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ORIGINAL RESEARCH

Isolation of a novel lectin from the dorsal spines of the devil stinger, *Inimicus japonicus*

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Abstract A novel lectin was purified from the dorsal spines of the devil stinger, *Inimicus japonicus* using a combination of affinity chromatography techniques. A single band was detected on a native PAGE gel with a relative molecular mass of 97 kDa. The N-terminal partial amino acid of the intact 75 kDa subunit of the 97 kDa lectin was found to be DHEDS. The agglutination of rabbit erythrocytes by the 97 kDa lectin was inhibited most effectively by methyl α-D-mannoside. The 97 kDa lectin stimulated mitogenesis in murine splenocytes. This is the first study to examine the dorsal lectin of *I. japonicus* and one of the very few studies on venom lectins from venomous scorpaeniform fish. These results suggest that the devil stinger, *I. japonicus*, may be a novel resource for biologically active substances.

Keywords Devil stinger · *Inimicus japonicus* · Dorsal spine · Lectin · Agglutination · Mitogenic activity · Murine splenocytes

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Background

A large number of venomous and poisonous animals exist in aquatic environments worldwide. More than 200 of the approximately 22,000 species of fish in the ocean are considered to be venomous (Halstead 1988; Russell 1996). Most of these venomous fish are non-migratory, slow moving, and mainly live in shallow waters in protected habitats (Maretic 1988). Venomous scorpaeniform fish include the lionfish and scorpionfish from the family Scorpaenidae, devil stinger and stonefish from the family Synanceiidae, and waspfish from the family Tetrarogidae (Kiriake et al. 2013). These fish possess 11–17 dorsal, 2 pelvic, and 3 anal spines, with the venom secretory complex being located within the anterolateral grooves of these spines (Russell 1965; Halstead 1988; Haddad et al. 2003; Smith and Wheeler 2006; Andrich et al. 2010).

The devil stinger *Inimicus japonicus*, which belongs to the family Synanceiidae, has 17 dorsal, 1 pelvic, and 2 anal spines, which contain venom glands that are covered by an integumentary sheath (Tange 1954). *I. japonicus*, a valuable demersal scorpaenid fish, is widely distributed along the coastal areas of eastern Asia at a depth of between 10 and 200 m (Wang et al. 2013). The body of *Inimicus* is covered in warts or skin lumps, with many skin tubercle glands similar to the stonefish. Envenomation occurs when people carelessly handle or step on these fish, and are stung by the dorsal spines. Envenomation appears immediately as intense, sharp, and persisting local pain, and swelling around the sting (Auerbach 1991; Yamamoto et al. 2010). Symptoms depend on the amount of venom injected. Systemic effects including dizziness, fever, and delirium have been reported (Auerbach 1991). However, only a limited number of studies have investigated the toxicity of *I. japonicus*. Therefore, we herein examined the dorsal venom of the devil stinger, *I. japonicus* using column chromatography and, for the first time, separated a novel lectin that induced mitogenic activity.

Methods

Isolation of a dorsal lectin

Inimicus japonicus (18 specimens, average size of 20 cm) were collected by local fishermen from the coast of Hiroshima Prefecture and Tokushima Prefecture, Japan in May 2003 (Fig. 1a, b). The collected fish were transported alive or frozen to our laboratory. The dorsal spines (a total of 17) of *I. japonicus* were cut from their base, and the dorsal venom protein was extracted with 0.15 M NaCl as reported previously (Nagasaka et al. 2009). Briefly, in the first step of purification, the venom protein was applied to a Phenyl Sepharose CL-4B (GE Healthcare, Uppsala, Sweden) affinity chromatographic 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 containing 0.01 M NaCl at a flow rate of 20 ml/h (Fig. 1a). The 2-ml elution fractions were collected and analyzed for absorption at 280 nm and agglutinating activity. Each of the unbound and bound fractions was pooled and

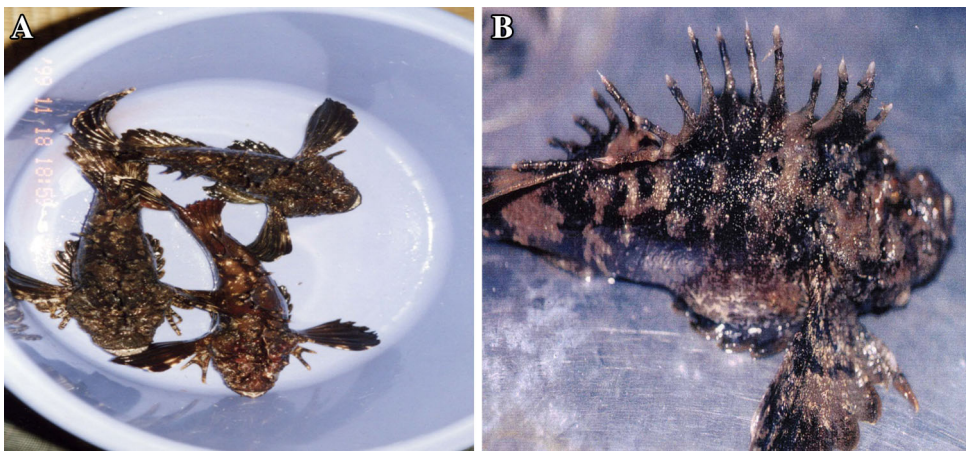

Fig. 1 The devil stinger *Inimicus japonicus* as seen from above (**a**) and a specimen with erect dorsal spines (**b**)

analyzed for electrophoresis. The final step of purification was achieved using a Concanavalin A-Sepharose 4B (Sigma-Aldrich, Missouri, USA) column (2 ml) equilibrated with 20 mM Tris-HCl buffer containing 0.4 M NaCl (pH 7.4). The unbound fraction (the PS-I fraction) was rinsed and washed with the same buffer, and eluted with the same buffer containing 100 mM methyl-α-D-mannoside in the buffer at a flow rate of 20 ml/h (Fig. 1b). Elution fractions (2 ml) were collected and analyzed for absorption at 280 nm and agglutinating activity. Each of the unbound (PS-I-ConA-I fraction) and bound (PS-I-ConA-II fraction) fractions was pooled and analyzed for electrophoresis. PS-I-ConA-II was then used as the purified lectin. Protein content was measured according to the method of Bradford (1976) using bovine albumin as a standard.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed 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. Protein samples were heated in the presence 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 the SDS-PAGE gel using the GelCode Glycoprotein kit (Pierce Biotech., Inc., IL, USA). This 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 on microtiter plates. Twenty-five microliters of a 2 % (v/v) suspension of erythrocytes in 6.4 mM phosphate-buffered saline (PBS) was added to 50 µl of a serial twofold dilution of the sample. The plates were incubated at room temperature for 1 h. The results obtained were expressed by the minimum concentration of the sample (µg/ml) required for positive agglutination. The inhibition of agglutination was expressed as the minimum concentration of each sugar required to inhibit the agglutinating activity of the sample.

Mitogenic activity

Mitogenic activity in murine splenocytes was determined using a cell culture assay with a dye, the tetrazolium salt 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Nakagawa et al. 1997). Splenocytes were collected from female ddY mice and suspended in RPMI-1640 medium supplemented with penicillin and streptomycin (100 µg/ml and 100 U/ml). Splenocytes (5×10^6 cells/ml) with or without concanavalin A (1 µg/ml) and samples were plated on flat-bottom microplates and incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 for 68 h. Ten microliters of the MTT tetrazolium salt solution (5 mg/ml) was then introduced into each well, and formazan was extracted from the cells with 10 % sodium sulfate (SDS) after 4 h. The optical density of each well was measured spectrophotometrically with a microplate reader (Thermo Fisher Scientific, MultiskanGo, Yokohama, Japan) at 570 nm.

N-terminal amino acid sequencing

Approximately, 3 µg of the sample protein was subjected to SDS-PGE, followed by electroblotting on a polyvinylidene difluoride membrane. The membrane was then stained with Ponceau S and destained. The protein band was excised and subjected to automated Edman degradation using the Shimadzu Model PPSQ-30 protein sequencer (Shimadzu Corp., Kyoto, Japan).

Statistical analysis

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

Results and discussion

Dorsal venom was previously reported to induce weak agglutination in rabbit erythrocytes and stimulated mitogenesis in murine splenocytes (Nakagawa et al. 2011). The venom possessed a glycoprotein that was stained by SDS-PAGE using the GelCode Glycoprotein kit, indicating that it contained one band corresponding to a protein with an apparent mass of 75 kDa (data not shown). Therefore, we herein attempted to isolate a lectin from the dorsal spines of *I. japonicus* using a combination of affinity chromatography techniques (Fig. 2a, b). Dorsal venom 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 with the same buffer and then eluted with the same buffer containing 0.01 M NaCl at a flow rate of 20 ml/h. Figure 2a shows the elution pattern with two protein peaks. The first peak (the PS-I fraction) induced agglutinating activity at a dose of 6.25 μg/ml, while the second peak (the PS-II fraction) induced this activity at a higher dose of 200 μg/ml (data not shown). SDS-PAGE analysis of the PS-I fraction showed two main bands that corresponded to proteins with apparent masses of 30 and 97 kDa (Fig. 2a). The PS-I fraction was applied to a Concanavalin A-Sepharose 4B column (2 ml) equilibrated with 20 mM Tris-HCl buffer containing 0.4 M NaCl (pH 7.4) for purification. The column 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.

As shown in Fig. 2b, a native PAGE analysis of the unbound fraction (the PS-I-ConA-I fraction) identified two bands corresponding to proteins with apparent masses of 30 and 97 kDa. On the other hand, the bound fraction (the PS-I-ConA-II fraction) showed a single discrete band corresponding to a protein with an apparent mass of 97 kDa. The PS-I-Con A-II fraction was a glycoprotein fraction that contained mannose residues. This glycoprotein fraction was designated as a 97 kDa lectin. The recovery of the 97 kDa lectin in terms of protein content accounted for 0.35 % of the dorsal venom. SDS-PAGE showed that the 97 kDa lectin was mainly composed of 75 and 30 kDa subunits (Fig. 2b). The 75 kDa subunit of the 97 kDa lectin was subjected to a partial amino acid sequence analysis. The N-terminal amino acid of the 75 kDa subunit was aspartic acid. The partial amino acid sequence was determined up to 5 residues, and found to be DHEDS. In the case of the waspfish *Hypodytes rubripinnis*, Karatoxin, a novel cytotoxic protein (110 kDa), was successfully purified from the dorsal spines of this fish (Nagasaka et al. 2009; Shinohara et al. 2010). The N-terminal partial amino acid sequence of the 76 kDa subunit of Karatoxin was shown to be DQHDDxPxxAPDPG. As the partial amino acid sequence of the 75 kDa subunit of the 97 kDa lectin was only analyzed up to 5 residues, it is currently difficult to determine whether it shares homology with the 76 kDa subunit of Karatoxin. Therefore, we are now attempting to improve the purifying method as well as the recovery of the 97 kDa lectin from the dorsal venom. Kiriake et al. (2013) recently identified the toxin of *I. japonicus* as a 160 kDa heterodimer composed of 80 kDa α- and β-subunits by cDNA cloning. This toxin was very similar to those of the lionfish *P. lunulata* and waspfish *H. rubripinnis*. The venoms of most venomous fish have been proposed to have similar toxic properties and molecules with similar structures (Saunders 1960; Russell 1965; Church and Hodgson 2002). Therefore, more detailed studies on the structure of the 97 kDa lectin are needed to elucidate its sequence homology to Karatoxin and piscine venoms such as those from the stonefish.

The agglutinating activity of the 97 kDa lectin was inhibited most effectively by methyl α-D-mannoside and, to a lesser extent, by D-mannose, and D-Glucose, 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). Figure 3 shows the results of mitogen responses in murine splenocytes in the presence of the dorsal venom and PS-I-ConA fractions (the PS-I-ConA-I fraction and PS-I-ConA-II fraction). As shown in Fig. 3a, the dorsal venom induced mitogenic activity in murine splenocytes at doses ranging from 25 to 50 μg/ml. However, a higher dose (100 μg/ml) of

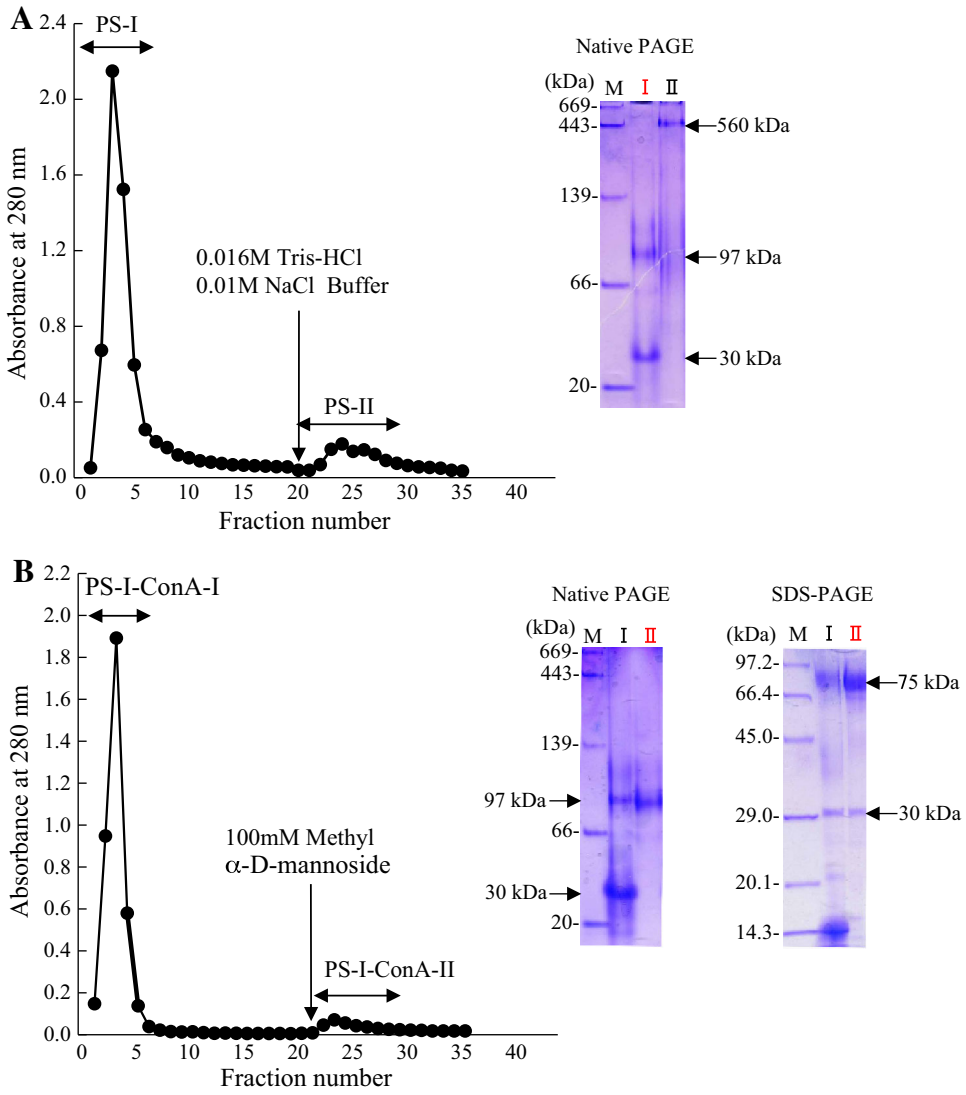

Fig. 2 Isolation of a novel lectin from dorsal spines of *Inimicus japonicus*. The isolation procedure was described in detail in "Methods". **a** The first purification step used Phenyl Sepharose CL-4B. **b** The second purification step used Concanavalin A-Sepharose 4B. Inset panels show native PAGE and SDS-PAGE of affinity chromatographic fractions. *M* mol wt markers

Table 1 Sugar inhibition of agglutinating activity of 97 kDa lectin

Sugar	Minimum effective concentration (mM)
D-Mannose	3.1
Methyl a-D-mannoside	0.78
D-Glucose	12.5
N-Acetylglucosamine	12.5
Others[a]	NI 100

NI non-inhibitory

[a] D-Galactose, D-Fucose, L-Glucose, D-Galactosamine, Lactose

the dorsal venom significantly decreased mitogenic activity, suggesting the presence of another biologically active component in addition to lectin component(s) in the venom. Most piscine venoms have been shown to exhibit potent cytolytic activity (Shier 1988; Church and Hodgson 2002). We also observed cytotoxic activity in human leukemia cells (K562) by the dorsal venom (unpublished data). The PS-I-ConA-I fraction and

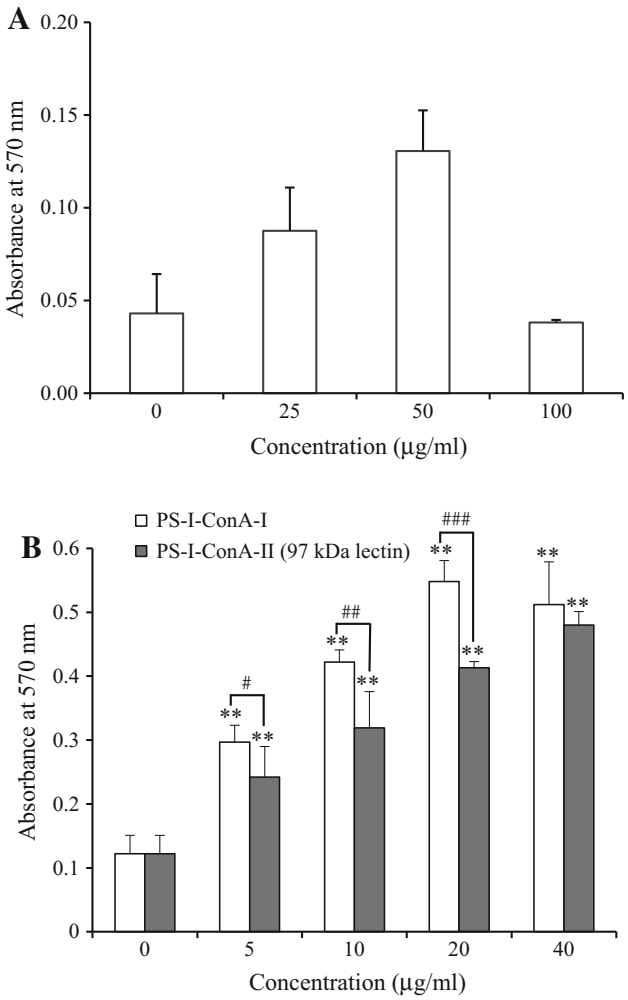

Fig. 3 Mitogen responses of the dorsal venom (**a**) and PS-I-ConA fractions (**b**) in murine splenocytes. Splenocytes (5×10^6 cells/ml) were incubated with the dorsal venom, PS-I-ConA-I and PS-I-ConA-II (97 kDa lectin), for 68 h and the incubation was continued with MTT for 4 h in a CO_2 humidified atmosphere. Data show the mean ± SD of two experiments performed in triplicate (**a**) or the mean ± SD of 3–4 experiments performed in triplicate (**b**). $^{**}P < 0.01$, significantly different from the negative control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, significant difference between two groups according to the Student's unpaired *t* test

97 kDa lectin effectively induced mitogenesis in a dose-dependent manner in murine splenocytes (Fig. 3b). The mitogenic activity induced by the PS-I-ConA-I fraction was slightly higher than that induced by the 97 kDa lectin. This suggests that there is a quantitative and/or qualitative difference in the lectin component between the two fractions. Further biological and structural studies on the PS-I-ConA-I fraction may lead to the identification of more novel lectin(s).

Lectins are a large group of proteins that reversibly bind specific carbohydrates and possess at least one non-catalytic domain (Drickamer 1988). Although many lectins have been isolated from various plants, bacteria, and animals, including invertebrates (Drickamer and Taylor 1993; Hatakeyama et al. 1994; Nakagawa et al. 2003; Sharon and Lis 2004; Malagoli et al. 2010), very few have been detected in marine vertebrates, particularly venomous fish (Satoh et al. 2002; Nagasaka et al. 2009). In the present study, we successfully isolated and partially characterized the 97 kDa lectin from *I. japonicus* as a novel piscine lectin. The 97 kDa lectin from *I. japonicus* venom exhibited mitogenic activity in murine splenocytes, suggesting that it may contribute to the local and systemic effects observed on envenomation such as swelling, severe pain, and fever. We more recently observed chemotactic activity in guinea-pig neutrophils (unpublished data). Thus, the 97 kDa lectin may affect inflammatory and immunomodulatory processes. The agglutination induced by the 97 kDa lectin was inhibited by D-mannose and methyl α-D-mannoside. These results suggest that the 97 kDa lectin may induce mitogenic activity by binding the mannose-containing carbohydrate chains present on the surface of murine splenocytes. Further studies are needed to clarify the mechanisms underlying the biological activities of the 97 kDa lectin. Moreover, an investigation of the structural features of this lectin is needed to elucidate the physiological significance of venom from *I. japonicus*. The present results suggest

that the 97 kDa lectin from *I. japonicus* venom is a source of biologically active substances, which may have applications as research tools.

Conclusions

A novel lectin was isolated from the dorsal spines of *I. japonicus* using a combination of affinity chromatography techniques. Native PAGE showed that the dorsal lectin was a glycoprotein with a molecular mass of 97 kDa.

The agglutinating activity of the 97 kDa lectin was effectively inhibited by methyl α-D-mannoside. The 97 kDa lectin may exhibit mitogenic activity by binding to a specific carbohydrate chain, such as mannose moieties on the cell surface. Our results suggest that the venom of the devil stinger, *I. japonicus* is a potent resource for piscine lectins.

Author contributions HN prepared the manuscript, and supervised the experiments. KN participated in the experiment design and manuscript writing. HS conducted the purification of dorsal lectin. KE conducted the mitogenic activity and agglutination activity assay. MS participated in the interpretation of the results. KO participated in the study design. All authors read and approved the final manuscript.

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Conflict of interest The authors declare that they have no competing interests.

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