A Novel Mitogenic Lectin from the Globiferous Pedicellariae of Sea Urchin, *Toxopneustes Pileolus*

H. NAKAGAWA*, C. YAMAGUCHI, F. TOMIYOSHI AND H. HAYASHI
Department of Life Science, Faculty of Integrated Arts and Sciences, University of Tokushima, Tokushima 770, Japan

Summary: A galactose-specific sea urchin lectin-I (SUL-I) was isolated from the large globiferous pedicellariae of *Toxopneustes pileolus* by gel chromatography, affinity chromatography and reverse-phase high performance liquid chromatography. SUL-I is a monomeric protein with a molecular mass of 32 kDa. The N-terminal amino acid is alanine. The partial amino acid sequence was determined up to 35 residues. SUL-I stimulated mitogen response on murine T lymphocytes. The mitogen response to SUL-I in T lymphocytes was effectively inhibited by D-galactose. Our study suggests that SUL-I is a novel mitogenic lectin from marine invertebrates.

Introduction

Some sea urchins are known to have external appendages (pedicellariae and/or spines) which contain biologically active substances. *Toxopneustes-pileolus* and *Tripneustes gratilla*, beloging to the family Toxopneustidae have well-developed globiferous pedicellariae with bioactive substances. Some of the bioactive substances produce deleterious and pharmacological effects [1-9].

The globiferous pedicellariae of *T. pileolus* have flexible heads containing pincers which function as seizing organs [10].

There are two main sub-types of pedicellariae: globiferous pedicellaria and large globiferous pedicellaria [11]. Although a comparison of venom proteins in the globiferous pedicellariae of sea urchins, *Toxopneustes pileolus* and *Tripneustes gratilla* has been reported preliminarily [12,13] there is little information available about a lectin from the pedicellariae of sea urchins.

More recently, in comparative study on lectin components from the globiferous pedicellariae and large globiferous pedicellariae of *T. pileolus*, we found that the crude extracts from the pedicellariae of the two sub-types exhibited agglutinating activity with rabbit erythrocytes in a Ca^{2+} independent manner. In addition, we have purified a novel sea urchin lectin-I (SUL-I) from the large globiferous pedicellariae of *T. pileolus*. SUL-I is a galactose-binding protein with a molecular mass of 32 kDa [14].

In this paper we describe the mitogen responses to the crude extract and SUL-I from the large globiferous pedicellariae of *T. pileolus*.

Results and Discussion

Scanning Electron Microscopy

Electron microscopic observations showed distinct features based on size and shape between globiferous pedicellaria and large globiferous pedicellaria (Fig. 2A and 2B). The head of large globiferous pedicellaria was much globular in shape. Each of the two sub-types of the pedicellariae had a sharp incurved spine referred to as a fang (Fig. 2C and 2D). Our present observation suggests the possibility that there may be functional differences in the two sub-types of venomous pedicellariae of *T. pileolus*.

Biological Activities of the Crude Lectin Extract

The crude lectin extract from the large globiferous pedicellariae caused agglutination with horse and rabbit erythrocytes. The agglutination with rabbit erythrocytes caused by the extract was significantly more intense at concentrations up to 0.31 μg/ml, while it showed a mild agglutination with horse erythrocytes at concentrations up to 15.6 μg/ml. Furthermore, the extract in higher concentrations (80 μg/ml) showed a weak hemolytic activity when incubated with rabbit erythrocytes (data not shown).

*To whom all correspondence should be addressed.

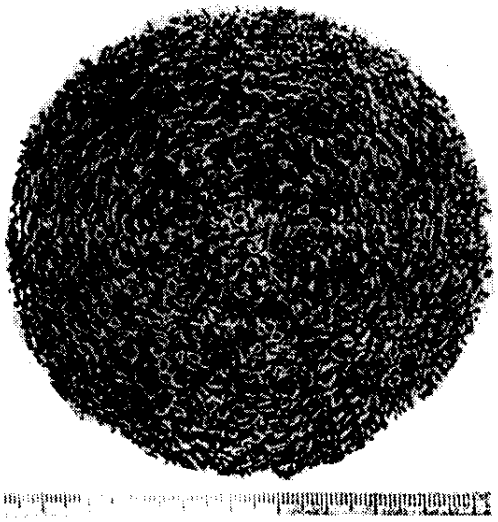

Fig. 1. Dorsal view of the sea urchin *Toxopneustes pileolus*. Diameter of test 10 cm. Living specimen taken in the coast of Shikoku Island, Japan. Note some of the globiferous pedicellariae with the jaws open.

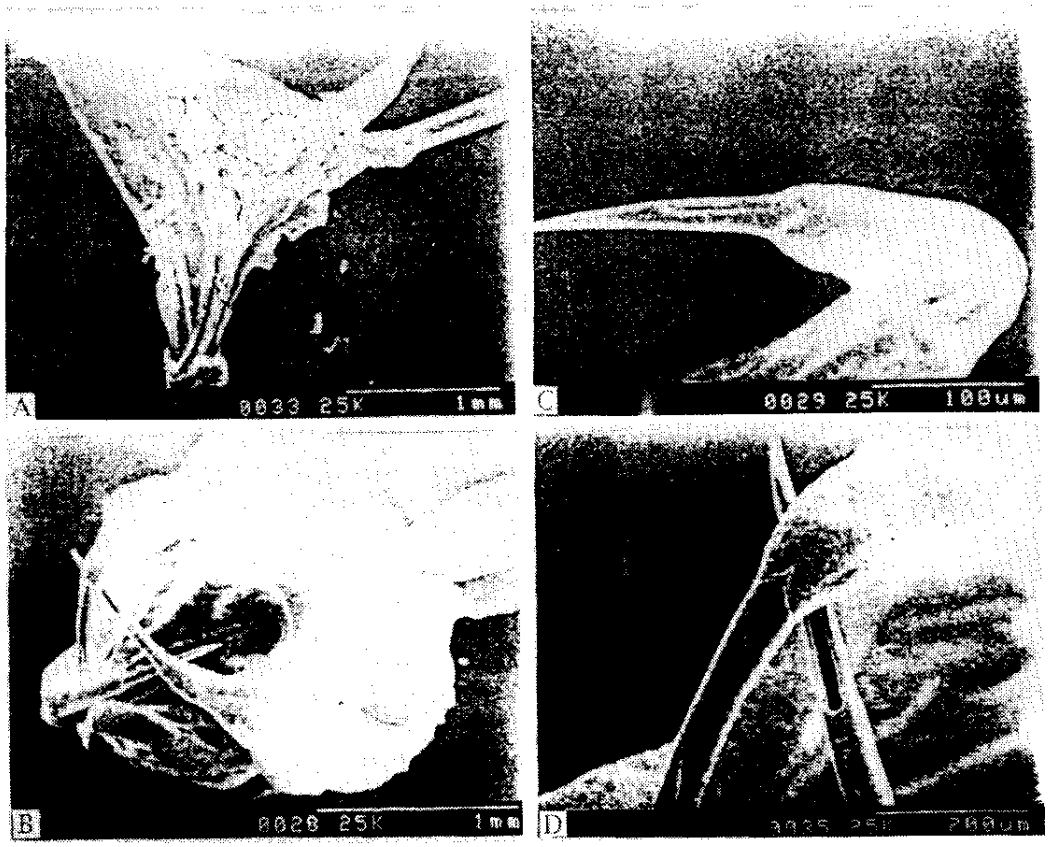

Fig. 2. Scanning electron micrograph showing the pedicellariae of *T. pileolus*. (A) a globiferous pedicellaria, x 30;(B) a large globiferous pedicellaria, x 40; (C) a fang of a globiferous pedicellaria, x 300; (D) a fang of a large globiferous pedicellaria, x 150.

One of the most dramatic effects of the interaction of lectins with cells is mitogenic stimulation [15]. Therefore, we examined whether the crude lectin extract exhibits mitogenic stimulation on murine splenocytes and mesenteric lymphocytes. As shown in Fig. 3, the extract had mitogenic stimulation in murine splenocytes in dose response manner. In mesenteric lymphocytes the extract also stimulated mitogen response in the same dose ranges (data not shown).

Isolation and N-terminal Sequence of SUL-I

A galactose-specific sea urchin lectin-I (SUL-I) was isolated from the crude lectin extract of *T. pileolus* by gel chromatography, affinity chromatography and reverse-phase high performance liquid chromatography. SUL-I consists of 294 amino acid residues with a molecular mass of 32 kDa, which is a monomeric protein [14]. Sequence analysis of 100 pmol of intact SUL-I indicated the N-terminal sequence from Ala-1 to Ile-35. SUL-I is rich in glycine and shows 5 glycine residues in the sequenced region. This is equivalent to 14 % of the residues (upto residue no. 35 from the N-terminal, Table-1). Residues 6 and 15 are probably cysteine.

As shown in Table-1, the amino acid sequence of SUL-I is only related to SUEL [16] except for echinoidin [17] SPLs and SJL-I [18-19]. If more lectin components of different sea urchin and sea cucumber species are identified in the future, some amino acid sequence homology should occur.

Mitogenic Activity of SUL-I

We have found that the crude lectin extract showed mitogenic stimulation on murine splenocytes. Therefore, the mitogenic activity of SUL-I was also investigated. SUL-I stimulated mitogen response of T cells separated from murine splenocytes in lower dose ranges (Fig. 4). The mitogen response to SUL-I was about 10 times higher than that to the crude lectin extract (Fig. 3). SUL-I may be a novel mitogenic lectin. We are presently studying the mitogen response to SUL-I on murine B-lymphocytes.

SUL-I is a galactose-specific lectin. Furthermore, we examined the effect of D-galactose on the mitogen response to SUL-I. As shown in Fig. 5, the mitogen response to SUL-I in murine T lymphocytes was effectively inhibited by D-galactose. The results

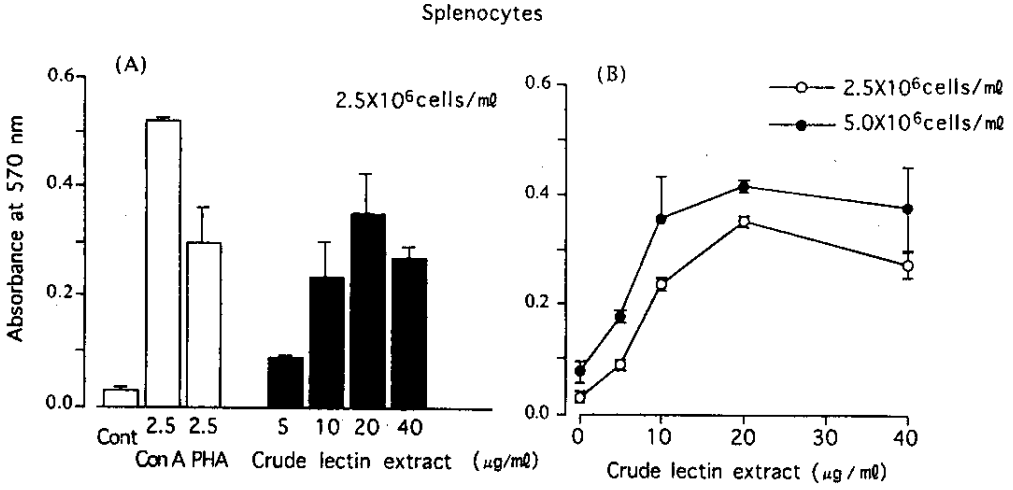

Fig. 3. Mitogen responses of the crude lectin extract from the large globiferous pedicellariae of T. *pileolus* on murine splenocytes. (A) Comparison of mitogen responses to plant lectins (Con A and PHA) and the crude lectin extract. (B) Dose effects of the crude lectin extract on mitogen response. The splenocytes were incubated with Con A, PHA or the extract for 68 hr and continued to incubate with MTT for 4 hr in a 5% CO_2 humidified atmosphere. The values show means Å S ±.D. of three experiments of triplicate determinations. Table 1. Partial amino acid sequence of sea urchin lectin-I (SUL-I) and comparison with other marine lectins Identical amino acid residues are shaded. Numbers in parentheses indicate positions of amino acid residues from the N-terminus.

Table 1.
Partial amino acid sequence of sea urchin lectin-I (SUL-I) and comparison with other marine lectins

Lectin	Organism	Sequence
		5 10 15 20 25 30 35
SUL-I	*Toxopneustes pileolus* (sea urchin)	A V G R T - E G K S L D L E - P E G Y I I S V N Y A N Y G R N S P G I
SUEL	*Anthrocidaris crassispina* (sea urchin)	(5) K K E R V C E D S S L T I S C P E G E G I V I Y D A I Y G R K R G E V
Echinoidin		(12) S N C Y R F F A V S L T W A E G E Q F C Q S F S V P S R G D I D S I G
SPL-I	*Stichopus japonicus* (sea cucumber)	(24) H C Y R F f N n P Q L D w V S A E Q Y C N q F G w V h d g - - - - - -
SPL-II		(14) G K C Y R L f H D H L T F T E A E H A C R A F K L R S C N g N d s - -
SJL-I		(18) G C Y K Y V D T - P K T F E E A E S T C R H F S P G P Y G - - - - F G

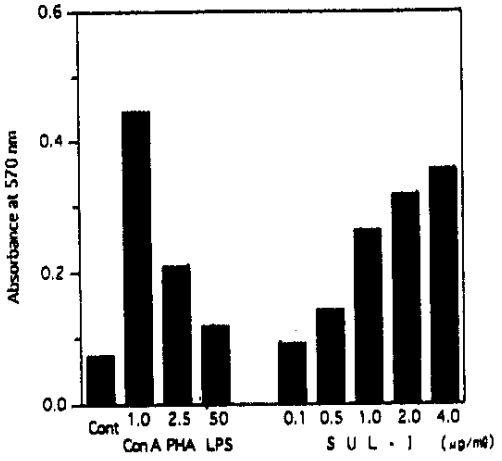

Fig. 4. Mitogen response of SUL-I on murine T lymphocytes separated from murine splenocytes. T lymphocytes were incubated with Con A, PHA, LPS or SUL-I for 68 hr and continued to incubate with MTT for 4 hr in a 5% CO_2 humidified atmosphere. The data show mean of triplicate determinations.

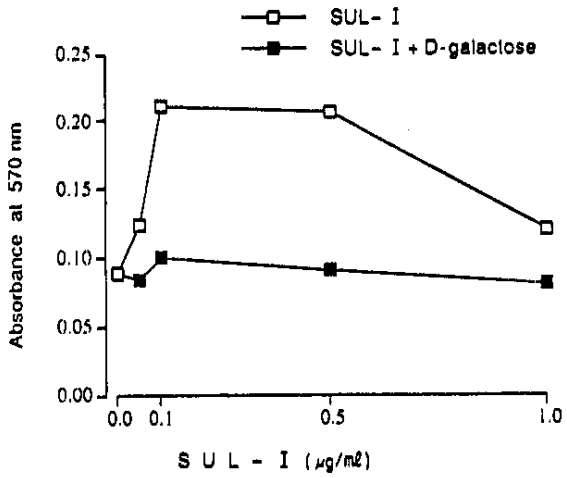

Fig. 5. Effect of D-galactose on mitogen response stimulated by SUL-I in murine T lymphocytes. T lymphocytes were incubated with D-galactose (50 mM) and SUL-I (0.1 to 1.0ug/ml) for 68 hr and continued to incubate with MTT for 4 hr in a 5 % CO_2 humidified atmosphere. The data show the means of 2 experiments of triplicate determinations.

suggest that mitogen response is caused by SUL-I binding to galactose-containing carbohydrates on murine T lymphocyte surface. Our recent studies reveal that SUL-I induces chemotactic response and superoxide production on guinea-pig macrophages. The responses to SUL-I are in lower dose ranges (0.625-10 μg/ml) (unpublished data). These findings suggest an intracellular function for SUL-I. Thus, SUL-I may be a broad spectrum biologic modifier.

Recent results show that galectins [20,21] formerly known as animal S-type or S-Lac lectins [22] are a new family of ß-galactoside-binding lectins, which recognize ß-galactoside sugars and share characteristic amino acid sequences. SUL-I is a calcium-independent lectin which exhibits specificity for galactose/lactose. In the present study, it is not clearly if SUL-I can be classified as a family of galectins in respect of the amino acid sequences. Further structural studies on SUL-I would facilitate insights into the physiological functions of sea urchin lectins.

Experimental

Toxopneustes pileolus sea urchins (139 specimens) were collected along the coast of Tokushima Prefecture, Shikoku Island, Japan, from 1993 through 1995 (Fig. 1). Rabbit blood sample was obtained from Nippon Bio-Test Laboratories (Tokyo, Japan). Sephadex G-200 was from Pharmacia Biotech (Uppsala, Sweden). Immobilized D-galactose gel was from Pierce (Illinois, U.S.A.). All the other chemicals were reagent grade.

Isolation of Sea Urchin Lectin-I (SUL-I)

Thirty large globiferous pedicellariae per sea urchin specimen (8-10 cm in diameter were removed with fine forceps. They were extracted with 20 ml of 0.15 M NaCl at 4°C for 24 hr. The 20 ml aliquot of each was centrifuged at 12,000 g for 20 min and the supernatant was used as the crude lectin extract [14]. Briefly, for the first step of purification, the crude lectin extract was applied to a Sephadex G-200 column (2.6 x 70 cm) equilibrated with 0.15 M NaCl solution and was eluted with the same solution at a flow rate of 15 ml/hr. Fractions of 10 ml each were collected and analyzed for absorption at 280 nm and agglutinating activity. For the second step of purification, the gel chromatographic fraction (the third protein peak) was dissolved in 150 mM phosphate buffer and placed on an immobilized D-galactose column (1 x 2 cm). The sample was washed with the same buffer and was eluted with 100 mM D-galactose in the same buffer. The 2 ml elution fractions were collected and analyzed for absorption at 280 nm and agglutinating activity. The second peak fraction was collected. The final purification was achieved by HPLC using a reverse-phase C_8 column. Two solvents, 0.1 % trifluoroacetic acid (TFA) and acetonitrile containing 0.08 % TFA, were used. The fraction was monitored at 230 nm. The main peak was pooled and analyzed for agglutinating activity, and used as the purified sea urchin lectin-I (SUL-I).

Scanning Electron Microscopy

The globiferous pedicellariae and large globiferous pedicellariae with stalks were fixed in 1 % osmium tetra-oxide containing filtered sea water, and dehydrated in an ascending series of ethanol and critical point dried. The pedicellariae were then mounted on aluminium stubs with silver paint and sputter-coated with gold for 3 min. They were examined under a Hitachi-SEM scanning electron microscope operated at 10 kV and 25 kV.

Amino Acid Sequence

The amino acid sequence was determined directly on a Shimadzu PPSQ-10 system protein sequencer.

Agglutinating Activity and Hemolytic Activity

The agglutinating activity was assayed by using rabbit erythrocytes in microtiter plates. 25 µl of 4 % (v/v) suspension of erythrocytes in 6.4 mM phosphate buffer saline, pH 7.2 (PBS) was added to 50 µl of serial 2-fold dilutions of the lectin fractions and SUL-I in PBS. The plates were incubated at room temperature for 1 hr. The assay of hemolytic activity was carried out using 2 % (v/v) suspension in PBS. The results were expressed by the minimum concentration of the test solution (µg/ml) required for positive agglutination or hemolysis.

Mitogenesis Assay

The mitogenic activity by murine lymphocytes was determined by the cell culture assay using the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide). Fresh splenocytes and mesenteric lymphocytes were taken from female ddY mice and suspended in a RPMI-1640 medium supplemented with penicillin and streptomycin (100 µg/ml and 100 U/ml). Splenocytes and mesenteric lymphocytes (2.5×10^6 cells/ml or 5×10^6 cells /ml) with or without mitogens such as concanavalin A (Con A), phytohemagglutinin (PHA), lipopolysaccharide (LPS) and lectin fractions, were plated in flat-bottomed microplates incubated at 37°C in a humidified atmosphere containing 5 % CO_2 for 68 hr. MTT (5 mg/ml) was then introduced in each well and the formazan in the cells was extracted with 10 % sodium dodecyl sulfate (SDS). The optical density of each well was measured spectro-photometrically with microplate reader (Bio-Rad, model 450) at 570 nm.

Gel Electrophoresis

Electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [23] using a

15-25 % gradient gel. The protein samples were heated in the presence of 2-mercaptoethanol for 4 min at 98°C. Gels were stained with Coomassie Brilliant Blue. The molecular weight standards (Amersham) used were ovalbumin (46,000), carbonic anhydrase (30,000), trypsin inhibitor (21,500), lysozyme (14,300), aprotinin (6,500) and insulin (b) chain (3,400). The lectin fractions were analyzed for the purity in each step of the purification.

Protein Concentrations

Protein concentration in fractions was determined by a colorimetric assay [24] using bovine serum albumin as a standard.

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