

Study on a pedicellarial venom lectin from the sea  
urchin *Toxopneustes pileolus*, in the coast of  
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## Summary

Sea urchins are common marine invertebrates that belong to the phylum Echinodermata. Urchins have globular or flattened bodies covered by regularly arranged spines and delicate triple-jawed pedicellariae. There are approximately 1,000 species of urchins on the world, 200 species of which are found on the coast of Japan. Six species of Japanese sea urchins serve as important source of food for people, while several species of echinoids cause injury to humans by virtue of their spines, their globiferous pedicellariae, or their poisonous flesh. The toxopneustid sea urchins, *Toxopneustes pileolus*, *Tripneustes gratilla* and *Lytechinus variegatus* have extremely well-developed globiferous pedicellariae with bioactive substances. In my search for biologically bioactive substances, I have been investigating mitogenic activity and/or chemotactic activity from the large globiferous pedicellariae of *T. pileolus*. A novel lectin from the large globiferous pedicellariae of *T. pileolus*, was isolated by a combination of gel chromatography and affinity chromatography techniques. On an SDS-PAGE, the purified lectin was detected with a relative molecular mass of 32 kDa in the presence or absence of 2-mercaptoethanol, suggesting that this lectin is a monomeric protein. The agglutination with rabbit erythrocytes by the 32 kDa lectin was effectively inhibited by D-galactose and, to a lesser extent, by lactose. The 32 kDa lectin appeared to have sequence homology to SUL-I, a D-galactose-specific lectin from *T. pileolus*. This lectin is named SUL-IA. The N-terminal 7 amino acid sequence of SUL-IA was shown to be AVGRSCE.

SUL-IA not only induced mitogenic stimulation on murine splenocytes but also had the ability to exhibit chemotaxis for guinea-pig neutrophils and macrophages. SUL-IA also induced mitogenic stimulation on murine T-lymphocytes. In addition, SUL-IA produced a Th1-related cytokine, IFN- $\gamma$  in a higher dose range, but not a Th2-related cytokine, IL-4 in the presence of murine T-lymphocytes. Thus, SUL-IA appears to be an immunomodulatory lectin. These results suggest that the toxopneustid sea urchin, *T. pileolus*, may be a novel resource for biological active substances such as useful probes for investigating the process involved in cell function. Moreover, I plan to perform more studies of the structural features of SUL-IA, which is one of the multiple lectins from the globiferous pedicellariae of *T. pileolus*.

## List of abbreviations

• BSA	Bovine serum albumin
• Con A	Concanavalin A
• DC	Dendritic cells
• ELISA	Enzyme-linked immunosorbent assay
• FMLP	Formly-methionyl-leucyl-phenylalanine
• HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
• IL-4	Interleukin-4
• IFN- $\gamma$	Interferon- $\gamma$
• MTT	3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
• PBS	Phosphate buffer saline
• PVDF membrane	Polyvinylidene difluoride membrane
• SAL	<i>Silurus asotus</i> egg lectin
• SDS	Sodium dodecyl sulfate
• SDS-PAGE	SDS-polyacrylamide gel electrophoresis
• TMB solution	3,3',5,5'-tetramethylbenzidine solution
• SUL-I	Sea urchin lectin-I
• SUL-IA	Sea urchin lectin-IA
• SUL-II	Sea urchin lectin-II
• Th1	T- helper 1 cell
• Th2	T- helper 2 cell

- RPMI-1640                      Roswell park memorial institute medium-1640
- 2-ME                                2-Mercaptoethanol

## Introduction

Echinoderms comprising the five extant classes (Asteroidea, Crinoidea, Ophiuroidea, Echinoidea and Holothuroidea) are a very diverse group of marine animals that have sparked the interests of scientists for over a century. Significant discoveries in the fields of cell biology, developmental biology and immunology have been made using echinoderms (Ramirez-Gomez and Garcia-Ararras 2010).

Of the five classes of echinoderms, sea urchins (Echinoidea) and sea cucumbers (Holothuroidea) are popular name for marine invertebrates. It is the sea urchins and the sea cucumbers that are both commercially fished and heavily overexploited (Kelly 2005). In the case of sea urchins, intoxication may result from ingestion of their poisonous gonads. However, in most cases, sea urchin envenomations are due to stings from either spines or venomous pedicellariae (Fujiwara 1935; Okada et al.1955). Echinoids have globular or flatted bodies covered by regularly arranged spines and delicate triple-jawed pedicellariae. There are approximately 1,000 species of sea urchin in the oceans, roughly 200 species of which are found along the coast of Japan. Six species of Japanese sea urchin serve as important sources of food for people, while several species of echinoid are capable of inflicting injury on humans (Fujiwara 1935; Kimura et al.1975). The toxopneustid sea urchins, *Toxopneustes pileolus*, *Tripneustes gratilla* and *Lytechinus variegatus* have extremely well-developed globiferous pedicellariae, which contain bioactive substances and cause deleterious effects (Alender et al.

1965; Feigen et al. 1966; Fujiwara 1935; Kimura et al. 1975; Mebs 1984). In the Japanese sea urchin *T. pileolus*, Contractin A, a mannose containing protein with a molecular mass of 18 kDa has been purified from globiferous pedicellariae (Nakagawa et al. 1991).

Lectins are proteins and/or glycoproteins possessing at least one non-catalytic domain that binds reversibly to specific carbohydrates inside and outside cells (Drickamer 1988; Kilpatrick 2002; Sharon and Lis 2004). In recent years, marine animal lectins have been identified in various marine invertebrates including echinoderms (Renwantz and Stahmer 1983; Giga et al. 1987; Dam et al. 1992; Himeshima et al. 1994; Kawagishi et al. 1994; Hatakeyama et al. 1995; Marques and Barracco 2000; Nair et al. 2000). In the sea urchin, the two D-galactose-binding lectins (SUL-I and SUL-II) and heparin-binding lectin (TGL-I) have been isolated from the large globiferous pedicellariae of *T. pileolus* and the globiferous pedicellariae of *T. gratilla*, respectively (Nakagawa et al. 1996, 1997, 1999a).

Therefore, in this study, I describe purification and partially characterization of a novel pedicellarial venom lectin from *T. pileolus* by a combination of gel chromatography and affinity chromatography techniques. I propose that the toxopneustid sea urchin, *Toxopneustes pileolus* may be novel sources for biologically active substances, such as new lectins with interesting mechanism of action.



## Materials and methods

### Isolation of a pedicellarial venom lectin

One hundred seven specimens of *T. pileolus* (8-11 cm in diameter) were collected along the coast of Tokushima Prefecture, Shikoku Island, Japan, in December 2006, and throughout December 2008 to December 2010 (Figure 1). In the case of globiferous pedicellariae, two hundred of the pedicellariae were removed and extracted with 20 ml of distilled water at 4°C for 48 hr, and in the case of large globiferous pedicellariae, eight to one hundred of the

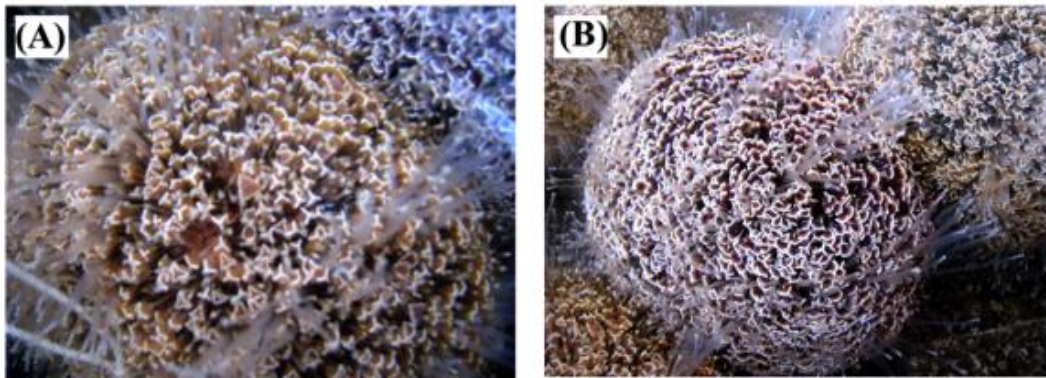
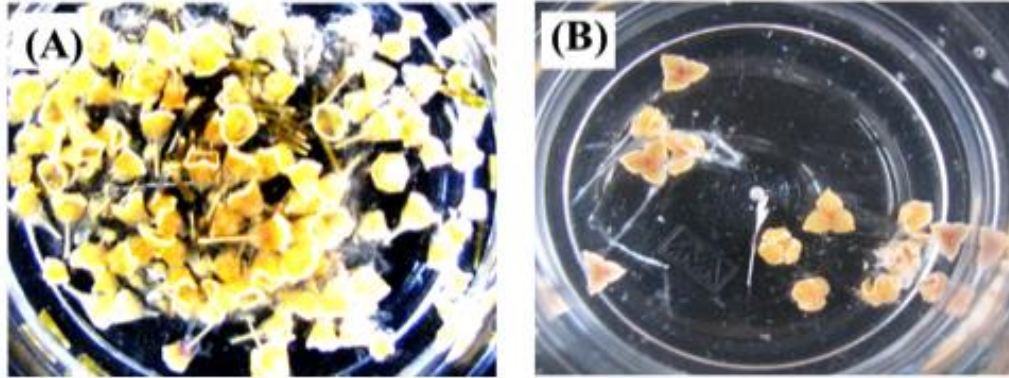


Figure 1 *Toxopneustes pileolus* sp. (A) Yellow type ; (B) Red type.

pedicellariae were removed and extracted (Figure 2). The protein levels of globiferous pedicellariae and large globiferous pedicellariae were determined. For the purification step, the crude venom lectin was fractionated from the large globiferous pedicellariae as reported previously (Nakagawa et al.1996).



**Figure 2** Globiferous pedicellariae and large globiferous pedicellariae from *T. pileolus*. (A) Globiferous pedicellariae of yellow type; (B) Large globiferous pedicellariae of yellow type.

Briefly, for the first step of purification, the crude venom lectin was applied to a Superdex 200 (prep grade, GE Healthcare, Uppsala, Sweden) gel filtration column (1.6 x 50 cm) equilibrated with 0.15 M NaCl solution containing 100 mM D-galactose and was eluted with the same solution at a flow rate of 8 ml/hr. Fractions of 2 ml each were collected and analyzed for absorption at 280 nm and screened for agglutinating activity. The final purification was achieved using a Phenyl Sepharose CL-4B (GE Healthcare, Uppsala, Sweden) affinity chromatographic column. The gel chromatographic fraction (the P-IV fraction) was applied to a Phenyl Sepharose CL-4B column (2 ml) equilibrated with 16 mM Tris-HCl buffer containing 2 M NaCl (pH 7.4). The sample was rinsed and washed with the same buffer and eluted with the same buffer containing 0.01 M NaCl at a

flow rate of 20 ml/hr. The 2 ml elution fractions were collected and analyzed for absorption at 280 nm and screened for agglutinating activity. Each of the first peaks was pooled and analyzed for SDS-PAGE, and then used as the purified pedicellarial venom lectin (SUL-IA). Protein assays were performed employing the method of Bradford (1976) using bovine serum albumin as a standard.

### **Scanning electron microscopy**

Electron microscopic observation was performed as described by Nakagawa et al. (1999b). The globiferous 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 aluminum stubs with silver paint and sputter-coated with gold for 3 min. They were examined under a scanning electron microscope (Hitachi S-3500N, Tokyo, Japan) operated at 10 kV and 25 kV.

### **Determination of biological activity**

The biological activity (U) in the crude venom and purified venom lectin is expressed as the protein content that induced a mitogenic response equivalent to 50% of concanavalin A (1 µg/ml)-induced maximum mitogenic response. The specific activity (U/mg protein) was also calculated.

### **Electrophoresis**

Polyacrylamide gel electrophoresis (PAGE) was run as described by Davis (1964) using a 4%-20% gradient gel. Sodium dodecyl sulfate (SDS)-PAGE was carried out by the method of Laemmli (1970) using a 10%-20% gradient gel. The protein samples were heated in the presence or absence of 2-mercaptoethanol for 4 min at 98°C. The gels were stained with Coomassie brilliant blue.

### **Glycoprotein staining**

The glycoprotein sugar moieties of the sample protein were detected in SDS-polyacrylamide gel using GelCode Glycoprotein kit (Pierce Biotech., Inc., IL, USA). The kit detects sugars that occur in glycoproteins, including galactose, mannose, glucose, N-acetylglucose, N-acetylgalactosamine, sialic acid, fucose and xylose.

### **Assay of agglutinating activity**

Agglutinating activity was assayed using rabbit erythrocytes in microtiter plates. A total of 30  $\mu$ l of a 2% (v/v) suspension of erythrocytes in 6.4 mM phosphate-buffered saline (PBS) was added to 50  $\mu$ l of serial 2-fold dilutions of the sample. The plates were incubated at room temperature for 1 h. The results are expressed as the minimum concentration of the sample ( $\mu$ g/ml) required for positive agglutination. Agglutination inhibition was expressed as the minimum concentration of each sugar required for inhibition of agglutinating activity by the purified lectin.

### **Mitogenic activity**

Mitogenic activity on the murine splenocytes and murine T-lymphocytes was determined by cell culture assay using dye, a tetrazolium salt, 3(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Nakagawa et al. 1997). The splenocytes were taken from female ddY mice (30 g) and suspended in RPMI-1640 medium supplemented with penicillin and streptomycin (100 µg/ml and 100 U/ml). T-lymphocytes were separated from murine splenocytes by column filtration. Splenocytes ( $5 \times 10^6$  cells/ml) and T-lymphocytes ( $5 \times 10^6$  cells/ml), with or without concanavalin A (1 µg/ml) as the positive control, the venom lectin samples, and with or without D-galactose (50 mM) were plated in flat-bottomed microplates and incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 68 hr. A total of 10 µl of MTT tetrazolium salt solution (5 mg/ml) was then introduced into each well, and formazan in the cells was extracted with 10% SDS after 4 hr. The optical density of each well was measured spectrophotometrically with a microplate reader (Bio-Rad Lab., Model 680, Tokyo, Japan) at 570 nm.

### **Assay of chemotaxis**

Polymorphonuclear leukocytes (neutrophils) were prepared from peritoneal exudates collected from male guinea-pigs (350-400 g) that intraperitoneally received a 1% glycogen solution and underwent laparotomy at 24 hours, and then 50 ml of PBS was injected intraperitoneally into guinea-pigs that were sacrificed under anesthesia. Subsequently, after intraperitoneal washing, washings containing exudate cells were collected with a pipette. The

washings were centrifuged three times at 420 g for 10 min at 4°C to collect the washed cells. The washed cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) and adjusted to a neutrophil density of  $2 \times 10^6$  cells/ml. Chemotaxis was measured employing a membrane filter method using a 48-well chemotaxis chamber (Neuroprove, Gaithersburg, MD, USA) (Ohura et al. 1990). Briefly, 25  $\mu$ l aliquots from the negative control, the positive control ( $10^{-7}$  M FMLP), and each concentration of venom lectin samples were transferred to sets of four replicate rows in the bottom compartment of the chemotaxis chamber. The compartment was covered with a polycarbonate membrane filter (3 $\mu$ m pore size). The micro-chemotaxis chamber was assembled further to create an upper chamber of 48 micro-wells. A 25  $\mu$ l aliquot of  $2 \times 10^6$  cells/ml cell suspension was added to each of the micro-wells in the upper compartment. The chemotaxis chamber was then incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 60 min. After the migration period, the membrane was removed. The upper side of the membrane was rinsed and wiped two times; then, the polycarbonate membrane was fixed and stained with Diff-Quick (Sysmex Int. Reagents Co., Ltd., Kobe, Japan). The membranes were mounted on slides and cells were counted using a fluorescent microscope (BIOREVO BZ-9000; Keyence, Osaka, Japan). Macrophages were also collected in the same manner as for neutrophils. A 1% glycogen solution was administered intraperitoneally into guinea pigs (350-400 g). At 96 hours, 50 ml of PBS was injected intraperitoneally into guinea-pigs that were sacrificed under anesthesia. Subsequently, peritoneal exudates were collected. The washings

were centrifuged three times in the same manner as for neutrophils to collect the washed cells. The washed cells were resuspended in DMEM and adjusted to a macrophage density of  $1 \times 10^6$  cells/ml. For this purpose, a 5  $\mu$ m polycarbonate membrane filter was employed. The chemotactic response of macrophages was measured in the same manner as described above for neutrophils.

#### **IL-4 and IFN- $\gamma$ production**

Murine lymphocytes were separated from splenocytes (female ddy mice) using The Collect Mouse T-Cell Kit (Biotex Labs Inc., Alberta, Canada). Murine T-lymphocytes ( $1 \times 10^7$  cells/ml) were stimulated with the purified venom lectin or plate-bound anti-CD3 monoclonal (m)Ab (coated overnight at 1  $\mu$ g/ml) in a 48-well flat-bottomed plate at 37°C under 5% CO<sub>2</sub> for 72 hr (Nakamoto et al. 2011). After the culture, culture supernatant was collected and stored at -40°C until use. Interferon (IFN)- $\gamma$  and interleukin (IL)-4 in the supernatants were determined using a mouse IFN- $\gamma$  or IL-4 ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

All experimental procedures were approved by the Animal Research Committee of the University of Tokushima.

#### **N-terminal amino acid sequencing**

Approximately 3  $\mu$ g of the sample protein was subjected to SDS-PAGE, followed by electroblotting onto a polyvinylidene difluoride membrane. The

membrane was then stained with Ponceau S and destained. A protein band was excised and subjected to automated Edman degradation using the Shimadzu Model PPSQ-10 protein sequencer (Nagasaka et al. 2009).

### **Statistical analysis**

Data are expressed as mean  $\pm$  standard deviation (SD). The statistical analyses were performed using SPSS version 16.0 software package (SPSS, Chicago, Inc., IL., USA). The statistical analysis of the results was performed by Dunnett's multiple comparison test when various experimental groups were compared to the control groups, and Student's *t* test was used for paired or unpaired groups.  $P < 0.05$  was considered statistically significant.



## Results

### Collection of sea urchin specimen

By the support for local fishermen, I collected 107 specimens of *T. pileolus* along the coast of Tokushima Prefecture, Shikoku Island, Japan, in December 2006, and from December 2008 through December 2010 (Figure 1). Table 1 shows the number of individuals of male and female *T. pileolus* by the color (yellow and red) of pedicellariae. Differences in terms of pedicellarial color and sex were statistically insignificant among the collected individuals of *T. pileolus*.

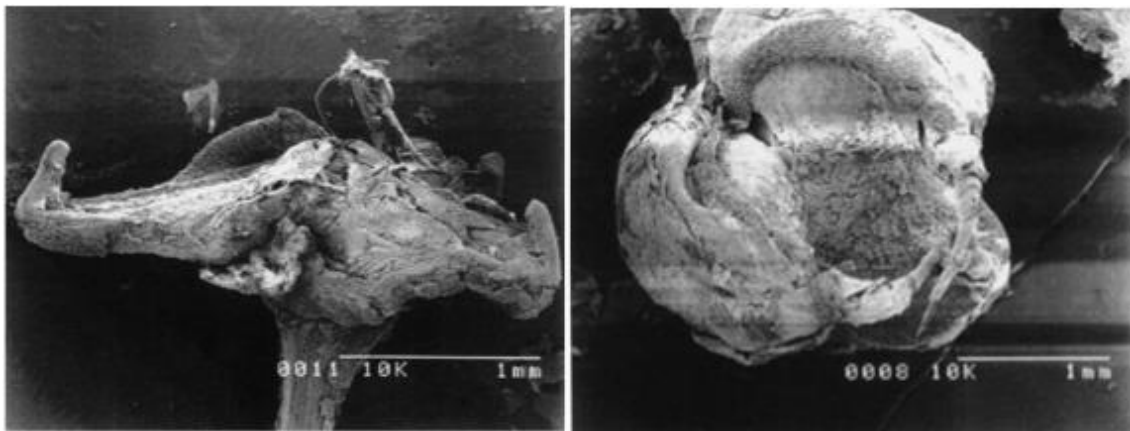
**Table 1 Comparison of two color types of *T. pileolus* specimen**

Year	Yellow type		Red type		
	male	female	male	female	
2006	6	8	10	6	
2008	6	7	6	5	
2009	9	5	4	8	
2010	7	8	5	7	
Total	28	28	25	26	(107)
	$7.0 \pm 1.4^a$	$7.0 \pm 1.4^a$	$6.3 \pm 2.6^a$	$6.5 \pm 1.3^a$	

One hundred seven specimens were collected along the coast of Tokushima Prefecture, Shikoku Island, Japan, in December 2006, and throughout December 2008 to December 2010. <sup>a</sup>The data show the mean  $\pm$  SD.

### Scanning electron microscopy

Electron microscopic observation showed distinct features based on size and shape between the globiferous pedicellaria and large globiferous pedicellaria of *T. pileolus* (Figure 3). The head of large globiferous pedicellaria was much globular in shape. The both pedicellariae had a sharp incurved spine referred to as a fang. The present observation suggests that there may be functional differences in the globiferous pedicellaria and large globiferous pedicellaria.



**Figure 3 Scanning electron micrographs showing the pedicellariae.** (A) a globiferous pedicellaria, ×40; (B) a large globiferous pedicellaria; ×30.

## Venom protein

The venom proteins were extracted from the globiferous pedicellariae and large globiferous pedicellariae of *T. pileolus* as shown in Figure 2. Table 2 shows a comparison of protein contents of globiferous pedicellariae and large globiferous pedicellariae. The protein contents of large globiferous pedicellariae was 11 times greater than that of globiferous pedicellariae. The venom protein from large globiferous pedicellariae induced agglutination of rabbit erythrocytes at a concentrations of 2.5 µg/ml in a Ca<sup>2+</sup>-independent manner (data not shown). *T. pileolus* venom causes an intense injury to humans via their large globiferous pedicellariae (Fujiwara 1935).

**Table 2 Comparison of protein content of globiferous pedicellariae and large globiferous pedicellariae from *T. pileolus***

Specimens of sea urchin	Total protein of globiferous pedicellariae	Protein content (mg/pedicellaria)	Total protein of large globiferous pedicellariae	Protein content (mg/pedicellaria)
No.1	2.8 / 200	0.014	8.4 / 40	0.210
No.2	2.0 / 200	0.010	4.2 / 16	0.263
No.3	2.8 / 200	0.014	3.0 / 15	0.200
No.4	3.5 / 200	0.018	22.4 / 95	0.236
No.5	5.6 / 200	0.028	8.4 / 43	0.195
No.6	5.0 / 200	0.025	13.8 / 53	0.260
No.7	4.9 / 200	0.025	6.0 / 34	0.176
No.8	4.0 / 200	0.020	15.2 / 87	0.175
No.9	3.2 / 200	0.016	3.5 / 21	0.167
No.10	2.4 / 200	0.012	3.0 / 17	0.176
	3.6 ± 1.0 <sup>a</sup>	0.018 ± 0.005 <sup>a</sup>	8.8 ± 5.0 <sup>a</sup>	0.206 ± 0.029 <sup>a</sup>

*T. pileolus* specimen was collected along the coast of Tokushima Prefecture in December 2008. <sup>a</sup>The data show the mean ± SD.

Figure 4 shows SDS-PAGE and glycoprotein sugar moieties of the crude venoms from the globiferous pedicellariae (N-type) and large globiferous pedicellariae (G-type). The crude venom from the globiferous pedicellariae showed glycoprotein sugar moieties.

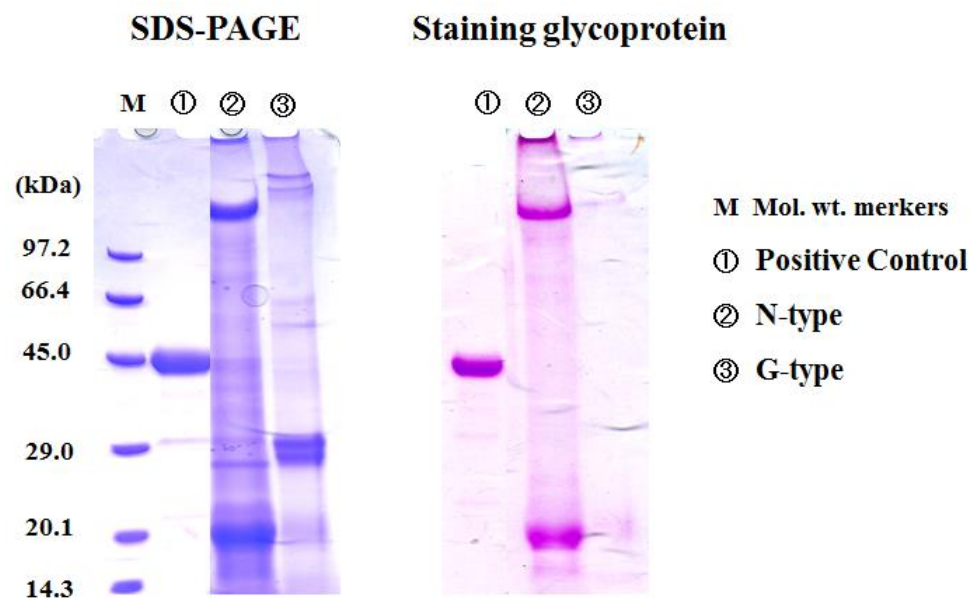


Figure 4 SDS-PAGE and staining glycoproteins in SDS-PAGE of the crude extract from the globiferous pedicellariae (N-type) and the large globiferous pedicellariae (G-type) of *T. pileolus*.

## Purification of a pedicellarial venom lectin

In this study, I attempted to isolate a novel lectin from the large globiferous pedicellariae of *T. pileolus*. The purification of a pedicellarial venom lectin from *T. pileolus* by gel chromatography and affinity chromatography is shown in Figure 5 and Figure 6. As shown in Figure 5, there is an elution pattern with four protein peaks. The P-IV fraction from a Superdex 200 column (1.6 x 50 cm) had relatively strong activity for agglutination of rabbit erythrocytes (minimum concentration: 0.625 µg/ml). From the analysis on

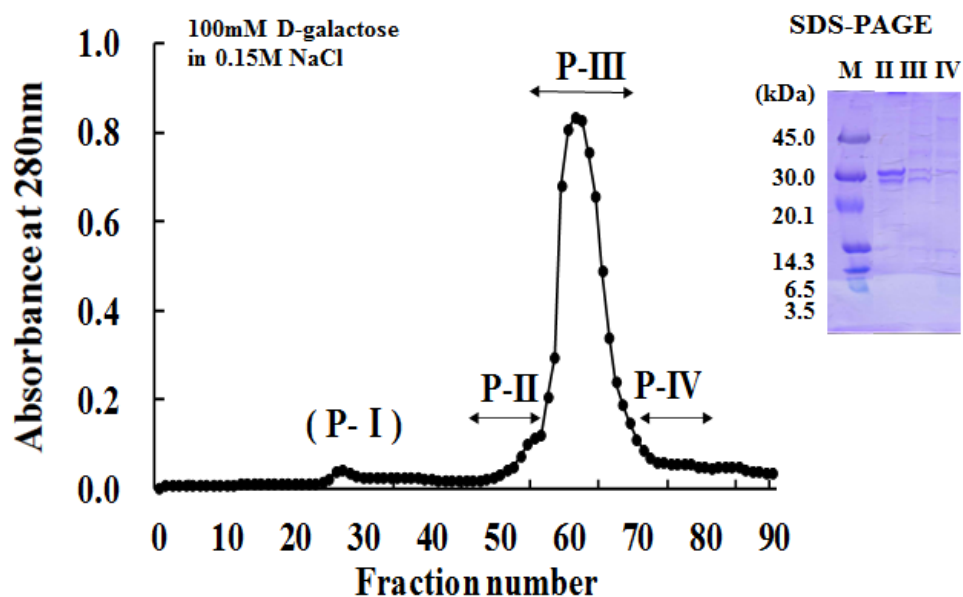
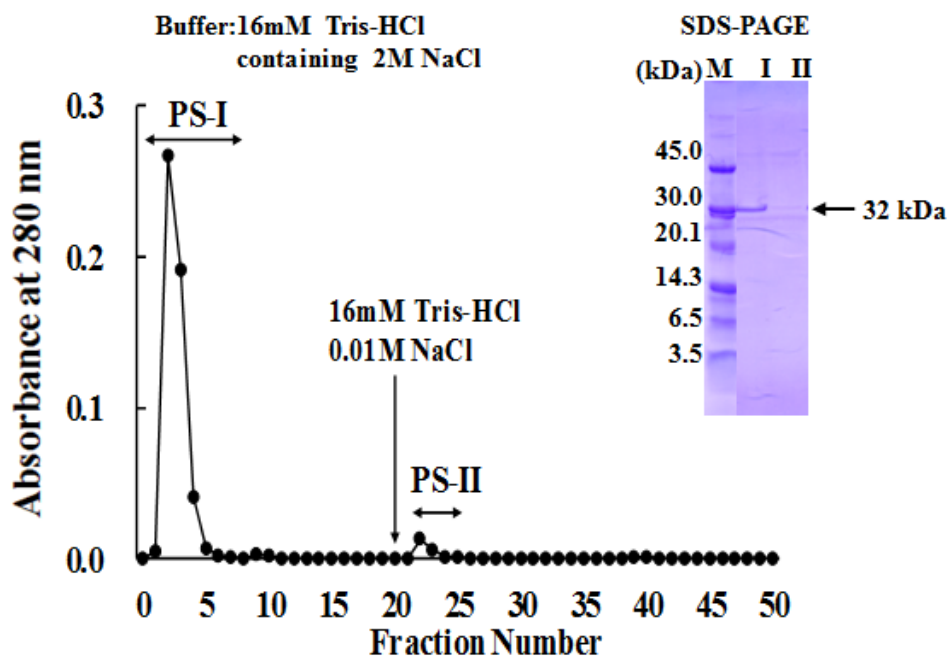


Figure 5 Elution patterns of Superdex 200 column chromatography of the crude extract from large globiferous pedicellariae in the presence of 100 mM D-galactose. Inset panel shows SDS-PAGE of the gel chromatography fractions. (M) Mol. wt. markers.

SDS-PAGE, the P-IV fraction appeared to have some proteins with molecular masses of 28 kDa to 32 kDa. As shown in Figure 6, for purification, the P-IV fraction was applied on a Sepharose CL-4B column (2 ml) equilibrated with 16 mM Tris-HCl buffer containing 2 M NaCl (pH7.4). The unbound fraction (the PS-I fraction) eluted with the same buffer showed a single band corresponding to a molecular mass of 32 kDa by SDS-PAGE (Figure 6). The purified lectin had intense agglutinating activity for rabbit erythrocytes, as well as the P-IV fraction.



**Figure 6** Affinity chromatography on a Phenyl Sepharose CL-4B column of P-IV gel chromatographic fraction. Inset panels show SDS-PAGE of the PS fractions. (M) Mol. wt. markers.

Figure 7A shows a single band corresponding to a molecular mass of 32 kDa by SDS-PAGE under reducing conditions and nonreducing conditions, suggesting that the purified lectin is a monomer. Furthermore, the 32 kDa lectin did not show sugar staining by SDS-PAGE using GelCode Glycoprotein kit, indicating that the purified lectin is devoid of carbohydrate (data not shown).

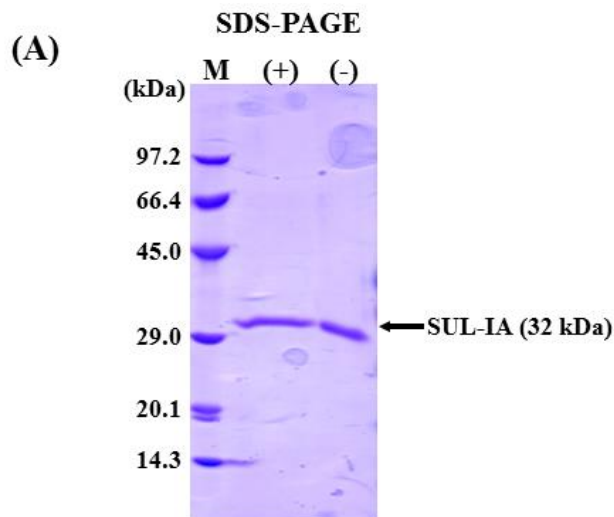


Figure 7 (A) SDS-PAGE of SUL-IA under reducing condition and nonreducing condition. (B) Partial amino acid sequences of SUL-I and SUL-IA. M = mol. wt. markers.

As shown in Figure 7B, the N-terminal amino acid of the purified lectin is alanine. The partial amino acid sequence was determined up to 7 residues. SUL-I, a D-galactose-specific lectin with a molecular mass of 32 kDa (Nakagawa et al., 1999b) from the large globiferous pedicellariae, showed sequence homology to the purified lectin. Therefore, the purified lectin was designed SUL-IA.

As shown in Table 3, the agglutination with rabbit erythrocytes by SUL-IA was most effectively inhibited by D-galactose, but not by D-glucose, methyl- $\alpha$ -D-mannose and D-fucose. Thus, SUL-IA appears to be a D-galactose-binding lectin. The recovery of SUL-IA in terms of protein content was 5.7% of the pedicellarial venom, and the specific activity (U/mg protein) increased fourfold compared with that of the venom in terms of mitogenic response on murine splenocytes.

**Table 3 Inhibition of agglutinating activity of SUL-IA by saccharides**

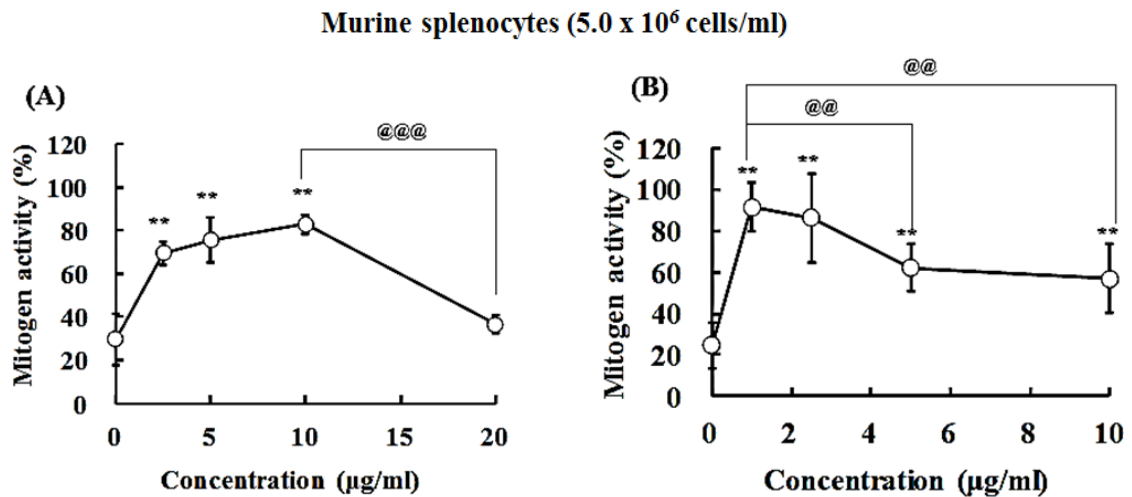
Saccharide	Minimum inhibitory concentration (mM)
D-galactose	25
N-acetyl-D-galactosamine	100
D-glucose	—
D-fucose	—
Methyl- $\alpha$ -D-mannoside	—
Lactose	50

Two percent of rabbit erythrocyte suspension was used with 2.5 $\mu$ g/ml of SUL-IA.



## Mitogenic activity of SUL-IA

Figure 8 shows the results of mitogenic response on murine splenocytes in the presence of the crude venom and SUL-IA. As shown in Figure 8A, the crude venom induced mitogenic activity on murine splenocytes in a dose-dependent manner in the dose range of 2.5 to 10  $\mu\text{g/ml}$ . A higher dose (20  $\mu\text{g/ml}$ ) of the crude venom showed a significant decrease in mitogenic

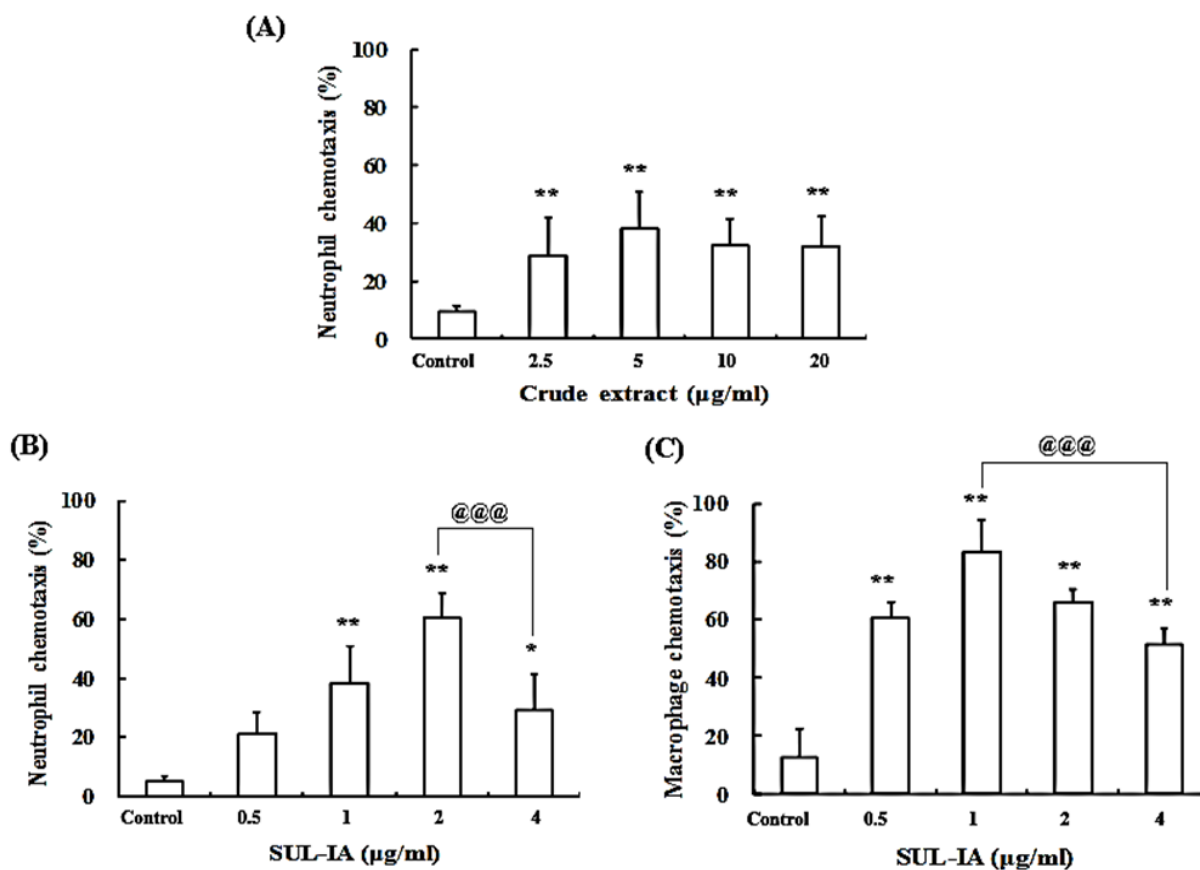


**Figure 8 Mitogenic effects of the crude venom (A) and SUL-IA (B) from *T. pileolus* on murine splenocytes.** The splenocytes ( $5 \times 10^6$  cells/ml) were incubated with the crude venom or SUL-IA for 68h and then the incubation was continued with MTT for 4h in a  $\text{CO}_2$  humidified atmosphere. The mitogenic response with concanavalin A ( $1\mu\text{g/ml}$ ) is expressed as 100 %. The data show the mean  $\pm$  SD of three experiments with triplicate determinations. \*\* $p < 0.01$ , compared with the negative control. @ $p < 0.01$ , @@@ $p < 0.001$ , statistically different between two groups according to Student's unpaired  $t$ -test.

activity, suggesting that it may contain pharmacologically active components such as catecholamines, peptides and proteins (Kimura et al., 1975; Church and Hodgson, 2002). SUL-IA had maximum mitogenic activity on murine splenocytes at a lower dose of 1.25 µg/ml (Figure 8B). However, in the dose range of 5 to 10 µg/ml, SUL-IA also showed a significant decrease in mitogenic activity.

### **Chemotactic activity of SUL-IA**

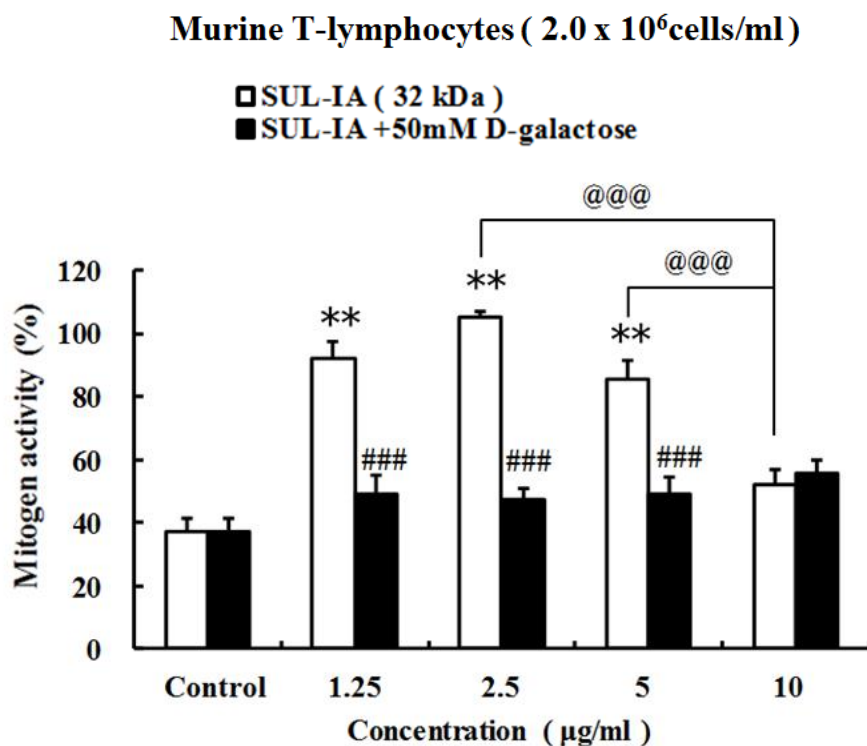
The crude venom induced chemotactic activity on guinea-pig neutrophils (Figure 9A). Therefore, the chemotactic activity of SUL-IA was also examined on guinea-pig neutrophils and macrophages. As shown in Figure 9A, the crude venom induced chemotactic activity on guinea-pig neutrophils at a lower dose of 2.5 µg/ml, and it had maximum activity at a dose of 5 µg/ml (38% compared with the positive control). Increases in the crude venom over the dose range of 10 to 20 µg/ml caused an increase in chemotactic activity on guinea-pig neutrophils. SUL-IA also exhibited effective chemotactic activity on guinea-pig macrophages, as well as on neutrophils, in the dose range of 0.5 to 2 µg/ml (Figure 9B and 9C). However, at a higher dose of 4 µg/ml, SUL-IA showed a significant decrease in chemotactic activity on guinea-pig neutrophils and macrophages (Figure 9B and 9C). The dual response to SUL-IA suggests that it may have wide-ranging effects on guinea-pig leukocytes as well as on murine splenocytes. In addition, guinea-pig macrophages were more sensitive to SUL-IA than neutrophils (Figure 9B and 9C).



**Figure 9** Effects of the crude venom (A) and SUL-IA (B) on guinea-pig neutrophil, and SUL-IA(C) on guinea-pig macrophage chemotaxis. Neutrophils ( $2 \times 10^6$  cells/ml) were incubated at  $37^\circ\text{C}$  for 60 min, and macrophages ( $1 \times 10^6$  cells/ml) were incubated at  $37^\circ\text{C}$  for 90 min, with or without SUL-IA. The chemotactic response with FMLP ( $10^{-7}$  M) is expressed as 100%. Data show the mean  $\pm$  SD of 3–5 experiments with triplicate determinations. \* $p < 0.05$ , \*\* $p < 0.01$ , compared with the control. @@@ $p < 0.001$ , statistically different between two groups according to Student's unpaired  $t$ -test.

## Immunoreactivity of SUL-IA

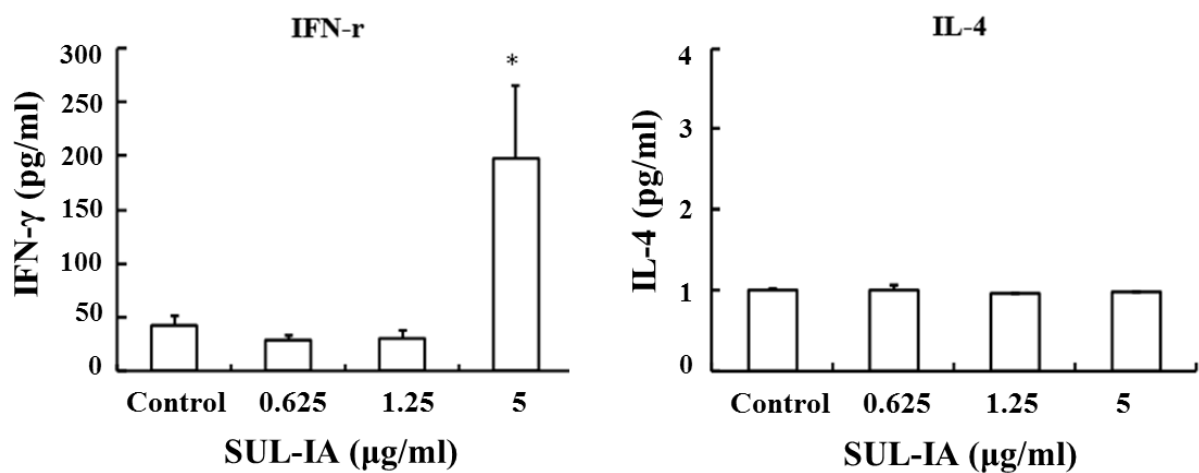
In this study, the D-galactose-specific lectin activity of SUL-IA was further examined in terms of its effect on murine T-lymphocytes. Murine T-lymphocytes were incubated with SUL-IA in the absence or presence of 50



**Figure 10 Effect of D-galactose on mitogen response to SUL-IA in murine T-lymphocytes.** T-lymphocytes were incubated with concanavalin A or SUL-IA for 68 h and the incubation was continued with MTT for 4h in a CO<sub>2</sub> humidified atmosphere. The data show the mean  $\pm$  SD of 3–5 experiments with triplicate determinations. \*\* $p < 0.01$ , compared with the control. ### $p < 0.001$ , statistically different between two groups according to Student's paired  $t$ -test. @@ $p < 0.01$ , @@@ $p < 0.001$ , statistically different between two groups according to Student's unpaired  $t$ -test.

mM D-galactose. As shown in Figure 10, SUL-IA prompted mitogenic response on murine T-lymphocytes in the dose range of 1.25 to 5  $\mu\text{g/ml}$ . However, a higher dose (10  $\mu\text{g/ml}$ ) of SUL-IA did not exhibit mitogenic activity on murine T-lymphocytes. The mitogen response to SUL-IA in the dose range of 1.25 to 5  $\mu\text{g/ml}$  was completely inhibited by 50 mM D-galactose. On the other hand, the inhibitory effect of SUL-IA at a higher dose (10  $\mu\text{g/ml}$ ) was not affected by 50 mM D-galactose (Figure 10). From these observations, it seems that even binding of SUL-IA to D-galactose residues on murine T-lymphocytes causes damage to cell functions, leading to biological consequences such as induction of apoptosis (Nakagawa et al. 2003; SundarRaj et al. 2009). Thus, SUL-IA may be a broad spectrum biologic modifier.

I have observed that SUL-IA has mitogenic stimulatory effects on murine T-lymphocytes. Therefore, the effect of SUL-IA on cytokine production was examined in murine T-lymphocytes. I measured the production of IFN- $\gamma$  and IL-4, which are indexes of the T helper (Th) 1/Th 2 balance. SUL-IA produced IFN- $\gamma$  in a higher dose range, but not IL-4 (Figure 11). These results suggest that SUL-IA promotes the development of IFN- $\gamma$ -producing T-lymphocytes compared with IL-4-producing T-lymphocytes. Helper T (Th) cells play an important role in cell-mediated immunity.



**Figure 11 Effect of SUL-IA stimulation on cytokine production from murine T-lymphocytes.** T-lymphocytes ( $1 \times 10^7$  cells/ml) were incubated at  $37^\circ\text{C}$  and stimulated with SUL-IA. After 72h incubation, culture supernatants were collected and used for determination of IFN- $\gamma$  and IL-4. Data show the mean  $\pm$  SD of three experiments with triplicate determinations. \* $p < 0.05$ , compared with the control.

## Comparison of N-terminal amino acid sequences

Table 4 shows a comparison of partial amino acid sequences of sea urchin lectins and catfish lectin. SUL-IA is related to SUL-I (Nakagawa et al. 1999b), but it did not show sequence homology to SUL-II (Sato et al., 2002), Contractin A (Nakagawa et al., 1991) and UT841 (Zhang et al., 2001). These results suggest the presence of multiple lectins from the globiferous pedicellariae and large globiferous pedicellariae of *T. pileolus*. Therefore, further structural studies on SUL-I, SUL-IA, SUL-II, Contactin A and UT841 are needed to elucidate the physiological functions of pedicellarial venom of *T. pileolus*.

**Table 4 Comparison of partial amino acid sequences of sea urchin lectins and catfish lectin**

Lectins	Organisms	Partial amino acid sequence							
		5	10	15	20	25	30	35	
SUL-I (32 kDa)	Sea urchin ( <i>T. pileolus</i> )	<u>AVGRT</u>	<u>XEGKS</u>	<u>LDLEX</u>	<u>PEGYI</u>	<u>ISVNY</u>	<u>ANYGR</u>	<u>NSPGY</u>	
SUL-IA (32 kDa)	Sea urchin ( <i>T. pileolus</i> )	<u>AVGRS</u>	<u>CE</u>						
SUL-II (23 kDa)	Sea urchin ( <i>T. pileolus</i> )	<u>SVINF</u>	<u>GWMS</u>	<u>SXVTXS</u>	<u>TSTRY</u>	<u>Y</u>			
Contractin A (18 kDa)	Sea urchin ( <i>T. pileolus</i> )	<u>SVINF</u>	<u>GWMS</u>	<u>SXVTXS</u>	<u>TSTRY</u>	<u>NGYGX</u>	<u>YXGFG</u>	<u>GSXTP</u>	<u>VD</u>
SAL (31.7 kDa)	Catfish ( <i>S. astous</i> )	(24) <u>ANMIT</u>	<u>CYGDV</u>	<u>QKLHX</u>	<u>CETGL</u>	<u>IIVKS</u>	<u>SLYGR</u>		

The amino acids marked by underline are identical residues compared with those of SUL-I.

## Discussion

Sea urchins are found on the seabed in all oceans. Although most sea urchins can be handled safely, some species are venomous. The venomous species, toxopneustid and diadematid sea urchins, are widely distributed throughout the Indo-Pacific area from East Africa to Japan. Species of needle-spined sea urchin, *Diadema setosum*, and flower sea urchin, *T. pileolus*, cause injury to humans via their spines or their flower-like pedicellariae. External injury and envenomation from the spines and/or pedicellariae are common and important problems. The pain is radiating and severe. For emergency treatment, physicians recommend infiltrations of the wound area with 0.5%-2% lidocaine and steroid ointment. In the event of shock, treatment includes the infusion of steroids (100-200 mg) (Auerbach 1991; Nagasaka et al. 2009). The toxopneustid sea urchin *T. pileolus* has well-developed globiferous pedicellariae with bioactive substances. Some of the bioactive substances produce deleterious and pharmacological effects (Fujiwara 1935; Okada et al. 1955; Kimura et al. 1975; Nakagawa et al. 1991; Kuwabara 1994).

In my research on novel sea urchin lectins as bioactive compounds, I have been investigating the mitogenic activity and chemotactic activity of the pedicellarial venom of *T. pileolus*. The level of venom protein from the large globiferous pedicellariae was 11 times greater than that of the globiferous pedicellariae (Table 2). In addition, the crude extract from the globiferous pedicellariae was determined to have sugar moieties (Figure 4).



Thus, these findings suggest that there is a difference in venom composition between the globiferous pedicellariae and large globiferous pedicellariae of *T. pileolus*. Furthermore, Some toxins and lectin such as Contractin A (Nakagawa et al. 1991), UT841 (Zhang et al. 2001) and SUL-I (Nakagawa et al. 1996) have been also purified from the globiferous and large globiferous pedicellariae of *T. pileolus*. It is likely that there are multiple bioactive substances from the globiferous pedicellariae and large globiferous pedicellariae of *T. pileolus*. In this study, I purified a novel lectin from the large globiferous pedicellariae of *T. pileolus*. The purification of a pedicellarial lectin from *T. pileolus* by gel chromatography and affinity chromatography is shown in Figure 5 and Figure 6. This lectin is a monomeric protein with a molecular mass of 32 kDa (Figure 7), which showed sequence homology to SUL-I, a D-galactose-binding lectin (Nakagawa et al. 1999b). The agglutination with rabbit erythrocytes by SUL-IA was effectively inhibited by D-galactose (Table 3). Therefore, the purified lectin is named SUL-IA (Figure 7B).

Sea urchins have a well-developed water duct system. While urchins have a poorly-developed vascular system, they contain coelomic fluid in body cavities. The coelomic fluid are considered to have lectins and cytokines that take part in physiologic functions (Malagoli et al. 2010). It has been reported that echinoidin, a multimeric protein from the coelomic fluid of the sea urchin *Anthrocidaris crassispina* is a Ca<sup>2+</sup>-dependent lectin (Giga et al. 1985, 1987). More recently, Nakagawa et al. (2012) reported that a lectin with a molecular mass of 300 kDa was purified from the coelomic fluid of *T.*

*pileolus*, and had agglutinating and mitogenic activity. The lectin in the coelomic fluid of *T. pileolus* appears to be related to defense mechanism of invertebrates, like vertebrate immune recognition. Thus, *T. pileolus* specimen may be a source of useful bioactive substances.

In the present study, SUL-IA induced chemotactic activity on guinea-pig neutrophils and macrophages. Guinea-pig macrophages were more sensitive to SUL-IA than neutrophils (Figure 9). From this, it appears likely that SUL-IA recognized different sets of D-galactose residues in guinea-pig macrophages in comparison with neutrophils. It has been reported that SUL-I had chemotactic and phagocytic activities for human polymorphonuclear leukocytes (Nakagawa et al. 2003). Chemotaxis and phagocytosis by leukocytes play an important role in the defense reactions against infection and injury in vertebrates. These results suggest that SUL-I and SUL-IA may be valuable tools for analyses of the inflammation and immune recognition of cells. It has been also reported that SUL-I showed not only mitogenic stimulation on murine splenocytes, but also cytotoxic effect on murine P388 cells (Sato et al. 2002). Similarly, SUL-IA had mitogenic activity on murine splenocytes and T-lymphocytes. At a lower dose SUL-IA prompted mitogenic response on murine splenocytes and T-lymphocytes. However, at higher doses SUL-IA showed a significant decrease in mitogenic activity on murine splenocytes and T-lymphocytes (Figure 8B and Figure 10). These results suggest that the venom lectin may induce cytotoxicity and apoptosis through binding D-galactose-containing carbohydrates that are present on the surface of murine splenocytes and T-lymphocytes (Nakagawa

et al. 2003; SundarRaj et al. 2009).

In addition, SUL-IA promoted the development of IFN- $\gamma$ -producing T-lymphocytes compared with IL-4-producing T-lymphocytes. Helper T (Th) cells play an important role in cell-mediated immunity. SUL-IA had potent chemotactic activity on guinea-pig leukocytes. Therefore, SUL-IA might affect inflammatory and immunomodulatory processes. Similarly, SUL-I, a D-galactose-specific lectin from *T. pileolus*, has the ability to induce differentiation of murine myeloid leukemic cells (Nakagawa et al., 2003). Recently, it has also been shown that SUL-I induces dendritic cell (DC) maturation from human monocytes (Takei and Nakagawa, 2006). Thus, it seems likely that SUL-IA is an immunomodulatory lectin. Although the effect of SUL-IA in vivo is not known yet, I am anticipating clinical applications, as well as for SUL-I (Fusetani and Kem 2009). These results suggest that SUL-I and SUL-IA may be valuable tools for analyses of the inflammation, differentiation and development of cells.

As shown in Table 4, SUL-IA is related to SUL-I, but it did not show sequence to SUL-II (Sato et al. 2002), Contractin A and UT841. On the other hand, SUL-I showed sequence homology to SAL, a rhamnose-binding lectin from catfish eggs (Hosono et al. 1999). Therefore, more detailed structural studies on SUL-I, SUL-IA, SUL-II, Contractin A and UT841 should provide insight into the biological functions of multiple lectins from the pedicellariae of *T. pileolus*.

## Conclusions

A novel lectin, SUL-IA, was purified from the large globiferous pedicellariae of the toxopneustied sea urchin, *Toxopneustes pileolus*, by gel permeation chromatography and affinity chromatography. SDS-PAGE showed that SUL-IA is a monomeric protein with a molecular mass of 32 kDa. The N-terminal partial amino acid sequence was determined up to 7 residues. The agglutinating activity of SUL-IA was effectively inhibited by D-galactose. SUL-IA was shown to have mitogenic activity on murine splenocytes and chemotactic activity. SUL-IA also induced mitogenic stimulation on murine T-lymphocytes. Furthermore, SUL-IA produced a Th1-related cytokine, IFN- $\gamma$ , in the presence of murine T-lymphocytes. Thus, SUL-IA appears to be an immunomodulatory lectin. These results suggest that *T. pileolus* venom lectin is a source of biologically active substances, which may have application as research and biomedical tools.

## Declaration of interest

The author declare that there are no conflicts of interest.

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## Publications

### Main publication

1. Kozue Edo, Hitomi Sakai, Hideyuki Nakagawa, Tamiko Hashimoto, Mitsuko Shinohara, Kiyoshi Ohura (2012) Immunomodulatory activity of a pedicellarial venom lectin from the toxopneustid sea urchin, *Toxopneustes pileolus*. *Toxin Rev* 31:54-60

### Sub publications

1. Hitomi Sakai, Kozue Edo, Hideyuki Nakagawa, Mitsuko Shinohara, Rie Nishiitsutsuji, Kiyoshi Ohura (2013) Isolation and partial characterization of a L-rhamnose-binding lectin from the globiferous pedicellariae of the toxopneustid sea urchin, *Toxopneustes pileolus*. *Int Aquatic Res* 5:12. At online: <http://www.intaquares.com/content/5/1/12>
2. Mitsuko Shinohara, Kuniko Nagasaka, Hideyuki Nakagawa, Kozue Edo, Hitomi Sakai, Koji Kato, Futoshi Iwaki, Kiyoshi Ohura, Haruhiko Sakuraba (2010) A novel chemoattractant lectin, Karatoxin, from the dorsal spines of the small scorpionfish *Hypodytes rubripinnis*. *J Pharmacol Sci* 113:414-417