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PURIFICATION AND CHARACTERIZATION OF β-GALACTOSIDE BINDING LECTIN FROM FROG (*Rana catesbeiana*) EGGS

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A β-galactoside-binding lectin, a homodimer composed of 14kDa subunits, was purified from unfertilized eggs of the frog *Rana catesbeiana* by asialofetuin-Sepharose 4B affinity column chromatography. The lectin was solubilized from eggs by addition of neither haptenic sugar nor detergent and showed a unique characteristic that it requires neither Ca^{++} nor SH-reagent for its hemagglutination activity. However, the partial amino acid sequence indicated that the lectin belongs to a family of soluble 14kDa β-galactoside-binding lectins (14K-lectin) widely distributed in vertebrates and classified as S type lectins. These results indicate that a 14K-lectin is present as the free form in unfertilized frog eggs, presenting the first structural evidence for the presence of a soluble 14K-lectin in the amphibian eggs.

β-Galactoside-binding 14kDa lectins (14K-lectin) are widely distributed in a variety of tissues of mammals, birds, and fish (1-5). Biological function of these lectins is still unclear, but they may function in cellular interactions such as cell adhesion, tissue construction, and differentiation (6-8). The presence of numerous lectins in frog tissues with different sugar specificities and molecular masses has been reported (4,10,11). Those include β-galactoside binding lectins composed of 14 and 16 kDa subunits from *Xenopus leavis* skin and *Bufo arenarum* ovary, respectively (9,10). However, there is no structural evidence that these lectins are members of the vertebrate 14K-lectin family.

Rana catesbeiana eggs are known to contain several soluble lectins. Two distinct lectins, which agglutinate human type A erythrocytes and cancer cells respectively,

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have been isolated and characterized (12,13). We observed that the extract of *Rana catesbeiana* eggs also strongly agglutinates trypsinized rabbit erythrocytes. Since this activity is inhibited by addition of β-galactoside, the presence of a β-galactoside-binding lectin has been predicted.

In this paper, we describe purification and characterization of a β-galactoside-specific lectin from *Rana catesbeiana* eggs and present an evidence by analysis of the partial amino acid sequence that it is a member of the vertebrate 14K lectin family.

MATERIALS AND METHODS

Purification of Rana catesbeiana Egg Lectin. Unfertilized frog eggs were obtained from the adult body cavity by laparotomy. Lipids were removed with cold acetone. Acetone powder of the eggs was used as the starting material for purification of the lectin. Asialofetuin (AF) obtained by mild acid hydrolysis of fetuin (Sigma)(14) was conjugated to BrCN-activated Sepharose 4B as described (15). One g of acetone powder of the eggs was homogenized with 10 ml of 100 mM NH_4HCO_3 (pH 8.0) at 0°C in a mortar with a pestle. After centrifugation at 27,500 x g for 60 min, the supernatant (crude extract) was applied to an AF-Sepharose 4B column (1 x 20 cm) equilibrated with 100 mM NH_4HCO_3 (pH 8.0) at 4°C. The column was washed with the same buffer until the absorbance at 280 nm reached the base line. The eluate obtained with the same buffer containing 0.1M lactose was exhaustively dialyzed against 100 mM NH_4HCO_3 and lyophilized.

Analytical Methods. Hemagglutination assay was performed as previously described (12). Apparent molecular mass was estimated by gel filtration on a Cellulofine GCL-2000m (Seikagaku Kogyo, Tokyo) column (1 x 100 cm) or by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)(16). Reduction and carbamoylmethylation of disulfide bonds and cleavage with BrCN were carried out as described (17,18). Peptides were separated by reversed-phase high performance liquid chromatography (HPLC) on a Cosmosil 5C8 (C8) column (4.6 x 100mm; Nacalai Tesque, Inc., Kyoto, Japan) with gradients of acetonitrile into dilute aquous trifluoroacetic acid. Amino acid composition was obtained with a Hitachi Model L8500 Amino Acid Analyzer. Sequence analysis was performed with an Applied Biosystems 470A Protein Sequencer connected on-line to a 120A PTH Analyzer.

Sequence homology was searched in the protein sequence database of the National Biomedical Research Foundation on a VAX 3600 computer by using the WORDSEARCH program (19) (version 6.0, April 1989). The alignment procedure used the SEGMENT program (20).

RESULTS

Purification and Characterization of Rana catesbeiana Egg Lectin. The crude extract of the eggs with 0.1M NH_4HCO_3(pH8.0) strongly agglutinated trypsinized and glutaraldehyde-fixed human type A, type O and rabbit erythrocytes, and weakly human type B (data not shown). This hemagglutination activity was specifically inhibited in

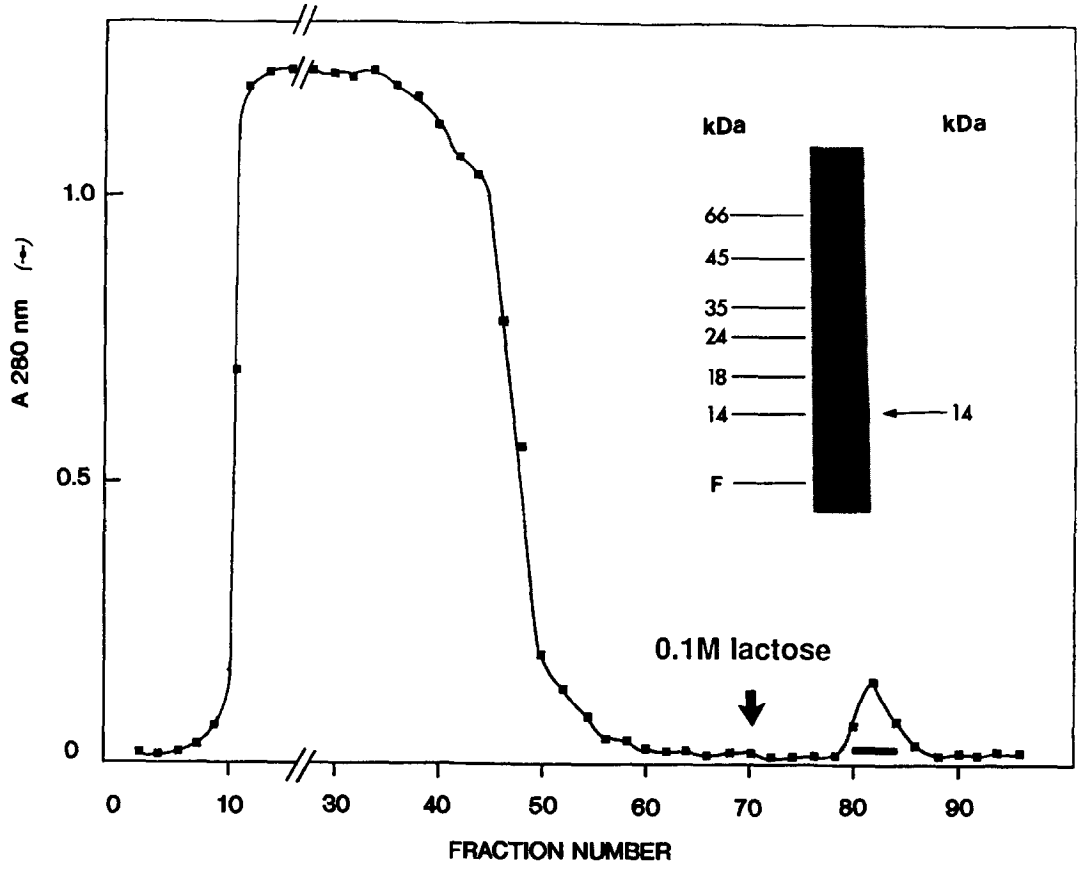

Figure 1. Purification of *Rana catesbeiana* egg 14 KDa lectin by AF-Sepharose 4B affinity column chromatography. Crude extract of acetone powder of the eggs was applied to AF-Sepharose 4B column equilibrated with 100 mM NH_4HCO_3 (pH 8.0). The column was washed with the same burffer and eluted with the same buffer containing 100 mM lactose (arrow). Eluate fractions shown by a bar were collected and, after dialysis, subjected to SDS-PAGE under reducing conditions (inset). Numbers indicated molecular masses of the lectin (right) and marker proteins (left); boving serum albumin (66 kDa), ovalbumin (45 kDa), pepsin (35 kDa), trypsinogen (24 kDa), β-lactoglobulin (18 kDa) and lysozyme (14 kDa). F, designates the gel front.

the presence of 1.0 mM lactose, 0.6 mM thiodigalactoside, or 0.04 mM AF. Since the total lectin activity extracted from a given amount of the acetone-powdered eggs was not affected by the addition of lactose (100 mM) and/or Triton X-100 (1.0 %), it was suggested that the lectin exists as a free and soluble form in the eggs.

The β-galactoside-specific lectin was easily purified to homogeneity from the crude extract by the single affinity chromatography as described in "Materials and Methods", resulting in 325 fold purification in the specific activity (titer/mg of protein). The molecular mass of the lectin was estimated to be 14 kDa by SDS-PAGE under both reducing and non-reducing conditions (Fig.1). Since the intact protein showed the

Table I . Inhibition of hemagglutination activity by saccharides and glycoproteins

Saccharide & Glycoprotein	Minimum inhibitory concentration
Asialofetuin	0.04 mM
Thiodigalactoside	0.6
Lactose	1.0
Melibiose	50.0
D-Galactose	50.0
N-Acetyl-D-galactosamine	50.0
N-Acetyl-D-glucosamine	>75.0
D-Glucose	>75.0
Fetuin	>6.3

Lectin titer was previously adjusted to 16. Trypsinized and glutaraldehyde-fixed rabbit erythrocytes were used for the assay.

apparent molecular mass of 30 kDa by the gel filtration in the presence of 100 mM lactose (data not shown), the lectin should be a homodimer composed of two non-covalently bonded 14 kDa subunits. Neither 20 mM $CaCl_2$ nor 10mM dithiothreitol affected the lectin-induced hemagglutination. Inhibition assay shown in Table I indicated that the purified lectin specifically recognizes β-galactoside.

Generation of Peptides and Sequence Analysis. Amino acid composition of the S-carbamoylmethylated (Cam) lectin is shown in Table 2. It resembles that of the16 kDa lectin purified from *Xenopus* adult skin (10). No amino-terminal sequence was

Table 2 . Amino acid composition of two frog β-galactoside binding lectins[a]

	Rana 14kDa	Xenopus 16kDa[b]		Rana 14kDa	Xenopus 16kDa[b]
Cys	2.2[c]	0.9	Met	1.5	1.8
Asp/Asn	10.8	14.5	Ile	6.4	8.3
Thr	4.3	5.3	Leu	6.9	7.3
Ser	9.0	6.5	Tyr	2.0	1.7
Glu/Gln	13.4	10.0	Phe	7.9	5.3
Gly	8.3	7.0	His	1.9	3.4
Pro	5.4	5.0	Lys	6.5	6.6
Ala	3.3	4.2	Arg	4.0	5.8
Val	6.2	6.4	Trp	N D[d]	N D[d]

[a] Results are expressed as residues per 100 residues.
[b] Taken from Bols et al (10).
[c] Analyzed as S-(carboxymethyl)cysteine.
[d] Trp was not determined.

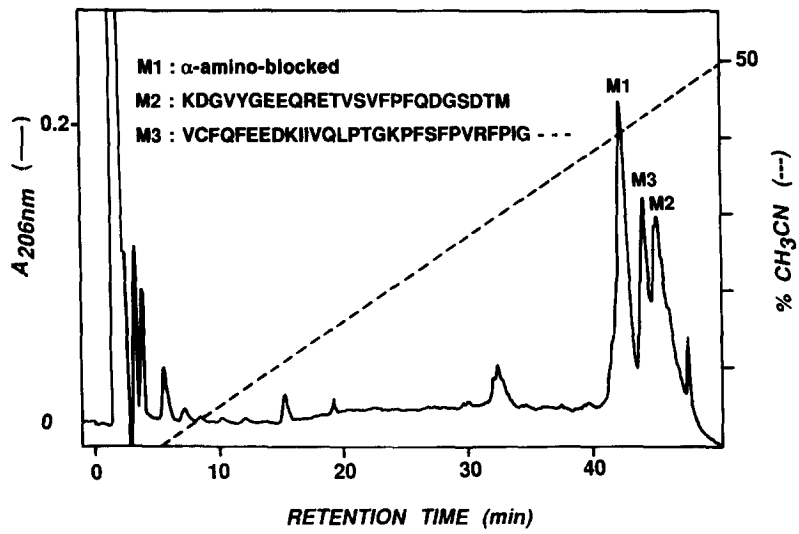

Figure 2. Separation of a CNBr digest of the Cam-protein by reversed-phase HPLC. Peptides were separated on a Cosmosil C8 column by a gradient of acetonitrile into 0.1% aqueous trifluoroacetic acid. Three peptides designated M1, M2, and M3 were collected and analyzed with a Sequencer as shown.

obtained with the intact Cam protein (1.0 nmol) by Sequencer analysis, indicating that the α-amino terminus is blocked. A digest of the Cam protein (1.0 nmol) with BrCN was separated by reversed-phase HPLC (Fig 2). Three major peptides M1, M2 and M3 were isolated and subjected to sequence analysis. Peptide M1 was α-amino-blocked as was the intact protein, indicating that it was derived from the amino-terminal portion. Sequences of peptides M2 and M3 were compared with those of other known proteins. The partial amino acid sequence of the frog egg 14 kDa lectin shows a high similarity to the vertebrate 14K-lectins (Fig. 3).

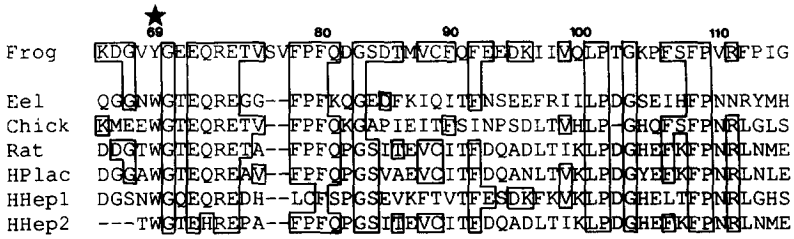

Figure 3. Sequence alignment of *Rana catesbeiana* egg 14K-lectin and vertebrate 14K-lectins. The partial sequence of *Rana catesbeiana* egg 14K-lectin was aligned to provide a miximum similarity with those of other known 14K-lectins. Asterisk indicates the position of Trp (residue-69 by the chicken 14K-lectin numbering system) conserved among the vertebrate 14K-lectin. Frog, *Rana catesbeiana* egg lectin; Eel, eel lectin (21); Chick, chicken lectin (26); Rat, rat lectin (27); HPlac, human placenta lectin (28); HHepl, human hepatoma cell lectin cDNA clone 1 (1); HHep2, human hepatoma cell lectin cDNA clone 2 (1).

DISCUSSION

Widely distributing vertebrate 14K-lectins have some common properties such as similar sugar specificity, SH-reagent requirements and amino acid sequence resembrance. The partial amino acid sequence of the frog *Rana catesbeiana* egg 14K-lectin purified in the present study indicated that it belongs to the vertebrate 14K-lectin family. This is the first report which clearly indicates the presence of a 14K-lectin in amphibia. The frog egg 14K-lectin showed most of common properties of the vertebrate 14K-lectins except for the SH-reagent requirements. Although the reason why the vertebrate 14K-lectins require SH-reagent for the full activity has not yet been clearly understood, SH-reagent is considered to protect a crucial tryptophanyl or cysteinyl residue in the active site from the spontaneous oxidation (21,22). The region around Trp-69 is highly conserved among the vertebrate 14K-lectins (Fig. 3) and thus considered as the carbohydrate recognition domain (21). In the frog egg lectin, however, Tyr substitutes for Trp-69, suggesting that Trp-69 in the vertebrate 14K-lectins is substitutable by Tyr without affecting the activity and also that this substitution may provide the frog egg lectin with the SH-reagent independency.

Most of the 14K-lectins are more effectively extracted from tissues in the presence of haptenic sugar such as lactose, suggesting the presence of endogenous ligand(s) for 14K-lectins (1-4). The expression of the 14K-lectins and/or the cell surface sugar chains has been known to change dramatically in the course of differentiation (23,24). The interaction between a lectin and the specific sugar chains may function as one of the molecular recognition systems in the cell (25). Our results indicated that the frog 14K-lectin is already expressed in the eggs and exists as the free-form. In the adult tissue, however, it is likely that it exists by binding to some glycoconjugates with an affinity to the 14K-lectin (our preliminary results). It seems important to study the expression levels of this lectin and its endogenous ligand(s) during development including differentiation and metamorphosis, for understanding the physiological role(s) of this lectin.

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