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PURIFICATION AND PROPERTIES OF EXTRACELLULAR α-GLUCOSIDASE OF A THERMOPHILE, *BACILLUS THERMOGLUCOSIDIUS* KP 1006

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Summary

An extracellular α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) of a thermophile, *Bacillus thermoglucosidius* KP 1006, was purified about 350-fold. The purified enzyme had a specific activity of 164 μmol of *p*-nitrophenyl-α-D-glucopyranoside hydrolyzed per min at 60°C and pH 6.8 per mg of protein. The molecular weight was estimated at 55 000. The pH and temperature optima for activity were 5.0—6.0 and 75°C, respectively. Below 40°C, the activity was less than 4.5% of the optimum. The enzyme showed a high specificity for α-D-glucopyranoside. The maximal hydrolyzing velocity per substrate diminished in the order: phenyl-α-D-glucopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, isomaltose, methyl-α-D-glycopyranoside. The respective K_m values were 3.0, 0.23, 3.2 and 27 mM. The activity was trace for turanose, and not detectable for sucrose, trehalose, raffinose, melezitose, maltose, maltotriose, phenyl-α-D-maltoside, dextran, dextrin and starch. Tris, *p*-nitrophenyl-α-D-xylopyranoside, glucose and glucono-δ-lactone blocked competitively the enzyme with respect to *p*-nitrophenyl-α-D-glucopyranoside. The K_i values were 0.12, 0.14, 2.2 and 2.4 mM, respectively. The activity was affected by heavy metal ions, but insensitive to EDTA, *p*-chloromercuribenzoate and iodoacetate. The enzyme was stable up to 60°C, and inactivated rapidly at temperatures beyond 72°C. The pH range for stability was 4.0—11.0 at 31°C, and 6.0—8.5 at 55.5°C. At 25°C, the enzyme failed to be inactivated in 45% ethanol, in 7.2 M urea, and in 0.06% sodium dodecyl sulfate, but the tolerance was extremely reduced at 60°C.

Introduction

α-Glucosidases (α-D-glucoside glucohydrolase, EC 3.2.1.20) from various origins have been extensively studied [1]. A few reports, however, have been pre-

sented on bacterial α-glucosidase [2—4]. Also, no work has been performed on α-glucosidase of thermophilic microbes. Recently, we have isolated from soil samples thermophilic bacteria capable of producing an extracellular α-glucosidase [5]. The bacteria isolated have been characterized as *Bacillus thermoglucosidius* sp. n., which is closely related to *Bacillus stearothermophilus* [5—7]. In *B. thermoglucosidius* KP 1006, a large accumulation of α-glucosidase takes place in the cytoplasm during logarithmic growth. α-Glucosidase appears in the culture at the mid-point of this period [5]. We have found that the thermophilic α-glucosidase has an extraordinary high resistance against denaturation [8]. In the present communication, purification and properties of this α-glucosidase are described.

Materials and Methods

Materials. Glucose, maltose, lactose, raffinose, sucrose, methyl-α-D-glucopyranoside, trehalose, cellobiose, D-(+)-turanose, melezitose, urea, sodium dodecyl sulfate, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-D-galactopyranoside and *p*-nitrophenol were purchased from Nakarai Chemicals, Ltd., Kyoto. *p*-Chloromercuribenzoate was purchased from Daiichi Pure Chemicals, Co. Ltd., Tokyo, DEAE-cellulose from Green Cross Industries, Co. Ltd., Osaka, hydroxyapatite, γ-globulin, egg albumin, *p*-nitrophenyl-α-D-mannoside and *p*-nitrophenyl-β-L-fucopyranoside from Seikagaku-kogyo Co. Ltd., Tokyo. Myoglobin, bovine serum albumin and glucose oxidase were purchased from Sigma Chemical Co., monoiodoacetate from E. Merck AG, Darmstadt, isomaltose from Pierce Chemical Co., *p*-nitrophenyl-α-D-xylopyranoside, *p*-nitrophenyl-β-D-xylopyranoside and *p*-nitrophenyl-β-D-galactopyranoside from Koch-Light Laboratories, and Sephadex G-100 and G-200 from Pharmacia. Phenyl-α-D-glucopyranoside, maltotriose and phenyl-α-D-maltoside were kindly supplied by Dr. Nobuyuki Suetsugu of this University. All other chemicals used were of analytical grade.

Assay of α-glucosidase

Method 1. *p*-Nitrophenol released from *p*-nitrophenyl-α-D-glucopyranoside by the action of α-glucosidase was determined photometrically with a Shimazu double beam spectrophotometer (model UV-200). This was the method used throughout the present investigation, except when the substrate specificity of the enzyme was being examined. The reaction mixture (1 ml) in a 1.0-cm light-path cell contained: 33.3 mM potassium phosphate (pH 6.8), 2 mM *p*-nitrophenyl-α-D-glucopyranoside and enzyme solution (0.1 ml). After 0.1 ml of the substrate was mixed with 0.9 ml of the complete medium lacked in the substrate, the reaction proceeded at 60°C for 1—3 min. The increase in absorbance at 400 nm due to *p*-nitrophenol was recorded continuously. The initial reaction velocity was assessed from the slope of the record running linearly.

The following extinction coefficients ($M^{-1} \cdot cm^{-1}$) of 400 nm *p*-nitrophenol at pH 6.8 were used to calculate the amount of product yielded: 9600 (30°C) [9], 10 000 (35°C), 10 500 (40°C), 11 000 (45°C), 11 500 (50°C), 11 900 (55°C), 12 400 (60°C), 12 900 (65°C), 13 300 (70°C), 13 800 (75°C), 14 300 (80°C), and 14 700 (85°C).

Method 2. Glucose produced by α-glucosidase from phenyl-α-D-glucopyranoside, methyl-α-D-glucopyranoside and the naturally occurring substrates was determined by using a Beckman oxygen electrode and glucose oxidase [10,11]. The electrode detected O_2 consumed in glucose oxidation by glucose oxidase. The potential applied between a rhodium cathode and a silver anode via a polarographic circuit (Beckman 100800 oxygen analyzer) was 0.53 V. α-Glucosidase reaction was allowed to proceed for 5—60 min at 60° C in the same medium as described in Method 1, except that it contained the various substrates (10 mM) instead of *p*-nitrophenyl-α-D-glycopyranoside. The reaction was stopped by heating for 3 min at 95° C. A 0.9-ml aliquot of the medium was mixed with 2 ml of 0.9 M Tris · HCl (pH 7.6), and subsequently equilibrated with air at 30° C to allow O_2 to dissolve to reach 230 μM in a vessel to which the electrode assembly was fitted. To the mixture was added 0.1 ml of glucose oxidase (3 mg protein, activity 59.4 units, dissolved in 1.5 M Tris · HCl, pH 7.0). The initial velocity of O_2 consumption was obtained from the linear slope of the record. Glucose was estimated from the reciprocal of the velocity on a Lineweaver-Burk plot made with known levels of glucose.

One unit of α-glucosidase activity was defined as the amount of enzyme needed for hydrolysis of 1 μmol *p*-nitrophenyl-α-D-glucopyranoside per min at 60° C. Protein was estimated by the method of Lowry et al. [12], or by a turbidimetry method which was applicable to the crude enzyme preparations in the early steps of the enzyme purification. In the latter procedure, 1 ml of the sample was mixed vigorously with 3 ml of 5% trichloroacetic acid. Protein was estimated from the absorbance at 660 nm of the suspension, with egg albumin as a standard (0.1—5 mg protein/ml).

Growth of B. thermoglucosidius KP 1006

Cells were cultivated at 60° C for 6 h with rotatory shaking (190 cycles/min) in 300-ml Erlenmeyer flasks containing 30 ml each of a medium (pH 7.0), which was composed of 1% (w/v) starch, 0.5% peptone, 0.3% meat extract, 0.3% yeast extract, 0.3% K_2HPO_4, 0.1% KH_2PO_4. 10 ml of the cell suspension was inoculated into 2-l Erlenmeyer flasks containing 200 ml each of a growth medium which had the same nutrients as the inoculum, except that the concentrations of starch, peptone and meat extract were 0.5, 2.5 and 0.2%, respectively. The flasks (25.6 l culture media) were shaked at 60° C for 48 h, after which the media were centrifuged at 0° C for 20 min (8000 × *g*). The supernatant fluid was used as the starting material for the enzyme purification.

Results

Purification of α-glucosidase

All operations were carried out at room temperature, unless otherwise mentioned. Centrifugation was conducted at 0° C for 20—30 min at 12 000 × *g*.

Concentration (Step 1). The culture broth (23 400 ml) was concentrated by evaporation at 45° C in vacuo to approximately 1/10 the initial volume, after which the resulting sediments were removed by centrifugation. In this step, there was no loss of α-glucosidase activity.

First $(NH_4)_2SO_4$ fractionation (Step 2). The supernatant liquid (2310 ml)

was supplemented with EDTA (final concn. 5 mM, pH 7.0), and brought with stirring to 40% saturation by slow addition of solid $(NH_4)_2SO_4$. After the solution had been stirred for 2 h, the precipitate was removed by centrifugation and discarded. Further $(NH_4)_2SO_4$ was added to the solution to give a final salt concentration of 70%, and then the mixture was stirred for 2 h. The precipitate recovered by centrifugation was suspended in 1 mM potassium phosphate (pH 6.8) containing 1 mM EDTA (referred to as Buffer A), and successively dialyzed against Buffer A (60 l, five days). This solution was centrifuged to remove the insoluble residues.

Chromatography on DEAE-cellulose (Step 3). A 500-ml portion of the dialyzed material (996 ml) was applied to a DEAE-cellulose column (4.5 × 35.2 cm) previously equilibrated with Buffer A. After washing with 1 l of Buffer A, the column was given a linear gradient of NaCl from 0 to 0.8 M, which was prepared by placing 1 l of Buffer A in a mixing chamber and 1 l of the same buffer containing 0.8 M NaCl in a reservoir. Fractions of 17.6 ml each were collected at a rate of 0.59 ml/min. The active fractions (147—176, 530 ml) were combined (Fig. 1). This chromatography was repeated once more using the remainder of the dialysate.

Second $(NH_4)_2SO_4$ fractionation (Step 4). The DEAE-cellulose eluate (1150 ml) was concentrated by evaporation, and then centrifuged to remove the coagulated protein residues. The $(NH_4)_2SO_4$ fractionation of the proteins (in 300 ml, containing 5 mM EDTA) was performed as described in Step 2. The precipitate resulting from 45—70% saturation was dissolved in Buffer A, followed by dialysis against the same buffer (2.5 l, 21 h).

Gel filtration on Sephadex G-200 (Step 5). The dialysate (460 ml) was concentrated at 40°C by evaporation, and subsequently the concentrate (10.1 ml) was passed through a Sephadex G-200 column (2.9 × 93 cm). 0.15 M potassium phosphate (pH 6.8) containing 1 mM EDTA (Buffer B) was utilized for

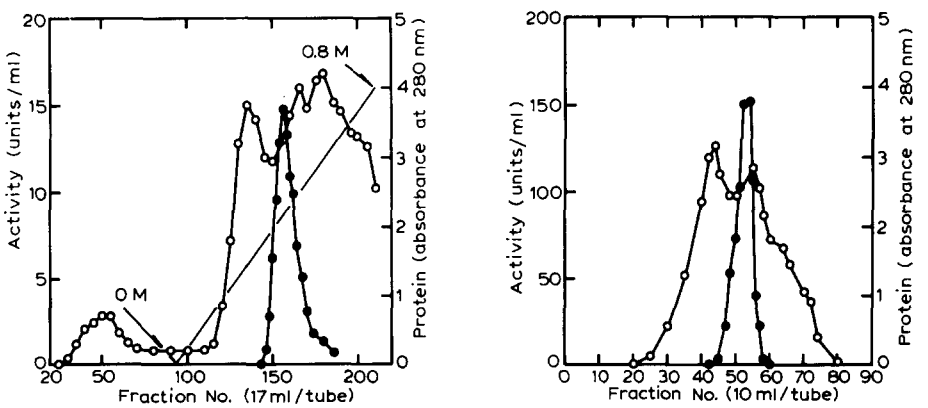

Fig. 1. Chromatography of α-glucosidase on a DEAE-cellulose column, with a concentration gradient of NaCl from 0 to 0.8 M. The activity is expressed in units per ml of the eluate. α-Glucosidase activity, ●———●; protein, ○———○; concentration change of NaCl, ———.

Fig. 2. Purification of α-glucosidase by gel-filtration on a Sephadex G-200 column. The enzyme activity, (unit/ml eluate), ●———●; protein, ○———○.

equilibration and elution. 10-ml fractions were collected with a rate of 0.22 ml/min. Fractions 47—57, which contained most of the enzyme activity, were pooled (Fig. 2).

Chromatography on hydroxyapatite (Step 6). EDTA (final concn. 5 mM) was added to the Sephadex G-200 eluate. The solution was saturated to 67% with $(NH_4)_2SO_4$ as in Step 2. The residues collected by centrifugation were dispersed in 1 mM potassium phosphate (pH 6.8) (Buffer C), and dialysed against the same buffer (7.5 l, 24 h) at 4°C. The 17.5-ml portions of the dialyzed material (35 ml) were subjected to chromatography on two separate columns of hydroxyapatite (3.0 × 8.5 cm) previously equilibrated with Buffer C. After the columns had been washed with 290 ml each of Buffer C, α-glucosidase was eluted at a rate of 0.5 ml/3 min with a linear elevation of the phosphate from 1 to 50 mM, in which the total volume of the gradient solution was 1 l. 10-ml fractions were collected. The active fractions (83—93) were combined (240 ml from the two columns) (Fig. 3).

Gel filtration on Sephadex G-100 (Step 7). The hydroxyapatite eluate was concentrated by dialysis against solid sucrose (24 h). The sucrose was removed by dialysis against Buffer A (6.5 l, 10 h). A 6-ml aliquot of the concentrate (48 ml) was placed on a Sephadex G-100 column (1.6 × 99 cm), which had been prepared with Buffer B. The elution was performed with this buffer at a rate of 0.19 ml/2 min, and 2.8-ml fractions were collected. As shown in Fig. 4, α-glucosidase activity was associated with the second protein peak (fractions 36—45). Fractions 39—42 (11.2 ml), which had higher specific activities of α-glucosidase, were combined. When this preparation was stored at −20°C, the activity was not lost for at least 1 year.

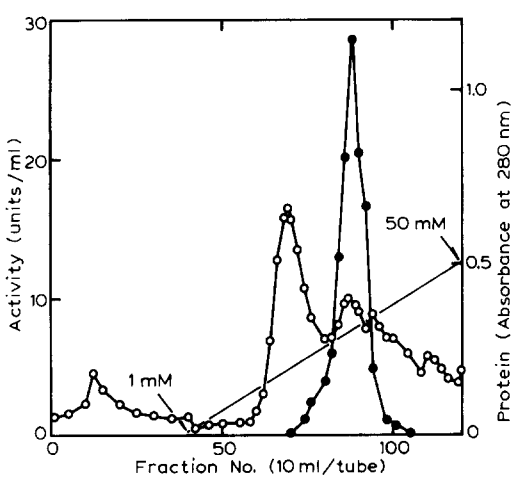

Fig. 3. Elution profiles of protein and α-glucosidase from a hydroxyapatite column with a concentration gradient system using phosphate buffer (pH 6.8) from 1 to 50 mM. The enzyme activity (units/ml eluate), ●———●; protein, ○———○; change of phosphate level, ———.

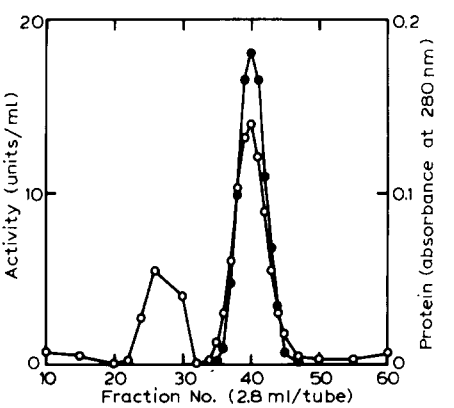

Fig. 4. Gel filtration of α-glucosidase on a Sephadex G-100 column. The enzyme activity (units/ml eluate), ●———●; protein, ○———○.

TABLE I

PURIFICATION OF α-GLUCOSIDASE FROM *BACILLUS THERMOGLUCOSIDIUS* KP 1006

Purification steps	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (-fold)	Yield (%)
1. Concentrate	2310	30 500	14 500	0.475	1	100
2. 40—70% $(NH_4)_2SO_4$ precipitate	996	5 830	11 500	1.97	4.1	79
3. DEAE-cellulose eluate	1150	3 220	10 900	3.39	7.1	75
4. 45—70% $(NH_4)_2SO_4$ precipitate	460	1 360	9 300	6.83	14.4	64
5. Sephadex G-200 eluate	110	763	8 480	11.1	23.4	58
6. Hydroxyapatite eluate	240	49.0	4 240	86.5	182	29
7. Sephadex G-100 eluate *	11.2 *	1.07 *	176 *	164	345	9.7

* 1/8 of the protein from Step 6 was purified in Step 7. The enzyme yield has been corrected for this change in amount, but all of the other values indicated refer to the aliquot.

A summary of the purification of α-glucosidase is given in Table I. The homogeneity of the purified enzyme was judged by disc electrophoresis on polyacrylamide gel [13]. The electrophoresis was done at 2 mA/tube (5.3 × 45 mm, 7.5% gel in Tris · HCl, pH 9.2; 30 μg protein applied/tube) for 3 h at 4°C with Tris · glycine buffer (pH 8.3). It was observed that two protein bands were present on the gel, one sharp at 2.8 cm from the start (the front, 4.2 cm) and the other only faint at 3.3 cm. α-Glucosidase activity absolutely coincided with the former band.

Characteristics of the purified enzyme

α-Glucosidase concentration was standardized to 0.173 μg protein/ml of the reaction mixture, except when otherwise stated.

Molecular weight. The molecular weight of α-glucosidase was estimated at 55 000 from its position of elution from a Sephadex G-200 column (1.6 × 96 cm), which had been prepared with 0.15 M potassium phosphate (pH 6.8) [14]. The gel-filtration was carried out at a rate of 0.7 ml/min with the same buffer (30 μg protein applied). The column was calibrated with blue dextran, myoglobin, egg albumin, bovine serum albumin and human γ-globulin.

Linearity and stoichiometry of reaction. The initial rate of hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside was directly proportional to α-glucosidase concentration. The reaction proceeded under a linearity during incubation for 10 min. One mol of the substrate was quantitatively hydrolyzed to yield one mol of D-glucose for each mol of *p*-nitrophenol liberated.

Effect of metal ions, EDTA and sulfhydryl reagents. The phosphate present in α-glucosidase preparation (0.7 ml) was removed by dialysis against 400 vols. of 0.2 M H_3BO_3/KCl/NaOH buffer (pH 7.5 at 23°C). Subsequently, the enzyme solution was mixed with 3.6 ml of the same buffer. A 0.1-ml aliquot (0.285 μg protein) was used for determination of the hydrolytic rate on *p*-nitrophenyl-α-D-glucopyranoside as described in Materials and Methods, except that the reaction mixture contained 2 mM heavy metal ions or EDTA in the borate buffer (20 mM). α-Glucosidase was completely blocked by Co^{2+}, Cu^{2+}, Fe^{3+}, Pb^{2+} and

TABLE II

SUBSTRATE SPECIFICITIES OF α-GLUCOSIDASE

The substrates (10 mM each; *p*-nitrophenyl-α-D-glucopyranoside, 2 mM) were allowed to react with α-glucosidase (10.1 μg protein) for 10 min at 60°C, and degrees of hydrolysis were calculated from glucose formed. The substrate levels were varied for K_m and V determination as follows: 0.05—5 mM *p*-nitrophenyl-α-D-glucopyranoside (the initial velocity was determined by Method 1 with 0.143 μg enzyme protein); 1—5 mM of phenyl-α-D-glucopyranoside (Method 2 with 2.60 μg protein); and 4—20 mM isomaltose or methyl-α-D-glucopyranoside (Method 2 with 5.19 μg protein).

Substrate	Hydrolysis (%)	K_m (mM)	V *
p-Nitrophenyl-α-D-glucopyranoside	100	0.23	183
Phenyl-α-D-glucopyranoside	93	3.0	314
Isomaltose	54	3.2	53.6
Methyl-α-D-glucopyranoside	7.3	27	13.0
Turanose	1.6	—	—

* μmol of substrate hydrolyzed/min/mg of protein.

Zn^{2+}, Mn^{2+}, Hg^{2+}, Ca^{2+}, Ba^{2+} and Mg^{2+} caused inhibitions of 96.6%, 86.3%, 37.2%, 13.5% and 11.8%, respectively. Preincubation of α-glucosidase at 60°C for 20 min with *p*-chloromercuribenzoate or monoiodoacetate (60 μM) before addition of the substrate failed to affect the enzyme activity.

Substrate specificity. As shown in Table II, α-glucosidase hydrolyzed *p*-nitrophenyl-α-D-glucopyranoside, phenyl-α-D-glucopyranoside, isomaltose, methyl-α-D-glucopyranoside, and turanose. But, the hydrolysis of turanose was only trace. Table II gives the K_m values and the maximal hydrolysis velocities (V) of these substrates at 60°C, which were determined by the method of Lineweaver and Burk [15]. α-Glucosidase had the highest affinity for *p*-nitrophenyl-α-D-glucopyranoside. Phenyl-α-D-glucopyranoside exhibited the highest V value. The following compounds (10 mM) were not substrates of α-glucosidase: sucrose, trehalose, melezitose, phenyl-α-D-maltoside, maltotriose, maltose, lactose, cellobiose, raffinose, dextran (1%), starch (1%), dextrin (1%), and the following *p*-nitrophenyl glycosides (3 mM); β-D-glucopyranoside, α- and β-D-galactopyranosides, α-D-mannopyranoside, β-L-fucopyranoside, and α- and β-D-xylopyranosides.

Inhibitors. Tris and histidine (5 mM) blocked hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside by 79.4% and by 14.8%, respectively. The following amines (5 mM) had no effect: aniline, hydrazine hydrate, NH_4Cl, glycine, serine and benzylamine. The rate of *p*-nitrophenyl-α-D-glucopyranoside hydrolysis was influenced by the following carbohydrates (10 mM); D-glucose, 35.9% inhibition; glucono-δ-lactone, 33.8%; glucosamine, 29.6%; isomaltose, 22.7%; gluconate, D-galactose and phenyl-α-D-glucopyranoside, 15.6%; D-xylose, 12.4%; maltotriose, 10.9%; trehalose, 9.4%; fuructose and mannitol, 7.7%; phenyl-α-D-maltoside, D-mannose, D-turanose, and melezitose, 6.3%; lactose, 4.7%; maltose, 3.0%; methyl-α-D-glucopyranoside, 2.6%; and cellobiose, 0%. Among the *p*-nitrophenyl compounds tested (3.33 mM), *p*-nitrophenol and its α-D-xylopyranoside were the most potent inhibitors (46.1% inhibition). The degrees of inhibition by other *p*-nitrophenyl glycosides were: α-D-mannopyranoside, 18.5%; β-

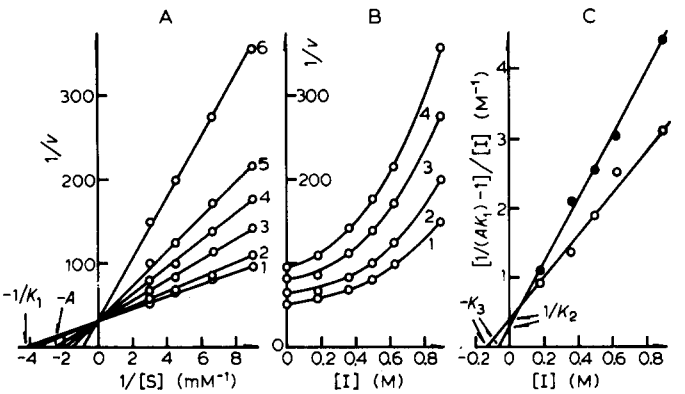

Fig. 5. Inhibition of α-glucosidase by sucrose. A: Lineweaver-Burk plots obtained at various sucrose levels (line 1, no sucrose; line 2, 0.18 M; line 3, 0.36 M; line 4, 0.50 M, line 5, 0.63 M; line 6, 0.90 M). —*A*, intercept value on the 1/[S] axis of the plot. B: Dixon plots at varying concentrations of *p*-nitrophenyl-α-D-glucopyranoside (curve 1, 0.333 mM; curve 2, 0.222 mM, curve 3, 0.150 mM; curve 4, 0.111 mM). C: Plots of $[1/(AK_1)-1]/[I]$ vs. [I] with sucrose (●———●) and maltose (○———○). *v*, initial reaction velocity (μmol per min); *V*, maximum initial velocity; [S], *p*-nitrophenyl-α-D-glucopyranoside concentration; [I], sucrose concentration (concentration of maltose or sucrose in C).

D-xylopyranoside, 10.9%; β-L-fucopyranoside, 9.2%; α-D-galactopyranoside, 3.1%; β-D-galactopyranoside and β-D-glucopyranoside, 0%. Glucose, glucono-δ-lactone, *p*-nitrophenyl-α-D-xylopyranoside and Tris inhibited α-glucosidase, competing with *p*-nitrophenyl-α-D-glucopyranoside. The respective K_i values were 2.2, 2.4, 0.14 and 0.12 mM, obtained by the Lineweaver-Burk method [15] and by the Dixon method [16].

Effect of sucrose and maltose. The hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside was affected by sucrose and maltose at their higher levels. As shown in Fig. 5A, the Lineweaver-Burk plots at various sucrose concentrations gave straight lines, intercepting at a point on the $1/v$ axis (v = initial reaction velocity). But the Dixon plots became parabolic curves (Fig. 5B). Furthermore, when $1/v$ was replotted against $[I]^2$ (I, sucrose), the plots obtained were found to linear, meeting at a point in the second quadrant of graph. Similar results were achieved when maltose was used as an inhibitor for α-glucosidase. These findings demonstrate a competition between 2 mol of the antagonist and 1 mol of the substrate for the catalytic site. The reactions involved in such a mechanism can be represented as:

$$E + S \rightleftharpoons ES \qquad (K_1) \qquad (1)$$

$$E + I \rightleftharpoons EI \qquad (K_2) \qquad (2)$$

$$EI + I \rightleftharpoons EI_2 \qquad (K_3) \qquad (3)$$

$$ES \rightarrow E + \text{Products} \qquad (4)$$

where E, S, and I denote α-glucosidase, *p*-nitrophenyl-α-D-glucopyranoside, and sucrose or maltose, respectively, and K_1, K_2 and K_3 are the respective dissociation constants of Eqns. 1—3. Assuming that Reactions 1—3 are in rapid equilibrium compared to Reaction 4, the reaction velocity can be formulated as fol-

lows:

$$\frac{1}{v} = \frac{1}{V} + \frac{1}{V}\frac{K_1}{[S]}\left(1 + \frac{1}{K_2} + \frac{1}{K_2 K_3}[I]^2\right) \quad (5)$$

where V is the maximal initial velocity. If the intercept on the 1/[S] axis of the Lineweaver-Burk plot is denoted by $-A$ Eqn. 6 can be derived from Eqn. 5:

$$\left(\frac{1}{AK_1} - 1\right)\frac{1}{[I]} = \frac{1}{K_2} + \frac{1}{K_2 K_3}[I] \quad (6)$$

On plotting $[1/(AK_1)-1]/[I]$ against [I], a straight line is obtained, cutting at $-K_3$ in the [I] axis and at $1/K_2$ in the $[1/(AK_1)-1]/[I]$ axis. K_1 corresponds to K_m of α-glucosidase for the substrate. As shown in Fig. 5C, the plots for maltose and sucrose according to Eqn. 6 both gave linear relationships. Respective values of K_2 and K_3 were calculated as 2.33 and 0.14 M with maltose, and 3.13 and 0.07 M with sucrose.

Effect of temperature on activity and stability. As shown in Fig. 6A, the rate of *p*-nitrophenyl-α-D-glucopyranoside hydrolysis was markedly increased by temperature elevation, exhibiting a maximum at 75° C, but abruptly dropped at the higher temperatures above this point. At 40° C, the velocity was only 4.5% of the maximal value. Arrhenius plots of the initial rates diverged from linearity, representing a biphasic curve at 30—75° C (Fig. 6B). The apparent activation energies were 28 000 cal/mol at 30—50° C and 5500 cal/mol at 65—75° C. As revealed in Fig. 6A, α-glucosidase was quite stable at 30—60° C. Indeed, heating for 2 h at 60° C caused no loss of activity. Inactivation developed slowly at temperatures beyond 60° C. The recovery of the enzyme activity was extremely diminished by exposure to the temperatures above 72° C (Fig. 6A).

Effect of pH on activity and stability. Fig. 7 shows that α-glucosidase func-

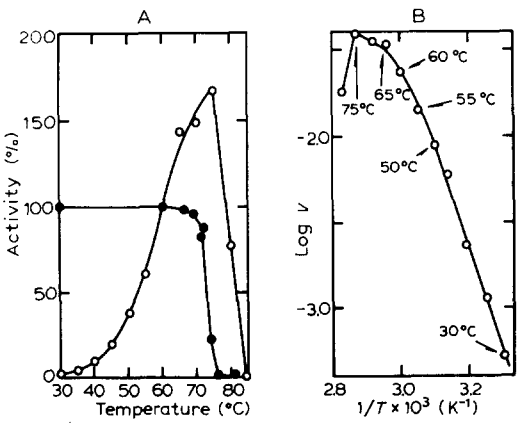

Fig. 6 Effect of temperature on stability and activity of α-glucosidase (A), and Arrhenius plots of the initial reaction velocities (B). In Fig. 6A, the activity observed at 60° C is expressed as 100%. Activity, ○———○. Stability, ●———●: 0.05-ml enzyme samples were heated for 10 min to different temperatures, after which they were chilled in ice water and mixed with 0.45-ml portions of cold 66.7 mM potassium phosphate (pH 6.8). The assay was conducted with 0.1-ml alqiuots (0.173 μg protein) of the diluted materials. T, absolute temperature; v, initial reaction velocity (μmol per min).

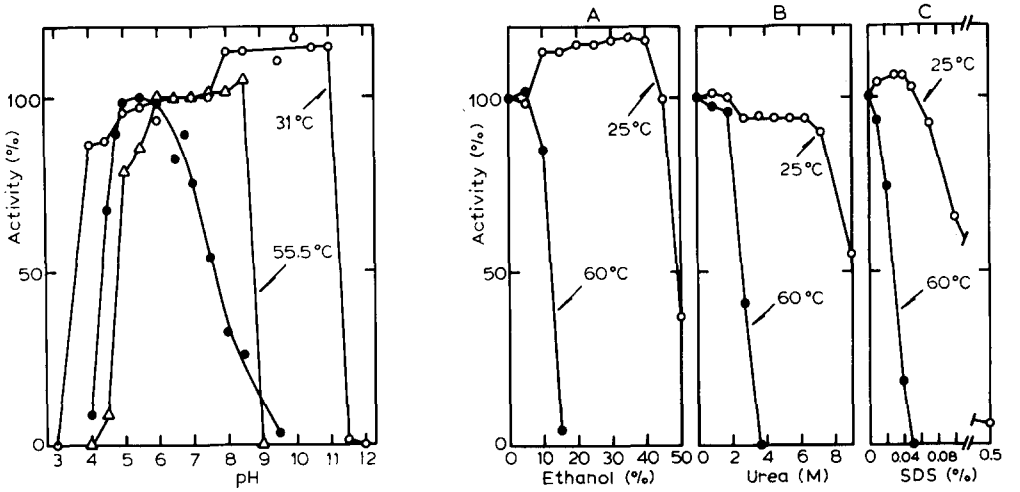

Fig. 7. Effect of pH on stability and activity of α-glucosidase. Stability at 31°C, ○———○; at 55.5°C, △———△. 0.05 ml enzyme solution was mixed with an equal volume of the following buffers: McIlvain's buffer (pH 3.0—8.5), 0.2 M Na_2CO_3/$NaHCO_3$ (pH 9.0—10.5), Ringer's buffer (pH 11.0—12.0). After incubation for 16 h at 31°C or 13 h at 55.5°C, the solution was mixed with 0.4 ml of cold 66.7 mM phosphate (pH 6.8). 0.1-ml portions (0.173 μg protein) were assayed for the activity recovered. The activity found at pH 7.0 before incubation is taken as 100%. Activity, ●———●; incubation was for 5 min at 60°C in the same medium as described in the text, except that the pH was altered by using 0.4 ml of the above buffers. At the end of incubation, to the medium were added 1.5 ml of 66.7 mM phosphate buffer (pH 6.8), after which pH and absorbance increase at 400 nm were measured. Amounts of *p*-nitrophenol were determined using its photometric titration curve at 400 nm (pK_a = 7.0 at 25°C). The data are expressed as percentages against the activity at pH 5.5.

Fig. 8. Effects of ethanol (A), urea (B) and sodium dodecyl sulfate (SDS) (C) on stability of α-glucosidase. Stability: at 25°C, ○———○; at 60°C, ●———●. The enzyme was treated with various concentrations of the reagents for 5 h at 25°C or at 60°C. At the end of incubation, the enzyme solutions (0.2 ml, 1.73 μg protein) containing ethanol or SDS were diluted with 0.8 ml each of 66.7 mM potassium phosphate (pH 6.8). The enzyme solutions (0.5 ml, 0.865 μg protein in 60 mM phosphate, pH 6.8) containing urea were used undiluted. 0.1-ml aliquots from these solutions were used for assay of the activity recovered. The activity found in the absence of the reagents is taken as 100%.

tioned optimally at pH 5.0—6.0, when *p*-nitrophenyl-α-D-glucopyranoside was used as a substrate. The activity fell suddenly at pH values below 5.0, but gradually above pH 6.0. The half-maximal activity was achieved at pH 4.3 and 7.6. α-Glucosidase was stable at 31°C over a wide pH range between 5.0 and 11.0, whereas the pH range became narrowed to 6.0—8.5 at 55.5°C (Fig. 7). An about 15% activation in the activity resulted, when α-glucosidase was treated in pH 8.0—11.0 at 31°C.

Effects of ethanol, urea, and sodium dodecyl sulfate on stability. As shown in Fig. 8, α-glucosidase was fairly stable at 25°C in 45% ethanol, in 7.2 M urea, and in 0.06% sodium dodecyl sulfate. Storage of the enzyme in 10—40% ethanol or in 0.01—0.05% sodium dodecyl sulfate brought about an increase by 14—17% or by 2—6% in the enzyme activity recovered (Fig. 8, A and C). But, such an activation was not observed with urea (Fig. 8B). α-Glucosidase was conspicuously labile at 60°C, and received a complete inactivation in 15% ethanol, in 3.6 M urea, and in 0.05% sodium dodecyl sulfate. The enzyme was stable at 60°C in 0.5% ethanol, and in 2 M urea.

Discussion

An α-glucosidase of *Bacillus thermoglucosidius*, which is responsible for hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside, has been highly purified from culture filtrate. The enzyme is able to hydrolyze, in addition to this chromogenic substrate, phenyl- and methyl-α-D-glucopyranosides. But, among the naturally occurring saccharides tested, isomaltose is only an effective substrate for the purified α-glucosidase. The enzyme fails to cleave the α-1,1-linkage of trehalose, α-1,2-linkages of sucrose and melezitose, or α-1,4-linkages of maltose, maltotriose, dextrin and starch. The α-1,3-linkage of turanose is attacked very slightly, but the same linkage in melezitose is not attacked at all. Although the α-1,6-linkage of isomaltose is split, dextran is not acted upon by the α-glucosidase. The α-glucosidase of *B. thermoglucosidius* strongly resembles an isomaltase isolated from a strain of *Saccharomyces cerevisiae* in its substrate specificity [17,18]. Although α-glucosidases purified from the cell-free extracts of *Pseudomonas* sp. [2], *Saccharomyces italicus* [9,19], and *S. cerevisiae* [20] can utilize as substrate *p*-nitrophenyl-α-D-glucopyranoside, these enzymes are differentiated from the *B. thermoglucosidius* enzyme by their abilities effectively to hydrolyze maltose and a number of disaccharides.

The molecular weight of α-glucosidase of *B. thermoglucosidius* is quite similar to that of the isomaltase of *S. cerevisiae* (M_r = 50 000) [18]. The former enzyme functions optimally at pH 5.5, the latter enzymes at pH 6.8. Both enzymes are strongly inhibited by heavy metal ions [17,18]. However, the *B. thermoglucosidius* enzyme is not affected by thiol reagents such as *p*-chloromercuribenzoate, but the isomaltase is irreversibly blocked by this reagent [18]. Tris is strongly inhibitory for the *B. thermoglucosidius* α-glucosidase and the *S. cerevisiae* isomaltase, as has been commonly observed with other α-glucosidases from various origins [3,9,17,18,21–24].

The hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside by the action of α-glucosidase of *B. thermoglucosidius* is prevented by the reaction products, glucose and *p*-nitrophenol. The inhibition by glucose is competitive with the substrate. But, the inhibition caused by *p*-nitrophenol has been found to appear abruptly at higher concentrations of the inhibitor than about 0.2 mM [25]. The reciprocal plots of the initial reaction velocities against the *p*-nitrophenol levels give sharply parabolic curves, which suggests multiple binding of *p*-nitrophenol to the enzyme. An elimination of C_6 from glucose in *p*-nitrophenyl-α-D-glucopyranoside produces a potent competitive inhibitor, *p*-nitrophenyl-α-D-xylopyranoside. The enzyme has a low affinity for maltose and sucrose, which is like the isomaltose of *S. cerevisiae* [18]. Binding of two molecules of these weak inhibitors to the catalytic site of the *B. thermoglucosidius* α-glucosidase is demonstrated in the present study.

α-Glucosidase of *B. thermoglucosidius* functions well at extraordinarily higher temperatures than isomaltase of *S. cerevisiae*. A maximal activation of the α-glucosidase is achieved at 75°C. However, the activity of this enzyme is negligible at temperatures below 40°C, at which the isomaltase exhibits its activity [17,18]. The α-glucosidase is fairly stable below 60°C, while the isomaltase is 96% inactivated by being kept at 52.5°C for only 15 min [17]. Arapid inactivation of the *B. thermoglucosidius* enzyme occurs above 72°C. The *S. cerevisiae*

isomaltase is stable between pH 6.2 and 7.5 at 30°C [18], while the α-glucosidase of *B. thermoglucosidius* is stable at pH 4.0—11.0 at 31°C. The pH range for stability of the α-glucosidase becomes narrowed to pH 6.0—9.0 at 55.5°C, which is wider than the pH range at 30°C of the isomaltase. The α-glucosidase is not denatured at 25°C in high concentrations of ethanol (45%), urea (7.2 M) and sodium dodecyl sulfate (0.06%). But, such a high tolerance towards these reagents is conspicuously reduced at 60°C. These observations suggest that the *B. thermoglucosidius* α-glucosidase takes a rigid protein conformation at temperatures around 25°C, which confers resistance against denaturation induced by the above reagents.

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