Discussion
DISCUSSION

Earlier investigations have revealed the presence of soluble carbohydrate binding proteins called lectins which agglutinated desialylated rabbit erythrocytes in the brain tissues of various mammalian species (Simpson et al., 1977; Kobiler and Barondes, 1977; Joubert et al., 1985; Zanetta et al., 1985; Joubert et al., 1986). A growing number of β-galactoside specific lectins have been characterized from brains, cerebellum (Kuchler et al. 1989; Caron et al., 1990; Gabius and Bardosi, 1990; Zanetta et al., 1992; Jaison and Appukuttan, 1994; Ola et al., 2001) as well as from other tissues like spleen, heart, lung etc. (Barondes, 1984; Harrison et al., 1984; Sparrow et al., 1987; Southan et al., 1987 Leffler et al., 1989; Hirabayashi et al., 1992).

The present study demonstrated the presence of a soluble lectin capable of agglutinating trypsinized rabbit erythrocytes in the tissue extract of sheep brain. This agglutination activity was specifically inhibited by lactose. This protein was purified through the procedure adopted for human brain galectin (Avellana-Adalid et al., 1990) with slight modification using a combination of ammonium sulphate fractionation and affinity chromatography on lactosyl-sepharose 4B column. The ammonium sulphate fractionation prior to affinity chromatography resulted in a partial purification and enrichment of specific activity of the lectin. Affinity chromatography was effective in eliminating all of the contaminating proteins and the bound preparation was eluted as a single peak using 0.2 M lactose (Fig. 5). This
resulted in purification and significant enhancement in the specific activity as evident from Table III. The total yield of the purified lectin was 0.04%, and was comparable to that reported by others (Bladier et al., 1991, Caron et al., 1987, Ola et al., 2001).

Affinity purified sheep brain lectin moved as a single protein band in native PAGE suggesting the homogeneity of the preparation (Fig. 6). The brain lectin was also found to be electrophoretically homogeneous in SDS-PAGE under reducing conditions (Fig. 7). Interestingly the subunit molecular weight of the galectin was found to be ~ 14.1 kDa (Fig. 9), which is similar to the brain galectins isolated from bovine, rat, human and caprine (Caron et al., 1987; Avellena-Adalid et al., 1990; Ola et al., 2001).

The molecular weight of sheep brain galectin under native conditions was elucidated using gel filtration chromatography (Fig. 8 and Table IV) and was found to be 28.5 kDa, while when the sheep lectin was electrophoresed in polyacrylamide gel in the presence of sodium dodecyl sulphate under reducing as well as non-reducing conditions, both preparations showed only monomer of molecular weight of ~ 14.1 kDa (Fig. 9 inset). Thus the sheep brain galectin is a dimer of two identical subunits, each of ~ 14 kDa, as was reported for other galectins isolated from different mammalian tissues including human, bovine and rat brain galectin (Oda and Kasai, 1983; Cerra et al., 1985; Hirabayashi et al., 1987 Caron et al., 1987; Bladier et al., 1989), this also implies that it is a homodimeric protein with subunits held together by non-covalent interactions. For
most galectins, the dimeric form provides the potential to bind to glycoconjugates (Drickamer, 1988; Hirabayashi and Kasai, 1993).

Most galectins bind lactose and N-acetylactosamine (LacNac) but there are subtle differences in their carbohydrate specificities (Leffler and Barondes, 1986; Oda et al., 1993; Ahmed and Vasta, 1994). The sugar binding specificity of sheep brain galectin for β-galactosides resembles that of known brain galectins from bovine, rat, goat and human (Leffler and Barondes, 1986; Caron et al. 1987; Bladier et al. 1991; Ola et al., 2001). Although it binds to free galactose, it has higher affinity for disaccharides, in particular, for lactose. The minimum inhibitory concentration required for other saccharides was also different, suggesting that the sheep brain galectin has its own unique and fine specificity (Sparrow et al., 1987; Solis et al., 1996; Kasai and Hirabayashi, 1996). The lactose structure is included in a variety of glycoconjugates, such as fibronectin, laminin, leukocyte common antigen (CD 45) and leukoadhesin (CD43), and has been proposed as the key glycocode for galectins (Kasai and Hirabayashi, 1996).

The results of inhibition studies using a number of saccharides shown in Table V and Fig. 11. lead to the conclusion that sheep brain galectin is specific for saccharides bearing non-reducing terminal D-galactose linked in a β-configuration. This is further supported by the observation that the methyl-α-D-galactopyranoside and p-nitrophenyl-α-D-galactopyranoside are weak inhibitors as compared to methyl-β-D-galactopyranoside and p-nitrophenyl-β-D-galactopyranoside. However,
D-galactose and D-galactosamine were inhibitors which indicate that a free hydroxyl, or a free amino group at C-2 is required for monosaccharides to cause inhibition. Moreover, the configuration at C-4 is also important, since neither glucose nor glucosamine was an inhibitor. The fact that lactose is far more inhibitory for hemagglutinating activity of the sheep brain galectin suggests that the carbohydrate binding site of the galectin could have extended geometry which is only partially occupied by galactose molecule.

As indicated in the table VI, the sheep brain galectin did not agglutinate any type of native human erythrocytes, however it agglutinated trypsin-treated human blood cells, with a preference for type A trypsin treated erythrocytes, rather than O, B and AB. Thus, it is possible that sheep galectin may bind to blood group A determinants GalNac instead of Gal and GlcNac (Sparrow et al., 1987). However, the ability of sheep galectin to agglutinate blood group O erythrocytes is probably mediated by the polylactosaminoglycans found on the human erythrocyte surface (Fukuda, 1985). The sheep brain galectin, however, could not agglutinate any of the other mammalian erythrocytes (sheep, goat, buffalo) possibly due to the absence of glycocodes that are recognised by sheep galectin.

The complete amino acid sequence of the 14kDa β-galactoside-binding protein isolated from sheep brain (Fig. 13,14) is clearly similar in sequence to galectin-1 characterized in human, rat, ovine, bovine and mouse species. This galectin shows greater identity
(95%) with the ovine placental galectin than with the human brain galectin (92%) and rat brain galectin (86%). This result is in agreement with the suggestion that the 14 kDa galectin-1 is species-specific rather than organ-specific (Bladier et al., 1991). In addition, the high degree of homology between sheep brain galectin and other mammalian galectins (86-95%) suggests the high conservation throughout evolution, which in turn, shows that they are likely to have evolved from common ancestor protein (Hirabayashi et al., 1992). The low value of identity with eel electric organ lectin (41%) may be due to the phylogenetic distance.

These homologies also suggest that those peptide sequences that appear to be highly conserved in all the galectins, would correspond to essential structural determinants. For example, the tetrapeptide 68-71, which is conserved under the form of W-G-T/A-E (Fig. 14), is thought to be part of the saccharide binding site (Levi and Teichberg, 1981).

Moreover, the conservation of amino acid residues that interact with the carbohydrate ligands (His 44, Asn 46, Arg 48, His 52, Asp 54, Asn 61 Trp 68, Glu 70 and Arg 73) suggests the sheep brain galectin is having type I (conserved) CRD (Fig. 14). Like other members of this subfamily, SBG galectin-1 contains only one CRD domain, and hence can be classified as proto-type according to its molecular architecture (Hirabayashi and Kasai, 1993). Besides, it shows the characteristics of cytoplasmic proteins, such as an
acetylated N-terminal amino group and the lack of a hydrophobic signal sequence.

As can be seen in Fig. 14 some of these galectins have a possible site of glycosylation: in sheep brain, ovine placenta and bovine heart (Asn92- Gln93- Thr94), chicken skin lectin (Asn92-Pro93- Ser94) and eel (Asn16- Leu17- Thr18) but none of these have been reported to be glycosylated.

Therefore, it can be concluded that there is likely to be one gene for the galectin-1 subfamily in sheep as well as in other mammals. But this does not exclude the existence of one or more other genes coding for more or less related molecules (Gitt and Barondes, 1986). Further, the high degree of homology of the mammalian galectin-1 subfamily members and their ubiquitous organ distribution suggests that they must be involved in some basic function.

Although the exact function of the brain galectin is not known, it is interesting to observe that it is a potent inductor of the mammalian brain cell aggregation (Fig. 15, Table VIII). It is, important to note that galectin - carbohydrate binding need not be the only, or even the main, determinant of specificity in brain cell interaction. A requirement for dual recognition involving other sets of complementary molecules, is consistent with the lectin recognition hypothesis (Caron et al, 1987). This result may also imply the involvement of the brain galectins in the neuro-transmission process.
The number of total thiol groups in the sheep brain galectin as determined by amino acid sequence was six moles/mole (Table VII). The presence of 1.42 moles/mole of free thiol groups in the galectin clearly indicate the presence of intrachain disulfide bonds as observed in rat lung galectin (Whitney et al., 1986). In order to investigate whether free thiol groups were necessary to maintain the galectin in its active form, the native sheep brain galectin was alkylated by iodoacetate, iodoacetamide, N-ethylmaleimide and p-hydroxymercuribenzoate respectively. This led to a wide range in the rates at which the thiol groups were alkylated and resulted in inactivation of hemagglutinating activity (Fig. 16). The inactivation of hemagglutination activity indicates that the reduced form of cysteine is required for the maintenance of the galectin in its active form. The loss of galectin activity thus could be due to a possible conformational change that occurred during modification of cysteine residues (Clerch et al., 1988), the slow rate of galectin inactivation suggests that the functionally relevant cysteine residues could be partially buried inside the protein and possibly not involved directly in carbohydrate binding but present at a relatively distant site (Whitney et al., 1986). This explains why being most hydrophobic thiol blocking reagent, pHMB was most effective to abolish the hemagglutinating activity followed by the less hydrophobic NEM and iodoacetate respectively. It is important to note that most galectins are not inactivated by iodoacetamide treatment (Hirabayashi et al., 1987; Ali and Salahuddin, 1989), infact some modified galectins showed higher
activity (Whitney et al., 1986; Clerch et al., 1988). Surprisingly, sheep brain galectin was readily inactivated by iodoacetamide.

In order to account for the requirement of reducing agents for the maintenance of activity, the galectin was oxidised by $\text{H}_2\text{O}_2$ (Fig. 17). This resulted in a significant loss of hemagglutinating activity, the oxidative inactivation of sheep brain galectin can be accounted for by the formation of intrachain disulfide bond (Whitney et al., 1986), which locks the protein into new conformation that cannot form the required secondary structure for carbohydrate binding (Clerch et al., 1988).

The sheep brain galectin was irreversibly inactivated by exposure to high temperature during relatively short period of time (Fig. 18), similar to other galectins such as bovine spleen galectin (Ahmed et al., 1996).

The pH range for optimal binding activity of the sheep brain galectin was rather wide (6.5 - 8.5) (Fig. 19), and overlapped with that of its natural physiological environments as reported earlier for bovine spleen galectin (Ahmad et al., 1996).

The ultraviolet absorption studies showed that aromatic chromophores in sheep brain galectin include tryptophan residue (Fig. 20). When sheep brain galectin was oxidized in the absence of reducing agent. The uv absorption peak of tryptophan at 280 nm shifted to 250 nm. The shift in peak suggests oxidation of tryptophan residue to an oxindole moiety which absorbs maximally at 250 nm (Levi and Teichberg, 1981). Thus, the reducing agent not only
prevents oxidation of cysteine residues in the galectin but also protects tryptophan from oxidation. The X-ray studies on bovine spleen galectin have shown that Trp-68 is found to make stacking interaction with galactose moiety (Abbott and Feizi, 1991; Hirabayashi and Kasai, 1991; Rini, 1995). Although there is no direct evidence that tryptophan is crucial for hemagglutination activity in case of sheep galectin, but it may play some role in maintenance of the carbohydrate binding site of the galectin molecule.

The fluorescence spectrum of sheep brain galectin-1 with maximum at 330 nm (Fig. 21) is typical of a tryptophan residue in hydrophobic environment (Levi and Teichberg, 1981). The exposure of sheep brain galectin-1 to oxidation caused quenching in the fluorescence which was concomitant with the loss of activity. The findings that lactose enhanced the fluorescence of SBG-1 and prevented the deleterious effects of oxidizing agent indicated that the emitting fluorophore is located within or in the vicinity of the lactose binding site and may be readily accessible to lactose (Levi and Teichberg, 1981). This suggestion is supported by quenching experiments carried out with H$_2$O$_2$ in the presence of disaccharide in the galectin solution, which also took place concomitant with the finding that Trp 68 is among the highly conserved amino acids and involved in binding with lactose by hydrophobic interactions (Hirabayashi et al., 1993; Lobsanov et al., 1993; Liao et al., 1994).

The far-uv CD spectrum of native sheep brain galectin (Fig. 23) is consistent with the large extent of $\beta$-sheet structure as described for
other galectins by X-ray crystallography (Liao et al., 1994). Addition of lactose (Fig. 23) produced no significant change, thus suggesting that the presence of this sugar did not induce any modification on the secondary structure of the galectin, while the exposure of the galectin to oxidation caused marked change in the CD spectrum with great loss in the β-sheet (Fig. 24), which is consistent with the loss of the activity upon oxidation that is indicative of a substantial change induced in the secondary structure (Yoshimasa et al., 2000). The spectral change is also consistent with the disruption of regular secondary structure (α-helix and β-sheet) to a random coil. Perhaps the intramolecular disulfide bond formation locks the protein into a new, inactive conformation that can not form the usual secondary structures and can not bind saccharides (Clerch et al., 1988). This suggests that the regular secondary structure is a vital part of maintaining the active galectin conformation.

The near-uv circular dichroism spectrum shown in Fig. 25 clearly shows that the change in the tertiary structure of the protein in the presence of lactose is negligible, while the spectra shown in Fig. 26 may not be the true representation of major conformational changes in the protein. The spectra clearly indicated the structural alterations of the galectin in the presence of oxidizing agent (H₂O₂) as compared to native sheep brain galectin. This may be due to the change in the microenvironment of the aromatic amino acid residues (Creighton, 1991).
In view of the possible common role of brain galectins, it was of interest to see whether the isolated galectin is phylogenetically/structurally related to other galectin or is a distinct protein sharing only a similar saccharide-binding specificity. Antibodies were raised against pure sheep brain galectin in rabbits, the antisera thus obtained gave a single precipitin band upon immunodiffusion with the antigen i.e. sheep brain galectin. This indicated the homogeneity of the preparation. The high titer of the antibody obtained by ELISA (Fig. 27) suggested that the galectin is highly immunogenic.

The crossreactivity between sheep, goat and buffalo brain galectins was examined by immunodiffusion method (Fig. 28). The anti-sheep galectin antibody recognized both goat and buffalo brain galectins, but the degrees of cross-reactivity were found to be different. The sharing of common antigenic determinants between galectins was further studied by competition ELISA (Fig. 30). A high degree of inhibition in anti-sheep galectin antibody binding to antigen (sheep brain galectin) was observed by galectins from goat and buffalo, which were used as inhibitors, however, the extent of binding was lower than that observed for sheep galectin. In particular, the goat brain galectin seems to show a remarkable structural or conformational homogeneity with the sheep brain galectin as it inhibited the binding of antibodies to an extent of 86%. The antibodies were highly specific for the immunogen, since only 4 μg/ml of sheep galectin was required to achieve 50% inhibition, while 5 μg/ml was required in the case of goat galectin to attain 50% inhibition. This is
also evident from the percent relative affinity of sheep galectin which was taken as 100% being the immunogen compared to 80% for goat galectin (Table IX). This is, however, expected since both are phylogenetically related and would therefore, be expected to have common conserved epitopes (Southan et al., 1987; Abbott et al., 1989; Ali et al., 1996, Ola et al., 2001). In case of buffalo galectin it has shown 67% inhibition with concentration of 10 µg/ml to attain 50% inhibition and relative affinity of 40%, compared to goat galectin buffalo galectin is a weaker inhibitor, similar results were observed in case of anti-goat galectin antibodies (Ola et al., 2001). Thus, the competition ELISA data indicated that the galectins from sheep, goat and buffalo share common antigenic determinants/epitopes which were recognized by the anti-sheep galectin antibodies. The presence of cross reacting lectin was detected in the lung, liver and heart tissues of sheep by dot blot analysis (Fig. 29). Extensive crossreaction was observed for all of them, clearly indicated antigenic cross-reactivity between brain galectins of different species, as well as lectins of other organs of same species.

The observations suggested that these galectins may be structurally related and may share a common fundamental biological role. Thus, the ubiquitous distribution and similarity in structure shows that the galectins may be part of independent separate functions carried out by the same protein, or they may be part of the same functional system.