SUMMARY
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. In the present study a buffalo heart lectin was purified and extensively characterized and the effect of deglycosylation on the properties of the purified galectin was studied. The immunological properties of the purified galectin were studied; its role in oxidative burst and degranulation was investigated, and erythrocyte membrane studies were performed.

**Purification and characterization of buffalo heart galectin**

The purified buffalo heart galectin (BfHG-1) was found to be a metal-independent 14.5 kDa protein. Stokes radius and diffusion coefficient determination revealed its homo-dimeric nature. BfHG-1 retained 100% activity up to 48°C and showed pH stability in the range of 4.5-10.5.

BfHG-1 agglutinated native human erythrocytes showed marked preference for the blood group A and the overall order of preference was A>O>AB>B. Exposed thiol group analysis of BfHG-1 indicated a molar ratio of 2.9, thus indicating the presence of at least three moles of sulfhydryl group per mole of BfHG-1.

Conformational changes were induced by the interaction of the BfHG-1 with thiol blocking reagents, denaturants and detergents. Among thiol blocking reagents, the most hydrophobic pHMB maximally inhibited the activity of BfHG-1 followed by lesser hydrophobic NEM, iodoacetate and iodoacetamide. The inhibitory effect of various chaotropic denaturants showed that urea caused maximum loss in BfHG-1 activity, followed by GdnHCl and thiourea. SDS, a well known ionic detergent denatured BfHG-1 even at a very small concentration, while, Tween-20 and Triton X-100 being neutral detergents were less inhibitory in nature.

The slope of linear Scatchard plot drawn using equilibrium dialysis data of BfHG-1 with lactose was used to calculate binding constant value, which suggested the presence of two binding sites per BfHG-1 molecule.

BfHG-1 exhibited an UV maximum at 280 nm, corresponding to the presence of single Trp residue and a large number of other aromatic residues. However, oxidation of BfHG-1 in the presence of 5mM H$_2$O$_2$ (without β-ME) resulted in the shift of absorption peak to 250nm, suggesting oxidation of Trp residue to an oxindole moiety, which absorbs maximally at 250nm.
The sequence alignment of BfHG-1 with other Gal-1 showed more than 87% identity, in agreement with its high conservation throughout evolution. The conservation of amino acid residues that interact with carbohydrate ligands allowed us to classify it as having type 1 conserved CRD.

When excited at 280 nm, BfHG-1 exhibited fluorescence emission spectrum maximum around 332 nm, typical to that of a Trp group in hydrophobic environment. The fluorescent profile of the oxidized galectin showed a remarkable quenching in the fluorescence intensity, accompanied with a decreased activity. However, in the presence of lactose, oxidation of Trp was prevented, also revealed by a slight decrease in the fluorescence of protein-carbohydrate complex.

Exposure to pHMB, urea and SDS resulted in remarkable quenching in the fluorescence intensity, indicating their damaging effect on the structural integrity of BfHG-1. A shift in far and near UV-CD, and FTIR spectra showed a major transition of native secondary structure of BfHG-1 from β-pleated form to a more open and enriched α-helix conformation, thus clarifying the reason for the loss of activity upon oxidation.

The far UV-CD spectra of BfHG-1 showed a low intensity spectrum with minimum around 218 nm, consistent with the presence of large extent of β-sheet structural profile as reported for other Gal-1. The FTIR spectrum also correlates with the CD analysis; consistent with the presence of large extent of β-sheet structure in BfHG-1 as suggested by maximum absorbance peak around 1630 cm⁻¹. BfHG-1 FTIR spectra were truncated between 1652 and 1576 cm⁻¹. Treatment of lactose with the native galectin did not cause any significant change in its secondary topology. However, exposure of BfHG-1 to H₂O₂, urea, pHMB and SDS caused very marked changes in far and near UV-CD, and FTIR spectra, concomitant with the loss of β-sheet structure and its hemagglutination activity.

Deglycosylation studies

Chemical analysis using Dubois phenol-sulphuric acid method revealed the presence of 3.55% sugar residues in the purified buffalo heart galectin. In order to study the role of glycosylation in galectin functioning, we removed the sugar residues using periodate oxidation method. This method completely removed the sugar moieties, as confirmed by the Dubois analysis, PAS staining and the difference in the
mobility of the native and deglycosylated galectin on SDS-PAGE. Deglycosylated galectin eluted later than the native glycosylated protein.

Deglycosylation resulted in a decrease in the Stokes radius and a corresponding increase in the diffusion coefficient of the purified galectin. The thermal and pH stabilities of the deglycosylated galectin were also found to be lesser than the native glycosylated galectin.

Deglycosylation did not result in any changes in the preferential action of thiol blocking reagents, denaturants and detergents on the inhibition of BfHG-1 activity. Although the deglycosylated galectin exhibited a reduced activity profile both before and after oxidation, the effect of H$_2$O$_2$ was much more pronounced on the deglycosylated form.

When treated with increasing concentrations of urea, pHMB and SDS, the deglycosylated form exhibited higher fluorescence intensity and a greater red shift as compared to the native glycosylated galectin.

The far UV-CD and near UV-CD maxima of native and deglycosylated galectins remained unchanged, but the deglycosylated form exhibited decreased circular dichroism than the native galectin. When treated with urea, pHMB and SDS, the deglycosylated galectin exhibited decreased circular dichroism than the glycosylated galectin, thus highlighting the significance of lectin glycosylation.

The FTIR spectrum maximum of native and deglycosylated galectins remained unchanged, but the deglycosylated form exhibited lesser optical density than the native galectin.

**Immunological, oxidative burst and degranulation studies**

Double immunodiffusion revealed high immunogenicity of the purified galectin in rabbits, as they readily gave a single line of identity indicating homogeneity of protein preparations, and indicated that anti-BfHG-1 antibodies are quite specific to BfHG-1. The antiserum showed a high titre value (>128000), suggesting high immunogenic nature of the purified galectin. Dot blot analysis established immunological cross reactivity of BfHG-1 with other galectins.

A gradual increase in superoxide production and lysozyme release was observed when the buffalo neutrophils were treated with the BfHG-1 and/or neutrophil activators.
The results of lysozyme release by various treatments can be summarized as: 

\[(BfHG-1 + CB) < (BfHG-1 + CB + fMLP) < (BfHG-1 + CB + PMA) < (BfHG-1 + CB + fMLP + PMA) < (BfHG-1 + CB + fMLP + PMA),\]

showing a maximum release in the presence of lectin and neutrophil activators. However, lysozyme release was found to be approximately three times higher than superoxide production under similar conditions. Pre-treatment of buffalo neutrophils with HBSS and BfHG-1, followed by stimulation with varying concentrations of fMLP and PMA did not produce significant differences in superoxide production and lysozyme release from the galectin pre-treated and control neutrophils.

**Erythrocyte membrane studies**

Extent of hemolysis of trypsinized rabbit erythrocytes in the presence and absence of BfHG-1 showed that agglutinated erythrocytes were significantly hemolysed in comparison to unagglutinated erythrocytes.

A concentration, temperature and incubation time dependent rise in the hemolysis of trypsinized rabbit erythrocytes was observed in the presence of BfHG-1. The hemolysis of the erythrocytes in the presence of BfHG-1 showed a sharp rise with the increasing pH up to 7.5 and became constant till pH 9.5.

The exposure of BfHG-1 treated trypsinized rabbit erythrocyte suspension was exposed to various sachharides revealed that lactose and sucrose provided maximum protection against hemolysis, while glucose and galactose provided lesser protection. When exposed to superoxide radicals \((O^2-)\) generated from a pyrogallol auto-oxidation system, BfHG-1 treated trypsinized rabbit erythrocyte suspension released higher oxyhemoglobin in comparison to the unagglutinated erythrocytes. The extent of erythrocyte hemolysis was found to be directly proportional to HOCl concentrations.

No significant change was observed in the hemolysis of BfHG-1 agglutinated erythrocytes collected from pre-operated breast and prostate cancer patients as compared to the non-agglutinated, whereas significant increase was observed in the lectin agglutinated erythrocytes of normal healthy controls and both the post-operated cancer patients. In breast cancer patients, lectin agglutinated pre-operated erythrocytes showed 36% and 28% decrease in hemolysis as compared to the control and post-operated erythrocytes, respectively. While, in prostate cancer patients, lectin agglutinated pre-operated erythrocytes showed 49% and 28% increase in the hemolysis as compared to the control and post-operated erythrocytes, respectively.