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ORIGINAL ARTICLE

Purification, characterization, structural analysis and protein chemistry of a buffalo heart galectin-1

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Abstract A soluble β-galactoside-binding lectin was purified by gel filtration chromatography from *Bubalus bubalis* heart. Its metal-independent nature, molecular weight of 14.5 kDa, preferential affinity for β-D-lactose, and 87–92% identity with carbohydrate recognition domain of previously reported galectin-1 confirmed its inclusion in galectin-1 subfamily. Stokes radii determination using gel filtration under reducing and non-reducing conditions revealed its homo-dimeric nature, further confirming its Gal-1 nomenclature. The purified lectin was found to be the most stable mammalian heart galectin purified till date, suggesting its preferential use in various recognition studies. Treatment of the purified lectin with oxidizing agent, thiol blocking reagents, denaturants, and detergents resulted in significant changes in UV–VIS, fluorescence, CD and FTIR spectra, which strongly emphasized the important aspect of regular secondary structure of galectins for the maintenance of their active conformation. Reduction of the activity of the purified lectin after oxidation by H_2O_2, with remarkable fluorescence quenching, may suggest potential role for galectin-1 in free radical-induced, oxidative stress-mediated cardiovascular disorders. The predictions of bioinformatics studies were found to be in accordance with the results obtained in wet lab.

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Introduction

Galectins are the family of proteins defined by at least one characteristic carbohydrate recognition domain (CRD) with affinity for β-galactosides, sharing certain conserved sequence elements, and requiring a reducing environment but not divalent cations for their binding activity (Barondes et al. 1994). They are distinguishable from all other animal lectins by their molecular weight and variable sub-cellular location. The CRD of galectins consist of 134 amino acid residues tightly bound into a sandwich of a six stranded and a five stranded anti-parallel β-sheets that form extended β-sandwich with a typical jelly-roll topology (Liao et al. 1994). Fifteen members of this gene family have now been isolated, purified, and characterized from vital organs of almost all taxa of the living world (Hasan et al. 2007).

Galectin-1 (Gal-1) is the most abundant member of the galectin family ubiquitously distributed in animal tissues, including heart of mammals (Bardosi et al. 1990), and has been reportedly involved in a wide range of significant biological functions (Hasan et al. 2007). In mammalian heart, Gal-1 is localized mainly in endocardial tissue, myocardial cell constituents, connective-tissue elements, and vascular structures (Bardosi et al. 1990). The basic physiological function assigned to Gal-1 in a healthy individual heart includes adhesion, transport and apoptosis. Although galectin activity in mammalian heart was first reported almost four decades ago (Waard et al. 1976), only few mammalian heart galectins have been thoroughly characterized till date. However, Gal-1 involvement in

various cardiovascular disorders like Chagas' disease (Giordanengo et al. 2001), hypoxia-induced pulmonary hypertension (Case et al. 2007), atherogenesis (Chellan et al. 2007), atherosclerosis and restenosis (Moiseeva et al. 2003) strongly recommends detailed exploration of mammalian heart galectins.

In the present study, we purified and extensively characterized a soluble β-galactoside-binding lectin from *Bubalus bubalis*. The thermal and pH stability range of the purified lectin was determined, and the extent of conformational changes induced by interaction of the lectin with thiol blocking reagents, denaturants, and detergents were monitored. The effect of an oxidizing agent on the lectin activity was monitored to gain information regarding its possible functional involvement in free radical-induced, oxidative stress-mediated cardiovascular disorders (Elola et al. 2005; Aragno et al. 2008). Amino acid sequencing of the purified lectin confirmed its inclusion in Gal-1 subfamily, and its structural analysis helped understand the important aspect of secondary structures. Bioinformatics studies were carried out to further analyze the results obtained in wet lab.

Materials and methods

Materials

Molecular weight markers, sugars, Sephadex G_{100} and G_{50}, acrylamide, bisacrylamide, cysteine, sodium phosphate, sodium chloride (NaCl), ethylene diamine tetra acetic acid (EDTA), β-mercaptoethanol (β-ME), lactose, sodium azide, Coommassie brilliant blue G-250, and R-250 were purchased from Sigma Chemical Co, USA. Microtiter plates (V-shaped, 96 wells) were purchased from Laxbro, India. All the other chemicals used were of analytical grade and purchased from Merck India Ltd, Qualigens Fine Chemicals, India.

Isolation and purification of buffalo heart lectin

Freshly obtained heart tissue (100 g) was removed from its pericardium, minced into small pieces, and suspended in 200 ml ice-chilled PBS 'A' [75 mM sodium phosphate buffer pH 7.2, containing 0.15 M NaCl, 10 mM EDTA, 5 mM β-ME, 200 mM lactose, and 0.02% (w/v) sodium azide]. Homogenization was carried out at 4°C in a stainless steel vessel using mixer table homogenizer. The homogenate was centrifuged in a Beckman C-24 BL cooling centrifuge (JA-20 rotor) for 30 min at 10,000 rpm at 4°C; the supernatant thus collected was centrifuged twice for 30 min at 15,000 rpm at 4°C and subjected to 30–70% ammonium sulfate fractionation. The precipitate obtained was dissolved in PBS 'B' [75 mM sodium phosphate buffer pH 7.2, containing 0.15 M NaCl, 5 mM β-ME and 0.02% (w/v) sodium azide]. After extensive dialysis against PBS 'B', the samples were centrifuged for 30 min at 15,000 rpm at 4°C to remove any aggregate formed, and the clear supernatant was subjected to Sephadex G_{50} gel filtration column packed according to the method of Laurent and Killander (1964) with some modifications. A purification column (2 × 60 cm) was prepared with sufficient amount of pre-swelled, fines removed Sephadex G_{50} gel (56 cm length). The dialyzed sample obtained after ammonium sulfate fractionation was applied at room temperature to 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. (1951). Hemagglutination activity of the lectin samples were determined with trypsinized rabbit erythrocytes by twofold serial dilution on microtiter V-shaped plates (Lis et al. 1994).

Polyacrylamide gel electrophoresis (PAGE)

To test homogeneity of the purified lectin, both native and sodium dodecyl sulfate (SDS)-PAGE were performed by the method of Laemmli (1970) using slab gel apparatus manufactured by Genei Pvt. Ltd., Bangalore, India. Routinely 12.5 and 15% acrylamide gels were run. Stock solutions of 30% acrylamide, containing 0.8% bisacrylmide, 1.0 M Tris–HCl (pH 6.8), 37 µl TEMED, and a pinch of ammonium persulfate were mixed in specific proportion to give desired percentage of acrylamide gel. The lectin 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 traces 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 till the tracking dye reached the gel bottom.

The lectin 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 and 2.5% (v/v) 2-mercaptoethanol and traces 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 till the tracking dye reached the gel bottom. The electrophoresed gels were then stained with CBB R-250 dye.

Molecular weight determination

Molecular weight of the purified lectin was determined using Sephadex G_{100} gel filtration column (2 × 60 cm)

under native (without β-ME) as well as reduced (with β-ME) conditions. The purified lectin 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. (1951). The data were analyzed according to the theoretical treatment of Andrews (1965). The linear plot between elution volume (V_e)/void volume (V_o) and log M was used and calculated the molecular weight of the lectin.

The subunit molecular weight of the purified lectin was calculated by the procedure of Weber and Osborn (1969), 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 log M and relative mobility (Rm), and the plot was used for calculating molecular weight of the lectin.

Stokes radius determination

Gel filtration data were used to determine Stokes radius of the purified lectin. 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 logarithm of K_{av} and Stokes radii of the marker proteins was used for calculating Stokes radius of the purified lectin.

Determination of diffusion coefficient

Diffusion coefficient (D) of the purified lectin was computed by the equation, $D = KT/6\pi\eta r$, where, K is the Boltzman constant (1.386×10^{-16} erg/deg), T is the absolute temperature (303 K), η stands for the coefficient of viscosity of the medium (0.0100P for water and dilute aqueous salt solutions at 20°C) and r is Stokes radius.

Carbohydrate-binding specificity

Standard saccharide solutions (100 mM each) were prepared with appropriate concentration in 0.15 M NaCl. To determine the minimum concentrations required for the inhibition of hemagglutination by these sugars, 25 µl of serially diluted test sugars were added to each well containing 25 µl of lectin samples containing four agglutinating units. Fifty microliters of trypsinized erythrocytes were added into each well, and the contents were mixed by gentle shaking and incubated for an hour at room temperature. Highest dilution of the test saccharides required for complete inhibition was noted.

Sequencing of tryptic and chymotryptic peptides: enzymatic digestion and purification

Purified lectin (2 mg in 3 ml of PBS containing 5 mM β-ME and 0.5 M NaCl) was dialyzed against 0.01 M ammonium hydrocarbonate and lyophilized. The lyophilized lectin was dissolved in 400 µl of 8 M deionized urea/0.05 M Tris–HCl pH 8.3, reduced with 0.045 M dithiothreitol (40 µl) at 50°C for 30 min, and then carboxamidomethylated with 0.1 M iodoacetamide (80 µl) under nitrogen atmosphere at room temperature for 2 h. The carboxamidomethylated galectin was diluted to 2 ml with distilled water and digested with trypsin and high specificity chymotrypsin at 37°C for 20 h using a 1:20 enzyme/substrate ratio. The tryptic and chymotryptic peptides were separated by reverse phase HPLC (Pharmacia LKB, Sweden) on a Vydac C18 column (4.7 × 250 mm) equilibrated with 0.1% trifluoroacetic acid in water (v/v). The column was eluted at a flow rate of 1.0 ml/min with 0–60% acetonitrile linear gradient in 0.1% trifluoroacetic acid (v/v) during 10 min.

Amino acid sequencing and computerized sequence comparison

The peptide fragments were then applied to a polybrene-coated glass filter and sequenced in an Applied Biosystems model 477A automatic sequencer run according to the manufacturer's instructions. Searches for similarities to the determined sequences were performed with the aid of the Swiss-Prot protein sequence data bank and ExPASy tools. The peptides were then aligned by their similarities.

Effect of divalent metal cations

To examine the effect of divalent metal cations on the activity of the lectin, demetallization of purified lectin (100 µg/ml) was carried out using 0.1 M EDTA followed by remetallization of the sample with 0.1 M $CaCl_2$ and $MnCl_2$, [$Sr(CH_3COO)_2$], $MgCl_2$, and $NiCl_2$ in PBS 'B'. Hemagglutination activity of each sample was tested by using a microtiter plate assay.

Human blood group specificity

Hemagglutination ability of the purified lectin was also scanned using trypsinized as well as untrypsinized preparations of human erythrocytes (blood groups A, B, AB and O) by the twofold serial dilution method.

Determination of sulfhydryl groups

The exposed thiol (–SH) groups were determined according to the procedure of Ellman (1959). The number of free

thiol groups was calculated in 'millimoles' using cysteine as standard.

Thermal stability

The purified lectin (100 μg/ml) was incubated in PBS 'B' at various temperatures (30–80°C) for 30 min. The samples were then cooled on ice, and hemagglutination activity of each sample was tested by using a microtiter plate assay.

pH stability

For determining the pH stability, the purified lectin (100 μg/ml) in 50 μl of normal saline containing 5 mM β-ME were incubated for 24 h at 4°C 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). Hemagglutination activity of each sample was tested by using a microtiter plate assay.

Effects of thiol blocking agents

The rates of reaction of the purified lectin (100 μg/ml) with 70 mM each of iodoacetamide, iodoacetate, *p*-hydroxymercuric benzoate (*p*HMB), and *N*-ethylmaleimide (NEM) were determined at room temperature in PBS 'B'. After the designated time, the hemagglutination activity of each sample was tested by using a microtiter plate assay.

Effects of denaturing agents

The effects of the denaturants on the purified lectin (100 μg/ml) were determined by incubating the lectin samples with increasing concentrations (0–8 M) of urea, guanidine HCl (GdnHCl), and thiourea in PBS 'B' at 37°C for 1 h. Hemagglutination activity of each sample was tested by using a microtiter plate assay.

Effects of detergents

The effects of different detergents were monitored by incubating the purified lectin sample (100 μg/ml) 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 using a microtiter plate assay.

Equilibrium dialysis

The binding of lactose to the purified lectin was quantitatively studied in PBS 'B' by equilibrium dialysis in dialysis bags (3 ml capacity) made from Sigma cellulose membrane. The dialysis bags containing 100 μg/ml of the lectin solution 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 lectin-free compartment, and its carbohydrate content was estimated (Dubois et al. 1956). The amount of lactose bound per mole of galectin was calculated according to Scatchard analysis.

UV–VIS spectroscopy

The requirement of a reducing agent for maintenance of lectin activity was further investigated by measuring the time-course effect of an oxidizing agent on native lectin by adding 5 mM H_2O_2 in PBS 'B' in the absence of β-ME. The UV spectra of native (100 μg/ml in PBS 'B' containing β-ME) and oxidized lectin (100 μg/ml in PBS 'B' containing 5 mM H_2O_2 in the absence of β-ME) were measured with an UV-1700 Pharmaspec UV–VIS double beam spectrophotometer (Shimadzu Corp., Kyoto, Japan) in the wavelength range of 220–320 nm.

Fluorescence spectroscopy

Intrinsic fluorescence of the purified lectin (100 μg/ml) was measured at 25°C in a Hitachi F-200 spectrofluorometer (Hitachi, Tokyo, Japan) equipped with a DR3 recorder. The lectin 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 fluorescence spectra in the presence of 0.1 M lactose, 5 mM H_2O_2, 8 M urea, 70 mM *p*HMB, and 5 M SDS solutions were also recorded. Appropriate controls containing the substances used for treatment were also evaluated. Each spectrum was the average of five scans.

Circular dichroism (CD) spectroscopy

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 ±0.10°C. The instrument was equipped with a microcomputer and pre-calibrated with (+)-10-camphorsulfonic acid. CD measurements were taken in the far UV region (200–250 nm) as well as the near UV region (250–350 nm) using purified lectin concentration of 250 μg/ml and 1 mg/ml, respectively. Spectra of the native lectin were recorded with a scan speed of 100 nm/min with a response time of 1 s. Changes in the CD spectra in the presence of 0.1 M lactose, 5 mM H_2O_2, 8 M urea, 70 mM *p*HMB, and 5 M SDS solutions were also recorded. Appropriate controls containing the

substances used for treatment were also evaluated. Each spectrum was the average of five scans.

Fourier transform infrared (FTIR) spectroscopy

The FTIR measurements were carried out on NICOLET (ESP) 560 spectrophotometer (USA) equipped with a transmission OMNIC ESP 5.1 software and a DTGS detector. Spectra of the native galectin (150 μg/ml in PBS 'B') were recorded at 37°C with a resolution of 4 cm^{-1} and 128 scans. Changes in the FTIR spectra in the presence of 0.1 M lactose, 5 mM H_2O_2, 8 M urea, 70 mM *p*HMB, and 5 M SDS solutions were also recorded. The changes in the peak frequency and intensity were then assigned to the conformational change within the lectin. Appropriate controls containing the substances used for treatment were also evaluated. Each spectrum was the average of five scans.

Bioinformatics studies

The amino acid sequence of the purified lectin was formatted using DNA tools (http://biology.semo.edu/cgi-bin/dnatools.pl). BLAST-P (http://www.ncbi.nlm.nih.gov/BLAST) and CLUSTALW (http://www.ebi.ac.uk/clustalw) tools were used to further corroborate the amino acid sequence. The Peptide cutter program of ExPASy tools was used to predict the digestion pattern of the purified galectin by trypsin and high specificity chymotrypsin in reverse phase HPLC. The theoretical average mass and monoisotopic mass of the purified lectin were predicted using ExPASy tools and compared with the findings of wet lab. Probable secondary structure of the purified lectin was explored using the 3D-JIGSAW Comparative Modeling Server program of ExPASy tools.

Results and discussion

About 70% of total active protein was precipitated between 30 and 70% of ammonium sulfate saturation, resulting in 3.95-fold purification. After extensive dialysis against PBS 'B', the salt-fractionated protein was chromatographed on Sephadex G_{50} gel filtration column equilibrated with the same buffer. Table 1 shows the purification of lectin from 100 g buffalo heart tissue. The purified lectin yield of 0.97 mg represented 63% recovery with a 1,715.3-fold purification. The purified lectin moved as a single band on both the native (Fig. 1, inset 'a') and SDS-PAGE (Fig. 1, inset 'b'), suggesting homogeneity of the preparation with respect to charge and mass.

On Sephadex G_{100} gel filtration column, the purified lectin eluted as a monomer of 14.5 kDa under reducing conditions, whereas it eluted as a dimer of 29 kDa under non-reducing conditions (Fig. 1), which revealed its homo-dimeric nature. SDS-PAGE also demonstrated the molecular weight of the purified lectin as 14.5 kDa (Fig. 1, inset 'b'). The Stokes radii of the monomer and the dimer lectin were found to be 17.8 and 26.3 Å, respectively. The diffusion coefficients of the monomeric and dimeric forms of the lectin corresponding to their respective Stokes radii were computed to be 12.5×10^{-15} and 8.47×10^{-15} cm^2/s, respectively. These findings further confirmed the homo-dimeric nature of the purified buffalo heart lectin.

Structural variations in the CRD topology of galectins have been reported to contribute to their extraordinary sensitivity toward corresponding sugar residues (Reuter and Gabius 1999), and formed the basis of our studies regarding their specificity toward various saccharides. Hemagglutination inhibition studies using a number of saccharides (Table 2) led to the conclusion that the purified lectin was specific for saccharides bearing non-reducing terminal D-galactose linked in a β-configuration. This was further supported by the observation that methyl-α-D-galactopyranoside and *p*-nitrophenyl-α-D-galactopyranoside are weak inhibitors (>100 mM required for inhibition) as compared to methyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-galactopyranoside (5.4 and 5.6 mM, respectively, required for inhibition). The affinity for β-galactosides satisfied the first major criterion for inclusion of the purified buffalo heart lectin in Gal-1 subfamily. The fulfillment of second major criterion (conserved 134 amino acid CRD) for inclusion of

Table 1 Purification table of B_fHG-1

Step fraction	Total protein (mg)[a]	Total activity (titer)[b]	Specific activity (titer/mg protein)	Purification fold	Yield (%)
Crude homogenate	2,730	19,888	7.03	1.0	100
Ammonium sulfate fraction	586	13,952	27.75	3.95	70
Gel filtration chromatography	0.97	12,544	12,058.8	1,715.3	63

Values are means of three different preparations from 100 g fresh buffalo heart tissue

Yield (%) of the fraction = (total activity of the fraction × 100)/total activity of crude homogenate

[a] Determined by the method of Lowry et al. (1951)

[b] The titer of the tested galectin is expressed as reciprocal of the highest dilution showing agglutination of trypsinized rabbit erythrocytes

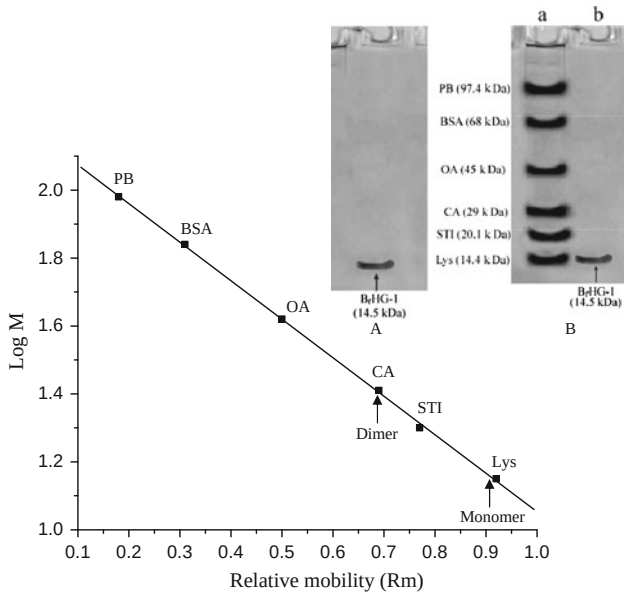

Fig. 1 Purification and molecular weight determination of B_fHG-1: *Inset A* shows native PAGE of the purified protein (35 μg). *Inset B* shows SDS-PAGE of the purified protein: *lane a* contains molecular weight markers (35 μg) in descending order of their molecular weights, and *lane b* contains purified protein (35 μg). Molecular weight of the purified protein was determined by gel filtration chromatography under native (without *β*-ME) as well as reduced (with *β*-ME) conditions. The purified protein along with standard molecular weight markers were loaded on the column calibrated with PBS 'B' and eluted with a flow rate of 10 ml/h. The linear plot between elution volume (V_e)/void volume (V_o) and log *M* was drawn using linear fit and used for calculating the molecular weight. The elution position of monomer and dimer B_fHG-1 are indicated by *arrows*. The molecular weight markers used were: *PB* phosphorylase *b* (97.4 kDa); *BSA* bovine serum albumin (68 kDa); *OA* ovalbumin (45 kDa); *CA* carbonic anhydrase (29 kDa); *STI* soyabean trypsin inhibitor (20.1 kDa); *Lys* lysozyme (14.4 kDa). The relative mobilities (Rm) of the marker proteins were plotted against their molecular weight using least square analysis of the data

the purified lectin in Gal-1 subfamily required its amino acid sequencing.

For determining the amino acid sequence the purified lectin was subjected to tryptic and chymotryptic hydrolysis. The peptide fragments thus obtained were separated by reversed phase HPLC (Fig. 2). The peaks T_1–T_9 representing pure peptides (Fig. 2a) were subjected to amino acid analysis and automated sequencing (Fig. 3), except for T6. The presence of an acetyl group was deduced from blocked N-terminus of the T6 fragment, as reported for other Gal-1 (Shahwan et al. 2004). Figure 2b represents the HPLC profile of fragments obtained by chymotryptic digestion. The sequence not established by tryptic digestion (T6) was subjected to Edman degradation, and the resulting fragments were designated C_1–C_3. Sequence alignment of the purified lectin with other Gal-1 characterized from cattle myelin sheath (Abbott et al. 1989), bovine heart, human brain, rat lung, and sheep brain (as referred in

Table 2 Effect of saccharides on hemagglutination activity of B_fHG-1

Saccharides	Minimum inhibitory concentration (mM)
β-D-Lactose	0.5
D-Galactose	3.0
Methyl-*β*-D-galactopyranoside	5.4
p-Nitrophenyl-*β*-D-galactopyranoside	5.6
D-Galactosamine	27.0
Methyl-α-D-galactopyranoside	>100
p-Nitrophenyl-α-D-galactopyranoside	>100
D-Glucose	>100

Standard solution of various saccharides (100 mM) were prepared in 0.15 M NaCl, and four agglutinating units of the B_fHG-1 was used in twofold serial dilution method to determine the minimum concentration required for inhibition of hemagglutination by various sugars

The following substances were tested and had no inhibitory activity at 100 mM: D-mannose, D-fucose, D-sucrose (Glc-α-1,2-Fuc), D-melibiose, D-glucosamine, D-cellobiose, D-fructose

Shahwan et al. 2004) showed a significant degree of identity (87–92%), in agreement with its high conservation throughout evolution. These results suggested that the 14.5-kDa Gal-1 was species specific rather than organ specific (Bladier et al. 1991). In addition, the conservation of amino acid residues that interact with carbohydrate ligands (His44, Asn46, Arg48, His52, Asp54, Asn61, Trp68, Glu71, and Arg73) allowed us to classify it as having type 1 conserved CRD (Fig. 3). The conserved 134 amino acid CRD satisfied the second major criterion for inclusion of the purified buffalo heart lectin in Gal-1 subfamily.

The amino acid sequencing allowed us to explore the molecular determinants of saccharide-binding specificity. Inhibition of the lectin activity by D-galactose and D-galactosamine indicated that a free hydroxyl or a free amino group at C2 was required for a monosaccharide to cause inhibition. Moreover, the configuration at C4 was also important, since neither D-glucose nor D-glucosamine caused inhibition. The fact that lactose was far more potent inhibitor for hemagglutination activity of the purified lectin, suggesting that carbohydrate-binding site of the galectin could have extended geometry, which was only partially occupied by the galactose molecule. It appeared that the aromatic side chain of the conserved Trp68 stacks adjacent to the galactose ring. Such van der Waal interactions between sugar and aromatic side chains are quite common in protein–carbohydrate complexes (Kasai and Hirabayashi 1996). The axial 4-OH of galactose, a main determinant of the galectin specificity, formed two key electrostatic interactions: one with the N atom of Arg48 and the other with the N atom of His44 (Shahwan et al. 2004). Both are found as invariant residues in the

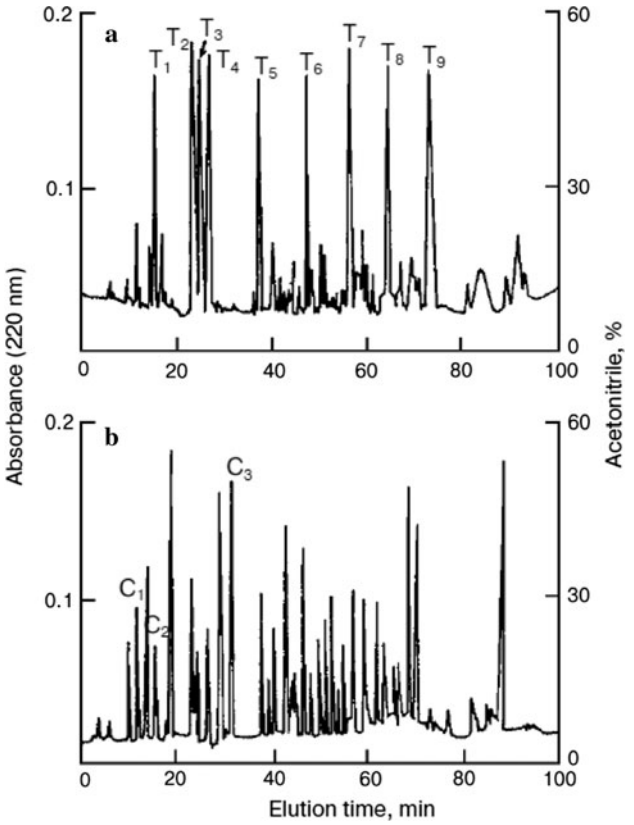

Fig. 2 HPLC profile of peptides obtained by tryptic (**a**) and chymotryptic (**b**) digestion of B_fHG-1. Peptides were separated by reversed phase HPLC on a Vydac C18 column equilibrated with 0.1% (v/v) trifluoroacetic acid in water. Elution was performed at a flow rate of 1.0 ml/min with 0–60% acetonitrile linear gradient in 0.1% (v/v) trifluoroacetic acid for 10 min, and the elution was monitored at 220 nm. *Peaks* T_1–T_9 represent tryptic peptides, and C_1–C_3 represent chymotryptic peptides, whose sequence was determined by Edman degradation and established from amino acid sequence

sequences of various galectins (Fig. 3). An ammonium group on C2 (D-galactosamine, D-glucosamine) decreased the inhibitory properties, likely because of its positive charge. Unlike human brain galectin-1, which required low concentrations of *β*-D-galactose, *p*-nitrophenyl-*β*-D-galactoside and D-fucose (1.4, 0.4, and 25 mM, respectively) for complete hemagglutination inhibition (Bladier et al. 1989), the purified buffalo heart lectin required higher concentrations of these saccharides (3.0, 5.6, and >100 mM, respectively), suggesting its own unique specificity.

Like other members of Gal-1 subfamily, the purified buffalo heart lectin was also found to be a homo-dimer composed of two subunits having one CRD each of 134 amino acids. It can also be classified as 'prototype' according to its molecular architecture as it neither had a link peptide joining two CRDs nor had the CRD joined to different types of domains as found in 'tandem repeat' and 'chimera' topologies, respectively. Besides, the purified lectin showed the characteristics of cytoplasmic proteins, such as an acetylated N-terminal amino group and the lack of hydrophobic trans-membrane segment and secretion signal peptide similar to other Gal-1 (Barondes et al. 1994). These findings confirmed that the purified lectin belongs to Gal-1 subfamily, and hence can be named as buffalo heart galectin-1 (B_fHG-1). The metal-independent nature of the purified lectin activity further confirmed its inclusion in Gal-1 family, similar to other Gal-1 (Ola et al. 2007).

The concentration of purified lectin required for hemagglutination varied markedly with the type of human erythrocytes. B_fHG-1 agglutinated native human erythrocytes with marked preference for the blood group A, with the overall order of preference being A > O > AB > B. Exposed thiol group analysis of B_fHG-1 indicated a molar ratio of 2.9, suggesting the presence of approximately 3 moles of sulfhydryl group per mole of lectin. The hemagglutination activity of the purified lectin was fully retained up to 48°C, and then gradually dropped and completely lost at 68°C. The purified lectin was found to be quite stable in the pH range of 4.5–10.5; however, it showed maximum hemagglutination activity near physiological pH 7.4. These findings suggested that B_fHG-1 may be the most stable mammalian heart galectin that have been purified till date.

The structural and functional integrity of B_fHG-1 was assayed in the presence of various thiol blocking reagents, chaotropic denaturants, and detergents in order to highlight its susceptibility to them. The need of reduced form of cysteine for the maintenance of B_fHG-1 in its active form (Shahwan et al. 2004; Ola et al. 2007) was further confirmed by its differential inhibition in the presence of various thiol blocking reagents. The lost of galectin activity might be due to the possible conformational changes that occurred during modification of cysteine residues (Liao et al. 1994). However, relatively slower rate of inactivation pointed toward the possibility of cysteine molecules being partially buried inside the lectin, and possibly not involved directly in carbohydrate binding, rather being present at a relatively distant site (Ali and Salahuddin 1989). This perhaps explained the effectiveness of *p*HMB, the most hydrophobic thiol blocking reagent, which most readily inhibited the hemagglutination activity (50% inhibition in 20 min) followed by lesser hydrophobic NEM (50% inhibition in 30 min), iodoacetate (50% inhibition in 47 min), and iodoacetamide (50% inhibition in 60 min).

As the chaotropic denaturants urea caused maximum lost in the B_fHG-1 activity (50% decrease at 3.0 M), followed by GdnHCl (50% decrease at 5.0 M) and thiourea (50% decrease at 6.0 M). These findings indicates that B_fHG-1 is also stabilized mainly by hydrogen bonding and hydrophobic interactions like other lectins (Nelson and Cox 2001), which are susceptible to denaturant induced alteration.

Fig. 3 Comparison of B_fHG-1 amino acid sequence of with other Gal-1. B_fHG-1 nomenclature of the peptides was found to be consistent with Fig. 2. B_fHG-1 sequence was compared with other Gal-1: *CMG-1* cattle myelin Gal-1; *BHG-1* bovine heart Gal-1; *SBG-1* sheep brain galectin; *HBG-1* human brain Gal-1; *RLG-1* rat lung Gal-1. Identities to B_fHG-1 sequence are shown at the *bottom right*. Amino acids conserved in all Gal-1 are designated with *asterisks*. Amino acids from other Gal-1, which differ from those present at the same position in B_fHG-1, are indicated by *dots*. Critical amino acids shown to be involved in the interaction with lactose and *N*-acetyllactosamine are designated with (*colons*) for hydrogen bonding and with (*boxes*) for hydrophobic interaction, and these residues correspond to the type 1 CRD invariant amino acids

The SDS, a well-known ionic detergent, denatured B_fHG-1 even at a very low concentration, possibly by changing the order of protein water molecules and also by intercalating into hydrophobic clefts of the lectin with its long hydrocarbon chain (Lanio et al. 2003). Tween-20 and Triton X-100 being neutral detergents showed less inhibition on lectin activity for they do not bind to galectins in detectable amount, and were found not very efficient in breaking non-covalent bonds, which are responsible for the quaternary structure of multimeric proteins (Helenius and Simons 1972).

The B_fHG-1 readily reacted with lactose according to the following reaction:

$$B_f\text{HG-1} + n(\text{Lac}) \rightarrow B_f\text{HG-1}(\text{Lac})n \quad (1)$$

$$K_a \rightarrow \frac{[B_f\text{HG-1 Lac}n]}{[B_f\text{HG-1}]\,[\text{Lac}]n} \quad (2)$$

$$\upsilon \rightarrow \frac{nK_a[\text{Lac}]}{1 + K_a[\text{Lac}]} \quad (3)$$

where n is the number of carbohydrate-binding sites in the B_fHG-1 dimer, 'Lac' designates lactose, 'υ' stands for the amount of lactose bound per mole of galectin, and K_a is the association constant for interaction of Lac with B_fHG-1.

Equation 3 can be rearranged as follows:

$$\frac{\upsilon}{[\text{Lac}]} \rightarrow -K_a\upsilon + nK_a \quad (4)$$

'υ' was determined in triplicate and plotted according to Eq. 4.

The linear Scatchard plot of equilibrium dialysis data of B_fHG-1 in PBS 'B' with lactose at 37°C was drawn. The slope of the plot gave a binding constant value, $K_a = 7.08 \times 10^3\ M^{-1}$. The number of binding sites was found to be 2.1 per lectin molecule.

Cerra et al. (1985) reported that a reducing agent is necessary for initial purification of galectins, but is not required for their continued activity. Stability studies also demonstrated the role of lactose in maintaining active conformation of the purified protein even in the absence of a reducing agent, possibly by preventing its oxidative inactivation due to formation of intra-molecular disulfide linkages (Cho and Cummings 1995). Since galectins remain in active conformation in the presence of reducing agents, we speculate that they might contain a susceptible oxidizable residue whose integrity is crucial for their activity. B_fHG-1 showed a UV spectrum maximum at 280 nm, corresponding to the presence of a single Trp residue and a large number of other aromatic residues.

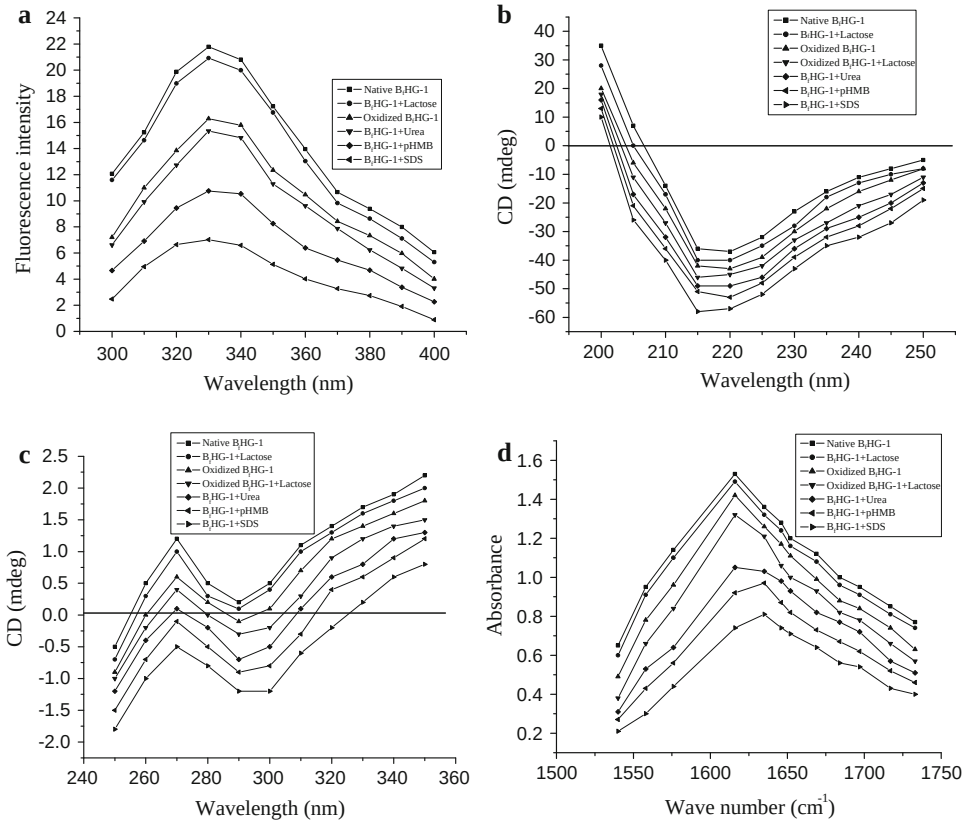

Fig. 4 **a** Fluorescence spectra. Intrinsic fluorescence of B_fHG-1 (100 µg/ml) in of PBS 'B' was measured in the wavelength range of 300–400 nm. **b** Far UV-CD spectra. Far UV-CD spectrum of B_fHG-1 (250 µg/ml) was recorded between 200 and 250 nm using 0.1-cm path length. **c** Near UV-CD spectra. Near UV-CD spectrum of B_fHG-1 (1 mg/ml) was recorded between 200 and 350 nm using 0.1-cm path length. **d** FTIR spectra. FTIR spectrum of B_fHG-1 (150 µg/ml) at 37°C was recorded with a resolution of 4 cm^{-1} and 128 scans. Changes in the fluorescence, far UV-CD, near UV-CD and FTIR spectra in the presence of lactose, H_2O_2, urea, and SDS were also recorded

However, oxidation of B_fHG-1 in the presence of 5 mM H_2O_2 (without β-ME) resulted in the shift of the absorption peak to 250 nm suggesting oxidation of Trp residue to an oxindole moiety, which absorbs maximally at 250 nm (Levi and Teichberg 1981). This result possibly suggested that the reducing agents not only prevent oxidation of Cys residues in galectins but also protect Trp from oxidation.

When excited at 280 nm, B_fHG-1 exhibited maximum fluorescence emission at 332 nm (Fig. 4a), typical of Trp residue in a hydrophobic environment. The fluorescence profile of the oxidized lectin showed a remarkable quenching in the fluorescence intensity accompanied with a blue shift from 320 to 342 nm. Oxidized lectin also exhibited a decreased activity, thus implicating intramolecular disulfide bonding as the basis of active conformation of B_fHG-1 (Whitney et al. 1986). However, the presence of lactose prevented the oxidation of Trp which was supported by a slight decrease in the fluorescence of the protein–carbohydrate complex. These findings stress upon the relevance of a reducing environment which is probably needed to reduce molecular oxygen normally present in the solutions to prevent it from oxidizing Trp residues (Levi and Teichberg 1981). It is also indicated that probably the fluorophore is present within or in the vicinity of lactose-binding site and may be readily accessible to lactose (Levi and Teichberg 1981). This finding has been supported by quenching experiments carried out with H_2O_2 in the presence of disaccharides in galectin solution. The involvement of Trp68 aromatic ring (a highly conserved amino acid) in an interaction with the hydrophobic part of the side chain of the conserved Lys63 assured its optimal orientation for interaction with lactose residues (Shahwan et al. 2004). The fluorescence emission spectrum of B_fHG-1 in the presence of urea, *p*HMB, and SDS showed a decrease in the fluorescence intensity indicating that the Trp residue in native lectin is located near an intramolecular quenching group, and lectin denaturation increased this interaction. The effect of the oxidant was also monitored by CD and FTIR analyses. A shift in far UV-CD (Fig. 4b), near UV-CD (Fig. 4c), and FTIR (Fig. 4d) spectra showed a major transition of native secondary structure of B_fHG-1 from β-pleated form to a more open conformation enriched in α-helix, thus clarifying the reason for the loss of activity upon oxidation (Lis and Sharon 1993).

The far UV-CD spectrum of native B_fHG-1 showed a low intensity spectrum with minimum around 218 nm (Fig. 4b), consistent with a predominantly β-sheet structure profile in accordance with other Gal-1 (Shahwan et al. 2004). The FTIR spectrum also correlates with CD analysis, consistent with the presence of a large percentage of β-sheet structure in native B_fHG-1 as suggested by

maximum absorbance peak at 1,630 cm^{-1} (Fig. 4d). The FTIR spectrum of native B_fHG-1 was found to be truncated between 1,652 and 1,576 cm^{-1}. The presence of lactose did not result in any significant change in secondary structure of native B_fHG-1. However, exposure of B_fHG-1 to H_2O_2, urea, *p*HMB, and SDS caused a very marked change in far UV-CD (Fig. 4b), near UV-CD (Fig. 4c), as well as FTIR spectra (Fig. 4d), concomitant with the loss of β-sheet structure. The spectral change was consistent with the disruption of regular secondary structures (25–50% α-helix and 35–60% β-sheet) to a random coil structure. It may be possible that the formation of disulfide bonds locks the lectin into a new inactive conformation, that neither can form the usual secondary structure and nor can bind saccharides. This strongly emphasizes the requirement of the regular secondary structure and of the presence of the complete polypeptide chain for maintaining the active B_fHG-1 conformation.

The predictions of bioinformatics studies were soundly in agreement with the results obtained in wet lab. BLAST-P and CLUSTALW established B_fHG-1 amino acid sequence similarity with other Gal-1. The predictions for trypsin and high specificity chymotrypsin digestion of the purified lectin using the Peptide cutter program of ExPASy tools were in accordance with the results obtained for reverse phase HPLC (Table 3). ExPASy tools predicted average mass as well as monoisotopic mass of the purified lectin as 14.5 kDa, thus corroborating our findings. Secondary structure prediction of B_fHG-1 using 3D-JIGSAW Comparative Modeling Server of ExPASy tools (Fig. 5) was in accordance with the known secondary structures of other Gal-1.

Table 3 Peptide cutter program of ExPASy tools was performed to validate the result obtained in reverse phase HPLC for digestion of B_fHG-1 by high specificity chymotrypsin and trypsin

Name of enzyme	No. of cleavages	Positions of cleavage sites
Chymotrypsin-high specificity (C-term to [FYW], not before P)	10	30 45 49 68 79 91 106 119 126 133
Trypsin	11	20 28 36 48 63 73 99 107 111 127 129

Conclusion

We for the first time report the purification and extensive physicochemical characterization of a Gal-1 from *Bubalus bubalis* heart. The finding that B_fHG-1 is the most stable mammalian heart galectin purified till date recommends its preferential use in various recognition studies. The susceptibility of carbohydrate-binding activity of B_fHG-1 to

Fig. 5 Secondary structure prediction of B_fHG-1. Secondary structure prediction of B_fHG-1 using 3D-JIGSAW Comparative Modeling Server program of ExPASy tools was in accordance with the known secondary structure of other Gal-1, and thus corroborated the results obtained in wet lab

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REMARK ACCI:  Relative solvent accessibility for each residue of the template; from low to
              high *,1,2,3,4,5,6,7,8,9,?
REMARK SS_QP: Predicted secondary structure of query (PSIPRED; Jones D.T.(1999)J.Mol.Biol.
              292,195-202)
REMARK SS_TP: Predicted secondary structure of template
REMARK SS:    Known secondary structure of template
REMARK SCORE: Accuracy of query to template sequence alignment (95 percent of alignments
              over 2 are accurate)
REMARK EVALUE: Parent confidence (expected number of random sequences with the same
               similarity in our database)

REMARK BfHG-1  ACGLVASNLNLKPGECLKVRGEVAPDAKSFLLNLGKDDNNLCLHFNPRFNAHGDINTIVCNSKDGGA
REMARK 1slc_D ACGLVASNLNLKPGECLRVRGEVAADAKSFLLNLGKDDNNLCLHFNPRFNAHGDVNTIVCNSKDAGA
REMARK ACCI   7865537825155941*314*4128724212111*53642***1*1*11728#2651*1*1126755
REMARK SS_QP  CCCCEEECCCCCCCCCEEEEEEEECCCCCEEEEEEECCCCCEEEEEEEEECCCCCCCEEEEEEEECCC
REMARK SS_TP  CCCEEEECCCCCCCCCEEEEEEEECCCCCEEEEEECCCCCCEEEEECCCCCCCCCCCEEEEEEECCE
REMARK SS     CCCCEEEEEEECCCCEEEEEEEECCCCCCEEEEEEEECCEEEEEEEEEECCEEEEEEEEECCE

REMARK BfHG-1  WGAEQREVAFPFQPGSVVEVCISFNQADLTVKLPDGHEFKFPNRLNLEAINYMSAGGDFKIKCVAFD
REMARK 1slc_D WGAEQRESAFPFQPGSVVEVCISFNQTDLTIKLPDGYEFKFPNRLNLEAINYLSAGGDFKIKCVAFE
REMARK ACCI   5575352842416544413111214373*2*4159#4415*414261731421111631436533356
REMARK SS_QP  CCCCEEECCCCCCCCCCEEEEEEECCCEEEEECCEEEEEEECCCCHHHEEEEEECCEEEEEEEEC
REMARK SS_TP  ECCEEEECCCCCCCCCCEEEEEEECCCEEEEECCCEEEEECCCCHHHEEEEEECCEEEEEEEEC
REMARK SS     ECCCEEECCCCCCCCCEEEEEEEECCCEEEEECCCCEEEEECCCCCCCEEEEEEECEEEEEEEEC

REMARK Seq_ID 92.54
REMARK SS_ID  91.79 N(134) EE(66) HH(3) CC(54) EH(0) EC(11) HC(0)
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oxidative inactivation might serve as an important parameter for the measurement of the effect of oxidative assault on the lectin expression under various pathological conditions. Thus, Gal-1 might have been evolved as a simple tool, which cells could use to temporarily modify local interaction with laminin, for themselves as well as for neighboring cells. Moreover, a marked reduction in the activity of oxidized lectin may suggest its potential role in free radical-induced, oxidative stress-mediated cardiovascular disorders. Thus, the present detailed findings regarding B_fHG-1 are important to gain a deeper understanding of the role of galectins in potentially life-threatening cardiovascular diseases.

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