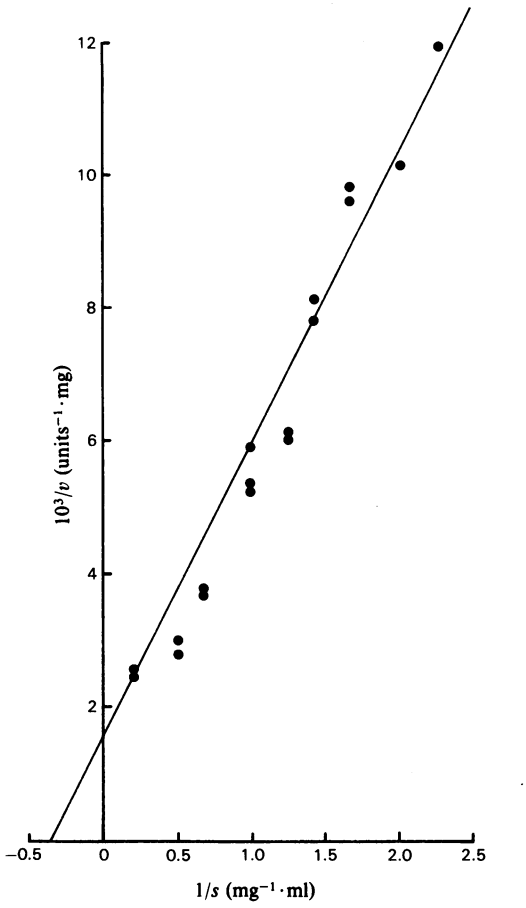

Fig. 6. *Double-reciprocal plot for β-1,6-glucanase*

β-1,6-Glucanase activity was measured at pustulan concentrations varying from 0.44 to 5.0mg/ml to obtain a double-reciprocal plot, from which $V_{max.}$ and K_m values were calculated.

of β-1,6-glucanases purified from other sources shows the preparations to have variable activities towards β-1,3-glucans (Table 2). In particular, the β-1,6-glucanase from *Rhizopus chinensis* and the lytic enzyme from *Bacillus circulans* had β-1,6-/β-1,3-glucanase activity ratios of approx. 3 and 5 respectively. Clearly either the enzymes were not pure or each single enzyme has a broad specificity towards β-1,3-linked glucans. Rombouts *et al.* (1978) pointed out that the products of laminarin degradation by the *B. circulans* lytic β-1,6-glucanase were gentiobiose and glucose, and that gentiobiose could only arise from cleavage of the β-1,3 linkages adjacent to β-1,6-linked glucose units at branch-points. Thus this glucanase acts as a debranching enzyme on β-1,3-glucans. The β-1,6-glucanases isolated from *P. italicum*, *Acinetobacter* sp., *Mucor hiemalis* and *B. circulans* (non-lytic enzyme) all exhibited some activity towards β-1,3-glucans. In general, the β-1,6-glucanases did not exhibit β-glucosidase activity (Table 2).

There is variation in the K_m values reported for β-1,6-glucanases; generally values of 1–3mg of substrate/ml were published. The $V_{max.}$ for the *P. brefeldianum* β-1,6-glucanase was considerably higher than those reported for the enzymes from other sources. The catalytic-centre activity of 471 mol of reducing equivalents/s per mol of enzyme is in the same range as those found for other glucan hydrolases such as cellulase (Tong *et al.*, 1980).

The pH and temperature optima for the purified β-1,6-glucanase found in the present study were similar to those for other β-1,6-glucanases (Table 2). The molecular-mass values reported for β-1,6-glucanases are in the range 30–50kDa, and all enzymes are monomeric. The value of 44kDa determined in the present study for the *P. brefeldianum* β-1,6-glucanase was a minimum molecular mass, but on the basis of results from other sources we conclude that the enzyme is probably monomeric.

Table 2. *Properties of purified microbial β-1,6-glucanases*

A plus sign (+) in the 'homogeneity' column indicates that adequate documentation was provided for a pure preparation. Abbreviation used: n.d., not determined.

Organism	Reference	β-1,6-Glucanase homogeneity	K_m (mg/ml)	$V_{max.}$ (μmol of reducing equivalents/min per mg of protein)	pH optimum	Temperature optimum	Molecular mass (kDa)	Activity relative to β-1,6-glucanase activity (100%)	
								β-1,3-Glucan	*p*-Nitrophenyl β-D-glucoside
P. brefeldiamum	The present paper	+	2.78	624	4.2	50	44	0.015	0.014
P. brefeldianum	Reese *et al.* (1962)	−	0.4–1.3	n.d.	4.0	50	n.d.	n.d.	n.d.
Acinetobacter sp.	Katohda *et al.* (1979)	+	n.d.	n.d.	5.5	n.d.	55	1.8	0
Penicillium italicum	Santos *et al.* (1979)	−	3.8	n.d.	5.0	60	40	1.25	1.9
Mucor hiemalis	Miyazaki & Oikawa (1976)	−	1.73	22.4	4.5	55	n.d.	2.7	0
Gibberella fujikuroi	Shibata & Fukimbara (1973)	+	0.08	4.6	5.0	n.d.	37	0	0
Rhizopus chinensis	Yamamoto *et al.* (1974)	+	1.69	0.9	5.5–6.0	60	30	33.6	n.d.
Bacillus circulans Lytic enzyme	Rombouts & Phaff (1976); Rombouts *et al.* (1978)	+	0.29	8.8	6.0–6.5	n.d.	54	20	+
Non-lytic enzyme	Rombouts *et al.* (1978)	+	0.83	101.1	5.0	n.d.	52	0.13	n.d.

The preparation of β-1,6-glucanase which we describe, and which is essentially free from β-1,3-glucanase and β-glucosidase activities, can be used for specifically degrading β-1,6-glucans from cell walls of *C. albicans* and for producing spheroplasts of organisms containing β-1,6-glucans in the cell wall.

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