*Biochemistry (Moscow), Vol. 68, No. 10, 2003, pp. 1097-1100. Translated from Biokhimiya, Vol. 68, No. 10, 2003, pp. 1365-1369.*


# Guanidine Hydrochloride Denaturation of Glycosylated and Deglycosylated Stem Bromelain

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Received January 5, 2003
Revision received March 10, 2003

**Abstract**—Glycosylation is one of the major naturally occurring covalent modifications of proteins. We have used stem bromelain, a thiol protease with a single, N-glycosylated polypeptide chain as a model to investigate the role of glycosylation of proteins. Periodate oxidation was used to obtain the deglycosylated form of the enzyme. Denaturation studies in the presence of guanidine hydrochloride (Gn·HCl) were performed using fluorescence and circular dichroism spectroscopy. The glycosylated stem bromelain was found to be stabilized by 1.9 kcal/mol as compared to the deglycosylated one. At a given concentration of denaturant, the fraction of denatured protein was higher in the case of deglycosylated stem bromelain. In short, deglycosylated bromelain showed more susceptibility towards guanidine hydrochloride denaturation, indicating the contribution of the carbohydrate part of the glycoprotein to the stability of the enzyme.

*Key words*: stem bromelain, deglycosylation, stability, guanidine hydrochloride

Glycosylation is the most common covalent modification in newly synthesized proteins [1, 2]. It 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 residues. N-Glycosylation is a cotranslational 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 [3, 4]. This suggests that N-glycosylation precedes the folding and maturation of the nascent glycoprotein to its native state.

The function of glycoproteins in biological systems is clear but the role of the carbohydrate moiety is still debatable. It is clear that there is no unifying specific function of carbohydrates in glycoproteins. Potential functions of the glycosyl moieties include stabilization of tertiary structure, role as sorting signal for directing proteins to specific cellular organelles and tissues, protection from proteolytic degradation, and organization of macromolecules into oligomeric forms. For a number of glycoproteins, the influence of carbohydrate depletion on structural and functional properties was investigated and different effects were observed [5-10]. Carbohydrate chains located on the surface of individual folding domains can play an important role during the late stages in the maturation of oligomeric proteins. Chu et al. have shown that the carbohydrate moiety of the secreted form of invertase from *Saccharomyces cerevisiae* promotes correct refolding of the unfolded enzyme *in vitro* [11]. However, Schulke and Schmid [12] have stated that the stability of yeast invertase is not significantly influenced by glycosylation.

In the present study, we used stem bromelain (EC 3.4.22.32) from *Ananas comosus* as a model system to elucidate the role of N-glycosylation towards the stability of small single-chain proteins. It exists as a single polypeptide chain of 212 residues, with a molecular weight of 23.8 kD [13, 14]. Stem bromelain is a glycoprotein with one oligosaccharide moiety of 1.0 kD per molecule, which is covalently attached to the peptide chain at -Asn-Asn117-Ser- [15, 16]. For comparative study, a non-glycosylated form of the enzyme was obtained by periodate oxidation [17]. We measured the stability profiles of glycosylated and deglycosylated forms of bromelain over a wide range of the denaturant (guanidine hydrochloride) concentrations. The effect of Gn·HCl on the two forms of the enzyme helped to elucidate the role of the carbohydrate moiety in stabilizing the native tertiary structure of the enzyme.

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## MATERIALS AND METHODS

**Materials.** Bromelain (EC 3.4.22.32) from *A. comosus* was obtained from Sigma (USA). Sodium periodate was purchased from SLR (India). Ethylene glycol was obtained from S. D. Fine Chemicals Ltd. (India). Guanidine hydrochloride was from Qualigens Fine Chemicals (India). All other reagents used were of analytical grade.

**Purification of the enzyme.** Gel electrophoresis of stem bromelain under denaturing conditions [18] revealed a single band, indicating homogeneity of the protein preparation. The purity of the enzyme was also assessed 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 (Fig. 1). The specific activity of the enzyme was found to be 0.62 μmol/min per mg protein, as calculated from the hydrolysis of casein at pH 7.0 and 37°C, a value similar to that reported earlier by Arroyo-Reyna et al. [19].

**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 sodium periodate at a molar ratio of 5 : 1. The reaction mixture was incubated for 15 min at room temperature in the 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.

**Protein estimation.** Protein concentration was determined spectrophotometrically or alternatively by the method of Lowry et al. [20]. Bromelain concentration was measured using a predetermined value of specific extinction coefficient $\varepsilon^{1\%}_{1cm,280nm}$ = 20.1 [21]. The absorbance of protein solution at 280 nm was measured on a Cecil model CE-594 double beam spectrophotometer. Light absorption measurements were performed in the visible range on an AIMIL Photochem-8 colorimeter.

**Carbohydrate estimation.** The phenol–$H_2SO_4$ method of Dubois et al. [22] was employed for determining the carbohydrate content of stem bromelain.

**Fluorescence measurements.** Fluorescence emission measurements were performed on a Hitachi model F2000 spectrofluorometer. Fluorescence emission spectra in the presence of varying concentrations of Gn·HCl were recorded at an enzyme concentration of 37.8 μM. The excitation and the emission slits were set at 10 nm each. Excitation wavelength was set at 280 nm and emission spectra were taken in the range of 300-400 nm.

**Circular dichroism (CD) measurements.** CD measurements were carried out with a Jasco model J720 spectropolarimeter equipped with a microcomputer. All the CD measurements were carried out at 30°C. Far UV and near UV CD spectra in the presence of varying amounts of Gn·HCl, were taken using a protein concentration of 12.6 and 37.8 μM, respectively. The protein samples were filtered using a Millipore filter (0.45 μm) prior to use. The changes in the Gibbs free energy and the midpoints of transition during unfolding were calculated according to Tayyab et al. [23].

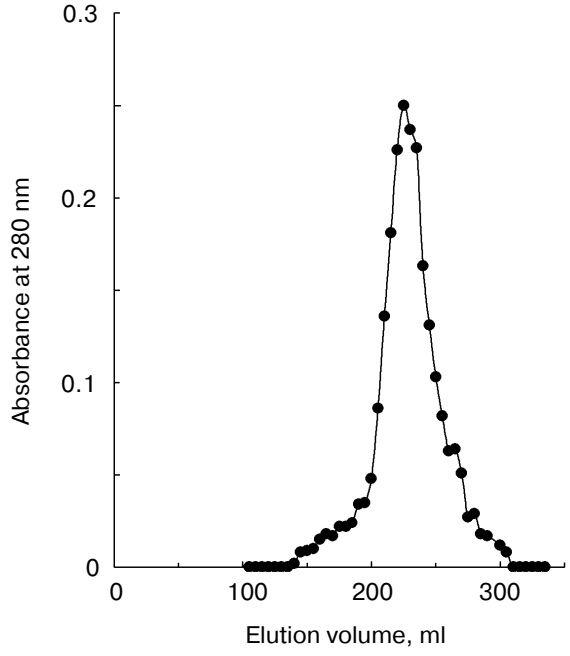


**Fig. 1.** Elution profile of stem bromelain on Seralose-6B column (74 × 1.15 cm) equilibrated with 0.02 M citrate-phosphate buffer, pH 6.0.

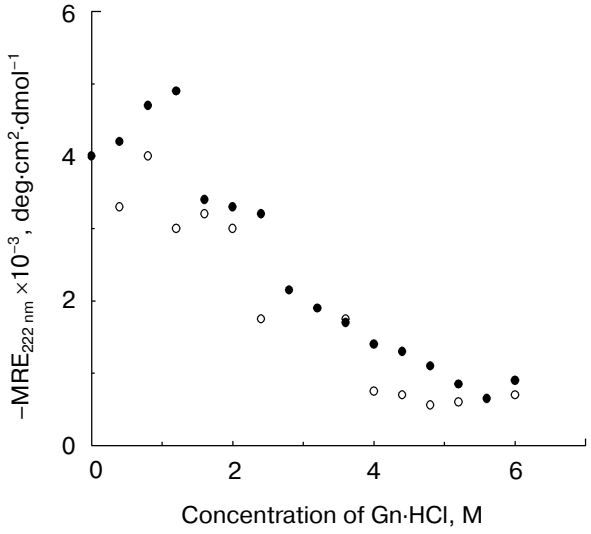


**Fig. 2.** Guanidine hydrochloride induced unfolding of glycosylated (•) and deglycosylated (○) stem bromelain. The unfolding transition was monitored by mean residue ellipticity at 222 nm.

Midpoint of transition ($C_m$) and Gibbs free energy change ($\Delta G$) for glycosylated and deglycosylated stem bromelain

| Analytical method | $C_m$, M | | $\Delta G$, kcal/mol | |
|---|---|---|---|---|
| | glycosylated | deglycosylated | glycosylated | deglycosylated |
| Far UV CD (222 nm) | 2.86 ± 0.03 | 2.28 ± 0.02 | −2.8 ± 0.07 | −0.9 ± 0.06 |
| Near UV CD (279 nm) | 3.52 ± 0.02 | 3.49 ± 0.01 | −1.8 ± 0.04 | −2.0 ± 0.05 |
| Fluorescence (340 nm) | 2.84 ± 0.04 | N.D.* | −3.1 ± 0.09 | N.D. |

* N. D., not determined.

## RESULTS AND DISCUSSION

Unlike the native glycosylated form of the enzyme, the periodate oxidized preparation of enzyme does not give any precipitation with *Cajanus cajan* and ConA lectins. Since both lectins interact specifically with proteins having high mannose content [24], the removal of carbohydrate moiety from the native enzyme was confirmed. Deglycosylation was reconfirmed by the phenol–sulfuric acid method.

The unfolding of glycosylated and deglycosylated preparations of bromelain was studied by far UV and near UV circular dichroism and fluorescence spectroscopy in the presence of varying concentrations of Gn·HCl. The far UV mean residue ellipticity (MRE) value as a function of denaturant concentration was monitored at 222 nm. For both the glycosylated and deglycosylated forms of the protein, there is a gradual decrease in the MRE values with increasing concentration of Gn·HCl (Fig. 2). As can be seen from the table, the midpoints of transition for glycosylated and deglycosylated forms were found to be 2.86 and 2.28 M, respectively. The change in free energy for glycosylated and deglycosylated preparations was also calculated and the glycosylated preparation was found to be stabilized by 1.9 kcal/mol compared to the deglycosylated one. Similar observations were made in the near UV region by monitoring ellipticity at 279 nm. Figure 3 shows the effect of the denaturant on the secondary structure of the protein. There is a loss in the secondary structure content with increasing concentration of Gn·HCl. At every concentration of Gn·HCl, the MRE value for glycosylated preparation is higher than that for deglycosylated one. Hence, we infer that the glycosylated form is more resistant to denaturation. Both the near UV as well as far UV CD data show that the carbohydrate-free protein loses its tertiary as well as secondary structures at lower concentrations of the denaturant. In both cases, the fraction of protein denatured at a given concentration of denaturant is higher for the deglycosylated preparation. Similar

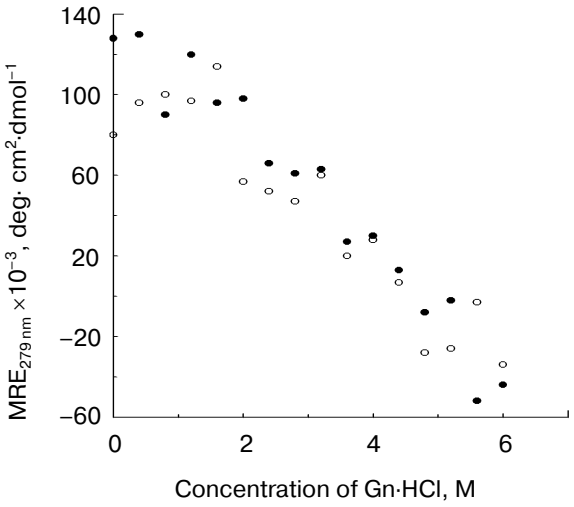


**Fig. 3.** Guanidine hydrochloride induced unfolding of glycosylated (●) and deglycosylated (○) stem bromelain. The unfolding transition was monitored by mean residue ellipticity at 279 nm.

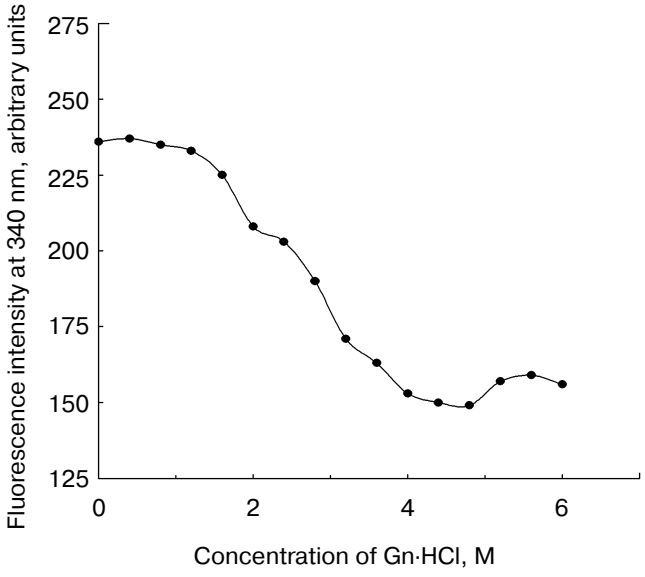


**Fig. 4.** Guanidine hydrochloride induced unfolding of glycosylated stem bromelain. The unfolding transition was monitored by fluorescence intensity at 340 nm.

investigations carried out with external and cytoplasmic forms of invertase have indicated the significance of carbohydrate for the stability of the glycoprotein [11, 25-29]. The unfolding of glycosylated bromelain was also monitored by fluorescence spectroscopy at 340 nm (Fig. 4) at increasing Gn·HCl concentration. A decrease in fluorescence intensity by 40% is observed in the completely denatured enzyme in the presence of 6 M Gn·HCl, and the emission maximum is shifted from 350 to 357 nm. Change in free energy was found to be −3.1 kcal/mol by this technique, which is comparable to that obtained from the far UV CD studies (table).

The data obtained in the present study on stem bromelain suggest a probable role of the carbohydrate moiety in stabilizing the glycosylated structure of the enzyme.

Financial assistance in the form of a minor research project (ref. No. Acad/D-1109) and other facilities provided by Aligarh Muslim University are gratefully acknowledged. Thanks also go to FIST-Department of Science and Technology, India, for their financial support. S. Rasheedi is a recipient of a fellowship sponsored by the Department of Biotechnology, Government of India. S. K. Haq acknowledges the Council of Scientific and Industrial Research, New Delhi for financial assistance in the form of a Junior Research Fellowship.

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