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Reactivity of Lectin from *Xenopus laevis* Eggs towards Tumor Cells and Human Erythrocytes

KAZUO NITTA,* GIICHI TAKAYANAGI and HIROAKI KAWAUCHI

Cancer Research Institute, Tohoku College of Pharmaceutical Sciences, 4–4–1 Komatsushima, Sendai 983, Japan

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Lectin from eggs of *Xenopus laevis* showed strong agglutination of various tumor cells such as Ehrlich ascites cells and Sarcoma 180 ascites cells. Agglutination of human erythrocytes by the lectin occurs only after digestion of these cells by sialidase. Since lectin-induced cell agglutination was inhibited by D-galactose (the most effective monosaccharide inhibitor), and since the inhibitory activity of lacto-*N*-biose I was much higher than that of *N*-acetyllactosamine, the lectin seems to recognize the terminal D-galactosyl residues and particularly 3-*O*-*β*-D-linked oligosaccharide chains involving the Gal-GlcNAc-X- sequence. Trypsin or pronase treatment of sialidase-treated human erythrocytes converted them from an agglutinable to a nonagglutinable form. This result demonstrates that the lectin receptor site(s) exists in trypsin-labile cell surface glycoprotein(s).

Keywords——lectin; *β*-galactoside-binding protein; frog; *Xenopus laevis*; tumor cell; cell surface glycoprotein

A number of carbohydrate-specific proteins, lectins, have been purified from various plant and animal sources. They are widely used as powerful tools in cell surface and complex carbohydrate research owing to their unique ability to bind specific sugars or sugar-containing macromolecules.[1,2] Various lectins with different specificities have been isolated from eggs of various frogs.[3,4] Among these lectins, the lectin from *Rana japonica* eggs agglutinated various tumor cells such as Ehrlich ascites cells, Sarcoma 180 ascites cells and rat ascites hepatoma cells (AH109A),[3a] but did not agglutinate nontransformed fibroblasts[3b] or human erythrocytes, even after trypsin or sialidase treatment.[5]

We have found that a lectin from eggs of *Xenopus laevis* (*X. laevis*) showed strong agglutination of various tumor cells as well as sialidase-treated human erythrocytes. This lectin should be useful as a probe of cell surface carbohydrate structure.

Experimental

Saccharides were purchased from Sigma, Fluka and Nakarai Chemicals, Ltd. (Kyoto, Japan). Synthetic samples of *N*-acetyllactosamine, lacto-*N*-biose I, 6′-*β*-D-galactosyllactose and lacto-*N*-tetraose, and lacto-*N*-tetraose obtained from urine were generous gifts from Dr. S. Tejima (Faculty of Pharmaceutical Sciences, Nagoya City University) and from Dr. M. Koseki (Fukushima Prefecture Institute of Public Health and Environment), respectively. Sialidase from *Arthrobacter ureafaciens* was obtained from Nakarai Chemicals, Ltd., and trypsin was from Sigma.

Preparation of *X. laevis* Lectin——Step 1. The acetone-dried powder of *X. laevis* eggs was homogenized with 10 volumes of ice-cold physiological saline and centrifuged. The clear supernatant fluid was dialyzed extensively against distilled water and then lyophilized.

Step 2. The crude lectin fraction (1.2 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. 7—24 (S1) were pooled, concentrated to a small

volume, dialyzed against distilled water, and lyophilized (Fig. 1A).

Step 3. S1 (700 mg) from step 2 was applied to a column (2.0 × 30 cm) of DEAE-cellulose equilibrated with phosphate buffer (0.001 M, pH 6.8), and elution was performed with a stepwise increase of the molarity of phosphate buffer (pH 6.8). Fractions of 5 ml each were collected and the absorbancy at 280 nm was determined for each fraction. The eluates in tubes No. 305—400 (S1-D4) were pooled and treated in the same way as described above (Fig. 1B).

Step 4. S1-D4 (200 mg) from step 3 was dissolved in phosphate-buffered saline (PBS; 0.05 M, pH 6.8) (10 ml) and centrifuged to remove insoluble materials. The supernatant was applied to a column (1.0 × 20 cm) of lactamyl-Sepharose 6B[6] equilibrated with the same solvent. Elution was performed first with a large amount of PBS, then with PBS containing 0.1 M lactose, and finally with PBS containing 0.1 M lactose and 1.0 M NaCl. Fractions of 3 ml each were collected and the absorbancy at 280 nm was determined for each fraction. The eluates in tubes No. 5—35 (S1-D4-L1) were pooled and treated in the same way as described above (Fig. 1C).

Electrophoresis——The purity of the lectin was determined by gel electrophoresis in sodium dodecyl sulfate (SDS) according to the method of Laemmli[7] using 4.5% polyacrylamide stacking gel and 10% polyacrylamide sample gel, and by electrophoresis on cellulose acetate using barbiturate buffer (pH 8.6, $\mu=0.05$) (Fig. 2).

Agglutination and Inhibition Assay for Tumor Cells——Agglutination activity was determined by using serial 2-fold dilutions of sample solution in small test tubes. Each tube contained 100 μl of cell suspension (1×10^7 cells/ml) and 100 μl of sample solution diluted in Ringer's solution. Inhibition of agglutination activity by haptens was determined by studying the effect of serial 2-fold dilutions of the hapten inhibitors. Saccharides were diluted in small test tubes and incubated with 3 agglutination doses (three times the minimum concentration for agglutination) of lectin for 30 min followed by addition of cell suspension.

Hemagglutination and Inhibition Assay——Hemagglutination and inhibition assay were performed in Ringer's solution by the use of a microtiter plate as described previously.[8]

Enzymic Digestion of Viable Cells——Cells were washed 3 times with physiological saline and 3 times with the buffer used for treatment with trypsin, pronase or sialidase, then treated with the enzyme. The legend to Fig. 3 gives the conditions of these enzymatic digestions. After incubation, cells were washed 3 times with Ringer's solution and tested for agglutinability.

Chemical Modification of *X. laevis* Lectin——Reductive methylation; Reductive methylation was carried out according to the method described by Means and Feeney.[9]

Acetylation; The acetylation of the lectin was carried out according to the method described by Fraenkel-Conrat.[10]

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.*[11]

Results

The elution patterns of fractions separated from the crude lectin fraction by gel filtration on Sephadex G-75, ion-exchange chromatography on DEAE-cellulose and affinity chromatography on lactamyl-Sepharose 6B are shown in Fig. 1. The presence or absence of lectin activity in each fraction is indicated by plus (+) or minus (−) in the chromatographic patterns (Fig. 1).

The electrophoretic profiles of fractions that possessed agglutinating activity, S1, Sl-D4 and S1-D4-L1, are shown in Fig. 2. When the lectin activity of crude extract is taken as 1.0, those of S1, S1-D4 and S1-D4-L1 are 2—3, 50—100 and 50—100, respectively. Although Roberson *et al.* prepared the lectin from *X. laevis* oocytes and blastula stage embryos by affinity chromatography on a column of melibiose coupled to aminoethylated polyacrylamide gel beads,[4] the tumor cell agglutinating activity was found in the unbound fraction (S1-D4-L1), which was not purified, in affinity chromatography on a lactamyl-Sepharose 6B column.

Table I gives the agglutinability of various cells by the lectin. The lectin agglutinated Ehrlich ascites cells and Sarcoma 180 ascites cells, but did not agglutinate rat ascites hepatoma cells (AH109A). Although intact and trypsin-treated blood group A-, B- and O-erythrocytes were not agglutinated by the lectin, they become strongly agglutinable after treatment with sialidase. Thus, the lectin nonspecifically agglutinated sialidase-treated human erythrocytes.

The time dependent changes of agglutinability of cells caused by trypsin and sialidase are shown in Fig. 3. Agglutinability of tumor cells by the lectin increased when the cells were

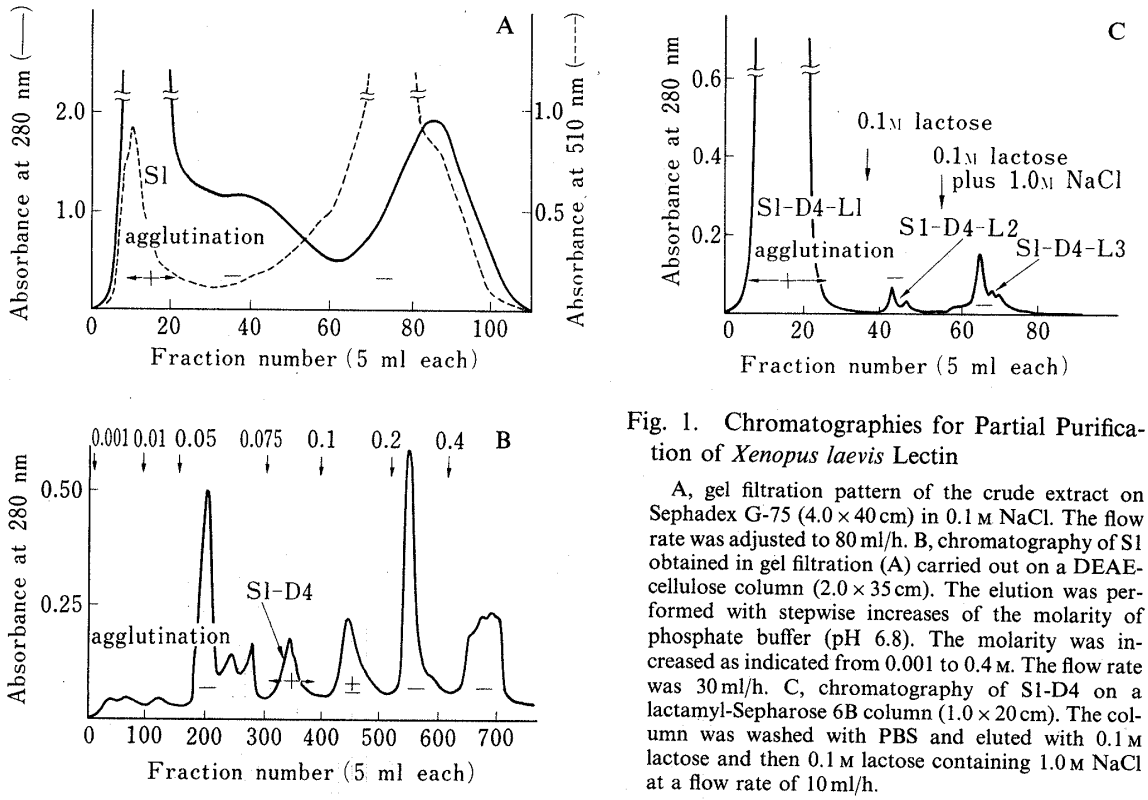

Fig. 1. Chromatographies for Partial Purification of *Xenopus laevis* Lectin

A, gel filtration pattern of the crude extract on Sephadex G-75 (4.0 × 40 cm) in 0.1 M NaCl. The flow rate was adjusted to 80 ml/h. B, chromatography of S1 obtained in gel filtration (A) carried out on a DEAE-cellulose column (2.0 × 35 cm). The elution was performed with stepwise increases of the molarity of phosphate buffer (pH 6.8). The molarity was increased as indicated from 0.001 to 0.4 M. The flow rate was 30 ml/h. C, chromatography of S1-D4 on a lactamyl-Sepharose 6B column (1.0 × 20 cm). The column was washed with PBS and eluted with 0.1 M lactose and then 0.1 M lactose containing 1.0 M NaCl at a flow rate of 10 ml/h.

incubated with 1 μg/ml trypsin for 10 min, but decreased when the incubation time was prolonged (see Fig. 3A). After treatment with 10 μg/ml trypsin for 15 min, agglutinability decreased (data not shown). Agglutinability of Ehrlich ascites cells slightly increased after treatment with sialidase for 10 min, but decreased when the incubation time was prolonged. However, agglutinability of Sarcoma 180 ascites cells increased on incubation of the cells with sialidase for 10 min, and the increment of agglutinability was maintained after incubation with sialidase for 60 min (see Fig. 3B). The changes of agglutinability of human O-erythrocytes by sialidase are shown in Fig. 3C. Trypsin treatment of sialidase-treated erythrocytes remarkably decreased the agglutinability of the red cells (see Fig. 3D). The results of pronase treatment under the same conditions were similar (data not shown).

Table II presents the inhibition data. Generally, the specificity of the binding site of the lectins is probed by means of such inhibition tests. The most effective monosaccharide inhibitor was D-galactose. High inhibitory activity was also exhibited by D-fucose and L-arabinose, whereas 2-acetoamido-2-deoxy-D-galactose was not inhibitory. Methyl or phenyl α-D-galactopyranoside was 1.5—3.0 times as effective as methyl or phenyl β-D-galactopyranoside, respectively. L-Rhamnose, D-lyxose, D-ribose and L-mannose had one-half to one-quarter of the effect of D-galactose. Among the oligosaccharides examined for their capacity to inhibit the lectin, lacto-*N*-tetraose was the best inhibitor. The order of inhibitory capacity for Ehrlich ascites cells was lacto-*N*-tetraose ≧ lactose > lacto-*N*-biose I = melibiose ≧ 6′-β-D-galactosyllactose > *N*-acetyllactosamine > lactulose ≧ stachyose > raffinose, and for Sarcoma 180 ascites cells it was lacto-*N*-tetraose ≧ lactose > lacto-*N*-biose I = 6′-β-D-galactosyllactose = stachyose ≧ melibiose > lactulose > raffinose. Agglutination of sialidase-treated erythrocytes by the lectin was inhibited by lacto-*N*-tetraose > lactose > lacto-*N*-biose I = melibiose > 6′-β-D-galactosyllactose > lactulose ≧ stachyose > raffinose.

Table III summarizes the effect of chemical modification on the tumor cell agglutinating activity of the lectin. Reductive methylation of the available lectin amino groups only slightly

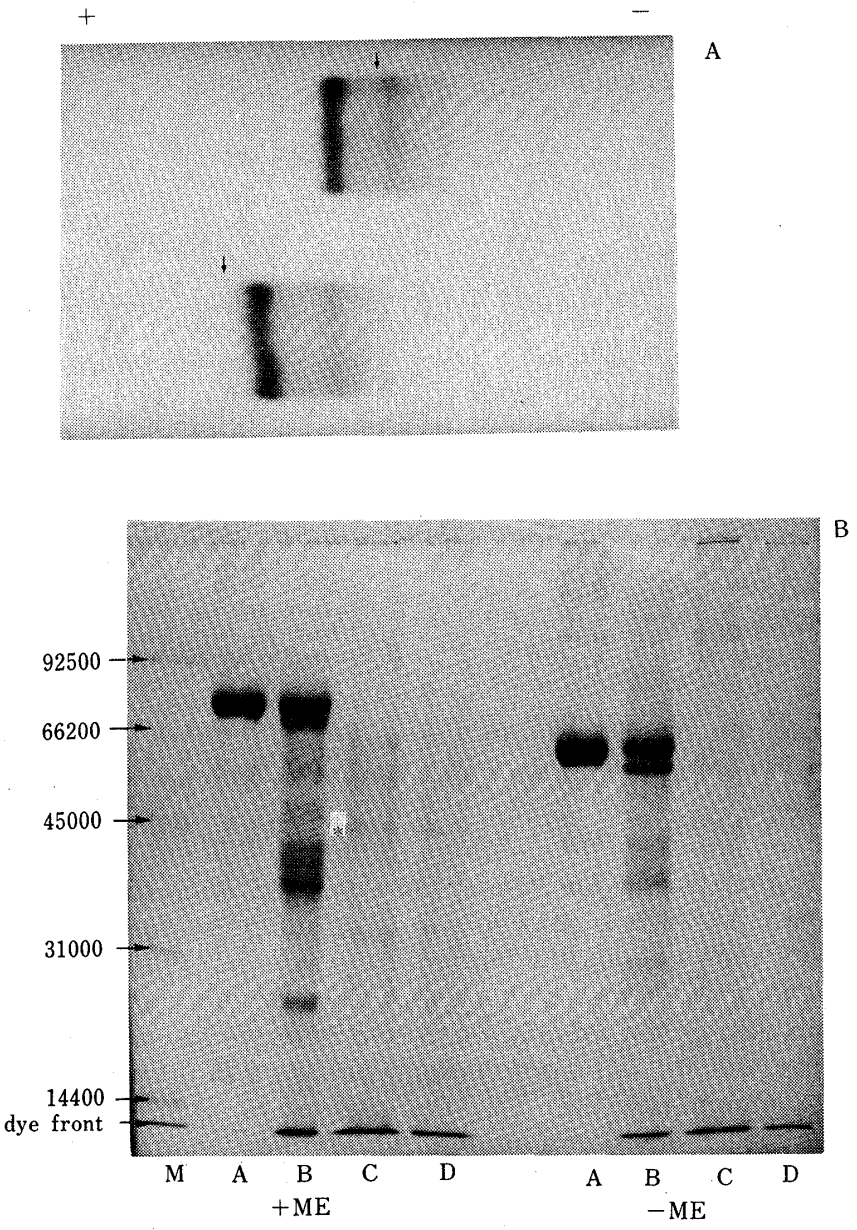

Fig. 2. Electrophoresis Patterns of the Fractions Derived from Column Chromatographies

A, cellulose acetate electrophoresis pattern of S1-D4 (10 μg) in barbiturate buffer with a current of 0.9 mA/cm for 45 min. The arrow indicates the origin. B, SDS gel electrophoresis pattern of (A) S1-D4, (B) S1-D4-L1, (C) S1-D4-L2, (D) S1-D4-L3 and (M) molecular weight standard in the presence or absence of 2-mercaptoethanol (+ME or −ME). A (50 μg protein), B (50 μg protein), C (100 μg protein) and D (100 μg protein). *) This band corresponds to the lectin reported by Roberson *et al.*[4]

reduced its ability to induce Ehrlich or Sarcoma 180 cell agglutination, and the acetylated or succinylated lectin-induced Sarcome 180 cell agglutination activity was slightly reduced. In contrast, acetylation or succinylation of the protein markedly reduced its ability to agglutinate Ehrlich cells.

Discussion

A cortical granule lectin of *X. laevis* eggs has been reported by Wyrick *et al.* to have some

TABLE I. Agglutinability of Various Cells by Partially Purified *Xenopus laevis* Lectin

Cells and treatment	Agglutinability (μg lectin)
Ehrlich tumor cells	10—20[a]
Sarcoma 180 cells	15—30
Ascites hepatoma cells (AH109A)	>500
A, intact	>1000[b]
B, intact	>1000
O, intact	>1000
A+trypsin	>1000
B+trypsin	>1000
O+trypsin	>1000
A+neuraminidase	10—20
B+neuraminidase	10—20
O+neuraminidase	5—10

a) Minimum quantity of lectin per 0.1 ml that caused obvious agglutination of a suspension of cells.
b) Minimum quantity of lectin per 0.025 ml that caused obvious agglutination of a suspension of erythrocytes.

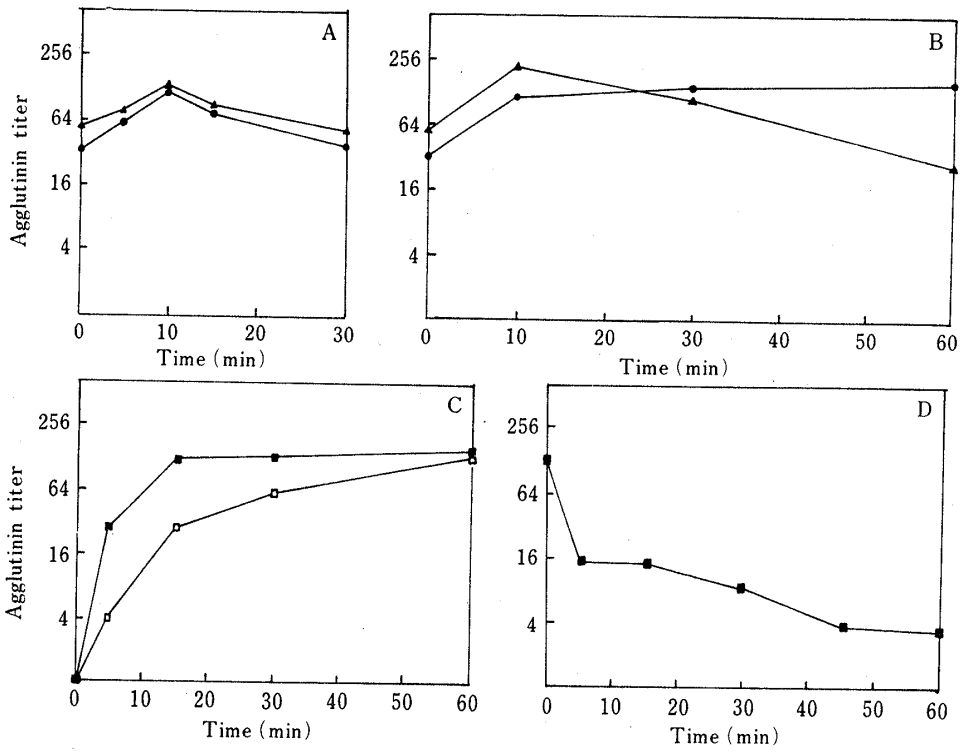

Fig. 3. Effect of Enzymatic Digestion of Ehrlich Cells (▲), Sarcoma 180 Cells (●) and Human O-Erythrocytes (■, □) on Their Agglutination

A, Trypsin treatment: Cells (4×10^6) were suspended in 1 ml of phosphate-buffered saline (pH 7.0) and incubated with trypsin (1 μg/ml). B, Sialidase treatment: Cells (1×10^6) were suspended in 1 ml of NaCl–CH_3COONa–$CaCl_2$ (pH 6.0) and incubated with sialidase (1 unit/ml). C, O-Erythrocytes (0.2 ml packed cell volume) were treated with sialidase (■, 0.1 unit; □, 0.01 unit). D, Sialidase-treated O-erythrocytes were incubated with trypsin (0.1%). When the minimum agglutination quantity per 200 μl is 1000 μg, the agglutination titer is taken as 1.0.

role at fertilization in blocking polyspermy.[12] The precipitin reaction of the lectin with the material from the isolated jelly layer of the eggs was blocked by methyl α-D-galactoside but not by methyl β-D-galactoside, and required Ca^{2+}. Nishihara subsequently reported that two

TABLE II. Comparison of Agglutination Inhibitory Activities of Monosaccharides and Oligosaccharides (mM)

Saccharides	Ehrlich	S-180	O-Erythrocyte[a]
D-Glucose	50	>100	30—60
L-Glucose	>100	>100	>100
N-Acetyl-D-glucosamine	>100	>100	>100
D-Glucosamine	>100	>100	>100
D-Glucuronic acid[b]	50—100		
D-Galactose	4—8	4—8	1.5—3
L-Galactose	>100	>100	>100
N-Acetyl-D-galactosamine	>100	>100	>100
D-Galactosamine	>100	>100	>100
Phenyl-α-D-galactoside	20—40	6—12	2.5—10
Phenyl-β-D-galactoside	30—60	12—24	8.5—17
Methyl-α-D-galactoside	4.5—9	6—12	1.1—2.2
Methyl-β-D-galactoside	25—50	12—24	6—12
α-D-Galacturonic acid[b]	12—24		
D-Mannose	>100	>100	50—100
L-Mannose	50	16—32	14—28
D-Mannosamine	>100	>100	>100
D-Fructose	60	90	25—50
L-Sorbose	>100	35—70	
D-Fucose	7	7—28	2.5—5
L-Fucose	>100	>100	>100
L-Rhamnose	30	40—80	6—12
D-Xylose	70	50	25—50
D-Lyxose	60	100	8—16
L-Lyxose	90	>100	14—28
D-Arabinose	>100	>100	100
L-Arabinose	15	10—20	1.5—3
D-Ribose		35—70	12—24
Lactose	3.5—7	4—8	0.4—0.8
Melibiose	6—12	14—28	0.9—1.8
Lactulose	50	45	6.5—13
N-Acetyllactosamine[c]	20	>25	>25
Lacto-*N*-biose I[c]	6—12	5—10	0.75—1.5
6′-β-D-Galactosyllactose[c]	13	6.5—13	1.8—3.6
Raffinose	90	45—90	15—30
Stachyose	40—80	5—10	10—20
Lacto-*N*-tetraose[c]	2	1—2	0.1—0.2
Lacto-*N*-tetraose[d]	7	3—6	0.5—1

a) Erythrocytes treated with sialidase for 1 h at 37 °C.
b) Inhibitory activities were determined after neutralization.
c) The samples were synthesized by Tejima *et al.*
d) The sample was isolated from urine by Koseki.

TABLE III. Chemical Modification of *Xenopus laevis* Egg Lectin

Method of modification	Lowest modified lectin concentration for agglutination (μg)	
	Ehrlich	Sarcoma 180
Reductive methylation	60—120	100
Acetylation	>500	250
Succinylation	300—600	100—200

lectins with molecular weights of 567000 or 695000 were separated by affinity chromatography on a column of J_1 material coupled to Sepharose 4B and the activity was inhibited by D-galactose, melibiose and lactose but not by raffinose or stachyose.[13] Lactose was twice as effective as D-galactose or melibiose. Roberson *et al.*[4] reported that the lectin activity in oocytes, embryos and liver was readily purified by affinity chromatography on an affinity column containing covalently linked melibiose, and the trypsin-treated, glutaraldehyde-fixed rabbit erythrocyte agglutination activities of these lectins were blocked by methyl α-D-galactoside 2—20 times as effectively as by methyl β-D-galactoside, and by lactose as well as melibiose. The lectin from oocytes has a molecular weight of 480000. Although Wyrick *et al.*[12] reported that the cortical granule lectin is secreted with fertilization and becomes incorporated into the fertilization envelope, Roberson *et al.* pointed out that the lectin in the embryo is not confined to the fertilization envelope.

The sugar specificity of the lectin reported by Roberson *et al.* is similar to that reported in this paper. Wyrick *et al.* reported that stachyose and raffinose were not inhibitors. Roberson *et al.* did not refer to this, but stachyose was found to be a potent inhibitor of the lectin-induced Sarcoma 180 cell agglutination in the present work. Although the lectin activity from oocytes and embryos was readily purified on a column of aminoethylated polyacrylamide gel beads conjugated with melibiose, the lectin reported here has no affinity for lactamyl-Sepharose, even though lactose is a potent inhibitor. Based on these findings, it is possible that heterogeneous lectins may be present in *X. laevis* eggs. As the agglutinating activity of the sum of heterogeneous lectins, if all of them were contained in this fraction (S1-D4-L1), was assayed and compared by the use of different species of cells, we must point out, differences of affinity of each lectin to each cell line and of the lectin content might complicate the situation.

The results suggest that the lectin seems to recognize the terminal D-galactosyl residues of cell surface oligosaccharide chains for the following reasons:

1) the lectin agglutinates Ehrlich ascites carcinoma cells and Sarcoma 180 ascites cells, and furthermore the agglutinability of these cells increases after brief treatment with sialidase;

2) agglutination of erythrocytes by the lectin occurs only after digestion of these cells by sialidase;

3) D-galactose is the most effective monosaccharide inhibitor.

Since trypsin or pronase treatment of sialidase-treated erythrocytes converted them from an agglutinable to a nonagglutinable form, the lectin receptor site exists in trypsin-labile cell surface glycoprotein(s).

Of the monosaccharides tested, D-galactose was the most effective inhibitor, whereas 2-amino-2-deoxy-D-galactose and 2-acetamido-2-deoxy-D-galactose were inactive, suggesting the importance of the 2-hydroxyl group of sugars. While methyl α-D-galactoside was 3 times as effective as methyl β-D-galactoside, lactose (Galβ1→4Glc) was twice as effective as melibiose (Galα1→6Glc). In addition, lactose was 5—8 times more active than *N*-acetyllactosamine, indicating that the lectin must recognize penultimate residues and the sugar linkage. Stachyose is more potent than raffinose in inhibiting the binding to Sarcoma 180 cells. This suggests that the lectin recognized not only the nonreducing terminal D-galactosyl residue but also the penultimate D-galactosyl residue. Since 6′-β-D-galactosyllactose gave higher inhibition than stachyose, the lectin preferentially binds the β-D-linkage. A preference for 3-*O*-β-D-linkage over 4-*O*-β-D-linkage is suggested by comparison of the activity of lacto-*N*-biose I with that of *N*-acetyllactosamine.

Based on the lack of Ehrlich cell agglutinating activity with both acetylated and succinylated lectin, it is possible that amino groups are present in the vicinity of the carbohydrate-binding site(s) of the lectin molecule. On the other hand, it is surprising that the acetylated or succinylated lectin-induced sarcoma 180 cell agglutinating activity was only slightly reduced. The findings presented here raise the possibility that the active fraction

reported by us may consist of heterogeneous lectins or that the lectin may react with several lectin receptors, which may consist of many oligosaccharide chains that differ in affinity for the lectin. Further work is necessary to purify the lectin and to determine the specificity by oligosaccharides assuming well difined structure and conformation.

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