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New source of the thermostable α-glucosidase suitable for single step starch processing

Anna Zdziebło, Józef Synowiecki*

Department of Food Chemistry and Technology, Faculty of Chemistry, Technical University of Gdańsk, ul Gabriela Narutowicza 11/12, 80-952 Gdańsk, Poland

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Abstract

Thermus thermophilus contains α-glucosidase that is located in the cell cytoplasm. The highest enzyme activity was achieved after 25–30 h of microorganism cultivation at 70 °C in a medium containing 0.8% peptone, 0.4% yeast extract and 0.2% Na Cl. Addition of 2% of starch, maltose or glucose to the medium enhanced the enzyme activity by 83, 72 and 31%, respectively. The enzyme was purified 7.2-fold by combination of ammonium sulphate precipitation and ion-exchange chromatography on Sepharose CL-6B. The enzyme preparations exhibited highest specific activity for pNPG hydrolysis (1.94 U mg^{-1}) at pH 6.2 and 85 °C. The activity of partially purified enzyme was almost unchanged during 5 h of incubation at 75 °C in phosphate-citrate buffer (pH 6.2) and the half-life of this enzyme incubated at 85 °C was 2 h. Rate of pNPG cleavage catalysed by the α-glucosidase was 9.5-times higher than that in the case of maltose hydrolysis. Furthermore, this enzyme shows remarkable activity toward maltose and maltotriose. With maltotetraose, maltopentaose and maltohexaose the reaction rate decreased with increase in molecular weight of the substrate.

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Keywords: α-glucosidase; *Thermus thermophikus*; Starch processing; Thermostable enzymes

1. Introduction

α-Glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) catalyses the release of D-glucose residues from the nonreducing end of a variety of substrates, including disaccharides, oligosaccharides and aryl- and alkyl-α-glucopyranosides. This enzyme, produced intracellularly by many microorganisms, is involved in the metabolism of starch and other carbohydrates into glucose. Thermostable α-glucosidases, isolated from a variety of thermophiles or hyperthermophiles, are suitable for the improvement of industrial starch processing into glucose syrup. At present, this process is carried out in two steps: liquefaction and saccharification. The thermostable α-amylase from *Bacillus licheniformis*, used for starch liquefaction is active at 95 °C and pH 5.9 and these conditions are not compatibile with those of the amyloglucosidases used in the next step, which are less thermostable and work at a temperature of about 60 °C. The necessity of temperature and pH adjustment before saccharification increases the cost of the process and requires additional ion-exchange refining of the final product for removal of the salt. Legin, Copinet, and Duchiron (1998a,b) demonstrated the feasibility of glucose syrup production in a single-step using thermostable α-glucosidase, e.g. from *Thermococcus hydrothermalis*, cooperating with α-amylase and pullulanase, possessing similar working temperature and pH values. Moreover, increased temperature of the process enhances the saccharification rate. This paper presents a study concerning α-glucosidase activity of the thermophilic, obligate aerobe, *Thermus thermophilus*, with an optimal growth temperature of about 70 °C (Marteinsson, Birrien, Raguénés, da Costa, & Prieur, 1999; Oshima & Imahori, 1974). This organism is a producer of several hydrolytic enzymes of remarkable thermostability. The reported properties and yield of α-glucosidase isolation suggest suitability of *Thermus thermophilus* as a source of enzyme for one step starch processing.

* Corresponding author.

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2. Materials and methods

2.1. Microorganism and culture conditions

Thermus thermophilus HB-8 (ATCC 27534) was cultivated on media composed of 0.4% of yeast extract (Difco Laboratories, USA), 0.8% peptone bio-Trypcase (bio Merieux, France), 0.2% NaCl and water. The cultures were grown in 200 ml Erlenmeyer flasks containing 100 ml of liquid medium (pH 7.5) autoclaved for 30 min at 121 °C. The flasks were inoculated with 1 ml of a *Thermus thermophilus* cell suspension (OD_{600} 1.2) and stirred in a water bath at 150 rpm at 70 °C for 2–50 h without additional aeration.

To examine the induction of thermostable α-glucosidase, 2.0% of maltose, starch or glucose was added to the medium after 8 h of *Thermus thermophilus* cultivation. After the desired growth time the cells were harvested by centrifugation at 12,000×*g* for 15 min. The pellet was washed with 5 mM Na_2HPO_4 solution, centrifuged, and stored at −18 °C until use.

2.2. Partial purification of α-glucosidase

Frozen cells (10 g) were disrupted for 15 min in a refrigerated mortar with 20 g of Alumina A-5 (Sigma, St. Louis, MO, USA) and 70 ml of 0.01 M sodium phosphate buffer (pH 7.2) containing 1 mmol $MgSO_4$ and 1 mmol of β-dithiothreitol per 1 l of the buffer, which was gradually added during extraction. The resulting suspension was centrifuged at 12,000×*g* for 15 min. Solid $(NH_4)_2SO_4$ was slowly added to the cell-free extract to 80% of saturation and the mixture was left overnight at 4 °C. The precipitate formed was collected by centrifugation at 12,000×*g* during 15 min, dissolved in 8 ml of 0.1 M phosphate citrate buffer pH 6.2 and concentrated by ultrafiltration on a Centriplus Centrifugal Filter Device with Ultracel-YM membrane 50-kDa cutoff (Millipore Corporation). The concentrated crude enzyme solution was applied to a DEAE Sepharose CL-6B column (2.8×38 cm) pre-equilibrated with 50 mM acetate buffer (pH 6.0). The column was eluted with 0.25 M NaCl in the same buffer, at a flow rate of 60 ml/h and 2 ml fractions were collected for determination of enzyme activity and protein content. This purification procedure resulted in 6-fold increase of enzyme specific activity measured at 70 °C using *p*-nitrophenol-α-D-glucopyranoside (pNPG) as substrate.

2.3. Determination of α-glucosidase activity

α-Glucosidase activity was determined by release of *p*-nitrophenol from 5 mM pNPG solution from Sigma, USA in 0.1 M phosphate citrate buffer (pH 6.2 adjusted at 70 °C) using the procedure of Legin et al. (1998b). The assays were initiated by the addition of 0.5 ml of enzyme solution to 2.5 ml of substrate, preincubated for 2 min at 70 °C. The reaction at 70 °C was terminated after 2 min by the addition of 1 ml of 1 M Na_2CO_3 solution. A blank, containing buffer instead of enzyme solution, was used to correct the thermal hydrolysis of pNPG. Absorbances at 405 nm were converted to *p*-nitrophenol concentration using a molar absorption coefficient of $16.86\times10^3\times M^{-1}$. One unit of α-glucosidase activity is defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol from pNPG per minute under the described conditions. Specific activity is α-glucosidase units per mg of protein, determined as by Lowry, Rosenbrough, Farr, and Randall (1951), using bovine serum albumin as a standard.

α-Glucosidase activity in the whole cells was assayed according to above procedure, using, instead of enzyme solution, an appropriate volume of the cell suspension in 0.1 M phosphate citrate buffer (pH 6.2), preincubated 30 min at 70 °C with 2 drops of trichloromethane and 1 drop of 0.1% SDS solution. Before absorbance measurement, the samples were centrifuged at 12,000×*g* for 10 min.

The amount of glucose released during carbohydrate hydrolysis was determined according to the method of Hugget and Nixon (1955), using the GOPOD reagent containing 125 mg of glucose oxidase, 5 mg peroxidase, 0.5 ml of 1% solution of *o*-dianisidine in 96% ethanol and 0.5 M phosphate buffer (pH 7.0), added to a final volume of 100 ml. The assays were initiated by addition 0.5 ml of α-glucosidase solution to 0.5 ml of 0.25% solution of maltose, maltotriose, maltotetraose, maltopentaose or maltohexaose in 0.1 M phosphate citrate buffer (pH 6.2). The reaction at 70 °C was stopped by addition of 3 ml of GOPOD reagent and incubation was continued for 30 min at 40 °C. The absorbance was measured at 415 nm and the amount of liberated glucose was calculated from the regression equation of the standard curve, determined at glucose concentrations up to 0.12 μmoles ml^{-1}.

2.4. The pH profile, optimal temperature and enzyme thermostability

The effect of pH on α-glucosidase activity was determined at 70 °C using 5 mM pNPG solution in 0.1 M phosphate citrate buffers in the pH range 5.0–8.0 adjusted at 70 °C. The temperature-dependence of enzyme activity was assayed in the range 50–95 °C, using the same substrate solution at pH 6.2. The α-glucosidase thermostability was determined by subjecting the enzyme solution in 0.1 M citrate phosphate buffer (pH 6.2) in sealed tubes to temperatures between 56 and 95°C. Samples were withdrawn at various times, cooled in ice and assayed for remaining activity at 70 °C and pH 6.2 using pNPG as substrate. The enzyme half-life values were calculated from the graph of the log residual activity versus time.

2.5. Effect of various compounds on enzyme activity

Partially purified α-glucosidase was preincubated 0.5 h at 20 °C in 0.1 M phosphate citrate buffer (pH 6.2) containing 1.0 mM of various cations in the form of chlorides, or 5.0 mM of inhibitors or other compounds listed in Table 3. The activity towards pNPG was determined using the standard assay method and was expressed as a percentage of that of control. In the control, each compound was added after termination of the enzymatic reaction.

2.6. Identification of the products of maltose hydrolysis

The assays were initiated by addition 0.5 ml of partially purified α-glucosidase to 2.0 ml of 1.0% solution of maltose (Sigma, Germany) in 0.1% phosphate-citrate buffer (pH 6.2). After 0.5 h, 1.0 h or 2.0 h of reaction, 5 μl of aliquots were removed and subjected to thin-layer chromatography on plates (10×20 cm) of silica gel 60 (Merck, Germany). The chromatograms were developed in a solvent system composed of 2-propanol, acetone and 1% aqueous solution of lactic acid (4:4:2, v/v). The sugar spots were visualized by spraying with reagent consisting of aniline (4 ml), diphenylamine (4 g), acetone (200 ml) and 85% H_3PO_4 (30 ml), followed by incubation at 105 °C for 1 h (Hansen, 1975). As standards, 1.0% solutions of glucose, maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose were used.

3. Results and discussion

Microbial α-glucosidases can be either intracellular, extracellular or membrane-bound (Urlaub & Wöber, 1978). Most of α-glucosidase activity was detected in the cell extract and only 3.9±1.6% of total activity was found in the culture broth after cultivation of *Thermus thermophilus* HB-8 on the medium containing 0.8% peptone, 0.4% yeast extract and 0.2% NaCl. The release of trace amounts of the enzyme to the medium was probably associated with the cell lysis. The sonication step increased the amount of extracted proteins by about 60% as compared to that after mechanical cell disruption. However, the enzyme activity in the sonicated samples was enhanced by only 7.7%. This suggests, that the α-glucosidase was not membrane bound. The relationship between biomass yield and changes in the ratio of peptone and yeast extract and mineral salt contents in the medium was reported earlier (Maciuńska, Czyż, & Synowiecki, 1998). The cell concentration and α-glucosidase production are correlated ($r = 0.99$) during the exponential growth phase, which reached the end after 25–30 h of microorganism cultivation at 70 °C (Fig. 1). Final density of the culture achieved during this time was about 1.6 g of dry cells per litre of medium. The cell-free extract from *Thermus thermophilus* growing without inducers had a specific activity 0.27±0.06 U mg^{-1} protein. Addition of 2.0 starch, maltose or glucose to the medium increases the α-glucosidase production by about 83, 72 and 31%, respectively. The yield of α-glucosidase extracted from *Thermus thermophilus* cultures reached the satisfactory level 170 U/l of medium, which was about 11-times higher than that reported for *Thermococcus hydrothermalis* by Legin et al. (1998b). Higher productivity of α-glucosidase (302 U/l of medium) is shown by *Pyrococcus furiosus* cultures (Constantino, Brown, & Kelly, 1990). However, large-scale cultivation of this hyperthermophilic anaerobe is difficult.

The enzyme was purified from the cell extract as described above. Specific activity of the cell-free extract increased to 0.47 U mg^{-1} protein after ammonium sulphate precipitation (Table 1). The total activity yield of about 72% was quite similar to the values measured by Maciuńska et al. (1998) and Cowan, Daniel, and Morgan (1984) during isolation of β-galactosidase from *Thermus thermophilus* HB-8 (75%) and *Thermus* 4–1A strain (74%). After anion-exchange chromatography on DEAE Sepharose CL-6B, the α-glucosidase activity was enhanced about seven times (Table 1). Furthermore, the elution profile of the crude enzyme from *Thermus thermophilus* shows the presence of some proteins without enzyme activity (Fig. 2).

Table 1
Changes of specific activity during purification of α-glucosidase from *Thermus thermophilus* (HB-8)[a]

Purification steps	Specific activity (U mg^{-1} protein)	Purification (fold)
Cell extract	0.27±0.06	1.0
$(NH_4)_2SO_4$ precipitation	0.47±0.15	1.7
Dialysed enzyme solution	0.48±0.09	1.8
DEAE Sepharose CL-6B	1.94±1.37	7.2

[a] The results are mean values of two purification procedures ±standard deviation.

Table 2
Substrate specifity of partially purified α-glucosidase from *Thermus thermophilus* (HB-8)[a]

Carbohydrate	Rate of hydrolysis (10^{-3} U mg^{-1})	Relative rate of hydrolysis (%)
Maltose	28.0±0.95	100
Maltotriose	27.8±0.23	99.1
Maltotetraose	15.9±0.13	56.8
Maltopentaose	8.55±0.10	30.5
Maltohexaose	5.69±0.23	20.3

[a] The results are the mean values of data from three determinations ±standard deviation.

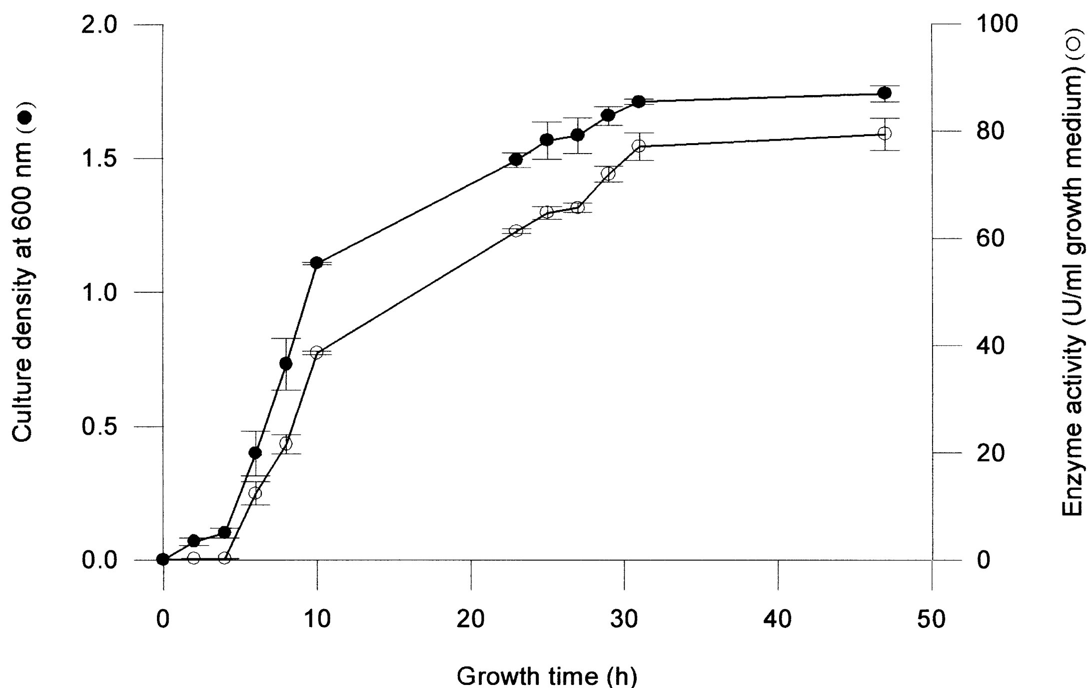

Fig. 1. Cell productivity and changes of α-glucosidase activity during growth of *Thermus thermophilus* (HB-8) at 70 °C in a medium listed in Section 2. The results are the mean values of three replicates.

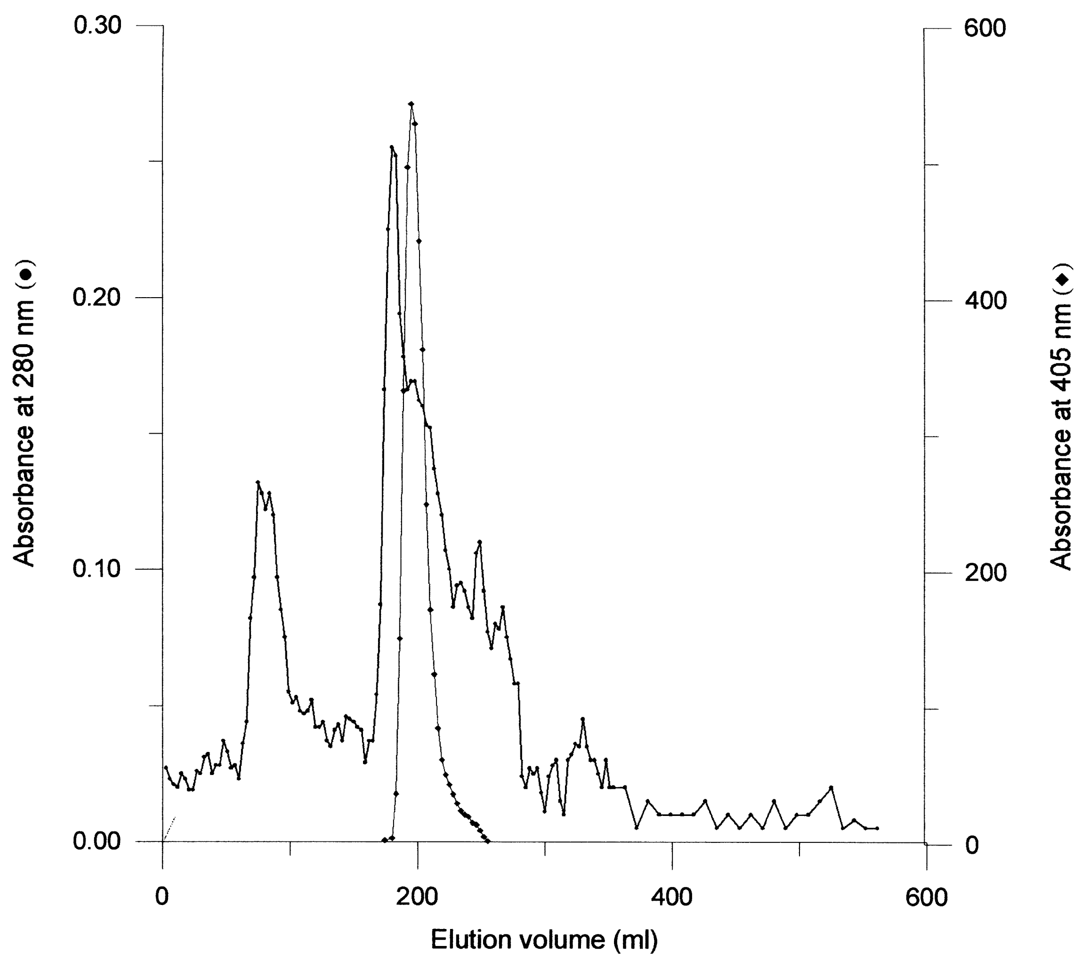

Fig. 2. Ion-exchange chromatography of α-glucosidase on the column (2.8×38 cm) with Sepharose CL-6B. Absorbances at 280and 405 nm represent changes in protein content and enzyme activity, respectively, between collected fractions (2 ml).

Microbial α-glucosidases exhibit significant diversity in their substrate specifity and are classified into those with highest specific activity toward maltose and those with highest activity toward aryl-D-glucosides (Kelly & Fogarty, 1983). The rate of pNPG cleavage catalysed by α-glucosidase from cell extract of *Thermus thermophilus* HB-8 was 9.5 times higher than that of maltose hydrolysis. Maltose and maltotriose were also efficiently hydrolysed by the enzyme (Table 2). For other carbohydrates tested, the reaction rate decreased with increase in molecular weight of the substrate. Maltohexaose was hydrolysed at 20.3% of the rate of maltose. The hydrolysis products caused by action of the crude α-glucosidase on maltose were analysed by thin-layer chromatography (Fig. 3). Glucose was the main end-product. However, the presence of small amounts of maltotetraose and other oligosaccharides was also detected. The formation of oligosaccharides was probably due to condensation reactions or transferase activity of the enzyme, which was also observed in the case of some other α-glucosidases (Kelly & Fogarty,1983; Nakao et al., 1994).

The data given in Table 3 suggest that α-glucosidase from *Thermus thermophilus* may not require metal ions for activity. This was confirmed by the lack of inhibitory effect of EDTA, which usually decreases metaloenzyme activity. Furthermore, low influence of 4-chloromercuribenzoate and monoiodoacetate on the α-glucosidase activity implies that cysteine residues are

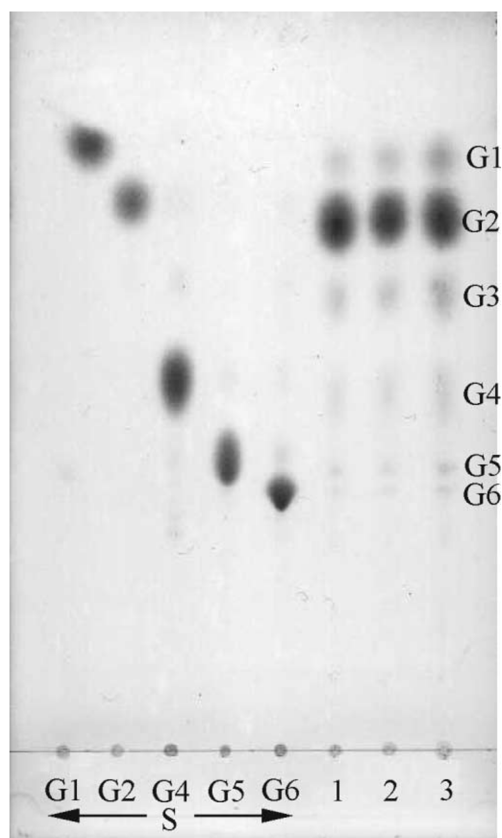

Fig. 3. Thin-layer chromatography of the products of maltose hydrolysis catalysed by α-glucosidase from *Thermus thermophilus* (HB-8) performed according to procedure described in Section 2. Abbreviations: G1, Glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; S, standard markers; 1, 2 and 3, samples hydrolysed for 0.5 h, 1 h and 2 h, respectively.

Table 3
Effects of various cations on the activity of paritially purified α-glucosidase from *Thermus thermophilus*[a]

Cation	Residual activity (%)
K^{+}	98.2±2.9
Ca^{2+}	99.5±2.0
Cu^{2+}	98.9±3.9
Mg^{2+}	100.1±2.3
Mn^{2+}	94.1±2.4
Fe^{2+}	90.3±2.9
Co^{2+}	95.9±4.4
Ba^{2+}	96.8±1.9
Hg^{2+}	27.6±0.9
Zn^{2+}	89.2±4.1
Cd^{2+}	113±1.9

[a] Means represent six determinations±standard deviation. The cation concentrations are 1 mM.

Table 4
Effect of various inhibitors and other agents on the activity of α-glucosidase from *Thermus thermophilus*[a]

Compound	Residual activity (%)
4-Chloromercuribenzoate	99.8±4.0
Monoiodoacetate	95.7±1.2
Phenylmethanesulphonyl fluoride	95.4±2.9
EDTA	110±2.1
2-Mercaptoethanol	136±3.5

[a] Means represent six determinations±standard deviation. The inhibitor concentrations are 5 mM.

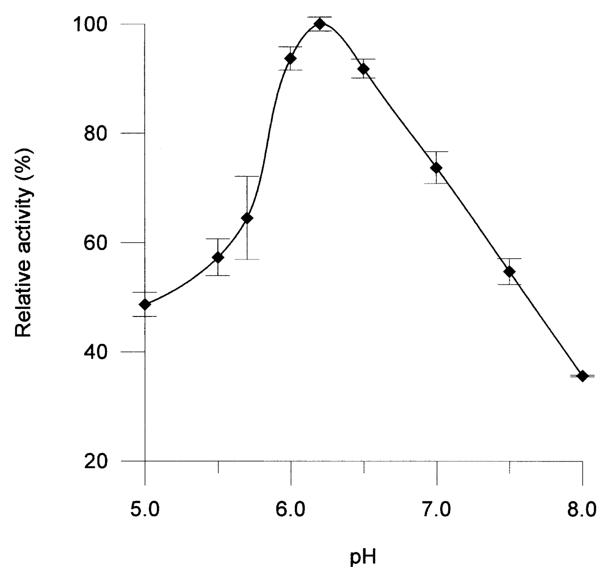

Fig. 4. Effect of pH at 70 °C on the activity of α-glucosidase from *Thermus thermophilus* (HB-8). The results are the mean values of three replicates.

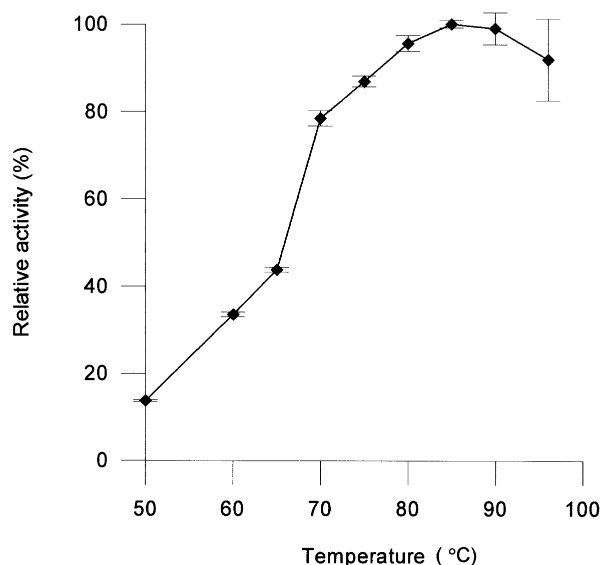

Fig. 5. Effect of temperature at pH 6.2 on the activity of α-glucosidase from *Thermus thermophilus* (HB-8). The results are the mean values of three replicates.

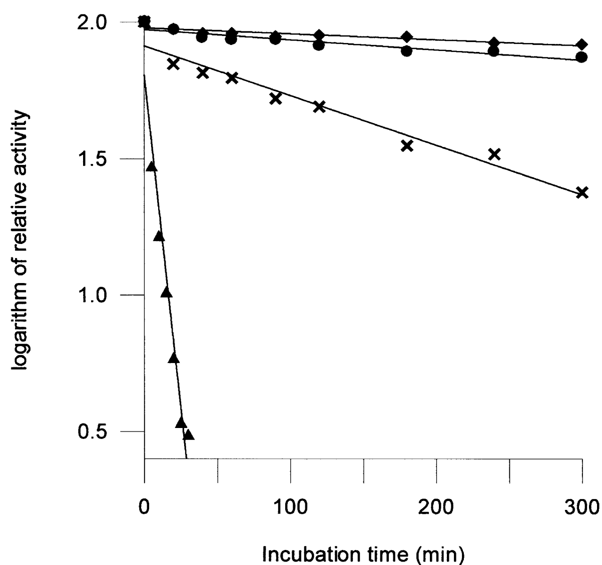

Fig. 6. Thermal stability of α-glucosidase from *Thermus thermophilus* (HB-8) incubated in M phosphate citrate buffer (pH 6.2) at 65 °C (◆), 75 °C (●), 85 °C (×) and 95 °C (▲). The results are mean values of three replicates. Standard deviations did not exceed 5% of the recorded values.

not involved in the enzyme active site (Table 4). This shows that the strong inhibition of the α-glucosidase by Hg^{2+} was not related to interaction of this cation with cysteine in the enzyme molecules. This finding is similar to the effect of these reagents on the thermostable α-glucosidases from *Clostridium thermohydrosulphuricum* and *Bacillus* sp. SAM1606 (Nakao et al.,1994; Saha & Zeikus,1991). Phenylmethanesulphonyl fluoride, which acts as an efficient inhibitor of enzymes with histidine and serine residues in the active site, did not significantly affect the activity of *Thermus thermophilus* α-glucosidase (Table 4).

The optimum pH for the hydrolysis of pNPG was 6.2 and the enzyme retained about 80% of maximal activity at pH values 5.8–6.9 (Fig. 4). The reported pH values are similar to those necessary for efficient starch liquefaction by α-amylase from *Bacillus licheniformis* (Termamyl®). This makes possible the application of both enzymes in a one-stage process.

The optimal temperature for *Thermus thermophilus* α-glucosidase activity (85 °C) given in Fig. 5 is similar to that (87 °C) determined for β-galactosidase isolated from this bacterium (Maciuńska et al.,1998). Only few α-glucosidases, e.g. from *Pyrococcus furiosus*, *Sulfolobus solfataricus* and *Thermococcus hydrothermalis*, show maximum activities at higher temperatures: 98, 95 and 96 °C, respectively (Constantino, Brown, & Kelly, 1990; Leveque, Janeček, Haye, & Belarbi, 2000; Rolfsmeier & Blum,1995). The measurement of *Thermus thermophilus* α-glucosidase thermostability as a function of temperature showed that the activity of partially purified enzyme did not change significantly during 5 h of incubation at 75 °C in phosphate citrate buffer (pH 6.2). Furthermore, the half-life of activity of this enzyme, incubated at 85 °C, was about 2 h and only at 95 °C did no measurable activity remain after 30 min of heating (Fig. 6). These properties of α-glucosidase suggest that *Thermus* thermophilus is a good source of the gene encoding synthesis of the enzyme suitable for one step starch processing. Cloning of this gene to a mesophlilic host might increase the α-glucosidase productivity. Experiments in this direction are now in progress.

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References

Constantino, H. R., Brown, S. H., & Kelly, R. M. (1990). Purification and characterization of an α-glucosidase from a hyperthermophilic bacterium *Pyrococcus furiosus*, exhibiting a temperature optimum of 105 to 115 °C. *Journal of Bacteriology*, *172*, 3654–3660.

Cowan, D. A., Daniel, R. M., & Morgan, H. W. (1984). Some properties of β-galactosidase from an extremely thermophilic bacterium. *Biotechnology and Bioengineering*, *26*, 1141–1145.

Hansen, S. A. (1975). Thin-layer chromatography method for identification of oligo-saccharides in starch hydrolysates. *Journal of Chromatography*, *105*, 388–390.

Huggett, A. S. C. & Nixon, D. A. (1955). Glucose oxidase method for measurement. *Biochemistry Journal*, *66* 12–19.

Kelly, C. T., & Fogarty, W. M. (1983). Microbial α-glucosidases. *Process Biochemistry*, *18*, 6–12.

Legin, E., Copinet, A., & Duchiron, F. (1998a). A single step high temperature hydrolysis of wheat starch. *Starch/Stärke*, *50*, 84–89.

Legin, E., Copinet, A., & Duchiron, F. (1998b). Production of thermostable amylolytic enzymes by *Thermococcus hydrothermalis*. *Biotechnology Letters*, *20*, 363–367.

Levêque, E., Janeček, Ŝ., Haye, B., & Belarbi, A. (2000). Thermophilic archaeal amylolytic enzymes. *Enzyme Microbial Technology*, *26*, 3–14.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Maciuńska, J., Czyż, B., & Synowiecki, J. (1998). Isolation and some properties of β-galactosidase from thermophilic bacterium *Thermus thermophilus*. *Food Chemistry*, *63*, 441–445.

Marteinsson, V. T., Birrien, J. L., Raguénés, G., da Costa, M. S., & Prieur, D. (1999). Isolation and characterization of *Thermus thermophilus* Gy1211 from a deep-sea hydrothermal vent. *Extremophiles*, *3*, 247–251.

Nakao, M., Nakayama, T., Harada, M., Kakudo, A., Ikemoto, H., Kobayashi, S., & Shibano, Y. (1994). Purification and characterization of a *Bacillus* sp. SAM1606 thermostable α-glucosidase with transglucosylation activity. *Applied of Microbiology and Biotechnology*, *41*, 337–343.

Oshima, T., & Imahori, K. (1974). Description of *Thermus thermophilus* a nonsporulating thermophilic bacterium from a Japanese thermal spa. *International Journal of Systematic Bacteriology*, *24*, 102–104.

Rolfsmeier, M., & Blum, P. (1995). Purification and characterization of maltase from the extremely thermophilic crenarchaeote *Sulfolobus solfataricus*. *Journal of Bacteriology*, *177*, 482–485.

Saha, B. C., & Zeikus, J. G. (1991). Characterization of thermostable α-glucosidase from *Clostridium thermohydrosulfuricum* 39E. *Applied Microbiology and Biotechnology*, *35*, 568–571.

Urlaub, H., & Wöber, G. (1978). α-Glucosidase, a membrane-bound enzyme of α-glucan metabolism in *Bacillus amyloliquefaciens*, purification and partial characterization. *Biochimica et Biophysica Acta*, *522*, 161–173.