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Distribution and Molecular Evolution of Rhamnose-binding Lectins in *Salmonidae*: Isolation and Characterization of Two Lectins from White-spotted Charr (*Salvelinus leucomaenis*) Eggs

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L-Rhamnose-binding lectins were isolated from white-spotted charr (*Salvelinus leucomaenis*) eggs to understand the distribution and molecular evolution of the lectins in *Salmonidae*. Only two L-rhamnose-binding lectins, named WCL1 and WCL3, were isolated from white-spotted charr eggs, though three lectins, named STL1, STL2, and STL3, had been obtained from steelhead trout (*Oncorhynchus mykiss*) eggs. The cDNAs of WCL1 and WCL3 included 1,245 and 838 bp nucleotides with open reading frames of 933 and 651 nucleotides, respectively, and encoded for the complete amino acid sequences of mature proteins consisted of 288 (WCL1) and 195 (WCL3) residues, and signal sequences of 23 and 22 residues, respectively. WCLs were composed of three (for WCL1) or two (for WCL3) tandemly repeated homologous domains, which consisted of about 95 amino acid residues, and showed 91 and 93% sequence identities to STL1 and STL3, respectively. The mRNAs of WCL1 and WCL3 were detected exclusively in liver and ovary, respectively, however, neither a protein nor mRNA corresponding to STL2 could be identified in white-spotted charr. The phylogenetic tree of the 16 sequences encoding carbohydrate recognition domains of 7 lectins from 4 species shows 5 functional clusters and their evolutional process. These results indicate that multiple L-rhamnose-binding isolectins have diverged by gene duplication and exon shuffling to play various biological roles in each species.

Key words: rhamnose-binding lectin; *Salmonidae*; fish egg; white-spotted charr; molecular evolution

L-Rhamnose-binding lectins (RBLs) have been found not only in fish eggs but also in sea urchin eggs.[1-4] RBLs from fish eggs are composed of two or three tandemly repeated characteristic carbohydrate-recognition domains (RBL CRDs), which consist of about 95 amino acid residues.[1-3] A L-rhamnose-binding lectin (SUEL) from sea urchin eggs is composed of a disulfide-linked homodimer of the RBL CRD,[4] indicating that RBLs comprise a novel animal lectin family, and may play important roles in animal eggs.

Three RBLs, named STL1, STL2, and STL3, were isolated from the steelhead trout (*Oncorhynchus mykiss*) eggs and their tissue distribution showed that STL1 had different distribution and expression profiles from those of STL2 and STL3.[5,6] Although STL1 could be detected in several tissues and cells such as spleen, thrombocytes, blood leukocytes, and serum of both male and female steelhead trout as well as in the ovary, the STL1 mRNA was restricted to the liver. In contrast, proteins and mRNAs of STL2 and STL3 were detected only in the ovary. STL2 and STL3 mRNAs were detected in the cytoplasm of oocytes, but not in other ovarian tissues. STLs were mainly localized in the cortical vesicles in the oocytes of all stages, and were then released into the perivitellin space just after fertilization.[5] These results indicate that STL1 expressed in the liver is transported to the immune system and oocytes via the blood stream. On the other hand, STL2 and STL3 are ovary-specific proteins, which are expressed specifically in the oocytes and accumulate in the cortical vesicles.

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Abbreviations: AUFS, absorbance unit full scale; CAM, *S*-carboxamidomethylated; CRD, carbohydrate recognition domain; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; RACE, rapid amplification of cDNA ends; RBLs, L-rhamnose-binding lectins; SAL, *Silurus asotus* lectin; STL, steelhead trout egg lectin; SUEL, sea urchin egg lectin; TBS, 20 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl; WCL, white-spotted charr egg lectin

STLs agglutinated Gram-negative and Gram-positive bacteria by recognizing the structures of lipopolysaccharide (LPS) and lipoteichoic acid (LTA) on their surfaces, respectively.[7] STLs showed much higher binding activities to smooth LPSs containing L-rhamnose in the repeating unit of O-antigen than to rough LPSs and lipid A lacking O-antigen, indicating that the chemical structure of O-antigen was important for LPS-binding of STLs. STLs also inhibited the growth of some bacteria. With consideration of these results, it is probable that RBLs may function as non-self recognition molecules in the innate immunity not only in the eggs but also in the adult fishes.

In this study, RBLs were isolated from white-spotted charr eggs to understand the distribution and the biological significance of RBLs in *Salmonidae*. From the comparison of molecular properties and primary structures of RBLs, we discuss the characteristics and molecular evolution of the RBL family.

Materials and Methods

Materials. The eggs were obtained from mature white-spotted charr cultured in Teikyo University of Science and Technology, Uenohara, Japan. *Achromobacter* protease I, *Staphylococcus aureus* V8 protease, and sugars were purchased from Wako Chemicals (Osaka, Japan). Endoproteinase Arg-C was purchased from Roche Diagnostics (Mannheim, Germany). Sepharose 6B and Hi-Trap Q columns were purchased from Amersham Pharmacia Biotech. (Uppsala, Sweden). Sepharose 6B was epoxy-activated by chloromethyloxirane and combined with L-rhamnose according to the protocol of Pharmacia. All other reagents were the purest grade commercially available.

Isolation of white-spotted charr egg lectins. Eggs (1 kg) were homogenized in 500 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl (TBS) and centrifuged at 15,000 × *g* for 30 min at 4°C. The supernatant was incubated with L-rhamnose-Sepharose 6B gel (~50 ml) at 4°C overnight. Unadsorbed substances were removed by washing the gel with TBS. The adsorbed substance was eluted with 0.2 M L-rhamnose in TBS. The fractions with significant absorption at 280 nm were collected and dialyzed against distilled water, and then lyophilized. The lyophilisate was dissolved in 8 M urea and diluted with 10 mM Tris-HCl buffer (pH 8.0) just before anion exchange chromatography on a Hi-Trap Q column (5 ml) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) and eluted with a linear gradient of 0 to 1 M NaCl in the same buffer. Each peak was collected, dialyzed extensively against distilled water, and lyophilized. During the purification steps, protein concentration was measured by the Micro BCA Protein Assay Reagent Kit (Pierce, IL, USA) using bovine serum albumin as a standard.

Hemagglutination assay and inhibition assay. A 4% suspension of rabbit erythrocytes was prepared in 0.15 M NaCl. The samples (50 μl) (2-fold serial dilutions in 0.15 M NaCl) were mixed with 50 μl of cell suspension in round-bottom microtiter plates (96-well) by agitation for 30 s and hemagglutination was measured after incubation for 30 min at room temperature. The titer was defined as the reciprocal value of the end point dilution causing hemagglutination. For a sugar inhibition test, sugars or glycoproteins in 0.15 M NaCl (25 μl) were mixed with 25 μl of WCLs by agitation for 15 min before adding the rabbit erythrocyte suspension. The inhibitory effect was expressed as the minimum concentration required for complete inhibition of the hemagglutinating activity with titer 8.

Molecular mass measurements of proteins. The molecular masses of lectins were measured by SDS-PAGE and matrix-assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometry (Kompact MaldiI I, Shimadzu, Kyoto, Japan). SDS-PAGE was done by the method of Laemmli[8] using a 15% separating gel in the presence or absence of 2-mercaptoethanol (2-ME), and protein bands were stained with Coomassie Brilliant Blue R-250.

Amino acid sequencing. Lectins were reduced, *S*-carboxamidomethylated (CAM) with monoiodoacetamide, and then digested with *Achromobacter* protease I, *Staphylococcus aureus* V8 protease, or endoproteinase Arg-C, separately. Each digest was separated by reversed-phase HPLC, and the amino acid sequences of isolated peptides were analyzed by a gas-phase protein sequencer (PPSQ-10, Shimadzu, Kyoto, Japan).

DNA sequencing. Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Poly $(A)^+$ RNA was purified with a Micro-FastTrack mRNA Isolation Kit (Invitrogen, Tokyo, Japan). cDNA libraries from the liver and ovary of white-spotted charr were constructed with the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). To amplify the cDNAs of WCLs, oligonucleotide primers were designed from the STLs nucleotide sequences.[2] F and R indicate sense and anti-sense primers, respectively. WCL1F1, 5′-CCAGGAAGGGACTCTGGCACTC-TGGTCACA-3′; WCL1R1, 5′-GCTGGTTGAGCC-CTCGCAGGTGATACTTGTCTTAGCAGGAA-3′; WCL3F1, 5′-TCGCCCTGATCACCAACTCACA-GACACCAACT-3′; WCL3R1, 5′-CTCGATCACA-TAGTAGTTGAGAATCAGAGCCTTCACA-3′. PCR was done with a combination of primers,

WCLsF1 and WCLsR2, and liver and ovary cDNA library as a template for WCL1 and WCL3, respectively, and *Pfu* Turbo DNA polymerase (Stratagene, CA, USA) on a Takara PCR Thermal Cycler Personal as follows. DNA was denatured at 94°C for 3 min, followed by three-step cycles (30 cycles); 92°C for 0.5 min, 50°C for 1 min, and 72°C for 2 min, and further extended at 65°C for 15 min. Amplified DNA fragments generated by PCR with primers WCLsF1 and WCLsR1 were, 0.9 kbp for WCL1 and 0.6 kbp for WCL3, respectively, and were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). Inserted DNA was sequenced on an ABI DNA sequencer (Model 373) by cycle sequencing using T7 and SP6 primers and the DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The flanking regions of the cloned cDNA fragment were obtained by the 5′- and 3′-RACE methods. The nucleotide sequence data reported in this paper are available from DDBJ, EMBL, and Genbank databases with accession numbers of AB077045 and AB077046 for cDNAs encoding WCL1 and WCL3, respectively.

Sequence data processing. Multiple sequence alignment was done by Clustal W program.[9] Homologous sequences were searched for by the FASTA program[10] accessed by Genome Net WWW. A phylogenetic tree was constructed by the neighbor-joining algorithm[11] based on evolutionary distance matrix constructed by Kimura's method.[12] The degrees of confidence for internal lineages in phylogenetic trees were calculated by the bootstrap procedure.[13]

Northern blot analysis. Total RNAs were prepared from tissues of white-spotted charr in the vitellogenic stage using Isogen (Nippon Gene). Twenty micrograms of total RNAs were separated on a formaldehyde-agarose gel, and transferred onto a Hybond N^+ (Amersham Pharmacia Biotech). The membrane was hybridized at 55°C overnight with digoxigenin (DIG)-labeled STLs antisense RNA probes in DIG Easy Hyb (Roche Diagnistics). After washing with 2 ×standard saline citrate (SSC)/0.1% SDS at room temperature and twice with 0.2×SSC/0.1% SDS at 65°C, the membranes were incubated with anti-digoxigenin-AP Fab' fragments (1:10000, Roche Diagnistics), and the signals were detected by using a chemiluminescent substrate, CDP-Star (Roche Diagnistics).

Genomic southern blot analysis. DNA was prepared from the liver of a female white-spotted charr. Ten micrograms of DNA were digested with *Eco* RI, *Hind* III, and *Bam* HI, respectively, separated on a 1.0% agarose gel, and transferred onto a Hybond N^+. The membrane was hybridized at 50°C overnight with DIG-labeled STLs antisense RNA probes. After washing with 2×SSC/0.1% SDS at room temperature and twice with 0.2×SSC/0.1% SDS at 50°C, the membranes were incubated with anti-digoxigenin-AP Fab' fragments (1:10000), and the signals were detected by using a chemiluminescent substrate, CDP-Star.

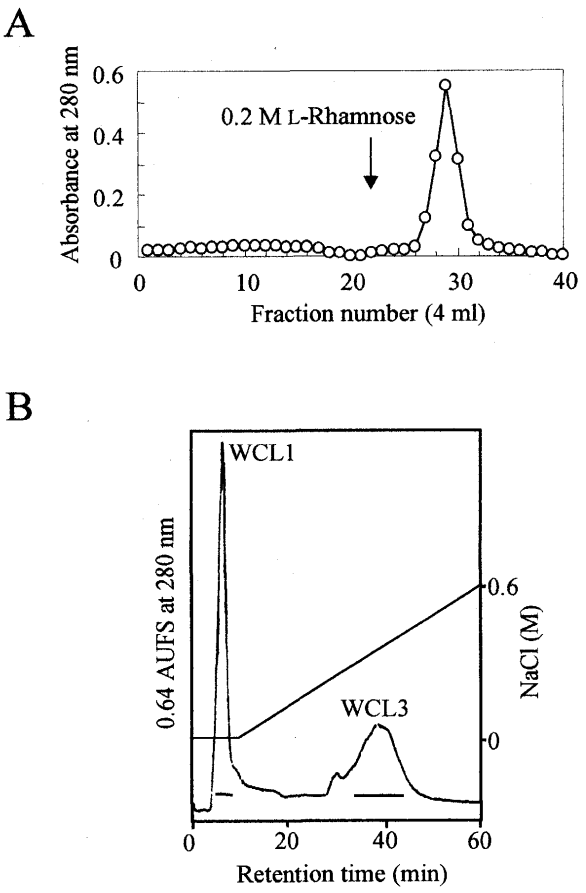

Fig. 1. Isolation of RBLs from White-spotted Charr Eggs.
WCLs were isolated by affinity chromatography on L-rhamnose-Sepharose 6B (A) and ion-exchange chromatography on Hi-Trap Q (B).

Results

Isolation and characterization of WCLs

Two RBLs were obtained from the egg extracts by affinity chromatography on L-rhamnose Sepharose 6B (Fig. 1A), followed by ion-exchange chromatography. WCL1 and WCL3 were isolated as an unadsorbed and an adsorbed fraction, respectively on a Hi-Trap Q column (Fig. 1B). The yields of WCL1 and WCL3 from 1 kg of ovulated eggs were 6.0 and 9.6 mg, respectively (Table 1). Each WCL gave a single band at 28 kDa (WCL1), and 22 kDa (WCL3) on SDS-PAGE in the absence of 2-ME (Fig. 2), and at 35 kDa (WCL1) and 26 KDa (WCL3) in the presence of 2-ME, respectively.

All of the WCLs and STLs showed hemagglutinat-

Table 1. Purification of RBLs from White-spotted Charr Eggs

Purification step	Protein (mg)	Total activity[a]	Recovery (%)	Specific activity[b]	Purification (fold)
Extract	94,000	1,280,000	100	13.6	1
L-Rhamnose Sepharose 6B	20	819,200	64	40,960	3012
Hi-Trap Q					
WCL1	6.0	20,480	1.6	3,413	251
WCL3	9.6	655,680	51	68,300	5022

[a] Obtained by multiplying the hemagglutination titer against 2% rabbit erythrocytes by the total volume of the solution.
[b] Total activity/mg protein.

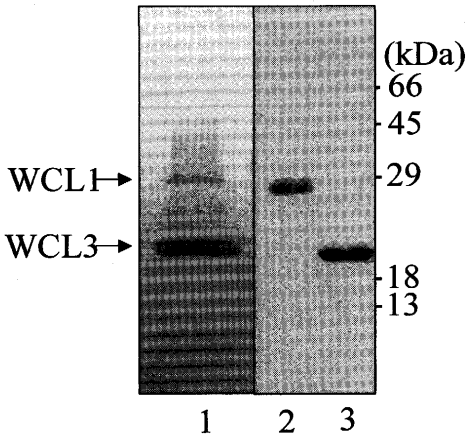

Fig. 2. SDS-PAGE of WCLs.
SDS-PAGE was done on 15% gel under nonreducing conditions. Lane 1 is WCLs isolated by affinity chromatography. Lanes 2 and 3 are WCL1 and WCL3, respectively, purified by ion-exchange chromatography. Bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21.5 kDa), myoglobin (18 kDa), and cytochrome *c* (13 kDa) were used as molecular markers.

Table 2. Hemagglutinating Activity of *Salmonidae* Egg Lectins

Erythrocyte	Minimum agglutinating concentration (μg/ml)				
	WCL1	WCL3	STL1	STL2	STL3
Human group A	>250	125	>250	>250	62.5
Human group B	>250	0.97	>250	0.39	0.9
Human group O	>250	125	>250	>250	125
Rabbit	7.8	0.12	31.2	0.39	0.9
Horse	>250	>250	>250	>250	250
Sheep	>250	>250	>250	>250	250
Chicken	>250	>250	>250	>250	62.5

Table 3. Inhibition of Hemagglutinating Activity of WCLs by Saccharides
2% intact rabbit erythrocytes and 8 hemagglutinating units of WCLs were used in each well.

Saccharide[a]	WCL1	WCL3
L-Rhamnose	0.05 mM	0.09 mM
Melibiose	1.6	3.1
L-Arabinose	6.3	25
D-Fucose	12.5	25
Raffinose	3.1	6.3
D-Galactose	6.3	25
Lactose	>100	>100
D-Arabinose	>100	>100
L-Fucose	>100	>100
Othersb	>100 (0.1%[c])	>100 (0.1%[c])

[a] Minimum concentration of saccharides required for complete inhibition.
[b] D-Glucose, D-mannose, D-xylose, D-N-acetylglucosamine, D-N-acetylgalactosamine, mucin type I, asialomucin type I, and fetuin.
[c] Glycoproteins such as mucin type I, asialomucin type I, and fetuin were tested at concentrations up to 0.1%.

ing activities to rabbit erythrocytes. WCL3, STL2, and STL3 also agglutinated human group B erythrocytes, however, WCL1 and STL1 did not agglutinate them (Table 2). RBLs showed no or very weak hemagglutinating activity to other erythrocytes tested in this study. L-Rhamnose was the most potent monosaccharide inhibitor for the hemagglutinating activities of WCLs as in the case of STLs (Table 3). Melibiose, L-arabinose, D-fucose, raffinose, and D-galactose, which have the same hydroxyl group orientation at C2 and C4 as L-rhamnose, had inhibitory effects. However, no other monosaccarides tested had any inhibitory activity even at a concentration of 0.1 M.

Amino acid and nucleotide sequences of WCL1

The enzymatic digests of CAM-WCL1 (50 nmol) with *Achromobacter* protease I and *Staphylococcus aureus* V8 protease were separated by reversed-phase HPLC, respectively (data not shown). The structures of isolated peptides were analyzed by amino acid sequencing, amino acid analysis, and MALDI-TOF mass spectrometry (Fig. 3). To identify the sequence of the remaining region, analysis of the nucleotide sequence was done using the method of rapid amplification of cDNA ends (RACE). The cDNA encoding the WCL1 subunit included 1,245 nucleotides with an open reading frame of 933 nucleotides. The open reading frame for the cDNA encoded for a mature protein of 288 amino acid residues, and a signal sequence of 23 residues with a typical hydrophobic core (Fig. 3). The stop codon at 934 was followed by a polyadenylation signal, AATAAA, starting at position 1,200. The amino acid sequences of the isolated peptides derived from WCL1 were exactly consistent

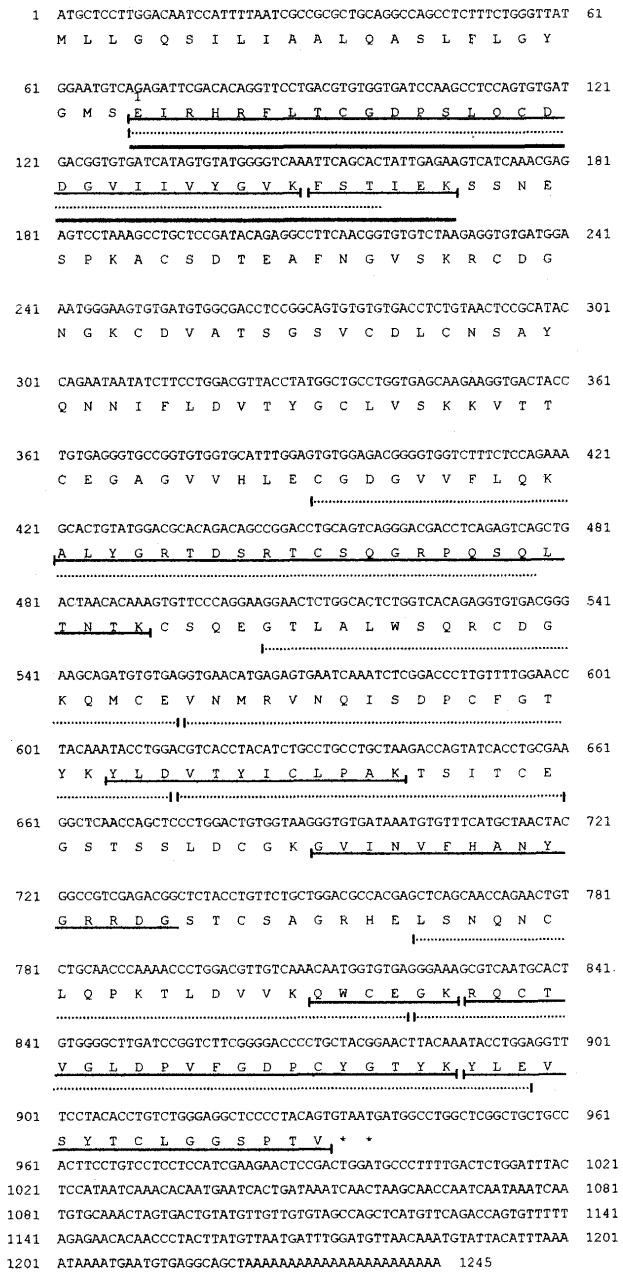

Fig. 3. Nucleotide Sequence and Amino Acid Sequence of WCL1.

Nucleotides and amino acid residues are numbered on the sides. Solid and dotted underlines represent the sequences determined by amino acid sequence analysis of the isolated peptides generated by cleavage of the CAM-WCL1 with *Achromobacter* protease I and *S. aureus* V8 protease, respectively. A bold solid underline represents N-terminal amino acid sequence.

with the sequence deduced from the nucleotide sequencing.

Amino acid and nucleotide sequences of WCL3

The enzymatic digests of CAM-WCL3 (50 nmol) with *Achromobacter* protease I, *S. aureus* V8 protease, and endoproteinase Arg-C were separated by reversed-phase HPLC and their structures were analyzed by amino acid sequencing, amino acid analysis, and MALDI-TOF mass spectrometry (Fig. 4).

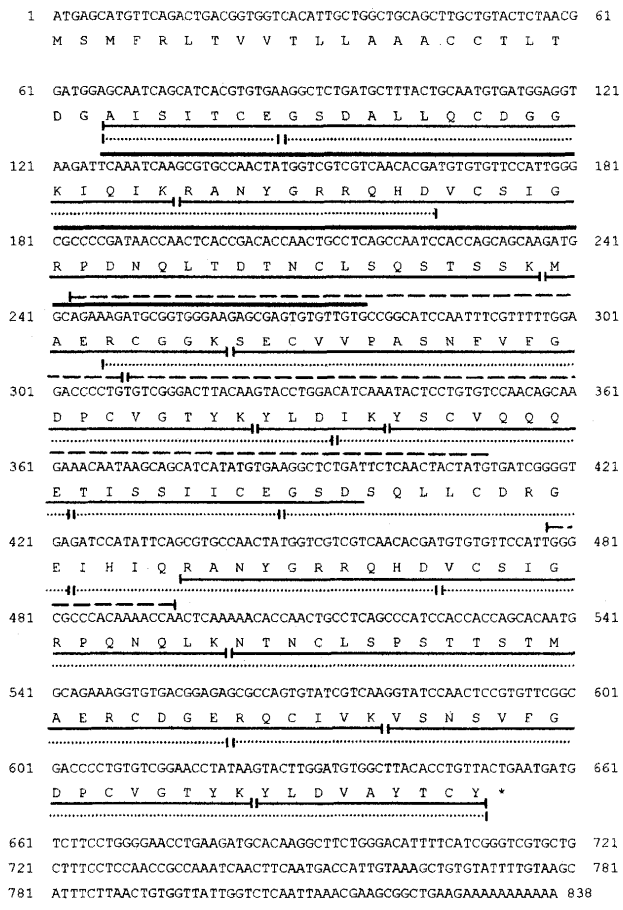

Fig. 4. Nucleotide Sequence and Amino Acid Sequence of WCL3.

Nucleotides and amino acid residues are numbered on the sides. Solid, dotted, and broken underlines represent the sequences determined by amino acid sequence analysis of the isolated peptides generated by cleavage of the CAM-WCL3 with *Achromobacter* protease I, *S. aureus* V8 protease, and Arg-C endoproteinase, respectively. A bold solid underline shows the N-terminal amino acid sequence.

To confirm the primary structure of WCL3, the nucleotide sequence was analyzed by the RACE method. The cDNA encoding WCL3 included 838 nucleotides with an open reading frame of 651 nucleotides. The open reading frame for the cDNA encoded a mature protein of 195 amino acid residues, and the signal sequence of 22 residues including a typical hydrophobic core. The stop codon at 655 was followed by a polyadenylation signal, ATTAAA, starting at position 808. The amino acid sequence of WCL3 obtained by sequencing of the isolated peptides is in good agreement with the sequence deduced from the nucleotide sequencing.

Northern blot analysis

Since WCL1 and WCL3 showed high sequence identities (>90%) to STL1 and STL3, respectively,

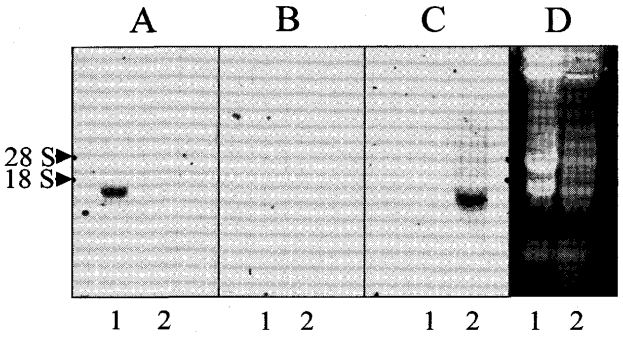

Fig. 5. Northern Blot Analysis of Tissue-specific Expression of WCLs.

Twenty micrograms of total RNA from liver and ovary was loaded onto a 1.5% agarose gel, transferred onto a membrane, and hybridized with DIG-labeled STL1 (A), STL2 (B), and STL3 (C) antisense RNA probes, respectively. (D) Ethidium bromide-stained pattern. Arrowheads indicate the positions of ribosomal RNA. Lane 1, Liver; lane 2, ovary.

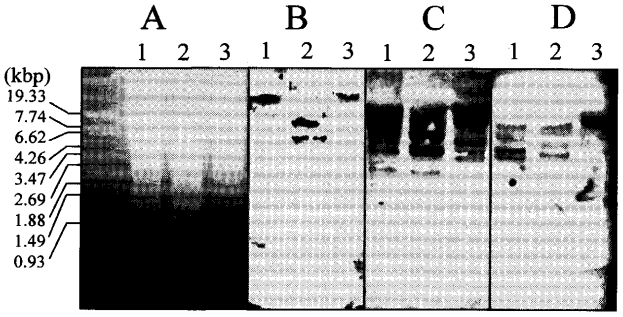

Fig. 6. Genomic Southern Blot Analysis of White-spotted Charr with the STLs Probes.

Ten micrograms of DNA digested with *Eco* RI (lane 1), *Hind* III (lane 2), and *Bam* HI (lane 3), respectively, were loaded onto a 1.0% agarose gel, transferred onto a membrane, and hybridized with DIG-labeled STL1 (B), STL2 (C), or STL3 (D) antisense RNA probes. (A) Ethidium bromide-stained pattern. The lines on the left show the positions of size markers (kilobases).

northern blot analysis of tissue-specific expression of WCLs was done using the STLs antisense probes (Fig. 5). The WCL1 mRNA was detected in the liver, but not in the ovary. In contrast, the WCL3 mRNA was detected in the ovary, but not in the liver. No signal was detected in these tissues with the STL2 antisense probe.

Genomic southern blot analysis

Positive signals using the STLs probes were detected in the white-spotted charr genome (Fig. 6). These patterns were similar to the results obtained with the steelhead trout genome by reacting with STLs probes.[6] The gene corresponding to the STL2 was detected, though no expression was observed at the protein and RNA levels.

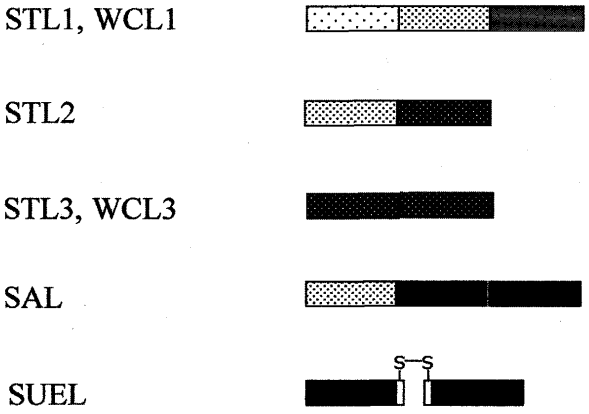

Fig. 7. Schematic Subunit Structures of RBLs.

Each box indicates the RBL CRD classified in Fig. 9.

Discussion

Two L-rhamnose-binding lectins, WCL1 and WCL3, were isolated from eggs of white-spotted charr and their amino acid sequences were analyzed by the combined use of protein sequencing and cDNA sequencing. The subunits of these lectins were composed of 288 and 195 amino acid residues, respectively, with 40% sequence identity. WCL1 and WCL3 showed 91 and 93% sequence identities to STL1 and STL3 from steelhead trout, respectively, with the same tandemly repeated structures; that is, three (for WCL1) or two (for WCL3) tandemly repeated RBL CRDs consisting of about 95 amino acid residues (Fig. 7).[2] All known RBLs from fish eggs are composed of two or three tandemly repeated RBL CRDs (Fig. 7).[2-4] The RBL CRD motif can be characterized by the highly conserved 8 half-Cys residues at the homologous positions and several highly conserved segments; Ala-Asn (Leu)-Tyr-Gly-Arg residues in the N-terminal region and Asp-Pro-Cys-X-Gly-Thr-Tyr-Lys-Tyr-Leu-Glu (Asp) in the C-terminal region (Fig. 8).[2-4] These regions must be related to L-rhamnose recognition activity. The presence of disulfide linkages formed by eight half-Cys residues supports the stabilities of RBLs in 8 M urea.

The carbohydrate-binding specificities of WCLs were similar to those of STLs, SAL from catfish (*Silurus asotus*) eggs, and SUEL.[2-4] The hemagglutinating activities of WCLs were most effectively inhibited by L-rhamnose and weakly inhibited by melibiose, L-arabinose, D-fucose, D-galactose, and raffinose, but not by L-fucose or D-arabinose. This result indicates that RBLs commonly recognize the hydroxyl group orientation at C2 and C4 of sugars. However, WCLs and STLs showed different hemagglutinating activities on several types of erythrocytes. Although WCL3, STL2, and STL3 agglutinated human group B erythrocytes, neither WCL1 nor STL1 agglutinated them. Both the different hemag-

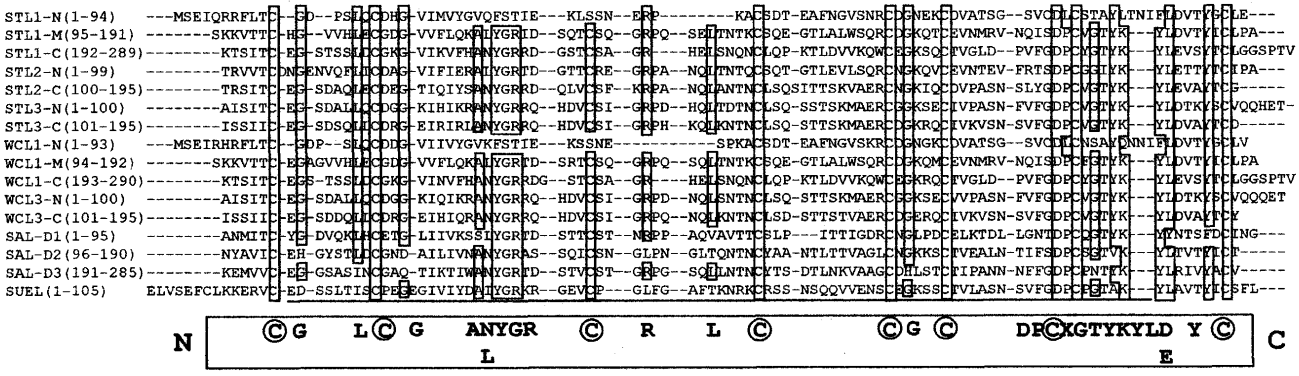

Fig. 8. Multiple Alignment of RBL CRDs.

Amino acid residues identical to STL-N, -M, and -C except for STL1-N are indicated by boxes. The bottom panel shows highly conserved amino acid residues of the RBL CRD motif. STL: Steelhead trout (*Oncorhynchus mykiss*) egg lectin; SAL: catfish (*Silurus asotus*) egg lectin; WCL: white-spotted charr (*Salvelinus leucomaenis*) egg lectin; SUEL: sea urchin (*Anthocidaris crassispina*) egg lectin.

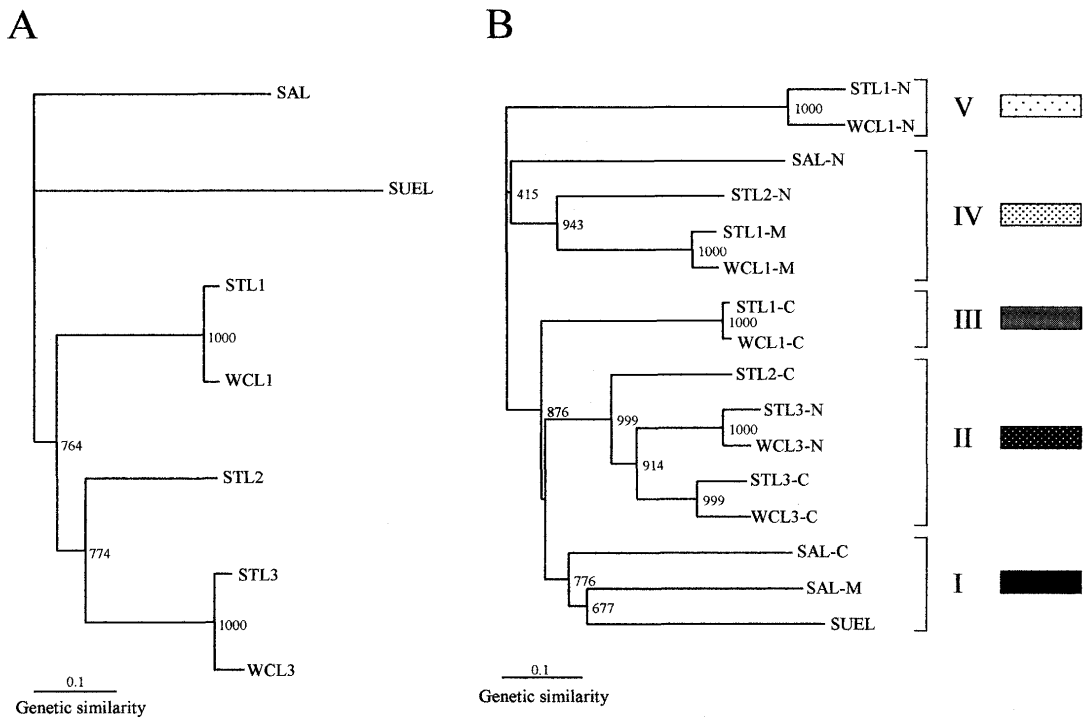

Fig. 9. Phylogenetic Tree of the RBL CRD.

A phylogenetic tree was constructed by the neighbor-joining algorithm based on an evolutionary distance matrix constructed by the Kimura's method. Panel A shows the phylogenetic tree constructed from full sequences of RBLs. Panel B shows the phylogenetic tree constructed from RBL CRDs. The CRD located in the N-terminal, middle, and C-terminal regions represented by N, M, and C, respectively. Each cluster is grouped into five classes; I ~ V. STL: Steelhead trout (*Oncorhynchus mykiss*) egg lectin; SAL: catfish (*Silurus asotus*) egg lectin; WCL: white-spotted charr (*Salvelinus leucomaenis*) egg lectin; SUEL: sea urchin (*Anthocidaris crassispina*) egg lectin.

glutinating activities and the different expression profiles of RBLs indicate that they have diversified to play different biological functions in fishes.[5)]

The phylogenetic trees of RBLs were constructed based on the amino acid sequences of 7 lectins from 4 species (Fig. 9A) and their 16 sequences of CRDs (Fig. 9B). The phylogenetic tree using the full sequence of RBLs shows that RBLs from *Salmonidae* (STLs and WCLs) separate into three groups corresponding to STL1, STL2, and STL3 (Fig. 9A). It is likely that the three different types of lectins have evolved in tandem after divergence into the different species. In contrast, the tree made from the 16 CRDs of RBLs shows 5 distinct clusters reflecting the evolutionary process of RBLs such as gene duplication and/or exon shuffling (Fig. 9B). STL3 and WCL3 appeared to be an ancient type of RBLs, because the cluster II containing STL3-N/-C and WCL3-N/-C were closer to the cluser I containing SUEL and SAL-M/-C than any other clusters (III-V) (Fig. 9B). On

Table 4. RBL CRD-like Domains (RCLDs) in Model Organisms

Organism	Protein	Amino acid	Accession No. (date base)
Homo sapiens	Latrophilin 1	40–134	T14324 (pir)
	Latrophilin 2	40–134	HSA1315811 (gp)
	Latrophilin 3	40–134	T14327 (pir)
	KIAA0821 protein	39–129	2506363C (prf)
	C21 orf 63	65–259	AF358258 1 (gp)
Drosophila	CG8639 gene product	26–114	AE003836 13 (gp)
C. elegans	Hypothetical protein B0457.1	44–135	T18759 (pir)
	F32A7.1	44–134	T21626 (pir)
	F32A7.3b	58–156	T21626 (pir)
	32A7.3a	56–160	T21628 (pir)
Arabidopsis	At2g32810/F24L7.5	486–574	AY039534 1 (gp)
	Putative β-galactosidase	788–876	ATH270305 1 (gp)
		777–838	C84685 (pir)
		790–851	ATH270304 1 (gp)
		768–848	ATH270299 (gp)
		743–825	T05771 (pir)
		757–839	ATH270307 1 (gp)
		767–846	AP001307 14 (gp)

the other hand, STL1 and WCL1 are constructed by three different types of CRDs, indicating that STL1 and WCL1 have evolved from an ancient gene to acquire new functions. The fact that STL1 and WCL1 showed different sugar specificities, hemagglutinating activities, and expression profiles more than STL2 and STL3/WCL3 supports this hypothesis.

Genomic sequencing of model animals such as vertebrates (*Homo sapiens*), invertebrates (*Drosophila melanogaster, Caenorhabditis elegans*), and plants (*Arabidopsis thaliana*) has revealed that a number of RBL CRD-like domains (RCLDs) are widely distributed in various organisms. RCLDs, which show 30–40% sequence identities to RBL CRDs and contain eight half-Cys residues and several common segments in the sequences, are summarized in Table 4. These are multi-domain proteins composed of a RCLD and other functional domains. Latrophilins, which are calcium-independent α-latrotoxin receptor family from bovine, rat, and human brains, comprise a RCLD in the N-terminal domain followed by a olfactomedin-like domain, a proline/threonine-rich domain, a transmembrane domain, and a cytoplasmic tail.[14–16] Latrophilin, which is a G-protein-coupled receptor is presumed to bind unidentified endogenous ligands and may regulate neurotransmitter release. The biological functions and sugar recognition activities of other RCLDs in model organisms have not been identified yet. It is probable that the intrinsic ligands for RCLDs are proteins, but not an L-rhamnose-containing glycoconjugate. These data support the idea that the ancestral gene of RBL CRD diverged and evolved by gene duplication and/or exon shuffling, producing new forms to play their own roles in various organisms.

Although three RBLs could be isolated from steelhead trout[2] and chum salmon eggs (unpublished result), only two RBLs corresponding to STL1 and STL3 were isolated from white-spotted charr. Although WCL1 and WCL3 mRNAs were detected in the liver and ovary, respectively, as in the case of STL1 and STL3, neither STL2-like protein nor mRNA could be detected in white-spotted charr (Figs. 2 and 5).[6] However, a STL2-like gene was detected on the white-spotted charr genome by the southern blotting analysis, indicating that the STL2-like gene was a pseudogene and its expression was suppressed. Although we do not know the biological significance of the lack of a STL2-like molecule in the white-spotted charr, WCL3 may perform both functions of STL2 and STL3 in white-spotted charr because STL2 and STL3 showed similar specificity for carbohydrates and expression profiles.[1,2,6]

RBLs have been isolated and partially characterized from the eggs of various teleost fish families such as *Salmonidae*,[17,18] *Percidae*,[19] *Cyprinidae*,[19,20] *Osmeridae*,[21–23] and *Clupeidae*.[24] Although RBLs have been found mainly in the primitive fishes (*Salmonidae, Cyprinidae, Osmeridae*), they were also found in advanced fish species such as *Percidae*[20,22] and play important roles in a variety of biological functions; prevention of polyspermy,[25] regulation of carbohydrate metabolism,[20] cross-linking of carbohydrate-rich proteins of the fertilization envelope,[25] bactericidal effects,[26] mitogenesis,[27] and macrophage-mediated tumor lysis.[28]

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