Purification and Characterization of *Silurus asotus* (Catfish) Roe Lectin

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A rhamnose-binding lectin isolated from *Silurus asotus* (catfish) roe by DEAE-cellulose ion exchange and galactose-Sepharose affinity chromatographies predominantly agglutinated human type B and rabbit erythrocytes. *S. asotus* lectin (SAL) also agglutinated sarcoma 180 ascites carcinoma cells, but not AH109A cells. The most effective saccharide in hemagglutination inhibition assay was L-rhamnose. The monosaccharides possessing steric similarity to the hydroxyl group orientation at C2 and C4 of the pyranose ring structure of L-rhamnose, such as L-mannose and L-lyxose, were also effective. The molecular weight of SAL was determined to be 38000 by size exclusion chromatography on TSK gel G3000SW and 33000 by SDS-polyacrylamide gel electrophoresis under reducing conditions. SAL did not require a Ca^{2+} ion or free thiol group for its agglutination activity. The N-terminal 29 amino acid sequence was determined by a gas-phase sequencer as follows, ANMITCYGDVQKLHXETGLIIVKSXLYGR (X: not determined). It has no homology to the sequences of well known vertebrate lectins.

Keywords fish roe; rhamnose-binding lectin; affinity chromatography; N-terminal sequence

Carbohydrate-binding proteins, lectins, have been found widely in nature.²⁾ To date, much information regarding the physiological significance of vertebrate lectins has been reported: (1) cell-cell or cell-tissue recognition³⁾ and adhesion,⁴⁾ (2) intracellular glycoprotein transport,⁵⁾ (3) modulation of lymphocyte migration,⁶⁾ (4) tumor cell metastasis,⁷⁾ (5) cell differentiation and formation⁸⁾ and (6) fertilization mechanism.⁹⁾ These biological functions were partially clarified, but it still remains unclear why the lectin is there.

In the last two decades, a number of fish roe lectins have been isolated from various fish families, and their physicochemical properties have been studied.¹⁰⁾ These fish roe lectins are classified into three types according to their carbohydrate-binding specificities: (1) L-rhamnose binding lectins from the families of Clupeidae, Osmeridae, Salmonidae, Cyprinidae, Cobitidae and Sparidae,¹⁰⁾ (2) D-glucose/L-fucose binding lectins from Cyprinidae, Percidae, Sparidae and Percichthyidae,¹⁰⁾ (3) sialoglycoconjugate binding lectin from Petromyzonidae.^{10,11)} Many L-rhamnose binding lectins preferentially or specifically agglutinate human type B erythrocytes.¹⁰⁾ Lectins possessing such a property have rarely been found in other species: e.g. *Griffonia simplicifolia* I (B4),¹²⁾ *Streptomyces* 27S5¹³⁾ and *Charybdis japonica* (CJA-B).¹⁴⁾

Many fish lectins have a high affinity to L-rhamnose, but this sugar is uncommon as an animal component, including in fish roe. The physiological significance of the existence of the L-rhamnose-binding proteins in fish roe has not yet been clarified. Moreover, it was reported that some of these lectins were localized in the cortical vesicles of the roe.¹⁵⁾

In this paper, we report the affinity purification of a rhamnose-binding lectin from *Silurus asotus* (belonging to the family of Siluridae) roe and its properties.

Experimental

Purification of *S. asotus* Lectin (SAL) The acetone-dried powder of *S. asotus* roe was homogenized with ice-cold 0.15 M NaCl containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and centrifuged at $10000 \times g$ for 30 min. The clear supernatant was dialyzed against distilled water for 3 d

and then lyophilized (saline extracted fraction; SEF). SEF (2 g) was dissolved in 10 mM phosphate buffer (PB) (pH 6.8, 30 ml), applied to the DE23 (Whatman) anion exchange column (2.2 $\times$ 40 cm), and equilibrated with the same buffer. Elution was performed with a stepwise increase in the molarity of PB, and each eluate was collected. The fraction eluted with 10 mM PB was treated in the same way as described above (D10). D10 (500 mg) was applied to a D-galactose-Sepharose 6B column equilibrated with 50 mM of an acetate buffer (pH 5.5) containing 0.15 M NaCl. The galactose-Sepharose was prepared according to the method of Fornstedt and Porath¹⁶⁾ by using divinyl sulfone as a resin activator. After the column was washed with the same buffer until no protein was detectable in the eluates, SAL was eluted with the same buffer containing 0.2 M D-galactose and treated in the same way as described above.

Determination of Molecular Weight and Chemical Analysis The purity and molecular weight of SAL were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and by size exclusion chromatography using TSK gel G3000SW. SDS-PAGE was performed according to the method of Laemmli¹⁷⁾ using 12.5% separation gel with standard protein markers. Protein bands were stained in Coomassie brilliant blue R-250 solution. The size exclusion chromatography was performed with 50 mM PB (pH 7.0) containing 0.1 M ammonium sulfate in the presence or absence of 0.2% 2-mercaptoethanol (2-ME) as a mobile phase by high performance liquid chromatography. Protein concentrations were determined according to the method of Lowry *et al.*¹⁸⁾ using bovine serum albumin (BSA) as a standard. Sugar concentration and free thiol analyses were performed by the phenol-sulfate method¹⁹⁾ and Ellman method,²⁰⁾ respectively.

Stability of SAL SAL was treated with trypsin (from bovine pancreas, type III, Sigma) (10 $\mu\text{g}/50 \mu\text{l}$) and pronase (Kaken Chemicals, Tokyo) (25 $\mu\text{g}/50 \mu\text{l}$) in 20 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl (TBS) at 37 °C for 30 min. Then the reaction mixture was diluted with TBS containing 0.1 mM PMSF and used for hemagglutination assay. After being heated at 60 °C for 30 min and 100 °C for 5 min, the sample solution was cooled in an ice bath and centrifuged before use.

Agglutination Assay and Inhibition Assay Agglutination assays of erythrocytes and tumor cells were performed as described previously.²¹⁾ The hemagglutination activities for human, bovine, horse, sheep, rabbit and chicken erythrocytes were determined in phosphate-buffered saline (PBS) (pH 6.8).^{21a)} Similarly, in tumor cell (sarcoma 180 and AH109A cells) agglutination, the sample solution (100 μl) and tumor cell suspension (100 μl) were mixed in a small test tube.^{21b)} The inhibitory activities of saccharides for human type B erythrocytes were tested in similar system as described above. The neoglycoproteins used, lactosyl-BSA (13.4 mol lactose/mol albumin) and melibiosyl-BSA (9.0 mol melibiose/mol albumin), were obtained from Sigma. Neohesperidosyl-BSA was synthesized by the method of Schwartz and Gray.²²⁾ BSA (34 mg), neohesperidose (Rhax1 $\rightarrow$ 2Glc) (50 mg) and sodium cyanoborohydride (50 mg) were mixed and reacted in 0.2 M PB (pH 8.0) (5 ml) at 37 °C for 10 d. The reaction mix-

ture was applied to a P-10 column (Pharmacia-LKB) to remove non-reacted saccharide. The absorbance at 280 nm (protein) and phenol-sulfate method reaction (saccharide) positive fractions, corresponding with neohesperidosyl-BSA, were collected, dialyzed against distilled water and lyophilized (14.0 mol neohesperidose/mol albumin).

Enzymatic Digestion of Erythrocytes Human erythrocytes were washed 3 times with 0.15 M NaCl and 3 times with the buffers used, as described below. The erythrocyte suspension (4%) was treated with 0.1% trypsin in 20 mM TBS (pH 7.4), 0.1% pronase in 20 mM TBS (pH 7.4) or 0.2 unit neuraminidase (from *Arthrobacter ureafaciens*, Nacalai Tesque, Tokyo) in 50 mM acetate buffer (pH 5.5) containing 0.15 M NaCl and 50 mM CaCl₂ at 37°C for 1 h. After enzyme treatment, the cells were washed 3 times with PBS and tested.

Amino Acid Composition and N-Terminal Sequence Analysis

Fig. 1. Affinity Chromatography of D10 on Galactose-Sepharose

SAL was eluted with 50 mM of an acetate buffer (pH 5.5) containing 0.15 M NaCl and 0.2 M galactose at a flow rate of 3 ml/min and collected fractions No. 120–140.

Fig. 2. Size Exclusion Chromatography and SDS-PAGE of SAL

(A) Size exclusion chromatography of SAL on TSK gel G3000SW. Elution was performed with 50 mM PB (pH 7.0) containing 0.1 M ammonium sulfate at a flow rate of 1 ml/min. The arrows indicate the retention time of marker proteins used: 1, γ -globulin (160000); 2, BSA (66200); 3, ovalbumin (45000) and 4, soybean trypsin inhibitor (21500). (B) Polyacrylamide gel (12.5%) electrophoresis in the presence of SDS. Lane 1, marker proteins used were: phosphorylase B (97400), BSA (66200), ovalbumin (45000), carbonic anhydrase (31000), soybean trypsin inhibitor (21500) and lysozyme (14400); lane 2, SAL.

acid composition of SAL was analyzed with a Hitachi model 835 amino acid analyzer. SAL was hydrolyzed in 6 N HCl containing 1% phenol or 4 N methane sulfonic acid containing 2% tryptamine hydrochloride (for Trp) under N₂ gas at 110°C for 24 h. The values of Thr, Ser and Tyr were extrapolated to zero time after 72 h hydrolysis. Cys was determined as cysteic acid after performic acid oxidation. Val and Ile had a 72 h hydrolysis value. The N-terminal amino acid sequence analysis was carried out after SDS-PAGE (described above) and electroblotting. Polyvinylidene difluoride membrane, Immobilon (0.45 μ m pore size), was from Millipore. Electroblotting was performed in a transfer buffer (25 mM Tris, 193 mM glycine, 20% (v/v) methanol) at 30 V overnight in a Bio-Rad (Mini Trans-Blot) apparatus. After the blotted membrane was stained by Coomassie brilliant blue R-250, the bands were cut and sequenced by automated Edman degradation in an Applied Biosystems 477A gas-phase sequencer. A computerized protein data base search used the FSTPCAN program by Lipman and Pearson.²³⁾

Mitogenic Assay Activation of human peripheral lymphocytes was measured as the incorporation of [*methyl*-³H]thymidine into the cells according to the method of Borrebaeck and Etzler²⁴⁾ with modification. Each well (final volume, 200 μ l) contained SAL (1–100 μ g/ml) and lymphocytes (1×10^5 cells/ml) in RPMI-1640 medium containing 10% fetal calf serum. After activation at 37°C for 48 h, the cells were pulsed for 24 h with [*methyl*-³H]thymidine (74 kBq/well). The cells were washed, harvested and their radioactivity counted.

Results

Purification of SAL The pattern of affinity chromatography on galactose-Sepharose is shown in Fig. 1.

TABLE I. Purification of SAL

Purification step	Protein (mg)	Total activity (HU) ^{a)}	Recovery (%)	Specific activity (HU/mg protein)	Purification (fold)
Saline extract	2295	—	100	—	—
Anion exchange chromatography	399	39.9	17.4	0.1	1
Affinity chromatography	35.2	2256	1.5	64	640

a) One hemagglutination unit (HU) is defined as the minimum amount of protein (μ g) required for positive agglutination of a 2% human type B erythrocyte suspension (1 ml).

Although SEF showed no agglutination activity (Table I), the lectin activity was concentrated in D10 eluted with 10 mM PB on a DE23 column. D10 was further purified by a galactose-Sepharose affinity column (Fig. 1). The lectin activity was eluted from the column with 50 mM of an acetate buffer containing 0.2 M galactose (SAL). Affinity chromatography showed a 640-fold increase in lectin activity when compared with D10 (Table I).

The size exclusion chromatography on TSK gel G3000SW showed that the approximate molecular weight (Mr) of SAL was 38000 in the absence (Fig. 2A) or presence of 2-ME (data not shown). On SDS-PAGE, SAL gave a homogeneous band with a Mr of 33000 in the presence of 2-ME (Fig. 2B).

Lectin Activity and Specificity Table II shows SAL-induced agglutination of various cells. Since the addition of EDTA (10 mM), 2-ME (10 mM), dithiothreitol (10 mM) or CaCl₂ (5 mM) did not influence human type B erythrocyte hemagglutination activity by SAL (data not shown), the agglutination assay was performed in PBS. SAL agglutinated rabbit, human (type B), and bovine erythrocytes, but not human (types A and O), horse, sheep and chicken erythrocytes. Treatment of human type A and O erythrocytes, as well as type B erythrocytes, with trypsin, pronase or neuraminidase led to strong agglutination. In addition, SAL agglutinated sarcoma 180 cells but not AH109A cells.

Table III shows the hemagglutination inhibition assay of intact human type B erythrocytes. L-Rhamnose proved to be the best monosaccharide inhibitor and was 160 times more potent than D-galactose. L-Mannose and L-lyxose were also good inhibitors. These monosaccharides, which are capable of inhibiting hemagglutination of type B erythrocytes, have a common configuration at C2 and C4 in their pyranose ring structures. The inhibition effect of methyl galactosides was not altered by the difference in their anomeric configuration, but phenyl α -D-galactoside was more effective than the β -configuration. *N*-Acetylgalactosamine listed in a footnote of Table III had no inhibitory activity. Melibiose, raffinose and stachyose were more effective than lactose and lactulose. Furthermore, neohes-

peridosyl- and melibiosyl-BSA were 16000 and 6000 times more potent than lactosyl-BSA, respectively.

To elucidate stability, the hemagglutination test was performed after enzyme treatment and heat denaturation of SAL. Proteinase treatment and heat denaturation of SAL caused a decrease in lectin activity (Table IV). Concerning mitogenic activity, SAL had no effect on [methyl-³H]-thymidine uptake to human peripheral lymphocytes from 1 μ g/ml to 100 μ g/ml (data not shown).

Chemical Properties of SAL The amino acid composition of SAL is shown in Table V. SAL was rich in Asp plus Asn, Thr, Gly and Leu but poor in Trp, Met and His. A free thiol group was not detected and sugar concentration was less than 1% (data not shown). According to the homology search based on the N-terminal 29 amino acid residues of SAL by the FASTPSCAN program, we could not find any homology between the primary structures of known lectins. The N-terminal sequence of perch lectin I from the roe of *Perca fluviatilis* (Percidae),¹⁰⁾

TABLE III. Inhibition of Hemagglutination Activity of SAL by Saccharides and Glycoproteins^{a)}

Saccharide and glycoprotein	Concentration (mM) ^{b)}
L-Rhamnose	0.009
Methyl- α -L-rhamnose	0.004
L-Mannose	0.075
L-Lyxose	0.75
D-Galactose	1.5
Methyl- α -D-galactoside	0.75
Methyl- β -D-galactoside	0.75
Phenyl- α -D-galactoside	0.375
Phenyl- β -D-galactoside	18.75
Thiodigalactoside	0.094
D-Fucose	1.5
L-Arabinose	1.875
D-Fructose	37.5
L-Glucose	37.5
L-Sorbose	150
L-Xylose	150
Neohesperidose	0.375
Lactose	7.5
Lactulose	15
Melibiose	0.375
Raffinose	0.375
Stachyose	0.187
Others ^{c)}	> 150
Neohesperidosyl-BSA	0.06 ^{d)}
Lactosyl-BSA	940 ^{d)}
Melibiosyl-BSA	0.15 ^{d)}

a) Used intact human type B erythrocytes and 8 HU of SAL/each well. b) Minimum concentration of saccharides required for complete inhibition. c) D-Glucose, L-galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-mannose, D-xylose, D-lyxose, D-arabinose, D-ribose, 2-deoxy-D-galactose and trehalose. d) Sugar concentration of neoglycoproteins are expressed in μ M.

TABLE II. SAL-Induced Agglutination

Erythrocyte or tumor cell	Specific activity
Human type A intact	<0.5
type A + trypsin	8
type A + pronase	32
type A + neuraminidase	32
type B intact	64
type B + trypsin	263
type B + pronase	263
type B + neuraminidase	556
type O intact	<0.5
type O + trypsin	8
type O + pronase	32
type O + neuraminidase	32
Bovine	8
Horse	<0.5
Sheep	<0.5
Rabbit	2500
Chicken	<0.5
Sarcoma 180	32
AH109A	<1

TABLE IV. Stability of SAL^{a)}

Factor	Specific activity
Control	64
Trypsin	8
Pronase	8
Heat (60 °C, 30 min)	2
(100 °C, 5 min)	1

a) Used intact human type B erythrocytes.

TABLE V. Amino Acid Composition of SAL

Amino acid	Residues/100 residues
Asx	15.8
Thr ^{a)}	12.5
Ser ^{a)}	6.9
Glx	4.8
Gly	8.7
Ala	6.6
Cys ^{b)}	7.7
Val ^{c)}	5.4
Met	0.6
Ile ^{c)}	5.4
Leu	8.0
Tyr ^{a)}	5.0
Phe	1.5
Trp ^{d)}	0.3
Lys	3.0
His	1.0
Arg	2.7
Pro	3.9

a) The value was extrapolated to zero hydrolysis time. b) Determined as cysteic acid. c) 72 h hydrolysis value. d) 24 h hydrolyzed with 4 N methanesulfonic acid.

Fig. 3. Alignment of Amino Acid Sequence of SAL, Glutaminase-Asparaginase

Sequences of glutaminase-asparaginase is referred from ref. 25. Identical residues are enclosed in boxes. Xaa is an undefined residue.

EPAXPPWGTEFG, was not similar to that of SAL. By searching other protein sequences (total 20024 proteins), the absolute homology score (435) and relative score (34.7%) were obtained between amino acid positions 10–22 in SAL with 261–273 in glutaminase-asparaginase from *Acinetobacter glutaminasificans*²⁵⁾ (Fig. 3).

Discussion

The eggs of various animals are a good source for lectin study. Amphibians or fish regularly produce a suitable material for investigation in quantities except during seasonal shortages. A number of fish lectins have been isolated and partially characterized.¹⁰⁾ The hemagglutination activity of SAL was similar to that of the other fish roe lectins.¹⁰⁾ SAL agglutinated intact human type B erythrocytes as well as rabbit erythrocytes. Although intact human types A and O erythrocytes were not agglutinated by SAL, they had become agglutinable after neuraminidase, trypsin and pronase treatment. (1) Neuraminidase treatment of erythrocytes led to increased hemagglutination activity of SAL (Table II), (2) the hemagglutination reaction was

inhibited by D-galactose (Table III), and (3) SAL was purified by D-galactose-Sepharose as described below (Fig. 1). Therefore, non-reducing end galactose residues of the carbohydrate chains on the erythrocyte membrane were concerned with SAL-induced hemagglutination. SAL did not agglutinate horse, sheep and chicken erythrocytes (Table II), but *Hypophthalmichthys molitrix* (Cyprinidae) lectin, which has a high affinity for L-rhamnose as well as SAL, agglutinates horse erythrocytes.¹⁰⁾ Another type of lectin was also found. D-Galactose/L-fucose or sialoglycoconjugates binding lectin^{10,11)} agglutinated human type O or horse erythrocytes, respectively. In comparing tumor cell agglutination of three fish roe lectins from *Hypomesus transpacificus*,²⁶⁾ *Misgurnus anguillicaudatus*²⁷⁾ and *Plecoglossus altivelis*²⁸⁾ with that of SAL reported here, the different agglutination patterns by these four lectins were obtained as follows: (1) *M. anguillicaudatus* lectin agglutinates Ehrlich and AH109A tumor cells, but not sarcoma 180 cells, (2) *H. transpacificus* and *P. altivelis* lectins agglutinate Ehrlich, but not sarcoma 180 and AH109A cells, and (3) SAL agglutinates sarcoma 180 but not AH109A cells. In addition, not only SAL but also the three lectins described above preferentially agglutinated human type B erythrocytes at similar concentrations. While all four lectins seem to recognize the same blood group antigen, galactosyl $\beta 1 \rightarrow X$ linkages, on human type B erythrocytes, the individual lectin may recognize partially different carbohydrate moieties as an inherent cell surface marker on each tumor cell.

Generally, purification of lectins is performed by using affinity adsorbents coupled with specific sugars. SAL had extremely high affinity for L-rhamnose (Table III), and was capable of binding to L-rhamnose-, L-mannose- and melibiose-Sepharose. Since these three sugar-Sepharose affinity chromatographies produced SAL (data not shown) in a similar manner as the D-galactose-Sepharose column chromatography described above, we attempted to use the D-galactose-Sepharose column (see Fig. 1) to isolate SAL because it was economically practical. SAL-induced hemagglutination was not altered by a reducing agent, Ca^{2+} ion or EDTA (data not shown), and no change was observed in the binding of SAL to galactose-Sepharose when chromatography was performed in the presence or absence of 2-ME (data not shown). The molecular weight of SAL was 38000 by size exclusion chromatography (Fig. 2A), and 33000 by SDS-PAGE. Since a single band was observed in SDS-PAGE under the reducing condition (Fig. 2B), the lectin was considered a monomer.

Sakakibara *et al.*²⁷⁾ reported that *P. altivelis* (ayu fish) lectin-induced hemagglutination was strongly inhibited by L-rhamnose, L-mannose and L-lyxose, and weakly by D-galactose, D-fucose and L-arabinose. The monosaccharides with a different steric configuration relative to the hydroxyl group orientation at the C2 and C4 of D-galactose did not inhibit *P. altivelis* lectin-induced hemagglutination. Although the carbohydrate-binding specificity of SAL was similar to that of *P. altivelis* lectin, the minimum inhibitory concentration of L-rhamnose for SAL-induced hemagglutination was lower than that of *P. altivelis* lectin. The order of inhibition activity of the C6 substituent was as follows, $\text{CH}_3 > \text{CH}_2\text{OH} > \text{H}$. Since melibiose, raffinose and stachyose were more potent inhibitors compared to lactose,

SAL preferentially recognized galactosyl α -linked sugar residues. In addition, neohesperidose and melibiose were 6250 and 2500 times more potent when coupled with BSA, respectively (Table III). Therefore, the protein moiety of ligands may influence carbohydrate recognition by SAL.

Fish roe lectins from powan, roach and perch possess mitogenic activity or indirect cytotoxicity mediated by macrophages or cytotoxins.^{10,29} SAL had no mitogenic activity for human peripheral lymphocytes (data not shown).

The complete amino acid sequence of fish roe lectins has not been determined yet. Amino acid composition of SAL was roughly similar to that of already known fish roe lectins, rich in Asp plus Asn, Thr, Gly and Leu, poor in Trp and Met (Table V). Since free thiol was not detected in the SAL molecule by the Ellman method (data not shown), Cys residues might participate in an intramolecular disulfide bond. The N-terminal 29 amino acid residues of SAL had no homology to the sequence of well-known lectins, not even to the N-terminal amino acid sequence of fish roe lectin, perch lectin I.

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