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Purification of a novel lectin from the dorsal spines of the stonefish, *Synanceia verrucosa*

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A novel lectin was purified from the dorsal spines of the stonefish, *Synanceia verrucosa,* using a combination of affinity chromatography techniques. A single band was detected on a native PAGE gel with a relative molecular mass of 45 kDa. The agglutination of rabbit erythrocytes by the 45 kDa lectin was inhibited most effectively by methyl *α*-D-mannoside. The 45 kDa lectin stimulated mitogenesis in murine splenocytes. This is the first study to examine the dorsal lectin of *S. verrucosa* and one of very few studies on venom lectin from stonefish. These results suggest that the reef stonefish, *S. verrucosa* may be a novel resource for biologically active substances. (J Osaka Dent Univ 2016 ; 50 : 55-61)

Key words : Stonefish ; *Synanceia verrucosa ;* Dorsal spine ; Piscine venom ; Lectin ; Mitogenic activity

INTRODUCTION

Fish that have sharp stinging spines with venom glands in the dorsal and pectoral fins are called venomous fish. About 200 species of venomous fish are known in the world,[1, 2] for example, rays, demon stingers, and scorpion fish, which inhabit Japanese waters. Venomous fish are generally slow-moving demersal fish with stinging spines as a defense organ.[3] Their venom is mainly composed of proteins, containing polypeptides or low-molecular-weight compounds. Symptoms caused by the venom include severe pain and swelling, the systemic symptom of fever, as well as convulsions or dyspnea in severe cases, and occasionally death.[4]

Stonefish (*Synanceia verrucosa*) belong to the family *Synanceiidae,* and are about 30 cm in body length. They are carnivorous fish that inhabit the coral reefs and sandy rocks of the tropics in the Indian and West Atlantic Oceans. In Japanese waters, they are found around Okinawa Island where they are consumed by the inhabitants. They are difficult to find because they lurk in reefs, seaweed or sand, and their bodies resemble their environment. The reef stonefish *S. verrucosa* has 13 dorsal and 3 anal spines, which contain venom glands that are covered by an integumentary sheath.

They have spines, with the longest one being about one-sixth of their body length. Among venomous fish with spines, *Synanceia verrucosa* has the strongest venom. Envenomation depends on the amount of injected venom. Severe pain and swelling, in addition to vasopermeable cardiotoxicity are caused by stabbing. Systemic symptoms, such as fever, hypotension and shock may occasionally cause death.[3, 5−7] The tips of the spines have venom glands, which rupture upon stabbing and inject venom. Common components and unstable proteins are presumed to be contained in the venom of all the fish species.[8, 9]

Although phylogenetic studies have been conducted on venomous spine structures and venom glands,[10] the first structural study on protein venoms was reported by Kiriake et al.[11] Thus, future advances in the molecular biological research on these fish venoms should facilitate their use as valuable biological resources that may be used as pharmaceuticals and physiological tools in life sciences.[12, 13] However, only a limited number of studies have investigated the toxicity of *S. verru-*

cosa.[14–16] Therefore, we purified lectin as a sugar-binding protein from the protein fraction derived from the dorsal fin venomous spines of *Synanceia verrucosa* in Okinawa.

MATERIALS AND METHODS

Isolation of dorsal venom lectin

Synanceia verrucosa (5 specimens : average size 30 cm) were collected by local fishermen from the coast of the islands of Okinawa Prefecture, Japan in August 2000 (Fig. 1). Venom fluid (300 μL) from venom sacs in the dorsal spines was pooled by syringe. The pooled venom was stored at −80°C until use. For the first step of purification, the dorsal venom was applied to a Concanavalin A-Sepharose 4 B (Sigma-Aldrich, St. Louis, MO, USA) affinity chromatographic column (2 mL) equilibrated with 20 mM Tris-HCl buffer containing 0.4 M NaCl at pH 7.4. The sample was rinsed and washed with the same buffer containing 100 mM methyl α-D-mannoside in the buffer at a flow rate of 20 mL/h, and then eluted with it (Fig. 2 A). The 2-mL elution fractions were collected and analyzed for absorption at 280 nm and assayed for agglutinating activity. Each of the unbound and bound fractions were pooled and analyzed by electrophoresis.

The final step of purification was achieved using a Phenyl Sepharose CL-4 B (GE Healthcare, Uppsala, Sweden) column (2 mL) equilibrated with a 16 mM Tris-HCl buffer containing 2 M NaCl at pH 7.4. The unbound fraction (the Con A-I fraction) was rinsed and washed with the same buffer containing 0.01 M NaCl at a flow rate of 20 mL/h, and then eluted with it (Fig. 2 B). Elution fractions of 2

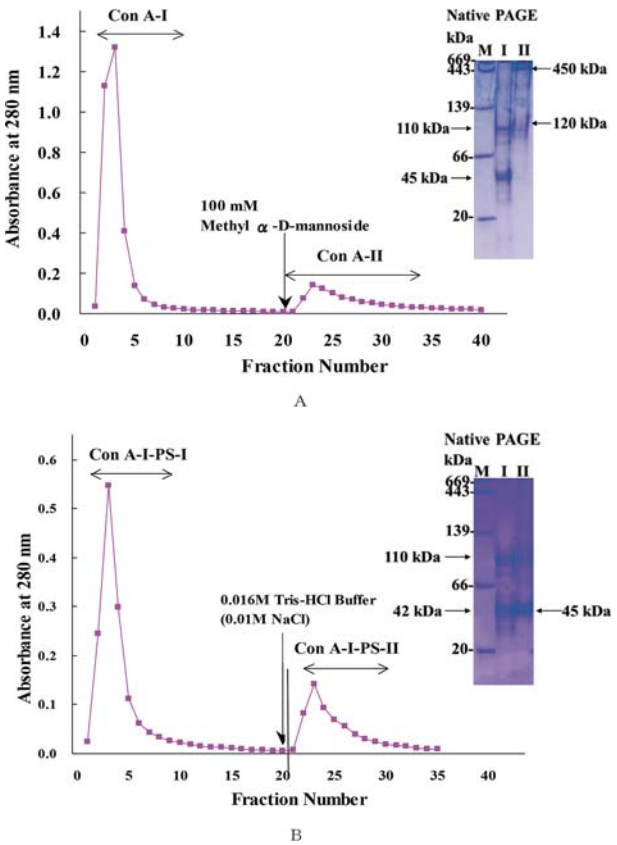

Fig. 2 Isolation of a novel lectin from the dorsal spines of *Synanceia verrucosa* as described in Materials and Methods. (A) The first purification step used Concanavalin A-Sepharose 4 B, and the second (B) used Phenyl Sepharose CL-4 B. Inset panels show the native PAGE of affinity chromatographic fractions. M : Molecular weight markers.

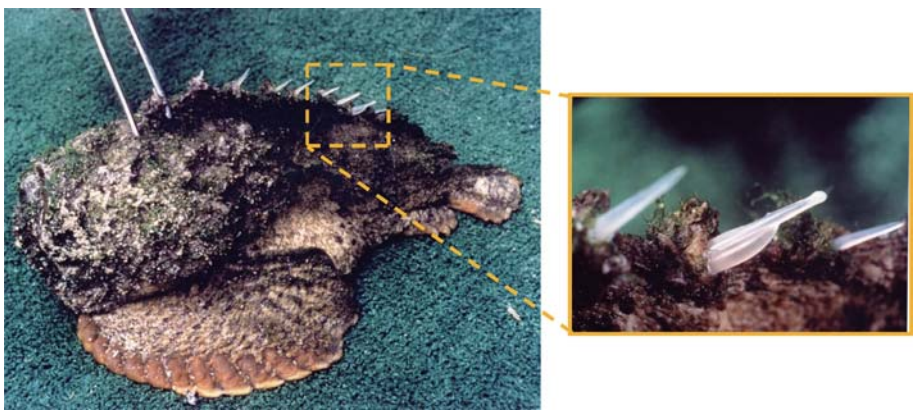

Fig. 1 The stonefish, *Synanceia verrucosa* with magnification of the dorsal spines with venom glands. The fish are about 30 cm in length. The specimens were collected along the coast of the island of Okinawa, Japan.

mL were collected and analyzed for absorption at 280 nm and assayed for agglutinating activity. Each of the unbound (Con A-I- PS-I) and bound (Con A-I -PS-II) fractions were pooled and analyzed by electrophoresis. The Con A-I-PS-II was then used as a purified lectin. The protein content was measured according to the method of Bradford[17] using bovine serum albumin as a standard.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was run as described by Davis[18] using a 4%-20% gradient gel. Sodium dodecyl sulfate (SDS)-PAGE was carried out by the method of Laemmli[19] using a 10%-20% gradient gel. The protein samples were heated in the presence of 2-mercaptoethanol for 4 min at 98°C and the gels were stained with Coomassie brilliant blue.

Assay of agglutinating activity

Agglutinating activity was assayed using rabbit erythrocytes in microtiter plates. A total of 25 μL of a 2% (v/v) suspension of erythrocytes in 6.4 mM phosphate-buffered saline (PBS) was added to 50 μL of a serial 2-fold dilution of the sample. The plates were incubated at room temperature for 1 h and the results were expressed by the minimum concentration of the sample (μg/mL) required for positive agglutination. Agglutination inhibition was expressed as the minimum concentration of each sugar required for inhibition of agglutinating activity of the sample.

Mitogenic activity

Mitogenic activity on the murine splenocytes was determined by cell culture assay using a dye, tetrazolium salt, 3(4,5-dimethyl thiazol-2-yl)-2-5-diphenyl tetrazolium bromide (MTT).[20] Splenocytes (5×10^6 cells per milliliter) with or without Concanavalin A (1 μg/mL), as positive control, and the samples were plated in flat-bottomed microplates and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 68 h. A total of 10 μL of MTT tetrazolium salt solution (5 mg/mL) was then added to each well, and formazan in the cells was extracted with 10% SDS after 4 h. The optical density of each well was measured spectrophotometrically at 570 nm using a microplate reader (Model 680 ; Bio-Rad Lab, Tokyo, Japan).

Cytotoxic activity

Cytotoxic activity on human leukemia cells (K562) was also determined by cell culture assay using MTT tetrazolium salt.[20] K562 (1×10^5 cells/mL) were plated into the flat-bottomed microtiter plates containing RPMI-1640 medium not supplemented with penicillin and streptomycin. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 with or without the samples. MTT tetrazolium salt solution (10 μL of 5 mg/mL) was added to each well, followed by the extraction of formazan with 10% SDS after 4 h. The optical density of the well was measured spectrophotometrically at 570 nm using the same microplate reader.

RESULTS

Purification of dorsal venom lectin

Dorsal venom lectin from *S. verrucosa* was purified using a combination of affinity chromatography techniques (Figs. 2 A and B). Dorsal venom was applied to a Concanavalin A-Sepharose 4 B column (2 mL) equilibrated with 20 mM Tris-HCl buffer containing 0.4 M NaCl at pH 7.4. The sample was rinsed thoroughly with the same buffer and then eluted with 100 mM methyl-α-D-mannoside in the buffer at a flow rate of 20 mL/h. Figure 2 A shows the elution pattern with two protein peaks. The first peak (the Con A-I fraction) induced agglutinating activity at a dose of 25 μg/mL, while the second peak (the Con A-II fraction) induced activity at a dose of 50 μg/mL. The mitogenic activity induced by the Con A-I fraction was slightly greater than that induced by the Con A-II fraction (data not shown).

Native PAGE analysis of the Con A-I fraction showed two main bands that corresponded to proteins with apparent masses of 45 and 110 kDa (Fig. 2 A). The Con A-I fraction was applied to a Phenyl Sepharose CL-4 B column (2 mL) equilibrated with 16 mM Tris-HCl buffer containing 2 M NaCl at pH

7.4. The column was rinsed thoroughly with the same buffer and then eluted with it. The elution was carried out using a linear gradient of NaCl from 2 M to 0.01 M in 16 mM Tris-HCl buffer at a flow rate of 20 mL/h.

As shown in Fig. 2 B, a native PAGE analysis of the unbound fraction (the Con A-I-PS-I fraction) identified three bands corresponding to proteins with apparent masses of 42, 100 and 110 kDa. On the other hand, the bound fraction (the Con A-I-PS-II fraction) showed a single band corresponding to a protein with an apparent mass of 45 kDa. This protein was designated as a 45 kDa lectin. The recovery of the 45 kDa lectin in terms of protein content, accounted for about 3% of the dorsal venom. The Con A-I-PS-I fraction and 45 kDa lectin both induced agglutinating activity at a dose of 25 μg/mL.

Sugar-binding specificity of 45 kDa lectin

The agglutinating activity of the 45 kDa lectin was inhibited most effectively by methyl α-D-mannoside (0.78 mM) and, to a lesser extent, by D-mannose (1.6 mM), suggesting that the hydrogen groups at C -1, C-3 and C-4 of the pyranose ring structure influenced sugar-binding to the lectin (Table 1).

Mitogenic activity of dorsal venom and 45 kDa lectin

We examined the mitogen responses of the dorsal venom and 45 kDa lectin in murine splenocytes. Splenocytes (5×10^6 cell/mL) were incubated with dorsal venom or 45 kDa lectin for 68 h and the incubation was continued with MTT for 4 h in a CO_2 humidified atmosphere. As shown in Fig. 3 A, the dorsal venom induced mitogenic activity in murine splenocytes in a dose-dependent manner (25-200 μg/mL). The 45 kDa lectin effectively induced mitogenesis in a dose-dependent manner in murine splenocytes (5-40 μg/mL) (Fig. 3 B). The mitogenic activity induced by the 45 kDa lectin was greater than that induced by the Con A-I-PS-I fraction (data not shown).

Table 1 Sugar inhibition of agglutinating activity of 45 kDa lectin

Sugar	Minimum effective concentration (mM)
D-Mannose	1.56
Methyl-α-D-mannoside	0.78
D-Glucose	25
D-Galactose	NI 100
D-Fucose	NI 100

NI : Non-inhibitory

Effect of the Con A-I-PS-I fraction and 45 kDa lectin on human leukemia cells (K562)

We examined the effect of the Con A-I-PS-I fraction and 45 kDa lectin on human leukemia cells (K562). It has been reported that the crude dorsal venom of *S. verrucosa* had weak cytotoxic activity on murine P 388 leukemia cells.[21] In this study, the cytotoxic activity of the Con A-I-PS-I fraction and 45 kDa lectin was investigated using K562 leukemia cells.

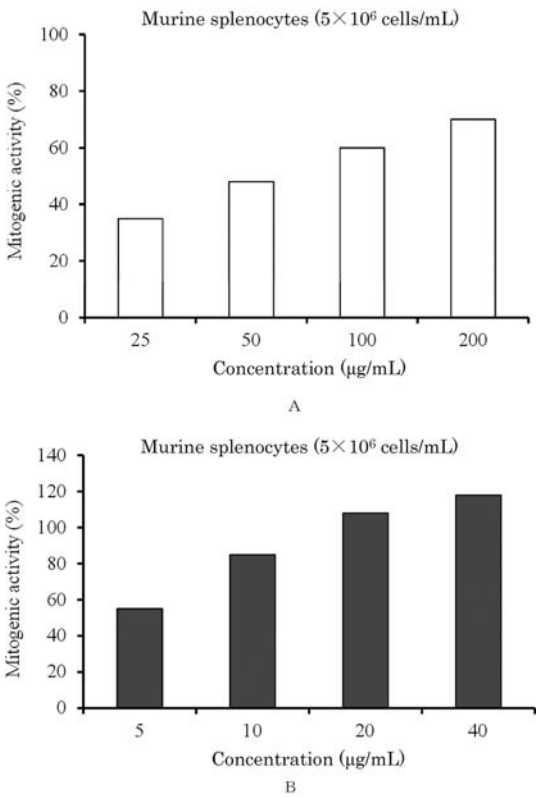

Fig. 3 Mitogen responses of the dorsal venom (A) and 45 kDa lectin (B) in murine splenocytes. Splenocytes (5×10^6cell/mL) were incubated with dorsal venom and 45 kDa lectin for 68 h and the incubation was continued with MTT for 4 h in a humidified CO_2 atmosphere. Data show the mean of the two experiments performed in triplicate.

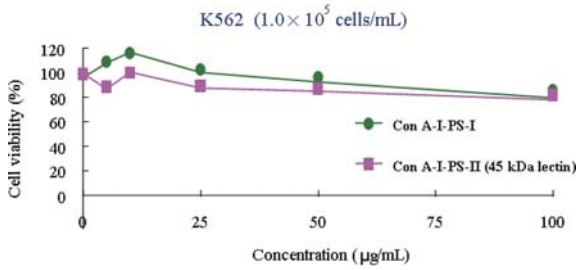

Fig. 4 Effects of the Con A-I-PS-I fraction and 45 kDa lectin on human leukemia cells (K562). The cells were incubated with the Con A-I-PS-I fraction or 45 kDa lectin. The control is expressed as 100%. Data show the mean of the two experiments performed in triplicate.

As shown in Fig. 4, the two fractions did not show cytotoxic activity on human K562 leukemia cells.

DISCUSSION

Dorsal venom from *S. verrucosa* was previously reported to induce weak agglutination in rabbit erythrocytes and to stimulate mitogenesis in murine splenocytes.[21] Therefore, in the present study we attempted to isolate lectin components from the dorsal spines of *S. verrucosa* using a combination of affinity chromatography techniques (Figs. 2 A and B). As shown in Fig. 2 B, a native PAGE analysis of the bound fraction (Con A-I-PS-II fraction) showed a single band corresponding to a protein with an apparent mass of 45 kDa. This protein was designated as a 45 kDa lectin. The agglutination with rabbit erythrocytes by 45 kDa lectin was effectively inhibited by methyl *α*-D-mannoside (Table 1). The 45 kDa lectin effectively induced mitogenesis in a dose-dependent manner in murine splenocytes (5 -40 *μ*g/mL) (Fig. 3 B). The mitogenic activity induced by the 45 kDa lectin was greater than that induced by the Con A-I-PS-I fraction (data not shown). This suggests that there is a quantitative and/or qualitative difference in the lectin components between the Con A-I-PS fractions.

Further biological and structural studies on the Con A-I-PS-I fraction may lead to the identification of more novel lectin(s). Most piscine venoms have been shown to exhibit potent cytotoxic activity.[9, 22] It has been reported that the crude dorsal venom of *S. verrucosa* had weak cytotoxic activity on murine P 388 leukemia cells.[21] Therefore, in the present study, the cytotoxic activity of the Con A-I-PS-I fraction and 45 kDa lectin was investigated using K562 leukemia cells. As shown in Fig. 4, the two fractions did not show cytotoxic activity on K562 leukemia cells. A dorsal lectin of the redfin velvetfish *Hypodytes rubripinnis* has been isolated by affinity chromatography.[23, 24] Karatoxin, a 110 kDa glycoprotein, exhibited mitogenic activity on murine splenocytes as well as cytotoxic activity on murine P 388 leukemia cells. The agglutination activity induced by Karatoxin was effectively inhibited by D-mannose.

In the present study, the agglutination induced by the 45 kDa lectin was inhibited by D-mannose and methyl *α*-D-mannoside. These results suggest that the 45 kDa lectin may exhibit mitogenic activity by binding the mannose-containing carbohydrate chains, which are present on the surface of murine splenocytes. Kiriake et al. recently identified by cDNA cloning the toxin of the devil stinger *Inimicus japonicus* as a 160 kDa heterodimer composed of 80 kDa *α*- and *β*-subunits.[11] This toxin is very similar to those of the lionfish *P. lunulata* and the waspfish *H. rubripinnis.* The venoms of most venomous fish are thought to have similar toxic properties, and molecules with similar structures.[8, 9, 25] More recently, it has been reported that 97 kDa lectin was purified from the dorsal spines of *I. japonicus.* The N-terminal partial amino acid of the intact 75 kDa subunit of the 97 kDa was found to be DHEDS.[26] More detailed studies on the structure of 45 kDa lectin of *S. verrucosa* are needed to elucidate its sequence homology to Karatoxin and piscine venoms such as those from the stonefish and scorpionfish.

Lectins are a large group of proteins that reversibly bind specific carbohydrates and possess at least one non-catalytic domain.[27] Although many lectins have been isolated from various plants, bacteria, and animals, including invertebrates,[28–32] very few have been detected in marine vertebrates, particularly venomous fish.[21, 23] In the case of the devil stinger *I. japonicus,* the 97 kDa lectin exhibited not only mitogenic activity in murine splenocytes,[26] but also chemotactic activity in guinea-pig neutrophils

(unpublished data). It has been proposed that the venoms of most poisonous fish are chemically and pharmacologically similar, and that their effects only differ quantitatively. Further studies are needed to clarify the mechanisms underlying the biological activities of the 45 kDa lectin of *S. verrucosa.* Moreover, an investigation of the structure of this lectin is needed to elucidate the biological significance of the dorsal venom from *S. verrucosa.* The present results suggest that the reef stonefish *S. verrucosa* venom may be a resource for pharmacologically active substances such as piscine lectins.

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The authors have no competing interests.

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