COMPARISON OF N-TERMINAL AMINO ACID SEQUENCE OF FISH ROE RHAMNOSE-BINDING LECTINS

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ABSTRACT

A 26-KDa rhamnose-binding lectin was purified from roe of shishamo smelt (*Osmerus lanceolatus*) by sequential chromatographies of DE23 anion exchange, D-galactose-Sepharose and TSK gel CM-5PW. This lectin (OLL) agglutinated rabbit and human ABO-type erythrocytes and sarcoma 180 cells. The lectin-induced hemagglutination was inhibited by L-rhamnose. The amino acid composition of OLL was similar to that of *Osmerus eperlanus mordax* roe lectin-4 (OML-4). The N-terminal 34 amino acid sequence of OLL was VTDDIXEGQQATLNXGSSVINVSANYGRTRVT (X: not defined) and similar to that of OML-4. Therefore, OLL and OML-4 may be the same lectin existing in the different species of fish roe.

Lectins are carbohydrate-binding proteins which agglutinate erythrocytes and other cells (6). Clinical applications of lectins at the present time are multiple, including (i) classification of various blood groups or types (4, 20), (ii) detection of polyagglutination induced by T-antigen (14, 15), (iii) evaluation of the immunocompetence of patients including AIDS (23), (iv) detection of tumors by recognition of tumor markers (24), and (v) histochemical approach toward the clinical investigations (5). In recent years, an interest has been taken in the relation between lectins and cell adhesion molecules. There are a great number of reports on selectins (1, 2, 9) which mediate adhesive interactions between circulating leukocytes and the vascular endothelium at a part of inflamed or injured venules with a carbohydrate-recognition mechanism.

Grillon *et al.* (7) reported that human granulocytes express rhamnose-binding lectin on their cell surface and their expression is modulated by mononuclear cells and granulocyte/macrophage colony-stimulating factor, but not by phorbol

myristate acetate. Rhamnose-binding lectins have been found mostly in fish roe (10). One of the biological roles of these lectins is thought to participate in defense mechanisms against pathogenic bacterium. Ozaki *et al.* (19) reported on a rhamnose-binding lectin from amago (*Oncorhynchus rhodurus*) roe and its receptor on macrophages. They suggested that a lectin receptor was expressed on peritoneal macrophages of amago after inflammatory stimulation.

In this paper, we describe the purification and characterization of shishamo smelt (*Osmerus lanceolatus*) roe lectin (OLL), and compare the N-terminal amino acid sequence between OLL and other fish roe rhamnose-binding lectins.

MATERIALS AND METHODS

Materials

Shishamo smelt (*Osmerus lanceolatus*) and olive rainbow smelt (*Osmerus eperlanus mordax*) were obtained in Hokkaido Prefecture, and catfish (*Silurus asotus*) in Fukushima Prefecture. Sepharose 6B was purchased from Pharmacia-LKB, standard proteins used for molecular weight estimation by sodium dodecyl sulfate-polyacrylamide

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Fig. 1 Affinity chromatography of D10 derived from roe of *O. lanceolatus* on galactose-Sepharose. D10 (500 mg) was applied to the column (2.2×20 cm). The column was washed with 50 mM acetate buffer (pH 5.5) containing 0.15 M NaCl. GSA-2 indicated with bar was eluted with the same buffer containing 0.2 M galactose at a flow rate of 3 ml/min and fractions No. 116–125 were collected.

gel electrophoresis (SDS-PAGE) from Bio-Rad and DE23 anion exchange resin from Whatman. All other chemicals used were of the highest purity available.

Purification of Fish Roe Rhamnose-Binding Lectins

The main purification procedure of fish roe lectins was described previously (8). Briefly, *Silurus asotus* lectin (SAL) was purified by DE23 anion-exchange chromatography and D-galactose-Sepharose affinity chromatography. DE23 column was equilibrated with 10 mM phosphate buffer (PB, pH 6.8) and eluted with a stepwise increase in the molarity (10, 100 and 200 mM) of PB. Galactose-Sepharose column was equilibrated with 50 mM acetate buffer (pH 5.5) containing 0.15 M NaCl and SAL was eluted with the same buffer containing 0.2 M D-galactose. Four *Osmerus eperlanus mordax* lectins (OML-1~4) were purified by a combination of procedures described above and high performance liquid chromatography (HPLC) on a column of

TSK gel CM-5PW (7.5×75 mm, Toso) (unpublished data). In case of purification of OLL, the affinity chromatography of the active fraction eluted with 10 mM PB from DE23 column (D10), produced two adsorbed fractions (Fig. 1). The second fraction, GSA-2, was further purified by HPLC on a same column described above equilibrated with 10 mM PB (pH 6.0) using a linear gradient of NaCl (0–0.6 M) at a flow rate of 1.0 ml/min, and then rechromatographed with 0.2–0.6 M NaCl gradient (Fig. 2). All the fractions obtained from each purification step described here were thoroughly dialyzed against distilled water and then lyophilized.

Agglutination Assay and Inhibition Assay

Hemagglutination assay and tumor cell agglutination assay were performed in 10 mM PB containing 0.15 M NaCl as described previously (17, 18). One hemagglutination unit (HU) is defined as the minimum amount of protein (μ g) required for positive agglutination of a 2% rabbit erythrocyte suspension (1 ml). The inhibitory activities of saccharides were

Fig. 2 HPLC pattern of GSA-2 on a column of TSK gel CM-5PW. (A) GSA-2 (500 mg) was applied to the column. The active fraction eluted with 0.49–0.52 M NaCl (27–32 min) in a gradient profile (----) at a flow rate of 1 ml/min. Eluate indicated with bar was collected. (B) The fraction obtained in (A) was further purified by the same column. OLL was eluted with 0.47–0.50 M NaCl (19–21 min).

tested in a similar system as described above.

Analytical Methods

SDS-PAGE were performed according to the method of Laemmli (12) using 12.5% separation gel with standard protein markers. Protein bands were stained with Coomassie Brilliant Blue R-250 solution. Protein concentration was determined according to the method of Lowry *et al.* (16), using bovine serum albumin as a standard.

Amino acid composition was analyzed with a Hitachi model L-8500 amino acid analyzer. Sample was hydrolyzed in 6 N HCl containing 1% phenol or under N_2 gas at 110°C for 24 h.

Sugar composition was determined by the method of Lehnhardt and Winzler (13) using a Finnigan TSQ-700 GC-MS apparatus.

N-Terminal amino acid sequence analysis was carried out by automated Edman degradation in an Applied Biosystems 477A gas-phase sequencer. Secondary structure of the N-terminal sequence was predicted by the method of Chou and Fasman (3).

RESULTS AND DISCUSSION

In recent studies, we have purified and characterized the rhamnose-binding lectins from roe of *Silurus asotus* (8) and *Osmerus eperlanus mordax* (unpublished data). The molecular masses of SAL, OML-1, -2, -3 and -4 are 33 KDa, 32 KDa, 25 KDa, 26 KDa and 26 KDa, respectively. These all lectins strongly agglutinated rabbit erythrocytes and this agglutination was inhibited by L-rhamnose and other related saccharides. However, OML-4 agglutinates human ABO-type erythrocytes in contrast with SAL and OML-1~3 that possessed anti-B-like hemagglutination activity.

In our experiment, the sequential chromatographies of anion-exchange, affinity and cation-exchange were suitable method for the purification of fish roe lectins. Generally, when saline-extracted fractions (SEFs) from fish roe were applied to the DE23 anion exchange column, the lectin activity was eluted with 10 mM PB. Then rhamnose-binding lectins were adsorbed to the galactose-Sepharose resin. The yield of sugar-binding proteins obtained by using galactose- and rhamnose-Sepha-

Fig. 3 SDS-PAGE pattern of OLL. Lane 1, OLL, Lane M, marker proteins. Marker proteins used were: phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). BPB, Bromophenol Blue

rose column were the same (data not shown). Further purification could be done by HPLC on the column of TSK gel CM-5PW. This procedure was applied to the purification of OLL. As shown in Fig. 1, two peaks were observed in adsorbed fraction on the affinity column. Since the hemagglutination activity of the second peak fraction (GSA-2) was higher than the first peak fraction, we have further purified GSA-2. Fig. 2 shows elution profiles of GSA-2 on TSK gel CM-5PW. The last peak eluted with 0.49–0.52 M NaCl was collected (Fig. 2A) and then rechromatographed on the same column with different gradient profile indicated in Fig. 2B. OLL was eluted with 0.47–0.50 M NaCl and a 95-fold purification was achieved when OLL was compared with SEF because HUs of SEF and OLL were 27 and 2,560, respectively. On SDS-PAGE, OLL

migrated as a single band with a molecular weight of 26 kDa under reducing condition (Fig. 3). This molecular weight of OLL is the same to those of OML-3 and -4. Kubo and Kawasaki (11) reported a 26-kDa Ca^{2+} -dependent rhamnose-binding lectin, which participates in a construction of epithelial tissue from body fluid of American cockroach (*Periplaneta americana*). Although both OLL and *P. americana* lectin were 26-kDa rhamnose-binding protein, OLL did not require Ca^{2+} for its lectin activity (data not shown).

OLL agglutinated rabbit and human A, B and O-type erythrocytes and sarcoma-180 murine ascites tumor cells at the minimum concentration of 0.39, 400, 25, 400 and 15 $\mu\text{g}/\text{ml}$, respectively, for obvious agglutination (data not shown). Table 1 shows the sugar-binding specificity of OLL. L-Rhamnose and L-rhamnose-containing disaccharides, neohesperidose and rutinose, strongly inhibited the OLL-induced hemagglutination. Since L-rhamnose, L-mannose, L-lyxose, D-galactose and D-fucose were potent inhibitors, and since the inhibitory activities of melibiose, raffinose and stachyose were significantly higher than those of lactose and lactulose (Table 1), OLL is thought to recognize hydroxyl orientations at C2 and C4 positions of the pyranose ring and α -galactosyl structures. Since L-rhamnose is not a naturally occurring sugar in vertebrates, the physiological significance of the rhamnose-binding lectins is still unclear. However, Ozaki *et al.* (19) reported that amago lectin may participate in host defense mechanisms and, in human immune system, Grillon *et al.* suggested that the rhamnose-binding lectin may have relevance to the antibacterial effect against microorganisms which have α -L-rhamnosyl residue in their surface glycoconjugates (7).

Table 2 shows amino acid compositions of OLL and OML-4. These two lectins contained high amounts of Asp plus Asn, Thr and Ser, and similar amounts of other amino acids except for Cys, His and Arg. As shown in Fig. 4, the N-terminal sequences of OLL and OML-2–4 were similar and YGR (-Tyr-Gly-Arg-) sequence at the position of 27–29 was conserved in these six lectins. Sugar contents of OLL and OML-4 were both less than 1% (w/w). Glucose, mannose and galactose were detected (Table 3), and the trace amounts of other sugars such as fucose, glucosamine and galactosamine were also detected (data not shown). Since the similar sugar compositions have been observed in the roe lectins of *Misgurnus anguillicaudatus* (22) and *Plecoglossus altivelis* (21), this sugar composi-

Table 1 *Inhibition of Hemagglutination Activity of OLL by Saccharides^a*

Saccharide	Concentration (mM) ^b
L-Rhamnose	0.004
D-Galactose	0.75
L-Galactose	>200
Methyl- α -D-galactoside	0.094
Methyl- β -D-galactoside	0.188
N-Acetyl-D-galactosamine	>200
D-Mannose	>200
L-Mannose	0.03
D-Fucose	0.75
L-Fucose	25
D-Xylose	50
L-Xylose	100
D-Lyxose	25
L-Lyxose	0.188
Neohesperidose	0.002
Rutinose	0.002
Lactose	12.5
Lactulose	12.5
Melibiose	0.094
Raffinose	0.094
Stachyose	0.094

^aIn the presence of 2% rabbit erythrocyte suspension and 8 HU of OLL in 50 μ l. ^bMinimum concentration of saccharides required for complete inhibition

tion may be typical in the fish roe rhamnose-binding lectins.

Therefore, it was suggested that OLL and OML-4, derived from different species of fish roe, are similar in respect to (i) molecular weight, (ii) cell agglutination profile, (iii) sugar specificity, (iv) amino acid and sugar composition, and (v) N-terminal sequence. Moreover the predicted secondary structure suggested that OLL and OML-1~4 may have typical β -turn repeats in the N-terminal region (Fig. 4). Since the relationship between secondary structure and biofunctional role(s) of these lectins is still unknown, the determination of complete amino acid sequences is necessary, and it is interesting to examine whether or not some consensus amino acid sequences are conserved in rhamnose-binding lectin molecules.

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Table 2 *Amino Acid Compositions of OLL and OML-4*

Amino acid	Residues in 100 residues	
	OLL	OML-4
Asx	13.7	11.9
Thr ^a	11.7	11.7
Ser ^a	10.7	12.0
Glx	4.8	5.5
Gly	8.0	9.4
Ala	5.0	5.4
Cys ^b	2.7	1.0
Val ^c	7.4	8.6
Met	1.1	1.4
Ile ^c	5.6	6.6
Leu	6.8	5.6
Tyr	4.8	6.3
Phe	2.3	2.3
Trp ^d	ND ^e	ND
Lys	2.2	2.2
His	3.0	0.9
Arg	5.5	3.3
Pro	4.5	5.9

^aCorrected to zero hydrolysis time values based on the 24, 48 and 72 h hydrolysis value. ^bDetermined as cysteic acid after performic acid oxidation. ^c72 h hydrolysis value. ^dHydrolyzed in 4 N methane sulfonic acid containing 2% tryptamine hydrochloride for 24 h. ^eND, not detected

	1	10	20	30
OLL	V T T D I	X E G Q Q A T L N X	G S S V I N V V S A N Y G R T D R V T	
OML-1	V M T V I	X E G Q Q E T L N X	G S S V V N V V S A N Y G R T N H V T	
OML-2	V T T D I	X E G Q Q A T L N X	G S S V I N V V S A N Y G R T D R V T	
OML-3	V T T D I	X E G Q Q A T L N X	G S S V I N V V S A N Y G R T D R V T	
OML-4	V T T D I	X E G Q Q A T L N X	G S S V I N V V S A N Y G R T D R V T	
SAL	A N M I T	X Y G D V Q K L H X	E T G L I I V K S X L Y G R	

Fig. 4 Comparison of N-terminal amino acid sequences between OLL, OML-1~4 and SAL (see text). Identical residues are enclosed in boxes. Predicted β -turn structure regions were indicated with underlines. X represents undefined residue.

Table 3 Sugar Compositions of OLL and OML-4

Sugar	Content (w/w %)	
	OLL	OML-4
Mannose	0.12	0.26
Glucose	0.35	0.48
Galactose	0.01	0.06

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