Biochemistry (Moscow), Vol. 69, No. 5, 2004, pp. 506-512. Translated from Biokhimiya, Vol. 69, No. 5, 2004, pp. 623-630.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM03-170, February 29, 2004.

Physicochemical Properties and Amino Acid Sequence of Sheep Brain Galectin-1

M. Shahwan, M. T. Al-Qirim, S. M. K. R. Zaidi, and Naheed Banu*

Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh 202002, India;
E-mail: naheednrizvi@rediffmail.com

Received July 7, 2003
Revision received September 30, 2003

Abstract—A β-galactoside-specific soluble 14-kD lectin from sheep brain was isolated, sequenced, and compared with similar galectins from other species. Percent identities of amino acid sequence and the carbohydrate recognition domain (CRD) revealed that the isolated galectin shares all the absolutely preserved and critical residues of the mammalian galectin-1 subfamily. The isolated sheep brain galectin (SBG) showed more than 90% amino acid sequence (92%) and carbohydrate recognition domain identity (96%) with human brain galectin-1. Conformational changes were found induced by interaction of the protein with its specific disaccharide and oxidizing agent (hydrogen peroxide). Upon oxidation a drastic change in the environment of aromatic residues and conformation of the galectin was observed with the loss of hemagglutination activity, while no significant change was observed upon addition of D-lactose (Gal(β1-4)Glc) in the far-UV and near-UV spectra, suggesting no significant modification in the secondary as well as tertiary structures of sheep brain galectin. But the functional integrity of the CRD is found to be affected in the presence of oxidizing agent, indicating intramolecular disulfide bonds and requirement of complete polypeptide chain for functional integrity of the carbohydrate recognition domain.

Key words: amino acid sequence, lectin, galectin-1, sheep brain galectin

Galectins are a family of proteins defined by having at least one characteristic carbohydrate recognition domain (CRD) with affinity for β-galactosides, sharing certain conserved sequence elements requiring a reducing environment but not divalent cations for their binding activity [1]. The available primary structure of a considerable number of galectins confirms that they do constitute a defined family of proteins with substantial degree of similarity [2]. They are distinguishable from all other animal lectins by their low molecular weight, ranging from 14 to 36 kD, and their variable subcellular location. Ten members of this gene family have now been identified [3]. The function of these molecules is largely unknown but evidence suggests their role in cell–cell interaction during development, cell–substratum adhesion [4, 5], and regulation of immune response [6], neoplastic transformation [7], and apoptosis [8].

Most of the galectins have not yet been crystallized, so the relative inhibitory activity of various mono and oligosaccharides still remain a valuable source of information on their carbohydrate specificities, particularly with regards to the orientation of hydroxyls at positions critical for binding [9-12]. Moreover, methods based on chemical modification of amino acids have provided insight into the nature of the amino acid residues that are directly involved in ligand binding [13].

Galectins have been classified into several groups based on their primary structures and subunit architecture. The galectin CRD consists of about 135 amino acids tightly folded into a sandwich of a 6 stranded and a 5 stranded antiparallel β sheets that form extended β sandwich with a typical jelly-roll topology [14]. Within the same peptide chain, some galectins have a CRD with only a few additional amino acids, whereas, others have two CRDs joined to different types of domain. These structural types have been named prototype, tandem repeat, and chimera, respectively [2], but they do not, however, reflect evolutionary relationship between galectins.

The three-dimensional structures of a limited number of galectins have been resolved [14-16], providing reliable information on the amino acid residues that interact with a carbohydrate ligand and the nature of the bonds involved.

The amino acid sequence of galectin-1 has been determined in a variety of species, including chicken, man, sheep, rat, mouse, and cattle; they are found to have 87-95% amino acid sequence identity with each other [2].

* To whom correspondence should be addressed.

0006-2979/04/6905-0506 ©2004 MAIK "Nauka/Interperiodica"

Within the CRD the percent identity among the so far characterized galectin-1 ranges from 77-93%, and the amino acids involved in ligand binding are found to be identical [2].

To gain further insight into the possible structural–functional relationship among the sheep brain and other 14 kD galectins expressed in different species, the present study was undertaken to characterize the sheep brain galectin-1 and to determine its complete amino acid sequence and sugar binding specificity. Moreover, the conformational changes induced by its interaction with D-lactose and oxidizing agent H_2O_2 were also analyzed by measuring intrinsic fluorescence and circular dichroism.

MATERIALS AND METHODS

Sugar, lactosyl-Sepharose-4B, β-mercaptoethanol, and sodium dodecyl sulfate were purchased from Sigma (USA). Sequencing grade reagents and solvents for protein sequencing, amino acid analysis, and HPLC were from Applied Biosystems (USA). All other chemicals and reagents were of analytical grade.

Purification of β-galactoside-binding lectin. The sheep brain galectin (SBG) was purified as described [17]. Briefly, the lectin was isolated by affinity chromatography using a lactosyl-Sepharose-4B column equilibrated with 75 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl, 5 mM 2-mercaptoethanol. The column was eluted with the same buffer supplemented with 200 mM lactose, and the purified galectin was dialyzed against 75 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 5 mM 2-mercaptoethanol and lyophilized. The purity and molecular weight of the eluted material was monitored by SDS-PAGE according to standard method of Laemmli [18].

Hemagglutination assay. Hemagglutination activity was determined with trypsin-treated rabbit erythrocytes [19]. Twofold serial dilutions of 50 μl samples were made in 50 μl of sodium phosphate buffer containing 0.15 M NaCl and 5 mM 2-mercaptoethanol on microtiter "U" plates. To 50 μl remaining in each well, 50 μl of the trypsinized erythrocyte suspension (40% v/v) was added. The titer (agglutinating unit) is defined as the reciprocal of the highest dilution giving visible agglutination. The specific activity is defined as the ratio of the titer to protein concentration. Various sugars and their derivatives tested for lectin inhibition were serially diluted in the same buffer. To each well four agglutinating units of lectin and 50 μl of trypsinized erythrocytes were added. The highest dilution of the test sugar required for inhibition was noted.

Sequencing of tryptic peptides: enzymatic digestion and purification. Purified sheep brain galectin (2 mg in 3 ml of PBS containing 5 mM mercaptoethanol and 0.5 M NaCl) was dialyzed against 0.01 M ammonium hydrocarbonate and lyophilized. The lyophilized galectin 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 a nitrogen atmosphere at room temperature for 2 h. The carboxamidomethylated galectin was diluted to 2 ml with water and digested with trypsin/chymotrypsin at 37°C for 20 h using 1 : 20 enzyme/substrate ratio. The tryptic peptides were separated by reverse-phase HPLC (Pharmacia LKB, Sweden) on a Vydac C18 column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid in water (v/v). The column was eluted at a flow rate of 0.8 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 major peptides selected were applied to a polybrene-coated glass filter and sequenced in an Applied Biosystems model 477A automatic sequencer (Applied Biosystems) run according to the manufacturer's instructions. Searches for the similarities to the determined sequences were performed with the aid of the Swiss Prot protein sequence data bank. The peptides were aligned by similarity.

Fluorimetry. The intrinsic fluorescence of the galectin was measured at 25 ± 0.2°C in a Hitachi F-2000 spectrofluorometer (Hitachi, Japan) equipped with a DR-3 recorder. The protein was excited (λ_{ex}) at 280 nm. Excitation and emission slit widths were 10 nm. Appropriate control containing substances used for the treatment were run and corrections made wherever necessary. Each spectrum represents an average of three scans. The change in intrinsic fluorescence of native galectin in PBS containing 5 mM 2-mercaptoethanol and oxidized galectin (by adding 5 mM H_2O_2 in the absence of 2-mercaptoethanol), in the presence as well as in the absence of 0.1 M lactose, was followed by measuring the emission spectra in the range 300-400 nm.

Circular dichroism. All the circular dichroism (CD) measurements were carried out at 25°C on Jasco spectrophotometer model J-810 using a SEKONIC XY plotter (model SPL-4301A) with a thermostatically controlled 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 precalibrated with (+)-10-camphorsulfonic acid. The spectra were recorded with a scan speed of 100 nm/min and with a response time of 1 sec. Each spectrum was recorded as an average of four scans. Far-UV CD spectra (200-250 nm) and near-UV CD spectra (250-300 nm) were taken at protein concentration of 250 μg/ml with 1- and 10-mm pathlength cells, respectively. Appropriate controls containing the substances used for treatment were also taken. The same protein concentration (250 μg/ml) was used in all CD measurements.

RESULTS AND DISCUSSION

Hemagglutination inhibition studies using a number of saccharides (table) led to the conclusion that SBG 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 (required >100 mM for inhibition) as compared to methyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-galactopyranoside (required 5 mM for inhibition). The inhibition by D-galactose and D-galactosamine indicates that a free hydroxyl or a free amino group at C2 is required for a monosaccharide to cause inhibition. Moreover, the configuration at C4 is also important, since neither D-glucose nor D-glucosamine caused inhibition. The fact that lactose is far more potent as inhibitor for hemagglutinating activity of SBG suggests that the carbohydrate-binding site of the galectin could have extended geometry, which is only partially occupied by the galactose molecule. It appears that the aromatic side chain of the conserved Trp68 stacks adjacent to the galactose ring. Such van-der-Waals interactions between sugar and aromatic side chains are quite common in protein–carbohydrate complexes [20]. The axial 4-OH of galactose, a main determinant of the galectin specificity, forms two key electrostatic interactions; one with the N-atom of Arg48 and the other with the N-atom of His44 [16]. Both are invariant residues in the sequences of galectin (Fig. 2). An ammonium group on C2 decreases the inhibitory properties (D-galactosamine, D-glucosamine), 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 [21], SBG required higher concentrations of these saccharides (2.5, 5, and >100 mM, respectively), suggesting its own unique specificity.

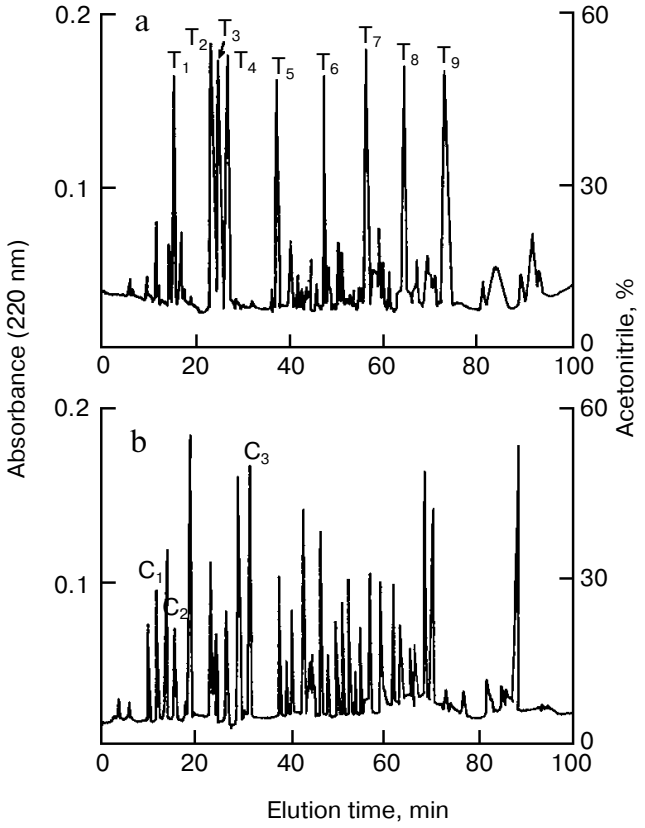

Fig. 1. HPLC profile of peptides obtained by tryptic (a) and chymotryptic (b) digestion of sheep brain galectin-1. Peptides were separated by reversed-phase HPLC on a Vydac C18 column equilibrated with 0.1% (v/v) trifluoroacetic acid in water. Elution was performed at a flow rate of 0.8 ml/min with 0-60% acetonitrile linear gradient in 0.1% (v/v) trifluoroacetic acid for 10 min, the elution being monitored at 220 nm. Peaks T1-T9 and C1-C3 represent peptides whose sequence was determined by Edman degradation or inferred from amino acid sequence.

Amino acid sequence. To obtain the amino acid sequence of SBG, fragments were generated by enzymatic cleavage of the purified galectin. Both tryptic and chymotryptic peptides were separated by reversed-phase HPLC (Fig. 1a) and subsequently submitted to amino acid analysis and/or automated sequencing (Fig. 2). The

Effect of saccharides on the hemagglutinating activity of sheep brain galectin

Saccharides	Minimum concentration of saccharides giving complete hemagglutination inhibition, mM
β-D-Lactose (Gal(β1-4)Glc)	0.4
D-Galactose	2.5
Methyl-β-D-galactopyranoside	5.0
p-Nitrophenyl-β-D-galactopyranoside	5.0
D-Galactosamine	25.0
Methyl-α-D-galactopyranoside	>100
p-Nitrophenyl-α-D-galactopyranoside	>100
D-Glucose	>100

Note: 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.

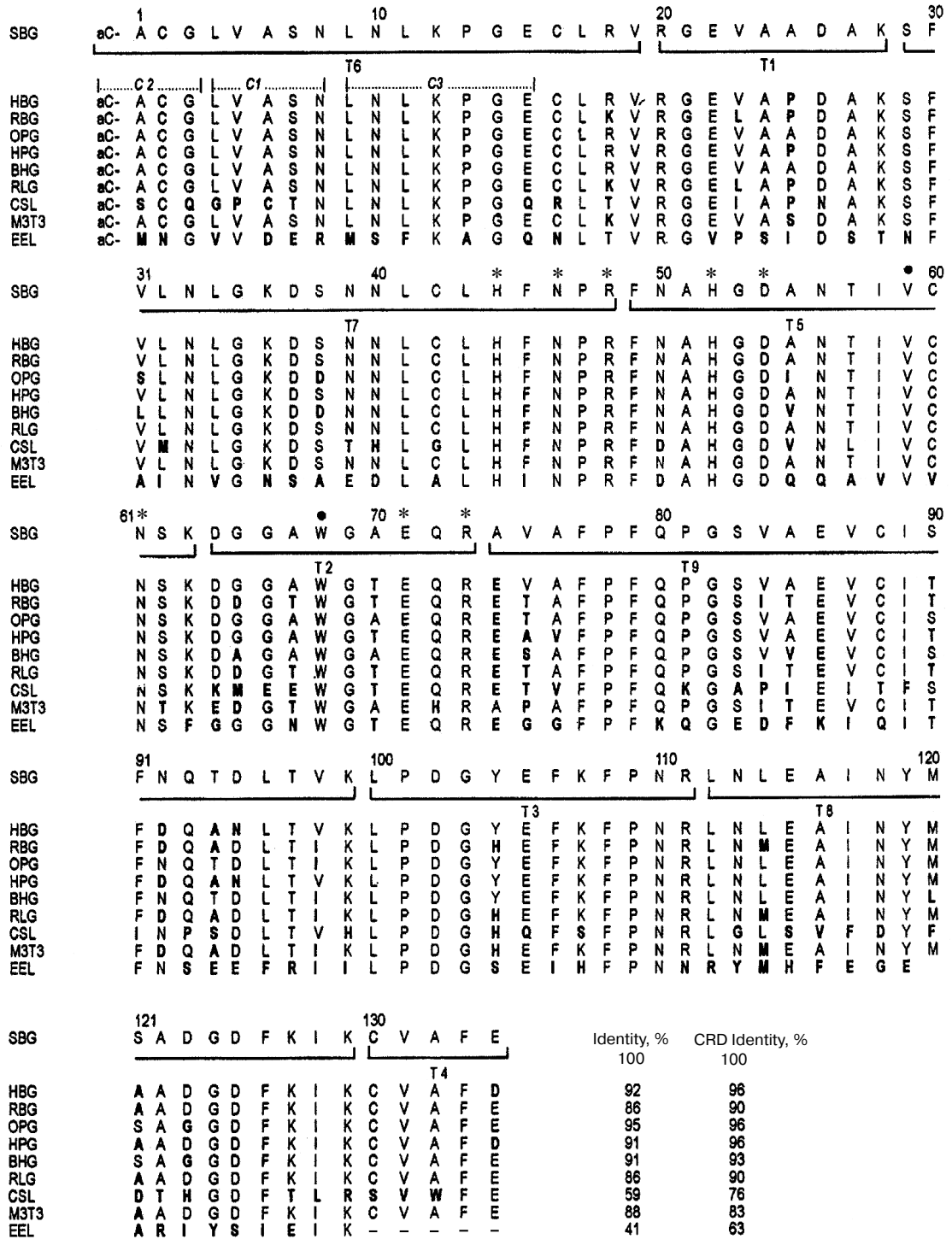

Fig. 2. Comparison of amino acid sequence of representative animal galectins. SBG (sheep brain galectin), present study, nomenclature of the peptides is consistent with Fig. 1. HBG, human brain galectin; RBG, rat brain galectin; OPG, ovine placental galectin; HPG, human placenta galectin; BHG, bovine heart galectin; RLG, rat lung galectin; CSL, chicken skin galectin; M3T3, murine 3T3 fibroblast; EEL, eel electro-lectin. Identities to SBG sequence are shown at the bottom right. Amino acids from other galectins, which differ from those present at the same position of SBG, are indicated in bold. Critical amino acids, which have been shown to be involved in the interaction with lactose [3] and N-acetyllactosamine [4, 5], are designated with asterisks (hydrogen bonding) or black circles (hydrophobic interaction), and these residues correspond to the type 1 CRD invariant amino acids.

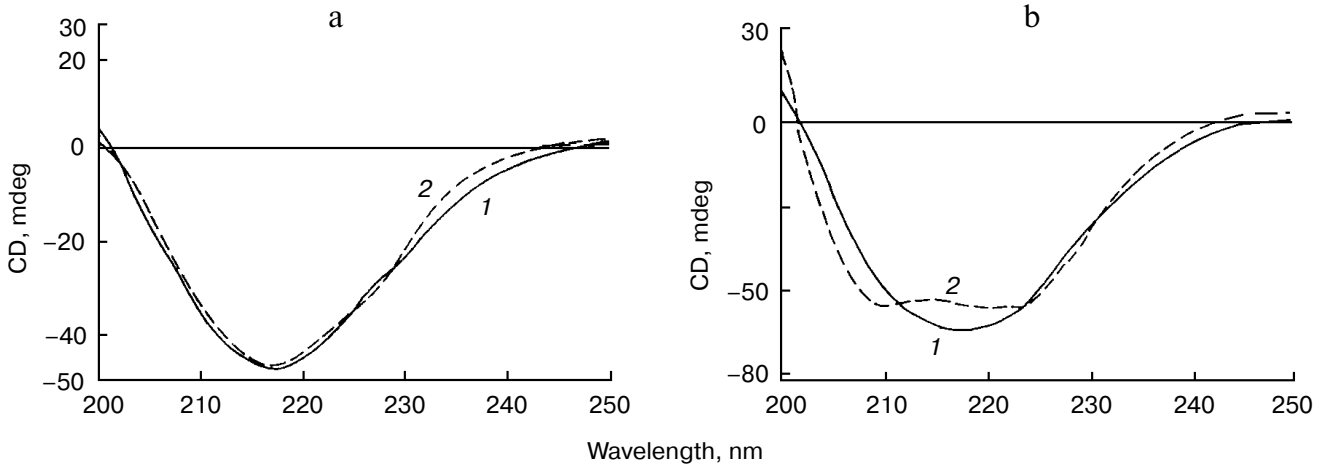

Fig. 3. Far-UV CD spectra of sheep brain galectin were obtained in 20 mM sodium phosphate buffer, pH 7.2. a) CD spectra of sheep brain galectin recorded in the absence (*1*) and presence of 3 mM lactose (*2*). b) CD spectra of native (*1*) and oxidized (*2*) sheep brain galectin in the wavelength range of 200-250 nm after addition of 5 mM H_2O_2.

peaks designated T1-T9 represent pure peptides. The amino acid sequences of all these peptides were determined except for T6, for which the amino acid composition was determined. The presence of an acetyl group was deduced from its blocked N-terminus as reported for other galectin-1 [17, 21]. Figure 1b represents the HPLC profile of fragments obtained by chymotryptic digestion. The sequences not established by tryptic digestion (C1-C3) were selected for Edman degradation.

The sequence alignment of SBG-1 with other galectin-1 characterized from human brain [22], rat brain [23], ovine placenta [24], human placenta [25], bovine heart [26], rat lung [27], chicken skin [28], murine 3T3 fibroblast [29], and eel electric organ [30] showed identities ranging between 41 to 95% (Fig. 2). Moreover, the CRD also showed a significant degree of identity (63-96%), in agreement with its high conservation throughout evolution. Sheep brain galectin-1 shows greater identity (95%) with the ovine placental galectin-1 than the human brain galectin (92%) and the rat brain galectin (86%). These results suggest that the 14.5-kD galectin-1 is species specific rather than organ specific [22]. 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. 2). Like

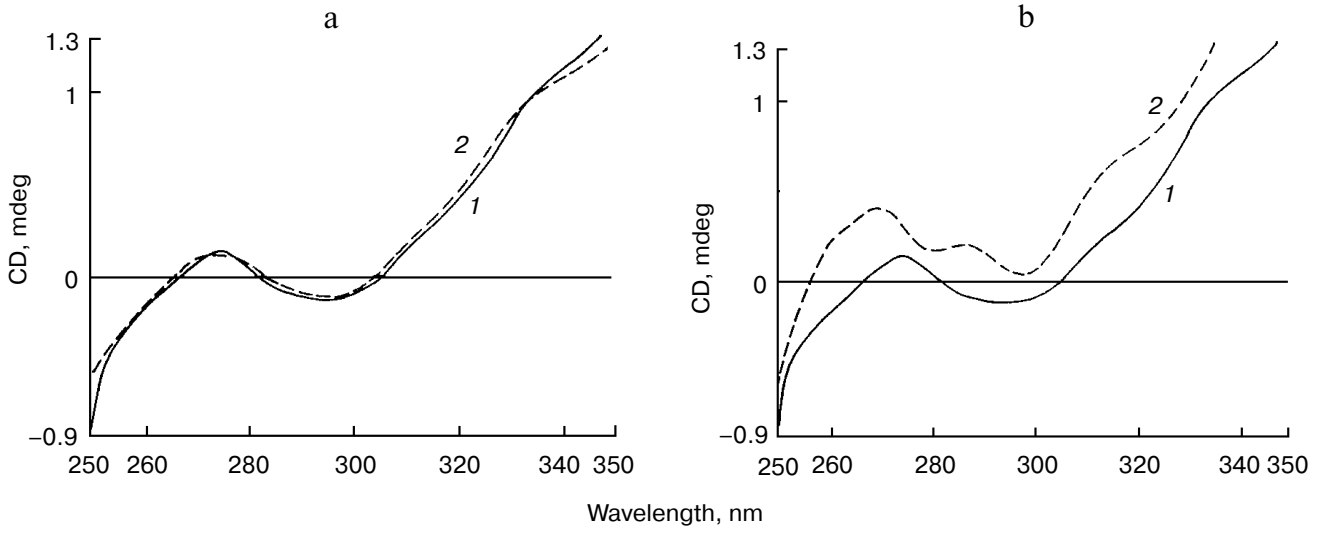

Fig. 4. Near-UV CD spectra of sheep brain galectin were performed in 20 mM sodium phosphate buffer, pH 7.2. a) Near-UV CD spectra of sheep brain galectin in the absence (*1*) and presence of 3 mM lactose (*2*). b) Near-UV CD spectra of native (*1*) and oxidized (*2*) sheep brain galectin in the wavelength range of 250-350 nm after addition of 5 mM H_2O_2.

other members of this subfamily, the SBG-1 is also a homodimer (unpublished data). It is composed of subunits of 134 amino acids, containing only one carbohydrate recognition domain, and it can also be classified as prototype according to its molecular architecture [2], as it neither has a link peptide joining two CRDs nor is the CRD joined to a different type of domain as found in tandem repeat and chimera, respectively. Besides, it shows the characteristics of cytoplasmic proteins, such as an acetylated N-terminal amino group and the lack of hydrophobic transmembrane segment and secretion signal peptide similar to other galectin-1 [1].

The far-UV CD spectra of native sheep brain galectin shows a low intensity spectrum with minima in the 215-217 nm range, consistent with the large extent of β-sheet structural profile as reported for human galectin-2 [15] and bovine galectin-1 [14]. Addition of lactose produced no significant change in the far-UV CD spectrum (Fig. 3a). This suggests that the presence of lactose does not induce modification of the secondary structure of the galectin, and the small differences in the spectra obtained in the presence and in absence of lactose could be attributed to the contribution of Trp residues to the far-UV CD spectra [31]. Figure 3b shows that exposure of sheep brain galectin to oxidizing agent (5 mM H_2O_2) caused very marked changes in the CD spectrum with the loss of the β-sheet structure. The spectral change is consistent with the disruption of regular secondary structures (25% α-helix and 38% β-sheet) to a random coil structure. It is possible that the formation of disulfide bonds locks the protein into a new, inactive conformation that cannot form the usual secondary structure and cannot bind saccharides. This suggests that the regular secondary structure is vital for maintaining the active galectin conformation.

The CD spectra of sheep galectin in the near-UV range (Fig. 4a) clearly show that the tertiary structure of the galectin did not undergo any significant change upon interaction with 10 mM lactose. However, addition of oxidizing agent (5 mM H_2O_2) resulted in subtle changes in the near-UV CD spectra of oxidized galectin indicating a remarkable change in the tertiary structure of the galectin upon oxidation (Fig. 4b), which is consistent with the dramatic alterations induced in the secondary structure of oxidized galectin [27].

The fluorescence spectra of native sheep galectin were measured in 75 mM sodium phosphate buffer containing 5 mM 2-mercaptoethanol over the wavelength range 300-400 nm with excitation at 280 nm (Fig. 5). Sheep galectin showed an emission spectrum with maximum at 337 nm, a typical spectrum of tryptophan in a hydrophobic environment [32]. Exposure of sheep galectin to oxidizing agent (5 mM H_2O_2) resulted in quenching of the fluorescence compared to the native galectin. Similar experiments of exposure to H_2O_2 carried out in galectin solution in the presence of 0.1 M lactose resulted in slight decrease in the fluorescence of the protein–carbohydrate complex. However, the presence of lactose in the galectin solution enhanced the fluorescence slightly. The fact that lactose increased the fluorescence indicates that the fluorophore is present within or in the vicinity of the lactose-binding site and may be readily accessible to lactose [32]. This suggestion is supported by quenching experiments carried out with H_2O_2 in the presence of disaccharides in the galectin solution as the aromatic ring of the Trp68, a highly conserved amino acid, is involved in interaction with the hydrophobic part of the side chain of the conserved Lys63 and assures its optimal orientation for interaction with lactose residues [14-16].

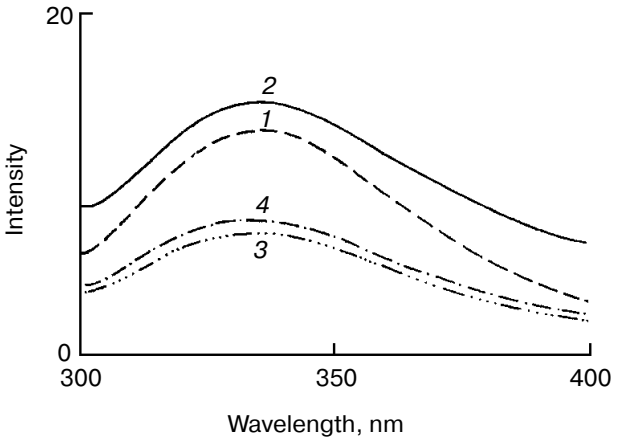

Fig. 5. Fluorescence spectra of sheep brain galectin. Experiments were performed in 75 mM sodium phosphate buffer, pH 7.2. The spectra of sheep brain galectin (100 μg/ml) alone (*1*) and in the presence of 0.1 M lactose (*2*), of oxidized (by adding 5 mM H_2O_2) galectin in the absence (*3*) and in the presence of lactose (*4*).

The sensitivity of galectin-1 carbohydrate-binding activity to oxidative inactivation might serve to limit the duration and physical range of its influence. Thus, galectin-1 might have evolved as a simple tool, which cells could use to temporarily modify local interaction with laminin both for themselves and for neighboring cells.

Facilities provided by Aligarh Muslim University are gratefully acknowledged. Thanks are due to University Grants Commission, New Delhi for financial support to two of the authors (M. S. and T. A.). The authors are also thankful to DST(FIST) and UGC-DRS for providing lab facilities.

REFERENCES

1. Barondes, S. H., Castronova, V., Cooper, D. N. W., Cummings, R. D., Drickamer, K., Feizi, T., Gitt, M. A., Hirabayashi, J., Huges, C., Kasai, K., Leffler, H., Liu, F., Lotanm, R., Mercurio, A. M., Mosingy, M., Pillai, S., Poirer, F., Raz, A., Rigby, P. W. J., Rini, J. W., and Wang, J. L. (1994) *Cell*, **76**, 597-598.

2. Hirabayashi, J., and Kasai, K. (1993) *Glycobiology*, **3**, 294-304.
3. Barondes, S. H. (1997) *Trends Glycosci. Glycotechnol.*, **9**, 1-7.
4. Barondes, S. H. (1984) *Science*, **223**, 1259-1264.
5. Joubert, R., Caron, M., and Bladier, J. (1987) *Dev. Brain Res.*, **36**, 146-150.
6. Kuwabara, I., and Liu, F. T. (1996) *J. Immunol.*, **156**, 3939-3944.
7. Le Marer, N., and Hughes, R. C. (1996) *J. Cell Physiol.*, **168**, 51-58.
8. Yang, R. Y., Hsu, D. R., and Liu, F. T. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 6736-6742.
9. Leffler, H., and Barondes, S. H. (1986) *J. Biol. Chem.*, **261**, 10119-10126.
10. Sparrow, C. P., Leffler, H., and Barondes, S. H. (1987) *J. Biol. Chem.*, **262**, 7383-7390.
11. Abbot, W. M., Hounsell, E. F., and Feizi, T. (1988) *Biochem. J.*, **252**, 283-287.
12. Ahmed, H., Allen, H. J., Sharma, A., and Matta, K. L. (1990) *Biochemistry*, **29**, 5315-5319.
13. Ahmed, H., and Gabius, H. J. (1989) *J. Biol. Chem.*, **264**, 18673-18678.
14. Liao, D. I., Kapadia, G., Ahmed, H., Vasta, G. R., and Herzberg, O. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 1428-1432.
15. Lobsanov, Y. D., Gitt, M. A., Leffler, H., Barondes, S. H., and Rini, J. M. (1993) *J. Biol. Chem.*, **268**, 27034-27038.
16. Borne, Y., Bilgiano, B., Lio, D. I., Strecker, G., Cantau, P., Herzberg, O., Feizi, T., and Combillau, C. (1994) *Nature Struct. Biol.*, **1**, 863-870.
17. Ola, M. S., Shahwan, M., and Banu, N. (2001) *J. Biol. Mol. Biol. Biophys.*, **5**, 1-9.
18. Laemmli, U. K. (1971) *Nature (London)*, **227**, 680-685.
19. Nawak, T., Kobilen, D., Roel, L., and Baronde, S. H. (1997) *J. Biol. Chem.*, **252**, 6026-6030.
20. Kasai, K., and Hirabayashi, J. (1996) *J. Biochem. (Tokyo)*, **119**, 1-8.
21. Bladier, D., Joubert, R., Adalid, V. A., Kememy, J. L., Doirrel, C., Amouroux, J., and Caron, M. (1989) *Arch. Biochem. Biophys.*, **269**, 433-439.
22. Bladier, D., le Coer, J. P., Joubert, R., Caron, M., and Rossier, J. (1991) *Neurochem. Int.*, **18**, 275-281.
23. Hynes, M., Buck, L. B., Gitt, M., Barondes, S. H., Dodd, J., and Jessell, T. M. (1988) in *Foundation Symposium*, London, Willey, New York, pp. 189-218.
24. Iglesias, M. M., Rabinovich, G. A., Ianovic, V., Sotomator, C., and Wolfenstein-Todel, C. (1998) *Eur. J. Biochem.*, **252**, 400-407.
25. Hirabayashi, J., and Kasai, K. (1988) *J. Biochem.*, **104**, 1-4.
26. Southan, C., Aitken, A., Childs, R. A., Abbott, W. M., and Feizi, T. (1987) *FEBS Lett.*, **214**, 301-304.
27. Clerch, L. B., Whitney, P., Hass, M., Brew, K., Miller, T., Werner, R., and Massaro, D. (1988) *Biochemistry*, **27**, 692-699.
28. Oyama, Y., Hirabayashi, S. H., Oda, Y., Ohno, S., Kawasaki, H., Suzuki, K., and Kasai, K. I. (1986) *Biochem. Biophys. Res. Commun.*, **134**, 51-56.
29. Wilson, T. J. G., Firth, M. N., Powell, J. T., and Harrison, F. L. (1989) *Biochem. J.*, **261**, 847-852.
30. Parataud, P., Levi, G., Teichberg, V. I., and Stroberg, A. D. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6345-6348.
31. Freskgard, P. O., Martenson, L. G., Jonason, P., Jonason, B. H., and Carlson, U. (1994) *Biochemistry*, **33**, 14281-14288.
32. Levi, G., and Teichberg, V. I. (1981) *J. Biol. Chem.*, **256**, 5735-5740.