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Full Length Research Paper

Purification and some kinetic properties of β-glucosidase from *Aspergillus terreus NRRL 265*

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An intracellular β-glucosidase (EC 3.2.1.21) from *Aspergillus terreus* NRRL 265 grown on whey permeate was purified to homogeneity as indicated by disc acrylamide gel electrophoresis with an apparent molecular mass of about 116 kDa. Optimal activity was observed at pH 5.0 and 60°C. The β-glucosidase had K_m values of 2.5, 3.7 and 5.5 mM for *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG), cellobiose and salicin, respectively. Glucose and glucono-δ-lactone were found to be competitive inhibitors with apparent K_i of 13.6 and 1.9 mM, respectively, when *p*-NPG was used as the substrate. Different metal cations had little or no effect on the enzyme activity, while Ag^+ and Hg^{2+} had an inhibitory effect when used at high concentrations. In addition, β-glucosidase was found to be a glycoprotein containing 69% carbohydrate by weight and free from any myco-toxins. SH groups do not seem to play a role in the catalytic action of β-glucosidase as addition of iodoacetate, reduced glutathione or mercaptoethanol did not affect the activity.

Key words: *Aspergillus terreus,* β-glucosidase, metal cations, iodoacetate.

INTRODUCTION

The enzymatic degradation of cellulose by cellulases has been the focus of several studies for their use in the bioconversions of agricultural wastes, in the improvement of the manufacture of recycled paper, in the production of food and fuel and in many various industries (Lambert et al., 2003 and Oyekola et al., 2007). Such enzymatic degradation requires the coordinated action of several cellulosome enzymes comprising exo-1,4-β-D-glucanase (EC 3.2.1.91), endo-1,4-β-D-glucanohydrolase (EC 3.2.1.4) and β-D-glucosidase (EC 3.2.1.21). Endo-glucanases cleave the internal glycosidic bonds of cellulosic chains and act synergistically with exoglu-canases and β-D-glucosidases during the degradation of crystalline cellulose (Oyekola et al., 2007). β-Glucosidase hydrolyses cellobiose to glucose, removing a strong inhibitor (cellobiose) of cellobiohydrolase from the reaction mixture (Tomaz and Queiroz, 1999). Although, numerous microorganisms are known to be able to produce high levels of extracellular cellulases, their β-glucosidase activities are often low and the inhibitory effect of cellobiose is one of the big obstacles to increase the efficiency of the enzymatic reactions. One of the well-known cellulase producers of *Trichoderma* sp. are also fairly deficient in β-glucosidase, causing the accumulation of cellobiose, which causes repressions on cellulase expressions and end-product inhibitions of the enzyme activities (Zaldivar et al., 2001). One of the approaches to solve this problem is the screening of wild strains with a high β-glucosidase activity and the optimization of the cultivation condition (Solovyeva et al., 1997). These authors reported that *Aspergillus* sp. were the most promising fungi with respect to β-glucosidase production. On the other hand, Juhász et al. (2003) reported that the cellulolytic system of *Trichoderma reesei* could be successfully supplemented with β-glucosidase from *Aspergillus* cultures**.** The aim of this study was to purify the crude intracellular β-glucosidase produced by *Aspergillus terreus* NRRL 265*.* Some of the enzyme properties and kinetics were also be studied.

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MATERIALS AND METHODS

Microorganism

A. terreus NRRL 265 was obtained from Northern Regional Research Laboratory United States Department of Agriculture (Peoria, IL, U.S.A.). The organism was grown and maintained by weekly transfer on slants of modified Czapek Dox's agar medium (Anon, 1972) adjusted at pH 6.0 and supplemented with 2% D-lactose as the only carbon source for growth.

Chemicals

p-Nitrophenyl-β-D-glucopyranoside (*p*-NPG) was purchased from Sigma Chemicals Company (St. Louis, MO, U.S.A.). Cellobiose was obtained from BDH Laboratory Supplies (Poole, England). Salicin was purchased from Riedel De Haen AG (Seelze-Hannover, Germany). Column chromatography adsorbents (Sephadex G100 and Sephadex G200) are fine grade beads purchased from Pharmacia Biotech (Uppsala, Sweden). Glucose oxidase/peroxidase (GOD/POD) kit was purchased from Biodiagnostic Company, Egypt. Molecular mass markers were purchased from Sigma Chemicals Company.

Culture conditions

Stock cultures of *A. terreus* were inoculated under aseptic conditions into a whey permeate, a by-product of the cheese production (Egyptian Dairy Industry El-Amiria, Cairo, Egypt) supplemented with 0.075% NH_4Cl and 0.05% yeast extract. The initial pH of the medium was adjusted to 6.0 before autoclaving. The inoculated flasks with 50 ml of sterile whey permeate medium in 250 ml Erlenmeyer flasks were then incubated statically at 30°C for 4 days.

Preparation of cell-free extracts

The mycelial biomass were harvested by filtration, rinsed thoroughly with distilled water, blotted dry with absorbent paper then ground with approximately twice its weight of cold washed sand in a chilled mortar and extracted with cold 0.05 M Na-citrate buffer pH 5.0 according to the method presented by Sebald et al. (1979). Thereafter, the slurry obtained was centrifuged at 5500 rpm for 15 min. The supernatant was used as the crude endocellular enzyme preparation.

β- Glucosidase activity assay

β-Glucosidase activity was determined photometrically using *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) as a substrate. The *p*-nitrophenol (*p*-NP) produced from the degradation of *p*-NPG by enzyme activity was measured according to the method of Bergham and Pettersson (1974). The enzyme units were calculated from standard curve using different concentrations of *p*-nitrophenol. One unit of enzyme activity expressed as International Units/ml (IU/ml) was defined as the amount of enzyme liberating one micromole (μmole) *p*-nitrophenol or glucose per minute under the standard assay conditions (Mamma et al., 2004). β-Glucosidase activity was also determined using cellobiose or salicin (as substrate) according to the method of Tomaz and Roche (2002). The reaction was performed at the optimum temperature for 30 min, where 0.1 ml of properly diluted enzyme was added to 0.4 ml of 0.05 M cellobiose or salicin solution in 0.05 M citrate buffer (pH 4.8). A reagent blank containing 0.1 ml of buffer instead of diluted enzyme and the optical density of the contents of each test tube was estimated colorimetrically by a glucose oxidase/peroxidase (GOD/POD) kit, Biodiagnostic, Egypt, against the blank test tube in 1cm path light cuvette by measuring the absorbency of the released glucose using a spectrophotometer at a wavelength of 510 nm.

Measuring protein concentration

Protein concentration in the crude enzyme preparation was determined according to Lowry et al. (1951). Proteins in the purified fractions were monitored by the method of Schleif and Wensink (1981).

Purification of β-glucosidase

All purification steps were performed at room temperature unless otherwise stated. The salting out with ammonium sulphate was done according to the method described by Jakoby (1971). Finely powdered ammonium sulfate was added to the cell free extract until the required saturation was reached (20%) with constant stirring, and then incubated overnight at 4°C. The observed precipitate was collected by centrifugation at 12.000 rpm for 30 min and dissolved in a minimal amount of 0.05 M Na-citrate buffer (pH 5.0), then dialyzed against the same buffer for 24 h. Further ammonium sulphate was added to the supernatant fluid and the process repeated until the final saturation of ammonium sulphate reached (40, 60, 80 and 100%).

Fractional precipitation with organic solvent (ethanol or acetone) was done according to the procedure of Kaufman (1971). The cold acetone or ethanol at -20°C was used as protein precipitants. Each percipitant was added separately to the cell free extract until the required concentration was reached (25%) with continuous stirring for 20 min at 4°C. The observed precipitate was collected by centrifugation at 12.000 rpm for 30 min and dissolved in a minimal volume of 0.05 M Na-citrate buffer (pH 5.0), then dialyzed against the same buffer for 24 h. Further protein precipitant was added to the supernatant fluid and the process repeated until the final concentration reached (50, 75 and 90%).

Determination of the carbohydrate content

The carbohydrate content of the purified enzyme was determined according to the method described by Dubois et al. (1956) on a sample of known protein concentration. The results showed that the purified β-glucosidase is a glycoprotein containing 69% carbohydrate by weight.

Determination of aflatoxins

Detection and determination of aflatoxins were performed by using HPLC according to AOAC official method. The results showed that the purified enzyme sample contains no aflatoxins.

Chromatographic purification on Sephadex G-100 followed by Sephadex G-200

This was done according to the method presented by De Palma-Fernandez ER et al. (2002). The dialyzed enzyme solution obtained with 50% (v/v) ethanol were applied to a Sephadex G-100 column (2.0 × 50 cm), which had been equilibrated with 0.05 M sodium-citrate buffer, pH 5.0. The column was eluted with the same buffer with flow rate of 30 ml/h. The active fractions (5 ml/tube) were combined, concentrated by freeze-drying lyophilizer (-50°C) and

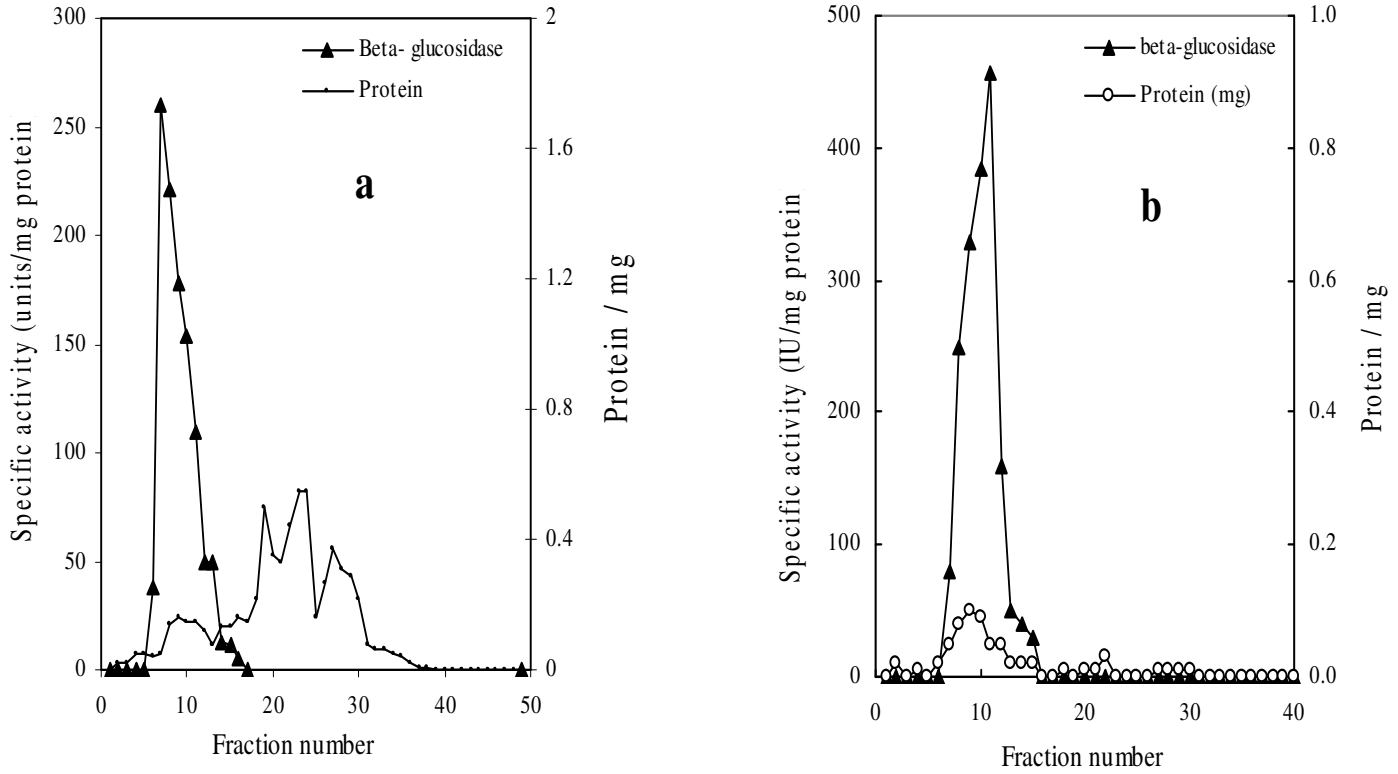

Figure 1. Elution diagram of β- glucosidase of *A. terreus* NRRL 265 from Sephadex G-100 (a) and Sephadex G-200 (b).

dialyzed against 0.05 M sodium-citrate buffer (pH 5.0). The dialyzed enzyme from Sephadex G-100 step was chromatographed on a Sephadex G-200 (2.0 × 50 cm) the column was eluted with 0.05 M sodium-citrate buffer, pH 5.0 with flow rate of 20 ml/h. Appropriate fractions (5 ml/tube) were combined, concentrated and dialyzed against the same buffer.

Polyacrylamide gel electrophoresis for protein separation

The efficiency of the purification process as a whole, as well as each step separately (that is, ethanol precipitation and gel filtration on Sephadex G-100 and Sephadex G-200) was evaluated through the polyacrylamide gel electrophoresis (PAGE) technique. SDS-PAGE was conducted using a 12.5% (w/v) polyacrylamide gel based on the protocol of Laemmli (1970). Protein bands were detected by Coomassie blue staining.

Statistical analysis

Statistical analysis was carried out according to the method described by Kenney and Keeping (1962) and the data were expressed as the mean ±S. D of three replicates.

RESULTS

Chromatographic separation of β- glucosidase on Sephadex G-100 and Sephadex G-200

The elution patterns of the intracellular β-glucosidase are illustrated graphically in Figure 1a and b from which it was noticed that the enzyme activity was high in the 5 (7^{th} to 11^{th} fraction) and 4 (8^{th} to 11^{th} fraction) fractions in case of using Sephadex G-100 and Sephadex G-200, respectively. These fractions were pooled, lyophilized and used for further purification studies. The data in Table 1 showed the purification steps, purification degrees and recovery yields of the intracellular β-glucosidase enzyme produced by *A. terreus* NRRL 265, from which it could be concluded that the enzyme could be purified about 138 times as compared to the crude enzyme.

Analysis of protein content by polyacrylamide gel electrophoresis

The resolution pattern of four enzyme samples from each enzyme purification step was tested (Figure 2). It was obvious that in case of crude enzyme, clear tailing proteins were shown along the whole gel. Most of these tailing proteins disappeared in the ethanol fraction, whereas two bands could be detected in Sephadex G-100 purified enzyme sample. Subsequent gel filtration on Sephadex G-200 showed only a single protein band with an apparent molecular mass of about 116 kDa indicating that the intracellular β-glucosidase enzyme of *A. terreus* NRRL 265 was effectively purified through the previous purification steps.

Table 1. Purification steps, purification folds and recovery yields of intracellular β-glucosidase enzyme of *A. terreus.*

Purification step	Total activity (IU)[a]	Total protein (mg)	Sp. activity (IU/mg protein)	Recovery (%)	Purification Fold
Crude extract	200 ± 0.4	60 ± 0.27	3.3	100.0	1.0
50% Eth-OH ppt.	160 ± 0.32	5.0 ± 0.21	32	80.0	9.7
Gel-filtration on:					
Sephadex G-100					
Fraction 7	13.0 ± 0.05	0.05	260.0	6.5	78.8
8	31.0 ± 0.04	0.14	221.4	15.5	67.1
9	28.5 ± 0.02	0.16	178.1	14.3	53.9
10	23.0 ± 0.01	0.15	153.3	11.5	46.5
11	16.5 ± 0.04	0.15	110.0	8.3	33.3
Sephadex G-200					
Fraction 8	20.0 ± 0.06	0.08	250.0	10.0	75.8
9	32.8 ± 0.04	0.10	328.0	16.4	99.4
10	34.6 ± 0.02	0.09	384.4	17.3	116.5
11	22.8 ± 0.01	0.05	456.0	11.4	138.2

[a]Activity was measured using p-NPG as substrate. Data is expressed as mean + S.D. of three replicate.

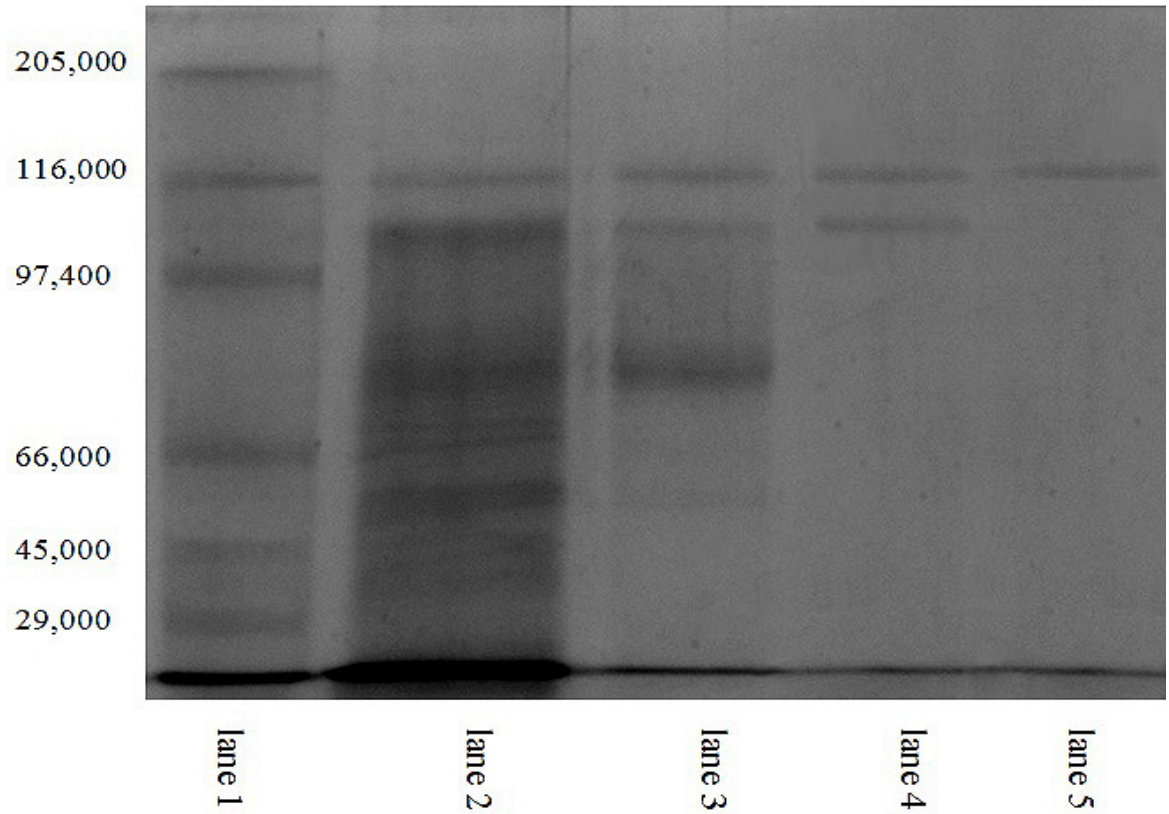

Figure 2. Electrophoretic analysis of β-glucosidase enzyme produced by *A. terreus* NRRL 265 at various stages of purification. Purification was performed on a 12.5 % (w/v) SDS-polacrylamide gel and stained with Coomassie brilliant blue. From left to right: Lane 1, molecular weight markers (myosin, 205,000; β-galactosidase, 116,000; phosphorylase, 97,400; bovine serum albumin, 66,000; albumin (egg), 45,000; and carbonic anhydrase, 29,000 D); lane 2, crude enzyme; lane 3, ethanol precipitated enzyme, lane 4 and 5 purified enzymes on Sephadex G-100 and Sephadex G-200, respectively.

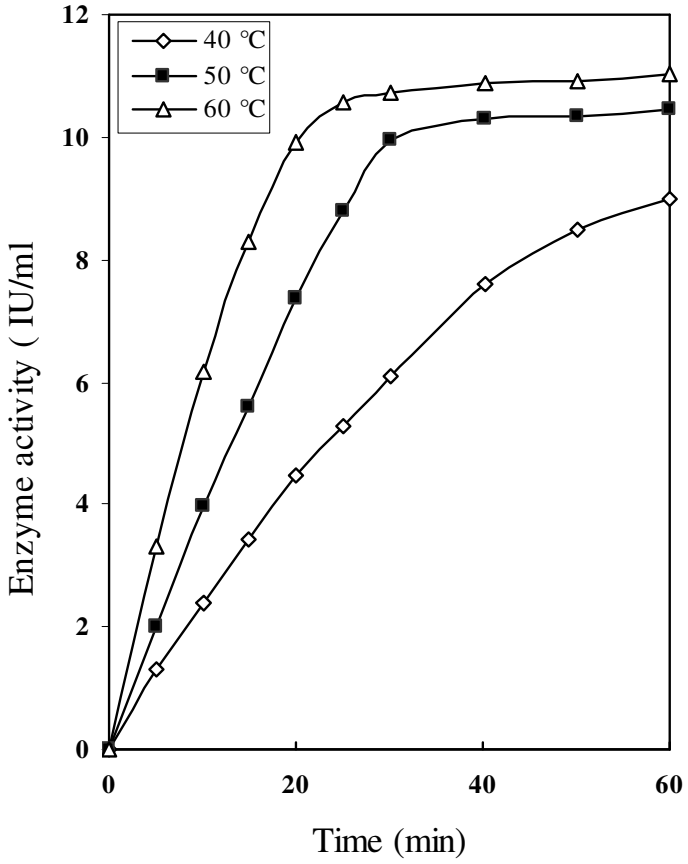

Figure 3. Effect of reaction time on the activity of purified intracellular β-glucosidase produced by *A. terreus*. Reaction mixture contained: *p*-NPG, 5 µmoles; sodium-citrate buffer pH 5.0, 50 µmoles; extract protein, 15 µg; total volume 1.1 ml; temp. and reaction time, as indicated.

Activity of purified β-glucosidase as a function of the reaction time

Three identical reaction mixtures were made. Each of them was incubated at a specific degree of temperature (40, 50 and 60℃). Samples were withdrawn at different intervals for a period of 1 h. Results obtained is graphically presented in (Figure 3). From this figure, it can be seen that enzyme activity was linear with time at least up to 20 min reaction time, after which the linearity of the reaction was not presented. It is also noticed that the enzyme activity expressed in term of enzyme units at each reaction time was higher at 60℃ incubation temperature for all reaction times used.

Temperature dependence

This study was made to find out the degree of temperature at which optimum hydrolytic activity of purified β-glucosidase could be achieved. This factor was studied by incubating the standard reaction mixture at a specific degree of temperature. The range used was from 10 to 90℃ for 10 min. The data obtained are graphically illustrated in Figure 4, from which it is clear that the purified enzyme showed progressive increased activity with the increase in reaction temperature. The figure also clearly demonstrates that 60℃ can be considered as the degree of temperature at which optimum activity occurs, while at higher temperatures a notable activity decrease was observed up to 70℃. In addition, the remaining observed activity at 90℃ indicates the thermophilic nature of the purified β-glucosidase produced by *A. terreus* NRRL 265.

Heat inactivation kinetics

This experiment was conducted to test the stability of β-glucosidase activity on subjecting the purified enzyme solutions (in absence of substrate) to a specific degree at and above the optimum temperature (50, 60, 70 and

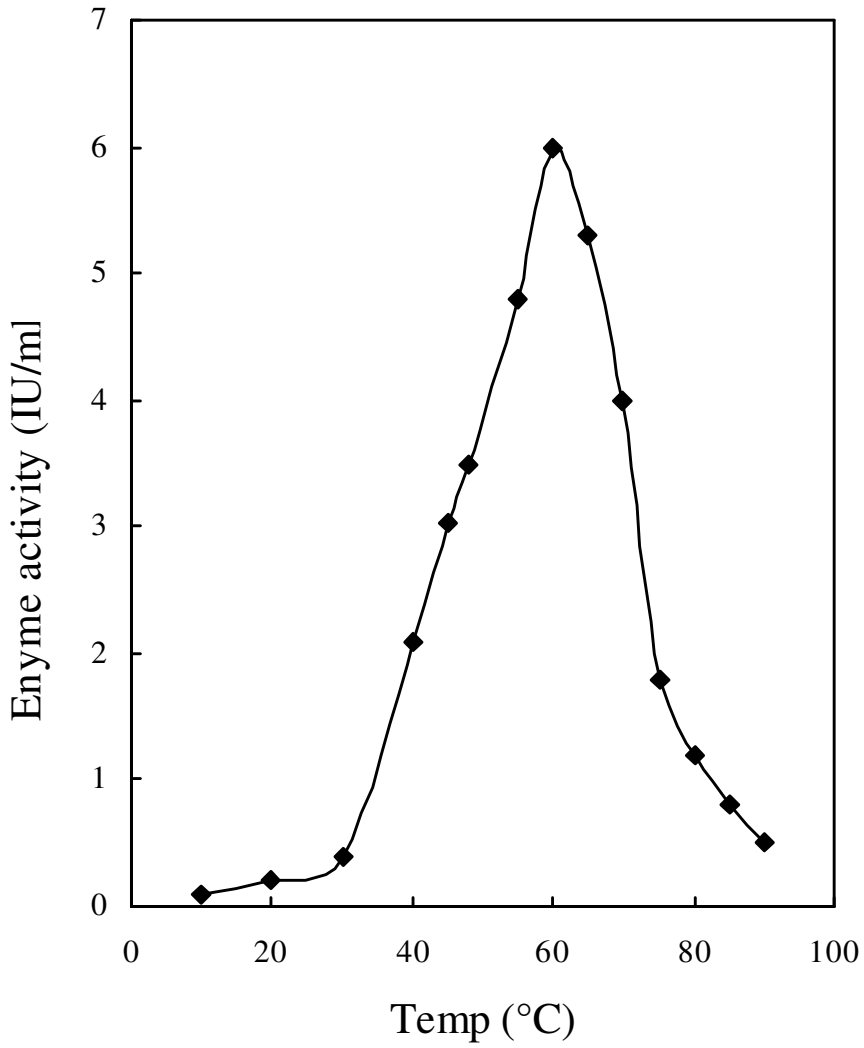

Figure 4. Temperature dependence of purified intracellular β-glucosidase produced by *A. terreus*. Reaction mixture contained: *p*-NPG, 5 µmoles; sodium-citrate buffer pH 5.0, 50 µmoles; extract protein, 15 µg; total volume 1.1 ml; temp., as indicated and reaction time, 10 min.

80°C) for different time intervals. Enzyme solutions (prepared in 0.05 M sodium-citrate buffer at pH 5.0) were incubated inside thin walled glass tubes at the designed temperature for 2 h. During this period, identical aliquots were removed at different time intervals, cooled and then assayed for β-glucosidase activity under standard conditions. Data obtained were cited as percentages of the remaining activities (Figure 5). As it appears from the figure, the purified enzyme was heat stable up to 2 h at 50°C. At 60°C, a slight decrease in the activity was observed after 30 min, while about 82% of the activity was still obtained after 60 min at 60°C. At 70°C, about 83% of the activity was observed after 5 min and then the activity was decreased rapidly, reaching 33 and 20% after 10 and 15 min, respectively. On the other hand, complete destruction of enzyme activity was observed at 80°C and higher temperatures. It could be concluded from the stated results that the higher thermal stability behaviour of β-glucosidase was purified from *A. terreus* NRRL 265.

Effect of storing, frequent freezing and thawing

To study these effects the purified enzyme preparation was put in the freezer at about -4°C, left for 7 days, after which it was warmed and an aliquot was withdrawn for assay of activity under the same experimental conditions. The remaining was put back in the freezer. This was repeated every 7 days for 3 months, from which it appears that by the end of the three months (13 cycles) only about 44% of the activity was lost (Data not shown).

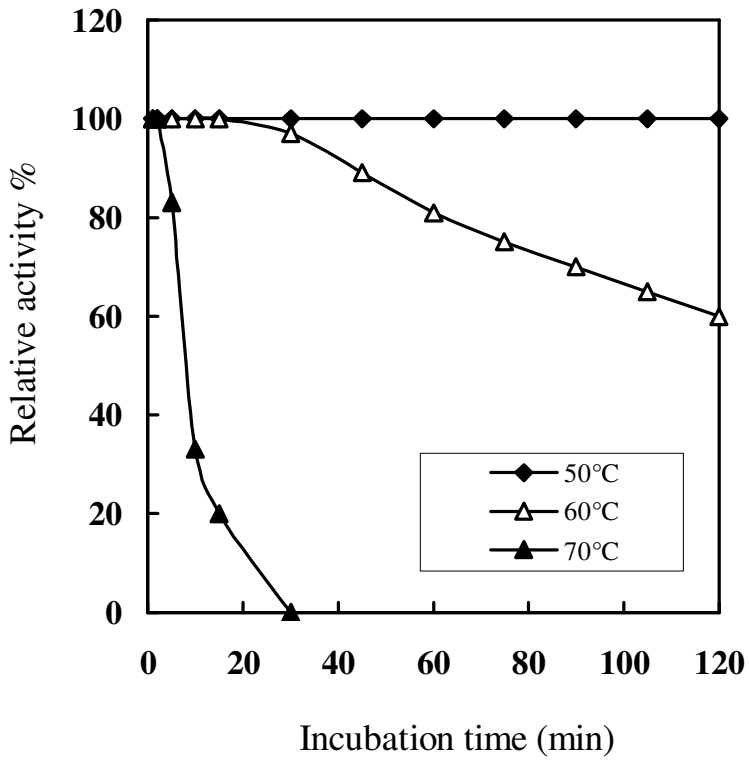

Figure 5. Heat inactivation kinetics of the purified β-glucosidase produced by *A. terreus*. Reaction mixture contained: *p*-NPG, 5 µmoles; sodium-citrate buffer pH 5.0, 50 µmoles; extract protein, 15 µg; total volume 1.1 ml; temp., 60°C and reaction time, 10 min.

pH value of the reaction mixture

Different buffers namely citrate, citrate-phosphate and phosphate with different pH values were used. In all reaction mixtures prepared, the same amount of protein, substrate (*p*-NPG) and buffer were added, after then, each was adjusted at a specific pH value. The reaction mixtures were incubated at 60°C for 10 min. Results obtained are presented as enzyme activity (IU/ml) in (Figure 6). The figure showed that pH 5.0 seems to be the most suitable pH for the enzyme activity irrespective of the type of buffer used. At pH 4.5, a slight decrease in the activity was observed and at pH 5.5 about 87% of the activity was still obtained. On the other hand, only about 30 and 47% of activity were found at pH 3.5 and pH 7.0, respectively. This means that β-glucosidase can occur over a wide range of pH values.

Effect of the nature and molality of the buffer

Three buffer systems namely sodium-citrate, sodium acetate and citrate-phosphate were used in this study. It appears that sodium-citrate buffer proved to be suitable for enzyme activity and almost more or less similar activities were obtained in the two buffer systems at pH 5.0. Six different molarities of sodium-citrate buffer were tested in this investigation ranging from 0.025 to 0.4 M. It was found that the most suitable molarity is that ranging from 0.025 to 0.1 M. Above this concentration, a decrease in the activity was obtained, reaching its lowest level (5.3 IU/ml) at 0.4 M (Data not shown).

Determination of pH stability

This experiment was achieved by incubating the enzyme preparation with sodium-citrate buffer to give pH range from 3.0 to 6.0, sodium-phosphate buffer from pH 6.0 to 8.0 and Tris-HCl of pH 8.0 and 9.0 at room temperature for 24 h. After the incubation period, the pH value of the enzyme solutions were readjusted to pH 5.0 and then added to the standard reaction mixture and the enzyme activity was then evaluated. Data in Table 2 presented as relative activities, showed that pH 5.0 seems to be the most suitable pH for the storage of the enzyme. A slight decrease in the activity was observed with the sample stored above or below this value. 62 and 38%, remaining activities were recorded with samples stored at pH 9.0 and 3.0, respectively. Indicating that β-glucosidase of *A. terreus* has higher pH stability over a wide range of pH values.

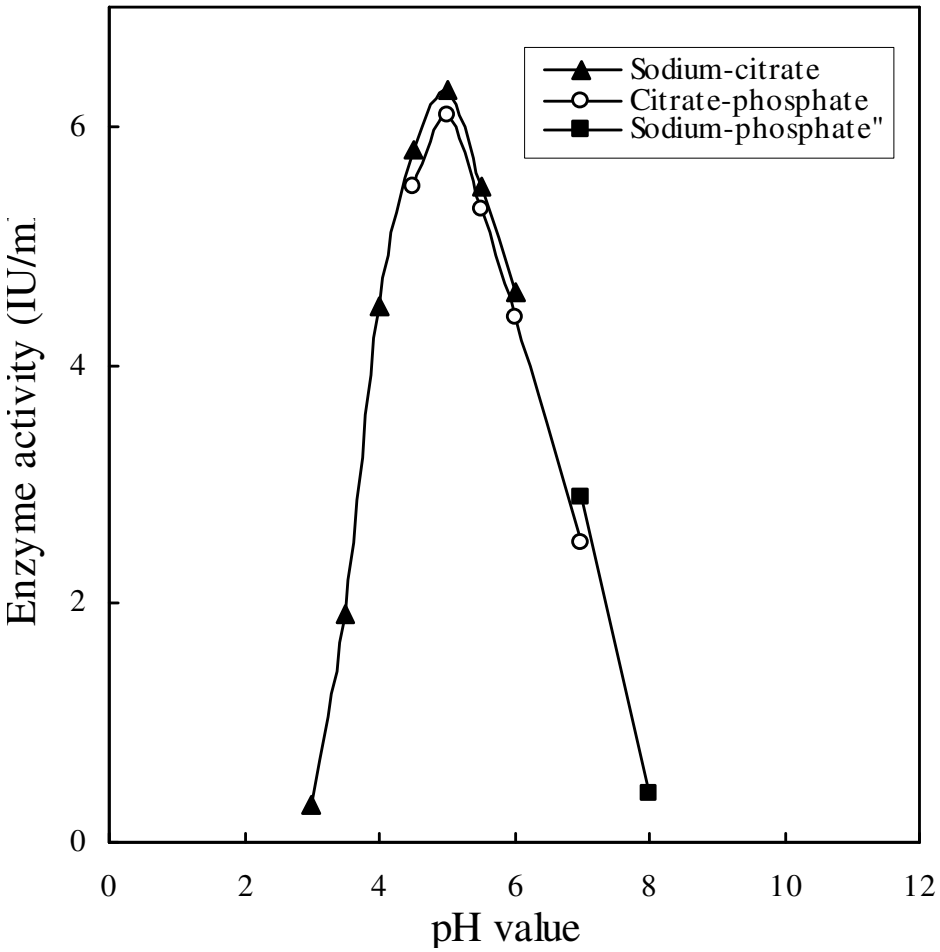

Figure 6. Activity of the purified β-glucosidase produced by *A. terreus* as a function of pH value of the reaction mixture. Reaction mixture contained: *p*-NPG, 5 µmoles; sodium-citrate buffer, 50 µmoles; pH value, as indicated; extract protein, 15 µg; total volume 1.1 ml; temp., 60°C and reaction time, 10 min.

Table 2. Effect of different metal salts on the activity of the purified ß-glucosidase.

Activator or inhibitor	Relative activity (%)		
	10^{-3}M	5 x 10^{-2}M	10^{-2} M
-	100	100	100
NaCl	123	117	105
KCl	117	115	104
$BaCl_2.2H_2O$	104	88	86
$HgCl_2$	75	54	27
$AgNO_3$	52	37	0.0
$MgSO_4.7H_2O$	112	91	88
$CoSO_4.7H_2O$	100	64	53
$CaCl_2.2H_2O$	102	93	91
$CuSO_4.7H_2O$	91	75	32
$FeCl_3$	93	85	48
$MnCl_2.4H_2O$	95	91	80

Reaction mixture contained: p-NPG, 5 µmoles; sodium-citrate buffer pH 5.0, 50 µmoles ; extract protein, 15 µg; metal, as indicated; total volume 1.1 ml; temp, 60°C; reaction time, 10 min.

Substrate specificity

Identical reaction mixtures containing the same amount of enzyme preparation were made, each received an equimolar amount of a specific substrate namely *p*-NPG, cellobiose, salicin, *p*-nitrophenyl-α-D-glucopyranoside and lactose. All the reaction mixtures were incubated at 60°C for 10 min for *p*-NP substrates, the reactions were monitored by the *p*-NP-releasing assay described in Materials and Methods section. For other substrates, glucose release was determined using the glucose oxidase-based kit. It was found that the highest activity was observed with *p*-NPG, among the various substrates tested followed by cellobiose and salicin. On the other hand, no activity was detected against *p*-NP-α-D-glucopyranoside and lactose.

Determination of K_m values

The apparent K_m (Michaelis constant) values of β-glucosidase for *p*-nitrophenyl-β-D-glucopyranoside, cellobiose and salicin were achieved through studies relating substrate concentrations to the velocities of the reactions. Different concentrations of each substrate were incubated with the same amounts of enzyme protein under constant conditions. Depending on the type of substrate, activity was determined by measuring the release of *p*-NP at 400 nm or of glucose (glucose oxidase-based kit). The apparent K_m values of β-glucosidase for each substrate were calculated from the Lineweaver-Burk plots relating 1 / V to 1 / [S] (Lineweaver and Burk, 1934). The K_m values of β-glucosidase for *p*-NP-β-G, cellobiose and salicin were found to be 2.5, 3.7 and 5.5 mM, respectively. The data obtained were illustrated graphically in Figures 7 to 9 for *p*-NPG, cellobiose and salicin, respectively. These values indicate that the affinity of β-glucosidase for the three substrates are in the order of magnitude *p*-NPG > cellobiose > salicin.

Inhibition of β-glucosidase activity by glucose and glucono-δ-lactone

The effect of addition of different concentrations of glucose and glucono-δ-lactone on the velocity of β-glucosidase-catalyzed reactions was tested. Different concentrations of glucose and glucono-δ-lactone were added separately to identical reaction mixtures. It was indicated that glucono-δ-lactone has an inhibitory effect on β-glucosidase activity, while glucose showed the same inhibition effect but in low levels (data not shown).

Determination of the type of K_i

Results obtained after 10 min of incubation at 60°C were graphically presented in Figure 10. The graphical method of Dixon (1953) was used to determine the K_i, the dissociation constants, of glucose and glucono-δ-lactone. Such results indicate that glucose and glucono-δ-lactone are competitive inhibitors of the enzyme as shown by Lineweaver-Burk plots, where at high substrate concentrations relief of inhibition was obtained; the apparent K_m for *p*-NPG decreased, whereas the maximum velocity remained the same. Enzyme-inhibitor dissociation constants or affinity constants (K_i) of β-glucosidase for glucose and glucono-δ-lactone were then calculated from the following equation:

$$K_i = (I)\ K_m / K_P - K_m$$

Where, (I) is the concentration of the inhibitor and K_P is the apparent K_m. The K_i values were calculated for glucose and glucono-δ-lactone and found to be 13.6 and 1.9 mM, respectively.

Effect of different metal cations and EDTA

This experiment was achieved by incorporating different mineral salts in the form of sulphates or chlorides of various cations (that is, Na^+, K^+, Ag^+, Ba^{2+}, Hg^{2+}, Mg^{2+}, Co^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+} and Fe^{3+}) at 10^{-3} M, 5 x 10^{-2} M and 10^{-2} M final concentration in standard reaction mixtures containing purified enzyme. Results obtained are tabulated in (Table 2) in the form of relative activities. From this table, it is clear that slight activation was noted for some metals indicating that the enzyme has no specific metal requirements for its activity. On the other hand, highest inhibition was exhibited by Ag^+, which inhibited the enzyme completely at a final concentration of 10^{-2} M. The effect of EDTA, known as a metal chelating agent, on β-glucosidase activity was tested to find out whether this enzyme is a metalloenzyme or not. It was found that the addition of EDTA at three different concentrations namely 10^{-3} M, 5 x 10^{-2} M and 10^{-2} M to the reaction mixture did not inhibit enzyme activity indicating that β-glucosidase is not a metalloenzyme (data not shown).

Absence of evidence for the involvement of an SH group in the catalytic site

The aim of this study was to test whether or not an SH group is involved in the catalytic action of the enzyme. This was carried out by testing the activity of the enzyme in the presence and absence of iodoacetate, reduced glutathione or mercaptoethanol at 10^{-3} M, 5 x 10^{-2} M and 10^{-2} M final concentrations. Results indicate the absence of evidence for the involvement of an SH group(s) in the catalytic action of β-glucosidase in agreement with the results obtained during studies with the crude extracts (data not shown).

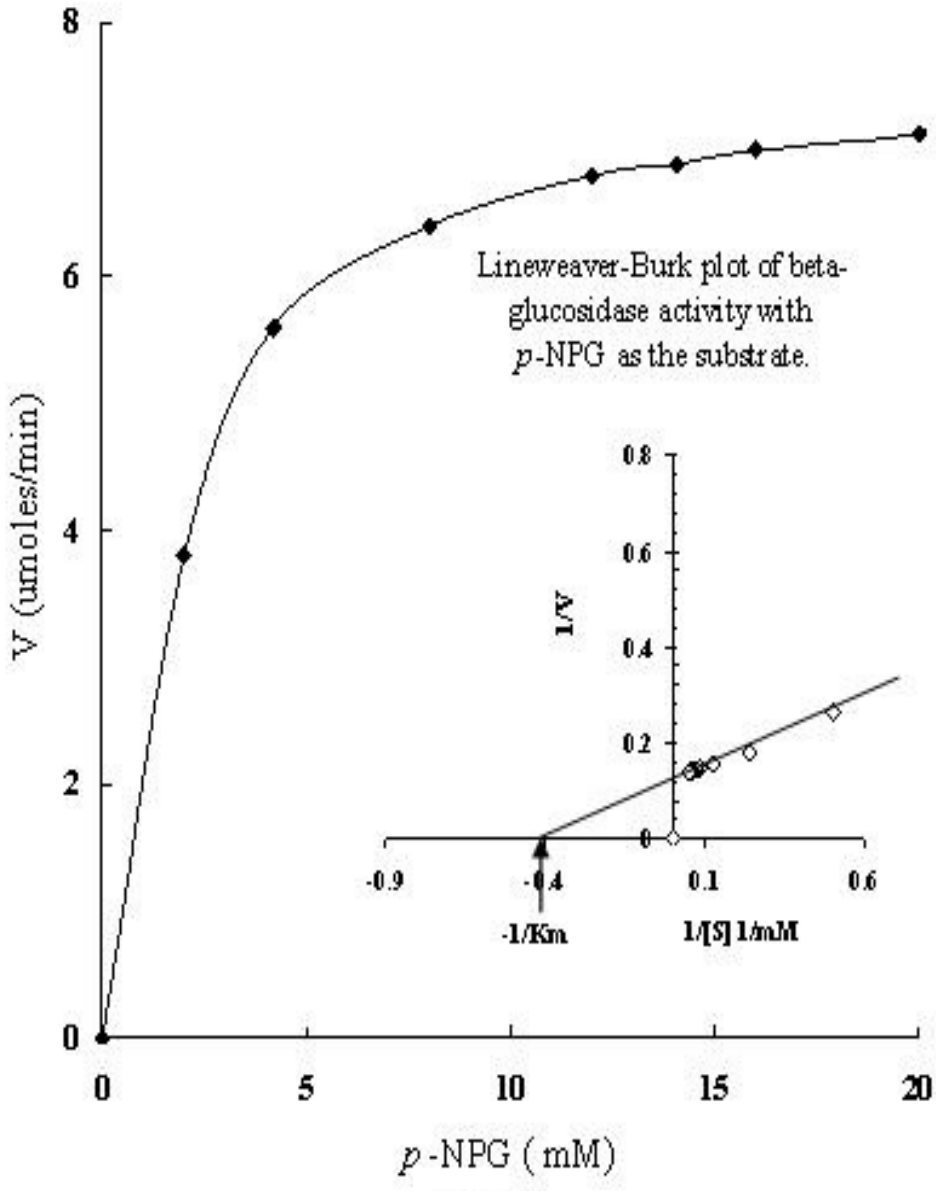

Figure 7. Effect of *p*-NPG concentration on β-glucosidase activity of *A. terreus*. Reaction mixture contained: mM *p*-NPG, as indicated; sodium-citrate buffer pH 5, 50 µmoles; extract protein, 15 µg; total volume 1.1 ml; temp., 60°C and reaction time, 10 min.

DISCUSSION

In this study, an intracellular β-glucosidase produced by *A. terreus* NRRL 265 grown efficiently on an inexpensive medium (whey permeate) was purified by different techniques to homogeneity including ethanol fractionation followed by gel filtration on Sephadex G-100 and Sephadex G-200 chromatographic columns. Results obtained indicate that β-glucosidase of *A. terreus* NRRL 265 had a maximal activity at 60°C and pH 5.0. In general, the optimal pH and temperature of *A. terreus* NRRL 265 β-glucosidase were more or less close to those reported for other fungal β-glucosidases namely *A. terreus* (Workman and Day, 1982), *Alternaria alternata* (Macris, 1984) and *Humicola grisea* (Peralta et al., 1997). The results also showed that the enzyme activity was fully stable at 50°C for 2 h. These results seemed to be much better than those reported for crude β-glucosidases from *Aspergillus phoenicis* (Deschamps and Huet, 1984) and *A. terreus* (Workman and Day, 1982). Thermostability studies showed that the enzyme was fully stable up to 2 h at 50°C. A slight decrease in the activity was observed after 30 min of incubation at 60°C. These results seemed to be more thermostable than other β-

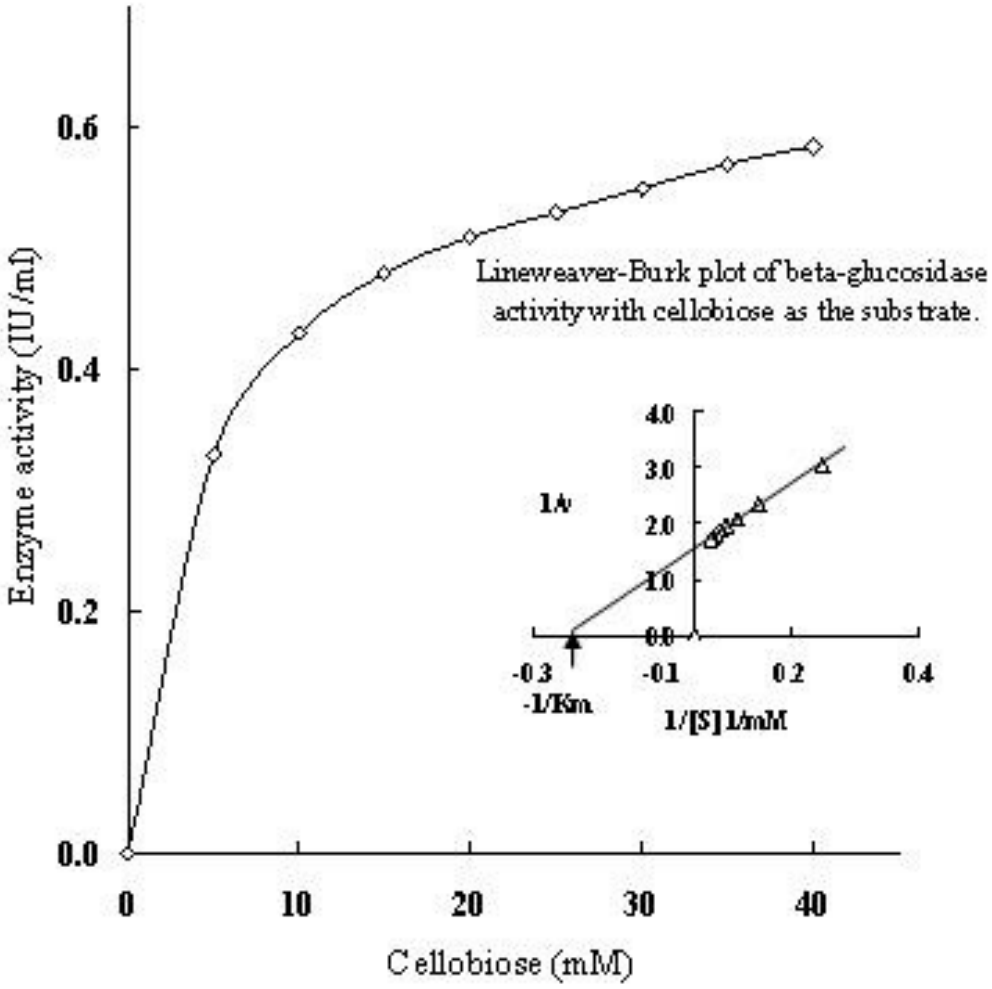

Figure 8. Effect of cellobiose concentration on β-glucosidase activity of *A. terreus*. Reaction mixture contained: mM cellobiose, as indicated; sodium-citrate buffer pH5, 50 µmoles; extract protein, 15 µg; total volume 0.5 ml; temp., 60°C and reaction time, 30 min.

glucosidases from *A. terreus* ATCC 52430 (Araujo and D`Souza, 1986), *Neurospora crassa* (Yazadi et al., 1990) and *A. niger* A20 (Abdel-Naby et al., 1999). A detectable inhibition was exhibited by Ag^{+} and Fe^{+3}, by about 14 and 48%, respectively, when added at a final concentration of 10^{-2} M, while Hg^{2+} and Cu^{2+} showed a slight inhibition. These results are in accordance with those reported for β-glucosidase produced by *Botrytis cinerea* (Gueguen et al., 1995) and *Rhizopus japonicus* (Chen and Fujio, 1997). Frequent freezing and thawing, of the purified enzyme for three months had no significant effect on enzyme activity which may be helpful with regard to storage of the enzyme preparation. The effect of EDTA, a chelating agent, on β-glucosidase activity indicates that the enzyme did not depend on a metallic co-factor at its active site. The results also revealed that sulfhydryl (SH) groups do not seem to play a role in the catalytic action of β-glucosidase as addition of iodoacetate, reduced glutathione and mercaptoethanol at any of the tested three concentrations (10^{-3} M, 5 x 10^{-2} M and 10^{-2} M) had no effect on enzyme activity. The β-glucosidase of *A. terreus* NRRL 265 is a broad specificity type since it showed high reactivity towards *p*-NPG, cellobiose and salicin. This is the most commonly observed type in cellulolytic microorganisms (Saha et al., 1995; Abdel-Naby et al., 1999). In general, the high specificity of *A. terreus* NRRL 265 β-glucosidase justifies its suitability for enriching cellulolytic complexes defective in β-glucosidase, especially that of *Trichoderma*. The apparent K_m value of this enzyme was found to be 2.5, 3.7 and 5.5 mM for *p*-NPG, cellobiose and salicin, respectively, indicating that the affinity of the enzyme for the three substrates are in the order of magnitude *p*-NPG > cellobiose > salicin. Obtaining hyperbolic rather than sigmoid saturation kinetics indicates that β-glucosidase seems not to be an allosteric enzyme. The results also showed that the purified β-glucosidase was competitively inhibited by glucose and glucono-1,5-lactone. The

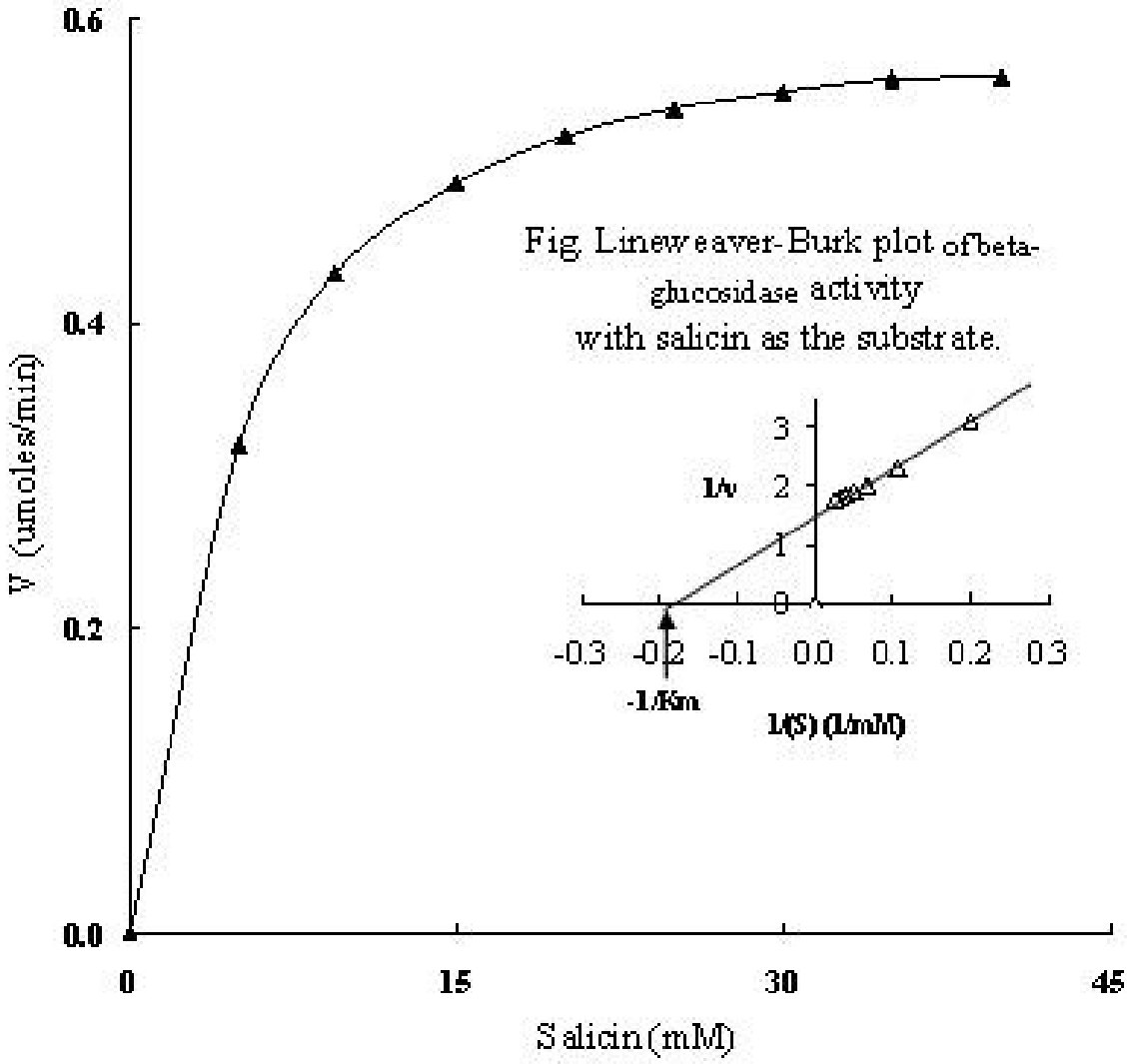

Figure 9. Effect of salicin concentration on β-glucosidase activity of *A. terreus*. Reaction mixture contained: mM salicin, as indicated; sodium-citrate buffer pH5, 50 µmoles; extract protein, 15 µg; total volume 0.5 ml; temp., 60°C and reaction time, 30 min.

corresponding dissociation constant (K_i) values determined from the Lineweaver-Burk plots were 13.6 and 1.9 mM, glucose and glucono-1,5-lactone, respectively, in agreement of the reported studies that competitive inhibition of glucose is a common characteristic of fungal β-glucosidases (Gueguen et al., 1995; Saha and Bothast, 1996).

In conclusion, the novel β-glucosidase purified from *A. terreus* NRRL 265 showed great potential as an industrial source of β-glucosidase. It is affected only slightly by glucose compared with other fungal β-glucosidases. The enzyme is extremely stable and is not affected significantly by cations, as well as, it could be produced efficiently on an inexpensive medium (whey permeate). One of its most important attributes is that its pH and temperature optima match those reported for *Trichoderma* cellulase, indicating a potential for supplementing *Trichoderma* cellulase with β-glucosidase from *A. terreus* NRRL 265.

Conclusion

An intracellular β-glucosidase from *A. terreus* NRRL 265 was purified to homogeneity with an apparent molecular mass of about 116 kDa. The purified enzyme was found to be a glycoprotein containing 69% carbohydrates by weight and free from mycotoxin. Optimal enzyme activity was observed at pH 5.0 and 60°C. K_m values were calculated and found to be 2.5, 3.7 and 5.5 for *p*-NPG, cellobiose and salicin, respectively. Glucose and glucono-δ-lactone were found to be a competitive inhibitor of the enzyme with apparent K_i of 13.6 and 1.9 mM, respectively. The enzyme lost about 44% of its

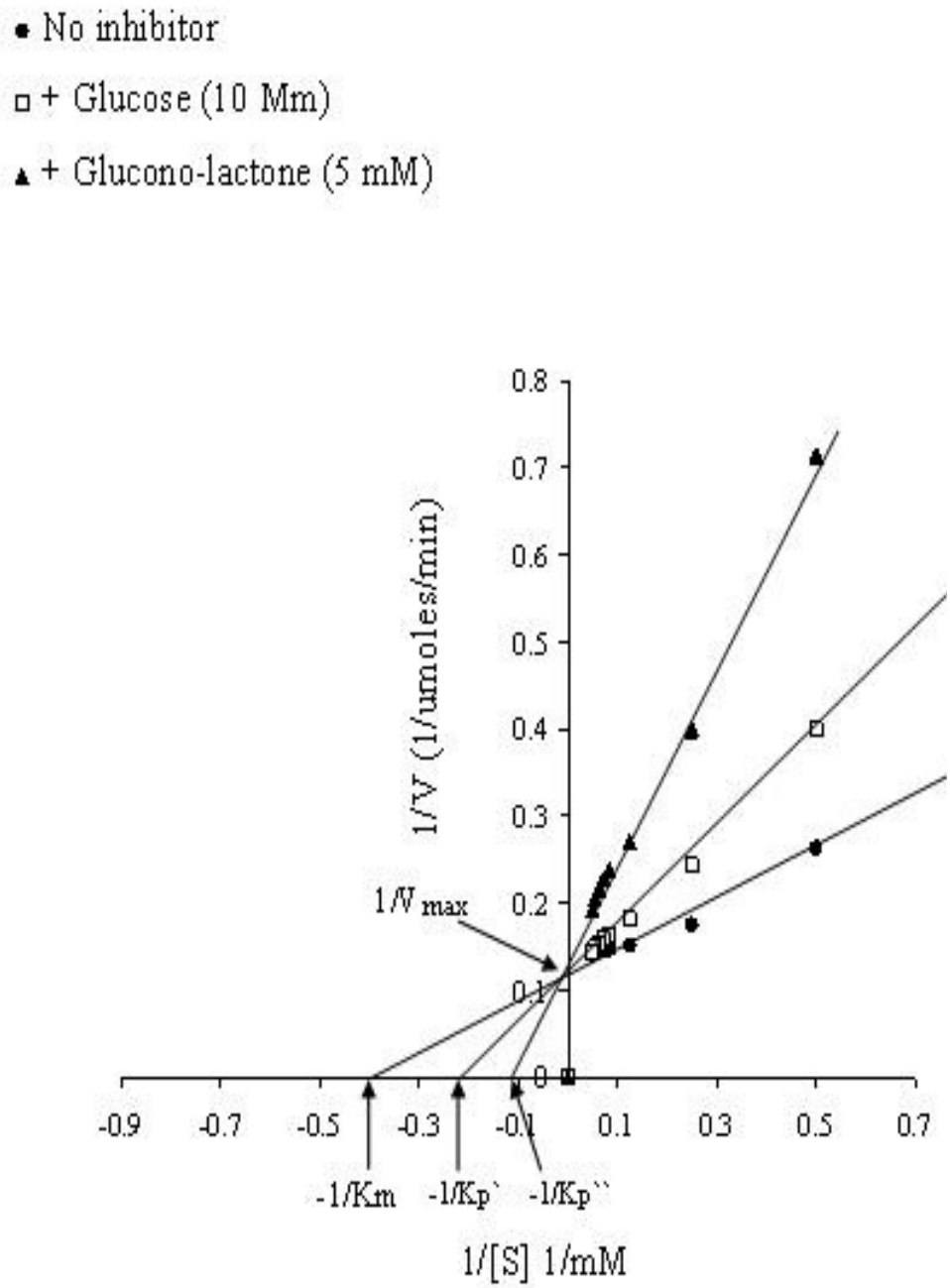

Figure 10. Competitive inhibition of *p*-NPG hydrolysis by β-glucosidase activity of *A. terreus*. Reaction mixture contained:, mM *p*-NPG and concentration of inhibitor as indicated; sodium-citrate buffer pH 5, 50 µmoles; extract protein, 15 µg; total volume 1.1 ml; temp., 60°C and reaction time, 10 min.

activity after 3 months of freezing and thawing. Thermal and pH stability studies indicated that the enzyme has higher thermal and pH stability over a wide range of temperature and pH values respectively. The enzyme has no specific metal requirement. EDTA did not inhibit enzyme activity indicating that β-glucosidase is not a metalloenzyme. SH-groups do not seem to play role in the catalytic sites of β-glucosidase as addition of iodoacetate, reduced glutathione or mercaptoethanol did not affect the enzyme activity.

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