Purification and Characterization of an Extracellular and a Cellular α -Glucosidase from *Bacillus licheniformis*

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α -Glucosidase has been purified from culture fluid and from lysed cells of *Bacillus licheniformis* NCIB 6346. The enzymes from these two sources were virtually identical in molecular weight as judged by SDS-PAGE (63 000) and catalytic properties. The enzymes were unstable at high temperature and lost all activity after incubation at 60 °C for 10 min. Of the substrates examined, isomaltose gave maximal activity, followed by maltotriose, *p*-nitrophenyl α -D-glucopyranoside, sucrose and maltose. With isomaltose or maltotriose as substrate, transglucosylation activity was evident.

INTRODUCTION

The final stage of starch metabolism in many micro-organisms is effected by α -glucosidase. This enzyme (α -D-glucosidase, EC 3.2.1.20) hydrolyses the (1→4)- α - and/or the (1→6)- α -linkages in the oligosaccharides remaining after degradation of starch by amylases. α -Glucosidase is distributed widely amongst micro-organisms and has been purified and characterized from bacteria, yeasts and moulds (reviewed by Kelly & Fogarty, 1983). The enzymes differ in their substrate specificities. Of those from *Bacillus* strains, the α -glucosidases from '*B. amylolyticus*' (Kelly *et al.*, 1980), *B. brevis* (McWethy & Hartman, 1979), *B. cereus* (Suzuki & Tanaka, 1981), *B. megaterium* (Kelly & Fogarty, 1983) and *B. subtilis* P-11 (Wang & Hartman, 1976) have been termed 'maltases' since they have a high activity towards maltose and little, if any activity towards *p*-nitrophenyl α -D-glucopyranoside (PNPG). The other group of α -glucosidases is more sparsely represented and includes those enzymes that hydrolyse PNPG more rapidly than maltose. The enzymes from '*B. thermoglucosidius*' (Suzuki *et al.*, 1979) and '*B. amyloliquefaciens*' (Urlaub & Wöber, 1978) are in this category but differ in that the former displays high activity towards isomaltose and has been designated as an oligo-(1→6)- α -D-glucosidase (EC 3.2.1.10) while the latter displays maximal activity towards sucrose.

An interesting feature of these enzymes is their cellular location. In '*B. amylolyticus*' (Kelly *et al.*, 1980), *B. brevis* (McWethy & Hartman, 1979) and *B. subtilis* P-11 (Wang & Hartman, 1976) the enzyme is extracellular. In *B. cereus* (Suzuki & Tanaka, 1981) and *B. megaterium* (Kelly & Fogarty, 1983) the enzyme is cytoplasmic and in '*B. thermoglucosidius*' it has been shown to accumulate in the cytoplasm during exponential growth before subsequent release into the culture fluid, possibly as a result of cell lysis (Suzuki *et al.*, 1976*a, b*). In '*B. amyloliquefaciens*' the enzyme is membrane bound (Urlaub & Wöber, 1978). The distribution of α -glucosidase in *B. licheniformis* NCIB 6346 appears to vary. In young cells it is largely cytoplasmic but, as the culture enters late exponential phase, the enzyme can be released by protoplast formation. Finally, in early stationary phase cultures, it can be detected in the culture fluid (Thirunavukkarasu & Priest, 1983). In this paper, we report the purification and characterization of the intracellular and extracellular α -glucosidases of *B. licheniformis* NCIB 6346. The two enzymes were essentially identical.

Abbreviation: PNPG, *p*-nitrophenyl α -D-glucopyranoside.

METHODS

Organism and growth conditions. *Bacillus licheniformis* NCIB 6346 was obtained from the National Collection of Industrial Bacteria, Aberdeen, UK. It was grown in a medium that had been optimized for the yield of intracellular and extracellular α -glucosidase and contained (g l^{-1}): KH_2PO_4 , 14; K_2HPO_4 , 6; trisodium citrate, 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; bacteriological peptone (Oxoid), 45 and waxy-maize starch, 15. The medium (3.5 l), in a 5 litre fermenter, was autoclaved at 121°C for 30 min. An inoculum was prepared by shaking cells in 100 ml nutrient broth (Oxoid no. 1) at 37°C for 16 h and 10 ml was inoculated into 50 ml of the optimized medium. After shaking for 7 h at 37°C , 25 ml of the cell suspension was transferred to the fermenter and incubated at 37°C for 16 h with constant stirring. Aeration was at 5 litres min^{-1} and foaming was controlled by the addition of silicone antifoam. The cells were harvested by centrifugation (5000 g, 20 min, 4°C) and used for purification of the intracellular enzyme; the supernatant was used for the purification of the extracellular enzyme.

Analytical procedures. α -Glucosidase activity was assayed by measuring the *p*-nitrophenol released from PNPG (Sigma). The reaction mixture contained phosphate buffer (2 ml, 0.1 M, pH 6.5), PNPG (250 μg) and enzyme solution (0.1 ml). After incubation at 37°C for 20 min, 1 ml Na_2CO_3 (5 M) was added and the absorbance measured at 420 nm. One unit of activity is the amount of enzyme that gives an increase of one absorbance unit under these conditions. Glucose was assayed using glucose oxidase (300 $\mu\text{g ml}^{-1}$, Boehringer) and horseradish peroxidase (30 $\mu\text{g ml}^{-1}$, Boehringer) dissolved in Tris/phosphate buffer (0.1 M, pH 7.0) with 2,2'-azino-di-(3-ethylbenzthiazoline sulphonic acid) (750 $\mu\text{g ml}^{-1}$, Sigma). This solution (1 ml) was incubated with 1 ml enzyme digest (see Table 2) for 20 min at 20°C , HCl (1 ml, 1 M) was added and the absorbance at 420 nm was related to glucose concentration with a standard curve. Protein was assayed using the Lowry procedure. TLC was done on silica gel G plates (Merck) developed in acetic acid/chloroform/water (35:30:5, by vol.). After 6 h the plates were dried and sprayed with phenol/sulphuric acid reagent (phenol, 3 g; H_2SO_4 , 5 ml; ethanol, 95 ml) and heated at 110°C for 10 min. Sugars appeared as brown spots. PAGE was done in tubes containing 7.5% (w/v) polyacrylamide at 5 mA per tube for 2 h. SDS-PAGE was done according to Weber & Osborn (1969) using 10% (w/v) polyacrylamide gels. The gels were stained with Coomassie blue.

Purification of extracellular enzyme. All steps were done at 4°C . $(\text{NH}_4)_2\text{SO}_4$ (313 g l^{-1}) was added slowly to the culture fluid. The solution was stirred for 6 h, then centrifuged (12000 g, 30 min) and the precipitate discarded. More $(\text{NH}_4)_2\text{SO}_4$ (214 g l^{-1}) was added to the supernatant and stirred overnight. The precipitate was recovered by centrifugation, dissolved in 25 mM-phosphate buffer (pH 6.5) and dialysed overnight against the same buffer. The enzyme solution was loaded onto a DEAE-cellulose (Whatman DE52) column (2.5 cm $\times$ 30 cm) that had been equilibrated with 25 mM-phosphate buffer (pH 6.5). It was eluted using a linear gradient of 0–0.5 M-NaCl at 20 ml h^{-1} and 120 fractions (5 ml each) were collected. α -Glucosidase was recovered in a single peak of activity in fractions 82 to 98. These were pooled, $(\text{NH}_4)_2\text{SO}_4$ (561 g l^{-1}) was added, and the solution was stirred for 6 h and centrifuged as before. The precipitate was dissolved in 25 mM-phosphate buffer (pH 6.5) and loaded onto a Sephadex G-200 (2.5 cm $\times$ 75 cm) column. Fractions (5 ml) were eluted at a flow rate of 10 ml h^{-1} and the α -glucosidase activity was recovered as a single peak of activity in fractions 30 to 52. Analysis of this material by PAGE revealed four bands stained by Coomassie blue. The enzyme was therefore concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (561 g l^{-1}); the precipitate was dissolved in 25 mM-phosphate buffer (pH 6.5) and loaded onto an Ultragel AcA 44 (LKB) column (3.5 cm $\times$ 42 cm). Fractions (3 ml) were eluted using the same buffer (20 ml h^{-1} flow rate) and the enzyme activity was recovered in fractions 30 to 40. Although this provided greater than 1000-fold purification (Table 1) the material still revealed two protein bands after PAGE. The pooled fractions from the AcA 44 column were concentrated by ultrafiltration and 400 μl was loaded onto preparative polyacrylamide gels (1.2 cm $\times$ 10 cm). After electrophoresis for 2 h at 4°C and 5 mA per tube, the gels were incubated at 37°C for 10 min in 5 mM-phosphate buffer (pH 6.5) containing PNPG (1 mg ml^{-1}). The region of the gel containing α -glucosidase, which was revealed as a yellow band, was removed, crushed and ground in water in a pre-chilled mortar. The gel solution was dialysed overnight against 5 mM-phosphate buffer (pH 6.5), and concentrated by ultrafiltration after removal of pieces of gel by centrifugation. The purified enzyme migrated as a single band after SDS-PAGE. It was stored at -20°C and was used for all assays.

Purification of the intracellular enzyme. Cells (5–10 g wet weight) were suspended in 45 ml phosphate buffer (0.1 M, pH 6.5) and lysozyme solution was added to a final concentration of 100 $\mu\text{g ml}^{-1}$. After incubation at 37°C for 15 min, DNAase (Sigma, 50 $\mu\text{g ml}^{-1}$) was added and incubation continued for a further 15 min. Cell debris was removed by centrifugation (12000 g, 30 min), streptomycin sulphate was added to the supernatant to 20 $\mu\text{g ml}^{-1}$ final concentration and the precipitate was removed by centrifugation at 12000 g for 30 min. The supernatant was dialysed against phosphate buffer (25 mM, pH 6.5) and purified in the same way as the extracellular enzyme but the AcA 44 chromatography step was omitted. The purified enzyme migrated as a single band after SDS-PAGE.

Molecular weight determination. The molecular weights of the purified α -glucosidases were determined by SDS-PAGE using (M_r in parentheses) bovine serum albumin (66000), pyruvate kinase (57000), lactate dehydrogenase (36000), lysozyme (14300) and RNAase (13700) for calibration.

Effects of temperature and pH on the activity and stability of α -glucosidase. The effect of temperature on enzyme activity was determined by doing the standard enzyme assay at a range of temperatures between 20 and 60 °C, with 5 °C increments, using 0.1 ml of the purified enzymes adjusted to 5 units ml⁻¹ at 37 °C (100% activity). The effect of temperature on enzyme stability was examined by incubating the purified enzymes (1 ml of 100 units ml⁻¹) in phosphate buffer (0.1 M, pH 6.5) at 20, 37, 45, 50 and 60 °C. After 60 min, 0.1 ml of the enzyme was removed and the residual activity was assayed at 37 °C. The effect of pH on enzyme activity was examined using citrate/phosphate (pH 3 to 5.5), sodium phosphate (pH 5.5 to 8.0) and glycine/NaOH (pH 8 to 10) buffers, all at 0.1 M, in place of the phosphate buffer in the standard assay mixture. Enzyme assays using 5 units purified enzyme ml⁻¹ were done at pH 3 to 10 using the appropriate blanks. To assess the effect of pH on enzyme stability, 20 units of enzyme were adjusted to 2 ml with the buffers used above and incubated for 20 min and for 24 h. The enzyme buffer solutions were assayed for residual activity at pH 6.5.

RESULTS

Purification of α -glucosidases

Summaries of the purification of the intracellular and extracellular α -glucosidases of *Bacillus licheniformis* NCIB 6346 are given in Table 1. The specific activities of the purified enzymes were 1017 and 960 units (mg protein)⁻¹ for the extracellular and intracellular enzymes respectively. The purified enzymes (50 µg protein) migrated as single bands in SDS-PAGE. The subunit molecular weights of both enzymes were 63 000 and, when electrophoresed together, they migrated as a single molecular species.

Characteristics of the purified enzymes

The two enzymes behaved almost identically with respect to temperature and pH (Figs 1 and 2). At 50 °C, enzyme activity was almost twice that at 37 °C but above this temperature it declined rapidly. At 55 °C activity was 65% of the 37 °C figure and at 60 °C activity was virtually undetectable. Similarly, the enzymes lost activity when incubated at elevated temperatures. The enzymes were stable without loss of activity for 60 min at 20 and 37 °C but at 50 °C, 40% of their activity was lost during this period. All activity was lost in 10 min at 60 °C.

The optimum pH for enzyme activity was around 6.0, although 90% activity was obtained at pH 7.0 (Fig. 2). Below pH 4.5 and above pH 9.5 there was no activity. The enzymes displayed a

Table 1. *Summary of purification of α -glucosidases*

Source	Fraction	Total enzyme activity (units)	Total protein (mg)	Specific activity [units (mg protein) ⁻¹]	Yield (%)
Extracellular	Supernatant	21 045	28 578	0.736	100
	Ammonium sulphate precipitation	18 600	3 131	5.94	88
	DEAE-cellulose chromatography	13 320	360	37.0	63
	Ammonium sulphate precipitation	8 816	117	75.5	42
	Sephadex G-200 gel filtration	8 305	40	208.1	39
	Ultragel AcA 44 gel filtration	7 917	9.6	821	38
	Preparative PAGE	4 908	4.8	1 017	23
	Lysed cells	54 400	1 350	40.3	100
	Ammonium sulphate precipitation	52 500	684	76.7	96
Intracellular	DEAE-cellulose chromatography	18 480	90.3	204	33
	Ammonium sulphate precipitation	15 900	39.7	399	29
	Sephadex G-200 gel filtration	9 628	22.3	431	18
	Preparative PAGE	5 392	5.6	960	10

Fig. 1

Fig. 2

Fig. 1. Effect of temperature on the activity (circles) and stability (squares) of the intracellular (open symbols) and extracellular (filled symbols) α -glucosidases.

Fig. 2. Effect of pH on the activity (circles) and stability (squares) of the intracellular (open symbols) and extracellular (filled symbols) α -glucosidases.

Table 2. *Substrate specificities of α -glucosidases from Bacillus licheniformis*

Substrates were incubated with purified enzyme ($100 \text{ units ml}^{-1}$) for 30 min at 37°C and the glucose released was assayed and compared to that released from *p*-nitrophenyl α -D-glucopyranoside (PNPG). There was no detectable activity with amylose (1%, w/v), amylopectin (1%, w/v), cellobiose, lactose, melibiose, methyl α -D-glucopyranoside, *p*-nitrophenyl β -D-glucopyranoside, raffinose, salicin or trehalose (all at 1 mM) as substrate.

Substrate (1 mM)	Relative hydrolysis rate (%)	
	Extracellular enzyme	Intracellular enzyme
<i>p</i> -Nitrophenyl α -D-glucopyranoside	100	100
Isomaltose	226	219
Maltotriose	142	156
Sucrose	56	51
Maltose	55	49
Phenyl α -D-glucopyranoside	20	21

wide range of pH stability. More than 80% residual activity was obtained between pH 5.5 and pH 10.0. The enzymes were unstable below pH 5.0 even when incubated for only 20 min.

The substrate specificities of the enzymes are shown in Table 2. Of the substrates examined, maximal activity occurred with isomaltose followed by maltotriose, PNPG, sucrose, maltose and phenyl α -D-glucopyranoside. The enzymes did not hydrolyse polysaccharides such as amylose, amylopectin or glycogen. They were inhibited by glucose, glucono- δ -lactone and Tris, and to a lesser extent by erythritol, xylose and inositol. They were not affected by EDTA (Table 3).

The products of hydrolysis of maltose, isomaltose and maltotriose by the purified extracellular and lysozyme-released enzymes were essentially the same. Maltose was slowly hydrolysed to glucose. Maltotriose was hydrolysed more rapidly to yield maltose and glucose, and the maltose produced was slowly hydrolysed to glucose. However, isomaltose was rapidly hydrolysed to glucose. In both the isomaltose and maltotriose reactions there was evidence of transglucosylase activity (Fig. 3).

Fig. 3. Thin layer chromatographic analysis of the action of the purified extracellular α -glucosidase (100 units in 1.5 ml 0.1 M-phosphate buffer, pH 6.5) on maltotriose (2.5 mg ml⁻¹). Samples (15 μ l) were analysed at the times shown. GX, oligosaccharides with X maltose units. IG2, isomaltose; S, standard reference mixture comprising a partial acid hydrolysis of starch that has been checked against authentic standards.

Table 3. *Inhibitory effects of various compounds on the activity of α -glucosidases from *Bacillus licheniformis**

Purified enzyme (12.5 units ml⁻¹) was incubated with inhibitor for 20 min at 20 °C and the residual activity was assayed and compared to enzyme in phosphate buffer (0.1 M, pH 6.5). Galactose, EDTA, fructose, methyl α -D-glucopyranoside and sorbitol at 5 mM (25 mM for EDTA) had no effect on enzyme activity.

Inhibitor	Concn (mM)	Relative activity (%)	
		Extracellular enzyme	Intracellular enzyme
Erythritol	1	98	100
	5	81	79
Glucose	1	44	43
	5	13	19
Glucono- δ -lactone	1	44	50
	5	23	19
Inositol	1	88	92
	5	77	76
Tris	1	21	20
	5	3	1
Xylose	1	100	100
	5	84	79

DISCUSSION

Purification and characterization of an intracellular and an extracellular α -glucosidase from *B. licheniformis* NCIB 6346 has revealed that both enzymes show virtually identical properties. Like the α -glucosidase of *B. cereus* (Yamasaki & Suzuki, 1974), *B. subtilis* (Wang & Hartman, 1976) and *B. brevis* (McWethy & Hartman, 1979) they showed maximal activity at about 50 °C.

The pH optima for activity of both α -glucosidases from *B. licheniformis* were about pH 6.0, similar to the pH optima for α -glucosidases from other *Bacillus* strains and yeasts (Kelly & Fogarty, 1983).

The two α -glucosidases from *B. licheniformis* NCIB 6346 had the same specificity for the substrates examined. The enzymes behaved more like an isomaltase than a maltase since they had a higher specific activity towards isomaltose than maltose. This is unusual, since a recent analysis of the substrate specificities of 12 bacterial α -glucosidases showed 10 to have highest activity towards maltose and of these only three showed positive activity towards (1 $\rightarrow$ 6)- α -glucosides (Kelly & Fogarty, 1983). The two remaining enzymes (from '*B. amyloliquefaciens*' and an unidentified *Bacillus* strain) showed highest activity toward sucrose and PNPG respectively. The former also possessed activity on PNPG and isomaltose (Urlaub & Wöber, 1978) and therefore is the most similar to the enzymes from *B. licheniformis*. Several α -glucosidases possess transglucosylation activity and generally synthesize (1 $\rightarrow$ 6)- α -linked oligosaccharides (Kelly & Fogarty, 1983). The enzymes from *B. licheniformis* showed transglucosylation activity when maltotriose or isomaltose was supplied as substrate.

α -Glucosidase, like the peripheral, membrane-associated alkaline phosphatase of *B. licheniformis*, can be removed by washing cells with NaCl or Triton (Spencer *et al.*, 1981; Kumar *et al.*, 1983; Thirunavukkarasu & Priest, 1983). This suggests a membrane-associated location for α -glucosidase, but all attempts to recover α -glucosidase activity from membranes have failed (G. Gammack & F. G. Priest, unpublished information). In common with alkaline phosphatase (Glynn *et al.*, 1977; Spencer *et al.*, 1982), the intracellular and extracellular α -glucosidases have essentially the same subunit molecular weights and could not be resolved by SDS-PAGE. This eliminates the involvement of a hydrophobic sequence containing a lipid moiety in the localization of these enzymes, as found in the membrane-bound penicillinase of this bacterium (Nielsen *et al.*, 1981). Thus, if the cellular location of α -glucosidase is largely associated with the membrane, as suggested by *in vitro* translation studies (Thirunavukkarasu & Priest, 1983), the localization process seems to resemble the alkaline phosphatase rather than the penicillinase of this organism.

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