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SJL-1, a C-type lectin, acts as a surface defense molecule in Japanese sea cucumber, *Apostichopus japonicus*

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ABSTRACT

The surface defense molecules of aquatic invertebrates against infectious microorganisms have remained largely unexplored. In the present study, hemagglutinins were isolated from an extract of body surface layer of Japanese sea cucumber, *Apostichopus japonicus*, by affinity chromatography with fixed rabbit erythrocyte membranes. The N-terminal sequence of a 15-kDa agglutinin was almost identical with that of SJL-1, a C-type lectin formerly identified in this species. Because cDNA sequence and tissue distribution of SJL-1 have not been reported, we performed cDNA sequencing, gene expression analysis, and western blotting and immunohistochemical evaluation with anti-recombinant SJL-1 (rSJL-1) antibodies. The hemagglutinin gene was transcribed mainly in the integument, tentacles, and respiratory tree. Western blotting revealed that SJL-I is present in a body surface rinse, indicating that SJL-1 is secreted onto the body surface. SJL-1-positive cells scattered beneath the outermost layer of the integument were detected by immunohistochemistry. Furthermore, rSJL-1 agglutinated Gram-positive and Gram-negative bacteria, and yeast. These results indicate that SJL-1 acts as a surface defense molecule in *A. japonicus*.

1. Introduction

A large number of microorganisms, including diverse disease-causing organisms, inhabit the aquatic environment, exploiting the absence of desiccation and ultraviolet radiation. The body surface of aquatic animals, therefore, is a crucial protective barrier against the invasion of infectious organisms.

An array of studies have revealed that the cutaneous mucus of fish contains multiple defense agents: lectins (Vasta et al., 2011), bactericidal peptides (Cole et al., 1997, Fernandes et al., 2002), proteases (Buchmann and Bresciani, 1988, Ross et al., 2000), and immunoglobulins (Zhao et al., 2008, Palaksha et al., 2008). The structure, distribution, and functions of diverse lectin molecules in piscine mucus have been well characterized, and fish cutaneous lectins are thought to eliminate microorganisms by their agglutinating activity (Kamiya et al., 1988, Tasumi et al., 2002, Tsutsui et al., 2006). In addition, some of them exhibit bacteriostatic (Tasumi et al., 2002) or opsonic activity (Nakamura et al., 2006, Tsutsui et al., 2007). In contrast, defense mechanisms at the body surface of aquatic invertebrates remain nearly unexplored. It is quite probable, however, that molecules involved in innate immunity, such as lectins, play pivotal roles in the body surface defense of invertebrates, which are devoid of adaptive immunity.

Among aquatic invertebrates, echinoderms are attractive subjects for comparative immunologists, because of their evolutionary proximity to the vertebrates. A considerable number of studies have reported cells, molecules, and genes potentially involved in the biodefense of echinoderm species, especially in sea urchins, as reviewed by Ramirez-Gomez and García-Arrarás (2010). However, biodefense agents acting at the body surface of echinoderms remain largely unknown, except for a limited number of studies on antifouling mechanisms at their body surface (McKenzie and Grigolava, 1996, Bavington et al., 2004, Guenther et al., 2009) and, lysozyme-like or anti-bacterial activities of starfish mucus (Canicatti and D'Ancona, 1990, Hennebert et al., 2015) and body wall extracts of echinoderms (Villasin and Pomory, 2000, Haug et al., 2002)

Holothurians, sea cucumbers, are echinoderms with soft integument, which makes them an attractive experimental model, allowing easy collection of integument tissues. Multiple lectin molecules of sea cucumbers have been identified, most of which were present in the coelomic fluid or animal extracts (Hatakeyama et al., 1993, Hatakeyama et al. 1994, Matsui et al., 1994, Bulgakov et al., 2007, Moura Rda et al., 2013, Gowda et al., 2008). AJCTL is the only exception (Han

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et al., 2012); this molecule is a C-type lectin, and is the same molecule as AJ-MBL, formerly found in the coelomic fluid of the Japanese sea cucumber, *Apostichopus japonicus*, by Bulgakov et al. (2007). Han et al. (2012) reported that the lectin gene is transcribed in the muscle, intestine, respiratory tree, as well as in the cells of the body wall, though the presence of the protein was not confirmed.

In the present study, we found that a body surface extract of *A. japonicus* exhibits a strong agglutinating activity toward rabbit red blood cells (RRBC). Two proteins (15 and 17 kDa) were detected in an eluate after affinity chromatography employing fixed RRBC membranes. The N-terminal amino acid sequence of the smaller protein was almost identical to SJL-1 (84% identity). SJL-1 was isolated from the tissues of *A. japonicus* by Hatakeyama et al. (1993). SJL-1 agglutinates RRBC, and shows affinity for *N*-acetylgalactosamine and galactose; its amino acid sequence was determined (143 amino acids, predicted molecular mass 15,837 Da) (Himeshima et al., 1994), but neither cDNA sequence nor tissue distribution of SJL-1 has been reported. We sequenced the cDNA, purified recombinant SJL-1 protein, and raised specific polyclonal antibodies against it. We then explored the tissue distribution of SJL-1 by western blotting, immunohistochemistry, and reverse-transcription PCR. We also examined the agglutinating activity of rSJL-1 against microorganisms. This approach revealed putative roles of SJL-1 as a defense molecule on the body surface of *A. japonicus*.

2. Materials and methods

2.1. Sample preparation

A. japonicus (blue-colored) was purchased from a fisherman in the Inland Sea of Japan and kept in a glass aquarium with artificial sea water (SEA LIFE, Marine Tech, Japan) at 13 °C. Animal body surface was scraped using a surgical knife and stored at −80 °C until use (Fig. 1). In the current study, the pigmented outer layer of the body that can be easily scraped off by a knife is referred to as the body surface; the thick white tissue beneath the body surface is referred to as the body wall. Five volumes of 0.01 M phosphate-buffered saline (PBS, pH 7.2) containing 0.1% NaN_3, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ [PBS(+)], and 1/500 vol of protease inhibitor cocktail (Sigma-Aldrich, USA) were added to the frozen samples collected from five animals, followed by homogenization. After centrifugation at 15,000 × *g* for 20 min, the supernatant was collected as the body surface extract (BS-Ext) and stored at −80 °C until use.

2.2. Hemagglutination test

RRBC were purchased from Nihon Bio Test (Japan). BS-Ext was serially diluted in PBS(+) in a microtiter plate. Equal volume of 4% RRBC suspension was added to each well and incubated for 1 h at 20 °C.

Sugar-inhibition test was performed to investigate sugar-specificity of the agglutinin in BS-Ext. BS-Ext, diluted in PBS(+) containing 0.2 M of mono- or di-saccharides, was examined for the ability to agglutinate RRBC.

2.3. Affinity chromatography with fixed red blood cell membranes

RRBCs were lysed using a hypotonic solution (1/20 diluted PBS) and centrifuged at 14,000 × *g* for 15 min. The supernatant was discarded, and this process was repeated several times until the supernatant became colorless. The precipitates were then fixed in 2% glutaraldehyde and 4% paraformaldehyde for 15 min at 4 °C. After washing thrice with PBS, 2 ml of the fixed RRBC membrane preparation was mixed with 6 ml of BS-Ext and incubated at 4 °C overnight. Fixed RRBC, mixed with PBS(+), were used as a control.

The fixed RRBC membranes that had been incubated with BS-Ext were centrifuged at 10,000 × *g* for 7 min and the supernatant was recovered as the non-adsorbed fraction. Following repeated washing of precipitates with PBS(+), proteins bound to the RRBC membranes were eluted with 0.2 ml of 0.1 M glycine-HCl buffer (pH 3.0). The same procedure was applied to the control. The collected fractions were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gels were stained with Coomassie Brilliant Blue (CBB) R250.

2.4. N-terminal amino acid sequencing

The eluate from Section 2.3 was concentrated approximately 10 times by ultrafiltration (Vivaspin 5k, Sartorius, Germany) and subjected to SDS-PAGE. After electrophoresis, the protein was electrically transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P^{SQ}, Millipore, USA) and stained with Ponceau-S stain. The N-terminal sequences of two proteins (migrating at 15 kDa and 17 kDa) were then established using a PPSQ 31A sequence analyzer (Shimadzu, Japan).

2.5. Sequencing the cDNA of the gene encoding a 15-kDa protein

Total RNA was extracted from the sea cucumber viscera using ISOGEN (Nippon Gene, Japan). cDNA was prepared using SMART™ RACE cDNA amplification kit (Clontech, USA) according to the manufacturer's instruction. All primer sequences and PCR conditions employed are listed in a supplemental data (Table A1). cDNA sequencing was performed as follows. First, 3′ RACE PCR was performed using cDNA from the intestine, Taq polymerase (Ex-Taq, Takara, Japan), a forward degenerate primer (F1; designed based on the obtained N-terminal amino acid sequence), and a reverse adapter primer (nested universal primer, NUP-A, Clontech). Nested PCR was done using primer F1 and a degenerate reverse primer (R1) designed based on amino acid sequences of SJL-1. The amplicon was ligated into a vector (pGEM-T Easy, Promega, USA), introduced into *Escherichia coli*-DH5α competent cells (HIT, RBC Bioscience, Taiwan), and sequenced using Big-Dye Terminator Cycle sequencing kit ver. 3.1 (Applied Biosystems, USA)

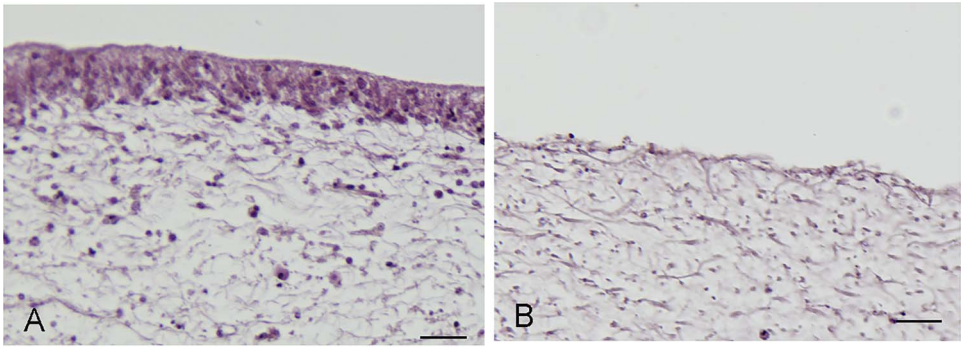

Fig. 1. Microscopic observation of the body surface of *A. japonicus* before (A) and after (B) scraping of the surface with a surgical knife. Paraffin sections (4-μm thick) stained with hematoxylin-eosin are shown. Scale bars, 50 μm.