
Effect of pH, temperature and alcohols on the stability of glycosylated and deglycosylated stem bromelain

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The biological significance of the carbohydrate moiety of a glycoprotein has been a matter of much speculation. In the present work, we have chosen stem bromelain from *Ananas comosus* as a model to investigate the role of glycosylation of proteins. Stem bromelain is a thiol protease which contains a single hetero-oligosaccharide unit per molecule. Here, the deglycosylated form of the enzyme was obtained by periodate oxidation. The differences in the glycosylated and deglycosylated forms of the glycoprotein have been studied at various temperatures and pH values, using probes such as loss of enzyme activity and by the changes in fluorescence and circular dichroism spectra. Deglycosylated bromelain showed decreased enzyme activity and perturbed fluorescence and circular dichroism spectra. In addition to this, a comparative study of their activities in different organic solvents showed a marked decrease in case of deglycosylated form of the enzyme. It is thus concluded that glycosylation contributes towards the functional stability of glycoenzymes.

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1. Introduction

Glycosylation is one of the most important post-translational modifications in newly synthesized proteins (Lis and Sharon 1993; Varki 1993). Glycosylation occurs without exception, in the integral membrane proteins of higher organisms and is quite common among secretory proteins. For instance, in blood serum, almost all proteins are glycosylated. Two different kinds of carbohydrate transfer are observed: O-glycosylation at hydroxyl groups of serine and threonine residues and N-glycosylation at asparagine (Asn) residues. N-glycosylation is a co-translational event where prefabricated oligosaccharide units are transferred from the lipid carrier dolichol diphosphate to Asn residues as soon as the growing polypeptide chain enters the lumen of the endoplasmic reticulum (Kobata 1992; Woods *et al* 1994). This suggests that N-glycosylation precedes the folding and maturation of the nascent glycoprotein to its native state.

In view of the rapidly growing store of information on the composition, structure, synthesis and biological signi-

ficance of glycoproteins, study of glycoproteins to understand the molecular consequence of glycosylation has become important. Various opinions have come up regarding the role of carbohydrate moiety in glycoproteins. On interaction with other macromolecules some of the non-reducing sugar residues are well known as specific determinants, as in blood-group substances, phytohemagglutinin receptor site of erythrocytes, etc. It has also been speculated that in secreted mammalian glycoproteins, glycosylation is somehow involved in the mechanism of secretion. Carbohydrate chains located at the surface of individual folding domains can play an important role during the late stages in the maturation of oligomeric proteins.

In the present study, to elucidate the role of glycosylation in case of small single-chain proteins, we used stem bromelain (EC 3.4.22.32) from *Ananas comosus* as a model system. It is a pineapple thiol protease; a glycoenzyme with a molecular weight of 23,800 (Vanhoof and Cooreman 1997) that includes a single asparagine-linked hetero-

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oligosaccharide unit per molecule (Yasuda *et al* 1970). Deglycosylated stem bromelain was obtained by means of periodate oxidation. Studies on the stability of the two forms towards guanidine hydrochloride-induced denaturation have been reported earlier (Rasheedi *et al* 2003). In the present communication, enzyme activities of the two forms of the glycoprotein have been compared over a wide range of temperature and pH as well as in the presence of organic solvents. Also, the stability profile of glycosylated and deglycosylated forms of bromelain was studied by observing the changes in fluorescence and circular dichroism spectra.

2. Materials and methods

2.1 Materials

Stem bromelain (EC 3.4.22.32) from *A. comosus*, was obtained from Sigma Chemical Company, St. Louis, MO, USA. Sodium periodate and casein were purchased from SRL, Mumbai, India. Methanol, ethanol and n-propanol were from E Merck India Limited, Mumbai, India. Ethylene glycol was obtained from SD Fine Chemicals Ltd., India. Trichloroacetic acid was from Qualigens Fine Chemicals, Mumbai, India. All other reagents used were of analytical grade.

2.2 Methods

2.2a Purification of enzyme: The purity of enzyme was checked by passing the protein through a pre-packed Seralose-6B (74 × 1.15 cm) column equilibrated with 0.02 M citrate-phosphate buffer, pH 6.0 and was found to elute as a single peak.

2.2b Preparation of periodate-oxidized stem bromelain: Stem bromelain (1 mg/ml) in 0.1 M sodium phosphate buffer (pH 7.0) was prepared and treated with 10 mM sodium periodate solution in a molar ratio of 5 : 1. Reaction mixture was incubated for 15 min at room temperature in dark. The oxidation process was stopped by adding 0.25 ml ethylene glycol per ml of sample. The quenched sample was then dialyzed at room temperature overnight against the same buffer.

2.2c Protein estimation: Protein concentration was determined spectrophotometrically or alternatively by the method of Lowry *et al* (1951). Bromelain concentration was measured using a predetermined value of specific extinction coefficient $E_{1\text{ cm}, 280\text{ nm}}^{1\%} = 20.1$ (Murachi *et al* 1965). The absorbance of protein solution at 280 nm was measured on a Cecil double beam spectrophotometer, model

CE-594. Light absorption measurements, in the visible range, were performed on AIMIL photochem-8 colorimeter.

2.2d Carbohydrate estimation: The phenol-H₂SO₄ method of Dubois *et al* (1956) was employed for determining the carbohydrate content of the two preparations.

2.2e Caseinolytic activity measurement: Proteolytic activity measurements were made using 2% casein as substrate and a stock enzyme concentration of 0.1 mg/ml. Casein (0.25 ml) was treated with enzyme solution (0.3 ml) in 0.1 M sodium phosphate buffer, pH 7.0 at 37°C for 15 min, and the reaction was stopped by the addition of 5% trichloroacetic acid (TCA) (0.25 ml). The precipitated material, from each reaction mixture, was removed by centrifugation and the TCA soluble fraction assayed by the method of Lowry *et al* (1951). An appropriate blank solution was also prepared and absorbance values at 700 nm were measured with reference to the blank. Enzyme activity was expressed in terms of $\Delta O.D_{700}$ of supernatant. The absorbance values obtained could be correlated with the amount of product formed.

2.2f Effect of substrate concentration: Proteolytic activity was determined at varying substrate concentrations ranging from 0–60.5 × 10⁻² mmol at 37°C in 0.1 M sodium phosphate buffer, pH 7.0.

2.2g Effect of temperature: Enzyme was exposed to a wide range of temperatures (25°C–90°C) and enzyme activity measured at pH 7.0. Thermal stability of glycosylated and deglycosylated stem bromelain was also checked at three different values of pH: pH 3.0, 7.0 and 9.0.

2.2h Effect of pH: The activity of the two forms of enzyme was also compared at different pH values: pH 2.0, 4.0, 6.0 and 8.0. Enzyme was prepared in 0.1 M glycine HCl buffer for pH 2.0, in 0.1 M sodium acetate buffer for pH 4.0 and 6.0 and in 0.1 M sodium phosphate buffer for pH 8.0.

2.2i Effect of alcohols: Enzymatic activity of glycosylated and deglycosylated stem bromelain was measured in the presence of methanol, ethanol and n-propanol (0%–90%).

2.2j Fluorescence measurements: Fluorescence emission measurements were performed on a Hitachi spectrofluorometer model F-2000. The fluorescence spectra were measured at different values of pH for both the forms of enzyme (glycosylated and deglycosylated) at a protein concentration of 5.4 μM with a 1 cm pathlength cell. The excitation and the emission slits were set at 10 nm each.

Excitation wavelength was set at 280 nm and emission spectra taken in the range of 300–400 nm.

2.2k Circular dichroism measurements: Circular dichroism (CD) measurements were carried out with a Jasco spectropolarimeter, model J-720 equipped with a micro-computer. All the CD measurements were carried out at 30°C. Spectral band-width was 1 nm. Near UV CD spectra of both the forms of enzyme were taken at a protein concentration of 25.2 μM . The protein samples were filtered using Millipore filter (0.45 μM) prior to use.

3. Results and discussion

Deglycosylation was confirmed by estimating for carbohydrate by phenol-sulphuric acid method. Besides this, the periodate oxidized preparation of enzyme does not give any precipitation with *Cajanus cajan* or with concanavalin A (ConA) lectin. Since lectin interacts specifically with the proteins having high mannose content (Siddiqui *et al* 1995), it confirms the removal of carbohydrate moiety from the native enzyme. This also needs to be stated here that to confirm the results, each experiment was repeated at least twice.

3.1 Effect of substrate

As can be seen from table 1, V_{max} for glycosylated stem bromelain is almost twice that of deglycosylated one, indicating higher rate of hydrolysis of substrate by glycosylated preparation. However, the deglycosylated enzyme shows a marginal increase in the value of K_m . These two data, taken together, suggest that the substrate binding sites might not have changed due to glycosylation and the catalytic activity may be altered due to topological effects.

3.2 Effect of temperature

Table 2 shows the effect of temperature on the residual enzymatic activity of glycosylated and deglycosylated preparations of bromelain. Glycosylated form shows maxi-

mum activity at 30°C and then decreases by 17% in the temperature range from 40°C–60°C. At higher temperatures, there is a sharp decrease in the enzymatic activity. The deglycosylated form of the enzyme shows a similar trend with an overall less activity at all temperatures.

Figure 1 shows the enzyme activity of glycosylated stem bromelain as a function of temperature. The effect of temperature on the activity of both the enzyme preparations was examined at three different pH values. As can be seen in figure 1, the glycosylated enzyme shows a similar thermal activity profile at pH 7.0 and 9.0 with temperature optimum (T_{opt}) around 40°C at pH 7.0 and 9.0 whereas at pH 3.0, the T_{opt} decreases. On the other hand, the deglycosylated preparation is more sensitive

Table 2. Residual activity of glycosylated and deglycosylated stem bromelain.

Temperature (°C)	Residual activity ($\Delta\text{OD}_{700}/\text{ml}$)	
	Glycosylated	Deglycosylated
30	0.60 \pm 0.03	0.26 \pm 0.02
40	0.48 \pm 0.03	0.22 \pm 0.04
60	0.48 \pm 0.02	0.22 \pm 0.03
80	0.20 \pm 0.02	0.16 \pm 0.02
90	0.16 \pm 0.02	0.14 \pm 0.02

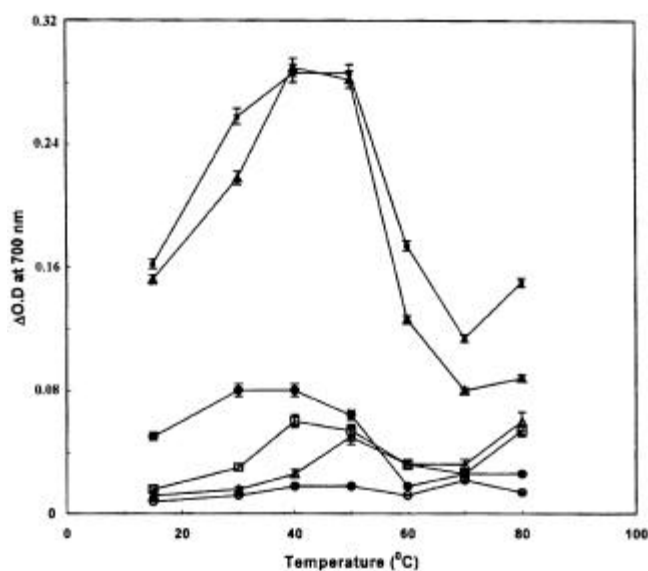


Figure 1. Effect of temperature on the stability of glycosylated and deglycosylated stem bromelain, at different pH values (in 0.1 M sodium acetate/sodium phosphate/glycine NaOH buffers) 3 (●, ○), 7 (▲, △), 9 (■, □), respectively. Enzyme (4.2 μM) was incubated at indicated pH values at different temperatures and then assayed for caseinolytic activity.

Table 1. Kinetic parameters (V_{max} and K_m) for glycosylated and deglycosylated stem bromelain.

	V_{max} ($\times 10^{-3}$ mM/min)	K_m (mM)
Glycosylated stem bromelain	1.213 \pm 0.07	0.14 \pm 0.01
Deglycosylated stem bromelain	0.653 \pm 0.05	0.17 \pm 0.01

towards pH changes both in the acidic as well as basic range. The T_{opt} of enzyme activity at pH 3.0 and 9.0 shifts towards lower temperature values as compared to that at pH 7.0.

3.3 Effect of pH

Figure 2 depicts the effect of pH on glycosylated and deglycosylated preparations. As can be seen from the figure, there is no change in the activity of the deglycosylated preparation while the glycosylated preparation shows optimal activity at pH value around 6.0. Interestingly, at pH 2.0, both the glycosylated and deglycosylated preparations show same extent of enzymatic activity while at the other extreme of pH i.e. pH 8.0, there is 3-fold difference in enzymatic activity of the two forms. These data are in support of our first data, indicating that at pH 3, the changes are much more significant for glycosylated enzyme.

Effect of pH was also monitored by fluorescence spectroscopy in pH range of 2–10. The fluorescence maxima were plotted with respect to pH (figure 3). The pH optima in both the cases lie in the range of 7–8. Both the preparations show the same extent of fluorescence intensity at extremes of pH values. At optimum pH, the deglycosylated preparation shows higher fluorescence intensity as compared to the glycosylated one. This may be because of more exposure of fluorophore of deglycosylated protein. As reported earlier, pH optima for bromelain lies

in a broad pH range, around pH 7.0 (Murachi and Neurath 1960).

3.4 Effect of alcohols

Figure 4 shows the effect of alcohols on the relative enzyme activity of the two forms of bromelain. It can be seen that 70% (v/v) methanol inhibits the glycosylated enzyme activity completely while ethanol concentration of 50% and n-propanol concentration of 30% show the same effect.

The steep decrease in the enzymatic activity in the presence of organic solvents is in the order of propanol followed by ethanol followed by methanol. Thus, it can be said that at a fixed concentration of an organic solvent, the enzymatic activity decreases with increase in the length of the hydrocarbon chain of the alcohol. Similar studies were made for deglycosylated preparation in the presence of all the above discussed alcohols and in all the cases there was no significant amount of enzymatic activity detected even at alcohol concentration as low as 30%. The mean of all the observations was taken and plotted as a single line.

3.5 CD spectra of glycosylated and deglycosylated enzyme

The effect of deglycosylation on the secondary as well as tertiary structures of stem bromelain was monitored by far UV as well as near UV CD studies, respectively. The

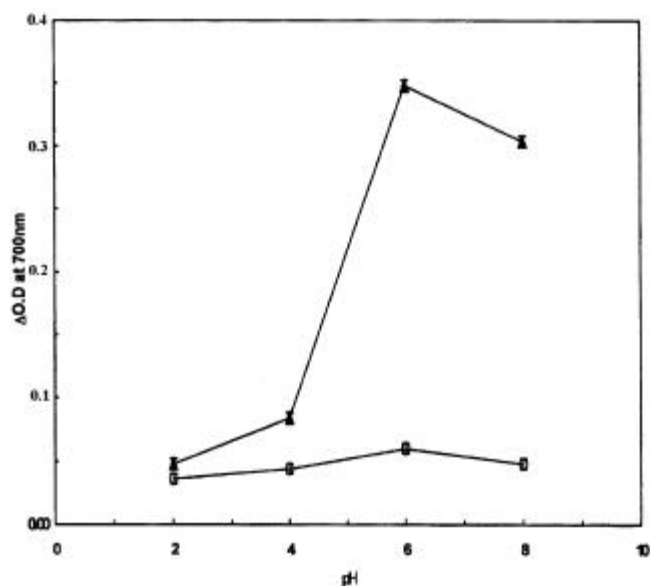


Figure 2. Effect of pH on activity of glycosylated (●) and deglycosylated (○) stem bromelain. The reaction was performed in 0.1 M glycine/acetate/phosphate buffers at the indicated pH values at 37°C.

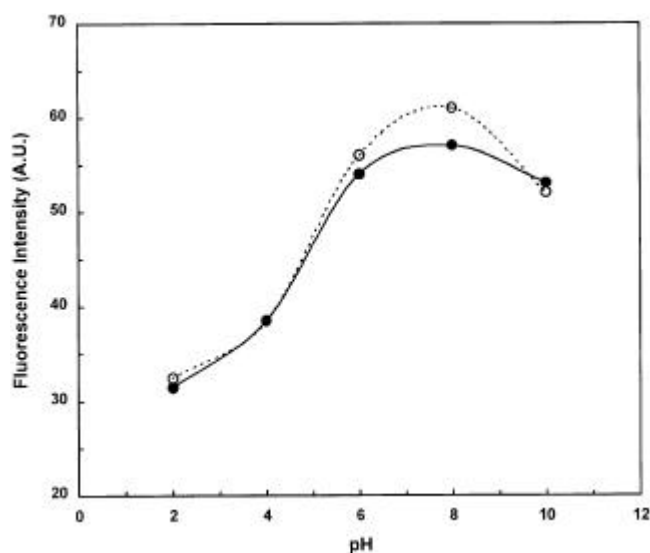


Figure 3. Fluorescence emission intensities of glycosylated (●) and deglycosylated (○) stem bromelain recorded at 340 nm as a function of indicated pH values. Enzyme concentration used was 5.4 μM.

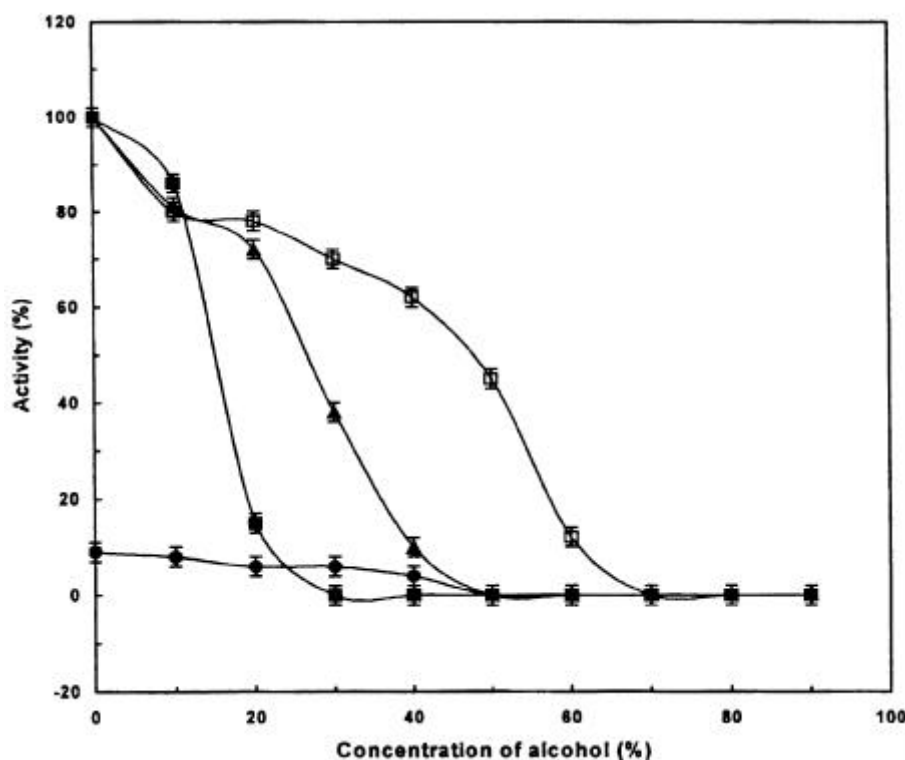


Figure 4. Effect of alcohols: methanol (□), ethanol (▲) and n-propanol (■) on activity of glycosylated and deglycosylated (●) stem bromelain at 37°C. 250 μ l of 2% casein was treated with 300 μ l of 4.2 μ M enzyme in 0–90% alcohols in 0.1 M sodium phosphate buffer, pH 7.0.

far UV CD spectrum was similar in both the cases as reported elsewhere (Reyna and Arana 1995; Haq *et al* 2002). Near UV CD spectrum for the glycosylated enzyme shows a positive peak at around 279 nm while there was a 6 nm red shift with a slight increase in the MRE value for the deglycosylated preparation (figure 5). The shift and the increase in MRE value may be due to change in the environment of the chromophoric group because of the removal of carbohydrate moiety.

The aim of this investigation was to elucidate the role of the carbohydrate moiety for the stability of stem bromelain, a single chain glycoprotein. We compared the activities and spectral properties of glycosylated and deglycosylated bromelain under various conditions by using pH, temperature and different alcohols. As stated earlier, the influence of carbohydrate depletion on structural and functional properties of a number of glycoproteins have been investigated and different effects were observed (Wormald *et al* 1991; Joao *et al* 1992; Joao and Dwek 1993; Rudd *et al* 1994; Lommerse *et al* 1995; Mer *et al* 1996). We found differences in stability of the two forms of bromelain. The temperature profiles of glycosylated

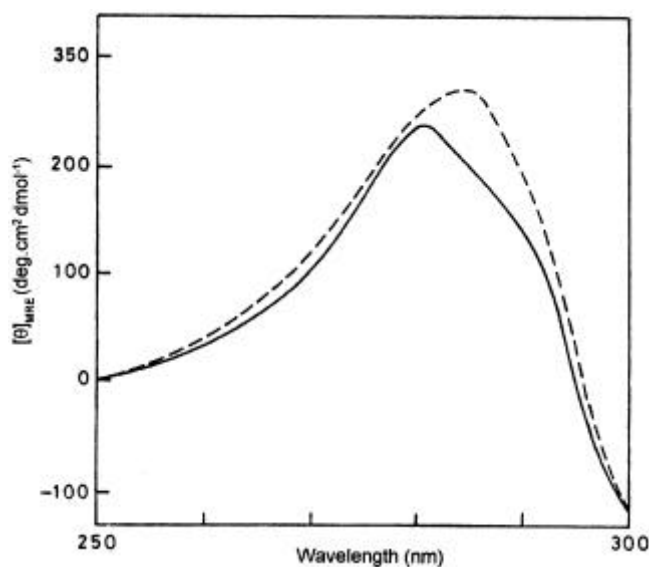


Figure 5. Near UV, CD spectra of glycosylated (—) and deglycosylated (---) stem bromelain. Concentration of enzyme taken was 25.2 μ M in 0.1 M sodium phosphate buffer, pH 7.0.

and deglycosylated enzyme clearly reflect that glycosylated form of enzyme shows greater stability at higher pH values compared to the deglycosylated enzyme.

Similar investigations carried out with external and cytoplasmic forms of invertase have indicated the significance of carbohydrate on the stability of the glycoprotein (Gascon *et al* 1968; Trimble and Maley 1977; Chu *et al* 1978, 1983, 1985; Williams *et al* 1985). In our study on stem bromelain, all the data obtained suggest a probable role of the carbohydrate moiety in stabilizing the glycosylated structure of the enzyme. The pH activity profile as well as the thermal stability studies at different pH values indicate that the glycosylated form of enzyme is comparatively more stable in the alkaline pH range. The slight differences observed in the fluorescence and near UV CD spectra of both the forms arise due to the removal of the carbohydrate component of the glycoprotein. All these data are reflective of the contribution of carbohydrate to the stability of the native protein, thus making it more resistant to different denaturants.

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