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Essential groups and stability of α-glucosidase of *Penicillium notatum*

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Abstract - α-Glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) was isolated from *Penicillium notatum*. The enzyme was induced by gibberellic acid (GA_3). The GA_3-mediated increase in the enzyme activity was repressed in presence of abscisic acid, cycloheximide and the antibiotics chloramphenicol, cordycepin, and rifampicin which are inhibitors of protein synthesis. α-Glucosidase was purified 440-fold with of 27.8-fold of purification. The enzyme was immobilized using chitosan gel. The optimal pH values were 6.5 and 7.5 for free and the immobilized enzymes, respectively. The optimal temperatures were 50 and 65 °C for free and immobilized enzymes, respectively. The enzyme hydrolyzed maltose, sucrose, isomaltose, maltotriose but not starch, amylopectin and amylose. Trehalose and glycerol protected the free and immobilized enzymes against inactivation at 70 °C, however trehalose was the better protector. Phytate protected the free enzyme against heat inactivation at both 65 and 70 °C. 2,4,6 Trinitrobenzenesulfonic acid, butanedione and diethylpyrocarbonate inactivated the enzyme and suggest that, lysyl, arginyl and histidyl groups are taking part in enzyme catalysis. The inactivation by the three compounds was protected by the substrate *para*-nitrophenyl-α-D-glucopyranoside. Treatment of the enzyme with 1-ethyl-3(3-dimethyl aminopropyl)-carbodiimide, *p*-chloromercuribenzoate, N-bromosuccinimide, N-acetlyimidazole revealing the involving of carboxyl, sulfhydryl, trptophenyl and tyrosyl groups, respectively in the catalysis of α-glucosidase. EDTA, *o*-phenanthroline, dipyridyl and 8-quinolinol inhibited the enzyme activity and the inhibition was higher in case of free enzyme compared with immobilized enzyme.

Key words: α-glucosidase; induction; purification; stability.

INTRODUCTION

α-Glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) catalyses the release of D-glucose residues from the non-reducing end of a variety of substrates, including disaccharides, oligosaccharides and aryl- and alkyl-α-glucopyranosides (Zdzieb and Synowiecki, 2002; Brazdova *et al*., 2009). Thermostable α-glucosidases isolated from a variety of microorganisms are suitable for the improvement of industrial starch processing into glucose syrup.

Most enzymes possess one or two amino acid residues in their active sites, which are involved in catalytic activity. Generally, the existence of tryptophan, cysteine, lysine, histidine, serine, arginine, and aspartate and glutamate residues has been reported at or near the active site of enzymes (Roig and Kennedy, 1992). Several methods have been described in the literature for the identification of the catalytically essential amino acid residues of enzymes: determination of amino acids involved in catalysis by measuring the kinetic parameters of enzymes at different pH values; X-ray structural analysis and substrate specificity studies are some examples of these methods. In the cases where the enzyme is available in limited amounts; the chemical modification of the enzyme molecule by amino acid specific reagents seems to be one of the most convenient approaches for identification of amino acids at or near the catalytic canter (Roig and Kennedy, 1992).

Immobilization of enzymes is now a widely used approach for obtaining reusable derivatives of enzymes. It is generally carried out by adsorption or covalent coupling to solid matrices, entrapment in polymeric substances like polyacrylamide or encapsulation. Often, immobilization also results in improvement of enzyme stability under process conditions. Immobilization also allows the use of enzymes in various reactor designs like packed or fluidized beds (Cheetham, 1985; Gupta and Mattiasson, 1992). Thus, lower capital/energy costs and better logistics are associated with a process using an immobilized system.

Use of stabilizing additives is a customary practice in enzyme technology and shelf life of enzyme products very much relies upon addition of such additives. These molecules exert stabilizing effects by inducing preferential hydration of proteins, i.e. the additive tends to be excluded from the vicinity of the protein molecule. At least in the case of polyols, the preferential hydration arises from

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an increase in the surface tension of the solvent water (Miroliaei and Nemat-Gorgani, 2001).

The present paper aims to describe induction, purification, kinetics, immobilization and stability of α-glucosidase from *Penicillium notatum*.

MATERIALS AND METHODS

Growth of organism. *Penicillium notatum* was grown on a liquid medium containing the following components: 2% corn steep liquor, 1.2% $(NH_4)_2H_2PO_4$, 0.07% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$ at pH 4.5. The liquid culture (100 ml medium in 250 Erlenmeyer flask) was grown at 27 °C in an orbital incubator for 3 days. Culture was inoculated from stocks kept on malt extract agar plates for 3 days.

Enzyme extraction. Twenty grams of freeze-dried mycelium collected from 400 ml of fungal culture were pulverized with an homogenizer (Nissei Excel Auto-Homogenizer; Nihonseiki Co., Tokyo) for 3 min in cold extraction buffer (100 mM Na phosphate buffer, pH 7.0, 5 mM DTT). Extracts were filtered through four layers of muslin and clarified by centrifugation at 5000 rpm for 20 min at 4 °C. The resulting supernatant was called the crude extract.

Purification of the enzyme. The crude extract was treated with ammonium sulphate to 80% saturation. The precipitated protein was taken up in 16 ml of extraction buffer with gentle constant stirring. The mixture was left on ice for 1 h and centrifuged at 2000 *x g* for 20 min. The eluate was applied directly on DEAE-cellulose column (1.9 x 7 cm) equilibrated with extraction buffer. The column was eluted with KCl gradient formed by 90 ml of 300 mM KCl, both in extraction buffer, at a flow rate of 1 ml min^{-1}. Active fractions were pooled, KCl was added (1 g 10 ml^{-1}) to bring concentration to 1.55 M and was then applied to a hydroxyapatite column (15 ml) equilibrated with extraction buffer containing 1.5 M. The column was eluted with a gradient formed by 30 ml of 1.5 M KCl and 30 ml of 50 mM KCl in extraction buffer. Active fractions were pooled and applied directly to a phenyl sepharose (0.5 x 5 cm). The column was eluted at room temperature with a KCl gradient (50-300 mM) over 20 min. Fractions of 1 ml were collected and kept on ice.

Electrophoresis. SDS–PAGE was performed on 7.5% gels according to the method of Laemmli (1970). After the run, the gels were stained for proteins with Phast Blue R (R-350).

Enzyme assay. α-Glucosidase was measured using *para*-nitrophenyl-α-D-glucopyranoside (PNPG) as substrate according to the method of Mala and Kralova (2000). The enzyme was incubated at 37 °C with 50 mM PNPG solution in 100 mM sodium phosphate buffer at pH 6.5 for 20 min. Reaction was terminated by adding 10% Na_2CO_3 (w/v) and the produced *p*-nitrophenol was detected spectrophotometrically at 405 nm. One unit of the activity was defined as the amount of the enzyme that produces 1 μmol of *p*-nitrophenol per 1 min at pH 6.5 and 37 °C.
Maltose, sucrose, maltotriose, isomaltose, starch, amylose and amylopectin were also tested as substrates at the same concentration of PNPG.

All values for the measured enzyme activities in the present investigation are the mean of three measurements ± SE.

Effect of phytohormones. Gibberellic acid (GA_3) and abscisic acid (ABA) (100 μM), either singly or in combination, were added to culture medium during the incubation for 72 h and then the enzyme activity was determined.

Effect of cycloheximide (CHI) and antibiotics on GA_3-mediated increase of α-glucosidase. To test whether the induced activity by GA_3 is due to *de novo* synthesis of α-glucosidase, 20 mg ml^{-1} of CHI, chloramphenicol, cordycepin and rifampicin were added with GA_3 in the growth medium.

Protein determination. Protein was measured by the Coomassie Blue binding method using bovine serum albumin as standard (Sedmak and Grossberg, 1977; Spector, 1978).

Modification of α-glucosidase by amino acid specific reagents. The enzyme was preincubated with amino acid reagents, which included butanedione (BD), N-bromosuccinimide (NBS), 2,4,6 trinitrobenzenesulfonic acid (TNBS), N-acetlyimidazole (NAI) and *p*-chloromercuribenzoate (PCMB) in 500 μl of 400 mM mannitol and 50 mM HEPES-Tris (pH 7.0) for 30 min at 25 ºC. Incubations with diethylpyrocarbonate (DEPC) and 1-ethyl-3(3-dimethyl aminopropyl)-carbodiimide (EDAC) were done in 500 μl of 400 mM mannitol and 20 mM-NaOH (pH 7.0). For DEPC the incubation medium contained ethanol at a final concentration of 1% (v/v), previously shown to have no effect on the enzyme under investigation. The preincubation reaction was stopped by diluting the mixture in buffer without substrate. The residual enzyme activity was then quantified by adding PNPG

Immobilization of α-glucosidase. The purified α-glucosidase was immobilized on chitosan gel.

Preparation of chitosan gel. Chitosan gel was prepared by adding 0.9 ml of chitosan flakes to 48 ml of distilled water and 2 ml of glacial acetic acid. The alginate solution was prepared by adding 9.5 ml of 4% (w/v) alginate gel, 85 ml 0.1 M phosphate buffer (pH 6.5) and 5 ml 30% glutaraldehyde that has been stirred gently for 1 h at room temperature. Fresh glutaraldehyde that has been stored at 4 °C was used.

Immobilization procedure. Two ml of purified α-glucosidase was added to 15 ml of the chitosan gel. This solution was stirred slowly for 5 min and allowed to stand until degassed. The gel was forced through needles dropwise into the alginate solution that was continuously stirred during the procedure. The beads were allowed to cure in solution with stirring for 30 min. The beads were rinsed with 100 mM phosphate buffer (pH 7.5) and stored in deionized water at 4 °C until needed.

RESULTS AND DISCUSSION

Production of α-glucosidase

Preliminary experiments were performed to determine the optimal time of α-glucosidase production by *P. notatum* and comparing the productivity in presence and absence of GA_3. Therefore, the fungus was grown for 5 days and the glucosidase activity was determined throughout the experimental period in presence and absence of GA_3. The

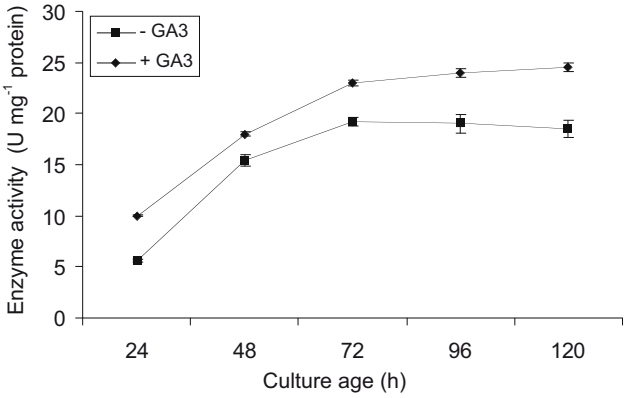

FIG. 1 - Specific activity of α-glucosidase during the growth of *Penicillium notatum* in the presence (+ GA_3) or absence (- GA_3) of gibberellic acid.

results in Fig. 1 indicate that a considerable appreciable level of α-glucosidase activity was produced after the third day of growth and the productivity of the enzyme was higher in presence of GA_3.

Effect of gibberellic acid (GA_3) and abscisic acid (ABA) on α-glucosidase

We examined possible interaction of GA_3 and ABA on α-glucosidase synthesis (Table 1). GA_3 included in the growth medium induced the enzyme activity whereas ABA repressed its activity. Furthermore, to test whether the induced activity by GA_3 is due to *de novo* synthesis of α-glucosidase, 20 mg ml^{-1} of cycloheximide (CHI) a protein inhibitor was added with GA_3 in the growth medium. The results show that the induced activity of α-glucosidase was repressed by CHI. This may indicate that induced α-glucosidase synthesis by GA_3 is due to *de novo* enzyme synthesis. Induction of the enzyme by GA_3 is in agreement with the results obtained for other enzymes such as phosphoenolpyruvate carboxylase (El-Shora, 1993; Bihzad and El-Shora, 1996) and NADP-glutamate synthase (El-Shora, 2001), phenylalanine ammonia lyase (El-Shora, 2002) and acid phosphatase (El-Shora and Metwally, 2008).

Effect of antibiotics on GA_3-mediated increase of α-glucosidase

The effect of antibiotic inhibitors of transcription and translation on the induction of α-glucosidase activity by GA_3 was investigated. The inhibitors reduced the mediated-increase by GA_3 particularly *in vivo* (Table 2). However, when the tested inhibitors were added directly to the assay mixture (*in vitro*) they showed a little or no effect. These results confirm further that the mediated-increase of α-glucosidase by GA_3 is through *de novo* synthesis.

Purification of α-glucosidase

A summary of the purification of α-glucosidase is presented in Table 3. This procedure included ammonium sulphate, DEAE-cellulose, hydroxyapatite and phenyl sepharose. This procedure resulted in specific activity of 440 U mg^{-1} protein and 27.8-fold. The purified enzyme was homogenous by SDS-PAGE (Fig. 2). The obtained specific activity in the present investigation is higher than that reported for the enzyme from *Bacillus thuringiensis* (Rowe and Margaritis, 2004) and comparable to that purified from *Geobacillus thermodenitrificans* (Ezeji and Bahl, 2006).

TABLE 1 - Effect of GA_3, ABA and cycloheximide on α-glucosidase of *Penicillium notatum*

Treatment	α-Glucosidase activity (U mg^{-1} protein)	Relative activity (%)
Untreated	40.3 ± 0.9	100
GA3 (100 μmol)	52.5 ± 0.8	130
GA3 (100 μmol) + ABA (100 μmol)	50.9 ± 0.6	125
GA3 (100 μmol) + CHI (20 mg ml^{-1})	43.6 ± 0.7	108

TABLE 2 - Induction of α-glucosidase of *Penicillium notatum* by 200 μmol of GA_3 and its negation reversal by antibiotics

Treatment	Enzyme activity			
	In vitro		*In vivo*	
	U mg^{-1} protein	Increase by GA_3 (%)	U mg^{-1} protein	Increase by GA_3 (%)
Control	16.6 ± 0.3	--	16.6 ± 0.3	--
GA_3	18.0 ± 0.4	8.4	30.6 ± 0.4	84.3
Chloramphenicol + GA_3	17.6 ± 0.5	6.0	27.0 ± 0.6	62.7
Cordycepin + GA_3	17.8 ± 0.6	7.2	27.6 ± 0.4	66.3
Rifampicin + GA_3	17.0 ± 0.5	2.4	23.0 ± 0.3	38.6

TABLE 3 - Purification of α-glucosidase of *Penicillium notatum*

Steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification fold
Crude extract	212.7	3360	15.8	100	1
$(NH_4)_2SO_4$ (45-80 %)	140.0	2520	37.2	75.0	2.4
DEAE-cellulose	20.0	920	46.0	27.4	2.9
Hydroxyapatite	2.2	504	229.1	15.0	14.4
Phenyl sepharose	0.5	220	440.0	6.5	27.8

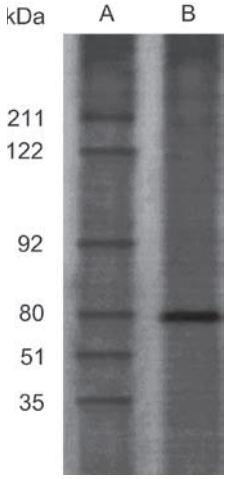

FIG. 2 - SDS-PAGE of purified α-glucosidase of *Penicillium notatum*. A: protein standards; B: α-glucosidase.

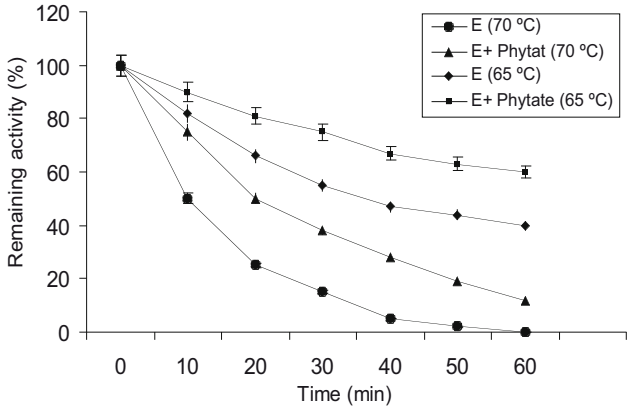

FIG. 3 - Thermostability of α-glucosidase of *Penicillium notatum* at 65 and 70 °C in the presence of sodium phytate.

Effect of various substrates on α-glucosidase activity

The pure α-glucosidase was examined using different possible substrates and the rate of hydrolysis of these substrates relative to that with PNPG were compared (Table 4). The enzyme hydrolyzed maltose, sucrose and maltotriose but did not hydrolyze isomaltose, starch, amylose and amylopectin. These results are in agreement with the results of other investigators (Ezeji and Bahl, 2006; Giannesi *et al*., 2006).

Effect of phytate on α-glucosidase activity

Phytate protected α-glucosidase activity when incubated at 65 °C and 70 °C for 30 min (Fig. 3). Phytic acid (myo-inositol hexakisphosphate), an anti-nutritional agent (Graf, 1983; Kerovuo *et al*., 1998) in monogastric animals (through chelating and forming complexes with proteins and various metal ions) conferred thermostability to the enzyme. These results are in harmony with those reported by Ezeji and Bahl (2006). However, it is not clear why the enzyme was more stable in presence of phytate. More studies are needed to find out the reason for the increase in thermostability of these enzymes in the presence of phytate. The resistance of α-glucosidase in this paper is of great potential industrial advantage to the corn processing and animal feed industry because phytate is the major storage form of phosphate in cereals, legumes, pollen and oilseed.

Active groups of α-glucosidase

Treatment of α-glucosidase in absence of its substrate PNPG with increasing concentration of TNBS, BD and DEPC resulted in a progressive decrease of the enzyme activity (Fig. 4). However, presence of PNPG significantly protected the enzyme from inactivation by the three compounds. Also, presence of PNPG partially protected the enzyme against EDAC, PCMB, NBS, NAI (Table 5). Faridmoayer and Scaman (2005) reported that histidine and tyrosine residues are involved in the catalytic activity of the enzyme from *Saccharomyces cerevisiae*.

Immobilization of α-glucosidase

α-Glucosidase was immobilized on chitosan gel. The yield percentage of immobilization in four experiments was calculated as:

$$\frac{\text{Immobilized activity}}{\text{Added activity}} \times 100$$

The calculated percentages were 97.2, 97, 95 and 90% for the first, second, third, and fourth experiments, respectively (Table 6). Results from repeated use of immobilized to break down the substrate are shown in Fig. 5. The data show that the immobilized enzyme retained 90% of its initial activity after 5 cycles of usage.

TABLE 4 - Substrate specificity of purified α-glucosidase of *Penicillium notatum*

Substrate	Specific activity (U mg^{-1} protein)	Relative activity (%)
PNPG	42.0 ± 0.7	100
Maltose	19.4 ± 0.9	46.2
Sucrose	15.2 ± 0.8	36.2
Maltotriose	9.6 ± 0.5	22.9
Isomaltose	0	--
Starch	6.8 ± 0.9	16.2
Amylose	0	--
Amylopectin	4.4 ± 0.7	10.5

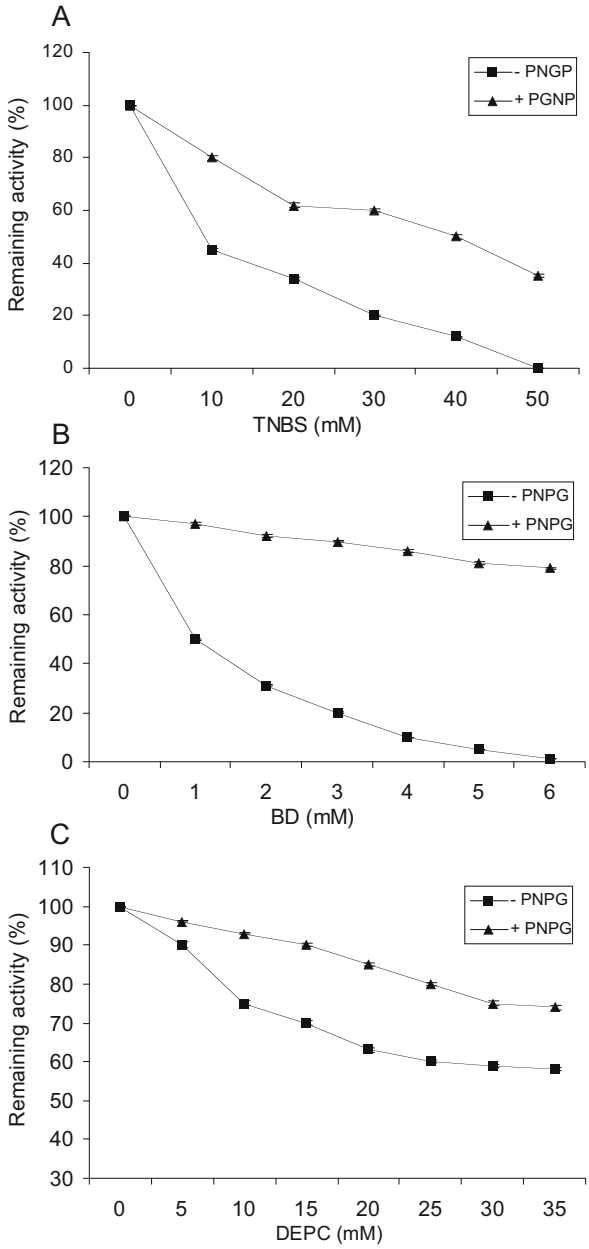

FIG. 4 - Stability of free α-glucosidase of *Penicillium notatum* against inactivation by TNBS (A), BD (B) and DEPC in the presence of PNPG (C).

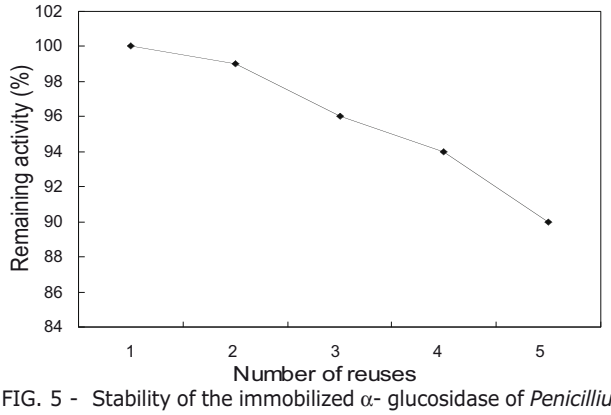

FIG. 5 - Stability of the immobilized α- glucosidase of *Penicillium notatum* after repeated use.

TABLE 5 - Protection of α-glucosidase of *Penicillium notatum* against inactivation by 5 mM PBMB, EDAC, NBS and NAI using as substrate PNPG

Inhibition system	Residual activity (%)
Enzyme	100
+ PCMB	29 ± 0.5
+ PNPG + PCMB	35 ± 0.8
Enzyme	100
+ EDAC	11 ± 0.2
+ PNPG + EDAC	64 ± 0.5
Enzyme	100
+ NBS	36 ± 0.7
+ PNPG + NBS	57 ± 0.5
Enzyme	100
+ NAI	19 ± 0.4
+ PNPG + NAI	52 ± 0.8

Optimal pH and temperatures of free and immobilized α-glucosidase

The free and immobilized enzymes showed optimal pH of 6.5 and 7.5, respectively (Fig. 6A) with a rapid decline as the pH increased above the optimal value. Immobilization of enzymes on charged supports often leads to displacements of the pH-activity profile to either alkaline or acidic regions (Leontievsky *et al*., 2001).

Also, the optimal temperatures were 50 and 65 °C for the free and immobilized enzymes, respectively (Fig. 6B). The shift in optimum temperature for α-glucosidase activity upon immobilization is due to minimization or prevention of conformational changes in the protein tertiary structure by increased temperature. This property of the immobilized enzyme will make it suitable for application in industrial processes carried out at elevated temperatures.

Thermal stability of free and immobilized α-glucosidase by polyols

The results in Fig. 7 show the thermal stability of free and immobilized α-glucosidase at 70 °C in presence of different concentrations of trehalose and sorbitol. Both enzymes showed significantly improved thermal stability as a result of entrapment particularly with sorbitol. The protection was more apparent in case of immobilized enzyme. Also, the thermal stability of both enzymes was studied in presence of 20% glycerol for 6 h (Table 7). The results indicate that the immobilized enzyme was more resistant to inactivation at 70 ºC compared to the free one. Many workers have reported that immobilization did lead to thermostabilization of enzymes (Ezeji and Bahl, 2006). Thermal inactivation of enzymes precedes the loss of the three-dimensional structure (Tsou, 1998).

It has been proposed that polyols such as sorbitol, trehalose and glycerol stabilize proteins by increasing the transition temperature of the proteins (Lin and Timashe, 1996) that is the most compact structure of a protein is favored and local backbone motions

Table 6 - Immobilization yield of α-glucosidase of *Penicillium notatum*

Experiment	Added enzyme (U)	Immobilized enzyme (U)	Immobilization yield (%)
1st	54.5	53.0	97.2
2nd	54.3	52.7	97.0
3rd	54.2	51.5	95.0
4th	52.9	47.5	90.0

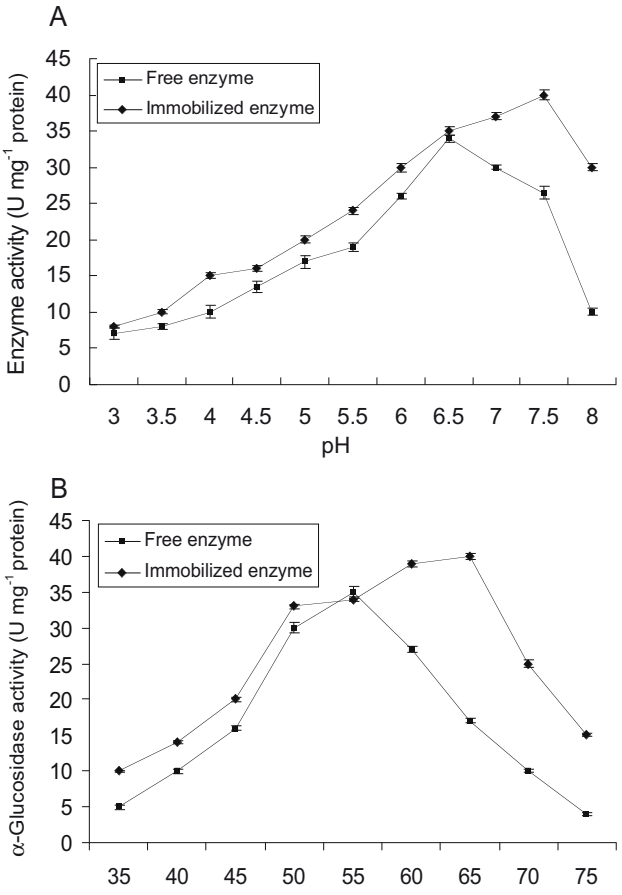

FIG. 6 - Effect of pH (A) and temperature (B) on free and immobilized α-glucosidase of *Penicillium notatum*.

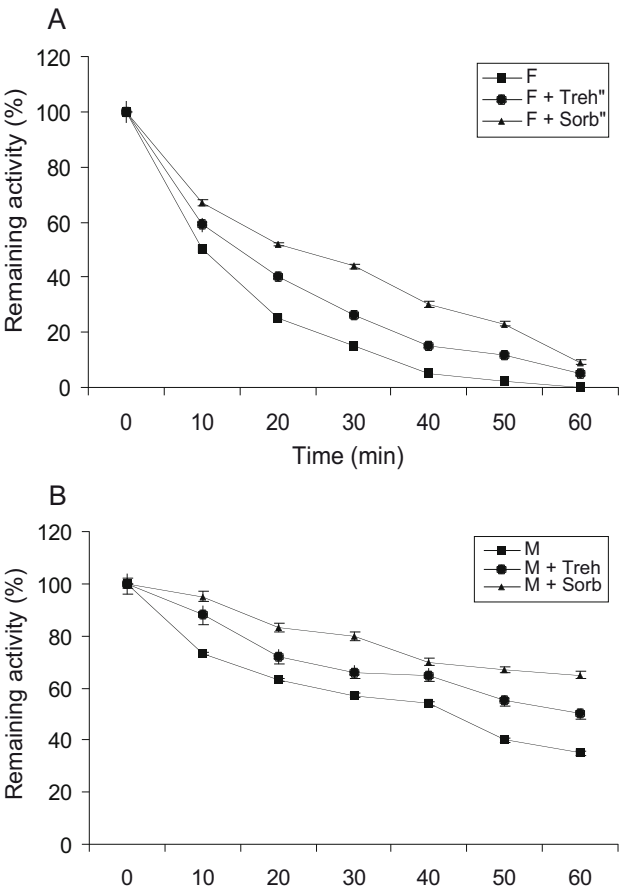

FIG. 7 - Thermal stability of free (A) and immobilized (B) α-glucosidase of *Penicillium notatum* at 70 °C in the presence of trehalose and sorbitol at 5 mM.

away from the fully folded state are inhibited (Butler and Falke, 1996). It is then possible that polyols inhibit both the first and second phases of inactivation through rigidization of the protein.

The stabilizing effect of additives is not an absolute effect valid for all enzymes, but it depends on the nature of the enzyme, on its hydrophilic and hydrophobic character and on the degree of interaction with the additive.

Effect of chelating agent on α-glucosidase

The effect of chelating agents EDTA, *o*-phenanthroline, EGTA, dipyridyl and 8-quinolinol on α-glucosidase was studied and the results are summarized in Table 8. The results show that all the tested agents inhibited the activities of the free and immobilized enzymes. The immobilized enzyme was more resistant to the inhibition by these compounds compared to the free one.

CONCLUSION

The enzyme was successfully immobilized in a system of chitosan and alginate. The induction of α-glucosidase by GA_3 from *P. notatum* as phytohormone is of great interest particularly for the production of the enzyme in high scale in industry. Also, the possibility of stabilizing α-glucosidase against thermal inactivation by maintaining in presence of phytate or polyols would be of great interest for biological applications as many industrial processes require elevated temperatures.

TABLE 7 - Thermostability of free and immobilized α-glucosidase of *Penicillium notatum* at 70 °C in presence of 20% glycerol

Time (h)	Relative activity (%)		
	No addition	Immobilized	Free
0	100	100	100
1	50 ± 0.5	100	100
2	23 ± 0.6	95 ± 0.5	91 ± 0.7
3	17 ± 0.7	90 ± 0.4	84 ± 0.5
4	12 ± 0.1	81 ± 0.6	74 ± 0.7
5	10 ± 0.01	74 ± 0.4	61 ± 0.4
6	5 ± 0.01	62 ± 0.5	54 ± 0.6

TABLE 8 - Effect of chelating agents on free and immobilized α-glucosidase of *Penicillium notatum*

Agents (5 mM)	Residual activity (%)	
	Immobilized	Free
None	100	100
EDTA	66 ± 0.5	27 ± 0.3
O-Phenanthroline	36 ± 0.9	7 ± 0.1
EGTA	84 ± 0.4	40 ± 0.4
Dipyridyl	55 ± 0.7	20 ± 0.8
8-Quinolinol	40 ± 0.8	14 ± 0.3

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