Vol. 107, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 30, 1982 Pages 451-457

GALACTOSE-SPECIFIC LECTIN IN THE HEMOLYMPH OF SOLITARY ASCIDIAN, *Halocynthia roretzi* : ISOLATION AND CHARACTERIZATION

Hideyoshi Yokosawa[1], Hitoshi Sawada[1], Yukichi Abe[1], Takaharu Numakunai[2] and Shin-ichi Ishii[1]

[1]Department of Biochemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan
[2]Marine Biological Station, Tohoku University, Asamushi, Aomori 039-34, Japan

Received June 9, 1982

SUMMARY: The lectin from the hemolymph of solitary ascidian, *Halocynthia roretzi*, has been isolated in an electrophoretically homogeneous form by affinity chromatography on a column of acid-treated Sepharose. It is a large protein with $s_{20,w}$=24 S, composed of subunits with a molecular weight of 41,000. D-Galactose and various disaccharides containing D-galactose inhibit the hemagglutinating activity of the lectin. Thus, *H. roretzi* lectin is a D-galactose-specific lectin.

INTRODUCTION

Lectins, divalent or multivalent carbohydrate-binding proteins (1), are known to occur in invertebrates (2) and vertebrates (3). In invertebrates, various lectins have been found in the hemolymph and tissue extract, and proposed to function in immune or other systems (2,4).

Ascidians are prochordates which are thought to occupy the phylogenetical position between vertebrates and true invertebrates. In spite of the phylogenetical interest in the evolution of immune systems, little is known about them in ascidians, except for a proposed role of lectin(s) in the hemolymph as opsonin or receptor for phagocyte (5). Several investigations have been reported on the presence of lectins in the hemolymph of

Abbreviations: TBS, 0.15 M NaCl buffered with 0.01 M Tris-HCl, pH 7.4; TBS-Ca^{2+}, TBS containing 10 mM $CaCl_2$.

0006-291X/82/140451-07$01.00/0

ascidians which agglutinate various vertebrate erythrocytes (6-11), but all the lectins reported have not been purified to homogeneity.

In this study, an electrophoretically homogeneous lectin was obtained first from the hemolymph of ascidian *Halocynthia roretzi* by using affinity chromatography, and several properties were characterized on the purified preparation. The lectin was a large protein and classified as a D-galactose-specific lectin.

MATERIALS AND METHODS

Solitary ascidians, *Halocynthia roretzi*, type A, were harvested in Mutsu Bay, Japan. Acid-treated Sepharose was prepared by incubation of Sepharose 6B(Pharmacia) with 0.2 N HCl at 50° for 3 hr.

The hemolymph was collected by cutting the tunic matrix without injuring the internal organs. After removing the cells by centrifugation, the hemolymph was pooled and stored at -20°. Alternatively, the pooled hemolymph was precipitated with ammonium sulfate at 75% saturation and resulting precipitate was collected by centrifugation, suspended in 0.15 M NaCl buffered with 0.01 M Tris-HCl, pH 7.4, (TBS), and stored at -20°.

Ammonium sulfate suspension was thawed and dialyzed against TBS containing 10 mM $CaCl_2$ (TBS-Ca^{2+}). After insoluble materials were removed by centrifugation, 6 ml of resulting supernatant was applied on a column of acid-treated Sepharose (1.1 x 11 cm) previously equilibrated with TBS-Ca^{2+}. After extensive washing with the same buffer, adsorbed materials were eluted by including 0.1 M lactose in TBS-Ca^{2+}. A portion of effluent fractions containing lactose was dialyzed against the buffer before the hemagglutinating assay. Active fractions were pooled, dialyzed and concentrated using an Amicon membrane PM 10.

The hemagglutinating activity was assayed by the serial dilution method on microtiter plates, using 25 µl of lectin solution and 25 µl of sheep erythrocytes which were washed with TBS-Ca^{2+} and suspended in the same buffer at 5 x 10^8 cells/ml. After incubation at room temperature, hemagglutinating end points and titers were determined. The inhibitory activity of sugars (Nakarai Chemicals) was measured by mixing 12.5 µl each of serial dilutions of the sugar with 12.5 µl of the lectin solution prior to the addition of 25 µl of the erythrocyte suspension and by determining the sugar concentration which halved the agglutinating titer.

Sodium dodecyl sulfate disc polyacrylamide gel electrophoresis was performed in a slab gel containing 12% polyacrylamide according to the method of Laemmli (12). Protein was stained with Coomassie brilliant blue R250.

Sedimentation velocity experiment was carried out in a Hitachi UCA-1A analytical ultracentrifuge equipped with a UV scanner system. A solution of lectin previously dialyzed extensively against TBS-Ca^{2+} was sedimented at 10° and 29,357 rpm. The value of partial specific volume, 0.715 ml/mg, estimated on the basis of amino acid composition data (see Table I) (13), was used for the calculation of sedimentation coefficient.

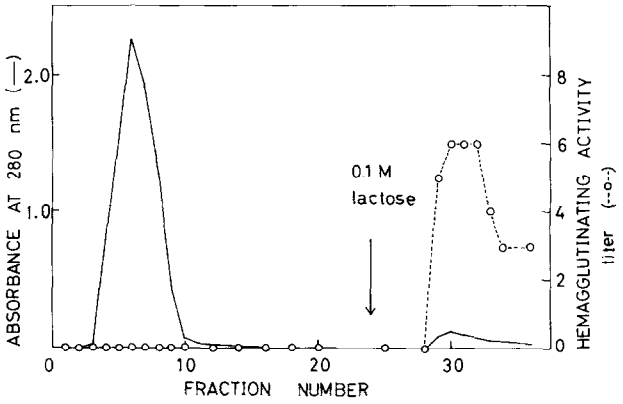

Fig. 1. Affinity chromatography on a column of acid-treated Sepharose. (——) Absorbance at 280 nm; (—o—) hemagglutinating activity.

An apparent sedimentation coefficient of the active lectin was estimated from the experiment of sucrose density gradient centrifugation. A solution of lectin in a volume of 0.2 ml was layered over 12 ml of 5-20% linear gradient of sucrose containing TBS-Ca^{2+} and centrifuged in a Spinco L8-55 ultracentrifuge for 14 hr at 4° and 32,000 rpm using an SW 40Ti rotor. Contents of the centrifugation tube were separated into 16 equal fractions and the hemagglutinating activity of each fraction was assayed after dialyzed against TBS-Ca^{2+}. Bovine serum albumin (Sigma, 4.3 S) and catalase (Milles, 11 S) were used as standards.

The amino acid composition of the lectin was determined after hydrolysis with 4 N methanesulfonic acid containing 0.2% of 3-(2-aminoethyl)indole for 24 hr at 115° according to the method of Simpson et al. (14).

RESULTS

H. roretzi hemolymph was found to agglutinate sheep erythrocytes. The hemagglutinating activity showed 4-to 8-fold enhancement on the addition of 5-10 mM $CaCl_2$, and was inhibited with melibiose most effectively among the sugars examined. D-Galactose, D-fucose and lactose also inhibited the activity though to a lesser extent. Other sugars did not exhibit detectable inhibitory activities. These results indicate that the hemolymph of *H. roretzi* contains D-galactose-specific lectin(s).

Sepharose, either with or without acid-treatment, has been used as an effective affinity adsorbent for D-galactose-specific lectin (1). As shown in Fig. 1, a column packed with acid-treated Sepharose was effective for the isolation of *H. roretzi*

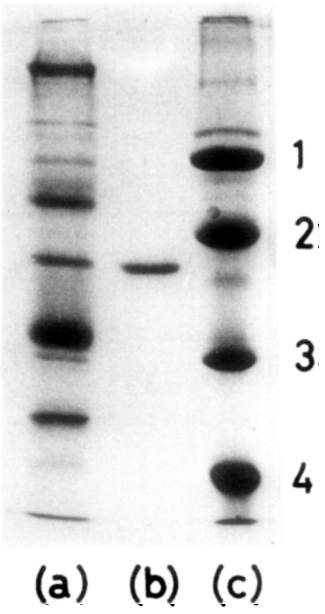

Fig. 2. Sodium dodecyl sulfate disc polyacrylamide gel electrophoresis of *H. roretzi* lectin. (a) sample before chromatography, (b) isolated lectin, (c) markers; 1. bovine serum albumin (Mr 68,000), 2. ovalbumin (45,000), 3. chymotrypsinogen (25,000) and 4. myoglobin (18,000).

lectin. The lectin adsorbed to the column in the presence of Ca^{2+} and was eluted with lactose. The purity of the lectin preparation was analyzed on the sodium dodecyl sulfate disc polyacrylamide gel under reducing conditions (Fig. 2). A single discrete band corresponding to a molecular weight of 41,000 was detected when stained with Coomassie brilliant blue.

The sedimentation velocity analysis of the purified lectin gave a single sedimentation boundary due to the material with an $s_{20,w}$ value of about 24 S. To ascertain whether the 24-S component had a hemagglutinating activity, the lectin was centrifuged through 5-20% linear gradient of sucrose and the distribution of hemagglutinating activity was analyzed. The activity showed an apparently single peak at the position corresponding to 20-22 S. Thus, *H. roretzi* lectin seems to have a large molecule which composes of subunits with a molecular weight of 41,000.

TABLE I

Amino Acid Composition of Purified *H. roretzi* Lectin

Amino Acid	Mol %	Amino Acid	Mol %
Asx	13.3	Ile	6.3
Thr	8.1	Leu	4.0
Ser	7.6	Tyr	5.5
Glx	10.2	Phe	2.8
Pro	4.7	His	5.0
Gly	9.0	Lys	4.8
Ala	6.7	Arg	2.7
Cys/2	1.3	Trp	2.9
Val	4.8		
Met	0.4	Sum	100.1

The purified *H. roretzi* lectin was heat-labile, demonstrating a complete loss of the hemagglutinating activity at 90° for 5 min. The UV spectrum of the lectin was typical of protein, showing an absorption maximum at 279 nm.

Table I shows the results of amino acid composition analysis. The lectin contains a moderately high content of glycine.

Various sugars were examined for their ability to inhibit the agglutination of sheep erythrocyte with the purified lectin. As shown in Table II, the inhibitory powers were in the order melibiose > lactose, D-galactose and D-fucose > L-fucose and stachyose. Other sugars including sialic acid showed no inhibi-

TABLE II

Effects of Various Sugars

on the Hemagglutinating Activity of Purified *H. roretzi* Lectin

Sugar	ID_{50} (mM)*	Sugar	ID_{50} (mM)*
Melibiose	3	D-Mannose	>50
Lactose	25	D-Glucose	>50
D-Galactose	25	N-Acetyl-D-Glucosamine	>50
D-Fucose	25		
L-Fucose	50	N-Acetyl-D-Galactosamine	>50
Stachyose	50		
Sialic Acid	>50		

* Concentrations of sugars required to halve the hemagglutinating activity.

tory activity at the concentration of 50 mM. Thus, *H. roretzi* lectin is a D-galactose-specific lectin, as tentatively assumed from the data using the hemolymph.

DISCUSSION

Halocynthia roretzi lectin isolated in this study appears to be different from the previously reported lectins of the same genus which have not yet been purified. Our lectin of *H. roretzi* is of a protein nature, as assessed from its heat-lability and UV spectrum, and assignable to a group of D-galactose-specific lectins, whereas the lectin of *H. hilgendorfi* has been reported to be a heat-stable polysaccharide (6) and that of *H. pyriformis* to be a sialic acid-specific lectin (8).

D-Galactose-specific lectins have been reported in the hemolymph of colonial ascidian, *Botrylloides leachii* (11). The hemagglutinating activity of *H. roretzi* lectin is inhibited with melibiose more effectively than with lactose, while the inversed order of their inhibitory effects has been observed with *B. leachii* lectins. One of *B. leachii* lectins has a large molecule, like *H. roretzi* lectin. Our preliminary experiments by the sedimentation equilibrium method showed a molecular weight of *H. roretzi* lectin to be about 600,000.

Recently, limulin, a sialic acid-specific lectin obtained from the hemolymph of *Limulus polyphemus*, has been identified as a C-reactive protein that is an acute phase protein found in man and other mammals, because of its ability for precipitating C-polysaccharide and binding with immobilized phosphorylcholine in the presence of Ca^{2+} (15). It has a large molecular weight of apparently 500,000, consisting of twelve subunits. From the resembrance between limulin and *H. roretzi* lectin in large molecularity, high glycine content, occurence in the hemolymph and Ca^{2+} requirement, it could be assumed that the *H. roretzi* lectin

has a similar biological role for example as a C-reactive protein. Investigations on the role of this lectin, as well as on its precise molecular structure, are now in progress.

REFERENCES

1. Goldstein, I.J., and Hayes, C.E. (1978) Adv. Carbohydr. Chem. Biochem. 35, 127-340.
2. Yeaton, R.W. (1981) Dev. Comp. Immunol. 5, 391-402.
3. Barondes, S.H. (1981) Ann. Rev. Biochem. 50, 207-231.
4. Yeaton, R.W. (1981) Dev. Comp. Immunol. 5, 535-545.
5. Coombe, D.R., Ey, P.L., Schluter, S.F., and Jenkin, C.R. (1981) Immunology 42, 661-669.
6. Fuke, M.T., and Sugai, T. (1972) Biol. Bull. 143, 140-149.
7. Wright, R.K. (1974) J. Invertebr. Pathol. 24, 29-36.
8. Anderson, R.S., and Good, R.A. (1975) Biol. Bull. 148, 357-369.
9. Parrinello, N., and Patricolo, E. (1975) Experientia 31, 1092-1093.
10. Wright, R.K., and Cooper, E.L. (1975) Amer. Zool. 15, 21-27.
11. Schluter, S.F., Ey, P.L., Keough, D.R., and Jenkin, C.R. (1981) Immunology 42, 241-250.
12. Laemmli, U.K. (1970) Nature 227, 680-685.
13. Cohn, E.J., and Edsall, J.T. (1943) Proteins, Amino Acids and Peptides, pp. 370-381, Reinhold, New York.
14. Simpson, R.J., Neuberger, M.R., and Liu, T.-Y. (1976) J. Biol. Chem. 251, 1936-1940.
15. Robey, F.A., and Liu, T.-Y. (1981) J. Biol. Chem. 256, 969-975.