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Heparin-Inhibitable Lectins: Marked Similarities in Chicken and Rat

Marie M. Roberson, Howard Ceri, Paula J. Shadle, and Samuel H. Barondes

Departments of Psychiatry and Biology, University of California, San Diego, La Jolla, California 92093 and Veterans Administration Medical Center, San Diego, California 92161

Extracts of young rat lung contain a heparin-inhibitable lectin that closely resembles one recently purified from chicken liver. Both lectins interact with heparin and N-acetyl-D-galactosamine, and were purified by gel filtration on Sepharose CL-2B followed by affinity chromatography on heparin-Sepharose. They both behave as high molecular weight aggregates that can be dissociated into two peptides with apparent molecular weights of 13,000 and 16,000 by gel electrophoresis in SDS. Samples of purified lectin contained up to 20% DNA by weight, and the degree of lectin aggregation and hemagglutination activity was greatly reduced by treatment with micrococcal nuclease without inhibiting heparin-binding activity. Association of lectin with DNA is an artifact of homogenization in high salt, since only 2% of the lectin is found associated with a purified nuclear fraction.

Key words: lectins (vertebrate), heparin, glycosaminoglycans

A number of lectins have been isolated from vertebrate tissues, especially those of chicken and rodents. Some, which are believed to participate in the clearance of partially degraded glycoproteins from the circulation, are integral membrane proteins that require detergent for solubilization [1, 2]. Others are solubilized in aqueous systems without detergent. Two such lactose-binding lectins have been purified from chicken tissues [3–5], one of which seems quite similar to a lactose-binding lectin found in mammalian tissues including bovine heart and lung [6] and rat lung [7]. Recently, another soluble chicken lectin that interacts with heparin and N-acetyl-D-galactosamine has been purified from embryonic chick muscle and adult chicken liver [8]. In the course of studies of a lactose-binding lectin in lung of young rats, we found evidence for a high concentration of heparin-inhibitable lectin in the lung extracts. In the present report we describe the purification of the heparin-inhibitable lectin from extracts of young rat lung, and show its striking similarities to the heparin-inhibitable lectin from chicken. We also provide evidence that the lectins from both chicken and rat are purified in association with DNA, with which they associate upon homogenization under conditions that disrupt nuclei.

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METHODS

Lectin Purification

Purification procedures were essentially the same as those used by Ceri et al [8] for the purification of the heparin-inhibitable lectin from chicken liver. Lungs were removed from 50–75-g male Long-Evans rats (Charles River Breeding Lab., Wilmington, Massachusetts), weighed, and homogenized for 1 min in a Sorvall Omnimixer at 4°C in 6 volumes (weight/volume) of a solution of 75 mM NaCl, 75 mM Na_2HPO_4/KH_2PO_4, pH 7.2, 4 mM β-mercaptoethanol, 2 mM EDTA (MEPBS) plus 1 M NaCl and 0.1 M lactose. The homogenates were centrifuged at 100,000g for 1 hr and the supernatants were applied to a Sepharose CL-2B column (3.0 × 26 cm) that had been equilibrated with 1 M NaCl plus 4 mM β-mercaptoethanol at 6°C. The lectin was eluted from the column with the same buffer. Active fractions were pooled and incubated for 10 minutes with two volumes heparin-Sepharose that had been prepared as described by Ginsberg et al [9]. The mixture was then dialyzed overnight against two liters of 0.01 M Tris, pH 8.6, 4 mM β-merceptoethanol containing 0.01 M NaCl. The dialyzed material was poured into a column that was eluted as described in the text. The purity of the eluted fraction was evaluated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate [10] using a 12.5% polyacrylamide sample gel and a 3% polyacrylamide stacking gel.

Hemagglutination Assays

Hemagglutination assays were performed with trypsinized, glutaraldehyde-fixed rabbit erythrocytes that had been treated with ethanol as described by Kobiler and Barondes [11]. As with the heparin-inhibitable lectin from chicken, the rat lectin is a relatively poor agglutinin of erythrocytes that have not been modified by this treatment. Agglutination activity was measured in microtiter V plates (Dynatech, Alexandria, Virginia) using serial twofold dilutions of lectin extracts in MEPBS, as described previously [11]. The fixed red blood cells were added last, after which the plates were shaken vigorously. The plates were scored after 60 min. Titer is the greatest dilution that produced agglutination (eg, 1/256). Specific activity is the titer^{-1} divided by the mg protein per ml of extract. Protein was measured by the method of Bradford [12].

Heparin-binding Assay

Heparin-binding assays were performed using MicroELISA plates (Dynatech, Alexandria, Virginia) by a modification of methods described by Butler et al [13]. Each well was incubated with 100 μl of a lectin solution for 16 hr at 6°C. The wells were washed four times with MEPBS before incubation with 0.03 μg ^{3}H-heparin containing 9,600 counts/min in 100 μl MEPBS for 30 min at room temperature. The specific activity of the ^{3}H-heparin (New England Nuclear, Boston) was 0.3 mCi/mg. Wells were washed four times with MEPBS before counting. ^{3}H-heparin binding varied linearly with the amount of protein applied to the wells over the range used in these experiments.

Subcellular Localization of Lectin Activity

Adult chicken liver was homogenized in nine volumes of 0.3 M sucrose containing 4 mM β-mercaptoethanol in a Potter Elvehjem homogenizer, and the extract was filtered through Nytex, then centrifuged at 12,000g for 10 min. The supernatant, here

designated cytoplasmic supernatant fraction, was collected, and the pellet was resuspended in 2 M sucrose in 4 mM β-mercaptoethanol. To separate nuclei from other particulate materials, a 3-ml fraction of this suspension was layered over 5 ml of 2 M sucrose containing 4 mM β-mercaptoethanol and centrifuged at 40,000g for 1 hr in a swinging bucket rotor. The top 3 ml was collected as the cytoplasmic particulate fraction, and the pellet was collected as the nuclear fraction.

For estimation of lectin activity by hemagglutination assay, aliquots of the particulate fractions were homogenized in MEPBS containing 1 M NaCl and 0.1 M lactose. Aliquots of the soluble fractions were mixed with concentrated stock solutions to arrive at similar solute concentrations. All fractions were promptly assayed for hemagglutination activity. In other experiments similar subcellular fractionation procedures were used for rat lung.

Nucleic Acid Measurements

Nucleic acid contamination of the purified heparin-inhibitable lectins was initially estimated spectrophotometrically at 260 and 280 mμ. The ratio of protein:nucleic acid was calculated from published tables [14]. Discrimination of DNA from RNA was made by degradation of samples with DNase I (bovine pancreas, Sigma type I), DNase II (porcine spleen, Sigma type IV), and RNase B (bovine pancreas, Sigma type III). DNA was also measured by the diphenylamine reaction [15]. Micrococcal nuclease was obtained from Worthington, Freehold, New Jersey.

To estimate DNA content of the subcellular fractions, a total nucleic acid extract was prepared with phenol [16]. The nucleic acids were then treated with 0.3 N NaOH at 37°C for 24 hrs and dialyzed to remove degraded RNA. The DNA, which is resistant to alkaline hydrolysis, was measured with a spectrophotometer.

RESULTS

The heparin-inhibitable lectin from rat lung behaved as a high molecular weight aggregate, and was excluded from a column of Sepharose CL-2B (Fig. 1) in a manner similar to that of the lectin isolated from chicken tissue [8]. All the lectin activity that bound to a heparin-Sepharose column was eluted with 1 M NaCl (Fig. 2). This differed from previous results with the chicken lectin, which required a somewhat higher salt concentration for complete elution [8]. Considerable purification was achieved with excellent recovery (Table I). The apparent increase in total activity with purification may reflect separation of lectin from an inhibitor. Based on the protein determinations of the starting and final material, the lectin was purified about 100-fold.

The material eluted with 1 M NaCl showed two major bands on SDS polyacrylamide gel electrophoresis (Fig. 3). These bands had molecular weights that were indistinguishable from those of the purified heparin-inhibitable lectin from chicken liver (Fig. 3) with apparent subunit molecular weights of 16,000 and 13,000. The purified rat lectin retained hemagglutination activity for several weeks when stored at 4°C, but activity was markedly reduced by freezing and thawing.

Hapten Inhibition Studies

There was a great similarity in the effect of hapten inhibitors on the lectins isolated from rat lung and chicken liver (Table II). N-acetyl-D-galactosamine inhibited 50% of lectin activity at concentrations as low as 5–9 mM. However, even at concen-

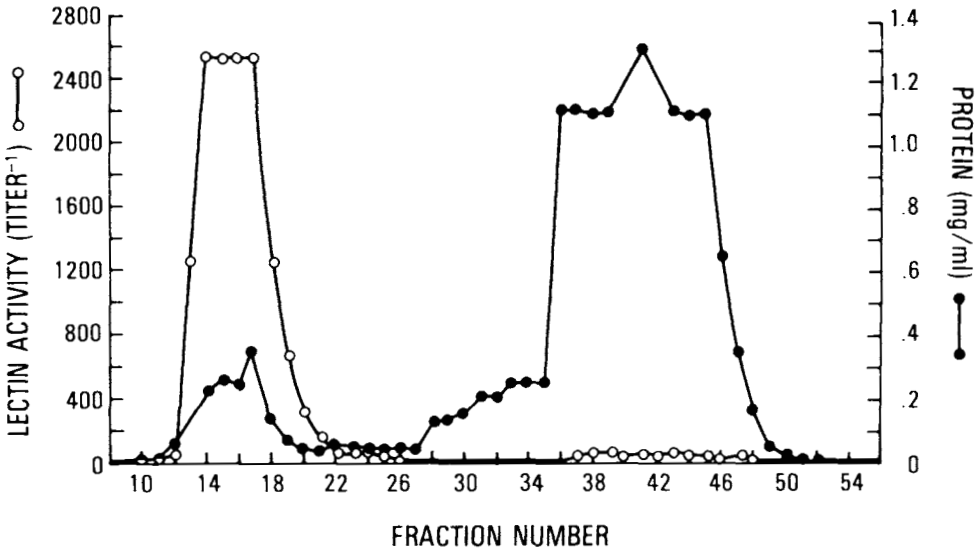

Fig. 1. Gel filtration of rat lung extract on Sepharose CL-2B. Extracts were prepared and applied to the column as described in the text. Fractions of 3.5 ml were collected. Fractions 13–19 were pooled for further purification on heparin-Sepharose.

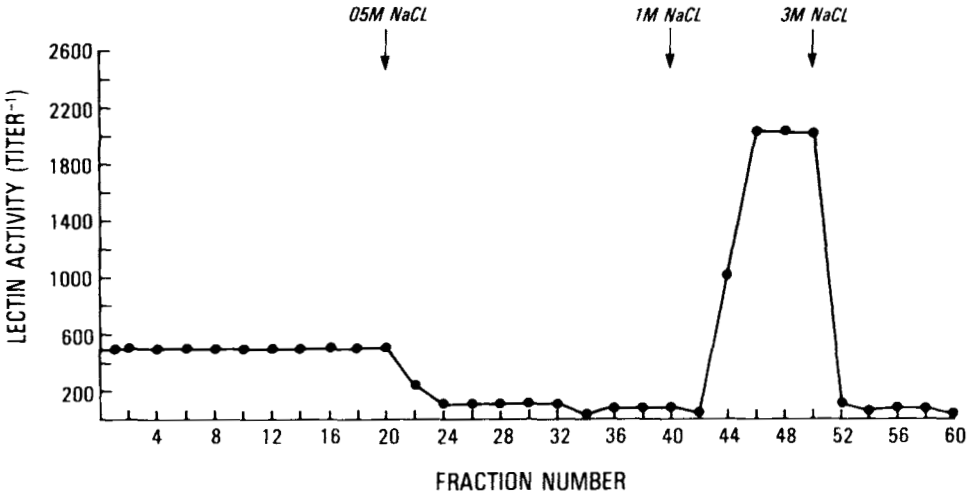

Fig. 2. Heparin-Sepharose affinity chromatography of the lectin that had been partially purified on Sepharose CL-2B. The arrows mark introduction of the indicated eluants, all of which were made up in 0.01 M Tris pH 8.1, 4 mM β-mercaptoethanol, 0.01 M NaCl. Fractions of 2.5 ml were collected.

TABLE I. Purification of Heparin-Inhibitable Rat Lung Lectin

Fraction	Specific activity	Total activity[a]	Total protein (mg)	% Recovery of activity
Crude extract	204	15,000	75	100
Sepharose CL-2B	13,000	47,000	3.5	306
Heparin-Sepharose	47,000	32,000	0.7	208

Experimental details are provided in the Methods section.
[a]$Titer^{-1}$ × ml of extract.

TABLE II. Inhibition of Lectin Activity by Sugars and Glycosaminoglycans

Inhibitor	Concentration inhibiting 50% of lectin activity	
	Rat lung	Chicken liver
N-acetyl-D-galactosamine	9 mM	5 mM
D-Mannose	>75 mM	>75 mM
D-Glucose	>75 mM	>75 mM
D-Galactose	>75 mM	>75 mM
L-Fucose	>75 mM	>75 mM
N-acetyl-D-glucosamine	>75 mM	>75 mM
D-Galactosamine	>75 mM	>75 mM
D-Glucosamine	>75 mM	>75 mM
Lactose	>75 mM	>75 mM
Melibiose	>75 mM	>75 mM
Heparin	0.31 μg/ml	0.37 μg/ml
Chondroitin-4-sulfate	>50 μg/ml	>50 μg/ml
Chondroitin-6-sulfate	>50 μg/ml	>50 μg/ml
Hyaluronic acid	>50 μg/ml	>50 μg/ml

The minimal concentration of inhibitor that reduced hemagglutination activity of the purified lectin by one step is taken to be the concentration that inhibits hemagglutination activity by 50%.

trations as high as 75 mM, only 87.5% of the activity could be inhibited. Heparin was a more potent inhibitor, completely blocking hemagglutination activity at 50μg/ml and inhibiting 50% of the lectin activity at concentrations as low as 0.31 μg/ml (Table II). Assuming a molecular weight of 10,000 for heparin for comparative purposes, its minimal inhibitory concentration is about 3×10^{-5} mM. The other glycosaminoglycans tested had no detectable effect (Table II).

Homogenization-Induced Association of Lectin With DNA

In the course of biochemical characterization of the purified lectin, we found that some preparations contained up to 20% nucleic acid by weight. The amount varied with different preparations, and tended to be greater with the material purified from rat lung than with that from chicken liver. The nucleic acid was identified as DNA since it was degraded by DNase I and DNase II but not RNase, and since the amounts measured spectrophotometrically were equivalent to those found by the diphenylamine assay, which is specific for DNA.

Fig. 3. SDS polyacrylamide gel electrophoresis of purified heparin-inhibitable lectin from (A) chicken liver and (B) rat lung. The apparent molecular weights of these bands are 15,800 ± 300 and 13,000 ± 900.

Association of the lectin with DNA influenced its physical state and hemagglutination activity. After treatment of purified rat lung lectin with 4 μg/ml of micrococcal nuclease for 20 hours at 4°C, lectin activity was no longer excluded by a Sepharose CL-2B column, and hemagglutination activity was diminished up to 90%. This marked reduction in hemagglutination activity did not appear to be due to a change in the binding activity of the lectin but rather to its state of aggregation. This was concluded from the finding that heparin-binding activity measured by a direct binding assay was not inhibited by the nuclease treatment. Using the radioactive heparin-binding assay described in Methods, nuclease-treated samples bound 1154 ± 206 counts/min of radioactive heparin, whereas nontreated lectin bound 1192 ± 131 counts/min. From these results we conclude that the marked diminution of lectin hemagglutination activity with nuclease treatment is related to the destruction of DNA–lectin complexes. Presumably the DNA, by forming such complexes, makes the lectin more polyvalent and, therefore, a more potent agglutinin.

Because of the association of lectin with DNA, we sought to determine if the lectin was associated with this nucleic acid in the nucleus. That some histones migrate like the lectin on SDS gels and bind heparin and DNA also prompted our interest in the localization of the lectin. However, we found that nuclear fractions prepared by standard subcellular procedures contained only about 2% of the total lectin activity (Table III). In contrast, about 98% of the total DNA of the tissue was found associated

TABLE III. Subcellular Localization of Lectin Activity in Chicken Liver

Fraction	Total lectin activity[a]	Percentage of total DNA
Whole cell extract	1,640,000	100
Cytoplasmic supernatant	205,000	1
Cytoplasmic particulate	1,440,000	1
Nuclear	31,000	98

The experimental procedures are described in Methods.
[a]$Titer^{-1}$ × ml of extract.

with the nuclear fraction (Table III), indicating that nuclei had not broken and released their contents during the subcellular fractionation procedure. From these results we conclude that the association of the lectin with the DNA is not physiological. It presumably occurs because the nuclei in the crude homogenates are disrupted by the high salt used for lectin extraction, with subsequent binding of the DNA to the lectin. This association may facilitate the purification of the lectin by generating the high molecular weight aggregates, which lead to its exclusion from Sepharose CL-2B. The lectin differs from histones not only in its subcellular localization, but in its insolubility in 0.5 N H_2SO_4, which readily solubilized histones that we prepared from chicken nuclei.

DISCUSSION

Previous studies [8] have demonstrated the presence of a heparin-inhibitable lectin in embryonic chick muscle and adult chicken liver. The present study describes the purification of a very similar lectin from rat lung tissue. The heparin-inhibitable lectin from rat lung was purified by a scheme similar to that used for the chicken materials and, like the latter, is composed of two polypeptide bands with apparent subunit molecular weights of 13,000 and 16,000. The hemagglutination activities of the purified materials from both animals are most sensitive to heparin. Of the simple saccharides tested, N-acetyl-D-galactosamine is the most potent inhibitor. In both species, this hapten does not totally inhibit lectin activity.

Heparin-inhibitable lectin in both rat and chicken was initially isolated as a very large molecular weight aggregate that voided on Sepharose CL-2B. In the present study we showed that this large molecular weight is the result of association of the lectin with DNA, since treatment of lectin with micrococcal nuclease leads to its inclusion in a Sepharose CL-2B column. Association of the lectin with DNA is apparently an artifact of the homogenization procedure, since only about 2% of the lectin activity is found associated with the nuclei. This association not only increases the apparent molecular weight of the lectin, facilitating its purification, but also augments its hemagglutination activity. However, heparin binding, as measured by a direct binding assay, is not influenced by nucleic acid association.

The function of the heparin-inhibitable lectins in chicken and rat tissues remains to be determined. Previous studies have shown that a heparin-inhibitable lectin is released into the medium of cultured chick myoblasts after fusion [17]. The lectin interacts strongly with a glycosaminoglycan component of the substrate attached material in

embryonic chick muscle cultures [17], suggesting the possibility that it may play a role in the organization of extracellular materials and/or the association of cells with these extracellular materials. The fact that a similar lectin is also present in rat may facilitate investigation of its function using the established tissue culture cell lines that are available.

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