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Glycosylation of Purified Buffalo Heart Galectin-1 Plays Crucial Role in Maintaining Its Structural and Functional Integrity

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Abstract—A buffalo heart galectin-1 purified by gel filtration chromatography revealed the presence of 3.55% carbohydrate content, thus it is the first mammalian heart galectin found to be glycosylated in nature and emphasizes the need to perform deglycosylation studies. Physicochemical comparative analysis between the properties of the native and deglycosylated proteins was carried out to understand the significance of glycosylation. The deglycosylated protein exhibited lesser thermal and pH stability compared to the native galectin. When exposed to thiol blocking reagents, denaturants, and detergents, remarkable differences were observed in the properties of the native and deglycosylated protein. Compared to the native glycosylated protein, the deglycosylated galectin showed enhanced fluorescence quenching when exposed to various agents. CD and FTIR analysis showed that deglycosylation of the purified galectin and its exposure to different chemicals resulted in significant deviations from regular secondary structure of the protein, thus emphasizing the significance of glycosylation for maintaining the active conformation of the protein. The remarkable differences observed in the properties of the native and deglycosylated galectin add an important dimension to the significance of protein glycosylation and its associated biological and clinical relevance.

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Galectin-1 (Gal-1) is the most abundant member of the galectin family characterized by affinity for β-galactosides and the presence of a conserved 134 amino acid carbohydrate recognition domain (CRD) [1]. Such is the abundance of Gal-1 that its expression has been reported from vital organs of almost all taxa of the living world, and it has been reportedly involved in a wide range of significant biological functions [2]. Gal-1 has nearly ubiquitous distribution in animal tissues including the heart of mammals, where it is localized mainly in endocardial tissue, myocardial cell constituents, connective-tissue elements, and vascular structures [3]. Gal-1 has recently been shown to be involved in various cardiovascular disorders like Chagas' disease [4], hypoxia-induced pulmonary hypertension [5], atherogenesis [6], atherosclerosis, and restenosis [7]. We therefore carried out the purification, characterization, structural analysis, and proteomic studies of buffalo heart galectin-1 (B_fHG-1) [8]. We then extended our study by performing some chemical analyses of the purified protein. While performing Dubois analysis, we found the purified B_fHG-1 to be significantly glycosylated. This finding came to us as a pleasant surprise as no glycosylated mammalian heart galectin have been reported to date, with the exception of a sponge galectin [9].

Glycosylation is an event known to be of paramount importance to cellular functioning and interactions. It has been reported to be one of the most important post-translational modifications for newly synthesized proteins [10] and exhibits great impact both on their physicochemical properties and on their biological functions [11]. Glycosylation has also been shown to act on unfolded proteins and is involved in the quality control of glycoprotein assembly in the endoplasmic reticulum [11]. Its aberrations have been found in all types of cancers, and several glycosyl epitopes function as tumor associated antigens [12]. Of particular interest is the cell surface molecule GM3, which has been found to be a ligand for Gal-8 [13]. This galectin has two CRDs [14] and is

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involved in cross-linking of its ligands. Extracellularly, it can organize cell adhesion molecules on the same cell as well as on different cells and the matrix [15], and a change in the ligands of such a cellular anchor may be very important, if not tantamount, to metastasis.

Despite such significance of galectin glycosylation, this aspect of galectin biology is still unexplored. This can be attributed to the lack of attention given to this aspect of galectin biology as compared with more attractive and rewarding avenues like genetic studies. The startling revelation of B_fHG-1 being significantly glycosylated makes it the first glycosylated galectin to have been reported from mammalian heart and prompted us to carry out deglycosylation of the purified galectin and observe the changes in its physicochemical properties post-deglycosylation, so as to understand the significance of galectin glycosylation.

MATERIALS AND METHODS

Materials. Proteins, sugars, Sephadexes G-50 and G-100, phenol–sulfuric acid, and Coomassie brilliant blue G-250 and R-250 were from Sigma (USA); microtiter plates (V-shaped, 96 wells) were from Laxbro (India). All other chemicals used were of analytical grade.

Isolation and purification of buffalo heart galectin (B_fHG-1). The buffalo heart galectin was purified essentially according to the method described in Ashraf et al. [8]. Hemagglutination activity of the galectin samples was determined with trypsinized rabbit erythrocytes by twofold serial dilution on microtiter V-shaped plates [16].

Polyacrylamide gel electrophoresis (PAGE). To test homogeneity of the purified galectin, both native and sodium dodecyl sulfate (SDS)-PAGE were performed by the method of Laemmli [17] using a slab gel apparatus manufactured by Genei Pvt Ltd (India). Routinely 12.5 and 15% acrylamide gels were run. The galectin samples to be loaded on the native PAGE were mixed with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 10% glycerol, 2.5% (v/v) 2-mercaptoethanol, and a trace of bromophenol blue as a tracking dye. Electrophoresis was carried out in Tris-glycine buffer (0.025 M Tris/0.2 M glycine) at 100 V until the tracking dye reached the bottom of the gel. The galectin samples to be loaded on the SDS-PAGE were mixed with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 2% SDS, 10% glycerol, 2.5% (v/v) 2-mercaptoethanol, and a trace of bromophenol blue as a tracking dye. Samples were then boiled for 5 min in a boiling water bath and electrophoresis was carried out in Tris-glycine buffer (0.025 M Tris/0.2 M glycine/0.02% SDS) at 100 V until the tracking dye reached the bottom of the gel. The electrophoresis gels were stained with CBB R-250 dye.

Molecular weight determination. Molecular weight of the purified galectin was determined using a Sephadex G-100 gel filtration column (2 × 60 cm) under native (without β-ME) as well as reduced (with β-ME) conditions. The purified galectin along with standard molecular weight markers was loaded on the column. The column was then eluted with PBS-B at a flow rate of 10 ml/h, 3 ml fractions were collected, and protein concentration was determined in each fraction by the method of Lowry et al. [18]. The data were analyzed according to the theoretical treatment of Andrews [19]. The linear plot between elution volume (V_e)/void volume (V_o) and logM was used for calculating molecular weight of the galectin.

The subunit molecular weight of the purified galectin was calculated by the procedure of Weber and Osborn [20] using its mobility on SDS-PAGE. The mobility of the marker proteins determined under identical conditions was plotted against the logarithm of molecular weight. The analysis of data indicated a linear relationship between logM and relative mobility (R_m), and the plot was used for calculating the molecular weight of the galectin.

Carbohydrate content determination and deglycosylation of B_fHG-1. The carbohydrate content of the purified galectin was determined using the phenol–sulfuric acid method [21], and deglycosylation of B_fHG-1 was carried out according to the method of Rasheedi et al. [22]. Protein sample (1 mg/ml) was prepared in PBS-B (75 mM sodium phosphate, pH 7.2, containing 0.15 M NaCl, 5 mM β-ME, and 0.02% (w/v) sodium azide) and treated with 10 mM sodium periodate solution in 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 0.5 ml ethylene glycol per ml of protein sample. The quenched sample was then dialyzed at room temperature overnight against PBS-B. The phenol–sulfuric acid method [21], PAS staining, and relative mobility of native glycosylated and dB_fHG-1 on SDS-PAGE were monitored to confirm the deglycosylation results.

Effect of deglycosylation on the electrophoretic pattern and molecular weight of B_fHG-1. The extent of deglycosylation on the electrophoretic pattern and molecular weight of B_fHG-1 was monitored by means of SDS-PAGE, where the migration of native B_fHG-1 was compared to that of the deglycosylated protein. The gels were stained with CBB R-250 dye, and the relative mobility (R_m) of each band was calculated.

The effect of deglycosylation on molecular weight of the purified galectin was also investigated by gel filtration chromatography. The molecular weights of native and dB_fHG-1 under native (without β-ME) as well as reduced (with β-ME) conditions were determined from its elution volume on a column containing Sephadex G-100 gel using standard molecular weight marker proteins. Fractions of 3 ml were collected, and protein concentration was determined in each fraction at 660 nm [18]. The data were analyzed according to the theoretical treatment

of Andrews [19]. The linear plot between elution volume (V_e)/void volume (V_o) and logM was used for calculating the molecular weight of B_fHG-1.

Effect of deglycosylation on Stokes radius and diffusion coefficient. Gel filtration data was used to determine Stokes radii of native and dB_fHG-1. The partition coefficient (K_{av}) value of each marker protein was calculated from the formula $K_{av} = (V_e - V_o)/(V_t - V_o)]$, where V_t stands for total bed volume. A linear plot between the square root of the negative algorithm of K_{av} and stokes radii of marker proteins was used for calculating Stokes radii of the native and deglycosylated galectins.

The diffusion coefficient (D) of native and dB_fHG-1 corresponding to the value of Stokes radii was computed using the equation $D = KT/6\pi\eta r$, where K is the Boltzmann constant ($1.386 \cdot 10^{-16}$ erg/deg), T is the absolute temperature (303 K), η is the coefficient of viscosity of the medium (0.01 P for water) and dilute aqueous salt solutions at 20°C, and r is Stokes radius.

Effect of deglycosylation on lactose binding. The binding of lactose to native and dB_fHG-1 was quantitatively studied in PBS-B by equilibrium dialysis in a dialysis bag (3 ml capacity) made from Sigma cellulose membrane. The dialysis bags containing 100 μg/ml of the galectin solutions were placed in plastic vials containing 1 ml of lactose solution in the range of 40-400 μM. After equilibrating for 24 h at 37°C, a portion was taken from the protein free compartment, and its carbohydrate content was estimated [21]. The amount of lactose bound per mole galectin was calculated according to Scatchard analysis.

Effect of deglycosylation on thermal and pH stability. The native and dB_fHG-1 (100 μg/ml each) were incubated in PBS-B at various temperatures (30-80°C) for 30 min. The samples were then cooled on wet ice, and hemagglutination activity of each sample was tested by microtiter plate assay.

The native and dB_fHG-1 (100 μg/ml each) in 50 μl of normal saline containing 5 mM β-ME were incubated with 50 ml of the following buffers for 24 h at 4°C: 0.1 M sodium acetate buffer (pH 3.5-5.5), 0.1 M sodium phosphate buffer (pH 6.5-7.5), 0.1 M Tris-HCl buffer (pH 8.5-9.5), and 0.1 M glycine-NaOH buffer (pH 10.5-11.5). Hemagglutination activity of each sample was tested by microtiter plate assay.

Effect of deglycosylation on reaction pattern of thiol blocking agents, denaturing agents, and detergents. The rates of reaction of native and dB_fHG-1 (100 μg/ml each) with 70 mM iodoacetamide, *p*-hydroxymercuribenzoate (*p*HMB), and N-ethylmaleimide (NEM) were determined at room temperature in PBS-B. After designated times, hemagglutination activity of each sample was tested by microtiter plate assay.

The effect of various denaturants on native and dB_fHG-1 (100 μg/ml) was determined by incubating protein samples with urea, guanidine HCl (GdnHCl), and thiourea in the concentration range of 0-8 M in PBS-B. Hemagglutination activity of each sample was tested by microtiter plate assay.

The effect of different detergents was monitored by incubating native and dB_fHG-1 (100 μg/ml each) with increasing concentrations (1-5 M) of SDS, Tween-20, and Triton X-100 in PBS-B at 37°C for 1 h. Hemagglutination activity of each sample was tested by microtiter plate assay.

Effect of deglycosylation on UV spectra. The requirement of reducing agent for maintenance of the native and dB_fHG-1 (100 μg/ml each) activity was further investigated by measuring the time course of effect of an oxidizing agent on native protein by adding 5 mM H_2O_2 in PBS-B in absence of β-ME. The UV spectra of native and dB_fHG-1 (100 μg/ml in PBS-B containing β-ME) and oxidized B_fHG-1 (100 μg/ml each containing 5 mM H_2O_2 in absence of β-ME) were measured on Shimadzu UV mini 1240 UV-VIS spectrophotometer in the wavelength region 220-320 nm.

Effect of deglycosylation on fluorescence spectra. Intrinsic fluorescence of native and dB_fHG-1 (100 μg/ml each) was measured at 25°C using a Hitachi F-200 spectrofluorometer (Hitachi, Japan) equipped with a DR3 recorder. The protein was selectively irradiated using an excitation wavelength of 280 nm with 10-nm band pass. Emission spectra were measured in the range of 300-400 nm. Changes in the intrinsic fluorescence of dB_fHG-1 in the presence of 5 mM H_2O_2, 8 M urea, 70 mM *p*HMB, and 5 M SDS solution were also recorded. Appropriate controls containing the oxidizing agent used for the treatment were run and correction made wherever necessary. Each spectrum was the average of five scans.

Effect of deglycosylation on structural and functional integrity of B_fHG-1. The effect of deglycosylation and that of various thiol blocking reagents, denaturants, and detergents on structural and functional integrity of the purified galectin was investigated by CD and FTIR analyses. All CD measurements were carried out on Jasco spectrophotometer model J-810 using a SEKONIC XY plotter (model SPL-4301A) with a thermostatically controlled (25°C) cell holder attached to a NESLAB model RTE-210 water bath with an accuracy of ±0.1°C. The instrument was equipped with a microcomputer and precalibrated with (+)-10-camphorsulfonic acid. The spectra were recorded with a scan speed of 100 nm/min with a response time of 1 sec. Measurements in the far UV (200-250 nm) as well as in the near UV (250-350 nm) regions were taken using 250 μg/ml and 1 mg/ml of B_fHG-1, respectively.

FTIR spectroscopy was done to see the changes in the secondary structure components (conformational changes) in B_fHG-1 in the presence of oxidizing agents. The equipment used was a NICOLET (ESP) 560 spectrophotometer (USA) equipped with transmission OMNIC ESP 5.1 software and a DTGS detector. B_fHG-1 solutions (150 μg/ml) were prepared in PBS-B. Original

Purification of B_fHG-1

Purification step	Total protein, mg*	Total activity, titer**	Specific activity, titer/mg protein	Purification fold	Yield***, %
Crude homogenate	2730	19 888	7.03	1.0	100
Ammonium sulfate precipitation	586	13 952	27.75	3.95	70
Gel filtration chromatography	0.97	12 544	12 058.8	1715.3	63

Note: Values are means of four different preparations from 100 g fresh tissue.
* Determined by the method of Lowry et al. [18].
** Titer of tested galectin is expressed as reciprocal of the highest dilution showing hemagglutination of trypsinized rabbit erythrocytes.
*** Yield of fraction (%) = (total activity of fraction × 100%)/(total activity of crude homogenate).

spectra of native and treated B_fHG-1 at 37°C were recorded with a resolution of 4 cm^{-1} and 128 scans. The changes in peak frequency and intensity were then assigned to conformational change within the protein. For both CD as well as FTIR measurements, appropriate controls containing native B_fHG-1 and the substances used for treatment were also taken. Each spectrum was the average of five scans.

RESULTS AND DISCUSSION

About 70% of total active galectin was precipitated after ammonium sulfate fractionation, resulting in 3.5-fold purification. After extensive dialysis against PBS-B, the salt-fractionated galectin was chromatographed on a Sephadex G-50 gel filtration column equilibrated with PBS-B. The results of purification from 100 g buffalo heart tissue are shown in the table. The purified galectin yield (1.09 mg) represented 66% yield with a fold purification of 1618.1. Figure 1 shows the elution profile of the purified buffalo heart galectin. The purified galectin moved as a single band SDS-PAGE (Fig. 1, gel photograph), suggesting homogeneity of the purified galectin with respect to charge and mass.

On the Sephadex G-100 gel filtration column purified galectin eluted as a monomer of 14.5 kDa under reducing conditions, whereas it eluted as a dimer of

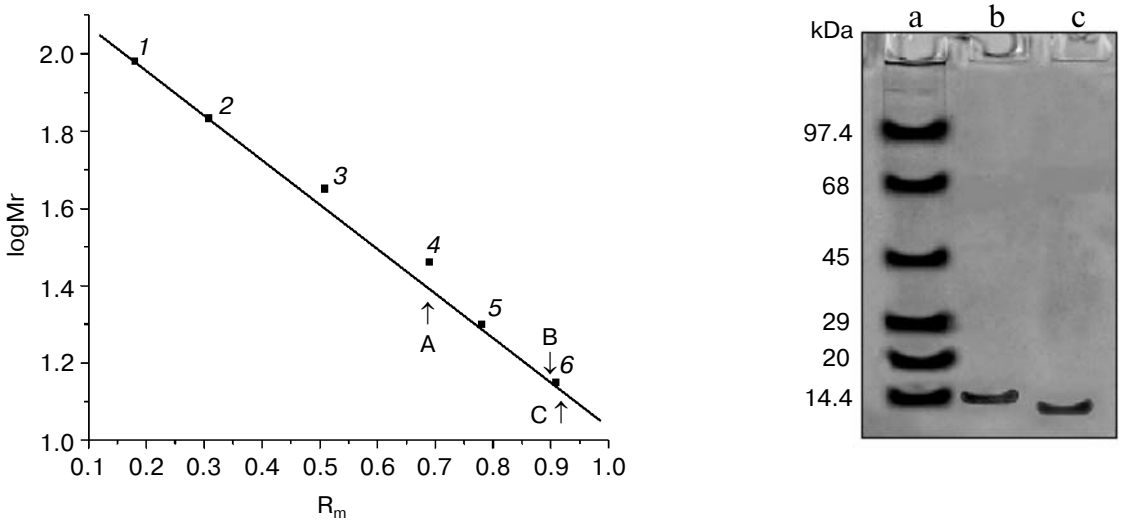

Fig. 1. Purification and molecular weight determination. Electrophoresis of the native and deglycosylated B_fHG-1 was performed on 12.5% acrylamide gel under reducing conditions. The relative mobilities (R_m) were plotted against their molecular weight using least square analysis of the data. The molecular weight markers used were: *1*) phosphorylase *b* (97.4 kDa); *2*) bovine serum albumin (68 kDa); *3*) ovalbumin (45 kDa); *4*) carbonic anhydrase (29 kDa); *5*) soybean trypsin inhibitor (20.1 kDa); *6*) lysozyme (14.4 kDa). The elution positions of dimer, monomer, and deglycosylated B_fHG-1 are indicated by arrows A, B, and C, respectively. The inset shows the electrophoretic pattern of native and deglycosylated B_fHG-1. Lanes: a) molecular weight markers (35 μg) in descending order of their molecular weight; b) 35 μg of purified native protein; c) 35 μg of deglycosylated protein. The gel was stained using Coomassie brilliant blue R250 dye.

29 kDa under non-reducing conditions (Fig. 1), thus, revealing its homo-dimeric nature. SDS-PAGE also demonstrated the molecular weight of purified galectin as 14.5 kDa (Fig. 1, gel photograph).

Although galectins are often reported to be present on cell surfaces or in extracellular matrix, they lack recognizable secretion signal sequences and do not pass through the standard endoplasmic reticulum/Golgi pathway [23]. As a result, most of the isolated galectins have characteristics typical of cytoplasmic proteins, such as having an acetylated N-terminus, free sulfhydryl groups, and lack of glycosylation [23]. Thus, galectins are usually non-glycosylated proteins. Interestingly, carbohydrate analysis revealed the presence of 3.55% sugar moiety of the total mass of B_fHG-1, suggesting it to be the first glycosylated Gal-1 to have been purified from mammalian heart tissues. The addition of large glycan structures to the protein backbone can dramatically alter the structure, and consequently the function of the polypeptide architecture to which they are attached [24]. Linked glycans can affect protein structure in two capacities: first, addition of carbohydrate to the partially folded nascent polypeptide can have an impact on or facilitate the protein-folding process; and second, the carbohydrate can stabilize the mature protein [24, 25]. Therefore, in order to study the role of carbohydrate residue on soluble B_fHG-1, we removed the sugar residues using the periodate oxidation method. This method completely removed the sugar moieties, which were confirmed by the Dubois analysis [21], PAS staining, and the difference in mobility of the native and deglycosylated protein on SDS-PAGE. This unique finding prompted us to investigate the role of glycosylation on the structure and functions of B_fHG-1 by a cooperative analysis of its activity and structural stability in both native and deglycosylated forms over a wide range of temperature and pH and in the presence of different thiol blocking reagents, denaturants, and detergents.

The deglycosylated protein eluted later than the native glycosylated protein, which corresponds to its enhanced mobility on SDS-PAGE (Fig. 1, gel photograph). On the Sephadex G-100 gel filtration column, the deglycosylated galectin eluted as a monomer of 14 kDa under reducing conditions and as a dimer of 28 kDa under non-reducing conditions. The reduced molecular weight of the deglycosylated galectin compared to native galectins (14.5 kDa under reducing conditions and 29 kDa under non-reducing conditions) can thus attributed to the effect of deglycosylation. These findings showed that deglycosylation does not disturb dimerization of the protein, hence suggesting that the sugar moieties removed in deglycosylation do not play any role in B_fHG-1 dimerization.

The gel filtration data were used to calculate the Stokes radii. Deglycosylation resulted in a decrease in Stokes radii and a corresponding increase in diffusion coefficients of the purified galectins. As a result of deglycosylation, Stokes radii of monomeric form of B_fHG-1 decreased from 17.5 to 17.2 Å and that of dimeric form of B_fHG-1 decreased from 26 to 25.8 Å. The corresponding diffusion coefficient of the monomeric form of B_fHG-1

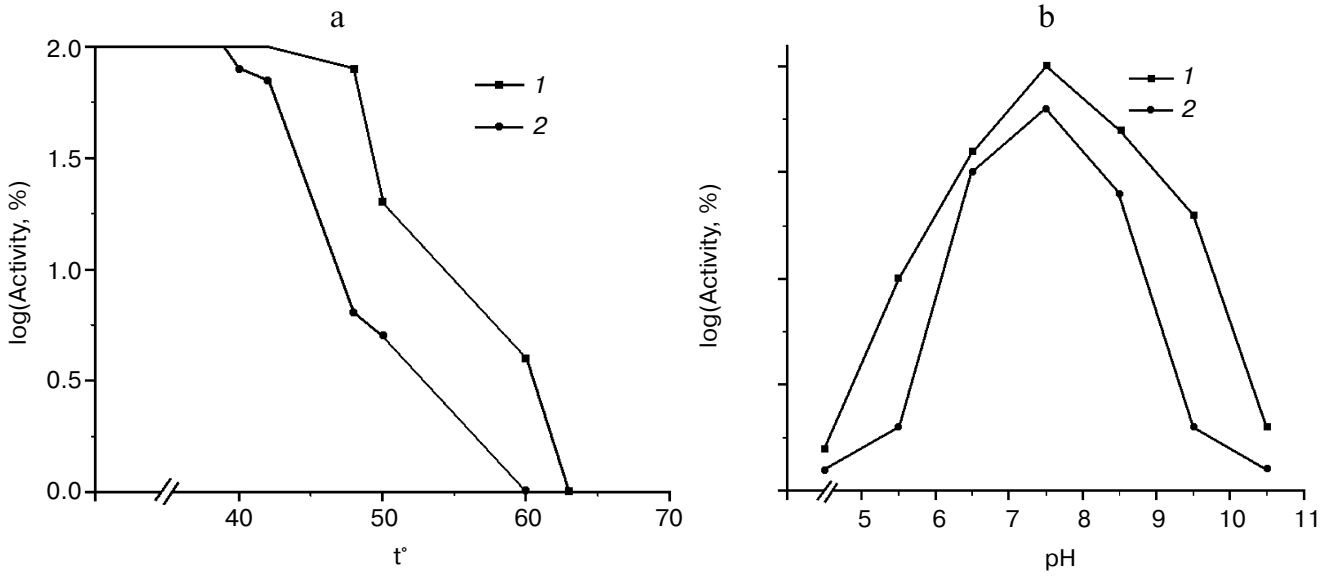

Fig. 2. Effect of deglycosylation on thermal (a) and pH (b) stability. a) The native (*1*) and dB_fHG-1 (*2*) (100 μg/ml) were incubated in PBS-B at various temperatures (30-80°C) for 30 min, samples were then cooled on ice, and hemagglutination activity of each sample was tested by microtiter plate assay. b) Native (*1*) and dB_fHG-1 (*2*) (100 μg/ml) in 50 μl of normal saline containing 5 mM β-ME were incubated with 50 ml of the following buffers: 0.1 M sodium acetate buffer (pH 3.5-5.5), 0.1 M sodium phosphate buffer (pH 6.5-7.5), 0.1 M Tris-HCl buffer (pH 8.5-9.5), and 0.1 M glycine-NaOH buffer (pH 10.5-11.5) for 24 h at 4°C. Hemagglutination activity of each sample was tested by microtiter plate assay.

increased from $12.7 \cdot 10^{-15}$ to $12.9 \cdot 10^{-15}$ cm^2/sec and that of dimeric form of B_fHG-1 increased from $8.57 \cdot 10^{-15}$ to $8.63 \cdot 10^{-15}$ cm^2/sec.

The thermal stability of B_fHG-1 (100% activity up to 42°C) was found to be higher than dB_fHG-1 (100% activity up to 39°C) (Fig. 2a). Thus, the presence of carbohydrate residues in the galectin backbone clearly indicates a role in thermal melting of proteins [26]. These results were found to be consistent with previous findings [27].

The pH stability range of B_fHG-1 (5.5-9.5) was found to be higher than the pH stability range of dB_fHG-1 (6.5-8.5) (Fig. 2b). Both native and deglycosylated forms exhibited maximum activity at pH 7.4. However, a decreased activity profile was observed for deglycosylated galectins. Hence, the presence of the carbohydrate moiety protected the purified galectins from extreme conditions of pH [22].

Deglycosylation did not result in any changes in the preferential action of thiol blocking reagents, denaturants, and detergents on the inhibition of galectin activities. Among the thiol blocking reagents, the more hydrophobic *p*HMB most readily abolished the hemagglutination activity of both B_fHG-1 and dB_fHG-1 (Fig. 3a), followed by lesser hydrophobic NEM and iodoacetamide. As per denaturants, urea most readily inhibited the hemagglutination activity of B_fHG-1 and dB_fHG-1 (Fig. 3b), followed by GdnHCl and iodoacetamide. Among the detergents, SDS most readily inhibited the hemagglutination activity of B_fHG-1 and dB_fHG-1 (Fig. 3c), followed by Tween-20 and Triton X-100. However, deglycosylated forms exhibited reduced activity profile compared to native galectins, consistent with earlier reports showing the role of glycosylation in imparting resistance to denaturant-mediated inactivation of galectins [22, 28].

The UV spectra of native and dB_fHG-1 showed maxima at 280 nm, with slightly higher absorbance for the deglycosylated forms (Fig. 4a). Oxidation of B_fHG-1 in the presence of 5 mM H_2O_2 (without β-ME) resulted in a shift of the absorption peak to 250 nm, due to oxidation of Trp residue to oxindole moiety [29]. Although the deglycosylated galectins exhibited a reduced activity profile both before and after oxidation, the effect of H_2O_2 was much more pronounced on the deglycosylated galectins. These findings suggest that glycosylation protects the galectins from oxidant-mediated inactivation.

When excited at 280 nm, the fluorescence emission maxima (332 nm) of B_fHG-1 and dB_fHG-1 (Fig. 4b) remain unchanged. However, the deglycosylated forms exhibited higher fluorescence intensity as removal of carbohydrate residues resulted in a greater exposure of the fluorophore, consistent with earlier reported studies relating to fluorescent behavior of glycosylated and non-glycosylated proteins [28]. When treated with increasing concentrations of urea, *p*HMB, and SDS, the deglycosylated forms exhibited higher fluorescence intensity and

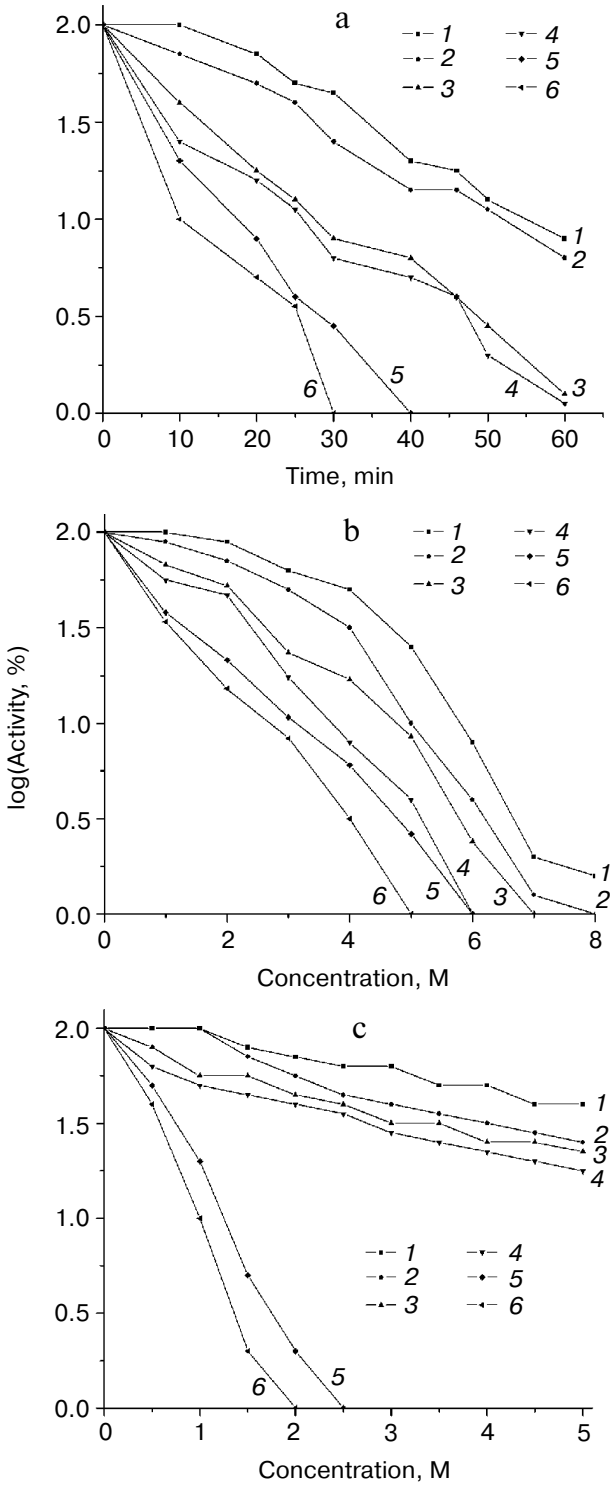

Fig. 3. Effect of deglycosylation on reaction pattern of thiol blocking reagents (a), denaturants (b), and detergents (c). a) The rates of reaction of native (*1*, *3*, *5*) and dB_fHG-1 (*2*, *4*, *6*) (100 μg/ml each) with 70 mM iodoacetamide (*1*, *2*), NEM (*3*, *4*), and *p*HMB (*5*, *6*) were determined at room temperature in PBS-B. b) The effect of various denaturants on native (*1*, *3*, *5*) and dB_fHG-1 (*2*, *4*, *6*) (100 μg/ml) was determined by incubating protein samples with thiourea (*1*, *2*), GdnHCl (*3*, *4*), and urea (*5*, *6*) in the concentration range of 0-8 M in 75 mM PBS-B. c) The effect of different detergents was monitored by incubating native (*1*, *3*, *5*) and dB_fHG-1 (*2*, *4*, *6*) (100 μg/ml each) with increasing concentrations (1-5 M) of Triton X-100 (*1*, *2*), Tween-20 (*3*, *4*), and SDS (*5*, *6*) in PBS-B at 37°C for 1 h.

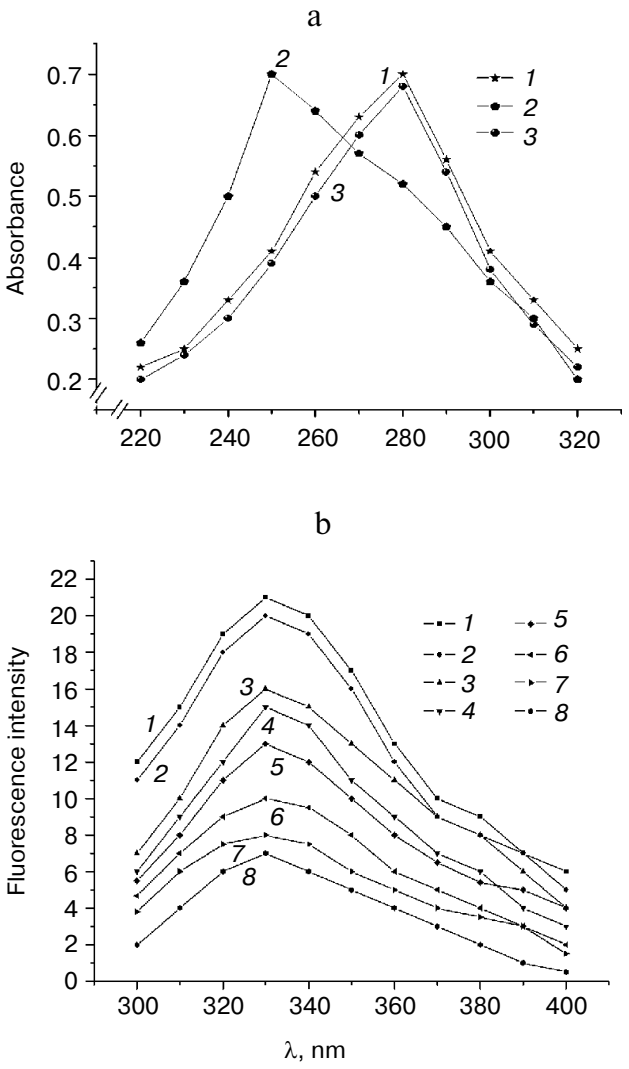

Fig. 4. Effect of deglycosylation on UV absorption (a) and intrinsic fluorescence (b) spectra. a) UV absorption spectra of native (*3*), deglycosylated (*1*), and oxidized B_fHG-1 (*2*) (100 μg/ml each) in PBS-B was measured in the wavelength range of 220-320 nm. b) Intrinsic fluorescence of native (*2*, *4*, *6*, *8*) and dB_fHG-1 (*1*, *3*, *5*, *7*) (100 μg/ml each) in 75 mM of PBS-B was measured in the wavelength range of 300-400 nm. Changes in the intrinsic fluorescence of the protein in the absence (*1*, *2*) and presence of 8 M urea (*3*, *4*), 70 mM *p*HMB (*5*, *6*), and 5 M SDS (*7*, *8*) solution were also recorded.

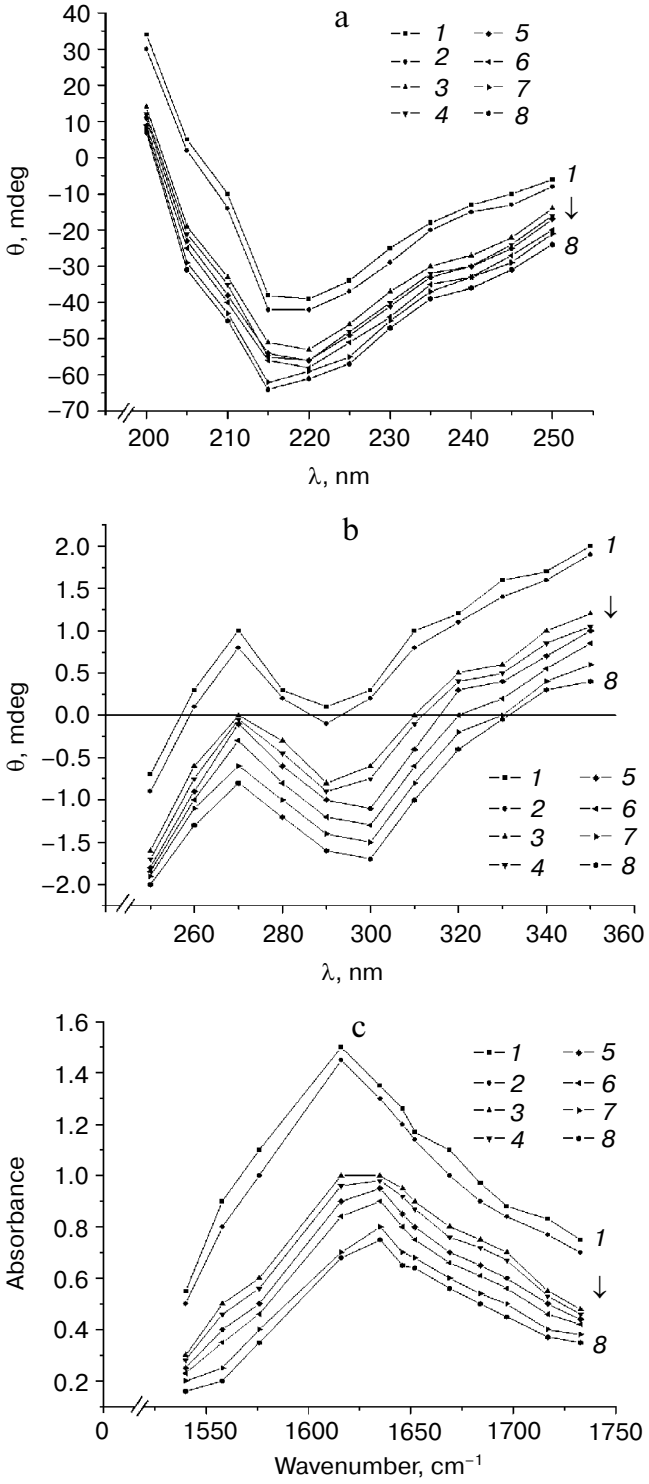

Fig. 5. Effect of deglycosylation on far UV-CD (a), near UV-CD (b), and FTIR (c) spectra. a) Far UV-CD spectra of native (*2*, *4*, *6*, *8*) and dB_fHG-1 (*1*, *3*, *5*, *7*) in the absence (*1*, *2*) and in presence of urea (*3*, *4*), *p*HMB (*5*, *6*), and SDS (*7*, *8*) (250 μg/ml each) in PBS-B was recorded between 200-250 nm using 0.1-cm pathlength. b) Near UV-CD spectra of native (*2*, *4*, *6*, *8*) and deglycosylated B_fHG-1 (*1*, *3*, *5*, *7*) in the absence (*1*, *2*) and presence of urea (*3*, *4*), *p*HMB (*5*, *6*), and SDS (*7*, *8*) (1 mg/ml each) in PBS-B was recorded between 200-350 nm using 0.1-cm pathlength. c) FTIR spectra of native (*2*, *4*, *6*, *8*) and dB_fHG-1 (*1*, *3*, *5*, *7*) in the absence (*1*, *2*) and in the presence of urea (*3*, *4*), *p*HMB (*5*, *6*), and SDS (*7*, *8*) (150 μg/ml each) in PBS-B were recorded.

greater red shift as compared to the native galectins. These findings suggest that the carbohydrate moieties due to steric hindrances play a significant role in shielding the Trp residues of native galectin from more polar microenvironment [28], thus placing emphasis on the importance of the glycan residues attached to the native protein, which stabilizes its structure and protects against the unfolding of the protein.

Glycosylation has been reported to increase the stability of a number of molecules against different denaturing agents [22, 24] and increased resistance against protease degradation [30]. Thus, there was an overall change

in the structure of both the forms and a reorientation of the tryptophan residues in the presence of different denaturants, which was more pronounced in the deglycosylated form. Based on these studies, it can be predicted that glycosylation influences the conformational dynamics of nascent polypeptides and confers their biological activity.

The far UV-CD (Fig. 5a) and near UV-CD (Fig. 5b) maxima of native and deglycosylated galectin remain unchanged, but the deglycosylated galectin exhibited decreased circular dichroism compared to the native galectins. When treated with urea, *p*HMB, and SDS, the deglycosylated galectins exhibited decreased circular dichroism compared to the glycosylated galectins, thus highlighting the significance of galectin glycosylation.

The FTIR spectra maxima of B_fHG-1 and dB_fHG-1 (1630 cm^{-1}) remain unchanged, but the deglycosylated galectins exhibited lesser optical density than the native galectins (Fig. 5c). When treated with urea, *p*HMB, and SDS, deglycosylated galectin exhibited decreased absorbance compared to glycosylated galectins, thus stressing the role of carbohydrate residues in protecting the galectins from inactivation caused due to oxidants, thiol blocking reagents, denaturant, and detergents.

The startling revelation of the purified buffalo heart galectin being glycoprotein in nature and remarkable differences observed in the physicochemical properties of native and deglycosylated galectins is nevertheless a breakthrough study in galectin biology, as no mammalian glycosylated heart galectin has been reported and the effect of deglycosylation has not been investigated so far.

The present study, therefore, holds promise as our findings strongly emphasize that glycosylation plays a vital role in maintaining the structural and functional integrity of purified buffalo heart galectin-1 (B_fHG-1). Hence, this places great emphasis on glycosylation as one of the most important posttranslational modifications for newly synthesized proteins and opens further avenues for future investigation in understanding the role of glycosylation in galectin function, and it adds an important dimension to the significance of galectin glycosylation and its associated biological and clinical relevance.

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