The Journal of Microbiology (2010) Vol. 48, No. 4, pp. 452-459
DOI 10.1007/s12275-010-9319-2

Purification and Characterization of the α-Glucosidase Produced by Thermophilic Fungus *Thermoascus aurantiacus* CBMAI 756

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(Received October 14, 2009 / Accepted April 23, 2010)

An α-glucosidase enzyme produced by the fungus *Thermoascus aurantiacus* CBMAI 756 was purified by ultra filtration, ammonium sulphate precipitation, and chromatography using Q Sepharose, Sephacryl S-200, and Superose 12 columns. The apparent molecular mass of the enzyme was 83 kDa as determined in gel electrophoresis. Maximum activity was observed at pH 4.5 at 70°C. Enzyme showed stability stable in the pH range of 3.0-9.0 and lost 40% of its initial activity at the temperatures of 40, 50, and 60°C. In the presence of ions Na^+, Ba^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, Al^{3+}, Zn^{2+}, Ca^{2+} this enzyme maintained 90-105% of its maximum activity and was inhibited by Cr^{3+}, Ag^+, and Hg^{2+}. The enzyme showed a transglycosylation property, by the release of oligosaccharides after 3 h of incubation with maltose, and specificity for short maltooligosaccharides and α-PNPG. The K_m measured for the α-glucosidase was 0.07 μM, with a V_{max} of 318.0 μmol/min/mg.

***Keywords*:** α-glucosidase, *T. aurantiacus*, termostable enzyme, transglycosylation reaction, purification

Thermoascus aurantiacus is a thermophilic fungus that belongs to the phylum Ascomycota. It grows optimally at 50°C and has been described as a great producer of enzymes with industrial interest such as β-glucosidases, proteases, amylases, and xylanases (Carvalho *et al*., 2006; Leite *et al*., 2007; Merheb *et al*., 2007; Brienzo *et al.*, 2008). Among the features that could be attributed to these enzymes, and that give them an enormous commercial potential, we cite a reasonable stability over a wide pH and temperature range.

Glucosidases belong to a group of glycoside hydrolases (EC 3.2.1.x) involved in the metabolism of oligossacarides, and biosynthesis and modification of glycoproteins (Melo *et al*., 2006). The enzymes α-glucosidase (EC3.2.1.20) and glucoamylase (EC3.2.1.3) are glucosidases that catalyze the hydrolysis of α-glucosidic *0*-linkage, releasing D-glucose from the non-reducing end side of target substrates. The distinction between them is based on the inversion of the anomeric configuration of the substrate (α → β or β → α), typical of β-amylase and glucoamylase, or its retention (α → α or β → β), which characterizes the α-glucosidase (Chiba, 1997). Thus, the glucoamylase releases β-glucose, while α-glucosidase forms α-glucose. The additional property of this retaining enzyme (α-glucosidase) is the transglycosylation activity that forms α-1,6 linkages (Chiba, 1997; Shimba *et al*., 2009).

Concerning the classification of glycoside hydrolases, α-glucosidases belong to families 13 and 31. Enzymes of family 13 are more active on heterogeneous substrates such as phenyl α-glucoside and sucrose than on maltose. Family 13 includes enzymes designated as type I. On the other hand, enzymes belonging to the family 31 have preference for homogeneous substrates such as maltose, maltotriose and maltotetraose. The α-glucosidases types II and III are classified as family 31. Finally, the type III α-glucosidase can hydrolyze polysaccharides such as amylose and starch (Chiba, 1997; Frandsen and Svensson, 1998).

The intracellular α-glucosidases are important for the synthesis of glycoproteins, a reaction that happens into the endoplasmic reticulum. α-Glucosidase I removes the outer α-1,2 linked glucose residue from its substrate, while the α-glucosidase II removes two α-1,3 linked glucose residues from peptides (Mehta *et al*., 1998). These enzymes can also operate in the biosynthesis and/or insertion of α-1,6 glucans into the fungus cell wall (Herscovics, 1999). Furthermore, the α-glucosidase in many bacteria represents the final stage of starch metabolism to generate glucose (Constantino *et al*., 1990).

The extracellular α-glucosidases isolated from many fungal strains are suitable for transglycosylation reactions from maltose, resulting in isomaltose (6-0-α-D-glucopyranosyl-D-glucopyranose) and panose (6-0-α-D-glucopyranosyl-maltose), which can be used in the food industry to increase the chemical properties of food (Kato *et al*., 2002). Moreover, the oligosaccharides and sugar conjugates generated have physiological functions with beneficial health effects such as being non-carcinogenic, they are prebiotics, they may improve gastrointestinal conditions, and promote mineral absorption (Murata and Usui, 2006).

In a previous work we reported the characterization of an α-glucosidase activity in a crude enzyme extract prepared from the *T. aurantiacus* CBMAI 756 strain (Carvalho *et al*., 2006). Here, we show the purification and characterization of a novel α-glucosidase enzyme produced by this thermophilic fungus, which generates oligosaccharides by transglycosylation reactions.

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Materials and Methods

Microorganism, cultivation, and enzyme production

The *T. aurantiacus* strain used in the present study was isolated from decaying lignocellulosic material collected in the State of Amazonas, Brazil, and was maintained as a stock culture at 7°C on potato dextrose agar. This strain was deposited in the Coleção Brasileira de Microrganismos para Ambiente e Indústria (CBMAI) at the CPQBA, Unicamp, Campinas, SP, Brazil, and received the code CBMAI 756. α-Glucosidase was produced by submerged fermentation (SmF) in Erlenmeyer flasks (250 ml) containing 50 ml medium consisting of 1.0% (w/v) soluble starch (Sigma, USA) and nutrient solution described by Carvalho *et al*. (2006). Strain inoculation was done according to Carvalho *et al*. (2006). Fermentation was carried out in a rotary shaker at 100 rpm for 216 h at 50°C. The biomass was separated by filtering through Whatman No. 1 paper in a Büchner funnel. The filtrate was used as a crude extract of α-glucosidase.

Measurement of enzyme activity

The hydrolysis activity was assayed at 70°C in a reaction mixture containing 0.1 ml of purified enzyme appropriately diluted and 0.4 ml of 0.20% (w/v) maltose (Sigma) in 250 mM sodium acetate buffer, pH 4.5. The glucose released was estimated by the peroxidase/glucose oxydase assay (Bergmeyer and Bernt, 1974). One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of glucose per minute under the assay conditions used.

When *p*-nitrophenyl-α-D-glucopyranoside (α-PNPG, Sigma) and *p*-nitrophenyl-β-D-glucopyranoside (β-PNPG) were used as substrates, the activity was measured in a mixture containing 0.25 ml of 100 mM sodium acetate, pH 4.5, 0.25 ml of 4 mM substrate solution, and 0.05 ml of purified enzyme. After 10 min of incubation at 70°C, the reaction was stopped with 2 ml of sodium carbonate 2 M, and the *p*-nitrophenol released was quantified spectrophotometrically at 410 nm (Palma-Fernandez *et al*., 2002). One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute in the reaction mixture.

Purification of the α-glucosidase

The α-glucosidase enzyme was purified as follows.

Ultra filtration

The crude extract containing the enzyme was concentrated by ultra filtration using Quixstand™ Benchtop (GE, England) with a cut-off of 50 kDa. At first, the crude enzyme extract (volume of 1,000 ml) was concentrated to 200 ml (concentration factor of 5×) and then mixed with 800 ml of buffer (10 mM sodium acetate, pH 4.0), followed by a new concentration. This procedure was repeated four times to remove pigments.

Ammonium sulphate precipitation

The filtered extract was precipitated with ammonium sulphate, which was added gradually to the extract under stirring up to 55% (w/v) of saturation. This solution was kept for 6 h at -20°C in order to precipitate the proteins, and then centrifuged at 12,000×g for 20 min. In a second stage, the supernatant was 90% (w/v) saturated with ammonium sulphate under slight stirring at 4°C, and maintained without stirring overnight at -20°C. The precipitate was recovered by centrifugation at 12,000×g for 20 min, and dissolved at a minimum volume (4 ml) of 10 mM sodium acetate buffer, pH 4.0. This suspension was dialyzed against the same buffer at 4°C, for 24 h. After dialysis, the material was filtered through a 0.22 μm pore size sterile filter (Millipore, USA).

Ion exchange chromatography

The dialyzed extract was loaded on to an anion exchange Q Sepharose Fast Flow Tricorn 10/50 column (Pharmacia Biotech, Sweeden), with a bed volume of 20 ml equilibrated with 50 mM sodium acetate buffer, pH 4.0. The column was washed with two column volumes 10 mM sodium acetate buffer, pH 4.0, and the bound proteins were eluted with the same buffer through a linear gradient of NaCl varying from 0 to 1.0 M, at a flow rate of 0.4 ml/min. Fractions of 1.5 ml were collected. The fractions with α-glucosidase activity were pooled and desalted overnight by dialysis against 10 mM sodium acetate buffer, pH 4.0, at 4°C, lyophilized and dissolved in 0.8 ml of 10 mM phosphate buffer, pH 7.0.

Gel filtration chromatography

Proteins were subsequently separated through gel filtration using a Sephacryl S-200 column (Sigma – 17-1166-01), and followed by a Superose 12 column (Sigma – 17-5173-01) using an ÄKTÄ purifier. The sample was applied in columns and eluted with 50 mM phosphate buffer containing 150 mM NaCl, pH 7.0, at a flow rate of 0.3 ml/min.

The molecular mass of the purified enzyme was tentatively determined by SDS-PAGE and gel filtration using a Sephacryl S-200 column. The standards used in column were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and carbonic anhydrase (29 kDa). The elution profiles were monitored by absorbance at 280 nm and those corresponding to α-glucosidase fractions were identified by enzyme activity.

Electrophoresis

The samples collected during the chromatography steps were analyzed by SDS-PAGE, according to Laemmli (1970). Prior to SDS-PAGE, each sample was treated by two different procedures: (1) without boiling and without the addition of β-mercaptoethanol (semi-denaturing; resolved through an 8% gel), and (2) sample was boiled for five minutes in the presence of β-mercaptoethanol (denatured sample; resolved in a 10% gel). Proteins were visualized by silver-staining (Blum *et al*., 1987). The determination of the α-glucosidase activity was carried out directly in the SDS-PAGE using samples prepared by the semi-denaturing procedure (Gabriel and Wang, 1969), and 1% (w/v) maltose as substrate. The identification of glycoproteins was also carried out in the same conditions. The glycosylated proteins were detected by staining the gel with periodic acid from Schiff (PAS) (Zacharius *et al*., 1969).

Protein and carbohydrate measurements

The Protein concentration was measured by the method of Hartree (1972) using bovine serum albumin (BSA) as a standard. The Carbohydrate content in the enzyme was measured by the method of Dubois *et al*. (1956), using standard curve of glucose as reference.

Properties of purified enzyme

The effects of pH and temperature on the α-glucosidase activity were determined by the methods described before (Carvalho *et al*., 2006).

The Michaelis constant K_m and V_{max} values were determined from Michaelis-Menten plots of enzyme activity measured with maltose (Sigma) as a substrate, at concentrations varying between 2.8×10^{-2} and 8.9 μM at optimum pH and temperature. The results were plotted using the program Grafit 5.0™.

The effects of metal ions (Ag^+, Na^+, Cu^{2+}, Ca^{2+}, Ba^{2+}, Co^{2+}, Hg^{2+}, Fe^{3+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Al^{3+}, Cr^{3+}), EDTA and β-mercaptoethanol on the activity of the enzyme were evaluated at concentrations of 1.7 to 10 mM in the reaction mixture. SDS, Tween 20, Tween

Table 1. Summary of the purification of *T. aurantiacus* CBMAI 756 α-glucosidase

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	1300	554.0	2.34	100.0	1
Ultra filtration	920	35.0	26.0	71.0	11
Ammonium sulphate precipitation	587	8.90	66.0	45.0	28
Q Sepharose	525	4.30	105.0	35.0	45
Sephacryl S-200	148	0.37	400.0	11.0	170
Superose 12	55.8	0.138	404.0	4.3	172

80, Triton-X, and Triton 100 at the concentration of 0.2% (v/v) were also evaluated.

Effects of inhibitors in the activity and substrate specificity of the α-glucosidase

The α-glucosidase activity inhibitors such as acarbose, voglibose (Watanabe *et al*., 2004), ginestein (Wang *et al*., 2004), diethylpyrocarbonate, castanospermine (Bravo-Torres *et al*., 2004), and iodoacetamide (Li and Chan, 1983) were tested in order to evaluate the inhibition level in the purified enzyme. The reaction mixtures were composed of 0.4 ml of 0.2% (w/v) maltose in 250 mM sodium acetate buffer, pH 4.5, 0.10 ml of enzyme solution and 10 µl of inhibitor solution at different concentrations, 250 mM, 10 mM, and 0.018 mM-1.5 mM; reactions were incubated at 70°C for 10 min. The IC_{50} was calculated using the program Grafit 5.0™ (Bravo-Torres *et al*., 2004).

The substrate specificity was evaluated using as substrates soluble starch, cellobiose, trehalose, sucrose, maltooligosaccharides (two to seven glucose units) at 2 mM (w/v) in 250 mM sodium acetate buffer, pH 4.5, and synthetic substrate (α-PNPG and β-PNPG) at 2 mM in the same buffer, under optimum conditions for enzyme activity.

Identification transglycosylation products by thin-layer chromatography

The profile of transglycosilation activities was evaluated at the optimum condition for the α-glucosidase activity, using a reaction mixture containing 5% (w/v) maltose and 0.2 ml (3 U) of the purified α-glucosidase, in 30 h at 70°C. Reaction products were analyzed by thin-layer chromatography ascents (TLC) on silica Gel G-60 using a mixture of n-butanol/ etanol/ water (5:3:2, v/v/v) as a mobile phase. Separated bands were visualized by spraying the matrix with 0.2% (w/v) orcinol in methanol and sulphuric acid (9:1, v/v) followed by heating at 100°C for 10 min (Fontana *et al*., 1988).

Results and Discussion

Purification of the α-glucosidase and determination of its molecular mass

A crude extract of *T. aurantiacus* containing the α-glucosidase activity (5 U/ml) was purified through a five-step purification procedure. At first, sample was filtered through a 50 kDa cut-off membrane leading to an increase of 11-fold in the specific activity of the enzyme, and also contributed to the removal of pigments. Next, filtered sample was subjected to ammonium sulphate precipitation (90%, w/v), which resulted in an increase of 28-fold in enzyme purity (Table 1). Precipitation was followed by fractionation through a Q-Sepharose anion exchange resin, in which two protein peaks were visualized. The second peak, eluted around 0.12-0.32 M salt, corresponded to the α-glucosidase activity (Fig. 1A). The fractions within this peak were dialyzed, lyophilized and applied to a Sephacryl S-200 column (Fig. 1B) giving a specific activity of 400 (U/mg protein). The active fractions pooled from this column were lyophilized for subsequent separation through a Superose 12 column, which resulted in a final specific activity

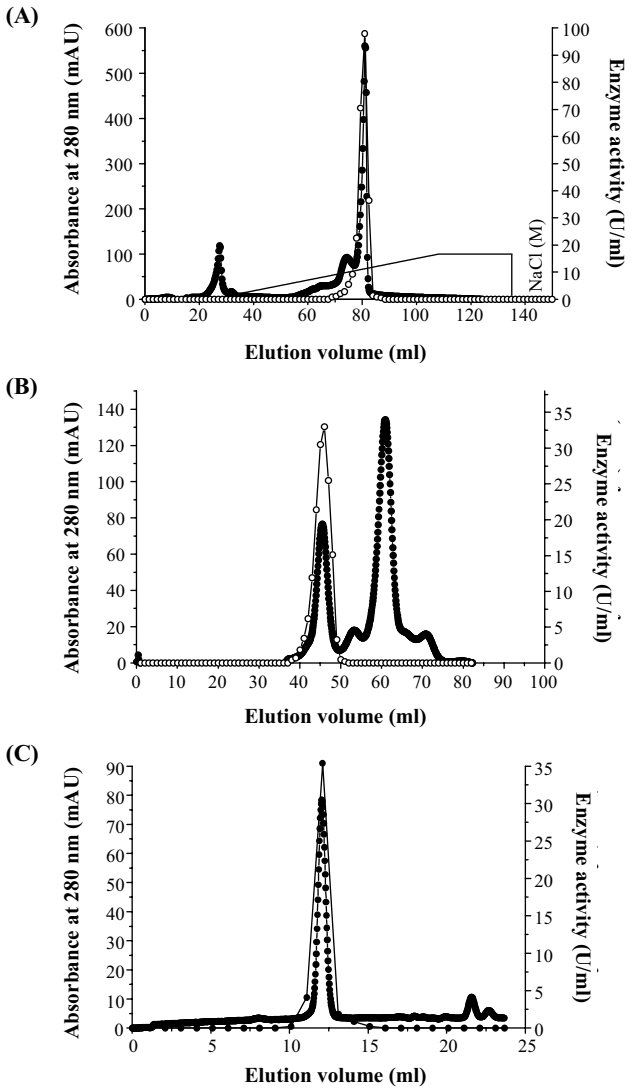

Fig. 1. Elution profile of α-glucosidase in (A) anion exchange chromatography through Q Sepharose and in gel filtration chromategraphy through Sephacryl S-200 (B) and Superose 12 (C). Symbols: (o) α-glucosidase activity; (•) absorbance at 280 nm; (-) concentration of NaCl (0-1.0 M).

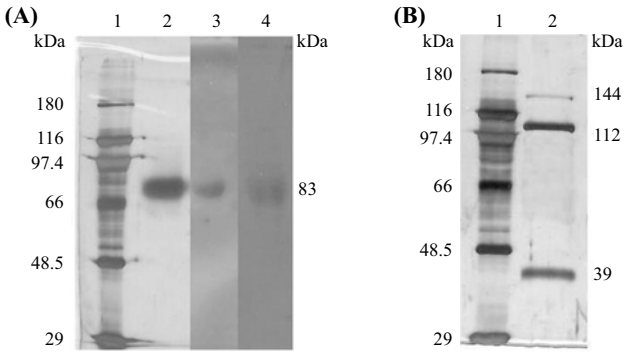

Fig. 2. Analysis of purified α-glucosidase in SDS-PAGE. (A) Lanes: 1, molecular weight markers 6.5-180 kDa (Sigma-M-6539); 2, sample was not boiled and without the addition of β-mercaptoethanol (semi-denatured sample); 3, staining for activity; 4, staining for glycoproteins. (B) Lanes: 1, molecular weight markers 6.5-180 kDa; 2, sample was boiled in the presence of β-mercaptoethanol (denatured sample).

of 404 (U/mg protein) and 172-fold enzyme purification (Fig. 1C and Table 1); after this step a single band of protein (M.W. 83 kDa) was detected in SDS-PAGE using semi-denatured sample (Fig. 2A, lane 2). The equivalent molecular mass was observed for bands obtained in gel of α-glucosidase activity and in a specific gel for glycoprotein (Fig. 2A, lanes 3 and 4). However, in the SDS-PAGE using denatured sample three protein bands were observed: 144, 112, and 39 kDa, which resulted in a total molar mass of 295 kDa (Fig. 2B, line 2), and suggesting that the enzyme is a trimeric protein. Oligomeric forms have been described for a number of microbial α-glucosidases (Kato *et al*., 2002; Tanaka *et al*., 2002; Giannesi *et al*., 2006). Another possibility is that our protein have two subunits (112 and 39 kDa), which remain associated during the purification steps, and ~144 kDa (112+39) as an unprocessed polypeptide.

The molar mass determined for the native enzyme using gel filtration though a S-200 column was 154 kDa (data not shown). The differences between the molecular mass estimated by gel filtration with native sample (154 kDa) and by SDS-PAGE using semi-denatured sample (83 kDa) may be due to the presence of carbohydrates in the enzyme that could interact with the resin. The α-glucosidase exhibited a carbohydrate content of 42%. Similar differences were also observed for the molar mass of α-glucosidases from *Chaetomium thermophilum* var. coprophilum, *Acremonium implicatum* IFO30538, the yeast *Schizosaccharomyces pombe*, and *Archachatina ventricosa*, which contained 14, 42.8, 91, and 25% of carbohydrates, respectively (Yamamoto *et al*., 2004; Okuyama *et al*., 2005; Giannesi *et al*., 2006; Soro *et al*., 2007).

Several procedures have been described for the purification of α-glucosidases from different origins and the molecular masses detected are in the range of 22-1,120 kDa, which indicate the enormous variability of these enzymes (Anindyawati *et al*., 1998; Kashiwabara *et al*., 2000; Nashiru *et al*., 2001; Kato *et al*., 2002; Faridmoayer and Scaman, 2004; Torre-Bouscoulet *et al*., 2004; Yamamoto *et al*., 2004; Okuyama *et al*., 2005; Ezeji and Bahl, 2006; Giannesi *et al*., 2006; Naested *et al*., 2006).

Biochemical characterization enzyme specificity

The α-glucosidase showed higher activity on maltose (28.7 U/ml) and the enzymatic activity decreased following an increase in the polymerization degree of short maltooligosaccharides (G3 to G7). In maltoheptaose, the activity was 10.2 U/ml, while no hydrolysis was observed when starch was used as a substrate. The enzyme was active on α-PNPG synthetic substrate (5.3 U/ml) but did not hydrolyze β-PNPG and cellobiose (Fig. 3), which show that the enzyme is a specific α-glucosidase that hydrolyzes α-1,4 bonds. The ability of the *T. aurantiacus* glucosidase to hydrolyze short maltooligosaccharides such as maltose faster than starch and other synthetic substrates is similar to α-glucosidases from other microorganisms such as *Aspergillus niger* (Sugimoto and Suzuki, 1994), *C. thermophilum* var. coprophilum (Giannesi *et al*.,

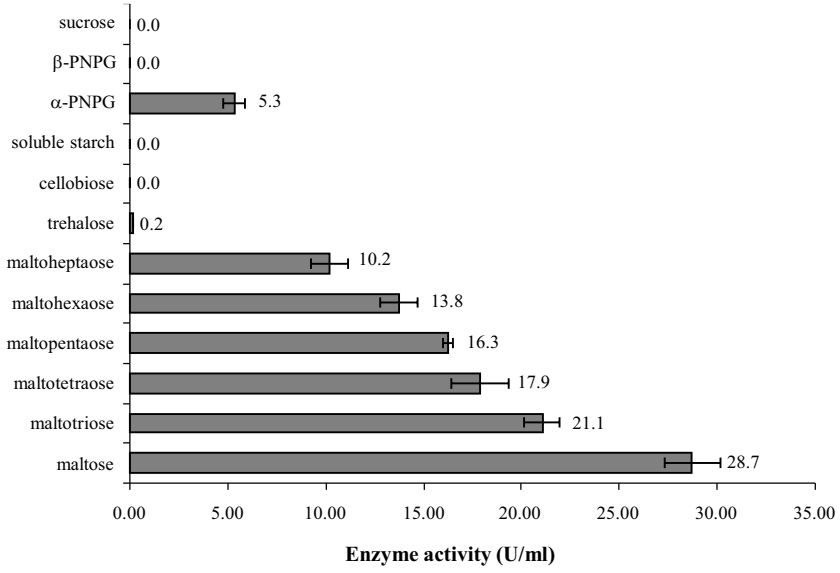

Fig. 3. Evaluation of the activity of *T. aurantiacus* CBMAI 756 α-glucosidase on different substrates.

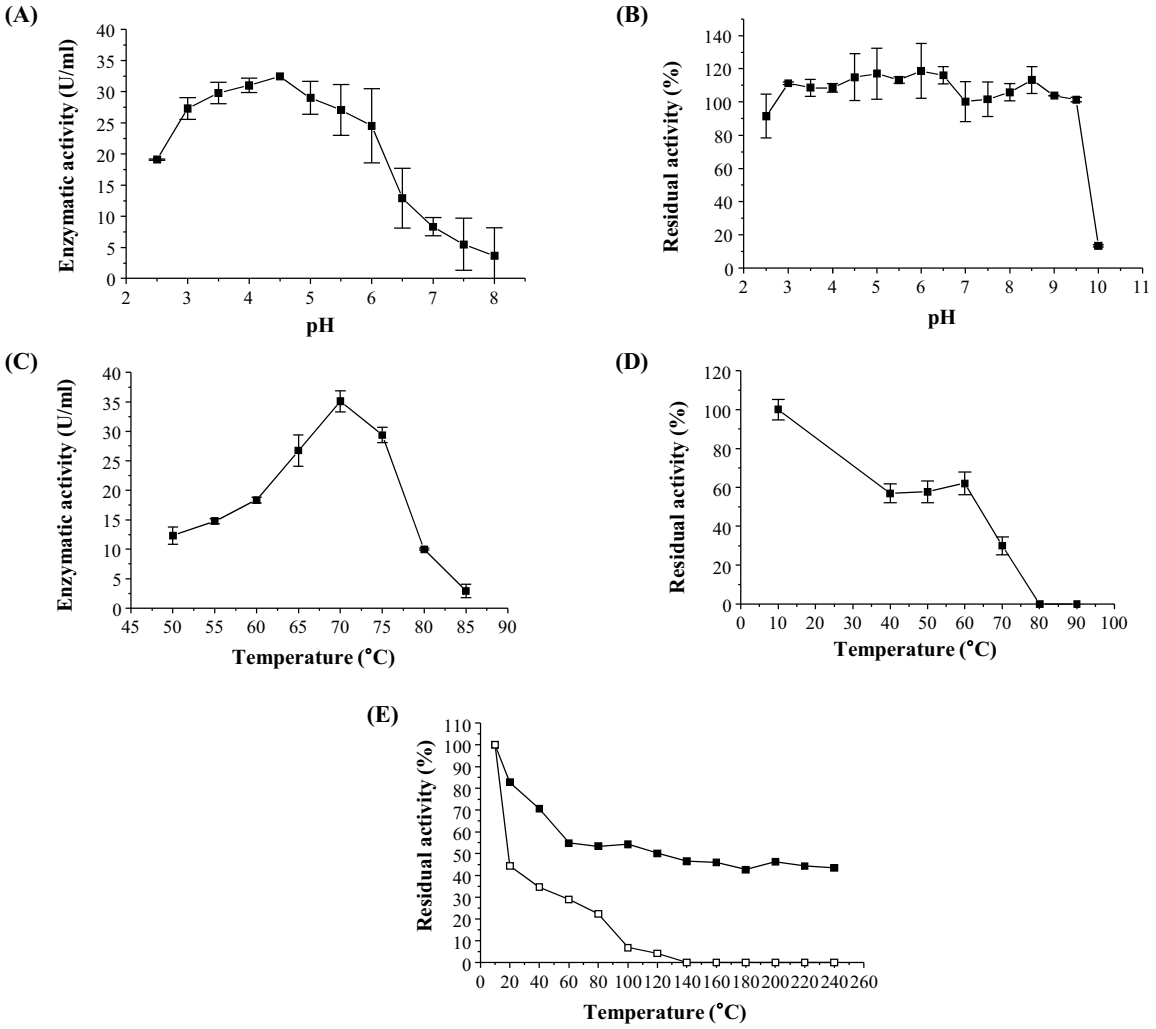

Fig. 4. α-Glucosidase activity during different physicochemical conditions. (A) Effect of pH on enzymatic activity, (B) Effect of pH on enzyme stability when in the absence of substrate, (C) effect of temperature on enzymatic activity, (D) effect of temperature on enzyme stability when in the absence of substrate, (E) enzyme stability at 60°C (■) and 70°C (□) when in the absence of substrate.

2006), *Geobacillus thermodenitrificans* HRO10 (Ezeji and Bahl, 2006), *Sulfolobus solfataricus* MT4 (Schiraldi *et al*., 2004), *Schizosacharomyces pombe* (Okuyama *et al*., 2005), and recombinant α-glucosidase (TtGluA) from *Thermoanaerobacter tengcongensis* MB4 (Zhou *et al.*, 2009).

Since the α-glucosidase was able to hydrolyze efficiently the homogeneous substrates with low molecular mass such as short maltooligosaccharides, as well as a heterogeneous substrate (α-PNPG), but failed to hydrolyze sucrose, it may be included into the type II group of α-glucosidases according to Chiba (1997). Therefore, it would belong to family 31.

Kinetic parameters

The K_m and V_{max} values for the α-glucosidase when maltose was used as a substrate were estimated to be 0.07 μM and 318.0 μmol/min/mg, respectively. The affinity for maltose was significantly higher than measurements reported for other purified α-glucosidases, which exhibited values of K_m varying from 40 μM to 17×10^3 μM and V_{max} between 23.0 μmol/min/mg to 34.2 μmol/min/mg (Okuyama *et al*., 2005; Ezeji and Bahl, 2006; Giannesi *et al*., 2006; Naested *et al*., 2006).

Effect of pH and temperature

α-Glucosidase showed high activity on maltose in a wide pH range (3.0 to 5.5) with a maximum activity at pH 4.5 (Fig. 4A). When in the absence of substrate the enzyme was also stable in a large range of pHs (3.0-9.0) (Fig. 4B). The optimum activity of the α-glucosidase was observed at 70°C (Fig. 4C). Several purified α-glucosidases from various sources showed optimum pH and temperature ranging from 4.0 to 7.5 and from 50 to 70°C, respectively (Anindyawati *et al*., 1998; Martino *et al*., 2001; Tanaka *et al*., 2002; Zdzieblo and Synowiecki, 2002; Iwata *et al*., 2003; Bravo-Torres *et al*., 2004; Yamamoto *et al*., 2004; Okuyama *et al*., 2005; Ezeji and Bahl, 2006; Giannesi *et al*., 2006; Zhou *et al.*, 2009).

The enzyme retained 60% of its original activity when incubated for 1 h at 40°C, 50°C, and 60°C in the absence of substrate (Fig. 4D). The half-life at 60°C and 70°C were 120 and 17 min, respectively (Fig. 4E). In our previous observations, the crude enzyme obtained from the culture medium of

T. aurantiacus showed a maximum of α-glucosidase activity in the same pH and temperature (Carvalho *et al*., 2006); however, the crude enzyme was more stable in relation to pH and temperature variations while in the absence of substrate. These results suggest that the decrease in stability of the purified enzyme could be related to some ions or stabilizing molecules which are removed during purification.

Enzymes from thermophilic organisms such as *C. thermophilum* var. *coprophilum* (Giannesi *et al*., 2006) and *G. thermodenitrificans* HRO10 (Ezeji and Bahl, 2006) usually display higher thermal stability than those from mesophiles such as *Mortierella alliacea* (Tanaka *et al*., 2002) and *A. implicatum* IFO30538 (Yamamoto *et al*., 2004). On the other hand, the glycosylated proteins exhibit even higher themostability and proteolysis resistance (Yamamoto *et al*., 2004; Okuyama *et al*., 2005). Probably the high carbohydrate contents of the *T. aurantiacus* α-glucosidase contribute to its stability.

Effect of ions, EDTA, β-mercaptoethanol, and detergents on enzyme activity

Ions such as Cr^{3+}, Ag^{+}, and Hg^{2+} strongly inhibited the α-glucosidase activity and Fe^{3+} and Cu^{2+} inhibited only 8-27% of its activity. The other ions tested such as Na^{+}, Ba^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, Al^{3+}, Zn^{2+}, Ca^{2+} did not alter the activity of the enzyme (Table 2).

EDTA at 10 mM and triton-X, Tween 80, and Tween 200 at 0.2% did not exert any effect on the enzyme activity while 10 mM of β-mercaptoethanol and 0.2% of SDS inhibited 76% and 47% of its activity, respectively (Table 2). Detergents are amphiphilic and have a critical role in the conformation of enzymes due to the formation of micelles, which could lead to enzyme instability. In opposite, detergents can reduce the surface tension and may facilitate the access of the enzyme to its substrate improving the enzyme action (Kumar and Satyanarayana, 2003).

The absence of inhibitory effects in the presence of EDTA is similar to what has been observed for other α-glucosidases from *C. thermophilum* var. *coprophilum*, *Thermus thermophilus*, *Thermococus* AN1, and *Mucor javanicus* (Yamasaki *et al*., 1973; Piller *et al*., 1996; Zdzieblo and Synowiecki, 2002; Giannesi *et al*., 2006). Since EDTA promotes the chelation of cations, our results lead to the suggestion that these enzymes do not require metal ions for enzymatic actions (Zdzieblo and Synowiecki, 2002; Kumar and Satyanarayana, 2003).

The inhibition observed by Hg^{2+} and β-mercaptoethanol suggests the presence of disulfide bridges in the enzyme, which can contribute to the stabilization of its structure, and improvement of thermostability (Vielle and Zeikus, 2001; Kumar and Satyanarayana, 2003).

Table 2. The effects of metal ions, EDTA, β-mercaptoethanol, and detergents on the activity of α-glucosidase.

	Concentration (mM)	
Control	100	100
Na^{+}	100	98
Ba^{2+}	104	103
Co^{2+}	101	102
Ni^{2+}	103	100
Mg^{2+}	101	100
Mn^{2+}	105	103
Al^{3+}	100	99
Zn^{2+}	93	93
Ca^{2+}	94	94
Cu^{2+}	84	73
Fe^{3+}	92	79
Hg^{2+}	3	0
Cr^{3+}	0	0
Ag^{+}	0	0
EDTA	104	97
β-Mercaptoethanol	80	24
SDS (0.2%)	53	-
Tween 20 (0.2%)	109	-
Tween 80 (0.2%)	109	-
Triton-X (0.2%)	100	-

Effect of chemical inhibitors

The effects of the well known α-glucosidase inhibitors idoacetamide, voglibose, acarbose, diethylpyrocarbonate, ginestein, and castanospermine were evaluated.

Iodoacetamide and diethylpyrocarbonate (DEPC) did not display any inhibitory effect on the α-glucosidase in concentrations below 250 mM (data not shown). These results might indicate that cysteine and/or histidine residues are not constituents of the catalytic and/or affinity sites of α-glucosidase (Bravo-Torres *et al*., 2004).

Genistiein has been implicated as a non-competitive and slow-binding inhibitor of some α-glucosidases and is susceptible to reversion. The use of this isoflavonoid at the concentration of 10 mM did not inhibit the enzyme activity, similar to the results reported for the α-glucosidases from *Shaccaromyces cerevisiae* and *Bacillus stearothermophilus* (Kim *et al*., 2008).

Acarbose, voglibose, and castanospermine at 1.5 mM inhibited the activity of the enzyme by 93%, 96%, and 100%, respectively, with IC_{50} of 0.11, 0.06, and 0.016 mM, respectively (Fig. 5). The sensitivity of this class of enzymes to voglibose, acarbose, and castanospermine has been demonstrated for α-glucosidases from different origins (Bravo-Torres *et al*., 2004; Watanabe *et al*., 2004), however, these inhibitors are not

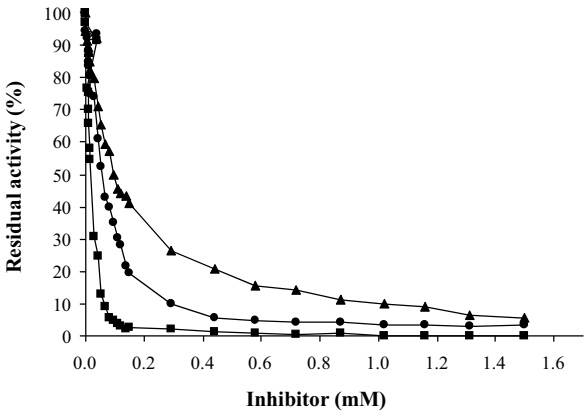

Fig. 5. The effect of inhibitors in the activity of *T. aurantiacus* α-glucosidase. Symbols (▲) Acarbose, (•) Voglibose, (■) Castanospermine.

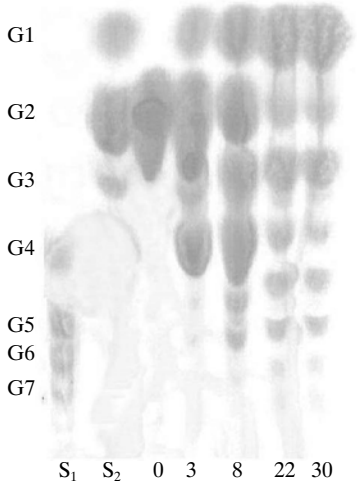

Fig. 6. Thin-layer chromatography of the products obtained by the transglycosylation reaction of *T. aurantiacus* α-glucosidase. Lanes: 1-5, transglycosylation reaction products at 0, 3, 8, 22, and 30 h, respectively; Standards: S_1 (G_7=maltoheptaose, G_6=maltohexaose, G_5=maltopentaose, G_4=maltotetraose,); S_2 (G_3=maltotriose, G_2=maltose, G_1=glucose).

specific to α-glucosidases, and are able to produce inhibitory effects on α-amylase and β-glucosidase too (Melo *et al*., 2006).

Transglycosylation activity

The profile of sugars released by the incubation of the purified α-glucosidase with 5% maltose evidenced the transglycosylation ability of the enzyme with the formation of oligosaccharides after 3 h of reaction (Fig. 6). In 8, 22, and 30 h it was observed the formation of pentasaccharides, hexasaccharides, and a small amount of heptasaccharides. According to Chiba (1997), only enzymes that produce α-anomer by "retaining" are capable of catalyzing transglycosylation.

Transglycosylation reactions have been reported for α-glucosidase isolated from *C. thermophilum* var. coprophilum (Giannesi *et al*., 2006), *Aspergillus nidulans* (Kato *et al*., 2002), *A. niger* (Shimba *et al*., 2009), *A. implicatum* IFO30538 (Yamamoto *et al*., 2004), *A. ventricosa* (Soro *et al*., 2007), *Xantophyllomyces dendrorhous* (Fernánez-Arrojo *et al*., 2007), and recombinant α-glucosidase (TtGluA) from *Thermoanaerobacter tengcongensis* MB4 (Zhou *et al.*, 2009). The transglycosylation reaction is an important property of α-glucosidases in the synthesis of sugars with commercial application, which highlights the importance of the α-glucosidase from *T. aurantiacus* studied in this article.

In conclusion, the transglycosylation ability shown by the enzyme isolated from *T. aurantiacus* indicates that it could be an α-glucosidase. The property of the enzyme to hydrolyze small maltooligosaccharides, as well as heterogenous substrates, but not sucrose and starch, may include it into the type II group of α-glucosidases. Divergences of molecular mass determined by two independent methods may be due to the presence of carbohydrates in its structure. Finally, the enzyme is apparently trimeric as judged by SDS-PAGE under denaturing conditions.

Acknowledgements

The authors wish to thank Fundação de Amparo à pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support.

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