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Isolation of a ß-galactoside-binding lectin from cat liver

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Abstract

A lectin from cat liver has been identified and purified by affinity chromatography on asialofetuin-Sepharose. One hundred micrograms of lectin was obtained from one cat liver with a purification factor of 1561. The lectin agglutinates trypsin-treated rabbit and cow erythrocytes. Hemagglutination was inhibited only by saccharides containing ß-galactosyl residues, of which the 1-amine-1-deoxy-ß-D-galactose was the most potent one by inhibiting hemagglutination at a concentration of 12.5 mM, followed by melibiose, trehalose and galactose. The lectin has a subunit molecular mass of 14.4 kDa determined by SDS-PAGE under reducing conditions and a pI of 4.85. Compared with the composition of lectins from calf heart and porcine heart, cat liver lectin contains approximately the same amount of cysteine, half the amount of glycine, twice as much arginine and threonine, and three times the amounts of tyrosine and methionine. Cat liver lectin contains four cysteine residues per subunit, all of them in the reduced form. Their lack of reactivity towards thiol-reactive supports suggests they are not exposed on the lectin surface. The protein apparently has a blocked N-terminus. The purified lectin was stable for up to 20 months stored at +4ºC in buffer supplemented with 4 mM ß-mercaptoethanol. Results indicated that this lectin belongs to the family of soluble ß-galactoside-binding lectins, also known as galectins, which are expressed in a wide range of vertebrate tissues.

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Introduction

Lectins are proteins that bind to specific carbohydrate structures and thus can recognize particular glycoconjugates in different tissues or on the surface of cells (1). Within the animal lectins there is a family of closely related ß-galactoside-binding lectins, which have been called S-type (2) and S-lac lectins and also, more recently, galectins (3). Galectins possess a conserved carbohydrate-recognition domain directed against ß-galactoside structures, in particular polylactosamine sequences; this binding requires the presence of thiol-reducing agents and is independent of divalent cations (2). They do not appear to be glycosylated, they do not require detergents for their solubilization and they have a predominantly cytoplasmic location, although under certain circumstances they accumulate on the cell surface, in the extracellular matrix, or even in the cell nucleus. Although galectins have been found in amphibians, fish, birds and in phyloge-

netically distant animals like nematodes and sponges, the literature predominantly describes galectins from mammalian sources (4) but a galectin has not been reported before in any cat organ. Mammalian galectins have been classified into ten families based on the amino acid sequence, but a more fundamental structural classification of subunits, which incorporates non-mammalian galectins, is the division into prototype, chimera-type and tandem-repeat-type galectins. Proto-type subunits consist of a single domain (Mr about 14 kDa) and usually form homodimers with hemagglutinating activity (4).

Galectins are involved in cell adhesion and the related function of regulation of cell proliferation and differentiation. They also participate in a number of other biological processes and thus it has been suggested that the function of a given galectin can vary from site to site depending on the nature of available ligands. Further research into the biological roles and a more complete characterization of these lectins will depend on the availability of reasonable amounts of highly purified material. To contribute to increasing the knowledge of the diversity, structure and functions of the galectins we report here the purification and partial characterization of a new ß-galactoside-binding lectin isolated from cat liver extracts.

Material and Methods

1,4-Dithiothreitol (DTT) and iodoacetamide were obtained from Fluka Chemie AG, Buchs, Switzerland. Aldrithiol-4 (4-pyridyl-disulfide, 4-PDS) was from Aldrich Chemical Company Inc., Milwaukee, WI, USA. Fetuin (from fetal calf serum, type III), bovine serum albumin (BSA), transferrin, ß-mercaptoethanol, trypsin and all carbohydrates were purchased from Sigma, St. Louis, MO, USA (except melibiose and galactose, which were from Merck, Darmstadt, Germany). Homogeneous 12.5 and 8-25 gradient gels, the pI broad calibration kit for isoelectrofocusing (IEF) 3-9, buffer strips for SDS-PAGE and native PAGE, low-molecular weight calibration kit, silver nitrate staining kit, CNBr-activated Sepharose 4B, thiopropyl-Sepharose 6B, epoxy-activated Sepharose 6B, and PD-10 columns (Sephadex G-25) were from Pharmacia-LKB, Uppsala, Sweden. Microtiter U-shape plates were from Sigma. PM10 membranes were from Amicon, Danvers, MA, USA. Fresh livers were obtained from European shorthaired cats, 8 months old, at the Swedish Department of Animal Services, National Veterinary Institute, Uppsala, Sweden, and stored frozen at -80ºC.

Preparation of asialofetuin-Sepharose

Fetuin was desialylated under mild acid conditions as described by de Waard et al. (5). Fetuin (300 mg) was dissolved in 25 ml 0.2 N HCl solution and heated at 80ºC for 1 h. The solution was then cooled down to 25ºC, neutralized with NaOH and dialyzed overnight against 0.2 M $NaHCO_3$ buffer, pH 7.9, to remove sialic acid. The resulting solution (31 ml) of asialofetuin was coupled to CNBr-activated Sepharose 4B according to the procedure recommended by the manufacturer (Pharmacia). Fifteen grams of the support (freeze-dried powder) was suspended in 1 mM HCl and washed for 15 min with 1 mM HCl on a sintered glass filter. The asialofetuin solution was adjusted to 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl and mixed with the gel in a glass tube with a screw cap. The mixture was rotated end over end for 2 h at room temperature. The excess of ligand was washed away on a glass filter with the coupling buffer and the remaining active groups were blocked with 1 M ethanolamine, pH 9.0, for 2 h at room temperature. The product was washed with three cycles of alternating pH consisting of a wash with 0.1 M acetate buffer, pH 4.5, containing 0.5 M NaCl, followed by a wash with

0.1 M Tris buffer, pH 8.0, containing 0.5 M NaCl.

The adsorbent thus prepared contained 123.85 mg asialofetuin per g of dry gel, as determined by total amino acid analysis.

Coupling of 1-amine-1-deoxy-ß-D-galactose to epoxy-activated Sepharose 6B

This procedure was performed according to manufacturer instructions (Pharmacia): 1 g of freeze-dried epoxy-activated Sepharose 6B was suspended in distilled water and washed on a sintered glass filter. Five hundred milligrams of 1-amine-1-deoxy-ß-D-galactose was dissolved in 0.1 M Na_2CO_3, pH 9.5, and mixed with the gel using a shaker for 16 h at 25ºC. The excess of ligand was washed away and the remaining excess groups were blocked with 1 M ethanolamine overnight. The gel was washed with the coupling solution, water and 0.1 M acetate buffer, pH 4.0, and stored at 4ºC until use.

Preparation of soluble protein extract from cat liver

The material was treated as described by Beyer et al. (6). Each cat liver was cleaned and rinsed with buffer consisting of 75 mM NaCl, 75 mM Na_2HPO_4/KH_2PO_4, pH 7.2, containing 2 mM EDTA, 4 mM ß-mercaptoethanol and 0.3 M lactose (Buffer A). The organ was cut into small pieces, mixed with 5 volumes of extraction buffer and homogenized with an UltraTurrax Ika-Werk mixer at 4ºC for 1 min/load. The homogenate was centrifuged for 30 min at 15,000 *g* at 4ºC and the supernatant was collected and centrifuged twice for 60 min at 30,000 *g*. The clear red supernatant fluid was stored at 4ºC.

Lactose-free extract

To eliminate the lactose from the crude extract, PD-10 columns for gel filtration were equilibrated with buffer consisting of 75 mM NaCl, 75 mM Na_2HPO_4/KH_2PO_4, pH 7.2, plus 2 mM EDTA and 4 mM ß-mercaptoethanol (Buffer B). The liver supernatant was submitted to gel filtration through the PD-10 columns by applying 1 ml and collecting 2 ml of the void volume. Alternatively, the lactose was removed by overnight dialysis against Buffer B. The lactose-free extract was ready to apply to asialofetuin-Sepharose or to be used for other purposes.

Affinity chromatography on asialofetuin-Sepharose

The lactose-free extract of cat liver (usually 30 ml) was applied to a column (9 ml packed gel) of asialofetuin-Sepharose at a flow rate of 15 ml/h and 2-ml fractions were collected. The column was washed with Buffer B until the absorbance at 280 nm of the effluent was negligible. Desorption of the bound lectin was performed by elution with 300 mM lactose in Buffer B. The fractions were collected, filtered through PD-10 columns as described above and assayed for hemagglutinating activity. The fractions containing hemagglutinating activity were pooled and concentrated with an Amicon-10 ultrafilter. The concentrate, which was kept at 4ºC, was used for SDS-PAGE IEF, inhibition studies and amino acid analysis, among others.

Affinity chromatography on 1-amine-1-deoxy-ß-D-galactosyl-Sepharose 6B

The lactose-free extract (10 ml) was applied to a column packed with 2 ml of the affinity gel at a flow rate of 15 ml/h and 2-ml fractions were collected. The column was washed with Buffer B until the absorbance at 280 nm of the effluent was negligible. The bound lectin was eluted with 300 mM lactose in Buffer B. The fractions were collected, filtered through PD-10 columns and assayed for hemagglutinating activity.

Hemagglutinating activity

Rabbit erythrocytes for the hemagglutination test were prepared from fresh blood, collected in Alsever's medium, and washed four times with 50 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS buffer). The packed cells were diluted in PBS to give 4% red cell suspensions. Trypsin treatment was carried out essentially as described by Nowak et al. (7): a 4% erythrocyte suspension in PBS containing 1 mg/ml trypsin was incubated for 1 h at 37ºC. The trypsin-treated cells were washed four times with PBS buffer.

Agglutination assays were carried out on microtiter U-shaped plates by adding 25 µl 0.15 M NaCl, 25 µl 1% BSA in 0.15 M NaCl, 25 µl sample, and 25 µl 4% red cell suspension in PBS. The well contents were mixed by gentle shaking and covered with plastic wrap. The plate was incubated for 30 min at room temperature and examined for agglutination against a white or illuminated background to determine the resulting titer. The hemagglutinating activity of all samples was quantified by the two-fold serial dilution assay. Titer per ml was defined as the reciprocal of the highest dilution giving visible agglutination of the trypsin-treated rabbit erythrocytes after 30 min of incubation. Specific hemagglutinating activity was defined as the ratio of the titer/ml and protein concentration (mg/ml).

Red cells of different origins were used to test hemagglutination: fetal calf, rabbit, cow and porcine erythrocytes were tested with and without trypsin treatment.

Inhibition of lectin binding to erythrocytes by carbohydrates

Saccharide specificity of lectin binding to erythrocytes was determined by sugar inhibition tests using 2-fold serial dilutions of the sugar solutions. To assess these inhibition tests, standard solutions of the saccharides were prepared at the appropriate concentration in 0.15 M NaCl. The lectin dilution used for these studies was the one able to cause 50% hemagglutination (the dilution before the last one able to cause clear hemagglutination). The sugars tested were: 50 mM *o*-nitrophenyl-ß-D-galactopyranoside and *p*-nitro-phenyl-ß-D-glucopyranoside; 100 mM D-glucosamine, 6-desoxy-L-galactose, D-ribose, α-L-rhamnose, 1-amine-1-deoxy-ß-D-galactose, D-fructose, *o*-nitrophenyl-ß-D-galactopyranoside-6-phosphate, N-acetyl-D-glucosamine, α-methyl-D-mannoside, D-saccharose, D-glucose, thioglucose, α-D-fucose; 200 mM D-trehalose and α-D-melibiose, and 300 mM D-galactose and α-D-lactose. Heparin, asialofetuin, fetuin and transferrin were also tested (all of them at 8 mg/ml concentration).

To determine the minimum concentrations required for inhibition of hemagglutination by these different carbohydrates, a volume of 25 µl 0.15 M NaCl was added to each well of the corresponding lane on the microtiter plate. A volume of 25 µl of the sugar solution was added to the first well and mixed, 25 µl was withdrawn and added to the second well, its content mixed, and 25 µl was withdrawn and added to the third well, etc. The extra 25 µl from the well containing the highest sugar dilution was discarded. To each well containing the sugar dilution, 25 µl of the corresponding lectin solution, 25 µl 1% BSA in 0.15 M NaCl and 25 µl 4% red cell suspension in PBS were added. The well contents were mixed by gentle shaking, covered with plastic wrap and analyzed.

Polyacrylamide gel electrophoresis

Electrophoretic analysis included SDS-PAGE under both reducing and nonreducing conditions, native PAGE and IEF analysis. The molecular mass of the SDS-denatured lectin sample was determined in homogeneous 12.5% Phast gels. The samples were diluted with sample buffer, i.e., 10 mM Tris/HCl buffer, pH 8.0, containing 1 mM EDTA, 2% SDS, 10% glycerol and 2.5% (v/v)

ß-mercaptoethanol, and boiled for 5 min. α-Lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30.4 kDa), ovalbumin (43 kDa), serum albumin (67 kDa) and phosphorylase b (94 kDa) were used as molecular mass calibration proteins. PAGE of the lectin was run using the 8-25 gradient Phast gels. Bromophenol blue was added to lectin samples as a migration marker. Proteins were silver stained, according to manufacturer instructions.

IEF was determined using a Pharmacia Phast System apparatus (Pharmacia). Isoelectric point was determined using the broad pI calibration kit run on Phast gel IEF 3-9: lentil lectin (basic, 8.65), lentil lectin (middle, 8.45), lentil lectin (acidic, 8.15), horse myoglobin (basic, 7.35), horse myoglobin (acidic, 6.85), human carbonic anhydrase (6.55), bovine carbonic anhydrase (5.85), ß-lactoglobulin (5.20), soybean trypsin inhibitor (4.55), and amyloglucosidase (3.50). The kit proteins were reconstituted in 35 µl of distilled water. The gel was run according to manufacturer instructions.

Protein determination

Protein concentration was determined by the method of Lowry et al. (8) using BSA as a standard, and by absorbance measurements at 280 nm.

Determination of amino acid composition

Amino acid analysis was performed at the University of Uppsala, Sweden. The lectin solution obtained by affinity chromatography on asialofetuin-Sepharose as described above was submitted to gel filtration through a PD-10 column with 10 mM ammonium acetate, pH 6.6, and hydrolyzed in 6 N HCl for 22 h at 110ºC in vacuum-sealed tubes.

Carboxymethylation of the cat liver lectin

Cat liver lectin (1.6 nmol) was incubated with 20-fold excess iodoacetamide in PBS buffer for 1 h at 24ºC. Excess iodoacetamide was removed by gel filtration through a PD-10 column with PBS buffer.

Titration of free sulfhydryl groups

This procedure was carried out by the method of Grassetti and Murray (9). The lectin was treated with 4-PDS and the release of 4-thiopyridone was monitored at 324 nm. The content of free thiol groups was determined using an extinction coefficient for 4-thiopyridone of 20.2×10^3 M^{-1} cm^{-1}.

Results and Discussion

Extraction of cat liver lectin

In this report we describe the purification to homogeneity of a lectin from cat liver. The effect of the composition of the extraction buffer on the recovery of the hemagglutinating activity was determined as follows: fresh cat liver was homogenized in 5 parts (w/v) of 75 mM NaCl and 75 mM Na_2HPO_4/KH_2PO_4 buffer, pH 7.2, containing 2 mM EDTA, with the following additions: i) none (control), ii) 4 mM ß-mercaptoethanol, iii) 300 mM lactose, and iv) 4 mM ß-mercaptoethanol and 300 mM lactose. We found hemagglutinating activity only when 4 mM ß-mercaptoethanol was present in the extraction buffer and even more activity was recovered when the buffer also contained 300 mM lactose. Thus, the presence of a reducing agent and lactose seems to be important for the initial extraction of the activity. Therefore, the tissue was homogenized and incubated with the buffer containing both ß-mercaptoethanol and lactose (Buffer A, see Material and Methods) to extract the soluble lectin. The sugar was removed from the supernatant obtained from the crude extract by gel filtration or dialysis. The lactose-free crude liver extract strongly agglutinated trypsinized rabbit erythrocytes, but did not

Table 1. Binding of various types of erythrocytes to cat liver galectin.

Source of erythrocytes	Treatment	Agglutination
Calf	None	–
	Trypsin	–
Cow	None	–
	Trypsin	+
Rabbit	None	–
	Trypsin	+
Porcine	None	–
	Trypsin	–

Clear evidence of hemagglutination is indicated by a "+". No detectable reaction is indicated by a " –".

Table 2. Purification of cat liver galectin by affinity chromatography on asialofetuin-Sepharose.

Step	Volume (ml)	Protein (mg/ml)	Activity (IU/ml)	Specific activity	Total activity	Purification -fold	Recovery (%)
Crude extract	30	7.8	2,560	328	76,800	1	100
Affinity chromatography	1	0.1	51,200	512,000	51,200	1561	66.7

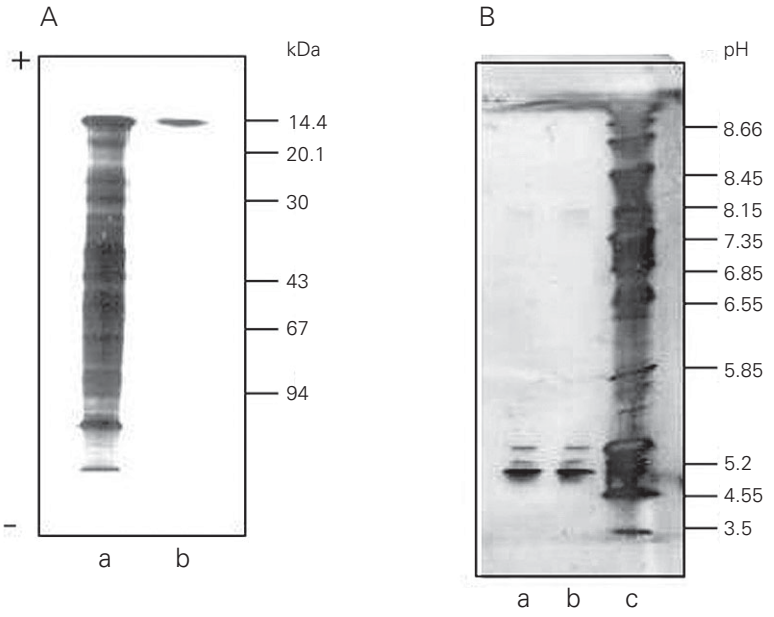

Figure 1. *A*, SDS-PAGE of cat liver galectin in 12.5% homogenous gel. *Lane a*, Crude extract of cat liver. *Lane b*, The galectin purified with asialofetuin-Sepharose. *B*, Isoelectric focusing pattern of cat liver galectin on Phast gel IEF 3-9. *Lanes a* and *b*, The lectin after purification with asialofetuin-Sepharose. *Lane c*, pI calibration standard proteins.

agglutinate the untreated rabbit red cells. The extent of binding of the lectin to erythrocytes from other species varied, depending on the source of the cells and the treatment (Table 1). Fresh cow erythrocytes were not agglutinated by the extract but were agglutinated after trypsin treatment. Porcine and calf red cells were not agglutinated by the extract even after trypsin treatment.

Purification of the lectin

Lectins have been purified by conventional procedures including ethanol precipitation, ion-exchange chromatography and gel filtration, or by affinity chromatography. The former methods rely on the physicochemical properties of proteins for separation while the affinity chromatography depends on the specific interaction between the lectin and a carbohydrate attached to a solid support. The lectin from cat liver was purified following general previously described affinity procedures to purify S-type lectins from other sources (5,10,11). The lactose-free crude extract was applied to either of two different affinity gels, i.e., the asialofetuin-Sepharose and 1-amine-1-deoxy-ß-D-galactosyl-Sepharose prepared as described in the Methods section. As the lectin was efficiently adsorbed onto the asialofetuin-Sepharose, the purification was performed using this affinity gel. Desorption was achieved using two different eluents, 300 mM 1-amine-1-deoxy-ß-D-galactose (the best inhibitor in solution) and 300 mM lactose. The best results were obtained with 300 mM lactose. The fractions containing the protein eluted with lactose from the column were pooled, submitted to gel filtration through a PD-10 column with a buffer containing 4 mM ß-mercaptoethanol (Buffer B, see Material and Methods) and concentrated using the Amicon ultrafilters. Data showing the recovery of the lectin after the different purification steps are presented in Table 2. Approximately 0.1 mg of lectin containing 51,200 units of hemagglutinating

activity towards trypsin-treated rabbit erythrocytes was obtained from one cat liver (60 g). The procedure resulted into a purification factor of 1561. That this one-step affinity purification method using asialofetuin-Sepharose yielded a highly purified lectin preparation was demonstrated by the fact that only one band was obtained when the preparation was analyzed by SDS-PAGE under reducing conditions and with silver staining (Figure 1A). The protein migrated with a mobility corresponding to the protein standard of 14.4 kDa. We also examined the behavior of the cat liver lectin upon SDS-PAGE analysis under nonreducing conditions. The electrophoretic mobility and apparent molecular mass were identical to that observed under reducing conditions, thus indicating that the dimer is not held together by disulfide bonds. When electrophoresis was performed under native conditions the lectin migrated as a single band close to the 30-kDa standard, indicating that the native form is dimeric (data not shown). Calf heart lectin and porcine heart lectin have been reported to exhibit some tendency to form multimeric complexes or aggregates (12). We did not find any high molecular mass band other than the one of about 30 kDa when the purified cat liver lectin was run under native conditions using the 8-25 gradient Phast gels.

Analysis of the purified lectin by IEF showed some heterodispersion in spite of the apparent subunit size homogeneity observed by PAGE and SDS-PAGE. The profile obtained comprised three bands: a major band at pI 4.85, and two faint bands located close together at pI 5.03 and 5.13 (Figure 1B). The former was by far the prevalent one, and the latter two more basic ones seemed to be present in equal but very low proportions.

Stability and storage

The crude extract showed no detectable loss of hemagglutinating activity when kept for up to three weeks in the buffer containing 4 mM ß-mercaptoethanol and 300 mM lactose at +4ºC, and when kept in PBS for one week. The purified lectin was stable for up to 20 months when stored at +4ºC in the buffer containing 4 mM ß-mercaptoethanol.

Carbohydrate specificity of the lectin

To investigate the carbohydrate specificity of the lectin, we screened a panel of monosaccharides, disaccharides, monosaccharide derivatives and glycoproteins for their ability to inhibit the hemagglutination of trypsin-treated rabbit erythrocytes. In the hemagglutination inhibition assay, the sugars, by specific interactions, bind to the lectin before the lectin can bind to the red blood cells. The result is no agglutination. The negative control shows positive agglutination because no sugars with specificity for the lectin are present to prevent the lectin from agglutinating the red cells. The results, reported as the minimum concentrations required for inhibition of the hemagglutination of cat liver lectin caused by different carbohydrates, are presented in Table 3. Among the saccharides tested, 1-amine-1-deoxy-ß-D-galactose was the most effective inhibitor, followed by melibiose, galactose and trehalose. Within the concentration range studied (up to 300 mM), most of the monosaccharides were ineffective as inhibitors, including α-D-fucose, D-ribose, α-L-rhamnose, D-fructose, N-acetyl-D-glucosamine, α-methyl-D-mannoside, D-glucose, *o*-nitro-phenyl-ß-D-glucopyranoside, and D-glucosamine. *o*-Nitro-phenyl-ß-D-galactopyranoside-6-phosphate did not inhibit agglutination, in contrast to *o*-nitro-phenyl-ß-D-galactopyranoside, which inhibited agglutination at a concentration of 25 mM. Thus, the hydroxyl group at position 6 of galactose is important for the lectin sugar binding. This is in agreement with what has been reported for the human dimeric galectin (13). We also inves-

Table 3. Effect of saccharides on the agglutinating activity of cat liver galectin.

	Concentration that inhibits hemagglutination by 50% (mM)*
1-amine-1-deoxy-ß-D-galactose	12.5
o-nitro-phenyl-ß-D-galactopyranoside	25.0
α-D-melibiose	25.0
D-trehalose	50.0
D-galactose	50.0
α-D-lactose	75.0
α-methyl-D-mannoside	>100.0
D-glucosamine	>100.0
α-D-fucose	>100.0
D-ribose	>100.0
α-L-rhamnose	>100.0
D-fructose	>100.0
p-nitro-phenyl-ß-D-glucopyranoside	>100.0
N-acetyl-D-glucosamine	>100.0
D-saccharose	>100.0
D-glucose	>100.0

*Corresponds to the dilution before the last one which caused agglutination of red cells in the two-fold serial dilution experiment.

Table 4. Comparison of the amino acid composition of cat liver lectin with other S-type mammalian galectins.

Amino acid	Cat liver	Porcine heart[a]	Calf heart[b]	Rat lung[c]	Calf spleen[b]
Asx	11.4	14.89	13.77	17.5	15.19
Gly	5.1	11.86	8.95	8.6	8.15
Glu	13.4	10.08	8.61	8.9	8.74
Leu	8.7	9.81	9.62	9.7	10.16
Ala	6.4	8.54	9.54	9.6	9.43
Phe	6.0	7.26	6.79	7.2	7.17
Ser	5.7	7.08	4.77	3.8	4.88
Lys	6.5	6.06	5.5	6.8	5.79
Val	6.3	5.92	6.5	3.5	6.65
Ile	4.1	4.41	3.51	3.5	3.39
Pro	5.0	3.6	6.03	4.8	4.39
Cys	3.2	2.99	3.89	6.2	4.52
Arg	6.0	2.49	3.67	2.5	3.62
Thr	5.5	2.32	2.18	4.7	2.04
His	2.2	1.61	1.59	2.8	1.48
Trp	–	0.97	2.89	–	2.89
Tyr	3.1	0.76	2.03	Trace	1.51
Met	1.1	0.35	0.15	Trace	–

Data are reported as the number of residues/100 residues (mol%).
[a]Ref. 12. [b]Ref. 11. [c]Ref. 10.

tigated heparin, fetuin, asialofetuin, transferrin and asialotransferrin as inhibitors. Asialofetuin inhibited at 1 mg/ml. None of the others inhibited hemagglutination at concentrations lower than 8 mg/ml.

Amino acid analysis of the galectin

The amino acid composition of the galectin was determined and compared with those reported for other S-type lectins such as porcine heart lectin, calf heart and spleen lectins and rat lung lectin (Table 4). Compared with the composition of galectins from calf heart (14) and porcine heart (12), cat liver galectin contains half the amount of glycine, approximately twice as much arginine and threonine, and three times the amounts of tyrosine and methionine. It has 3 cysteines per 100 residues and therefore the subunit consisting of about 134 amino acids probably contains 4 cysteine residues. The cysteine residues do not seem to be involved in the dimerization of the lectin via disulfide bonds, as indicated by SDS-PAGE analysis under reducing and nonreducing conditions (see above) and by titration of the free thiol groups with 4-PDS (see below).

An N-terminal blockage of the galectin is suspected to be responsible for the failure to determine its NH_2-terminal sequence.

Effect of sulfhydryl reagents on the galectin

A characteristic feature reported for the 14-kDa lectins from animal species that have been studied in detail is their dependence on a reducing medium to prevent oxidation and to maintain carbohydrate-binding activity after purification (12,15,16). Because of these repeated observations and after we established the need for ß-mercaptoethanol in the extraction buffer, we examined the requirements for reducing conditions for maintaining the hemagglutinating activity of the cat liver extract. ß-Mercaptoethanol was removed from the extract by gel filtration

through PD-10 columns equilibrated with PBS buffer. In order to ensure complete reduction and to prevent formation of mixed disulfides of the lectin and of mercaptoethanol in the crude extract after gel filtration, incubation with 4 mM DTT was performed, and then excess reducing agent was removed by gel filtration. The eluate was immediately assayed by the hemagglutination test and the rest of the eluate was kept at +4ºC and examined 8 days later. The lectin in the extract kept its carbohydrate-binding ability in the absence of reducing agents even when stored for 8 days; after this period the activity dropped to half the initial level.

We also tried to determine the requirement of the purified lectin for reducing conditions during storage. A solution of the lectin in PBS was incubated for 3 h with 4 mM ß-mercaptoethanol. The reducing agent was then removed by gel filtration of the sample on a PD-10 column with PBS and the void material was kept at +4ºC for 24 h. A parallel control was run with the constant presence of the reducing agent. Hemagglutination analysis at regular intervals showed that within the first 4 h the lectin lost part of its activity and 5-6 h after the removal of the reducing agent the lectin was completely inactive, while the activity of the control with the reducing agent remained active. We again added ß-mercaptoethanol at a concentration of 40 mM to the inactive sample and incubated the preparation for 24 and 48 h to determine whether the inactivation was reversible or not. The sample was still inactive after these time periods in the presence of the reducing agent, so the inactivation of the purified lectin appears to be irreversible.

The importance of thiol groups in maintaining the activity of these lectins (14,17,18) has been extensively discussed. Whitney et al. (14) have proposed that oxygen inactivates rat lung lectin by oxidation of cysteine residues, resulting in intrachain disulfide bond formation rather than disulfide bonds between the subunits of the dimeric lectin. In addition, it has been pointed out that no cysteine residues are found in the highly conserved region of these lectins and that the thiol groups therefore are not directly involved in the carbohydrate-binding function of the lectins (19).

Amino acid analysis revealed that the cat liver galectin has 3 cysteines per 100 residues. Assuming a number of 130-134 amino acids per subunit for the 14-kDa lectins (20), this means that the lectin probably has 4 cysteine residues per subunit. To determine whether the thiol groups were in free form or involved in disulfide bonds, we titrated the free sulfhydryl groups with 4-PDS reagent. The reaction was followed by reading the absorbance at 324 nm at regular intervals until it stabilized. The results indicated the presence of 4 mol of free sulfhydryl residues per mol of lectin. Thus, there are no disulfide bonds in the reduced cat liver lectin. Due to the linear appearance of the A_{324}/time progress curve, we can assume that the 4 sulfhydryl groups are equally reactive. These results are in agreement with those reported by Whitney et al. (14) for rat lectin. To determine whether these residues are exposed or not, we used a thiopropyl-Sepharose column. The active lectin was submitted to gel filtration through a PD-10 column equilibrated with 0.1 M Tris/HCl, pH 8.0, and 0.5 M NaCl, to remove ß-mercaptoethanol. Since the purified lectin without the reducing agent lost its activity within 5 h, we applied it to a thiopropyl-Sepharose column immediately after gel filtration. When we examined the material that passed through the column, about 75% of the applied protein was recovered. Thus, the sulfhydryl groups seem not to be highly exposed. It has been suggested before for the rat (17) that only one of the 6 cysteines is exposed on the surface of the lectin molecule and thus is more readily oxidized than the others. It also seems that the cat galectin loses its activity faster when passed through the column since the eluted material was completely inactive.

Different mechanisms have been proposed to account for the oxidative inactivation of the soluble galactose-binding lectins of the rat (14) and the electric eel (21). In both cases oxidation of the lectin solutions was accelerated by flushing them with oxygen or bubbling oxygen through them for 30 min. In the case of the rat 14-kDa lectin, it was proposed that intramolecular disulfide bonds are formed leading to a change in the secondary structure. This conclusion was based on the observations that, upon oxidation, the decrease in hemagglutinating activity was paralleled by a change in circular dichroism and a decrease in the number of free thiol groups, but without evidence of intermolecular cross-linking as assessed by SDS-PAGE in the absence of ß-mercaptoethanol. In the 14-kDa lectin of the electric eel, where cysteine residues have not been detected, it was suggested that inactivation could be due to oxidation of a tryptophan residue to form an oxindol. Abbott and Feizi (22) reported that oxidation of the bovine lectin was achieved by removal of the reducing agent during a period of 72 h by dialysis and their results were consistent with oxidative inactivation resulting from disulfide bond formation between a number of cysteine residues. Tracey et al. (15) demonstrated that intramolecular disulfide bond formation in the soluble ß-galactoside-binding lectin from bovine heart muscle is associated with oxidative inactivation. The effect of amino acid substitution by site-directed mutagenesis in human 14-kDa ß-galactoside-binding lectin has been studied (23), suggesting that oxidation of one cysteine could be the key to the inactivation of this lectin. It has been reported for other mammalian 14-kDa lectins of the galectin-2 type that one of these cysteine residues is located close to the sugar-binding site but does not participate in the actual sugar-lectin interaction. Nevertheless, the presence of reducing agents such as ß-mercaptoethanol is needed for these lectins to remain in a reduced state. It has been found, however, that the activity is preserved for a reasonable time even in the absence of reducing agents after alkylation of the lectin thiol groups with iodoacetamide (14,16). Consistent with this, we found that, after carboxymethylation, the cat liver galectin kept its activity for at least one week regardless of whether ß-mercaptoethanol was present or not. The carboxymethyl group is obviously too small to interfere sterically with the sugar binding but, on the other hand, it seems to protect the lectin from inactivation. A possible explanation for these results might be that the loss of activity is due to a conformational change of the lectin caused by oxidation of the thiol groups to disulfides.

This hypothesis is supported by the fact that reaction of the cat liver galectin with the thiol titration reagent 4,4'-dipyridyl disulfide led to instantaneous loss of hemagglutinating activity. This reagent is well known to react with free thiols with formation of mixed reactive disulfides, which in turn can react with other thiols to form disulfide bonds. Another possibility for the observed loss of activity might of course also be that the introduced 4-pyridyl-disulfide structure is large enough to block the sugar-binding site of the lectins.

These findings may contribute to the understanding of the biological role of this type of lectins since there is evidence that they function in an extracellular environment (4), which is generally considered to be less reducing than inside the cell. Cooper et al. (24) suggested that oxidative inactivation of the L-14 lectin carbohydrate-binding activity might be a way to limit the duration and physical range of its activity, which opens up interesting research possibilities *in vivo*.

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