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Galectin-1 from Bovine Spleen: Biochemical Characterization, Carbohydrate Specificity and Tissue-Specific Isoform Profiles[1]

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Selected biochemical properties, including the charge heterodispersity profile and carbohydrate specificity, of bovine galectin-1 were determined in detail. The lectin was purified through an improved purification protocol that yielded 35-40 mg/kg of wet tissue with a specific activity of 1.7-2×10⁴ mg^{-1}·ml. The galectin is a homodimer of approximately 14.5 kDa subunits with $E_{280}^{mg/ml}$ of 0.65 ml·mg^{-1}·cm^{-1}. When stored in the presence of its carbohydrate ligand, the lectin's binding activity remained stable in a non-reducing environment even at room temperature. The optimal pH for binding to the ligand was 6.5-8.0. The overall carbohydrate specificity of the bovine galectin-1 isolated from spleen is similar to that of the galectin isolated from heart and to other mammalian galectins that exhibit "conserved" (Type I) carbohydrate recognition domains (CRDs) [Ahmed, H. and Vasta, G.R. (1994) *Glycobiology* 4, 545-549], but differs from those from *Xenopus laevis* and rat intestine domain I. The fluorescence of 4-methylumbelliferyl α-D-galactopyranoside was quenched on binding to bovine spleen galectin-1. Scatchard plots of data obtained at 5, 15, and 30°C showed that the galectin has two sugar exothermic binding sites with association constants of 3.4×10⁵, 1.0×10⁶, and 0.3×10⁵, respectively. Chemical modification studies indicated that histidine, tryptophan, carboxylic acid, and arginine, but not lysine or tyrosine, are involved in the binding to the carbohydrate ligand. On isoelectric focusing, the spleen galectin-1 appeared as six isoforms ranging from pI 4.56-4.88 with main components at pI 4.63 (34.0%), 4.73 (42.6%), and 4.88 (16.6%). The galectin-1 isolated from heart yielded a quali- and quantitatively different profile with four isoforms ranging from pI 4.53-4.73, those with pIs of 4.56, 4.63, and 4.73 being common to the spleen homolog. Edman degradation of selected peptides purified from the spleen galectin-1 digest revealed amino acid sequences identical to those obtained for the heart galectin-1. This suggests that although point mutations in the subunit primary structure may not be the likely source of isolectins, as observed for *X. laevis*, tissue-specific co- or post-translational modifications may be the possible cause of the differences in the galectin isoform profile between bovine spleen and heart.

Key words: bovine spleen, carbohydrate specificity, conserved CRD, galectin-1, isoform.

Galectins are S-type β-galactosyl binding lectins that require a reducing environment but do not require divalent cations for their binding activity (*1*). The primary structures of a considerable number of galectins are currently available (*2*), and support the view that most galectins constitute a defined family of proteins with a substantial degree of similarity. Furthermore, the three-dimensional structures of a limited number of galectins (*3-5*) have been resolved, providing reliable information on the amino acid residues that interact with a carbohydrate ligand, and determine the architecture of the binding site and the nature of the bonds established. However, because most galectins have not yet been crystallized, the relative inhibitory abilities of various mono- or oligosaccharides still remain as a valuable source of information on their carbohydrate specificities, particularly with regards to the orientation of hydroxyls at positions critical for binding (*6-9*). Moreover, methods based on the chemical modification of amino acids (*10*) have provided insight into the nature of the amino acid residues that are directly involved in ligand binding.

Galectins have been classified into several subgroups

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Abbreviations: CRD, carbohydrate recognition domain; ASF, asialofetuin; HRP, horseradish peroxidase; ABTS, diammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate); PBS, phosphate-buffered saline (0.01 M Na_2HPO_4/0.15 M NaCl/0.01% NaN_3, pH 7.5); PBS/ME, PBS containing 0.01 M 2-mercaptoethanol; PBS(1:10), PBS diluted 10-fold with water; CAM-G, galectin reduced and carboxamidomethylated on a solid phase under mild conditions (see "EXPERIMENTAL PROCEDURES"); RP-HPLC, reversed phase microbore HPLC; Gal αOUmbMe, 4-methylumbelliferyl α-D-galactoside.

["proto," "tandem," and "chimera" (*2*); galectins-1-8 (*1*)] based on their primary structures and subunit architecture. Although all members of the galectin family bind lactose/*N*-acetyllactosamine, limited diversity exists in the carbohydrate specificity (*6, 8, 9, 11-13*). Based on the differences in specificity and the conservation of amino acid residues in their carbohydrate recognition domains (CRDs) that interact with a carbohydrate ligand, we have classified galectins into two types: "conserved" (Type I) and "variable" (Type II) (*13*). Most of the lectins in the galectin-1 group have "conserved" CRDs and exhibit very similar carbohydrate specificities (*6, 8, 9, 13*). The CRDs of lectins grouped as galectins-2, -3, -4, -5, -7, and -8 may have deletions or replacements at the relevant aforementioned amino acid sequence positions and are different from the "conserved" group in their carbohydrate specificities (*11-13*). Therefore, this classification may reflect not only common features of their carbohydrate specificities, but also possibly evolutionary aspects of their recognition functions.

The biological role(s) of galectins remain unclear but experimental evidence suggests that they mediate cell-cell and cell-extracellular matrix interactions that occur in tissue development (*14*), inflammation (*15*), apoptosis (*16*), and tumor metastasis (*17*). The developmental expression —and possibly function— of a particular galectin may vary from tissue to tissue (*18*), and extracellular polylactosaminoglycans, such as laminin, have been proposed as the major ligands (*14, 19*). While galectin-1 is believed to play a role in the embryogenesis in mammals and an amphibian, *Bufo arenarum*, galectin from another amphibian, *Xenopus laevis*, is probably involved in host defense, because the lectin is expressed abundantly in skin, poorly in muscle, and not at all in the embryo (*20*). Charge heterodispersity has been observed in purified galectins from different species (*11, 21, 22*) and although the biological significance of this observation is unknown, in the case of mouse galectin-3, post-translational modifications were proposed as the cause (*21*). In contrast, the heterodispersity of the *X. laevis* skin galectin was attributed to variability of the primary structure at two positions (amino acid residues 62 and 86) in the derived peptide sequences (*11*). However, no evidence of tissue-specific galectin heterodispersity within one particular species has been reported so far. Among the various groups of galectins examined at present, the galectins-1 from several vertebrate tissues have been extensively studied (*2, 18*), and they show 87-95% amino acid residue identity with each other (*2*). Within the CRD [residues 44-73, the numbers are for bovine spleen galectin-1 (*4*)], the percent identity among the galectins-1 characterized so far ranges from 77 to 93%, and the relevant amino acids that participate in ligand binding are identical.

Galectin activity in bovine tissues was first reported about two decades ago (*22, 23*). Among the several tissues examined (spleen, thymus, liver, and heart) the highest galectin activity was found in spleen (48 mg/kg), followed by heart (16 mg/kg) (*22*). Detailed characterization of the heart galectin, including its carbohydrate specificity and primary structure (*8, 24-26*), and its interaction with poly-*N*-acetyllactosamine and similar carbohydrate chains of laminin were also accomplished (*19, 27*), providing insight into its biological role(s). Calf spleen galectin has been shown to form a homogeneous cross-linked complex with asialofetuin (*28*). Recently, we determined the 3-D structure of bovine spleen galectin-1 complexed with *N*-acetyllactosamine (*4*). The 3-D structure of bovine heart galectin-1 in complex with biantennary saccharides of *N*-acetyllactosamine was reported simultaneously (*5*). The primary structure and carbohydrate specificity of bovine heart galectin-1 indicates that the lectin carries a conserved CRD (*13*).

In order to gain further insight into the possible structure-function relationships among bovine galectins expressed in different tissues, such as spleen and heart, we conducted studies aimed at the further biochemical characterization of the spleen galectin-1, including its detailed carbohydrate specificity and the thermodynamic parameters for the binding to its ligand. Surprisingly, our results suggest that although the bovine galectin-1 from spleen is identical in primary structure and carbohydrate specificity to that isolated from heart, its isoform profiles is tissue-specific and consistent through multiple preparations, suggesting that the presence of distinct galectin-1 isoforms in spleen and heart may be due to co- or post-translational modifications.

EXPERIMENTAL PROCEDURES

Reagents—Ampholine PAG plates for isoelectric focusing, gel permeation chromatography molecular weight standards and ribonuclease A were purchased from Pharmacia. The protein assay reagent was from Bio-Rad. The peroxidase substrate, diammonium 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), was from Kirkegaard & Perry Laboratories. Sequencing grade reagents and solvents for protein sequencing, amino acid analysis, and HPLC were from Applied Biosystems (Division of Perkin-Elmer). Sequencing grade trypsin was from Boehringer-Mannheim. Horseradish peroxidase (HRP), DEAE-Sepharose, sugars for fluorescence quenching studies and reagents for chemical modification studies were from Sigma Chemical. All other reagents were of the highest grade commercially available. Asialofetuin (ASF) was prepared according to Vasta and Marchalonis (*29*), and conjugated with CNBr-activated Sepharose according to the Pharmacia protocol. Lactosyl-Sepharose was made by coupling lactose with Sepharose CL 6B (Pharmacia) through divinyl sulfone (*30*).

Purification of Bovine Spleen and Heart Galectin-1—Fresh bovine spleens and hearts were obtained from a local slaughterhouse. In a typical purification experiment the tissue (110 g) was cut into small pieces and then homogenized with a Ultra-Turrax T 50 (Janke & Kunkel, IKA Labortechnik) homogenizer in cold (4°C) phosphate-buffered saline (diluted 1:10)/0.01 M 2-mercaptoethanol/0.1 M lactose [PBS (1:10)/ME/Lac] containing 0.1 mM phenylmethylsulfonyl fluoride (2 ml buffer/g wet tissue). The homogenate was centrifuged 27,000 × *g* for 1 h at 4°C, and the clear supernatant was mixed with DEAE-Sepharose (10 ml supernatant/1 ml resin) pre-equilibrated with PBS (1:10)/ME. After gentle mixing for 1 h at 4°C, the slurry was transferred to a fritted glass funnel, and the resin was washed with 10 bed volumes of cold PBS (1:10)/ME to remove lactose and unbound protein. The bound proteins were eluted with 500 ml of PBS/ME/0.002 M

EDTA/0.5 M NaCl. The eluate was adsorbed on a column of lactosyl-Sepharose or ASF-Sepharose pre-equilibrated with PBS/ME/0.002 M EDTA/0.5 M NaCl. The column was washed until the absorbance reached the baseline with equilibrating buffer followed by 5 bed volumes of PBS (1:10)/ME, and the bound protein was eluted with 0.1 M lactose in PBS (1:10)/ME. The fractions containing protein were pooled, and then aliquots were absorbed on DEAE-Sepharose columns (0.5 ml bed volume), overlaid with 50% glycerol in eluting buffer and stored at −20°C.

Analytical Procedures—Agglutination testing of the untreated (in PBS/ME) or chemically modified (in PBS) galectin was carried out on BSA-coated 96-well Terasaki plates (Robbins Scientific, Mountain View, CA) with glutaraldehyde-fixed protease-treated rabbit erythrocytes (*31*). Protein concentrations were determined on 96-well flat bottom plates with the Bio-Rad Protein Assay using BSA as a standard as described elsewhere[4]. In fluorescence studies, galectin concentrations were determined from the absorbance at 280 nm (0.65 $ml \cdot mg^{-1} \cdot cm^{-1}$). Analytical polyacrylamide slab gel electrophoresis in the presence of sodium dodecylsulfate (2%) was carried out on 15% (w/v) acrylamide gels under reducing conditions as reported elsewhere (*32*). The native molecular size of bovine spleen galectin-1 was determined by non-denaturing PAGE (3-27%) gel electrophoresis in 0.09 M Tris/0.08 M boric acid/0.0026 M EDTA (TBE) buffer (pH 8.3) according to the manufacturer (Jule, New Haven, CT). The sample was prepared in a non-denaturing sample buffer [TBE (diluted 1:10)/10% glycerol/0.01% Bromophenol Blue] and electrophoresed at a constant voltage (70 V) for 3 h. Gel permeation chromatography of the native galectin, galectin-HRP conjugate, and reduced and carboxamidomethylated galectin was carried out on a Pharmacia Superose 6 or 12 column (1 × 30 cm) as described elsewhere[4] (*31*).

Stability—The temperature stability of bovine spleen galectin-1 was determined by incubating 100 μl samples in PBS/ME (33 μg/ml) at various temperatures for 30 min, cooling them on wet ice and then titrating them against glutaraldehyde-fixed protease-treated rabbit RBC (*29*). To determine the stability of bovine spleen galectin-1, the purified lectin (100 μg) was absorbed on 1 ml each of lactosyl-Sepharose and asialofetuin-Sepharose, and then each matrix was thoroughly washed with aerated PBS (20 ml) and stored at 8°C. Control matrices contained the same amount of lectin in PBS/ME. After 30 days, the lactosyl-Sepharose and asialofetuin-Sepharose columns were eluted with 2 ml of PBS/ME/0.1 M lactose, and the eluates were dialyzed against PBS/ME in the presence of 2 mg of BSA. The hemagglutinating activity was measured with glutaraldehyde-fixed protease-treated rabbit RBC. In another set of experiments, 50 μg of active lectin (in 50 μl of PBS) was dialyzed against aerated PBS at room temperature for 18 h. An equal amount of lectin was blocked with 0.1 M lactose in 50 μl of PBS and then dialyzed against aerated PBS/0.1 M lactose at room temperature for 18 h. The control had the same amount of lectin in 50 μl of PBS/ME and was dialyzed against PBS/ME at room temperature for 18 h.

Characterization of the Carbohydrate Specificity—Preparation of the galectin-HRP conjugate: The purified bovine spleen galectin was carboxamidomethylated with iodoacetamide on a solid phase under mild conditions in the presence of excess ligand (0.1 M iodoacetamide/0.1 M lactose at 4°C for 1 h in the dark) as reported elsewhere[4], yielding carboxamidomethylated galectin (CAM-G). Unlike conventional methods (see "Sequencing of tryptic peptides"), this carboxamidomethylation procedure maximizes the retention of the lectin's carbohydrate binding activity in non-reducing environments. The CAM-G was conjugated to HRP through glutaraldehyde coupling as follows: to a mixture of CAM-G (1.8 mg) and HRP (4.0 mg) in 2.0 ml of PBS (azide-free)/0.5 M NaCl/0.1 M lactose, 240 μl of 1% glutaraldehyde was added. After overnight incubation at 4°C, the conjugation mixture was diluted 40-fold with cold water and then adsorbed to DEAE-Sepharose (0.5 ml) pre-equilibrated with azide-free PBS (1:10). The column was washed to remove lactose, and then the conjugate was eluted with 2 ml of PBS (azide-free)/1 M NaCl and purified by affinity chromatography on lactosyl-Sepharose. Finally, the conjugate was separated from unreacted galectin by gel permeation chromatography on a Superose 6 column as described above, and stored at −20°C in 1% BSA-50% glycerol.

Optimal pH for binding: The optimal pH for bovine spleen galectin binding was determined as follows: 6 ng of galectin-HRP conjugate in 60 μl of water containing 0.1% Tween 20 was mixed with 60 μl of various buffers (0.2 M), and 100 μl of each mix was subjected in triplicate to the binding assay described above. The buffers used were citrate-phosphate, pH 4.0-6.0; phosphate, pH 6.5-8.0; and carbonate-phosphate, pH 8.5-9.5.

Solid phase binding-inhibition assay: Binding of bovine spleen galectin-HRP to asialofetuin and its inhibition by sugars were determined as reported elsewhere[4]. Briefly, ASF (0.5 μg/100 μl /well) in 0.1 M Na_2CO_3/0.02% NaN_3 (pH 9.6) was adsorbed to the wells of microtiter plates (Sumilon) and then incubated at 37°C for 3 h. After aspirating off the residual ASF solution, fixation was carried out with 2% formaldehyde in PBS at 37°C for 30 min. The plates were washed three times with PBS (azide-free)/0.05% Tween 20, and then incubated with the galectin-HRP conjugate (for binding assays) or with pre-incubated mixtures of the conjugate and test ligands (for binding-inhibition assays). The pre-incubation of the galectin-HRP conjugate (12 ng in 60 μl of azide-free PBS-Tween 20 buffer) for binding-inhibition assays was carried out by mixing equal volumes of the conjugate and the test ligand at varying concentrations. After 1 h at 4°C, each conjugate-ligand mixture (100 μl) was added to wells in duplicate and the plates were incubated for 1 h at 4°C. The plates were washed with ice-cold azide-free PBS/Tween 20 buffer and then the bound peroxidase activity was assayed with ABTS. The amounts of galectin-peroxidase conjugate that bound to ASF were determined from a standard curve obtained with increasing amounts of galectin-peroxidase conjugate placed in uncoated wells and developed with ABTS under conditions (reaction volume, time and temperature) identical to those for the binding assay.

Fluorescence titrations: The quenching of the fluorescence of 4-methylumbelliferyl α-D-galactopyranoside (Gal

[4] Ahmed, H., Pohl, J., Fink, N.E., Strobel, F., and Vasta, G.R. The primary structure and carbohydrate specificity of a β-galactosyl-binding lectin from toad (*Bufo arenarum* Hensel) ovary reveal closer similarities to the mammalian galectin-1 than to the galectin from the clawed frog *Xenopus laevis*. *J. Biol. Chem.* (in press).

αOUmbMe) by bovine spleen galectin-1 was monitored with a Perkin-Elmer LS-5B luminescence spectrometer. The excitation was at 318 nm and the emission spectra were recorded above 330 nm. In a temperature-controlled cuvette (1 cm path length), a fixed concentration of lectin (0.87 μM) was mixed with different concentrations of Gal αOUmbMe (0.2–10 μM) at 5, 15, and 30°C, and then the quenching of Gal αOUmbMe fluorescence was recorded. A linear standard curve for Gal αOUmbMe in buffer only was obtained in the same concentration range at the same temperature. The relative fluorescence spectra in the presence of lectin were compared to the standard curve to obtain bound and free sugar, and the data were analyzed (*33*).

Chemical modification of amino acid residues: Chemical modification of the bovine spleen galectin was carried out using CAM-G as described (*10*). For each chemical modification the following controls were introduced: blocking of the galectin binding site with excess carbohydrate ligand (lactose); saturation of the activity of the modifying reagent with excess free amino acid; and substitution of the modifying reagent with an equal volume of buffer. Modification of amino groups of CAM-G was performed with acetic anhydride, whereas lysine residues alone were modified with *O*-methyl isourea. Tyrosine, histidine, and tryptophan residues were modified with tetranitromethane, diethyl pyrocarbonate and 2-hydroxy-5-nitrobenzyl-bromide, respectively. Carboxyl groups of CAM-G were amidated with the methyl ester of α-amino butyric acid in the presence of 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC), and arginine residues were chemically altered with either cyclohexane-1,2-dione or phenylglyoxal. After modification, all experimental samples and controls were mixed with 100 μg of BSA and then dialyzed separately against PBS (pH 7.3). The hemagglutinating activity of the dialyzed experimental samples and controls was assessed with glutaraldehyde-fixed protease-treated rabbit RBC. Fluorescence emission spectra (300–400 nm) of native and modified lectins were recorded with excitation at 280 nm. The CD spectra of the native and chemically modified bovine galectin were measured at 25°C with a Jasco J-720 recording spectrophotometer in a cell of 10 mm pathlength at 200–350 nm.

Isoelectric focusing: Analytical isoelectric focusing was carried out on a thin (1 mm) layer (5% polyacrylamide) Ampholine PAG plate (Pharmacia) (pH range, 4.0–6.5) in an EC 1001 electrophoresis unit (EC Apparatus) according to the manufacturer's instructions. The affinity-purified bovine spleen or heart galectin in PBS/ME/0.5 M NaCl was concentrated on a Centricon 10 (Amicon) and then dialyzed against 0.25% glycine/0.002 M dithiothreitol. The pH gradient was determined by measuring the pHs of the supernatant solutions obtained by grinding 5 mm slices of the gel in 1 ml of distilled water. Gels were fixed with sulfosalicylic acid-trichloroacetic acid-water, stained with 0.1% Coomassie Brilliant Blue R 250 in ethanol-acetic acid-water, destained with ethanol-acetic acid-water, and densitometrically scanned with a BioImage Gel Scanner (Millipore). Galectin isoform pIs were determined from a plot of marker pIs [glucose oxidase (pI 4.2), trypsin inhibitor (pI 4.6), β-lactoglobulin A (pI 5.1), and carbonic anhydrase II (pIs 5.4, 5.9)] *vs.* distance from the cathode.

Sequencing of tryptic peptides: Bovine spleen galectin (1.8 mg in 3 ml of PBS/ME/0.5 M NaCl) was dialyzed against 0.01 M ammonium hydrocarbonate and then freeze-dried. The freeze-dried 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 1.6 ml with water and digested with trypsin (67 μg) at 37°C for 18 h. The tryptic peptides were separated on a narrow bore Vydac C4 column (0.21×15 cm) by RP-HPLC using a Beckman-116 pump and a Beckman programmable detector module-166, with monitoring at 214 nm. Following injection, the column was washed with 100% of solvent A (0.1% trifluoroacetic acid in water) and the peptides were eluted with a linear gradient from 100% solvent A to 40% solvent B (0.08% trifluoroacetic acid in 70% acetonitrile) in 5 min, followed by 40–50% solvent B in 20 min and finally 50–100% solvent B in 5 min. The major peptides (based on detection at 214 nm) were rechromatographed on the same column or on a microbore Aquapore ODS-300 C-18 silica column (0.1×25 cm, d_p ~7 mm, 300 Å pore size; Applied Biosystems), and then sequenced by automated Edman degradation of the peptides (*34*) with an Applied Biosystems model pulsed-liquid 477A/120A sequencing system.

RESULTS

Galectin Purification—Following extraction with diluted PBS, the galectin was first absorbed on DEAE-Sepharose, washed and then eluted with a high salt buffer. The high salt eluate from DEAE-Sepharose was immediately loaded on a lactosyl-Sepharose column, the resin was washed until the absorbance reached the baseline, and the bound protein was eluted with lactose (Fig. 1A). The yield of the purified protein was 35–40 mg/kg of wet spleen with a specific activity of 1.7–2.0×10^4 mg^{-1}·ml (Table I). The galectins purified from spleen and heart gave a single polypeptide corresponding to approximately 14.5 kDa, as assessed by SDS-PAGE under reducing or non-reducing conditions (Fig. 2A). A similar value (14.6 kDa) was obtained on HPLC in 6 M Gdn-HCl (Fig. 1C). On gel permeation chromatography under non-denaturing conditions the spleen galectins appeared as a single peak corresponding to 28.5 kDa (Fig. 1B), suggesting that it is composed of two identical non-covalently linked 14.5 kDa subunits. However, on PAGE under non-denaturing conditions, the galectin mobility corresponded to 32 kDa (Fig. 2B), possibly because of differences in charge densities between the galectin and protein standards. Table II summarizes the physicochemical properties of bovine spleen galectin-1.

Stability—Bovine spleen galectin was considerably stable at high temperatures and retained 6% of the total activity after 30 min at 100°C (Fig. 3A). The stability of the lectin binding activity in a non-reducing environment was examined with the galectin adsorbed on affinity matrices and in solution, with and without its ligand. When adsorbed on lactosyl-Sepharose or asialofetuin-Sepharose in a buffer containing no reducing agent, the galectin retained full activity after 30 days at 8°C. The galectin also retained full activity in the absence of a reducing agent, if maintained in solution in the presence of excess ligand (0.1 M lactose). However, the activity was reduced almost 40-fold in

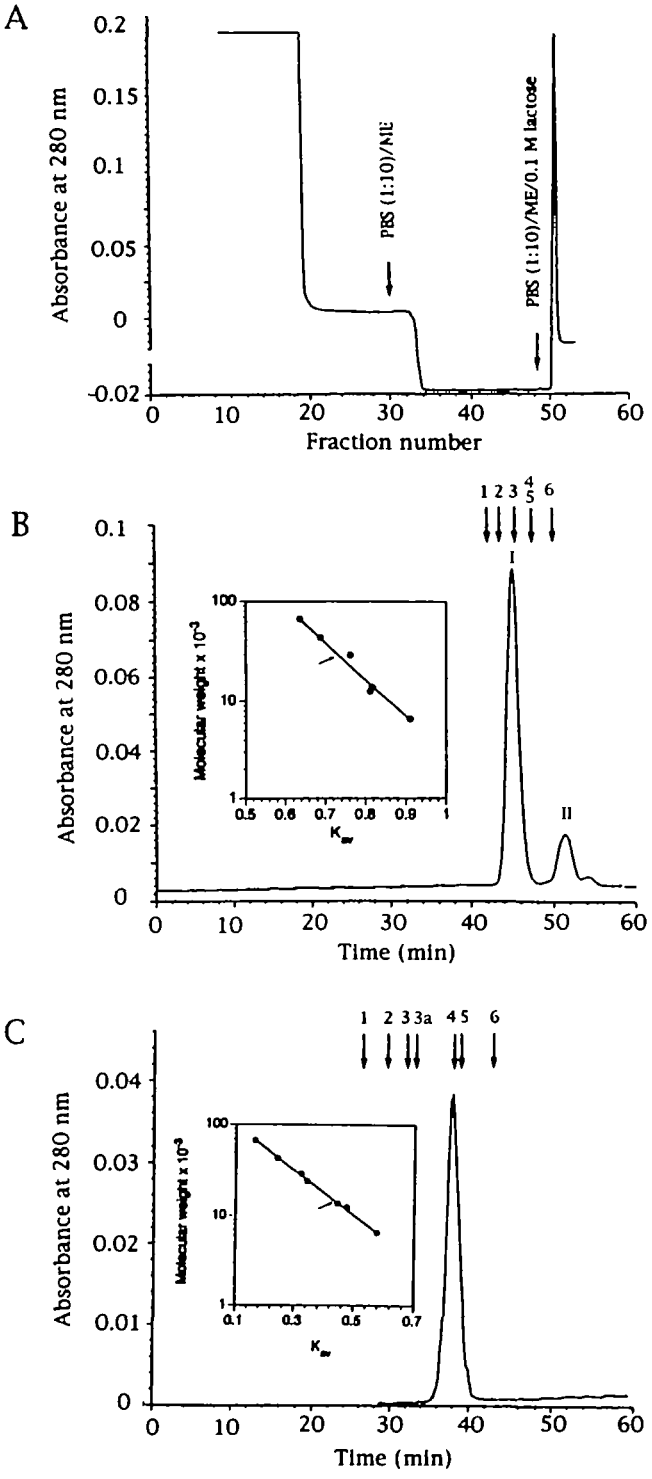

TABLE I. **Purification of bovine spleen galectin-1.** The crude extract was adsorbed on DEAE-Sepharose and then after washing the bed, the bound protein was eluted with high salt and purified on either ASF-Sepharose or lactosyl-Sepharose.

Step	Total protein (mg)	Specific activity[a]	Total activity[b]	Recovery (%)	Purification (fold)
Crude extract-1	3,500	32	112,000	100	1
NaCl eluate (DEAE-Sepharose)	1,456	50	72,800	65	1.6
Lactose eluate (ASF-Sepharose)	3.9	16,667	65,001	58	521
Crude extract-2	3,366	40	134,640	100	1
NaCl eluate (DEAE-Sepharose)	1,645	71	116,795	87	1.8
Lactose eluate (Lac-Sepharose)	4.47	20,000	89,400	66	500

One hundred ten grams of spleen was used for each extract. [a]Specific activity is expressed as titer/mg protein/ml. The titer was determined from the hemagglutination of the galectin with protease-treated fixed rabbit erythrocytes. [b]Total activity = specific activity × total protein.

Fig. 1. **A: Purification of bovine spleen galectin-1 on lactosyl-Sepharose (2.5×4.0 cm).** The column was washed with PBS/ME/0.002 M EDTA/0.5 M NaCl until the absorbance reached the baseline, followed by with 10 bed volumes of PBS (1:10)/ME (indicated by an arrow), and then the bound protein was eluted with 0.1 M lactose in PBS (1:10)/ME (indicated by an arrow). The fractions (each 10 ml) containing bound protein were pooled and stored on DEAE-Sepharose as described under "EXPERIMENTAL PROCEDURES." **B: Gel permeation chromatography of the native galectin (Peak I) on Superose 6 (1×30 cm) equilibrated with PBS/ME/0.25 M NaCl/0.01 M lactose, pH 7.5.** The molecular weight standards were: (1) BSA (66 kDa), (2) ovalbumin (43 kDa), (3) carbonic anhydrase (29 kDa), (4) ribonuclease A (13.7 kDa), (5) cytochrome c (12.4 kDa), and (6) aprotinin (6.5 kDa). Peak II corresponds to lactose. Inset: Estimation of the native molecular weight of the galectin (indicated by an arrow). **C: Gel permeation chromatography of the denatured galectin on Superose 12 (1×30 cm) equilibrated with 6 M guadinium-HCl.** The molecular weight markers were the same as in B plus (3a) chymotripsinogen (24 kDa). Inset: Estimation of the subunit molecular weight of the galectin (indicated by an arrow).

solution in the absence of both a reducing agent and the ligand, and could not be restored by adding reducing agents.

Preparation of the Bovine Spleen Galectin-HRP Conjugate and Optimization of the Solid Phase Galectin Binding Assay—The binding and enzyme activities of the galectin-HRP conjugate were monitored throughout its purification. After the conjugation procedure, approximately 61% of the total protein was recovered through its binding to lactosyl-Sepharose. Gel permeation chromatography on Superose 6 allowed separation of the galectin-HRP conjugate from unreacted galectin (Fig. 4A). Peak I corresponding to approximately 73 kDa represents the galectin-HRP conjugate resulting from equimolar cross-linking of galectin (29.2 kDa) and HRP (approximately 44 kDa), and exhibits both peroxidase and hemagglutinating activity. Fractions corresponding to the ascending slope of peak I were pooled and used for the binding assays. About 16% of the affinity-purified protein was recovered in the active galectin-HRP conjugate pool. In order to optimize the solid phase assay for the characterization of bovine galectin's carbohydrate specificity, the wells of 96-well ELISA plates were coated with varying concentrations (0.01-100 μg/well) of ASF, followed by fixing and extensive washing, and finally the addition of varying concentrations (2.5-40 ng/well) of the galectin-HRP conjugate. Figure 4B shows the binding profile for each conjugate concentration tested: increased binding was observed with increasing amounts of conjugate added for all concentrations of coated ASF. The maximum binding was achieved with approximately 0.5 μg of ASF per well, with absorbance values reaching a plateau beyond that concentration. Figure 4C shows the binding of variable amounts of the conjugate to ASF-coated wells (0.5 μg/100

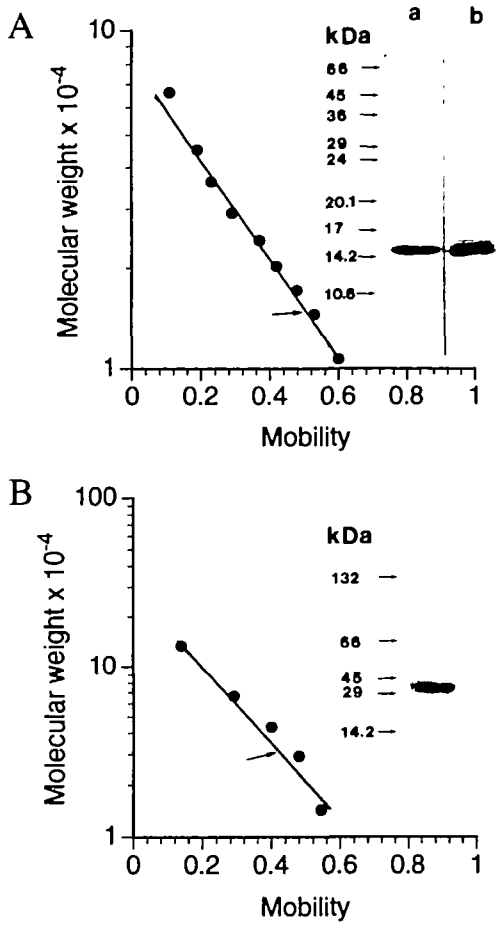

Fig. 2. **Estimation of the (A) subunit (spleen and heart) and (B) native (spleen) molecular weights of the bovine galectin on PAGE.** A: SDS-PAGE of (a) spleen and (b) heart under reducing conditions (0.5% 2-mercaptoethanol). Each sample (2 μg) was loaded on a 15% polyacrylamide gel using a discontinuous buffer system and stained with Coomassie Blue. The standards (Sigma Chemical), from higher to lower molecular weight, were: BSA, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, soybean trypsin inhibitor, myoglobin (1-153), α-lactalbumin, and myoglobin I+III. B: Non-denaturing PAGE in Tris-borate buffer (pH 8.3). The sample (10 μg) was loaded on a 3-27% polyacrylamide gel using a continuous buffer system and stained with Coomassie Blue. The standards, from higher to lower molecular weight, were: BSA dimer, BSA monomer, ovalbumin, carbonic anhydrase, and α-lactalbumin.

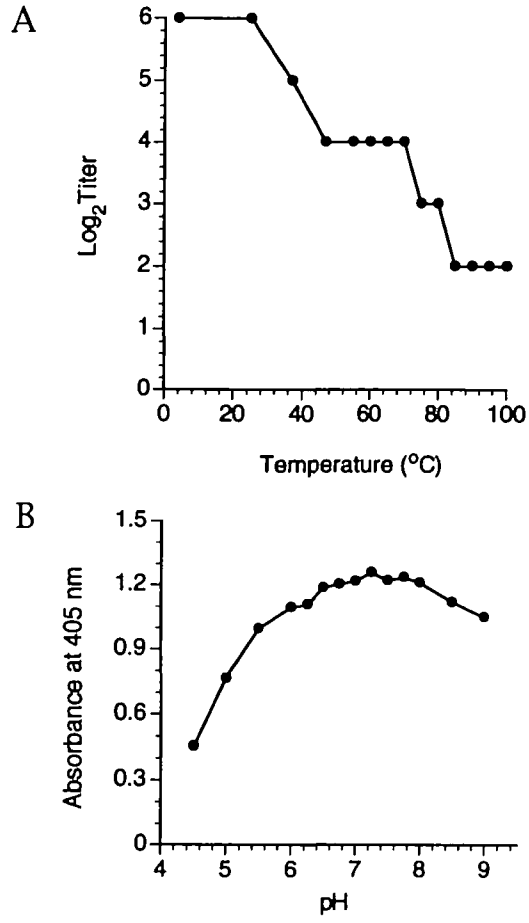

Fig. 3. **A: The thermal stability of the bovine spleen galectin was examined at the temperatures indicated as described under "EXPERIMENTAL PROCEDURES." B: pH-dependent binding of the bovine spleen galectin-HRP conjugate to asialofetuin.** The binding of the galectin-HRP conjugate to ASF was carried out at various pHs as described under "EXPERIMENTAL PROCEDURES."

TABLE II. **Macromolecular properties of bovine spleen galectin-1.**

Physical measurement	Spleen galectin
Subunit molecular weight on SDS-PAGE	
Reduced	14,500
Non-reduced	14,500
Subunit molecular weight on HPLC gel filtration (Reduced and carboxamidomethylated, in 6 M GdnHCl)	14,600
Native molecular weight on non-denaturing PAGE	32,000
Native molecular weight on HPLC gel filtration	28,500
Native molecular weight on sedimentation equilibrium	26,200±450
$E_{280}^{1\,mg/ml}$	0.65 ml·mg^{-1}·cm^{-1}
Excitation wavelength (maximum)	290 nm
Emission wavelength (maximum)	360 nm

μl/well) as a function of time. For all conjugate concentrations tested, the binding was approximately linear for incubation times up to 15 min. From these preliminary results, the optimal amount of ASF for coating the plates was established to be 0.5 μg/100 μl/well, and the amount of the galectin-HRP conjugate to be added for the binding and binding-inhibition experiments to be 10 ng/100 μl/well. The optimal substrate incubation time was determined to be 15 min. Approximately 2 ng of the galectin-HRP conjugate was bound to ASF under the optimal conditions. Although no further blocking of the ASF-coated wells was required, the addition of Tween 20 to the binding and washing buffers substantially reduced the background absorbance (results not shown). Reproducible sigmoid profiles were obtained for inhibition of the binding of the galectin-HRP conjugate to ASF by increasing concentrations of lactose, under the optimal conditions established (Fig. 4D). The lactose concentration required for 50% inhibition of the galectin-HRP conjugate binding to ASF varied from 64-86 μM. The binding activity of bovine spleen galectin as to ASF was optimum at pH 6.5-8.0 (Fig. 3B). The activity decreased drastically below pH 6.0 and thus all further experiments were carried out at pH 7.5.

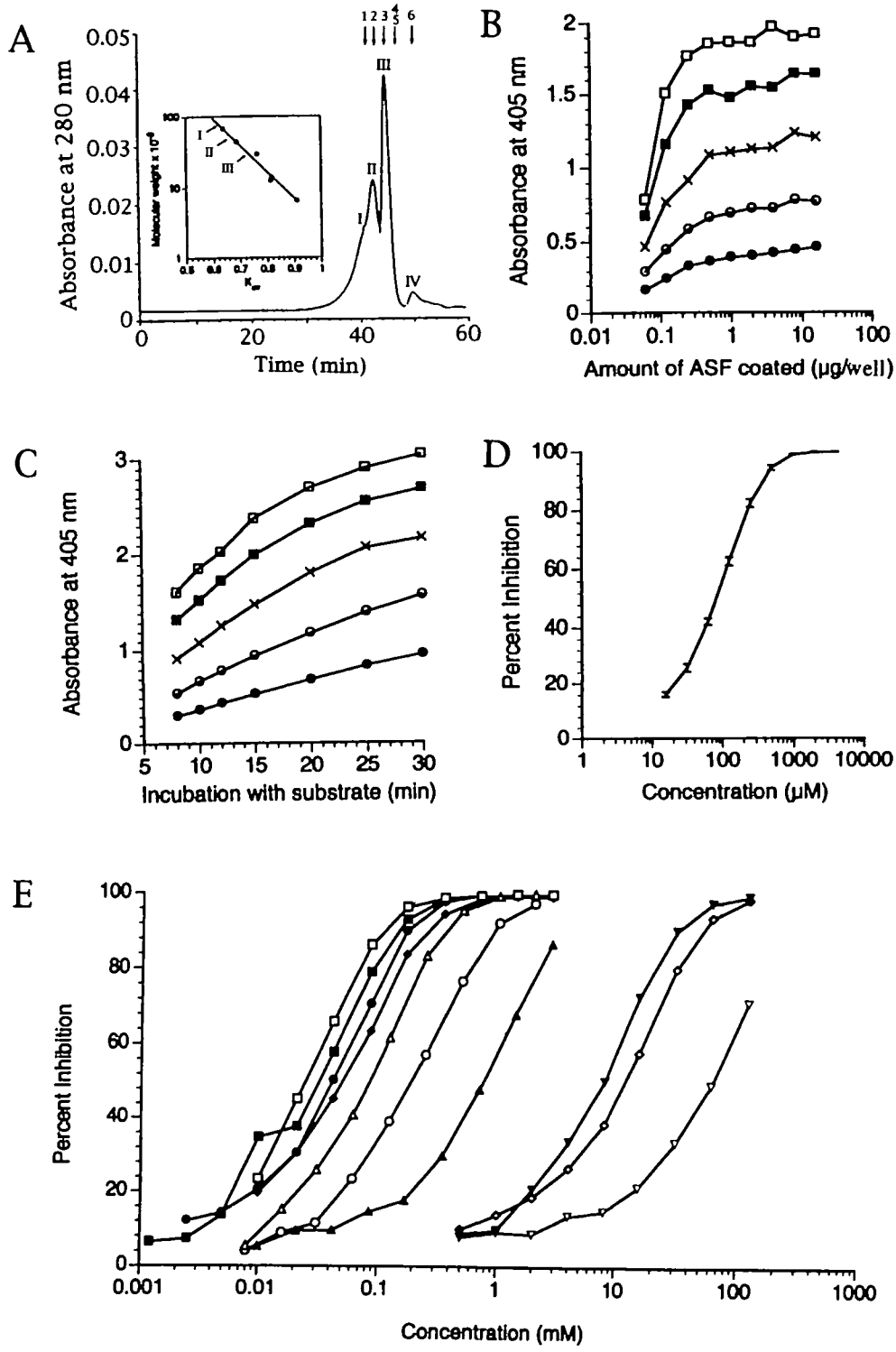

Fig. 4. **A: Elution profile of the bovine spleen galectin-peroxidase conjugate on Superose 6 (1×30 cm) in PBS (azide-free)/0.25 M NaCl/0.01 M lactose, pH 7.5 (flow rate, 0.4 ml/min).** I, the conjugate; III, unreacted galectin. The calibration markers were the same as in Fig. 1B. Inset: Estimation of the molecular weights of the conjugate (I) and the unreacted galectin (III). **B, C: Binding of various concentrations of the bovine spleen galectin-HRP conjugate (2.5-40 ng/100 µl/well) to (B) various amounts of ASF (0.015-16 µg/100 µl/well) for 20 min of development by substrate and (C) a fixed amount of ASF (0.5 µg/100 µl/well) for variable times of development by substrate;** ●, 2.5; ○, 5 ng; ×, 10 ng; ■, 20 ng; and □, 40 ng. **D: Reproducibility of lactose inhibition curves ($n=11$) for the galectin-HRP conjugate.** Error bars represent the inhibition values for n determinations. Each value represents the average of two wells. The inhibition of galectin-HRP conjugate binding to ASF by various concentrations of lactose was determined by a solid phase assay as described under "EXPERIMENTAL PROCEDURES." **E: Representative curves for the inhibition of galectin-HRP binding to ASF** for: ●, Galβ1,4Fruf; ○, Fucα1,2Galβ1,4Glc; ■, Galβ1,3Ara; □, MeOGalβ1,4Glc; △, Galβ1,4Glc; ◆, Galβ1,4Glcβ-OMe; ▲, Galβ1,6GlcNAc; ◇, Gal; ▽, GalNAc; and ▼, Galα-OMe.

Carbohydrate Specificity—The carbohydrate specificity of the bovine spleen galectin was determined by analyzing the binding of the galectin-HRP conjugate to ASF in the presence of several saccharides using the solid phase assay, under the optimal conditions established (Fig. 4E). Preliminary studies with this method for selected carbohydrate ligands yielded a relative inhibitory activity profile identical to that obtained through hemagglutination-inhibition assays with the unmodified bovine spleen galectin, indicating that the specificity of the galectin was not modified by the conjugation procedure. This validation of the solid phase method developed allowed comparisons of the relative inhibitory activities of carbohydrates tested with those estimated by other well established methods that involve unmodified (*9*) or radiolabeled (*6*) galectins. For each test saccharide, a complete inhibition curve was determined,

TABLE III. **Inhibition of bovine spleen galectin-1 binding to asialofetuin by a series of saccharides expressed as I_{50} of each compound relative to lactose I_{50}.**[a] Inhibition of the binding of the bovine spleen galectin-HRP conjugate to asialofetuin was examined for several saccharides. For each test saccharide, the molar concentration that inhibited the binding of the lectin conjugate to ASF by 50% (I_{50}) was calculated and normalized with respect to the lactose standard.

Saccharide	Relative inhibitory activity
Galβ1,4Glc	1.0
Galβ1,4Glcβ-OMe	1.3
Galβ1,4Glcβ-OPhNO$_2$(p)	1.5
Galβ1,4Glcβ-OPhNH$_2$(p)	1.5
Galβ1,4Glcβ-OPhNH$_2$(p)-ANS[b]	1.1
Galβ1,4Glcβ-OUmbMe	0.9
Galβ1,4Glcβ-OPhITC	0.4
Galβ1,4GlcNAc	5.5
Galβ1,3GlcNAc	2.4
Galβ1,3GlcNAcβ-OPhNO$_2$(p)	3.4
Galβ1S1βGal	5.9
Galβ1,3Ara	2.6
Galβ1,4Man	2.3
Galβ1,4Fru*f*	1.9
Galβ1,6GlcNAc	0.1
Galβ1,3GalNAc	<0.02[c]
Galα1,4Gal	<0.02
Galβ1,6Gal	<0.03
MeO-2Galβ1,4Glc	4.1
Fucα1,2Galβ1,4Glc	0.5
Fucα1,2[GalNAcα1,3]Galβ1,4Glc	0.2
NeuAcα2,3Galβ1,4Glc	0.4
Glcβ1,4Glc	<0.003
Galβ1,4Galβ1,4Glc	<0.05
NeuAcα2,6Galβ1,4Glc	<0.1
GlcNAcβ1,6Galβ1,4Glc	<0.04
Fucα1,3[Galβ1,4]Glc	<0.04

[a]Lac I_{50} varied from 64–86 μM in 11 experiments. [b]Galβ1,4Glcβ-OPhNH$_2$(p)-ANS was synthesized from (p) amino phenyl β-D-lactoside and 4-azido naphthalene sulfonyl chloride (Ahmed *et al.*, unpublished results). [c]50% inhibition of binding was not achieved at the highest concentration tested.

and the molar concentrations that inhibited the binding of the lectin conjugate to ASF by 50% (I_{50}) were calculated and normalized with respect to the lactose included on each plate as a standard (Table III). The test saccharide I_{50} values relative to galactose, the non-reducing terminal monosaccharide common to all, are summarized in Table IV. The inhibitory effectiveness of Galβ1,4GlcNAc and thiodigalactoside was about 6-fold higher than that of lactose, but the human blood group A tetrasaccharide, Fucα1,2[GalNAcα1,3] Galβ1,4Glc, was 5-fold less effective than lactose, and the effect of Galβ1,3GalNAc negligible (Table III). Therefore, the overall binding-inhibition pattern of the bovine spleen galectin suggests a specificity very similar to those observed for mammalian 14 kDa lectins exhibiting conserved (Type I) CRDs (*13*), but rather different from those of rat intestine RI36-I (*12*) and clawed frog *X. laevis* (*11*) with variable (Type II) CRDs (*13*). GalαOUmbMe and GalαOPhNO$_2$(p) were 15.5 and 10.6-fold better inhibitors than Gal (Table IV), possibly due to hydrophobic interaction between the galectin and the aglycon of the α-linked monosaccharide, but Galβ1,4GlcβO-PhNO$_2$(p), Galβ1,4GlcβO-PhNH$_2$(p), Galβ1,4GlcβO-PhNH$_2$(p)-ANS, and Galβ1,4GlcβO-UmbMe were almost as effective as lactose (Table III).

Thermodynamic Studies—The fluorescence of Gal αOUmbMe was quenched upon the addition of the bovine spleen galectin-1 (Fig. 5A). The binding was confirmed to be specific for the Gal moiety because (a) quenching could be reversed by the addition of lactose and (b) no quenching was observed with 4 methylumbelliferyl α-L fucoside (data not shown). Interestingly, the fluorescence of 4 methylumbelliferyl β-D lactoside (Galβ1,4GlcβO-UmbMe) was not quenched by the lectin (data not shown), although it was as inhibitory as lactose (Table III). The fluorescence quantum yield of Gal αOUmbMe bound to lectin was obtained graphically as the relationship:

$$F_o/(F_o-F)=q/(q-q')K_a[P]+q/(q-q')$$

where F_o and F are the measured fluorescence of sugar alone and in the presence of lectin at concentration [P], respectively, and q and q' are the quantum yields of Gal αOUmbMe and its complex with the lectin (*35*). The quantum yield of Gal αOUmbMe bound to the lectin was obtained by extrapolating a plot of $F_o/(F_o-F)$ *vs.* $1/[P]$ to $1/[P]=0$. The plot gave a straight line with a intercept on the ordinate, $F_o/(F_o-F)=0$, resulting in a quantum yield of zero (Fig. 5B), thereby indicating that the fluorescence quantum yield can be used as a measure of ligand binding to lectin on titration. Scatchard plots obtained from the results of fluorescence-quenching by various concentrations of Gal αOUmbMe of a fixed concentration of lectin at 5, 15, and 30°C are shown in Fig. 5C. The number of binding sites determined at each of the three temperatures was 2 per molecule. The first portion of each curve (Fig. 5C) may result from positive cooperativity, as observed in the binding of human spleen galectin-1 with B-lymphoblastoid cells (*36*). The association constants (K_a) obtained from these plots were 3.4×10^5 M^{-1} at 5°C, 1.0×10^5 M^{-1} at 15°C, and 0.3×10^5 M^{-1} at 30°C. The association constant decreased with the increase in temperature suggesting that the lectin-sugar interaction is exothermic. Free energy (ΔF), enthalpy (ΔH), and entropy (ΔS) were calculated from the K_a values as follows: $\Delta F=-RT\ln K_a$, where $R=$ gas constant and $T=$absolute temperature. ΔH was determined from two temperatures of the van't Hoff plot (Fig. 5D), according to the following equation:

$$\Delta H=\frac{4.576\ (\log K_2-\log K_1)}{1/T_1-1/T_2}$$

where K_1 and K_2 are the association constants at temperatures T_1 and T_2, respectively. ΔS was obtained with the

TABLE IV. **Inhibition of bovine spleen galectin-1 binding to asialofetuin by a series of saccharides expressed as I_{50} of each compound relative to galactose I_{50}.**[a]

Saccharide	Relative inhibitory activity
Gal[b]	1.0
Galα-OMe	1.2
Galα-OPhNO$_2$(p)	10.6
Galα-OUmbMe	15.5
Galβ-OMe	0.5
Galβ-OPhNO$_2$(p)	2.7
GalNAc	0.1
MeO6-Gal	<0.1
Galα1,6Glc	0.7
Galα1,6Glcβ1,2Fru*f*	1.0
Galα1,6Galα1,6Glcβ1,2Fru*f*	1.2
Galβ1,4Glc	135.0

[a]Gal I_{50} was 11.6 mM. [b]All sugars are in the D form.

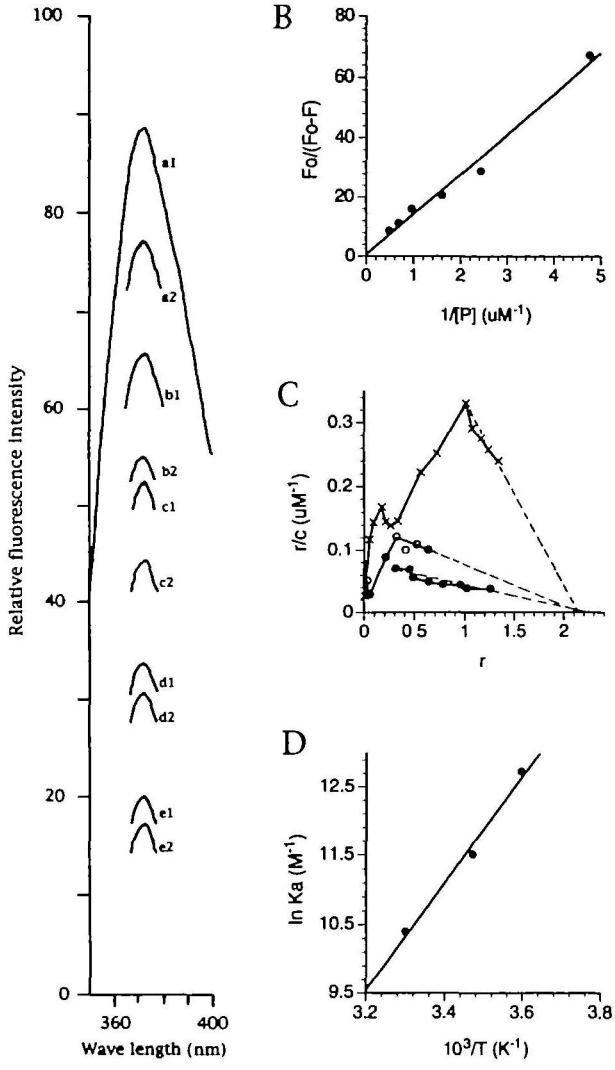

Fig. 5. **A: Fluorescence emission spectra of GalαOUmbMe in the (a1-e1) absence and (a2-e2) presence of bovine spleen galectin-1.** The samples were excited at 318 nm with 5-nm slits in both the monochromators in a Perkin Elmer LS-5B spectrofluorometer. The fluorescence of (a1), 1.17 μM; (b1), 2.1 μM; (c1), 3.46 μM; (d1), 4.58 μM, and (e1), 6.77 μM sugar was quenched to a2-e2, respectively, in the presence of 0.87 μM bovine spleen galectin-1. **B: Quantum yield of GalαOUmbMe bound to bovine spleen galectin-1. C: Scatchard plot for the binding of GalαOUmbMe to bovine spleen galectin-1.** The binding site values were obtained from the intercept on the *x*-axis, and association constants were determined from the slope. ●, 5°C; ○, 15°C; ×, 30°C. **D: van't Hoff plot for the binding of GalαOUmbMe to bovine spleen galectin-1.**

TABLE V. **Association constants and thermodynamic parameters for the binding of GalαOUmbMe to bovine spleen galectin-1.**

Temperature (°C)	K_a (M^{-1})	ΔF (kJ·mol^{-1})	ΔH (kJ·mol^{-1})	ΔS_U (J·mol^{-1}·K^{-1})
5	3.4×10^5	−29.4		56.6
15	1.0×10^5	−27.5	−15.9	48.3
30	0.3×10^5	−26.2		42.0

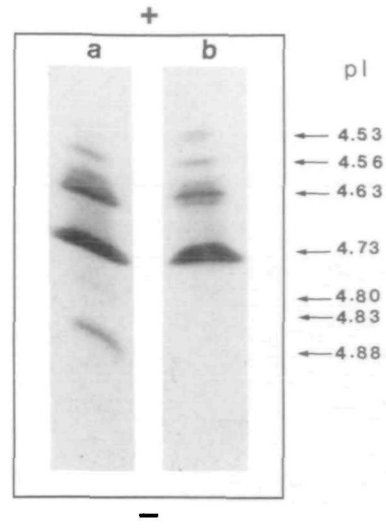

Fig. 6. **Isoelectric focusing of the (a) bovine spleen and (b) bovine heart galectins-1.** The experiment was carried out on a Ampholine PAG plate (pH 4-6.5) as described with "EXPERIMENTAL PROCEDURES."

TABLE VI. **Isolectin profiles of bovine spleen and heart galectins-1.** *Analytical isoelectric focusing was carried out on an Ampholine PAG plate (Pharmacia), and the pI of each isolectin was determined from a plot of marker pIs vs. their distances from the cathode, and quantitated densitometrically.*

pI	Percentage	
	Spleen	Heart
4.53	–	2.8
4.56	5.5	7.2
4.63	34.0	36.5
4.73	42.6	53.5
4.80	0.31	–
4.83	1.0	–
4.88	16.6	–

equation, $\Delta F = \Delta H - T\Delta S$. The entropy values were converted into unitary entropy changes by adding 7.98 to the entropy change values (*37*). The calculated ΔF, ΔH, and ΔS values are summarized in Table V. The free energy values of lectin binding to Gal αOUmbMe are negative at the three temperatures, indicating the spontaneous binding. The negative enthalpy suggests that the binding is exothermic in nature and thereby strengthens the binding at low temperature. ΔS_U was calculated as the sum of ΔS_L, ΔS_{WL}, and ΔS_{WP}, where ΔS_L represents the entropy change for ligand molecules, ΔS_{WL} the entropy change for the stripping of water molecules from the ligand molecules, and ΔS_{WP} the entropy change for the stripping of water molecules from the protein molecules. The positive value of ΔS_U obtained indicates strong binding between the lectin and sugar. In the event of protein-ligand interaction, the water molecules that were initially attached to the ligand or protein molecules are detached to join the bulk solvent, resulting in an increase in randomness. Therefore, ΔS_{WL} and ΔS_{WP} should contribute to the positive entropy values, and the net change in entropy (ΔS_U) reflects the strength of the binding. For the bovine spleen galectin, $\Delta S_{WL} + \Delta S_{WP}$ surpasses the negative effect of ΔS_L.

Chemical Modification—Treatment of CAM-G with

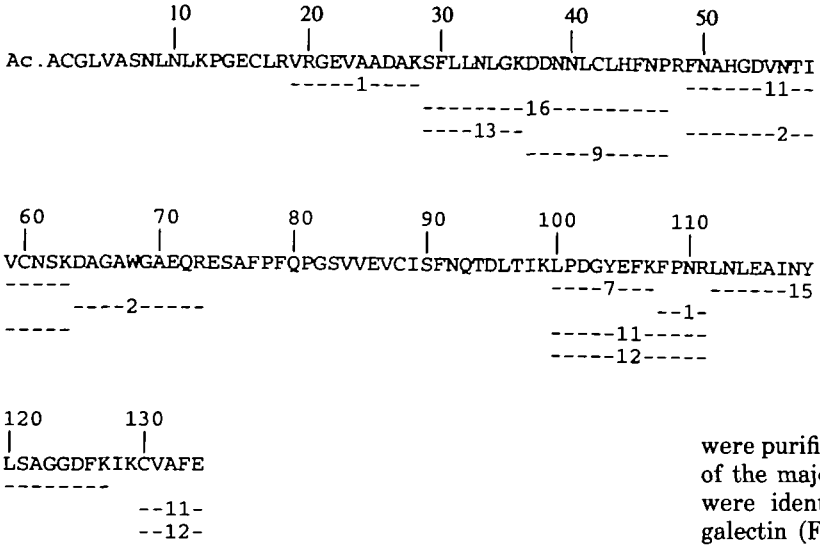

Fig. 7. **The partial amino acid sequence of the bovine spleen galectin.** The complete sequence for bovine heart galectin-1 is given with the residue numbers. The sequences for spleen galectin-1 peptides and their overlaps are shown. The peptide numbers were assigned based on their order of elution from a C4 column as described under "EXPERIMENTAL PROCEDURES."

diethyl pyrocarbonate, that reacts predominantly with histidine residues, resulted in a 92% loss of binding activity. In the presence of 0.1 M lactose or 0.17 M histidine, almost full activity was retained, suggesting the involvement of histidine residues in the sugar binding site. The modification of tryptophan and carboxylic acid by 2-hydroxy 5-nitrobenzyl bromide and methyl ester of α-aminobutyric acid, respectively, resulted in a partial (64 and 68%, respectively) loss of binding activity, suggesting the participation of tryptophan and carboxylic acid in sugar binding. Arginine residues are also important for sugar-binding, because the treatment of the galectin with cyclohexane-1,2-dione or phenyl glyoxal resulted in a partial (94 and 42%, respectively) loss of binding activity. The modification of lysine and tyrosine residues did not affect the binding activity, suggesting that these amino acids are not involved in sugar recognition.

Isoelectric Focusing—Despite the apparent subunit size homogeneity observed with both methods (SDS-PAGE and gel permeation chromatography), the purified bovine galectin-1 exhibited considerable heterodispersity on isoelectric focusing. The spleen galectin-1 gave a reproducible profile comprising unequal proportions of six isoforms ranging from pIs of 4.56 to 4.88 (Fig. 6a). The percentages of bovine spleen isolectin, as estimated on scanning in the visible wavelength range (Table VI), revealed three prevalent isoforms of pIs 4.63, 4.73, and 4.88. Surprisingly, the heart galectin-1, purified in parallel with the spleen equivalent, showed different isoform profiles (Fig. 6b) with two prevalent isoforms of pIs 4.63 and 4.73. The first three basic isoforms (pIs 4.88, 4.83, and 4.80) were not present in the heart preparation, and the most acidic isoform (pI 4.53) in bovine heart was undetectable in the spleen preparation (Table VI). For both preparations, the peak corresponding to pI 4.63 resulted from three near-overlapping bands, that were not resolved in densitometric scans. The isolectin profiles obtained with the CAM-Gs were similar to those for the untreated galectins (data not shown).

Primary Structure Analyses—Edman degradation of 100-200 pmol of the native bovine spleen galectin did not yield an N-terminal sequence, indicating that the protein N-terminus is not accessible. A number of tryptic peptides were purified by RP-HPLC and sequenced. The sequencing of the major peptides identified 87 residues, all of which were identical with the sequence of the bovine heart galectin (Fig. 7). A major peptide did not yield any sequence on repeated experiments, and thus most likely contained the N-terminal sequence.

DISCUSSION

An improved protocol for the purification of bovine galectin-1 was developed. The use of DEAE-Sepharose prior to affinity chromatography offers two advantages over conventional methods: the simultaneous partial isolation of the galectin and the removal of lactose, already present in the extraction buffer, which would block galectin binding during affinity chromatography. Therefore, the high salt eluate from DEAE-Sepharose can be immediately loaded onto a lactosyl-Sepharose or ASF-Sepharose column without the previous dialysis routinely performed for conventional purification protocols. Furthermore, repeated affinity chromatography of crude extracts prepared from mammalian tissues (*e.g.* spleen) that are rich in pigments such as hemoglobin or ferritin, which may aggregate and precipitate, usually reduces the lifetime of the affinity resin. The ion exchange fractionation of the crude extract, that we introduce prior to the affinity chromatography, results in a clear eluate that contains the galectin, whereas the pigment and most other proteins are separated in the flow-through fraction. The yield of the purified galectin obtained by this method is comparable to that obtained by a conventional method (*22*). The recovery of the galectin activity from lactosyl-Sepharose was 66% (Table I), *i.e.*, comparable to that from ASF-Sepharose (58%), although ASF was found to be a better ligand than lactose in solution (*9*). Thus, the optimized purification method represents an improvement over conventional protocols because it can be employed to purify any acidic galectin, allows faster processing of larger volumes of tissue extracts, and increases the lifetime of the affinity matrix.

Although multiple galectins have been isolated from human and rat lung, only one type of galectin could be detected in bovine spleen. The bovine spleen galectin is a dimer of two identical subunits, each 14.5 kDa, as evidenced by SDS-PAGE, and gel permeation chromatography under denaturing and non-denaturing conditions. Under non-denaturing conditions, gel permeation chromatography of the galectin at a concentration of 0.5 mg/ml (17 μM) gave a peak corresponding to the dimeric species

but failed to yield any that would correspond to the monomeric 14.5 kDa subunit. In solution, monomer-dimer equilibrium has been observed for CHO galectin-1 (*38*) and toad ovary galectin[4] at protein concentrations ranging from 0.5 to 80 μM. The absorption coefficient of the bovine spleen galectin-1 was found to be $E_{280}^{mg/ml} = 0.65 \cdot ml \cdot mg^{-1} \cdot cm^{-1}$, which was close to that of the human spleen equivalent ($0.55\ ml \cdot mg^{-1} \cdot cm^{-1}$).

Analyses of the optimal binding activity and stability of the bovine spleen galectin under a variety of experimental conditions revealed that this protein, when carboxamidomethylated or stored in the presence of a soluble or solid phase-bound ligand, can remain fully active for a long period in the absence of reducing agents. Similar results were obtained with CHO galectin-1 and toad ovary galectin[4] when bound to affinity matrices (*38*). For electric eel galectin, the loss of fluorescence on oxidation could be prevented by lactose (*39*). It has been proposed that the ligand, lactose or *N*-acetyllactosamine, maintains the galectin in the active conformation (*3-5*), probably by preventing oxidative inactivation due to the formation of intramolecular disulfide bonds (*40*). Interestingly, the galectin from *Rana catesbeiana* does not require a reducing agent for binding activity, although the structural basis of this observation has not been elucidated (*41*). The pH range for the optimal binding activity of the bovine spleen galectin was wider (6.5-8.5) than that of the toad ovary galectin[4], but similar to that of the human spleen galectin-1 (*9*). The bovine spleen galectin was relatively more resistant to thermal inactivation than the toad ovary galectin: the bovine spleen galectin retained 6% of the total activity even at 100°C for 30 min (Fig. 3A), whereas the toad ovary galectin[4] was completely inactivated at 70°C.

The overall carbohydrate-binding profile of the bovine spleen galectin-1 was similar to those of mammalian galectins-1 (*6, 7, 9, 24*) carrying "conserved" CRDs (*13*), but different from those with "variable" CRDs (*11-13*). The relative inhibitory efficiencies of four key oligosaccharide structures, lactose [Galβ1,4Glc], *N*-acetyllactosamine [Galβ1,4GlcNAc], Galβ1,3GalNAc, and a human blood group A-tetrasaccharide [Fucα1,2(GalNAcα1,3)Galβ1,4Glc], can provide the preliminary information required for the assignment of a galectin to either CRD type. The hydroxyls at C-4′ and C-6′ of galactose residues are critical for both conserved and variable CRDs. No epimerization (for OH at C-4′) nor substitutions (for both OH) are possible without considerable changes in binding affinity. The 3-OH of Glc/GlcNAc (in Galβ1,4Glc or Galβ1,4GlcNAc) or the 4-OH of GlcNAc (in Galβ1,3GlcNAc) cannot be epimerized or substituted for the conserved CRDs, but for variable CRDs epimerization is possible, since Galβ1,3GalNAc is an equally potent inhibitor to Gaβ1,4Glc in RI36-I (*12, 13*). The substitution of 2-OH of Glc residues by NHAc in Galβ1,4GlcNAc promoted binding 5-10 fold compared to Galβ1,4Glc for conserved CRDs, but for variable CRDs, various degrees of binding (negligible to 11-fold better) were observed (*13*). The substitution of OH at C-2′ or C-3′ individually or both did not affect the binding dramatically. The 2-3 fold weaker binding in the case of conserved CRDs was observed probably because of steric hindrance between the bulky substituents and the interacting amino acids of the proteins. Interestingly, for some galectins having variable CRDs, the substitutions at C-2′ and C-3′ (as in the case of A-tetrasaccharide) promoted binding 10-32 fold (*6, 7, 12*), probably due to deletions in the CRD domain that yield a structure that can accommodate the bulky substitutions (*13*). In summary, the results of analyses of sugar specificity suggested that the relative binding affinity of the conserved (Type I) CRDs for the oligosaccharides in question are in the following decreasing order: *N*-acetyllactosamine > lactose > A-tetrasaccharide > Galβ1,3GalNAc. The galectin-1 from bovine spleen showed the above order of specificity.

The presence of two binding sites per molecule of bovine spleen galectin-1, as revealed by quenching of the fluorescence of GalαOUmbMe at three temperatures, is consistent with the crystallographic data (*4*). The association constant increased at lower temperature suggesting exothermic binding, which is a general property of lectins. The lack of quenching of the fluorescence of 4-methylumbelliferyl β-D-lactoside with the galectin was probably due to the absence of contact between the fluorescent derivative present at the 1-C atom of the Glc residue (in Galβ1,4Glc) and the interacting amino acid residues of the lectin. This is supported by the 3-D structures of galectin-1 (*4*) and galectin-2 (*3*) complexed with *N*-acetyllactosamine and lactose, respectively, where the 1-C atom clearly projects away from the binding pocket, with the 1-OH group completely solvated (*4*). Based on the binding-inhibition data, it has been proposed that the 4′-OH and 6′-OH of the Gal residue, and the 3-OH of the GlcNAc residue are directly recognized by the galectin CRD (*6-9*). Resolution of the structures of galectin-sugar complexes (*3, 4*) confirmed the above interactions, and demonstrated a van der Waals interaction between W^{68} and the Gal ring. Furthermore, our data suggest that additional hydrophobic interaction between the α-linked aglycon and the galectin may be responsible for the 10.6- and 15.5-fold higher inhibitory effectiveness of Galα-OPhNO$_2$ and Galα-OUmbMe, respectively, relative to Gal (Table IV). This was not the case for Galβ-OPhNO$_2$, in which the aglycon is β-linked (Tables III and IV). Likewise, lactose derivatives with a β-linked aglycon, such as Galβ1,4Glcβ-OPhNO$_2$ or Galβ1,4Glcβ-OUmbMe, did not show increased inhibitory effectiveness relative to lactose (Table III), supporting this conclusion. It is noteworthy that for sheep spleen galectin, 50-fold stronger binding with GalαOUmbMe relative to Gal was observed on titration calorimetry, although the K_a for GalαOUmbMe was almost 10-fold lower than that observed in this study (*42*). This may reflect limitations of indirect approaches, such as the one employed herein, rather than actual differences in the K_a values of the galectins in question. The results of chemical modification studies on bovine spleen galectin suggest that histidine, tryptophan, arginine and carboxylic acid, but not lysine or tyrosine, are amino acid residues likely involved in carbohydrate recognition. The nature of the amino acid residues identified through this approach is consistent with information obtained for other related galectins by site directed mutagenesis (*43*), and resolution of the three dimensional structures (*3-5*) establishing the participation of H^{44}, N^{46}, R^{48}, H^{52}, D^{54}, N^{61}, W^{68}, E^{71}, and R^{73} in the interaction with the ligand.

The distinct isoform profiles for the bovine spleen and heart galectins were reproducible in multiple preparations isolated from several individual animals ($n = 4$). Therefore,

it is unlikely that these are artifacts, such as ones resulting from amidation of carboxyl residues, generated on the *in vitro* manipulation of the purified proteins. Furthermore, carboxamidomethylation of the native galectins did not modify their isoform profiles. The only two isolectins (pIs 4.75 and 4.85) detected by Briles *et al.* (*22*) for bovine galectin on isoelectric focusing in the pH range of 3.5-9.5 may be due to the different experimental conditions for galectin isolation and isoform resolution. The distinct isolectin profiles obtained for galectin-1 isolated from bovine spleen and heart led us to determine its amino acid sequence in order to elucidate the structural basis for this observation. All peptide sequences repeatedly determined were identical to those reported for the heart galectin (*24*). In this aspect the primary structure of the bovine galectin-1 resembles those of those isolated from human placenta, lung, spleen, and brain, for which all amino acid sequences were found to be identical regardless of the source tissue (*44-47*). The 3-D structure of bovine spleen galectin-1 is now available and the electron density is consistent with the sequence of heart galectin-1 (*4*). However, although the structure was resolved at 2.7 Å and refined at 1.9 Å, it cannot provide accurate information on relatively small substituents. Therefore we conclude that bovine spleen galectin-1 is identical to heart galectin-1, although tissue-specific co- or post-translational modification(s), as evidenced by the different isoforms profile(s), cannot be ruled out. Because peptide sequencing of the purified galectin-1 from either bovine heart (*48*) or spleen (present study) failed to reveal alternate residues at any position, that could provide a structural basis for the presence of isoforms, it is unlikely that point mutations, such as those observed for the *X. laevis* isoforms, are the mechanism responsible for the charge heterodispersity observed in the bovine galectins. Thus, it is possible that unequal derivatization of amino acids, such as phosphorylation, amidation, and sulfation, that may be specific or more or less prevalent in different organs or tissues, is the source of the heterodispersity observed in the bovine spleen and heart galectins. For the galectin-3 from Madin-Darby canine kidney cells, the unequal phosphorylation of serine residues was shown to be the cause of isolectin formation (*21*). However, in our preliminary studies involving the treatment of the galectin-1 from bovine spleen and heart with alkaline phosphatase from calf intestine, we failed to modify the heterodispersity profiles observed for the untreated controls. Further studies aimed at the identification of other co- or post-translational modifications responsible for the tissue specific isoform profiles are currently underway in our laboratory.

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