Rhamnose-binding Lectins from Steelhead Trout (Oncorhynchus mykiss)
Eggs Recognize Bacterial Lipopolysaccharides and Lipoteichoic Acid

Hiroaki Tateno,* Tomohisa Ogawa,* Koji Muramoto,*,† Hisao Kamiya,** and Mineo Sanyoshi***

*Department of Biological Resource Sciences, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan
**School of Fisheries Sciences, Kitasato University, Sanraku, Iwate 022-0101, Japan
***Department of Biological Sciences, Teikyo University of Science and Technology, Uenohara, Yamanashi 409-0193, Japan

Received October 12, 2001; Accepted November 12, 2001

The interaction between bacteria and three l-rhamnose-binding lectins, named STL1, STL2, and STL3, from steelhead trout (Oncorhynchus mykiss) eggs was investigated. Although STLs bound to most Gram-negative and Gram-positive bacteria, they agglutinated only Escherichia coli K-12 and Bacillus subtilis among the bacteria tested. The binding was inhibited by l-rhamnose. STLs bound to distinct serotypes of lipopolysaccharides (LPSs), and showed much higher binding activities to smooth-type LPSs of Escherichia coli K-12 and Shigella flexneri 1A than to their corresponding rough-type LPSs. STLs also bound to lipoteichoic acid (LTA) of Bacillus subtilis. These results indicate that STLs bound to bacteria by recognizing LPSs or LTA on the cell surfaces.

Key words: animal lectin; rhamnose-binding lectin; steelhead trout; lipopolysaccharide; lipoteichoic acid

Immunity is an important physiological mechanism in animals for protection against infection and the preservation of internal homeostasis. The immune response is generally mediated by innate and acquired systems. Although acquired immunity is found only in vertebrates, innate immunity is present in all multicellular organisms.1) Non-self-recognizing proteins involved in innate immunity recognize mainly carbohydrate moieties on pathogens, such as lipopolysaccharides (LPSs) of Gram-negative bacteria, lipoteichoic acids (LTAs) of Gram-positive bacteria, glycolipids of mycobacterium, and mannans of yeast.1) Lectins are typical carbohydrate-binding proteins, which have been suggested to play an important role in host-pathogen interactions by specific recognition with cell surface substances of bacteria. Animal lectins are classified into several families depending on their sequence similarities and sugar-binding specificities; C-type lectins, galectins, pentraxins, etc.2) Some of these lectin families have been isolated from fishes and characterized; C-type lectins from Oncorhynchus mykiss,3) galectins from Conger myriaster,4,5) and Oncorhynchus mykiss,7) and pentraxin from Anguilla japonica.8) A novel animal lectin family, which is characteristic in its l-rhamnose binding specificity, has been isolated from eggs of various species of the teleost fishes such as Salmonidae,9,10) Percidae,11) Cyprinidae,12,13,14) Siluridae,15) Osmeridae,16) and Clupeidae.17) We isolated and characterized three l-rhamnose-binding lectins, named STL1, STL2 and STL3, from steelhead trout (Oncorhynchus mykiss) eggs.18,19) They were composed of tandemly repeated homologous domains in the sequence. These repeated domains had approximately 95 amino acid residues, and showed a significant sequence homology to sea urchin (Anthocidaris crassispina) egg lectin.20) The carbohydrate recognition domain (CRD) contained eight half-Cys residues at homologous positions and several highly conserved segments. In spite of these characteristic structures and sugar binding specificities, little information is available about the biological functions of the rhamnose-binding lectin family.

In this paper, we report the interaction of STLs with bacteria, and their LPSs and LTA to explore the

1 To whom correspondence should be addressed. Koji Muramoto, Tel & Fax: 81-22-717-8807; E-mail: muramoto@biochem.tohoku.ac.jp

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HBS, 0.01 M HEPES buffer (pH 7.4) containing 0.15 M NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P-20; HRP, horseradish peroxidase; LPS, lipopolysaccharide; LTA, lipoteichoic acid; R:: rough-type; S:: smooth-type; STL, steelhead trout egg lectins; TBS, 20 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl
biological functions of the L-rhamnose-binding lectin family in innate immunity.

Materials and Methods

Materials. Smooth-type (S-) LPSs from Escherichia coli O26:B6, Escherichia coli O55:B5, Escherichia coli O111:B4, Escherichia coli O127:B8, Escherichia coli K-235, Klebsiella pneumoniae, Pseudomonas aeruginosa serotype 10, Salmonella minnesota, and Shigella flexneri 1A, rough-type (R-) LPS from Shigella flexneri (Re), lipid A from Shigella flexneri, and LTA from Bacillus subtilis were purchased from Sigma (St. Louis, MO, USA). S-LPSs from Escherichia coli K-12 LCD25 and R-LPSs from Escherichia coli K12, D31m4 (Re) were purchased from List Biological Laboratories (Campbell, CA, USA). Fish pathogens, Aeromonas salmonicida, Aeromonas hydrophilia, and Pasteurella piscicida were kindly provided by Dr. M. Sakai (Miyazaki University, Miyazaki, Japan).

Preparation of STLs and anti-STLs antiserum. STLs were isolated from the unfertilized eggs of steelhead trout by affinity chromatography, anion exchange chromatography, and reversed-phase high performance liquid chromatography successively as described. Anti-STL IgGs, which had been raised in rabbits and isolated by affinity chromatography on a Hi-Trap Protein A column (1 ml, Amersham Pharmacia Biotech, Uppsala, Sweden), were digested with pepsin to yield Fab', followed by conjugation with horseradish peroxidase (HRP) (Wako Chemical, Osaka, Japan) as described.

Bacteria agglutinating activity of STLs. The bacteria-agglutinating activity of STLs was examined using Gram-negative bacteria (Escherichia coli K-12, Escherichia coli B, Aeromonas salmonicida, Aeromonas hydrophilia, Pasteurella piscicida, and Salmonella typhimurium LT2) and Gram-positive bacteria (Bacillus subtilis and Staphylococcus aureus). Bacteria except fish pathogens were grown at 37°C overnight in Luria-Bertani medium. Fish pathogens, A. salmonicida, A. hydrophila, and P. piscicida, were grown at 23°C overnight in the same medium. The bacteria were then collected by centrifugation at 10,000 × g for 1 min, washed with 20 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl (TBS), and re-suspended in TBS to a final concentration of 3 × 10⁹ cells/ml. Two-fold serial dilutions of lectins (10 μl in TBS) were mixed with 10 μl of bacteria in a round-bottom microtiter plate (96-well) by agitation for 30 s, and the mixtures were left for 30 min at room temperature. A portion of the mixture was transferred onto a slide glass to measure the agglutination by light microscopy. The titer was defined as the reciprocal value of the end point dilution causing agglutination.

Antibacterial activity of STLs. Antibacterial activity was examined by the turbidimetric method using E. coli K-12 and B. subtilis. A mixture of 50 μl of bacterial suspension (5 × 10⁹ cells) and 50 μl of STLs (0.005–12.5 μg) was incubated in Luria-Bertani medium in a 96-well microtiter plate at room temperature with agitation. The bacterial growth was monitored by the turbidity measured at 630 nm.

Binding of STLs to LPS and LTA. An enzyme-linked immunosorbent assay (ELISA) was used to examine the binding of STLs to LPS and LTA. FALCON PRO-BIND immunoassay plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) were coated with 100 μl of LPS (5 μg/ml) or LTA (5 μg/ml) dissolved in the coating buffer (0.1 M NaHCO₃, pH 9.6) at 37°C for 1.5 h. After non-specific binding sites were blocked with 0.5% bovine serum albumin (BSA) in 10 mM sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl (PBS), the wells were washed with the washing buffer (0.05% Tween 20 in PBS) three times. The plates were then incubated at 37°C for 1.5 h with various concentrations of STLs (3-3,000 ng/ml) in 100 μl of PBS containing 0.5% BSA. The plates were washed with the washing buffer, and reacted with 100 μl of HRPlabeled anti-STL Fab' (1 μg/ml) in PBS containing 0.5% BSA at 37°C for 1.5 h. After the plates were washed, the bound enzyme activity was measured at 490 nm with o-phenylenediamine as a chromogen. Assays were done in triplicate.

Binding of STLs to bacteria. STLs (300 ng) were incubated with bacteria (1.5 × 10⁹ cells/ml) in 1 ml of TBS at 4°C overnight. After centrifugation at 10,000 × g for 1 min, unbound STLs in the supernatant were analyzed by ELISA.

Surface plasmon resonance analysis. The interaction between STLs and LPSs was analyzed by surface plasmon resonance using BIACore (Amersham). L-Rhamnose (1 mM, 10 μl) in water was incubated at 90°C for 1 h with 6-hydrazidohexyl d-biotinamide (BH2-AC5) (2 mM, 10 μl) in 30% aqueous acetonitrile. The biotinyl L-rhamnose (1 μM, 100 μl) was injected onto the sensor chip SA, on which streptavidin had been immobilized. The sensor chip was regenerated with 50 mM H₂PO₄ (4 μl) at the end of each measurement. STLs (100 μg/ml) dissolved in HBS (0.01 M HEPES buffer (pH 7.4) containing 0.15 M NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P-20) were injected onto the rhamnose-immobilized sensor chip at a flow rate of 20 μl/min. Various concentrations of LPS (0.03-500 μg/ml) were added to the STL solution for inhibition assay. An unmodified sensor chip was used as the control.
Sensogramms were analyzed by nonlinear least squares curve fitting using BIA evaluation software 3.0.

Results

Bacteria agglutinating activity of STLs

The agglutinating activities of STLs were examined using Gram-negative bacteria (E. coli K-12, E. coli B, A. salmonicida, A. hydrophila, P. piscicida, and S. typhimurium LT2) and Gram-positive bacteria (B. subtilis and S. aureus). STL3 strongly agglutinated E. coli K-12 and B. subtilis at the concentrations of higher than 0.08 and 0.7 μM, respectively. These agglutinations were completely inhibited by the addition of S-LPS from E. coli K-12 (0.1 mg/ml) or LTA from B. subtilis (0.1 mg/ml), and by 0.1 M D-glucose. STL1 and STL2 also agglutinated these bacteria at the concentrations of higher than 7.9 and 5.8 μM for E. coli K-12, and 3.9 and 0.7 μM for B. subtilis, respectively. In contrast, STLs did not show any significant agglutinating activity toward other bacteria tested in this study, but, STLs bound to the bacteria except for STL3 against E. coli B (Fig. 1).

STL3 showed 50% growth inhibition of E. coli K-12 at the concentration of above 60 μg/ml on steady phase. STL1 and STL2 showed 20 and 15% growth inhibition, respectively, at 250 μg/ml. STLs showed 40, 30, and 30% inhibition to the cell growth of B. subtilis at the concentration of 250 μg/ml.

Binding of STLs to LPSs

LPSs were immobilized on 96-well immunoassay plates for the binding with STLs. The amounts of LPSs immobilized on the plates were estimated from the unbound LPSs, in the coating buffer, which were measured by using a chromogenic endotoxin-specific assay kit (Seikagaku Kogyo, Tokyo, Japan). About 80-90% of the applied LPSs were immobilized on the plate.

![Fig. 1. Binding of STLs to Gram-negative and Gram-positive Bacteria.](image)

STLs (300 ng) were incubated with bacteria (1.5 × 10⁸ cell) in 1 ml of TBS at 4°C overnight. After centrifugation (10,000 × g), STLs in the supernatant were analyzed by ELISA to estimate the percentages of bound to bacteria. The data shown are means ± S.D. of three experiments.

---

Fig. 2. Binding of STLs to Various Types of LPSs.

LPSs immobilized on 96-well immunoassay plates were reacted with 100 μl of STLs (10 ng) in PBS containing 0.5% BSA at 37°C for 1.5 h. The bound STLs were analyzed by ELISA as described in the text. A: STL1; B: STL2; C: STL3. The amounts of LPSs immobilized in the wells were estimated by an endotoxin assay kit and the percentages of the immobilized LPSs are shown in the panel D. The data shown are means ± S.D. of three experiments.
plates (Fig. 2(D)). Each LPS showed no significant difference in the immobilizing efficiency. STLs showed different binding activities to various types of LPSs and lipid A (Fig. 2(A)–(C)); STL3 had higher specificity in the binding than STL1 and STL2. Although STLs showed high binding activities to E. coli K-12 S-LPS and S. flexneri 1A S-LPS, the activities were greatly reduced to R-LPSs from the rough mutants, suggesting that STLs could recognize the O-antigens and core polysaccharides of LPSs.

To confirm the abilities of STLs to recognize the O-antigens, the binding activities of STLs to S-LPSs and R-LPSs from E. coli K-12 and S. flexneri were compared. The amounts of STLs bound to both types of LPSs increased in concentration-dependent manner. STLs bound to S-LPSs much more than to R-LPSs that lack O-antigens and core polysaccharides (Fig. 3). Moreover, the binding of STLs to S. flexneri S-LPS was inhibited by P. aeruginosa S-LPS as well as by S. flexneri S-LPS and E. coli K-12 S-LPS, but not by S. flexneri R-LPS or E. coli K-12 R-LPS (Fig. 4). S-LPSs from E. coli O26:B6, E. coli O55:B5, E. coli O111:B4, E. coli O127:B8, E. coli K-235, K. pneumoniae, and S. minnesota showed only marginal inhibitory effects on the binding (data not shown).

Effects of sugars on the binding of STLs to LPSs
STLs (300 ng/ml) were reacted with S. flexneri S-LPS in the presence of various concentrations of sugars. The 50% inhibitory concentration (IC50) of L-rhamnose to the binding of STL1, STL2, and STL3 were 13, 12, and 133 μM, respectively (Fig. 5(A)). Melibiose, L-arabinose, D-galactose, raffinose, and D-fucose, which have the same orientation of hydroxyl groups at C2 and C4 as L-rhamnose, also showed inhibitory effects, though their effects were much weaker (Table 1). D-Arabinose and L-fucose did not show an inhibitory effect, at least up to 50 mM. This inhibitory profile of sugars is similar to that obtained from
Fig. 4. Inhibitory Effects of LPSs on the Binding of STLs to S. flexneri S-LPS.

S. flexneri S-LPS immobilized on the immunoassay plates were reacted with 100 μl of STLs (30 ng) in PBS containing 0.5% BSA in the presence of various concentrations of LPSs. The bound STLs were analyzed by ELISA as described in the text. (A): STL1; (B): STL2; (C): STL3. ⊗: E. coli K-12 S-LPS; ⊘: E. coli K-12 R-LPS; ◯: S. flexneri S-LPS; ◯: S. flexneri R-LPS; ▲: P. aeruginosa S-LPS. The bound STLs were analyzed by ELISA as described in the text. The data shown are means ± S.D. of three experiments.

the hemagglutinating activities of STLs.19) STLs lost their binding activities to LPSs upon heating at 70°C for 60 min (Fig. 5(B)).

Surface plasmon resonance analysis

The binding of STLs to L-rhamnose was also analyzed by surface plasmon resonance. By injecting STLs into the sensor chip on which L-rhamnose was immobilized, specific dose-dependent sensorgrams were obtained. The binding parameters were estimated by nonlinear least squares curve fitting on the basis of the subunit monomers of STL1 (Mr, 31.4 k), STL2 (Mr, 21.4 k) and STL3 (Mr, 21.6 k). The association constants (K_a) of STLs with L-rhamnose were 1.64 \times 10^5, 7.78 \times 10^4, and 7.09 \times 10^3 M, respectively. When STL3 (100 μg/ml) was co-injected with various concentrations of LPSs, the concentration-dependent inhibition of the binding to L-rhamnose was observed with S-LPSs from E. coli K-12 and S. flexneri 1A, but not with R-LPSs from E. coli K-12 and S. flexneri (Fig. 6). The IC_50 of LPSs to the binding between STLs and L-rhamnose were estimated by
Fig. 6. Effects of S- and R-LPSs on the Sensorgrams of STL3 to l-Rhamnose Immobilized onto the Sensor Chip.
STL3 (100 μg/ml) was co-injected with 500 (I), 125 (II), 31.3 (III), 15.6 (IV), 7.8 (V), 3.9 (VI) μg/ml of E. coli K-12 S-LPS (A), E. coli K-12 R-LPS (B), S. flexneri S-LPS (C), and S. flexneri R-LPS (D) on the sensor chip.

Table 2. The 50% Inhibitory Concentration (IC_{50}) of LPSs on the Binding of STLs to l-Rhamnose

<table>
<thead>
<tr>
<th>STL</th>
<th>IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STL1</td>
<td></td>
</tr>
<tr>
<td>E. coli K-12 S-LPS</td>
<td>39.4</td>
</tr>
<tr>
<td>E. coli K-12 R-LPS</td>
<td>&gt;167</td>
</tr>
<tr>
<td>S. flexneri S-LPS</td>
<td>11.2</td>
</tr>
<tr>
<td>S. flexneri R-LPS</td>
<td>&gt;167</td>
</tr>
</tbody>
</table>

4-parameter fitting on the basis of the molecular masses of 10 kDa for S-LPSs and 3 kDa for R-LPSs (Table 2). E. coli K-12 S-LPS was the most potent inhibitor for the binding between STLs and l-rhamnose.

Binding of STLs to LTA

LTA was immobilized on 96-well immunoassay plates under the same conditions as LPS, and the plates were used for the binding experiment with STLs. STLs bound to the LTA in a concentration-dependent manner similar to the LPS binding (Fig. 7(A)). STLs (300 ng/ml) were reacted with LTA in the presence of various concentrations of l-rhamnose. The IC_{50} of l-rhamnose to the binding of STL1, STL2, and STL3 were 27, 60, and 58 μM, respectively (Fig. 7(B)).

Discussion

This study demonstrated that STLs recognized LPS and LTA, which were major integral components of the outer membranes of Gram-positive and
Gram-negative bacteria, respectively. Binding studies of STLs with distinct serotypes of LPSs showed that STLs had higher affinity to S-LPSs from *E. coli* K-12 and *S. flexneri* 1A than R-LPSs. This indicates that STLs recognize LPSs through their O-antigens and core polysaccharides, at least in the case of these two LPSs. The O-antigen is the immunodominant structure exposed to the environment and is highly variable among bacterial strains, whereas the core polysaccharides and lipid A moieties are more conserved. Therefore, the differences of binding affinities of STLs to various serotypes of S-LPSs from *E. coli* might be due to the O-antigens. The inconsistency of the binding and agglutinating activities of STLs against bacteria may be due to the variable spatial arrangement of the multivalent binding sites.

The O-antigens of *E. coli* K-12 S-LPS and *S. flexneri* 1A S-LPS, which showed high affinities to STLs, include L-rhamnose residues in the molecules (Fig. 8). However, O-antigens of *P. aeruginosa* 10 S-LPS showed high affinity to STLs without L-rhamnose residue. Instead, the O-antigens are composed of rarely occurring trisaccharide repeating units containing derivatives of D-glucose, N-acetyl-D-fucosamine, and L-mannitolulose acid. It is not surprising because STLs recognize monosaccharides that have the same orientation of hydroxyl groups at C2 and C4 as L-rhamnose. The structure of *N*-acetyl-D-fucosamine (2-acetamido-2,6-dideoxy-D-galactose) is similar to that of L-rhamnose regarding to the orientation of hydroxyl groups at C4. The fact that D-fucose inhibited the binding between LPS and STLs supports this assumption. On the other hand, *E. coli* O26:B6 S-LPS failed to inhibit the binding between STLs and L-rhamnose in spite of the presence of α-L-Rha-(1→4)-α-L-FucNAc in the O-antigen. This result implies that the binding affinities of STLs against *E. coli* O26:B6 S-LPS are much lower than those against L-rhamnose itself and that the glycosidic linkage of L-rhamnose is important for the recognition of STLs. The linkage of the O-antigen of *E. coli* O26:B6 S-LPS is α1→4, whereas the linkages of other O-antigens are α1→2 or α1→3. Other LPSs used in this study do not contain L-rhamnose residues in the repeating units of O-antigens.

It should be noted that STLs interacted with not only S-LPSs but also R-LPSs and lipid A. Furthermore, STLs agglutinated a Gram-positive bacterium and bound to its LTA. The interactions were inhibited by L-rhamnose. These results indicate that STLs can recognize various pathogen-associated molecular patterns. STLs also inhibited the growth of *E. coli*
K-12 and B. subtilis. Our recent immunohistochemical study showed that STLs were located in the perivitellin space of fertilized eggs and STL1 was mainly localized in the tissues of the immune system; serum, spleen, thrombocytes, and blood leukocytes. With consideration of these results, it is probable that STLs may function as non-self-recognition proteins in the innate immune system in these tissues.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research (11460094) from the Ministry of Education, Science, Sports, and Culture of Japan. H. T. is a recipient of a Research Fellowship (No. 01541) of the Japan Society for the Promotion of Science for Young Scientists.

References

