Pakistan J. Zool., vol. 30(1), pp. 23-30, 1998.

Purification and Characterization of Chemically Modified Beta-Glucosidases from *Aspergillus niger* NIAB-280*

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Abstract.- Extracellular β-glucosidase was purified to homogeneity with 32 fold purification in four steps via ammonium sulphate precipitation, hydrophobic interaction chromatography, FPLCmono-Q anion exchange and gel filtration chromatography. The recovery of β-glucosidase after complete purification was 32%. β-Glucosidase was modified by EDC in the presence of glycinamide (GAM) and ethylenediamine dihydrochloride (EDAM) as nucleophiles for 60 min. Effects of neutralization with GAM and reversal with EDAM of surface carboxyl groups on the kinetic properties of the enzyme were studied. Native, GAM and EDAM had V_{max}/K_m values of 0.93, 1.22 and 0.60, respectively. The modification of surface carboxyl groups shifted the optimum pH range in both GAM and EDAM from 4-6 to 1.8-3.8. The optimum temperature of native β-glucosidase was 70°C and remained unchanged after chemical modification. GAM β-glucosidase was highly active in the presence of 25 mM $MnCl_2$ (V_{max} 2.69), however, it was less stable as compared to the native beta-glucosidase.

Key words: Enzyme purification, beta-glucosidase, EDC, glycinamide, ethyline diaminedihydrochloride, *Aspergillus niger*.

INTRODUCTION

Purification of enzymes is required studying the kinetic parameters regarding stability against various denaturants and activators (Esen and Gungor, 1993). Furthermore, monoclonal antibodies, structural analysis and chemical modification studies require the enzyme purity upto homogeneity level (Sanyal *et al.*, 1988; Heupel *et al.*, 1993; Himmel *et al.*, 1993). β-Glucosidases have been purified and characterized from *Aspergillus japonicus* (Sanyal *et al.*, 1988), *A. roseus* (Vodjdani *et al.*, 1992), *A. nidulans* (Hoh *et al.*, 1993), *A. fumigatus* (Ximenes *et al.*, 1996), *A. wentii* (Kvesitadze *et al.*, 1990) and different local strains of *A. niger* (McCleary and Harrington, 1988; Unno *et al.*, 1993; Himmel *et al.*, 1993).

The emergence of chemical modification has made new methods available for specifically altering the properties of enzymes so that their structure can be tailored to their intended use in bioreactors or food systems; for example stabilizing labile enzymes to operate in novel environments, altering enzyme specificity to bring about predetermined changes in food components, and introducing functional properties to existing or new molecules (Law, 1996). The selection of reagents for the chemical modification of amino acids should be reasonably specific. For example, acetic anhydride is most commonly used for the modification of amino groups of proteins and carbodiimides for activating carboxyl groups which can then be coupled to amines (Lundblad, 1995). The industrial processes can be made more economical by shifting the optimum pH and temperature of enzymes to be used in the bioreactor. The technique of chemical modification have also been used to alter the kinetic properties of the enzymes (Lundblad, 1995). Therefore, introduction of a new group into a protein has been known to cause changes in pH optimum, substrate specificity and susceptibility to protease digestion. For example, when a fast reacting thiol and an amino group of lysine in the vicinity of the active site of native rabbit liver fructose-1-6-bi-phosphatase was modified with O-phthalaldehyde, its pH optimum shifted from 6.8 to 8.5 (Dzugaj *et al.*, 1985). It has been observed that chemical modification reduces the capability of enzymes to catalyze reactions involving complex substrates, while their activity towards simple substrates is either retained or even enhanced (Wilchek *et al.*, 1967).

The technique of chemical modification has been used to enhance the stability of enzymes (Mozhaev and Martinek, 1984). There are reports of thermostabilization of enzymes by chemical modification. Acetylation of amino groups of

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0030-9923/98/0001-0023 $ 04.00/0

horseradish peroxidase (Miland *et al.*, 1996) and a-amylase from *Bacillus subtilis* (Urabe *et al.*, 1973) increased their stability toward heat. Charge reversal by chemical modification of carboxyl groups of glucoamylase from *A. niger* also increased its thermostability (Munch and Tritsch, 1990). Previously only couple of studies have been carried out on carboxyl group modification of ß-glucosidases from *Schizophyllum commune* (Clarke, 1990) and *Trichoderma reesei* (Mata *et al.*, 1993) and these studies were done to identify the active-site residues. There have been hardly any attempt to increase the thermal stability of ß-glucosidases by chemical modification of its carboxyl groups. It is highly desirable that the thermal stability of this industrially important enzyme be increased so that the hydrolysis of cellulose could be carried out at higher temperatures (Godfrey and West, 1996).

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Company, U.S.A. The ß-glucosidase obtained after culturing *A. niger* on wheat bran was concentrated and dialyzed against distilled water in Amicon concentrator model RA 2000. The concentrate was further processed for purification by ammonium sulfate precipitation and fast protein liquid chromatography.

Ammonium sulfate precipitation

Varying amounts of solid ammonium sulfate 10% to 90% (w/v) were added separately to one ml of ß-glucosidase concentrate in eppendorf tubes and left for overnight at 4°C. Then these tubes were centrifuged at 10,000 rpm for 15 minutes and the supernatant was assayed for ß-glucosidase activity. It was observed that complete precipitation occurred at 70% (w/v) concentration of $(NH_4)_2SO_4$. Therefore, solid $(NH_4)_2$ SO_4 was added to ß-glucosidase concentrate to give concentration of 55% (w/v) and the solution was kept overnight at 4°C. Then, after 30 minutes of centrifugation at 18000 rpm (39,200 x g), the supernatant was transferred to another tube and treated with 15% (w/v) $(NH_4)_2SO_4$ to give final concentration of 70% (w/v). The solution was again kept overnight at 4°C and then centrifuged under the same conditions as done for 55% concentration. This time, the supernatant was discarded and the pellet containing ß-glucosidase was dialyzed against distilled water.

Fast protein liquid chromatography (FPLC)

The crude extract after ammonium sulphate precipitation was subjected serially to hydrophobic-interaction chromatography on phenyl superose column, anion-exchange chromatography on Mono-Q column and gel filtration chromatography (Deutscher, 1990).

Hydrophobic interaction chromatography (HIC)

The ß-glucosidase after ammonium sulfate precipitation having 1.5 M urea, was then filled in superloop by peristaltic pump fitted on FPLC. The enzyme was loaded on phenyl superose column at a flow rate of 1 ml/min. The elution was carried out with a linear gradient (2-0M) of $(NH_4)_2SO_4$ in 100 mM sodium phosphate buffer pH 5.0. Two ml size fractions, containing ß-glucosidase, were pooled and dialyzed.

Mono-Q anion exchange chromatography

The dialyzed fractions of ß-glucosidase after HIC were then loaded on Mono-Q column at a flow rate of 1 ml/min and a linear NaCl gradient from 0-1 M in 20 mM Tris/HCl, pH 7.5 was used as elution buffer. Fractions of 2 ml were collected. Active fractions were pooled and dialyzed.

Gel filtration chromatography

Pooled fractions from Mono-Q column were finally loaded on superose column. The sample was loaded as 200 ml/run by using "loop TMS programme" of FPLC. 100 mM Tris/HCl, pH 7.0 having 0.15 M NaCl, was used as elution buffer. The flow rate was 0.5 ml/min. 1 ml size fractions were collected.

Beta-glucosidase assay

ß-Glucosidase was assayed using 15 mM paranitrophenyl ß-D glucopyranoside (pNPG) as substrate in 50 mM sodium acetate, pH 5 buffer at 40°C for 10 min (Wood and Bhatt, 1988).

Protein estimation

Proteins were estimated as given by Bradford (1976).

Sodium dodecyl sulfate denaturing PAGE

Purity of ß-glucosidase was checked by subjecting it to 7.5% SDS-PAGE (Laemmli, 1970). The proteins were stained by Coomassie blue R-250 (Merril, 1990) and destained by a solution of methanol, acetic acid and water (9:2:9) (v/v/v).

Carboxyl group modification of ß-glucosidase

The extracellular ß-glucosidase was chemically modified in two ways *viz.* by (i) neutralization of carboxyl groups with glycinamide, and (ii) charge reversal by ethylene diamine dihydrochloride.

For glycinamide modified -glucosidase (GAM) glycinamide (1 M) and glucose (100 mM) were added to five ml of purified ß-glucosidase (6.5 U/ml) solution and the pH was adjusted to 5.5 with 12M NaOH. The reaction was initiated by adding 0.075 g (75 mM) of EDC and maintained at 25°C. After 60 min, the reaction was quenched by adding five ml of 0.5 M sodium acetate buffer, pH 5.5, containing 1 M hydroxylamine for the regeneration of tyrosines. The modified enzyme was exhaustively dialyzed against 10 mM sodium acetate, pH 5.5 to remove reagents.

For ethylenediamine dihydrochloride modified ß-glucosidase (EDAM) ethylenediamine dihydrochloride (1 M) was used as nucleophile instead of glycinamide.

Effect of Substrate (pNPG)

The kinetic constants of Michaelis Menten (K_m and V_{max}) were determined by using various concentrations of pNPG (0.1-2.0 mM). ß-glucosidase was assayed at 40°C. The total volume of assay mixture was 3.0 ml, and comprised of 250 ml buffer + 250 μl ß-glucosidase + 500 μl substrate (pNPG) + 2ml 1M Na_2CO_3 solution (reaction stopper).

Effect of Mn^{++}

The Mn^{++} effect was determined by varying the pNPG concentrations (0.1-2.0 mM) at fixed Mn^{++} concentrations (0.025, 0.1 and 0.25 mM) (Dixon and Webb, 1979). Apoenzyme of ß-glucosidase was made by dialysing it against 5 mM EDTA in the presence of 0.1 mM PMSF (prepared in Dimethyl sulphoxide) for 14 hours. Then EDTA and PMSF were removed by dialysing ß-glucosidase against distilled water for 9 hours (Three changes).

Optimum temperature

Native and modified ß-glucosidases were assayed using 15 mM pNPG at different temperatures (10 to 80°C). The reaction mixture except enzyme, was incubated at required temperature for three minutes. Then ß-glucosidase was added and assayed for five minutes.

Optimum pH

Native and chemically modified ß-glucosidases were assayed at 30°C at different pH's ranging from 2-9.5. The buffers used were: pH 2-2.8 (40 mM glutamic acid/HCl), pH 3-3.6 (100 mM gly/HCl), pH 3.8-4.4 (50 mM glutamic acid/HCl), pH 4.6-5.4 (100 mM sodium acetate/acetic acid), pH 5.6-6.4 (100 mM MES/KOH), pH 6.6-7.4 (100 mM MOPS/KOH), pH 7.6-8 (100 mM HEPES/KOH), pH 8.2-8.6 (100 mM gly-gly/KOH) and pH 8.8-9.6 (100 mM gly/NaOH).

Thermostability

Thermal inactivation of native and modified ß-glucosidases were determined by incubating the enzyme solutions in 25 mM sodium acetate, pH 5.5 buffer at a particular temperature. Aliquots were withdrawn at different times, cooled on ice for 3 hours (Violet and Meunier, 1989) and then assayed for ß-glucosidase activity at 40°C. This procedure was repeated at four different temperatures ranging from 55 to 67°C.

RESULTS AND DISCUSSION

Purification of ß-glucosidase to homogeneity was completed in four steps, which resulted in 32-fold increase in purification factor. The recovery of ß-glucosidase after complete purification was 32 percent (Table I). The recovery of ß-glucosidase after ammonium sulfate precipitation was 88%. Precipitation of ß-glucosidase started at 55% (w/v) ammonium sulfate, while complete precipitation occurred at 70% (w/v) concentration. It was noted that ß-glucosidase activity decreased immediately after the addition of solid ammonium sulfate in enzyme solution. Due to this reason, ß-glucosidase was decided to dialyse at each step. At this stage 4-fold purification was observed (Table I).

After ammonium sulfate precipitate the ß-glucosidase was subjected to hydrophobic interaction chromatography. Percentage recovery of ß-glucosidase after HIC was 50% and was 13 fold purified. The purified ß-glucosidase from HIC was then applied on mono-Q column and 25 fold purification was achieved. The percentage recovery was 44%. The extracellular ß-glucosidases were purified by many researchers e.g., 275 fold purification in case of *Aspergillus japonicus* (Sanyal *et al.*, 1988), highly purified upto 99% from *Aspergillus niger* (Himmel *et al.*, 1993; UNNO *et al.*, 1993) and 157-fold purification in case of *Aspergillus nidulans* (Hoh *et al.*, 1993).

Table I.- Summary of the purification of beta-glucosidase from *Aspergillus niger*.

Treatment	Total units	Total protein (mg)	Specific activity (units/mg)	Purification factor	% recovery
Crude extract	205	485	0.42	1.00	100
$(NH_4)_2SO_4$ ppt.	180	112	1.61	3.83	88
Phenyl superose: hydrophobic interaction chromatography	102	18.25	5.59	13.31	50
Mono-Q: anion-exchange chromatography	90	8.5	10.59	25.21	44
Superose: gel filtration	65	4.49	13.26	31.57	32

All values were after dialysis against water. One unit of beta-glucosidase activity is equal to 1 μM of P-nitrophenol released/ml/min from PNGP in sodium acetate buffer pH 5.0 and 40 °C.

The purified ß-glucosidase from mono-Q was finally applied on superose column. Thirty two fold purification was achieved after gel filteration (Table I). ß-glucosidase was purified upto homogeneity level as there was only a single band on 7.5% SDS-PAGE (Fig. 1).

Fig. 1. 7.5% SDS-denaturing PAGE of purified β-glucosidase stained with coomassie blue R250.

Chemical modification of ß-glucosidase

The mechanism of catalysis of cellulases, xylanases, chicken egg lysozyme and ß-glucosidases involve two residues, one of which donates a proton to the substrate while the other negatively charged residue, stabilizes the oxo-carbonium ion (Clarke and Yaguchi, 1985; Clarke *et al.*, 1993). There is wide consensus on nucleophilic residue which is believed to be a carboxyl group of low pKa but the proton donor have been identified as either histidine or carboxyl group with high pKa. Kinetic and chemical modification studies of ß-glucosidases from *Botryodiplodia theobromae* (Umezurike, 1977; Umezurike, 1987) and *Trichoderma reesei* (Mata *et al.*, 1993) have implicated histidine as one of the residue that is involved in catalysis. On the other hand Clarke (1990) have identified carboxyl as proton donating residue in ß-glucosidase from *Schizophyllum commune* by the technique of chemical modification.

The modification by water soluble carbodiimide (EDC) requires that the carboxyl group must be protonated or in unionized form. Chan and co-workers (1988) found out that rate of modification reaction is retarded in the presence of an added nucleophile. In this case, modification reaction were always carried out in the presence of an added nucleophile in order to avoid the introduction of a bulky group into the enzyme. These bulky groups could cause unwanted conformational changes and the disruption of structure resulting in faster rate of inhibition. Furthermore, addition of a nucleophile helps to prevent the secondary modification of amino groups of the enzyme by consuming the activated

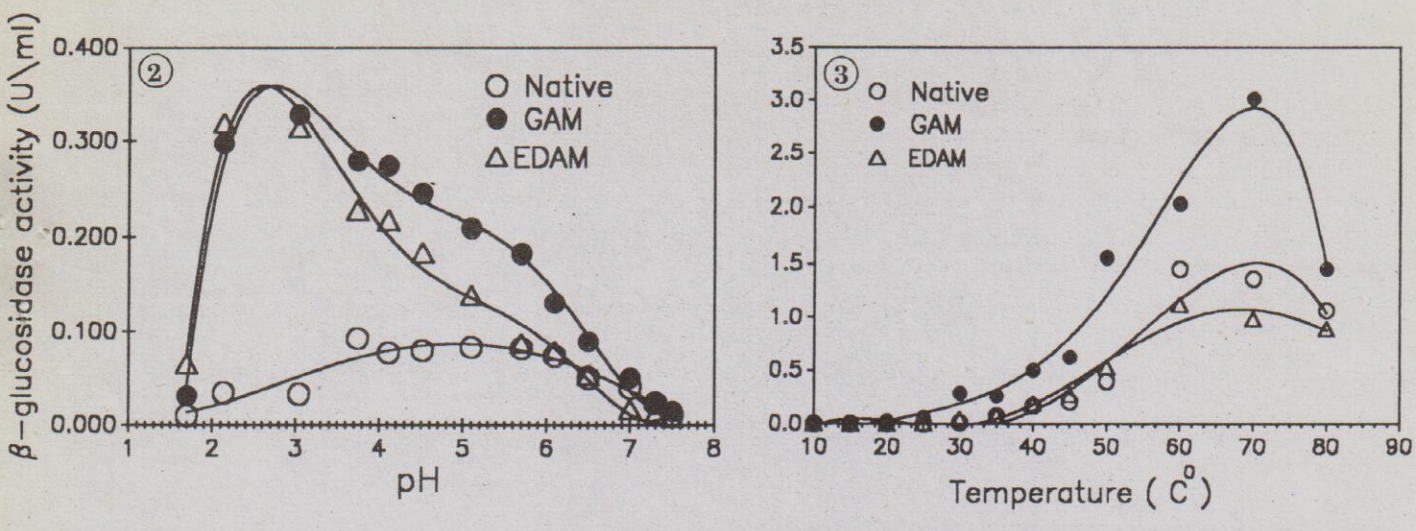

Fig. 2. The plot of pH optimum of native and chemically modified β-glucosidase at 30 °C.
Fig. 3. The plot of temperature optimum of native and chemically modified β-glucosidase at pH 5.

carboxyls generated as a result of reaction quenching.

As it is evident from the above reports that carboxyl groups are involved in catalysis. Therefore, protection of active site carboxyls from chemical modification must be required. 100 mM concentration of glucose (competitive inhibitor for ß-glucosidase) was used for active site carboxyl's protection. In case of carboxyl group modification of ß-glucosidase from *Trichoderma reesei* by 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline, no protection was afforded by pNPG or cellobiose, implying that no carboxyl is involved in substrate binding (Mata *et al.*, 1993). Deoxynojirimycin completely protected the essential active-site carboxyl of *Shizophyllum commune* from inactivation by EDC (Clarke, 1990).

Properties of modified ß-glucosidase

pH optimum

The pH optimum of native ß-glucosidase was between 4.6-5.3 (Fig. 2). Chemical modification shifted the optimum pH to 1.8-3.8 in both cases *i.e.*, charge neutralization (GAM) and reversal (EDAM) (Fig. 2). The pH optima of ß-glucosidases from different species of *Aspergillus* and various fungi were reviewed and found to be between 4-7 (Bennett and Klich, 1992; Godfrey, 1996). The pH optimum of various strains of *A. niger* have been worked out and found to be 3.4 (Bennett and Klich, 1992), 4 (McCleary and Harrington, 1988), 4.5-5.5 (Godfrey, 1996) and 3.5-4.5 (Busto *et al.*, 1995).

Temperature optimum

The optimum temperature of native enzyme was found to be 70 °C (Fig. 3) which was at least 5 °C higher than ß-glucosidases reported from other *Aspergillus* species (Sanyal *et al.*, 1988; Hoh *et al.*, 1993; Ximenes *et al.*, 1996). Optimum temperature remained unchanged after carboxyl group modification (GAM and EDAM). Optimum temperature for *A. niger* and *A. oryzae* was reported as 60-70 °C (Godfrey, 1996).

Kinetic properties

Effect of Mn^{++}

It was observed that native and GAM ß-glucosidase were activated by low (25mM) and inhibited by higher concentrations (100-250mM) of $MnCl_2$. While EDAM ß-glucosidase was inhibited by all concentrations (Fig. 4 and Table II). It is evident from Table II that for 25mM $MnCl_2$ increase in V_{max} was accompanied by an increase in the k_m. The specificity constant V_{max}/k_m for this concentration was low as compared to native indicating that Mn^{++} was not favourable for ß-glucosidase. High values of k_m for 25mM $MnCl_2$ showed that accessibility of pNPG to active site is very low. But V_{max} was very high. The K_m values of ß-glucosidases for pNPG from *A. roseus* (Vodjani et al., 1992), *A. niger* (Himmel *et al.*, 1993), *A. fumigatus* (Ximenes *et al.*, 1996), *A. phoenicis* (Bennett and Klich, 1992), *A. japonicus* (Sanyal *et al.*, 1988), and *A. niger* (Busto, *et al.*, 1995) were 1.33, 0.30, 0.075, 44, 2, and 3.13

mM, respectively. ß-Glucosidase from *A. Japonicus* (Sanyal *et al.*, 1988) was slightly activated whereas that from *A. nidulans* (Hoh *et al.*, 1993) was unaffected by 1 mM Mn^{++}.

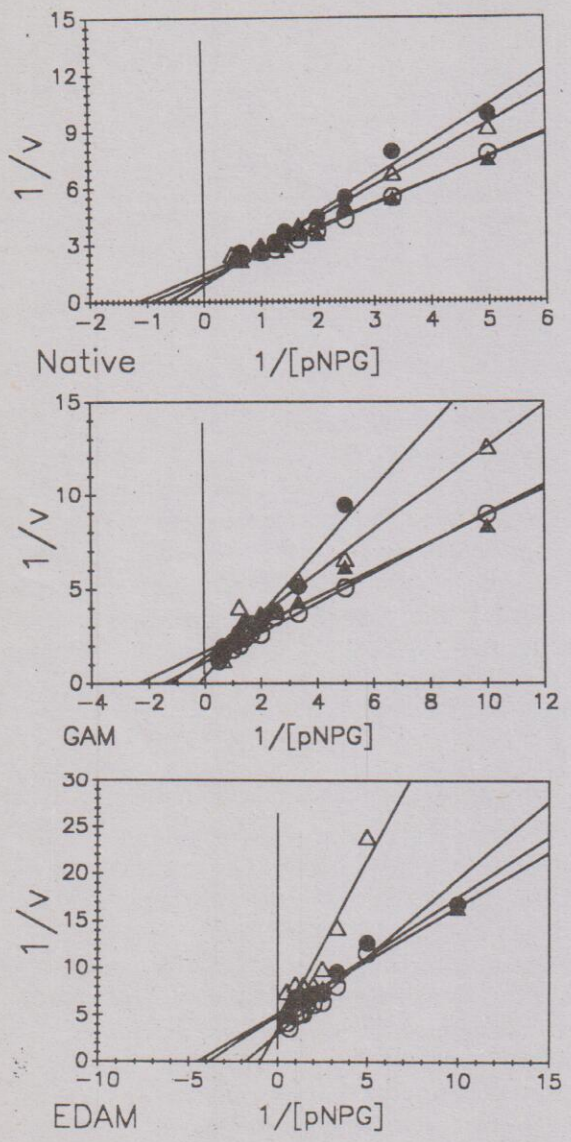

Fig. 4. Lineweaver-Burk plot showing β-glucosidase activation at low and inhibition at high Mn^{++} concentrations. Intercept on Y-axis = $1/V_{max}$ and intercept on X-axis = $1/K_m$. The Mn^{++} concentrations are: Open circle (0 mM), closed circle (25 mM), open triangle (100 mM), closed triangle (250 mM).

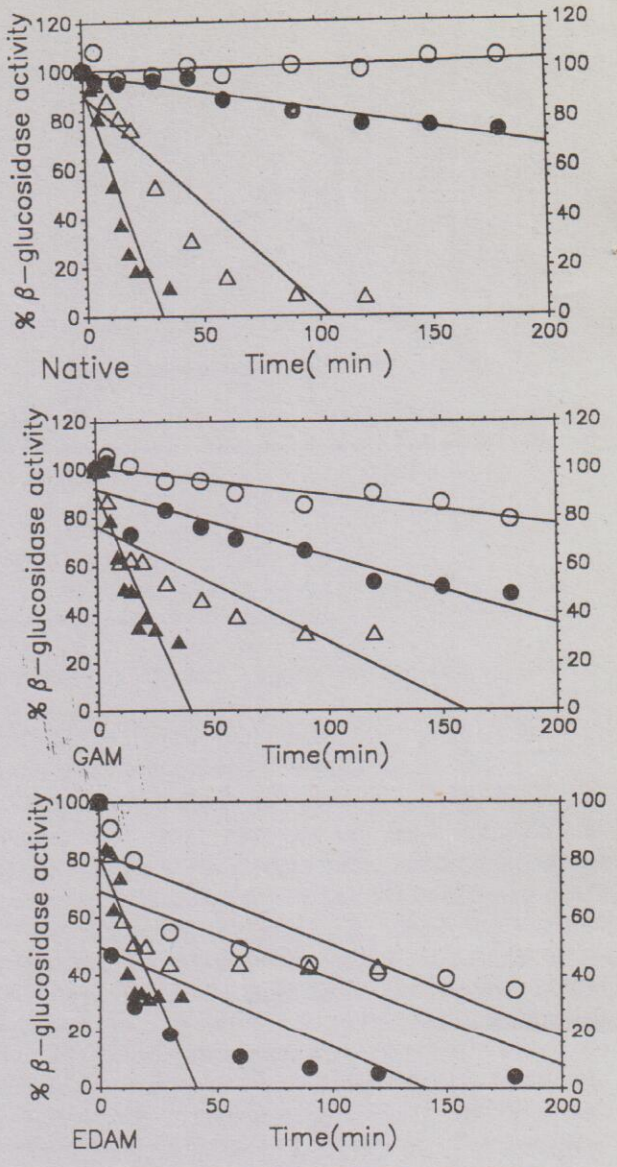

Fig. 5. Plots for thermostability of native and chemically modified β-glucosidase. Samples were incubated at 50°C (open circle), 55°C (closed circle), 60°C (open triangle), 64°C (closed triangle) and 67°C (open square) in 25 mM sodium acetate buffer, pH 5.5 and the aliquots withdrawn at different time intervals were cooled in ice before assaying for residual enzyme activity at 40°C.

The Michaelis-Menten kinetics of GAM and EDAM showed that the neutralization of negative charge increased the affinity of pNPG for the active-site and was increased further in case of charge reversal (Table II). On the other hand V_{max} of EDAM was reduced, while that of GAM was unchanged as compared with native enzyme. Specificity constants

Table II.- Effect of Mn^{2+} on the kinetic properties of native and chemically modified beta-glucosidases.

$MnCl_2$ kinetic parameters		beta-glucosidase		
		Native	GAM	EDAM
0 mM	V_{max} (Imol min^{-1} ml^{-1})	0.813	0.874	0.340
	k_m (mM)	0.874	0.714	0.560
	V_{max}/K_m	0.930	1.220	0.610
25 mM	V_{max} (Imol min^{-1} ml^{-1})	1.164	2.695	0.200
	k_m (mM)	2.695	5.000	0.260
	V_{max}/K_m	0.430	0.540	0.770
100 mM	V_{max} (Imol min^{-1} ml^{-1})	0.912	0.722	0.290
	k_m (mM)	0.721	0.833	1.000
	V_{max}/K_m	1.260	0.867	0.290
250 mM	V_{max} (Imol min^{-1} ml^{-1})	0.684	0.590	0.190
	k_m (mM)	0.590	0.426	0.220
	V_{max}/K_m	1.160	1.380	0.860

(V_{max}/K_m) reveal that overall GAM was found to be more and EDAM less efficient in hydrolyzing the substrate as compared with the native enzyme (Table II). In case of ß-glucosidase from *Schizophyllum commune* (Clarke, 1990) the k_{cat} was reduced and K_m was increased when the enzyme was modified with ethylenimine. The same trend was found while the determination of pH and temperature optima. Hence all the three types (native, GAM and EDAM) of ß-glucosidase have the same strength (6.5 U/ml), therefore, the results are comparable. GAM was highly active as compared to native (Figs. 2, 3).

Thermostability

Thermostability of chemically modified ß-glucosidase was determined at various temperatures and found that the stability in both cases GAM and EDAM was decreased as compared to native (Fig. 5). The neutralization of excessive negative or positive charges on the surface of enzymes by chemical modification have been shown to increase the thermostability of horseradish peroxidase (Miland *et al.*, 1996) and a-amylase (Urabe *et al.*, 1973), respectively.

ACKNOWLEDGEMENT

Work discussed is the part of Ph.D of Mr. M. Hamid Rasheed. The technical assistance of G. A. Waseer is appreciable. Special thanks are due to Mr. Tanvir Ahmed and Ahsan M. Shemsi for their help in computer work.

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(Received 17 June 1997)