Purification and Characterization of Two Ca$^{2+}$-Dependent Lectins from Coelomic Plasma of Sea Cucumber, Stichopus japonicus

Taei Matsui,*† Yasuhiro Ozeki,* Masami Suzuki,* Akiya Hino,* and Koiti Titani*

*Division of Biomedical Polymer Science, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-11; and †Department of Biological Sciences, Kanagawa University, Hiratsuka, Kanagawa 259-12

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Two structurally distinct lectins were purified from the coelomic plasma of holothurian, Stichopus japonicus, by affinity chromatography on a porcine stomach mucin-conjugated agarose column, gel filtration on a Superose 6 column, and ion-exchange chromatography on a HiTrap Q-FPLC. The two lectins showed apparent molecular masses of about 400 kDa (SPL-1) and 60 kDa (SPL-2) on gel filtration, but about 17 kDa on SDS-PAGE under reducing conditions. Both lectins showed hemagglutination activity toward rabbit erythrocytes in the presence of Ca$^{2+}$ ions. The N-terminal amino acid sequences were highly homologous to but distinct from those of a Ca$^{2+}$-dependent (C-type) lectin named SJL-I purified from the same species. In addition to porcine stomach mucin, the hemagglutination activity of SPL-1 was strongly inhibited by uronic acids such as galacturonic acid, and glucuronic acid, while the activity of SPL-2 was inhibited by GalNAc and galactosides. Both lectins were adsorbed on clotted coelomocytes in the presence of Ca$^{2+}$ but not in the presence of inhibitory sugars or EGTA, suggesting the presence of an endogenous carbohydrate ligand(s) for plasma C-type lectins in the clot. However, coelomocyte clotting occurred normally even in the presence of inhibitory sugars, but was strongly inhibited by synthetic GRGDSP peptide or EGTA, suggesting the participation of integrin but not the lectin–carbohydrate interaction in the clotting events.

Key words: clotting, coelomic plasma, C-type lectin, integrin, sea cucumber.

Lectins are widely distributed carbohydrate-recognition proteins other than glycosidases, glycosyltransferases and immunoglobulins (1). Most animal lectins can be classified into two groups, Ca$^{2+}$-dependent (C-type) and SH-group dependent (S-type) lectins, based on their biochemical characteristics and structural similarities (2). The interaction of certain lectins with specific carbohydrate structures (ligand) on glycoconjugates has been implicated in diverse cellular recognition systems, such as leukocyte adhesion to inflamed endothelial cells (3), clearance of asialoglycoproteins from the circulation (4) and sperm-egg binding during fertilization (5).

In invertebrates, a variety of hemagglutinins have been found in the coelomic plasma and coelomocytes (6-9). Biochemical analysis has revealed that some of these hemagglutinins are structurally homologous to vertebrate C-type lectins (8, 9). Moreover, a sea urchin coelomic fluid lectin known as echinodrin has been described as a unique C-type lectin containing the RGD sequence (9), one of the cell recognition signals found in cell adhesive proteins, including fibronectin, laminin, and fibrinogen (10). Little is known about the physiological function of echinoderm plasma lectins. We have recently demonstrated that echinodrin exhibits in vitro RGD-dependent cell adhesive activity (11), suggesting that echinodrin may play a role in cellular adhesion by recognizing both carbohydrates and integrins in the coelomic fluid. A coelomic fluid hemagglutinin from Paracentrotus lividus has also been reported to adhere to coelomocytes (12). The aggregation of coelomocytes or the coagulation of coelomic fluid are two processes that occur rapidly upon removal from the coelomic cavity. These phenomena are mostly Ca$^{2+}$-dependent, like mammalian hemostasis (13), but the activating and regulating mechanisms as well as the relation to plasma lectins have not been fully investigated except for the horseshoe crab (14). In the present study, we isolated and structurally characterized two lectins from echinoderm (holothuria) plasma. We also examined whether these lectins participate in coelomocyte clotting.

MATERIALS AND METHODS

Preparation of Coelomic Fluid—Approximately 100 ml of coelomic fluid was collected from the opened body cavity of 3 sea cucumbers, S. japonicus, and filtered through a nylon mesh (95 μm pore size). The fluid was mixed at 0°C with 1/10th volume of 150 mM EGTA, 100 mM Tris-HCl (pH 7.5), and a mixture of protease inhibitors (final 5 mM benzamidine, 2 mM N-ethylmaleimide, 1 mM phenyl-

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2 To whom correspondence should be addressed.

Abbreviations: PE, S-pyridylethylated; FSM, porcine stomach mucin; SPL-1 and -2, sea cucumber plasma lectin-1 and -2; TBS, Tris-buffered saline.
methanesulfonyl fluoride). Coelomocytes were removed by centrifugation (10,000 × g, 30 min at 4°C), and the supernatant (plasma) was stored at −80°C until use.

**Hemagglutination Assay**—Hemagglutination assay was performed using trypsinized and glutaraldehyde-fixed rabbit erythrocytes as described previously (7). Glutaraldehyde-fixed human type A, B, and O erythrocytes and formaldehyde-fixed human platelets were also examined. In the general assay method, hemagglutination was measured in the presence of 10 mM Ca²⁺ and 0.05% Tween-20 using microtiter V-plates and expressed as titer, defined as the reciprocal of the highest dilution giving positive hemagglutination. For the sugar inhibition assay, each sugar or glycoprotein solution (200 mM or 10 mg/ml) was serially diluted with Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl) and mixed with lectin solution previously adjusted to titer 16. The minimum inhibitory sugar concentration was scored. The sugars and glycoproteins were of specially pure grade, purchased from Seikagaku Kogyo, Wako Pure Chemicals, and Sigma Chemicals.

**Affinity Chromatography**—A column of porcine stomach mucin (PSM)-conjugated agarose gel (5 ml, EY Laboratories) was washed with 10 volumes of TBS containing 5 mM EDTA and equilibrated with TBS containing 2.5 mM CaCl₂. The coelomic plasma (100 ml) was dialyzed against 300 volumes of TBS containing 2.5 mM CaCl₂, and the supernatant obtained by centrifugation (27,000 × g, 30 min, at 4°C) was applied to the PSM-agarose column at 4°C. After washing with the same buffer, the column was eluted with TBS containing 5 mM EDTA. Eluates with hemagglutinating activity were collected and concentrated on a Centricon-10 (Amicon Grace).

**FPLC**—The PSM-agarose column-bound fraction was subjected to gel filtration on a Superose-6 FPLC column (HR 10/30, Pharmacia LKB) equilibrated with TBS. Fractions of 0.5 ml were collected and tested for hemagglutination using small aliquots. Ion exchange chromatography was performed on a HiTrap Q column (1 ml, Pharmacia LKB) with a gradient of salt concentration containing 10 mM Tris-HCl, pH 7.5, from 150 mM to 1 M NaCl.

**Other Procedures**—SDS-PAGE was performed according to Laemmli’s system (15) using 15% separation gel under reducing or unreducing conditions. Protein concentration was determined with BCA protein assay reagent (Pierce) using BSA as a standard. The concentration of plasma Ca²⁺ was determined as described previously (16).

**Reduction and S-Pyridylethylation of SPL**—SPLs were reduced with 2% tri-n-butylphosphine (Wako Pure Chemicals) and pyridylethylated with 1% 4-vinylpyridine (Tokyo Kasei) in 1 ml of 120 mM Tris-HCl (pH 8.3) containing 6 M guanidine hydrochloride at room temperature for 4 h (17). S-Pyridylethylated (PE) SPLs were purified by reversed-phase HPLC on a column (4.6 × 250 mm) of SynChropak RP8 (SynChrom) using a gradient of acetonitrile into aqueous 0.1% TFA.

**Amino Acid Analysis and Sequence Determination**—PE-SPLs were hydrolyzed in 6 N HCl containing 1% phenol at 110°C for 24 h by the vapor-phase method and analyzed on a Hitachi L8500 amino acid analyzer. Amino-terminal sequence determination was performed using an Applied Biosystems Model 470A protein sequencer connected online to a Model 120A PTH analyzer. Sequence homology search was performed using the protein sequence database of the National Biomedical Research Foundation (July, 1993) on a VAX 3600 computer with the WORDSEARCH program (18).

**Binding of SPL to Coelomocyte Clot**—Coelomic fluid collected without addition of EGTA was allowed to stand for 3 h at 0°C to undergo clotting (aggregation of coelomocytes). The coelomocyte clot obtained by centrifugation was washed several times with 50 volumes of TBS containing 5 mM EDTA until the supernatant showed no significant hemagglutinating activity. Then 25 mg of wet clot was suspended in 100 µl of either coelomic plasma or purified SPLs in TBS (titer 128) in the presence or absence of 10 mM Ca²⁺, 2 mM EGTA, 20 mM galacturonic acid, 2 mM GalNAc, 1 mg/ml PSM. After 1 h at 4°C, each suspension was centrifuged (15,000 rpm, 15 min at 4°C) and the precipitate was washed twice with 500 µl of TBS containing 2.5 mM Ca²⁺. Finally, the precipitate was incubated twice with 100 µl of TBS containing 10 mM EGTA for 5 min with gentle agitation and later centrifuged to obtain the supernatant. The hemagglutinating activity in each supernatant was assayed under Ca²⁺-fortified conditions.

**Coelomocyte Clotting In Vitro**—Freshly collected coelomic fluid (450 µl) was immediately transferred to a 24-well plastic plate containing 50 µl of 150 mM EGTA, 200 mM GalNAc, 200 mM lactose, PSM (10 mg/ml in TBS), synthetic GRGDSP or GREGSP peptide (5 mg/ml in TBS). The plate was gently agitated for 10 min at room temperature, and then the cells were fixed with 2% formaldehyde and examined under a phase contrast microscope.

### RESULTS

**Purification of Sea Cucumber Plasma Lectins (SPL)**—The coelomic fluid of *S. japonicus* strongly agglutinated fixed rabbit erythrocytes but weakly agglutinated human type A, B, or O erythrocytes. It also agglutinated fixed rabbit erythrocytes but weakly agglutinated human type A, B, or O erythrocytes. Since our preliminary experiments indicated that plasma-agglutinating activity was inhibited by EGTA, suggesting that the major hemagglutinin in the plasma was Ca²⁺-dependent.
induced Ca\(^{2+}\)-dependent hemagglutination was most effectively inhibited by PSM, we attempted to purify the hemagglutinin using affinity chromatography on a PSM-agarose column. As shown in Fig. 1, almost all Ca\(^{2+}\)-dependent hemagglutinating activity was bound to the PSM column and eluted with EDTA. The PSM-column bound fraction was shown to contain multiple components, that were separated into four active fractions by gel filtration on a Superose-6 column (Fig. 2). Specifically, two major fractions, designated as SPL-1 and SPL-2, accounted for about 80% of the total activity. These two fractions were selected and used for further analysis. Using ion exchange chromatography, we were able to remove trace amounts of contaminants on a HiTrap Q column (data not shown) to purify SPL-1 and -2 to apparent homogeneity on SDS-PAGE (Fig. 3). The purification steps are summarized in Table I.

Characterization of SPLs—Both SPL-1 and SPL-2 showed a single band at approximately 17 kDa in SDS-PAGE under reducing conditions, but displayed bands of 16 and 60 kDa, respectively, under non-reducing conditions (Fig. 3). Based on the apparent molecular masses of SPL-1 (400 kDa) and SPL-2 (68 kDa) calibrated by gel filtration (Fig. 2), SPL-1 may exist as an aggregate of non-covalently-bound 17 kDa subunits, while SPL-2 may be a tetramer composed of 17 kDa subunits covalently bound via S-S linkages.

We also determined the carbohydrate-binding specificities of the SPLs by measuring the minimum sugar concentration that inhibited SPL-induced hemagglutination (Table II). SPL-1 showed a high affinity to galacturonic and glucuronic acids in addition to PSM. However, a variety of galactosides and hexosamines showed little inhibitory effect. It is of interest that polyuronic acid compounds such as chondroitin sulfates, heparin, and hyaluronic acid had weak or no inhibitory effect. In contrast to SPL-1, SPL-2-induced hemagglutination was strongly inhibited by GalNAc as well as PSM. Lactose and other galactosides also had affinity for SPL-2, and the \(\alpha\)-anomers were a little more potent than the \(\beta\)-anomers. SPL-2 was bound to a lactose-conjugated affinity column owing to its high affinity for lactose, but SPL-1 was not retained by the column (data not shown).

The effects of Ca\(^{2+}\) on SPL-induced hemagglutination was examined. The concentrations of Ca\(^{2+}\) necessary for exhibiting 50% hemagglutination were 0.4 and 1.0 mM for SPL-1 and -2, respectively. Since the Ca\(^{2+}\) concentration in the plasma was higher than these values (10.7 ± 0.8 mM, \(n = 3\)), we suggest that both SPLs were fully active under in vivo conditions. Mg\(^{2+}\) ion did not produce the same effects as Ca\(^{2+}\).

Amino-Terminal Sequences of SPLs—The amino-termi-
Fig. 4. Amino acid sequence homology among SPLs and the known C-type lectins. Echinoidin, sea urchin coelomic fluid lectin (9); SJL-I, S. japonicus lectin I (19); ABL, acorn barnacle coelomic fluid lectin (8); FFL, fresh fly hemolymph lectin (20); BJL, Bothrops jararaca venom lectin (21); Bot (β), botrocetin β-subunit (17); RHL-1, rat hepatic lectin-1 (22); ASGP-H1, human asialoglycoprotein receptor-1 (23).

Numbers indicate positions of amino acid residues from the N-terminus. Small letters indicate tentative identifications. Gaps have been inserted to maximize homology. Identical residues to those of both SPLs are boxed. The RGD sequence in echinoidin is underlined.

TABLE II. Inhibition of S. japonicus plasma lectin-induced hemagglutination by various carbohydrates.a

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Minimum inhibitory concentration (mM)</th>
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<tr>
<td></td>
<td>SPL-1</td>
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<tr>
<td>GalNAc</td>
<td>&gt;40</td>
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<tr>
<td>GlcNAc</td>
<td>&gt;40</td>
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<tr>
<td>N-Acetylmannosamine</td>
<td>&gt;40</td>
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<tr>
<td>N-Acetylneuramic acid</td>
<td>&gt;40</td>
</tr>
<tr>
<td>Gal</td>
<td>&gt;40</td>
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<tr>
<td>Methyl-α-galactoside</td>
<td>N.D.</td>
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<tr>
<td>Methyl-β-galactoside</td>
<td>N.D.</td>
</tr>
<tr>
<td>Methyl-α-mannoside</td>
<td>&gt;40</td>
</tr>
<tr>
<td>Fuc</td>
<td>&gt;40</td>
</tr>
<tr>
<td>Glic</td>
<td>&gt;40</td>
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<tr>
<td>Glucuronic acid</td>
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<tr>
<td>Galacturonic acid</td>
<td>1.3</td>
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<tr>
<td>Lactose</td>
<td>40</td>
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<tr>
<td>N-Anthracose</td>
<td>N.D.</td>
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<tr>
<td>Thiodigalactoside</td>
<td>&gt;40</td>
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<tr>
<td>PSM</td>
<td>0.13 mg/ml</td>
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<tr>
<td>Fetsin</td>
<td>N.D.</td>
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<tr>
<td>Asialofetuin</td>
<td>N.D.</td>
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<tr>
<td>Chondroitin sulfate A, B, C</td>
<td>&gt;1.0 mg/ml</td>
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<tr>
<td>Hyaluronic acid (porcine skin)</td>
<td>0.5 mg/ml</td>
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<tr>
<td>Heparin</td>
<td>&gt;4.0 mg/ml</td>
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aThe minimum concentration required for inhibition of hemagglutination by the lectins of titer 16 was determined. *Not determined.

*Chondroitin sulfate A from whale cartilage, B from porcine skin, and C from shark cartilage and human umbilical cord were tested.

nal sequences of PE-SPLs are depicted in Fig. 4. Our results indicate that the lectins are structurally similar but clearly distinct. The two lectins contained three Cys residues at identical positions and showed approximately 37% identity in the amino-terminal regions. Identities between SPLs and SJL-I, a C-type lectin recently purified from the same species (19), were 22% (SPL-1) and 32% (SPL-2). Alignment with other C-type lectins indicated that both SPLs had a significant homology to C-type lectins such as echinoidin (41–45%), Bothrops jararaca venom lectin (BJL) (28%), and mammalian hepatic lectins (28%) (Fig. 4). It is of interest that SPLs have a homology to botrocetin, which is known to modulate von Willebrand factor to induce platelet aggregation in vitro (17).

Binding of SPLs to Coelomocyte Clot—When the intact plasma was incubated with EGTA-washed coelomocyte clot, approximately 75% of the hemagglutinating activity was adsorbed on the clot in a Ca²⁺-dependent manner, and was mostly released from it with EGTA (Fig. 5). When SPL-1 and -2 (titer 128) were used instead of fresh plasma, almost all activities were adsorbed to the clot and recovered by adding EGTA. Lack of Ca²⁺, and particularly the presence of galacturonic acid and GalNAc, totally inhibited the binding of SPL-1 and -2 to the clot. These results indicate that coelomocytes contain specific ligands for SPLs.

Effects of Sugars and RGD Peptides on the Coelomocyte Clotting—Effects of sugars on the clotting of coelomocytes were examined in vitro. Coelomocytes were agglutinated within 10 min after collection (Fig. 6a). Neither PSM (Fig. 6c), GalNAc (Fig. 6d), nor lactose interfered with the coelomocyte clotting. However, the clotting was inhibited in the presence of either EGTA (2 mM EGTA), or in the presence of 50 mM galacturonic acid (GalUA), or 2 mM GalNAc, and the activity eluted from each clot by EGTA was examined (eluate [EGTA], eluate [GalUA], and eluate [GalNAc], respectively). Data represent the mean of three (plasma) and two (SPL-1, -2) independent experiments.
Lectins from Coelomic Plasma of Sea Cucumber

Fig. 6. Effects of sugar and RGD-peptide on coelomocyte clotting of *S. japonicus*. Coelomic fluid was withdrawn and immediately transferred to plates (a) containing 15 mM EGTA (b), 1 mg/ml of PSM (c), 20 mM GalNAc (d), 0.5 mg/ml of GRGDSP (e) or GRGESP peptide (f). After 10 min, the cells were fixed with 1% formaldehyde and examined under a microscope.

**DISCUSSION**

In the present study, we purified two Ca\(^{2+}\)-dependent lectins, designated SPL-1 and -2, from the coelomic plasma of sea cucumber *S. japonicus*. Our results indicate that, although both lectins were present in soluble forms in the plasma and had a similar subunit molecular weight, they differed from each other in molecular mass of the native form, sugar specificity, Ca\(^{2+}\)-sensitivity, and primary structure. Although the deduced N-terminal sequences did not include the carbohydrate recognition motif, both SPLs may be classified as C-type animal lectins. Yamasaki and coworkers (24) have purified two lectins with molecular masses of 13 (SJL-I) and 15 kDa (SJL-II) from the same species using a lactosyl-Sepharose 4B column, and the primary structure of SJL-I has been recently elucidated (19). These investigators extracted lectins from tissues using TBS containing 0.3% Triton X-100, suggesting that the lectins exist in membrane-bound form. We recently observed that both Ca\(^{2+}\)-dependent and -independent hemagglutinating activities are present in the TBS-extract of body mucus and coelomocytes of the same species.

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Matsui, T., unpublished results). SPL-2 resembles SJL-I with regard to Ca\(^{2+}\)-dependency and sugar specificity (24), but is distinct from SJL-I in amino acid sequence, composition (data not shown) and molecular mass, suggesting that the two are isolectins.

Several soluble C-type lectins have been isolated from the plasma of invertebrates and vertebrates (25-28). Some of these humoral lectins are involved in self-defense mechanisms as opsonins, or may act as phagocytic activators or antibacterial factors. It remains unclear, however, whether echinoderm plasma lectins also possess these activities. It is of interest that several compounds such as saponins (29), hemolysin, and lysozyme (30) have been identified in the sea cucumber as antibacterial and defensive molecules. The SPLs showed no hemolytic activity toward freshly prepared rabbit erythrocytes (Matsui, unpublished observation).

In echinoderms, clotting events (aggregation of coelomocytes) are essential in preventing loss of hemolymph from the wound, in addition to shrinking of body walls. Furthermore, aggregation of coelomocytes may also encapsulate invading organisms. It is well known that holothurian amoebocytes show a morphological change (activation) during the early stage of clotting, followed by formation of a cell aggregate (clot) composed of amoebocytes and spherule cells (13, 30, 31). The agglutination process, but not the morphological change, requires Ca\(^{2+}\) (31). Although SPLs bind the coelomocyte clot in a Ca\(^{2+}\)- and carbohydrate-dependent manner, addition of PSM had no inhibitory effect on the clotting, suggesting that the SPL-carbohydrate binding is not essential for the clotting event. However, in the present work, we obtained the novel finding that the RGD-integrin recognition system may be involved in the clotting process. We have previously reported the RGD-dependent but carbohydrate-independent cell adhesive activity of echinodin (11), which contains the RGD sequence (9) (Fig. 4). Although the presence of a plasma component(s) responsible for the coelomocyte clotting other than SPLs is suggested, it is possible that SPLs are bifunctional lectins (32) recognizing both carbohydrate and integrin as well as echinodin. Further structural analysis and cell adhesion assay using SPLs are necessary to examine this possibility.

It is important to isolate and characterize endogenous ligands for SPL from coelomocytes in order to elucidate the physiological role of these plasma lectins in echinoderms. A variety of proteoglycans have been purified from the sea cucumber body wall (33, 34). Structural analyses have demonstrated that these proteoglycans are highly sulfated and Fuc-branched chondroitin sulfates, insusceptible to chondroitinase (33, 35). Although commercially available chondroitin sulfates are not potent inhibitor of SPLs (Table II), it is possible that certain unique proteoglycans with a high affinity to SPLs are present in coelomocytes of the sea cucumber. Plasma SPLs may interact with the ligand(s) on the activated coelomocytes or insoluble secretions from the coelomocytes during the clotting process, and may support "hemostasis" in echinoderms or have a secondary effect on the clot.

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