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Partial Purification and Properties of Lectin from *Rana catesbeiana* Tadpole[1)]

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Lectin from tadpoles of *Rana catesbeiana* was partially purified by gel filtration on Sephadex G-75 followed by successive ion-exchange chromatographies on diethylaminoethyl (DEAE)-cellulose and carboxymethyl (CM)-cellulose columns. Since intact and enzyme-treated erythrocytes showed no difference in agglutinability by the lectin, a β-galactoside-binding basic protein, this may indicate that the lectin recognizes glycolipid antigens rather than glycoprotein antigens of erythrocytes. In view of the lack of hemagglutinating activity with both acetylated and succinylated lectin, it is probable that amino groups are present in the visinity of the carbohydrate-binding site of the lectin molecule.

Keywords——lectin; β-galactoside-binding protein; frog; *Rana catesbeiana*; tadpole; human erythrocyte

A family of animal lectins has been isolated from various tissues, and the biological function of lectin activity has been studied by several investigators.[2-4)] Several vertebrate lectins are able to mediate receptor-specific endocytosis of glycoproteins[5)] while others are developmentally regulated in embryonic chick muscle,[6)] rat lung[7)] and chick embryo kidney.[8)] We reported that lectins isolated from frog eggs preferentially agglutinated various tumor cells.[9)] Three lectins isolated from *Rana catesbeiana* eggs could be divided into two groups.[9c)] One group showed preferential agglutination of blood group A erythrocytes, and its specificity seemed to be directed to the trisaccharide residue GalNAcβ1→ 3Galβ1→ 4 GlcNAc.[9c,10)] The other group showed strong agglutination of various tumor cells such as Ehrlich ascites tumor cells and Sarcoma 180 ascites cells.[9)]

The tadpole of *Rana catesbeiana* contains a β-D-galactoside binding protein which non-specifically agglutinates human erythrocytes. This agglutination is specifically inhibited by lactose. To determine whether the biological role of the frog egg lectin molecule is correlated with the blockage of polyspermy at fertilization or with developmental regulation, we have initiated a study of *Rana catesbeiana* tadpole lectin.

In this paper, we describe the partial purification of tadpole lectin and present some of its physicochemical properties.

Experimental

Saccharides were purchased from Sigma and from Nakarai Chemicals, Ltd. (Kyoto, Japan). *N*-Acetyllactosamine and lacto-*N*-biose I were generous gifts from Professor S. Tejima (Faculty of Pharmaceutical Sciences, Nagoya City University). Sialidase from *Arthrobacter ureafaciens* and pronase were obtained from Nakarai Chemicals, Ltd.; trypsin from Sigma.

Preparation of partially purified Lectin——Step 1: The acetone-dried powder (14.5 g) from *Rana catesbeiana* tadpole (stage 49—54)[11)] (80 g) was homogenized with 10 volumes of ice-cold physiological saline and centrifuged at 15000×*g* for 30 min. The clear supernatant fluid was dialyzed against distilled water and then lyophilized. A summary of the purification procedure is given in Table I.

Step 2: The crude lectin fraction (1.0 g) was dissolved in 0.1 M NaCl (20 ml) and centrifuged to remove insoluble materials. The supernatant was applied to a column (4.0×40 cm) of Sephadex G-75 equilibrated with the same solvent. Fractions of 5 ml each were collected with an automatic fraction collector and the absorbancy at 280 nm was determined for each fraction. The eluates in tubes No. 60—105 (S3) were pooled, concentrated to a small volume, dialyzed against distilled water, and lyophilized (Fig. 1A).

Step 3: S3 (1.0 g) from step 2 was applied to a column (2.0×35 cm) of DEAE-cellulose equilibrated with phosphate buffer (0.001 M, pH 6.8), and elution was carried out with a stepwise increase of the molarity of phosphate buffer (pH 6.8). Fractions of 5 ml each were collected. Since it was possible that S3 contained a very small amount of aromatic amino acids (Fig. 1A), the absorbancy at 230 nm based on peptide bonds was determined. The eluates in tubes No. 90—200 (S3-D2) were pooled and treated in the same way as described above (Fig. 1B).

Step 4: S3-D2 (60 mg) from step 3 was applied to a column (1.0×20 cm) of CM-cellulose equilibrated with the same buffer as described in step 3. The chromatography was carried out under the same conditions as described in step 3. Fractions of 5 ml each were collected and the absorbancy at 230 nm was determined for each fraction. The eluates in tubes No. 108—150 (S3-D2-C3) were pooled and treated in the same way as described above (Fig. 1C).

Hemagglutination Assay——Hemagglutination activity was determined using serial 2-fold dilutions of sample solution in microtiter U-plates. Each well contained 25 μl of 2% erythrocyte suspension and 25 μl of sample solution diluted in Ringer's solution.

Hemagglutination Inhibition Assay——To study the effects of saccharides on hemagglutination, saccharides were serially diluted in microtiter plates and incubated with 3 hemagglutination doses (three times the minimum concentration for hemagglutination) of lectin in 25 μl for 30 min followed by addition of 25 μl of 2% erythrocyte suspension.

Treatment of Erythrocytes with Enzymes——Erythrocytes were washed with physiological saline and treated with trypsin and pronase according to the method described by Finne[12] and with neuraminidase as previously described[10] for 1 hr at 37°C. After incubation, erythrocytes were washed three times with Ringer's solution and tested for agglutinability.

Physicochemical Properties of partially purified Tadpole Lectin——Lectin solution (1.0—1.5 mg/0.05 ml) was heated for 30 min, then cooled in an ice-bath and assayed. Lectin solution (1.0—1.5 mg/ml) was

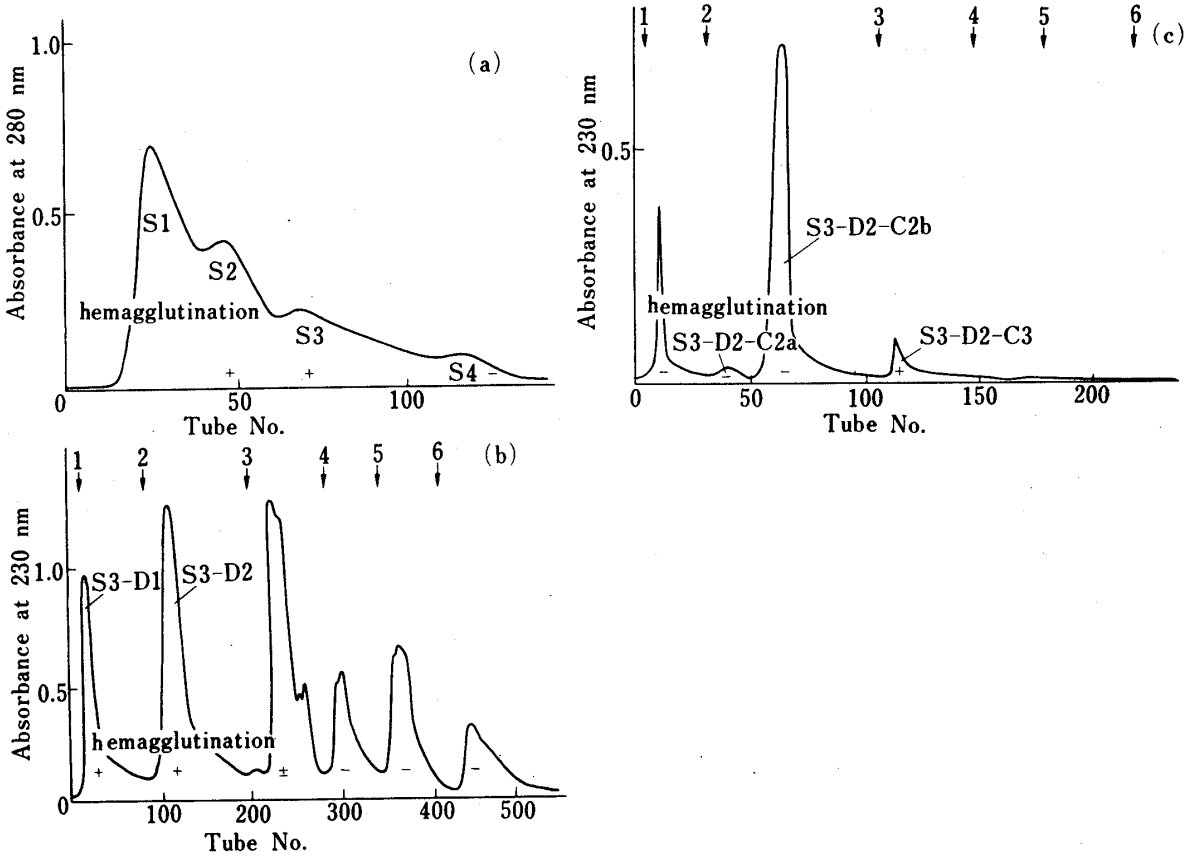

Fig. 1. Chromatographies for Partial Purification of *Rana catesbeiana* Tadpole Lectin

(a) Gel filtration pattern of the crude extract on Sephadex G-75 in 0.1 M NaCl. (b) Elution pattern on DEAE-cellulose chromatography of S3 with stepwise increases of molarity of phosphate elution buffer (pH 6.8) as indicated from 0.001 to 0.4 M. (c) Chromatography of S3-D2 on CM-cellulose with stepwise increases of molarity (0.001 to 0.4 M) of phosphate elution bufer (pH 6.8).

Eluent: 1; 0.001 M phosphate buffer, 2; 0.01 M phosphate buffer, 3; 0.05 M phosphate buffer, 4; 0.1 M phosphate buffer, 5; 0.2 M phosphate buffer, 6; 0.4 M phosphate buffer.

adjusted to pH values from 2 to 12 with 0.1 N HCl and 0.1 N NaOH. Each solution was incubated at 4°C for 24 h and dialyzed against distilled water, then the hemaggltinating activity was determined.

Reductive Methylation: Reductive methylation was carried out according to the method described by Means and Feeney.[13]

Acetylation: The acetylation of the lectin was carried out according to the method described by Fraenkel-Conrat.[14]

Succinylation: The succinylation was carried out in the presence of 1.0 M sodium bicarbonate buffer at pH 8.0 and at 25°C as described by Habeeb *et al.*[15]

Electrophoresis——The purity of the lectin was determined by electrophoresis in cellulose acetate using barbiturate buffer (pH 8.6, μ=0.05) at a constant current of 0.9 mA/cm.

Results and Discussion

Partial Purification of *Rana catesbeiana* Tadpole Lectin

The elution patterns of fractions separated from the crude lectin fraction by gel filtration on Sephadex G-75 and ion-exchange chromatographies on DEAE- and CM-cellulose columns are shown in Fig. 1. The steps of purification and the lectin activity of each fraction are shown in Table I. The yield of S3-D2-C3 was about 0.2% of the soluble protein. Cellulose acetate electrophoresis in barbiturate showed 3 bands for S3-D2-C3, and the lectin activity was localized in the fast-migrating band moving towards the cathode within these bands (Fig. 2), because S3-D2-C2b was inactive (Table I) and only the fast-migrating band contained in S3-D2-C3 was lacking in the electrophoretic pattern of S3-D2-C2b (data not shown). The lectin fraction was partially purified by the classical method, since poor results were achieved in initial attempts in which we passed S3 through a lactosyl-Sepharose column.[16]

Stability and Chemical Modification of *Rana catesbeiana* Tadpole Lectin

The lectin was labile when heated to 70°C for 30 min, but not at lower temperatures (data not shown). The optimum pH for activity was pH 7—8, and even after treatment at pH 2 or 12 for 24 h, the activity was nearly the same as that of non-treated lectin (data not shown). Table II summarizes the effect of chemical modification on the hemagglutinating activity of the lectin. Since chemical modification of the lectin by acetylation and succinylation of its amino groups abolished the hemagglutinating activity, it is probable that amino groups are present in the vicinity of the carbohydrate-binding site(s) of the lectin. As noted above, these

TABLE I. Partial Purification of *Rana catesbeiana* Tadpole Lectin

Step No.	Fraction	Total yield (%)	Lectin activity		Purification
			μg[a]	Agglutination titer/μg protein[b]	
1	Crude lectin fraction	100	250	ND	
2	S3	13.8	23	1.9	1.0
	S2	16.8	40	ND[c]	ND
3	S3-D2	4.5	16	4.3	2.3
	S3-D1	1.0	2—4	ND	ND
4	S3-D2-C3 (Partially purified lectin)	0.2	0.4	6250	3307
	S3-D2-C2b	2.7	>500	ND	ND

a) Minimum quantity of lectin per 50 μl that caused obvious agglutination of a 2% suspension of O-erythrocytes.
b) When the minimum agglutination quantity per 50 μl is 1000 μg, the agglutination titer is taken as 1.0.
c) ND; Not determined.

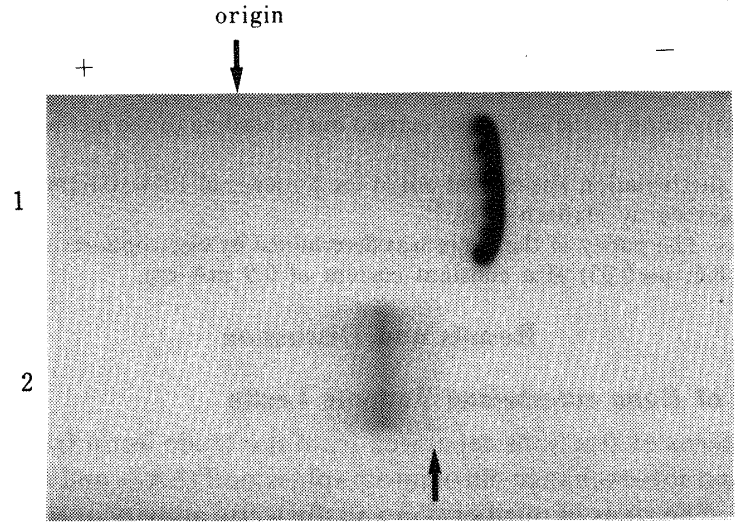

Fig. 2. Cellulose Acetate Electrophoresis Pattern of *Rana catesbeiana* Egg Agglutinin and partially purified Tadpole Lectin (S3-D2-C3)

1: *Rana catesbeiana* egg agglutinin agglutinated various tumor-cells (concentration, 10 μg/5 μl).
2: S3-D2-C3 (concentration, 5 μg/5 μl). The lectin activity was localized exclusively in the fraction indicated by the arrow.

TABLE II. Chemical Modification of Tadpole Lectin (S3-D2 Fraction)

Method of modification	Lowest modified lectin concentration for agglutination (μg)
Reductive methylation	100
Acetylation	>500
Succinylation	>500
S3-D2	16

properties of the lectin are similar to those of the agglutinin[9c] which was isolated from eggs of *Rana catesbeiana* and which preferentially agglutiated tumor cells such as Ehrlich ascites cells and rat ascites hepatoma cells.[17] Since the agglutinating activity of the agglutinin was not inhibited by lactose, chemical modification of the lectin was carried out in the absence of lactose for comparison of the properties of the lectin with those of the agglutinin.

Agglutinability of Erythrocytes by *Rana catesbeiana* Tadpole Lectin

The tadpole lectin strongly agglutinates human erythrocytes nonspecifically, and weakly agglutinates mouse, rat and guinea pig erythrocytes (see Table III); it does not agglutinate various tumor cells such as Ehrlich ascites cells, Sarcoma 180 ascites cells, L1210 cells, AH109A cells and AS653 cells. Trypsin degrades the major sialoglycoprotein and pronase degrades both the major sialoglycoprotein and band 3.[12] Since these enzyme treatments of human erythrocytes left them agglutinable to nearly the same extent as intact cells, the lectin may recognize glycolipid antigens rather than glycoprotein antigens of human erythrocytes.

Sugar Specificity of *Rana catesbeiana* Tadpole Lectin

The sugar specificity of the tadpole lectin is very similar to that of the egg lectin, showing an anti-A like property.[9c,10] Saccharide specificity was assessed by measurement of inhibition of hemagglutination. The results are given in Table IV. The most effective inhibitor was

lactose. While phenyl α-D-galactoside was twice as effective as phenyl β-D-galactoside, lactose was 5—6 times as effective as melibiose. Lactulose, *N*-acetyllactosamine[18] and lacto-*N*-biose I[19] are also potent inhibitors, indicating that the lectin preferentially binds the β-D-linkage. Since the inhibitory activity of *N*-acetyllactosamine was similar to that of lacto-*N*-biose I, the lectin seems to recognize not only the 4-*O*-β-D-linkage but also the 3-*O*-β-D-linkage.

TABLE III. Minimum Concentration of Tadpole Lectin (S3-D2 Fraction) giving a Positive Hemagglutination Reaction

Red-blood-cell type and treatment	Agglutinability[a] (μg lectin)
A, intact	32
B, intact	16
O, intact	16
A+trypsin	16
B+trypsin	8—16
O+trypsin	16
A+pronase	16
B+pronase	8
O+pronase	4—8
A+neuraminidase	16
B+neuraminidase	4
O+neuraminidase	16
Mouse	500
Rat	125
Guinea pig	500

a) See the footnote to Table I *a* for definition.

TABLE IV. Minimum Concentration of Inhibitor giving Complete Inhibition of Hemagglutination of Human O-Erythrocytes by Tadpole Lectin

Saccharide	Concentration (mM)[a]	Saccharide	Concentration (mM)[a]
D-Glucose	>200	D-Galactosamine · HCl	50—100
L-Glucose	>200	Methyl-α-D-galactoside	50—100
N-Acetyl-D-glucosamine	>200	Methyl-β-D-galactoside	150
D-Glucosamine · HCl	>200	Phenyl-α-D-galactoside	50
D-Xylose	>200	Phenyl-β-D-galactoside	100
L-Xylose	>200	2-Deoxy-D-galactose	>200
D-Arabinose	>200	Lactose	1.5—3.0
L-Arabinose	>200	Lactulose	6—12
D-Fucose	>200	*N*-Acetyllactosamine	6
L-Fucose	>200	Lacto-*N*-biose I	7.5
D-Galactose	50—100	Melibiose	50—100
L-Galactose	>200	Raffinose	>200
N-Acetyl-D-galactosamine	100	Stachyose	12

a) Minimum quantity of saccharide per 25 μl that inhibited hemagglutination of a 2% cell suspension.

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