Purification and Properties of (1→3)- α -Glucanases from *Bacillus circulans* WL-12

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Extracellular endo-(1→3)- α -glucanase (EC 3.2.1.59) was optimally induced in *Bacillus circulans* WL-12 when grown in a mineral medium containing intact cells of *Schizosaccharomyces pombe* or extensively purified (1→3)- α -glucan as the carbon source. The enzyme activity was separated from most other proteins, including the β -glucanases, by affinity adsorption of the enzyme on water-insoluble colloidal (1→3)- α -glucan. The enzyme was released by allowing it to hydrolyse this substrate and purified further by DEAE-agarose chromatography and polyacrylamide P-150 gel filtration. Passage over DEAE-agarose apparently also separated a minor (1→3)- α -glucanase component. The principal (1→3)- α -glucanase was specific for the (1→3)- α -glucosidic bond and hydrolysed (1→3)- α -glucan endolytically to a mixture of nigerose and glucose with a transient accumulation of nigerotetraose. The reaction proceeded at a constantly declining rate with either colloidal (1→3)- α -glucan or with the soluble carboxymethyl-(1→3)- α -glucan as substrate. Nigeran, containing alternating (1→3)- α - and (1→4)- α -linkages, was not hydrolysed. Substrate dependence showed Michaelis–Menten kinetics. There was a pH optimum of 7.5 to 8.5 with a pronounced shoulder at pH 5 to 7. The molecular weight was estimated by slab gel electrophoresis in sodium dodecyl sulphate to be 134000. The enzyme did not appear to require divalent metal ions because its activity was stimulated by EDTA. There was evidence for essential thiol groups. Carbohydrate was not detected in the enzyme.

INTRODUCTION

Some yeasts and many higher fungi contain (1→3)- α -linked glucan in their cell walls (Bacon *et al.*, 1968; Horisberger *et al.*, 1972; Kanetsuna *et al.*, 1969; Wessels & de Vries, 1973). In addition there are β -glucans that contribute significantly to the strength and rigidity of the cell walls (Phaff, 1971). A well characterized (1→3)- α -glucanase, free of other carbohydrases, would be extremely useful in removing (1→3)- α -glucan from other polysaccharides or in serving as an aid in the structural analysis of this polysaccharide. Additionally, such an enzyme, utilized in conjunction with other appropriate hydrolases, should improve the formation of protoplasts in fungi that contain this wall glucan. Snail digestive fluid, which is commonly employed for this purpose, contains a mixture of hydrolases but lacks (1→3)- α -glucanase activity.

There are relatively few reports of the occurrence of (1→3)- α -glucanases in fungi (Hasegawa *et al.*, 1969; Guggenheim & Haller, 1972; Reese *et al.*, 1972; Zonneveld, 1972; Walker & Hare, 1977) or in bacteria (Bacon *et al.*, 1968; Ebisu *et al.*, 1975; Pitts & Keele, 1973; Reese *et al.*, 1972; Tanaka & Phaff, 1966; Vrsanská *et al.*, 1974; Imai *et al.*, 1977*a, b*). Four (1→3)- α -glucanases have been purified and studied in some detail (Hasegawa *et al.*, 1969; Ebisu *et al.*, 1975; Imai *et al.*, 1977*a, b*; Walker & Hare, 1977) while another two have been partially purified (Zonneveld, 1972; Guggenheim & Haller, 1972).

Bacillus circulans WL-12, isolated as a lytic organism on yeast cell walls (Tanaka & Phaff,

1965), has been studied extensively with regard to its synthesis of a number of extracellular β -glucanases when grown on baker's yeast cell walls or on the alkali-insoluble baker's yeast glucan (Phaff, 1977; Tanaka *et al.*, 1978). Tanaka & Phaff (1966) noted that a culture filtrate of *B. circulans* WL-12 grown on *Schizosaccharomyces* cell walls caused complete clearing of these walls in an agar plate (cup plate test). Because cell walls of *Schizosaccharomyces* are known to contain (1 $\rightarrow$ 3)- α -glucan in addition to β -glucans (Bacon *et al.*, 1968), this indicated the possible presence of (1 $\rightarrow$ 3)- α -glucanase activity in *B. circulans*; this was later confirmed by Fleet & Phaff (1973).

The present paper describes the optimum conditions for synthesis of (1 $\rightarrow$ 3)- α -glucanase in *B. circulans* WL-12, its purification and properties.

METHODS

Organisms and culture conditions. *Bacillus circulans* WL-12 and *Schizosaccharomyces pombe* C-277 were obtained from the culture collection of the Department of Food Science and Technology, University of California, Davis, U.S.A. *Aspergillus niger* NRRL 326 was provided by Dr S. Kirkwood, Department of Biochemistry, University of Minnesota, U.S.A.

Bacillus circulans WL-12 was maintained on agar slants of Yeast Nitrogen Base (YNB) without amino acids (Difco) and 0.25% (w/v) (1 $\rightarrow$ 3)- α -glucan (or 0.1% glucose) in 0.1 M-sodium phosphate buffer, pH 6.5. For the production of (1 $\rightarrow$ 3)- α -glucanase, the bacteria were grown in liquid YNB medium (250 ml volumes in 1 l Erlenmeyer flasks) containing 0.1 M-sodium phosphate buffer, pH 6.5, and various concentrations of purified (1 $\rightarrow$ 3)- α -glucan or 0.5% (dry wt/v) of various *Sch. pombe* preparations. Flasks were inoculated with 24 h starter cultures (0.5% inoculum) which had been grown in the same medium, and then incubated at 30 °C on a rotary shaker (174 rev. min⁻¹) for periods up to 96 h. The cultures were centrifuged at 12000 g for 10 min at 2 °C to remove bacteria and cell debris and the supernatant culture fluid was dialysed for 48 h against 10 vol. distilled water (changed every 12 h) at 4 °C.

Aspergillus niger NRRL 326 was grown as described by Hasegawa *et al.* (1969) in 401 medium in a fermenter.

Schizosaccharomyces pombe C-277 was maintained and grown as previously described (Fleet & Phaff, 1973).

Cell count. Total cell counts were made in a Petroff-Häuser bacterial cell counter. Pairs of bacterial cells in which a cross-wall was distinctly visible were counted as two cells.

Preparation of (1 $\rightarrow$ 3)- α -glucan, yeast cells and yeast cell walls as growth substrates. (1 $\rightarrow$ 3)- α -Glucan was extracted from *A. niger* as described by Hasegawa *et al.* (1969) and Johnston (1965). The product ($[\alpha]_D^{25} + 254^\circ$, in 1 M-NaOH) gave no significant activity with endo-(1 $\rightarrow$ 3)- β -glucanase or endo-(1 $\rightarrow$ 6)- β -glucanase (Fleet & Phaff 1974b), or with a commercial α -amylase (Sigma). Partial acid hydrolysis (Johnston, 1965) followed by paper chromatography revealed nigerosaccharides in addition to glucose and a trace of maltose. Protein content was 0.16%. (1 $\rightarrow$ 3)- α -Glucan was also extracted from cell walls of *Sch. pombe* by the same procedure but not purified further.

Cell walls of *Sch. pombe* were prepared as described by Fleet & Phaff (1973) and then lyophilized. Fresh whole yeast cells, after washing with distilled water, were lyophilized and stored at 4 °C under vacuum.

Assay substrates. A highly purified preparation of (1 $\rightarrow$ 3)- α -glucan from *A. niger* was obtained from the laboratory collection (Fleet & Phaff, 1974a). The water-insoluble material was converted into a colloidal or a very fine suspension of swollen glucan that remained dispersed for several hours. A weighed portion of the glucan was dissolved in 1 M-NaOH and neutralized very slowly with 1 M-acetic acid with constant stirring. The colloidal glucan was washed four times with distilled water and then suspended in the assay buffer or distilled water to the appropriate concentration. Finally, the glucan was finely dispersed by repeatedly ejecting the suspension through a syringe (1 mm i.d.).

Carboxymethyl-(1 $\rightarrow$ 3)- α -glucan [CM-(1 $\rightarrow$ 3)- α -glucan] was prepared from the same insoluble (1 $\rightarrow$ 3)- α -glucan by a combination of the procedures established for other glucans (Clarke & Stone, 1962; Stone, 1972). The product had a degree of substitution of 0.17 (by the acid-wash method of Eyler *et al.*, 1947) and was soluble in water at 0.5% (w/v). A low degree of substitution was desirable in view of possible inhibition of activity with a substituted substrate (Almin & Erickson, 1968; Barras *et al.*, 1969; Nisizawa, 1973).

Saccharomyces cerevisiae cell walls, cellosaccharides, mannan, phosphomannan (*Hansenula holstii*) and isomaltosaccharides were obtained from the laboratory collection. Other substrates were amylose (hydrolysed) (Connaught Medical Research Laboratories, Toronto, Canada), *p*-nitrophenyl- α -D-glucopyranoside (Calbiochem), nigeran (Pierce Chemical Co., Rockford, Illinois, U.S.A.), gentiobiose (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), laminarin (Nutritional Biochemicals Corp.), pustulan (Calbiochem) and chitin (Calbiochem).

Enzyme assays. Glucanase activity was measured at 30 °C. The reaction was started by adding 0.25 ml enzyme to an equal volume of prewarmed substrate and stopped by adding 1 ml copper reagent (Nelson–Somogyi). Water (0.5 ml) was added to the inactivated mixture which was thoroughly mixed, and the reducing sugar concentration was determined. Assays were done in triplicate, with a reaction mixture containing no substrate as a control. Values that differed by not more than 2% were averaged. The final substrate concentrations used in standard assays were as follows. (a) (1→3)- α -Glucanase incubation mixtures contained 5 mg colloidal (1→3)- α -glucan ml⁻¹ or 3.5 mg CM-(1→3)- α -glucan ml⁻¹ in 75 mM-sodium succinate buffer, pH 6.0, or in 75 mM-glycine/NaOH buffer, pH 8.3; EDTA (1 mM) was normally included. (b) In a study of the effect of cations on the activity of (1→3)- α -glucanase a number of metal ions (as chlorides, except Pb²⁺ and Ag⁺ which were used as nitrates) were tested in concentrations of 1 and 0.01 mM at pH 6.0 (in 75 mM-sodium succinate buffer) and at pH 7.3 (in 75 mM-glycine/NaOH buffer) on CM-(1→3)- α -glucan. The enzyme was incubated for 15 min at 30 °C in buffer in the presence of the inhibitor before addition to the substrate. EDTA was omitted from the reaction mixtures. (c) Assay mixtures for (1→3)- β - and (1→6)- β -glucanase contained 5 mg of the appropriate substrate ml⁻¹ in 5 mM-sodium succinate buffer, pH 5.0. One unit of glucanase activity is defined as that amount of enzyme which releases 1 μ mol reducing sugar equivalent, expressed as glucose, min⁻¹ under the standard conditions.

Because the time course of (1→3)- α -glucanase activity of *B. circulans* WL-12 is not linear, all assays were incubated for a fixed time (10, 15 or 30 min) so that a pre-determined standard correction could be applied to obtain the initial rates.

Lytic activity on *S. cerevisiae* cell walls was determined as previously described (Rombouts & Phaff, 1976a). Phosphatase (Arnold, 1972) and proteolytic activities (Cabib & Ulane, 1973) were determined with *p*-nitrophenyl phosphate (Calbiochem) and Azocoll (Calbiochem) as the respective substrates, both at final concentrations of 5 mg ml⁻¹ in 25 mM-sodium succinate buffer, pH 6.0.

Trehalase and invertase activities were assayed by the formation of reducing sugars from trehalose and sucrose as the respective substrates, both at final concentrations of 5 mg ml⁻¹ in 50 mM-sodium succinate buffer pH 6.0. Other polysaccharide assays were incubated for 12 h and contained 5 mg substrate ml⁻¹ in 50 mM-sodium succinate buffer, pH 6.0.

Analytical measurements. Reducing sugars were determined by the method of Nelson–Somogyi (Spiro, 1966), using glucose as standard. Protein was determined by the Folin method of Lowry, with bovine serum albumin as standard. Total carbohydrate was estimated by the phenol/sulphuric acid method (Dubois *et al.*, 1956).

Paper chromatography. Products of enzymic digestion or partial acid hydrolysis were separated by descending paper chromatography using Whatman no. 1 paper at room temperature with propan-1-ol/ethyl acetate/water (6:1:3, by vol.; Johnston, 1965). Sugar spots were detected with alkaline silver nitrate (Trevelyan *et al.*, 1954).

Colloidal (1→3)- α -glucan (5 mg ml⁻¹ in 75 mM-sodium succinate buffer, pH 6.0) was incubated at 30 °C with a final concentration of 0.35 unit of purified enzyme ml⁻¹. [The CM-(1→3)- α -glucan assay was used at pH 6.0 to determine the enzyme concentration.] Samples were removed at intervals, heat-inactivated, and frozen. Products of the enzymic hydrolysis were identified by the R_{glucose} values and compared with oligosaccharide standards [prepared by partial acid hydrolysis of the purified (1→3)- α -glucan] chromatographed simultaneously and adjacently.

Column chromatography. DEAE-agarose (Bio-Rad) was equilibrated in a column (15×1.3 cm) with 5 mM-sodium phosphate buffer, pH 6.5. Elution of protein was done with a linear gradient to 0.5 M-NaCl in the same buffer (200 ml 5 mM-sodium phosphate buffer in the mixing chamber; 200 ml 0.5 M-NaCl in the same buffer in the reservoir). Fractions of 5.0 ml were collected by gravity flow each 10.5 min. A carboxymethyl (CM)-agarose (Bio-Rad) column (12.5×0.75 cm) was equilibrated with 10 mM-sodium succinate buffer, pH 5.0. Elution of protein was done with a linear gradient to 0.2 M-NaCl (75 ml salt/buffer solution and 75 ml buffer in the mixing chamber). The flow rate was 2.7 ml h⁻¹ and fractions of 1.26 ml were collected.

Molecular exclusion was done with polyacrylamide Bio Gel P-150 (column 69×2.6 cm; 385 ml bed volume; 104 ml void volume) or with agarose Bio Gel A-0.5 m (Bio-Rad) (column 73.5×2.2 cm; 278 ml bed volume; 104 ml void volume). Both materials were equilibrated with 50 mM-sodium succinate buffer, pH 6.0 and eluted with the same buffer by gravity flow. Fractions of 5.0 ml were collected at 17 ml h⁻¹ with the polyacrylamide gel and fractions of 2.5 ml at 18 ml h⁻¹ with the agarose gel. Samples applied to these columns were concentrated by ultrafiltration, using a PM-10 membrane (Amicon Corp., Lexington, Mass., U.S.A.).

Affinity adsorption of (1→3)- α -glucanase. Colloidal (1→3)- α -glucan was added (final concentration, 0.1% dry wt) to the crude culture filtrate which had been dialysed against distilled deionized water at 4 °C for 48 h. The suspension was slowly stirred for 15 min at approximately 10 °C, and then centrifuged at 14500 g for 10 min at 2 °C to collect the glucan. The adsorption step was repeated with another 0.1% of the glucan, and finally a third time with 0.05%. Assays of the culture filtrate between each step indicated that

60% of the original (1 $\rightarrow$ 3)- α -glucanase activity had been adsorbed in the initial application, 83% by the second step, and 91% after the third step.

The three glucan pellets were combined and diluted with 50 mM-sodium succinate buffer, pH 6.0, to approximately 40% of the original culture fluid volume. The buffer contained 0.01% sodium azide. This relatively high dilution of the pellet was used to minimize possible substrate inhibition during glucan digestion. The enzyme-glucan complex (40 ml) was dialysed at 30 °C against 200 ml of the same buffer. The digestion required 8 d with five changes of the buffer-oligosaccharide solution. When the ambient buffer no longer contained a significant concentration of reducing sugars, the enzyme solution was centrifuged to remove the small amount of insoluble material.

Gel electrophoresis. Discontinuous polyacrylamide gel electrophoresis by the thin slab technique (Ames, 1974) was used with the buffer system of Laemmli (1970). Molecular weights were estimated by the use of sodium dodecyl sulphate (SDS, 0.01%)–polyacrylamide (8%) gels according to the procedure of Weber & Osborn (1969). Protein standards were bovine serum albumin (mol. wt 68000; Sigma), phosphorylase-*a* (mol. wt 94000; Boehringer), β -galactosidase (mol. wt 130000; Worthington) and *Escherichia coli* RNA polymerase (β' sub-unit, mol. wt 165000; kindly provided by Dr R. H. Doi, University of California, Davis, U.S.A.). Protein (1 to 2 μ g, not exceeding 30 μ l in volume) was applied to each gel well, and a current was maintained for 2.5 to 3 h at 15 mA per slab. Protein bands within the slabs were detected by Coomassie Brilliant Blue (R-250) (Fairbanks *et al.*, 1971). Enzymic activity was detected in gels run with the same buffer system except that SDS, 2-mercaptoethanol and heating were omitted. During protein migration, the gel slabs were continually cooled by water (24 °C) on one side. At the conclusion of migration, adjacent triplicate samples were separated by slicing parallel to the direction of protein migration. The centre strip was stained for protein and the adjacent strips were used for glucanase determinations with CM-(1 $\rightarrow$ 3)- α -glucan (5 mg ml $^{-1}$ in 60 mM-sodium succinate buffer, pH 6.0) as substrate. Gels were scanned at 600 nm with a Gilford gel scanning device.

Enzyme storage. Enzyme solutions, containing 0.01% sodium azide as an antimicrobial agent, were stored at 4 °C.

RESULTS

Induction of (1 $\rightarrow$ 3)- α -glucanase

Selection of the best inducer substrate. When *B. circulans* WL-12 is grown on cell walls or on isolated wall glucan of *Saccharomyces cerevisiae*, only β -glucanases are synthesized (Fleet & Phaff, 1973). Several substrates containing (1 $\rightarrow$ 3)- α -glucan were investigated for their ability to induce (1 $\rightarrow$ 3)- α -glucanase in *B. circulans* WL-12. The highest activities were obtained with whole cells of *Schizosaccharomyces pombe* or with purified (1 $\rightarrow$ 3)- α -glucan from *Aspergillus niger*. With these substrates β -glucanases were synthesized at low levels when peak activities of (1 $\rightarrow$ 3)- α -glucanase were reached (usually after about 36 h growth). Impure (1 $\rightarrow$ 3)- α -glucan (extracted from *Sch. pombe*) or *Sch. pombe* cell walls gave significantly lower yields of (1 $\rightarrow$ 3)- α -glucanases and higher activities of β -glucanases. Purified (1 $\rightarrow$ 3)- α -glucan from *A. niger* was superior as inducer substrate and a concentration of 0.25% (w/v) produced the highest activity [0.31 unit ml $^{-1}$; 2.6 units (mg protein) $^{-1}$ using CM-(1 $\rightarrow$ 3)- α -glucan at pH 6.0 as the assay substrate]. (1 $\rightarrow$ 3)- α -Glucanase excretion into the culture fluid took place primarily during the second half of the exponential growth phase and stopped abruptly with the beginning of the stationary phase (Fig. 1).

Factors affecting the activity of the crude enzyme

(1 $\rightarrow$ 3)- α -Glucanase activity as a function of pH. The pH value for optimal activity with a 36 h culture fluid grown on 0.25% (w/v) of purified (1 $\rightarrow$ 3)- α -glucan showed highest activity between pH 7 and 9 with a sloping shoulder of activity between pH 5 and 7. The activities were assayed with CM-(1 $\rightarrow$ 3)- α -glucan in 75 mM-Tris/sodium succinate buffers, pH 3.7 to 9.8.

Salt concentrations for optimal activity determinations. Preliminary assays of (1 $\rightarrow$ 3)- α -glucanase activity by varying the sodium succinate buffer at pH 6.0 indicated that a final concentration of at least 75 mM was required before maximum activity was achieved. The presence of EDTA with the CM-substrate further enhanced the activity by about 10% or more, depending on the enzyme sample used.

Fig. 1. Production of extracellular glucanases by *Bacillus circulans* WL-12 grown at 30 °C on 0.25 % purified (1→3)- α -glucan. Activities are expressed as units ml⁻¹ in culture fluid after dialysis for 24 h against distilled water. Assay substrates were: colloidal (1→3)- α -glucan (○) and CM-(1→3)- α -glucan (at pH 6.0) (●) for (1→3)- α -glucanase; laminarin for (1→3)- β -glucanase (□); pustulan for (1→6)- β -glucanase (△); *Saccharomyces cerevisiae* cell walls (at pH 6.0) for lytic activity (▼). Final assay substrate concentrations were 5 mg ml⁻¹. Total bacterial cell counts ml⁻¹ (■).

Purification of (1→3)- α -glucanase

Production and concentration of culture filtrate. *Bacillus circulans* WL-12 was grown on YNB medium containing 0.25 % (w/v) of purified (1→3)- α -glucan as described in Methods. After 36 h growth, the culture filtrate was harvested by centrifugation and dialysed against distilled deionized water at 4 °C for 48 h. No loss in enzyme activity was experienced under these conditions.

Concentration of the dialysed culture filtrate by lyophilization usually resulted in a 60 % loss in activity. The most effective method of selective concentration involved affinity adsorption of the (1→3)- α -glucanase activity on insoluble (1→3)- α -glucan (see Methods). Assays of the recovered enzyme solution, after digestion of the glucan pellet, indicated that virtually all of the (1→3)- β - and (1→6)- β -glucanase activities had been eliminated, although approximately 2.5 % of the lytic activity on baker's yeast cell walls remained associated with the (1→3)- α -glucanase. The affinity purification step resulted in a four- to fivefold increase in specific activity (Table 1).

Chromatography on a column of DEAE-agarose gave a peak which contained (1→3)- α -glucanase activity and eluted sharply at 0.06 M-NaCl (Fig. 2). The use of the agarose matrix was superior to cellulose because the latter caused a small tailing effect. Active fractions (29 to 34) were combined.

In addition to the (1→3)- α -glucanase (designated I) derived during the salt elution from the DEAE-agarose, a minor (1→3)- α -glucanase (designated II) passed through the column with the solute during the initial loading. The residual lytic β -glucanase activity was associated with the (1→3)- α -glucanase II.

A 3 ml sample of the enzyme concentrated by ultrafiltration was applied to a Bio Gel P-150 column. Sephadex G-100 was unsuitable because of a considerable affinity of the enzyme for its matrix. The glucanase I was eluted as a single peak (Fig. 3) with an exclusion volume of 119 ml. Because the activity eluate was near the void volume (104 ml), the void volume samples could have overlapped with enzyme fractions. Consequently, another concentrated sample (2 ml) was applied to a Bio Gel A-0.5 m column. The specific activities of the active fractions (60 to 76; exclusion volume 164 ml) did not differ significantly from

Fig. 2. DEAE-agarose chromatography of (1→3)- α -glucanase from *Bacillus circulans* WL-12. After affinity adsorption and auto-digestion, the enzyme was dialysed for 24 h against 5 mM-sodium phosphate buffer at pH 6.5 and applied to a column (15 $\times$ 1.3 cm) of DEAE-agarose equilibrated with the same buffer. (1→3)- α -Glucanase activity (●); protein (○); NaCl concentration (---).

Fig. 3. Bio Gel P-150 gel filtration of (1→3)- α -glucanase I from *Bacillus circulans* WL-12 after chromatography on DEAE-agarose and concentration by ultrafiltration. The protein sample (3 ml) was applied to the column and eluted as described in Methods. Protein (○); (1→3)- α -glucanase I activity (●).

those obtained by polyacrylamide filtration. Active fractions (22 to 29) from the polyacrylamide column were combined. The enzyme solution contained no detectable carbohydrate.

Table 1 summarizes the purification procedure of (1→3)- α -glucanase I using the assay substrates at pH 8.3. The purification monitored at pH 6.0 gave similar results except for a slightly greater apparent extent of purification and yield. The yield of (1→3)- α -glucanase I was actually greater than Table 1 indicates since (1→3)- α -glucanase II was also present in the dialysed culture filtrate (total activity taken as 100%).

Properties of (1→3)- α -glucanase I

Substrate specificity. Purified (1→3)- α -glucanase I had activity only against insoluble (1→3)- α -glucan or CM-(1→3)- α -glucan. Nigeran [containing alternating (1→3)- α - and (1→4)- α -linkages], laminarin, pustulan, chitin, *S. cerevisiae* cell walls, mannan, phosphomannan, amylose, cellosaccharides, isomaltosaccharides, *p*-nitrophenyl- α -D-glucopyranoside, gentiobiose, *p*-nitrophenyl phosphate, Azocoll, trehalose and sucrose were not substrates.

Pattern of action. After 30 min and 3 h hydrolysis, the prominent oligosaccharide was

Table 1. *Summary of the purification of extracellular (1→3)- α -glucanase I from Bacillus circulans WL-12*

Activity was determined with both carboxymethyl-(1→3)- α -glucan (3.5 mg ml⁻¹) and with colloidal (1→3)- α -glucan (5 mg ml⁻¹, values in parentheses) as substrates in 75 mM-glycine/NaOH buffer at pH 8.3. Other assay conditions are described in Methods.

Procedure	Volume (ml)	Protein concn (mg ml ⁻¹)	(1→3)- α -Glucanase activity (units ml ⁻¹)	Specific activity [units (mg protein) ⁻¹]	Purification factor	Yield (%)
Dialysed culture filtrate	200	0.161	0.38* (0.30)*	2.3 (1.9)	—	100*
Affinity adsorption on colloidal (1→3)- α -glucan followed by auto-digestion	104	0.052	0.51* (0.43)*	9.8 (8.3)	4.2 (4.5)	70* (75)*
DEAE-agarose chromatography	30	0.091	1.17 (0.94)	12.9 (10.3)	5.5 (5.6)	59 (60)
Bio Gel P-150 gel filtration†	40	0.025	0.34 (0.28)	13.6 (11.2)	5.8 (6.1)	48 (53)

* Activity includes both (1→3)- α -glucanase I and (1→3)- α -glucanase II.

† Eluate from DEAE-agarose was concentrated by ultrafiltration and divided into two portions, each applied separately in the step. One application presented here with P-150, represented 55 % of that activity.

nigerotetraose followed by glucose, nigerose and traces of nigerotriose, -pentaose, and -hexaose. After 8 h, nigerotetraose and nigerose showed chromatographic spots of equal intensity, with glucose slightly less pronounced. Nigerotriose and the other larger oligosaccharides were still present at very low concentrations. The final sample at 72 h contained a greatly increased concentration of nigerose, followed by glucose and finally nigerotriose and -tetraose, in approximately equivalent spot intensity. The weak spots representing higher oligosaccharides (nigeropentaose and larger) did not appear to change much in intensity throughout the hydrolysis.

Stability and heat denaturation. The purified enzyme was stable for at least 2 years at 4 °C and pH 6 in 50 mM-sodium succinate containing 0.01 % sodium azide. The enzyme was stable for at least 1.5 h at pH 5.25 to 10.5 at 30 °C. Approximately 50 % of the activity was lost after less than 10 min at pH 3.8 or after 30 min at pH 4.2. Appreciable losses in activity occurred during freezing at -15 °C.

Heating of the enzyme (in 5 mM-sodium phosphate buffer, pH 6.5) for 90 min at 40 °C resulted in 5 % loss in activity; 90 min at 45 °C caused 25 % inactivation; and 20 min at 50 °C resulted in nearly complete inactivation.

The initial rates of hydrolysis of the colloidal substrate at pH 6.0 were determined at several temperatures by constructing tangents to the initial slopes of the curves showing sugar reducing-equivalents produced as a function of time. Plotting the logarithm of the specific rates against the reciprocal of the absolute temperature gave a straight line from which the activation energy was calculated to be 73 kJ mol⁻¹.

Electrophoretic studies. The molecular weight of the purified (1→3)- α -glucanase I was estimated by discontinuous polyacrylamide gel electrophoresis in SDS to be 134 000 (standard deviation 100; three runs). When the protein concentration was near the useful maximum capacity of the gel (2 μ g per sample well), a very faint protein band appeared with a relative mobility slightly greater (0.32) than that of the major band (0.28). Repeated freezing and thawing of the protein prior to electrophoresis increased the intensity and number of minor bands in the same area of the gel.

The purified enzyme migrated towards the anode during discontinuous polyacrylamide gel electrophoresis in Tris/HCl buffer (initial pH 8.8) without SDS. Only one protein band

Fig. 4. Time course of (1→3)- α -glucan hydrolysis. Portions of the purified enzyme were incubated with substrate in 75 mM-sodium succinate buffer, pH 6.0, at 30 °C. Samples of the reaction mixtures were withdrawn at intervals and the reducing sugars were determined as described in Methods. Reducing sugars produced with soluble CM-(1→3)- α -glucan (3.5 mg ml⁻¹, enzyme protein 0.013 mg ml⁻¹) (●) or with colloidal (1→3)- α -glucan (5 mg ml⁻¹, enzyme protein about 0.053 mg ml⁻¹) (▲).

Table 2. *Kinetic constants of the purified (1→3)- α -glucanase I from Bacillus circulans WL-12 at pH 6.0 and pH 7.7 for both the carboxymethyl- and colloidal (1→3)- α -glucan substrates*

Buffers were 75 mM-sodium succinate with 1 mM-EDTA at pH 6.0 and 75 mM-Tris plus 75 mM-sodium succinate with 1 mM-EDTA at pH 7.7. Assay conditions are given in the text. The constants were determined from Lineweaver–Burk plots of the results.

Substrate	pH	K_m (mg ml ⁻¹)	V_{max} [μ mol min ⁻¹ (mg protein) ⁻¹]
Carboxymethyl-(1→3)- α -glucan	6.0	0.43	9.0
	7.7	0.43	21.0
Colloidal (1→3)- α -glucan	6.0	2.13	9.1
	7.7	2.04	22.2

was evident when the gels were scanned at 600 nm; its mobility coincided with the band of activity detected by the reducing sugar assay of the enzyme released from the sectioned gel.

Kinetic properties. Reducing sugar equivalents during the hydrolysis of the CM-(1→3)- α - and colloidal (1→3)- α -glucans with the purified enzyme were not released linearly (Fig. 4). When the reaction data with the two substrates were applied to a first-order reaction equation, the curves were still non-linear, demonstrating that the non-linearity was not solely due to non-saturation of the enzyme by substrate.

The purified enzyme preparation in 75 mM-Tris/75 mM-sodium succinate buffers had a broad pH optimum of 7.5 to 8.5 with both the colloidal and CM-(1→3)- α -glucans as substrates, but the profiles were not symmetrical. The enzyme, with both substrates, exhibited a distinct shoulder of activity at pH values between 5 and 7 (about 70% and 85% of optimal activity, respectively), suggesting the possible presence of two (1→3)- α -glucanases. However, the rates of heat inactivation (e.g. at 48 °C) when the activity was followed at two pH values (6.0 and 8.3) with respect to time were identical with both assay substrates.

The kinetic constants V_{max} and K_m were determined by following initial reaction velocities at pH 6.0 and 7.7 as a function of substrate concentration with both the colloidal and CM-(1→3)- α -glucans (Table 2). The enzyme conforms to Michaelis–Menten kinetics as indicated by the ratio of 81 for substrate concentrations required for 90% and 10% of V_{max} (Koshland *et al.*, 1966) and by the slope of 1 when the data were plotted according to the Hill equation (Whitaker, 1972). While substrate inhibition could not be detected with the colloidal substrate, it was evident with the carboxymethyl substrate when concentrations

exceeded approximately 3 to 3.5 mg ml⁻¹. The insoluble substrate was not used at concentrations greater than 10 mg ml⁻¹ because the suspensions became excessively pasty.

Effect of inhibitors. A number of metal ions were tested on (1→3)- α -glucanase I as explained in Methods: Mg²⁺, Ca²⁺, Ba²⁺, Sr²⁺ and Co²⁺ showed no significant inhibition at either concentration or pH; Zn²⁺, Mn²⁺ and Cu²⁺ gave moderate to strong inhibition at 1 mM but not at 0.01 mM; Hg²⁺ and Ag⁺ were strongly inhibitory at 0.01 mM at pH 6.0 and 7.3; 1 mM-Pb²⁺ was strongly inhibitory at pH 6.0 and 7.3, whereas 0.01 mM-Pb²⁺ was not inhibitory at pH 6.0 but gave 42% inhibition at pH 7.3. 1,4-Dithiothreitol (4 mM final concentration) completely reversed the inhibition by 0.01 mM-Pb²⁺ at pH 7.3. Iodoacetamide gave strong inhibition at 10 mM.

Observations on (1→3)- α -glucanase II

(1→3)- α -Glucanase II (cf. Fig. 2) could be separated from the lytic β -glucanase(s) by its early elution (at 0.035 M-NaCl) from CM-agarose, but was rather rapidly and irreversibly inactivated at pH 5 used for this chromatographic procedure. The minor enzyme was subjected to discontinuous polyacrylamide gel electrophoresis in SDS after its elution from the CM-agarose. The mobility of the main protein band indicated a molecular weight approximately the same as that determined for the major enzyme. When (1→3)- α -glucanase II was mixed with (1→3)- α -glucanase I their activities at pH 6 or 8 were additive. The instability and small quantities of (1→3)- α -glucanase II available precluded further characterization of this enzyme.

DISCUSSION

Extracellular (1→3)- α -glucanase was induced in *Bacillus circulans* WL-12 by growth on (1→3)- α -glucan substrates. Maximum activities were obtained in 36 h at 30 °C, which is half the time required for this organism to reach optimal β -glucanase synthesis on β -glucan substrates (Fleet & Phaff, 1973). The enzyme was released into the culture fluid during the second half of the exponential phase of growth (before any visible evidence of sporulation) and release came to an abrupt halt when the stationary phase was reached (Fig. 1); this indicates that enzyme excretion is not connected with cell autolysis or with the sporulation process. The purity of the inducer (1→3)- α -glucan was important since small quantities of β -glucan led to increased synthesis of β -glucanases at the expense of (1→3)- α -glucanase yield. Purified (1→3)- α -glucan or whole cells of *Schizosaccharomyces pombe* proved to be superior to cell walls of this yeast as inducers of (1→3)- α -glucanase while producing low levels of β -glucanases. The suitability of whole cells suggests that the (1→3)- α -glucan is located on the exterior of the yeast cell, as previously suggested (Carbonell *et al.*, 1970; Fleet, 1973). The effect of β -glucan contaminants in the (1→3)- α -glucan substrate became evident when a partially purified (1→3)- α -glucan from *Sch. pombe* (0.25%, w/v) served as inducer. Manners & Meyer (1977) have shown that alkali extraction of cell walls of this yeast yields an alkali-soluble β -glucan in addition to (1→3)- α -glucan. With this contaminant present, the (1→3)- β -glucanase activity exceeded the (1→3)- α -glucanase activity, decreasing the latter to less than half that obtained with pure (1→3)- α -glucan. The simultaneous synthesis of (1→3)- β -glucanases has also been noted in the other organisms that produced (1→3)- α -glucanases during growth on (1→3)- α -glucans; however, the effects of possible β -glucan contaminants on (1→3)- α -glucanase yields were not analysed in these cases (Guggenheim & Haller, 1972; Imai *et al.*, 1977*a, b*). In our study (1→3)- β - and (1→6)- β -glucanase activities, determined by reducing group increases, remained very low throughout the growth period of 50 to 60 h on highly purified α -glucan, while the lytic activity on *S. cerevisiae* cell walls increased steadily and rose sharply toward the end (Fig. 1). This confirms the observation by Rombouts & Phaff (1976*a, b*) that there was no correlation between total β -glucanase activity and lytic activity when *B. circulans* was grown on cell walls of *S. cerevisiae*. It is noteworthy that in organisms where (1→3)- α -glucanases are

inducible, the specific activities of the enzymes in the culture fluid were much lower [0.73 unit (mg protein)⁻¹ in *Trichoderma viride* assayed at 40 °C (Hasegawa *et al.*, 1969); 0.6 unit (mg protein)⁻¹ in *Streptomyces* sp. at 40 °C (Imai *et al.*, 1977a); 1.9 units (mg protein)⁻¹ in *Bacillus circulans* WL-12 at 30 °C] than in organisms producing (1→3)- α -glucanases constitutively [5 units (mg protein)⁻¹ in *Flavobacterium* sp. at 37 °C (Ebisu *et al.*, 1975); 7.1 units (mg protein)⁻¹ in *Cladosporium resinae* at 50 °C (Walker & Hare, 1977)] even though β -glucanases were also formed by the latter.

A highly stable (1→3)- α -glucanase I was purified approximately sixfold, indicating the relatively pure state of the enzyme in the 36 h culture filtrate of *B. circulans*. The DEAE-agarose purification step resolved a second (1→3)- α -glucanase II activity, representing approximately 13 to 15% of the total activity after the (1→3)- α -glucan affinity step. The presence in the culture filtrate of *Trichoderma harzianum* of two (1→3)- α -glucanases with very similar properties has also been reported by Guggenheim & Haller (1972).

(1→3)- α -Glucanase I was specific for (1→3)- α -glucan (pseudonigeran) as substrate. This differs from the *Trichoderma viride* endo-(1→3)- α -glucanase which has been reported to hydrolyse nigeran [with alternating (1→3)- α - and (1→4)- α -bonds] as well as pseudonigeran (Hasegawa *et al.*, 1969). The initial products of hydrolysis of (1→3)- α -glucan by (1→3)- α -glucanase I were dominated by nigerotetraose, which is atypical during random hydrolysis of a linear polysaccharide by an endo-hydrolase. The initial accumulation of nigerotetraose may mean that polymers or polymer fragments are cleaved preferentially at the fourth (1→3)- α -glucosidic bond from the non-reducing ends of the chains. The tetramer was ultimately hydrolysed further, presumably into two dimers because nigerotriose never represented more than a minor product. Eventually, nigerose and glucose became major end-products. In endo-hydrolytic reactions where products in turn can serve as substrates, the new substrates most probably possess different kinetic constants. These products possibly serve as inhibitors of the initial reaction. Such factors may be responsible for the non-linear rate of (1→3)- α -glucan hydrolysis by this enzyme (Fig. 4).

(1→3)- α -Glucanase I has a relatively broad optimum pH (about 7.5 to 8.5) for activity. The asymmetrical profile caused by a pronounced shoulder between pH 5 and 7 suggested a non-homogeneous enzyme preparation; however, heat inactivation rates and electrophoretic studies indicated a single protein. One possible explanation for such a profile would be a higher solubility of the alkali-soluble (1→3)- α -glucan (e.g. weakening of hydrogen bonding between polymer molecules) at the higher pH values because the extent of the asymmetry was less pronounced with the soluble CM-(1→3)- α -glucan as substrate. The activity at high pH values is interesting in that endo-(1→3)- α -glucanases studied previously have much lower pH optima (Ebisu *et al.*, 1975; Guggenheim & Haller, 1972; Hasegawa *et al.*, 1969; Imai *et al.*, 1977b; Walker & Hare, 1977). However, retention of activity at higher pH values was reported by Ebisu *et al.* (1975) in the case of (1→3)- α -glucanase from a strain of *Flavobacterium*. An exo-(1→3)- α -glucanase from *Aspergillus nidulans* (Zonneveld, 1972) also possessed a lower pH optimum but still exhibited good activity at pH 8.0.

The relatively high K_m value (2.1 mg ml⁻¹) obtained with the colloidal (1→3)- α -glucan may be a reflection of the insoluble nature of the polysaccharide because a much lower value (0.43 mg ml⁻¹) was obtained with the soluble CM-(1→3)- α -glucan. Other investigators (Hasegawa *et al.*, 1969) also found a high K_m for (1→3)- α -glucanase from *Trichoderma viride* with the insoluble glucan. It is known that (1→3)- α -glucan tends to form aggregates of fibres (Carbonell *et al.*, 1970) and such a property would contribute significantly to high K_m values.

The reversibility of heavy metal ion inhibition of the enzyme by dithiothreitol and the strong inhibition by iodoacetamide suggest the involvement of thiol groups in the active site.

The ease of preparation and purification along with the stability of (1→3)- α -glucanase I should promote this hydrolase as a useful analytical tool in investigations of (1→3)- α -glucan-containing yeast or fungal cell walls.

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