Short Communication

A Novel Chemoattractant Lectin, Karatoxin, From the Dorsal Spines of the Small Scorpionfish *Hypodytes rubripinnis*

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Abstract. A chemoattractant lectin from the dorsal spines of the redfin velvetfish, *Hypodytes rubripinnis*, was isolated using a combination of affinity chromatography techniques. The glycoprotein, with a molecular mass of 110 kDa, is named Karatoxin. Karatoxin caused agglutination of rabbit erythrocytes. This agglutination was effectively inhibited by D-mannose. In addition, Karatoxin exhibited not only mitogenic activity in the presence of murine splenocytes, but also chemotactic activity in the presence of guinea-pig neutrophils and macrophages. Thus, Karatoxin appears to be a novel chemoattractant lectin. These results suggest that the redfin velvetfish *Hypodytes rubripinnis* may be a novel source of biologically active substances.

Keywords: chemoattractant lectin, Karatoxin, *Hypodytes rubripinnis*

The redfin velvetfish *Hypodytes rubripinnis* is a small congipodid fish that belongs to the Scorpaenidae group, known as scorpionfish. However, *H. rubripinnis* is dangerous to man. The venom organs of *H. rubripinnis* consist of 14 dorsal, 2 pelvic, and 3 anal spines that contain venom glands, covered by an integumentary sheath, like other Scorpaenidae (1). Envenomation usually occurs due to mishandling of the fish when the force of contact with a spine causes it to pierce the flesh and exert sufficient pressure on the venom glands; this results in the dislodging of the stored venom, which flows along the spine into the subcutaneous tissue of the victim. The main symptom of envenomation is intense pain with severe muscle ache, and the area surrounding the puncture wound is swollen. Systemic effects include dyspnea and restlessness. For emergent treatment, physicians recommend infiltrations of the wound area with 0.5% – 2.0% lidocaine and steroid ointment. In the event of shock, treatment includes the infusion of steroids (100 – 200 mg) (2). However, there is little information available

regarding the biochemical and physiological properties of the venom from *H. rubripinnis*. More recently, we observed that Karatoxin, a glycoprotein purified from the dorsal venom, shows cytotoxic activity against murine P388 leukemia cells (2).

Lectins isolated from numerous plants and animals including invertebrates are generally defined as non-enzymatic proteins that play an important role in physiological functions (3, 4). In recent years, many lectins have also been isolated from marine sources (5 – 7), but very few have been reported in marine animals, particularly fish (8). Here, we report the biological activities of Karatoxin as a novel chemoattractant lectin from the dorsal spine venom of spine *H. rubripinnis*.

Hypodytes rubripinnis (180 specimens, average size of 8 cm) were collected by local fishermen from the coast of Hiroshima Prefecture, Japan in August 1999 (Fig. 1). The collected fish were transported live or frozen to our laboratory. The dorsal spines (total of 14) from each *H. rubripinnis* specimen were cut from their base. The dorsal spines with the integumentary sheath containing a fusiform strand of glandular tissue were extracted with 0.15 M NaCl followed by centrifugation at $2,000 \times g$ for 20 min at 4°C. The resulting supernatant was lyophilized.

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Fig. 1. Redfin velvetfish, *Hypodytes rubripinnis*; average size: 8 cm.

The lyophilized venom was stored at -80°C until use. The protein content was measured according to the method of Bradford (9) using bovine serum albumin as a standard.

Agglutinating activity was assayed using rabbit erythrocytes in microtiter plates. A $25\text{-}\mu\text{l}$ aliquot of a 2% (v/v) suspension of erythrocytes in 6.4 mM phosphate-buffered saline (PBS) was added to $50\text{-}\mu\text{l}$ of a serial two-fold dilution of the sample. The plates were incubated at room temperature for 1 h. The results are expressed as the minimum concentration of the sample ($\mu\text{g}/\text{ml}$) required for positive agglutination.

Mitogenic activity in the presence of murine splenocytes was determined employing a cell culture assay using the MTT tetrazolium salt (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (10). Polymorphonuclear leukocytes (neutrophils) and macrophages were induced by the intraperitoneal injection of 1% glycogen solution into male guinea pigs and collected by centrifugation in PBS. The washed cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) and adjusted to a neutrophil density of 2×10^6 cells/ml and a macrophage density of 1×10^6 cells/ml. Chemotaxis was measured employing a membrane filter method using a 48-well chemotaxis chamber (Neuroprobe, Gaithersburg, MD, USA). A cell suspension was placed in the upper wells of a chamber separated by a polycarbonate membrane filter ($3\text{-}\mu\text{m}$ pore size for neutrophils and $5\text{-}\mu\text{m}$ pore size for macrophages). The lower wells contained the chemotactic stimuli or DMEM as a negative control. In the case of neutrophils, after a 60-min incubation at 37°C in 5% CO_2 , the filter was removed, fixed, and stained. In the case of macrophages, after 90-min incubation at 37°C in 5% CO_2 , the filter was removed, fixed, and stained. Filters for both cell types were mounted on slides and cells were counted using a fluorescent microscope (BIOREVO BZ-9000; Keyence, Osaka), respectively.

The dorsal venom was applied to a Concanavalin A-Sepharose column (2 ml) equilibrated with 20 mM Tris-HCl buffer containing 0.4 M NaCl (pH 7.4). 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. The bound fraction (Con A fraction) eluted by methyl- α -D-mannoside showed agglutinating and mitogenic activity. For purification, the Con A 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 with the same buffer and then eluted with the same buffer containing 0.01 M NaCl at a flow rate of 20 ml/h. The bound fraction eluted with 0.01 M NaCl (the PS fraction) showed a single discrete band corresponding to an apparent mass of 110 kDa on native PAGE, exhibiting agglutinating activity. The PS fraction (the glycoprotein) was designated Karatoxin (2). The recovery of Karatoxin as the protein content was about 1% of the dorsal venom, and the biological activity of Karatoxin was about 40 times higher than that of the venom in terms of agglutinating activity in the presence of rabbit erythrocytes.

We previously reported that dorsal venom protein from *H. rubripinnis* showed weak agglutination with rabbit erythrocytes and the degranulation of rat mesenteric mast cells (11). The agglutination caused by the venom was inhibited with D-mannose treatment. The venom also induced the mitogenic activity of murine splenocytes in the dose range of 10 to 100 $\mu\text{g}/\text{ml}$ (Fig. 2).

In this study, we reported the mitogenic and chemotactic activities of Karatoxin, a dorsal mannose-containing glycoprotein from *H. rubripinnis* (Figs. 2 and 3). Karatoxin is mainly composed of 76- and 30-kDa subunits. The N-terminal 14 amino acid sequence of the 76-kDa subunit was shown to be DQHDDxPxxAPDPG (2). The amino acid sequence of Karatoxin is unique and is not related to other fish venoms (12, 13). The 76-kDa subunit of Karatoxin contains mannose moieties (data not shown). Now, we are analyzing the primary structure of Karatoxin, and the total RNA has been isolated from the dorsal spines of this fish (unpublished data). While Karatoxin ($1.25\text{--}10\text{-}\mu\text{g}/\text{ml}$) did not exhibit hemolytic activity in the presence of rabbit erythrocytes, it showed agglutinating activity with rabbit erythrocytes. This agglutination by Karatoxin was effectively inhibited by D-mannose (data not shown). As shown in Fig. 2, Karatoxin had potent mitogenic activity. The mitogenic activity of Karatoxin was about 100 times higher than that of the crude venom. On the other hand, larger doses of Karatoxin showed a decrease in mitogenic activity. The dual response to Karatoxin suggests that it may have wide-ranging effects. Furthermore, Karatoxin exhibited

chemotactic activity in the presence of guinea-pig neutrophils and macrophages in a low-dose ranges ($0.625 - 5.0 \mu\text{g/ml}$) (Fig. 3). This is the first reported finding involving guinea-pig leukocytes. In the present study, we could not detect activity at the lowest dose of $0.1 \mu\text{g/ml}$ of Karatoxin. As shown in Fig. 3, guinea-pig neutrophils were more sensitive to Karatoxin than guinea-pig macrophages. Karatoxin may exhibit chemotactic activity through binding to D-mannose containing carbohydrates that are present on the surface of guinea-pig neutrophils and macrophages. Therefore, it appears that Karatoxin is likely to recognize different sets of D-mannose residues in guinea-pig neutrophils in comparison with macrophages. These results suggest that Karatoxin may be a valuable tool for analyses of the inflammation, differentiation, and development of cells. Thus, Karatoxin appears to be a unique lectin.

It has been proposed that the venoms of most poisonous fish are chemically and pharmacologically similar,

and that their effects differ only quantitatively. In addition, most piscine venoms exhibit hemolytic activity (14). In this study, Karatoxin showed no hemolytic activity. However, it exhibited not only cytotoxic activity (2), but also mitogenic activity, suggesting that the toxin may contribute to the local and systemic effects observed on envenomation such as severe pain, swelling, and fever. Moreover, Karatoxin had a chemotactic effect. Therefore, Karatoxin might affect inflammatory and immunomodulatory processes. Karatoxin appears to be a D-mannose-binding lectin. Based on the present results, Karatoxin may exhibit activity through binding to a specific carbohydrate chain, such as mannose moieties on the cell surface. More studies are necessary to clarify the biological activities of Karatoxin. Our data suggest that *H. rubripinnis* venom is a source of pharmacologically active substances such as lead compounds and compounds that have applications as research tools.

Fig. 2. Mitogenic effects of the crude venom (A) and Karatoxin (B) from *H. rubripinnis* on murine splenocytes. The splenocytes (5×10^6 cells/ml) were incubated with the crude venom or Karatoxin for 68 h and then underwent continued incubation with MTT for 4 h in a 5% CO_2 humidified atmosphere. The mitogenic response to concanavalin A ($1.0 \mu\text{g/ml}$) was expressed as 100%. Data represent the mean of two experiments of triplicate determinations.

Fig. 3. Effects of Karatoxin on neutrophil (A) and macrophage (B) chemotaxis. Neutrophils (2×10^6 cell/ml) were incubated at 37°C for 60 min, and macrophages (1×10^6 cells/ml) were incubated at 37°C for 90 min with or without Karatoxin. The chemotactic response with FMLP (10^{-7} M) is expressed as 100%. Data represent the mean $\pm$ S.D. of 3–4 experiments of triplicate determinations.

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