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Purification and characterization of a novel glucoamylase from *Fusarium solani*

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Abstract

Thermostable enzymes are currently being investigated to improve industrial processes of starch saccharification. A novel glucoamylase was purified to electrophoretic homogeneity from the culture supernatant of *Fusarium solani* on a fast protein liquid chromatographic system (FPLC). The recovery of glucoamylase after gel filtration on FPLC was 31.8% with 26.2-fold increase in specific activity. The enzyme had a molecular mass of 40 kDa by SDS-PAGE and 41 kDa by gel filtration. The glucoamylase exhibited optimum activity at pH 4.5. The K_{cat} and K_m were 441/min and 1.9 mg/ml, respectively, for soluble starch, specificity constant (K_{cat}/K_m) was 232. The enzyme was thermally stable at 50 °C and retained 79% activity after 60 min at this temperature. The half-life of the enzyme was 26 min at 60°C. The enzyme was slightly stimulated by Cu^{2+} and Mg^{2+} and strongly inhibited by Hg^{2+}, Pb^{2+}, Zn^{2+}, Ni^{2+} and Fe^{3+}.

Keywords: Amyloglucosidase; *Fusarium solani*; Specificity constant; Purification; Half-life

1. Introduction

Glucoamylase (EC 3.2.1.3, 1,4-α-D-glucan glucohydrolase) is an important industrial enzyme that removes the glucose units from the non-reducing chain-ends of starch and glycogen by hydrolyzing α-1,4 linkages consecutively. The primary commercial application of glucoamylase is the production of glucose syrups from starch. These syrups can be used for fermentation, production of crystalline glucose, or as a starting material for fructose syrups (Fogarty, 1983; Mase, Matsumyia, Mori, & Matsuura, 1996; Marlida, Hassan Saari, Radu, & Baker, 2000). Glucoamylases have also been applied to the brewing industry in the production of low calorie beer, where maltodextrins in the malted barley are hydrolyzed to simple sugars, which can then be completely fermented by brewer's yeast. Glucoamylase is second to proteases in worldwide distribution and sales among industrial enzymes (James & Lee, 1997).

Enzyme production and characterization is a growing field of biotechnology. Interest in thermostable amylases has increased tremendously, since resistance to thermal inactivation has become a desirable property in many industrial applications (Kumar & Satyanarayana, 2004). Several microbial sources have been investigated (Selvakumar, Ashakumary, Helen, & Pandey, 1996; Niaz et al., 2004a, 2004b; Chen, Li, Zang, & Zhou, 2005) for their ability to produce starch-hydrolyzing enzymes, though most of them occur in fungi. The enzymes used commercially originate from strains of either *Aspergillus* sp. (Pandey & Radhakrishnan, 1993) or *Rhizopus* sp. (Yu & Hang, 1991) where they are used for the conversion of malto-oligosaccharides into glucose. Each industrial application demands enzymes with specific kinetic properties, making it important to exploit new microbial sources of enzymes.

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The operating range of glucoamylase, in terms of pH, temperature and compatibility with other enzymes, must be investigated in various microbial sources to exploit its efficiency of action in a variety of food processing operations.

Fusarium solani is a novel fungal strain and there are scant reports of the characterization of glucoamylase from it. This paper reports the results of purification and characteristics of glucoamylase from this mould. Investigation of kinetic and thermostability properties of glucoamylase from *F. solani* will help to determine the suitability of this enzyme for food industry applications.

2. Materials and methods

2.1. Materials

All the chemicals used were of analytical grade and mainly purchased from Sigma Chem., USA, unless otherwise mentioned. Middle molecular mass of protein calibration kit was from Fermentas. Glucose kit was obtained from Biocon, Germany.

2.2. Organism and inoculum preparation

A pure culture of Fusarium solani was obtained from the National Fungal Culture Collection of Pakistan (NFCCP), Department of Plant Pathology, University of Agriculture, Faisalabad. It was maintained on potato dextrose agar (PDA) slants at 4 °C. Inocula were prepared by transferring spores from 5 to 6 day-old slant culture, into 500 ml Erlenmeyer flasks containing 150 ml of sterile Vogel's medium. The composition of inoculum medium was (g/l): glucose, 20.0; trisodium citrate, 2.5; KH_2PO_4, 5.0; NH_4NO_3, 2.0; $(NH4)_2\ SO_4$, 4.0; $MgSO_4 \cdot 7\ H_2O$, 0.2; peptone, 2.0; microelement solution, 10 ml and vitamin solution, 5.0 ml. pH of the medium was adjusted to 5.0 using 1 M HCl/1 M NaOH. The flasks were incubated on a rotary shaker at 150 rpm at 30 ± 1 °C for 48 h to get homogeneous spore suspension (10^6–10^7 spores/ml). The spore suspension was used as inoculum in the growth media for production of glucoamylase under solid state fermentation.

2.3. Production of glucoamylase

Glucoamylase was produced under solid state fermentation in 250 ml Erlenmeyer flasks containing 5 g wheat moistened with mineral salt solution (g/l; trisodium citrate, 2.5; KH_2PO_4, 5.0; NH_4NO_3, 2.0; $(NH4)_2SO_4$, 4.0; $MgSO_4 \cdot 7H_2O$, 0.2) to 70% moisture content. After sterilization, the flasks were cooled and inoculated with a 15% inoculum level and incubated at 35 ± 1°C for 96 h (optimum conditions). Then the fermented biomass was extracted with distilled water, filtered and then centrifuged at $15{,}300 \times g$ for 10 min at 4 °C. The clear supernatant was used as the crude enzyme source.

2.4. Enzyme activity

Glucoamylase activity was determined as described earlier (Iqbal et al., 2003). Appropriately diluted enzyme (100 μl) was reacted with 1% soluble starch solution in 50 mM MES monohydrate buffer (pH 5.5) at 40 °C for 40 min. The reaction was then stopped by boiling water for 5 min, and cooled on ice. The released glucose was measured using a glucose oxidase method. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose equivalent/ml/min at pH 5.5 and 40 °C.

2.5. Proteins assay

Proteins were estimated by the Bradford method (1976) with bovine serum albumin (BSA) as the standard.

2.6. Purification of glucoamylase

2.6.1. General

All steps were carried out at 4 °C.

2.6.2. Ammonium sulfate precipitation

Ammonium sulfate was added to make a 30% (w/v) saturation of the culture filtrate (2000 ml) and the solution was left overnight at 4 °C. After centrifugation at $15{,}300 \times g$ for 15 min, the pellet of the precipitated proteins was discarded whereas the supernatant was treated with 35% (w/v) ammonium sulfate to give a final concentration of 65% (w/v). The solution was again kept overnight and centrifuged as described above. This time the supernatant was discarded whereas the pellet containing glucoamylase was dialyzed against distilled water to remove salts.

2.6.3. FPLC anion-exchange chromatography on HiLoad column

Partially purified glucoamylases, after ammonium sulfate precipitation were loaded onto a HiLoad-Q Sepharose column using a super loop of 50 ml with a flow rate of

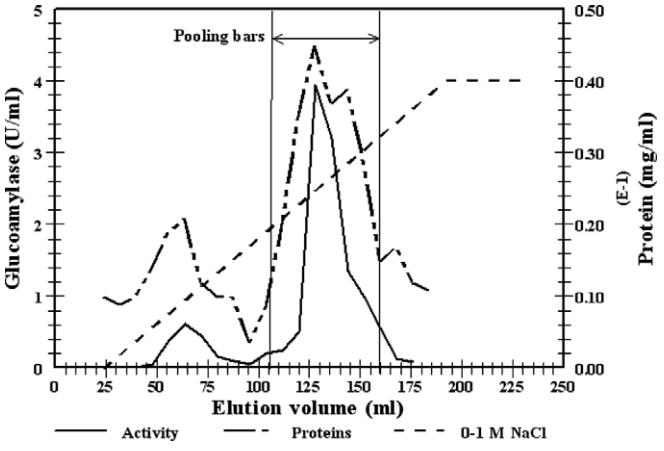

Fig. 1. HiLoad anion-exchange chromatography.

2 ml/min. A linear gradient of NaCl (0–1 M) in 20 mM Tris/HCl, pH 7.5, was used as elution buffer and fractions of 8 ml were collected. Two isoforms of glucoamylase were separately pooled and dialyzed to remove salts. The chromatogram is shown in Fig. 1.

2.6.4. FPLC hydrophobic interaction chromatography

The pooled fractions from the previous column corresponding to major form A of glucoamylase, were subjected to Phenyl Superose column chromatography at a flow rate of 1 ml/min (Fig. 2). The elution was carried out with a linear gradient of ammonium sulphate (0–2 M) in 50 mM phosphate buffer, pH 7. The fractions corresponding toglucoamylase were pooled and dialyzed to remove salts.

2.6.5. FPLC anion-exchange chromatography on mono-Q column

The purified glucoamylase from the previous step was loaded onto a Mono-Q column at a flow rate of 1 ml/min and a linear gradient of NaCl (0–1 M) in 20 mM Tris/HCl, pH 7.5, was used as elution buffer; 2 ml size fractions were collected (Fig. 3). The fractions corresponding to glucoamylase were pooled, dialyzed and concentrated.

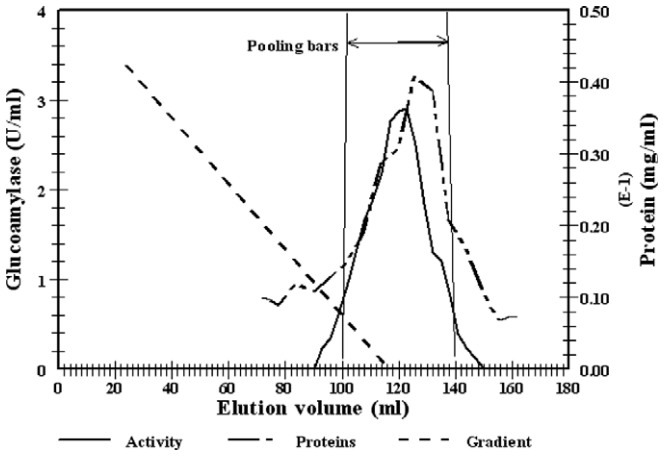

Fig. 2. Hydrophobic interaction chromatography.

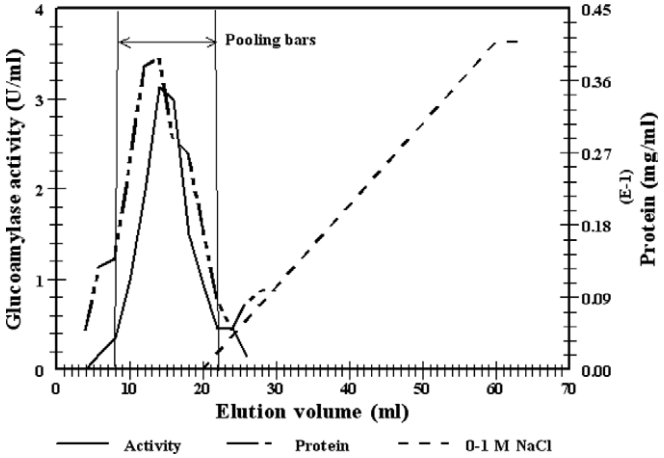

Fig. 3. Mono-Q anion-exchange chromatography.

2.6.6. FPLC gel filtration chromatography

The pooled fractions from Mono-Q were subjected to a FPLC gel filtration column with the dual purpose of purification and determination of native molecular weight. Sample (200μl/run) was loaded using a ''loop TMS program'' of FPLC with a flow rate of 0.5 ml/min. 100 mM Tris/HCl, pH 7, with 0.15 M NaCl, was used as elution buffer. Different molecular weight markers (carbonic anhydrase, 29 kDa; chicken egg albumin, 45 kDa; bovine serum albumin, 66 kDa; alkaline phosphatase, 100 kDa and alcohol dehydrogenase, 150 kDa) were used to calibrate the column. The chromatogram is shown in Fig. 4.

2.7. Sub-unit molecular mass

Purity of the purified enzyme and its sub-unit molecular mass were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli system (Laemmli, 1970) on 10% polyacrylamide gel. Standard proteins with a molecular mass ranging from 14.4–116 kDa (middle molecular mass calibration kit, Fermentas) were used for calibration. The gel containing different molecular weight markers was stained with Coomassie blue G-250 solution (Fig. 5).

2.8. Characterization of glucoamylase

2.8.1. Effect of pH

The effect of pH on glucoamylase activity was determined by assaying the enzyme as previously mentioned, with the difference that the activity was determined at different pH values ranging from 2 to 9, and in various buffer solutions (Marlida et al., 2000).

2.8.2. Optimum temperature and thermal stability

Glucoamylase was assayed at different temperatures ranging from 20 to 75 °C, at pH 5.5, as described previ-

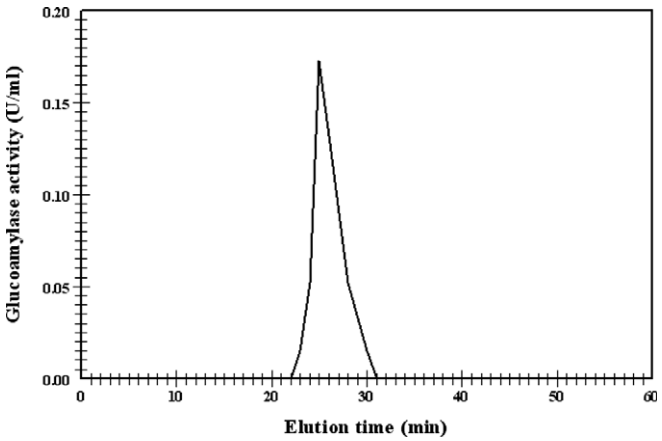

Fig. 4. Gel filtration chromatography.

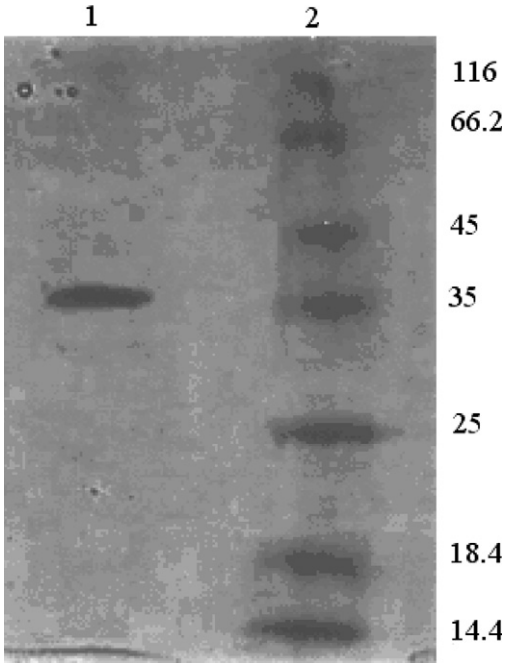

Fig. 5. 10% SDS-PAGE of glucoamylase from *F.solani*. Lane-1 (glucoamylase), Lane-2 (protein marker ladder). From top: *β*-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovulbumin (45 kDa), lactate dehydrogenase (35 kDa), RE Bsp981 (25 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa).

ously. Activation energy was determined from the Arrhenius plot, as described earlier (Siddiqui, Azhar, Rashid, & Rajoka, 1997). Thermal stability of glucoamylase was determined by incubating it in 10 mM Tris/HCl buffer, pH 7.0, at 50–60 °C for 60 min. Time course aliquots were withdrawn, cooled in an ice bath and assayed under standard conditions.

2.8.3. Effect of substrate

Glucoamylase from *F. solani* was assayed in the reaction mixtures containing variable amounts of soluble starch [0.025–0.15% (w/v)] at pH 5.5. The data were plotted (according to Lineweaver-Burk) to determine the values of kinetic constants (K_{cat} and K_m).

2.8.4. Effect of metal ions

The effect of metal ions (1 mM) on the activity of glucoamylase was determined by incubating the enzyme in the presence of metal ion solution at 40 °C for 40 min. The relative activity was monitored by the standard method. The relative activity assayed in the absence of metal ions was taken as 1.00.

3. Results and discussion

3.1. Purification of glucoamylase

An extracellular starch-degrading glucoamylase was purified to homogeneity from the culture filtrate of *F. solani* grown under solid state fermentation using wheat bran as substrate. The complete precipitation of glucoamylase was observed at 65% of ammonium sulfate at 0 °C. The HiLoad anion-exchange column separated the enzyme into two peaks (Fig. 1). The major portion of the enzyme activity was eluted as a sharp peak at 619 mM NaCl, while the minor portion was eluted just at the start of gradient. The major part was applied on subsequent columns for purification. The five step purification procedure of glucoamylase from *F. solani* resulted in an increase in specific activity of 26.2-fold with 31.8% recovery. The purification of this enzyme is summarized in Table 1. The glucoamylases from *Monascus purpureus* (El-Sayed, El-Aassar, & Abdel-Meguid, 2000) were purified up to 25.3-fold using a five step purification procedure.

3.2. Native and sub-unit molecular masses

The native molecular mass of glucoamylase was 41 kDa, as determined from the gel filtration chromatogram on a Pharmacia's FPLC unit. The SDS-PAGE for the determination of sub-unit molecular mass gave a single band of 40 kDa, making glucoamylase a monomer (Fig. 5). The molecular masses of monomeric glucoamylases from *Sclerotium rolfsii* (Kelkar & Deshpande, 1993) and *Arachniotus citrinus* (Niaz et al., 2004a, 2004b) were found to be 66 and 88 kDa, respectively.

3.3. Optimum pH and temperature

The activity of the purified glucoamylase towards soluble starch was determined at pH 2–9 at 40 °C. The enzyme exhibited optimum activity in a range of pH 3.0–5.5 with a maximum activity at pH 4.5 (Fig. 6). Below and above the optimum range, the activity of the enzyme decreases rapidly. Most of the fungal starch- degrading enzymes have optimum pH values of 4.0–6.0 (Ali, Malek, & Hossain, 1994; Amirul, Khoo, Nazalan, Razip, & Azizan, 1996; Marlida et al., 2000; Niaz et al., 2004a, 2004b). The change in pH affects the ionization of essential active site amino acid residues, which are involved in substrate binding and catalysis, i.e. breakdown of substrate into products. The ionization of these residues may cause distortion of active site cleft and hence indirectly affect the enzyme activity.

The activation energy and optimum temperature of glucoamylase from *F. solani* toward soluble starch were found to be 36.0 kJ/mol and 40 °C, respectively (Fig. 7). It is obvious from the Figure that the enzyme had a single conformation up to transition temperature. Temperature optima of 40–45 °C have been reported for glucoamylase from *Aureobasidium pulluan* (Fortina, Parini, & Nsengumulemyi, 1993) and *Aspergillus niger* ATCC 1015 (Abou-Zeid, 1999).

3.4. Kinetic constants

The K_m and V_{max} values, determined from Lineweaver-Burk plot, were 1.9 mg/ml and 665 U/mg proteins, whereas

Table 1
Purification of glucoamylase from *Fusarium solani*

Treatment	Total units	Total protein (mg)	Specific activity (U/mg)	Purification factor	% Recovery
Crude	50,304*	2180*	23.1*	1.00*	100*
$(NH_4)_2SO_4$ precipitation	36,851 42,536*	992 988*	37.4 43.1*	1.62 1.86*	73.3 84.6*
FPLC Hiload® chromatography	24,972 26,899*	432 427*	57.8 63.0*	2.50 2.72*	49.6 53.5*
Hydrophobic interaction chromatography	21,150 24,032*	125 123*	169 195*	7.33 8.47*	42.0 47.8*
FPLC Mono-Q® chromatography	19,055 20,187*	65 61*	293 331*	12.7 14.3*	37.9 40.1*
Gel filtration chromatography	15,993*	26.53*	603*	26.2*	31.8*

* Values after dialysis against distilled water.

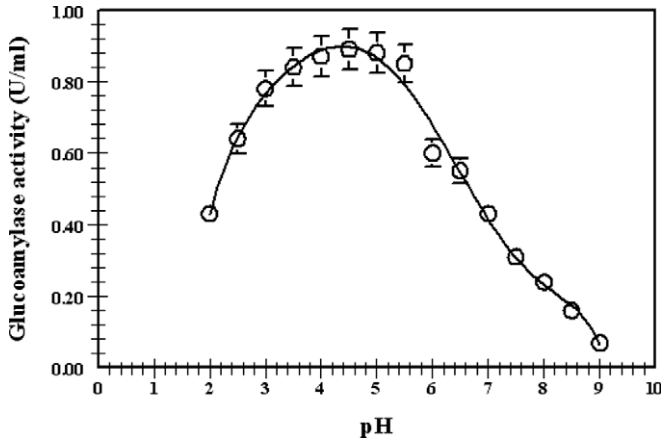

Fig. 6. Effect of optimum pH on glucoamylase activity. Error bars show standard deviation among three independent observations.

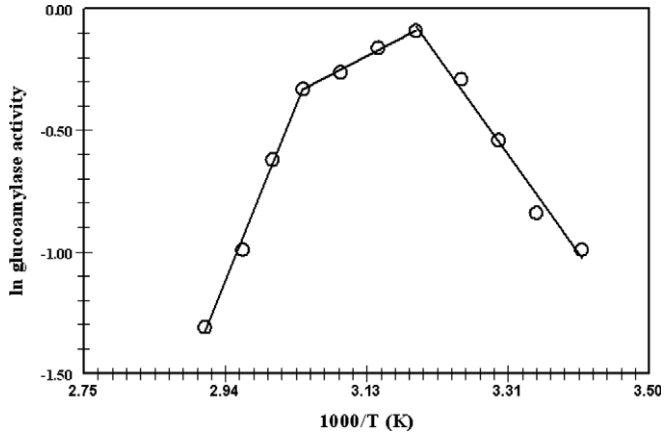

Fig. 7. Arrhenius plot for the determination of energy of activation for starch hydrolysis. The values of standard deviation among replicates were too small to be visible.

the K_{cat} value was 441/min at 40 °C and pH 5.5. The specificity constant (K_{cat}/K_m) was 232, indicating a high catalytic power of the enzyme. A low K_m value for the soluble starch reflects a higher number of hydrogen or hydrophobic interactions between the substrate and active site residues of the enzymes (Marlida et al., 2000). K_m values of 3.5 and 10.0 mg/ml for soluble starch have been reported for glucoamylases from *Aspergillus niger* – NCIM (Selvakumar et al., 1996) and *Acremonium* sp. (Marlida et al., 2000). A comparison with the above results shows that glucoamylase from *F. solani* has approximately 2- and 5-fold lower K_m values, respectively. This indicates that the enzyme has high affinity for the starch.

3.5. Thermal stability

Thermostability is the ability of enzyme to resist thermal unfolding in the absence of substrates, while thermophilicity is the capability of enzymes to work at elevated temperatures in the presence of substrate (Georis et al., 2000). The glucoamylase from *F. solani* was thermally stable at 50 °C with a half-life of 182 min. However, at 60 °C it was not thermally stable and displayed a half-life of 26 min (Fig. 8). Rapid denaturation occurred above 60 °C. Glucoamylase from *A. citrinus* (Niaz et al., 2004a, 2004b) was reported to be thermally very unstable because, after heating for only 7 min at 55 °C, more than 50% of its activity was lost. Similar findings have been reported by Itkor, Shida, and Tsukagoshi (1989). The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of activation (Daniel, 1996).

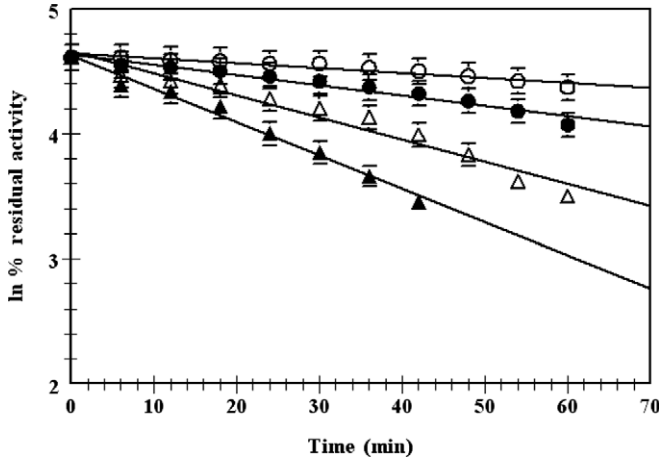

Fig. 8. Irreversible thermal denaturation of glucoamylase from *F. solani*. Error bars show standard deviations among three independent observations. Samples were incubated at 50 °C (open circle), 54 °C (closed circle), 57 °C (open triangle) and 60 °C (closed triangle) in 10 mM Tris/HCl buffer, pH 7.0.

Table 2
Effect of metal ions on glucoamylase activity

Metal salt (1 mM)	Relative glucoamylase activity
Control (H_2O)	1.00
K^+	1.08
Na^+	1.03
Cu^{2+}	1.46
Fe^{3+}	0.21
Pb^{2+}	0.08
Ca^{2+}	0.98
Hg^{2+}	0.06
Mg^{2+}	1.32
Ni^{2+}	0.22
Zn^{2+}	0.11

3.6. Effect of metal ions

The glucoamylase activity was determined at pH 5.5 and temperature 40 °C in the presence of different metal ion solutions. As shown in Table 2, Na^+, K^+ and Ca^{2+} ions did not inhibit the enzyme activity at a concentration of 1 mM. The results revealed that none of these ions was required for catalytic activity. The enzyme was slightly stimulated by Cu^{2+} and Mg^{2+} but strongly inhibited by Hg^{2+}, Pb^{2+}, Zn^{2+}, Ni^{2+} and Fe^{3+}. Hg^{2+}, Zn^{2+} and Ni^{2+} also inhibited glucoamylase activity in other microorganisms such as *Thermomucor indicae-seudaticae* (Kumar & Satyanarayana, 2003), while $Fe^{3+}$and Hg^{2+} inhibited glucoamylase activity in *Chaetomium thermophilum* (Chen et al., 2005). The effect of metal ions could be important in the use of raw materials with high salt content. Moreover it suggested the involvement of tryptophan and cysteine in catalytic activity and in maintaining the conformation of the enzyme.

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