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Purification, characterization and biological effect of lectin from the marine sponge *Stylissa flexibilis* (Lévi, 1961)

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ABSTRACT

SFL, a lectin from the marine sponge *Stylissa flexibilis* was purified by cold ethanol precipitation followed by ion exchange chromatography on DEAE Sepharose column and Sephacryl S-200 gel filtration. SFL is a dimeric glycoprotein of 32 kDa subunits linked by a disulfide bridge with a molecular mass of 64 kDa by SDS-PAGE and 65 kDa by Sephacryl S-200 gel filtration. SFL preferentially agglutinated enzyme treated human A erythrocytes. The activity of lectin was strongly inhibited by monosaccharide D-galactose and glycoproteins asialo-porcine stomach mucin and asialo-fetuin. The lectin was Ca^{2+} dependent, stable over a range of pH from 5 to 8, and up to 60 °C for 30 min. The N-terminal amino acid sequence of SFL was also determined and a blast search on amino acid sequences revealed that the protein showed similarity only with lectins from the marine sponge *Spheciospongia vesparia*. SFL caused agglutination of *Vibrio alginolyticus* and *V. parahaemolyticus* in a dose dependent manner and inhibited the growth rates of the virulent bacterial strains. Growth inhibition of *V. alginolyticus* and *V. parahaemolyticus* with SFL was not observed in the presence of D-galactose or asialo-porcine stomach mucin, suggesting that the lectin caused the agglutination through binding to the target receptor(s) on the surface of Vibrios. Thus, the marine sponge *S. flexibilis* could promise to be a good source of a lectin(s) that may be useful as a carbohydrate probe and an antibacterial reagent.

1. Introduction

Lectins, or carbohydrate-binding proteins, are present in various organisms from viruses to mammals and serve as recognition molecules between cells, cell and matrix, and organisms. Owing to the capability of discriminating carbohydrate structures, not only are lectins used as valuable biochemical reagents in many research fields, including glycomics, but they are promising candidates for medicinal and clinical application (Sharon and Lis, 2003).

Recently, a large number of lectins from marine sponges that possess various biochemical characteristics have been purified and identified including: galectins, C-type, tachylectin-like and F-type lectins (Gardères et al., 2015). Like other natural products isolated from marine organisms, lectins from marine sponges have shown great potential as candidates for new drugs, due to their wide range of biological activities, such as pro-inflammatory and antitumoral (Kawsar et al., 2011; Queiroz et al., 2009), mitogenic (Atta et al., 1989; Bretting et al., 1981a; Xiong et al., 2006; Dresch et al., 2012), chemotactic (Dresch et al., 2008; Queiroz et al., 2008), cytotoxic properties (Pajic et al., 2002), antibacterial activities (Schröder et al., 2003) and anti-protozoan effects against Leishmania chagasi (Moura et al., 2006; Medeiros et al., 2010). Thus, marine sponges may be dominant sources of useful lectins for basic research and applications.

Furthermore, antibacterial activities have been reported for lectins from various biological sources (Santi-Gadelha et al., 2006; Charungchitrak et al., 2011; Schröder et al., 2003; Moura et al., 2006; Kawsar et al., 2011). However, little is known about the effects of lectins from marine organisms towards marine vibrios, except the species-specific activities against vibrios have been reported for the lectins from the red algae, such as ESAs from *Eucheuma serra* and GMA from *Galaxaura marginata* (Liao et al., 2003), EDAs from *Eucheuma denticulatum* (Hung et al., 2015a), KSAs from *Kappaphycus striatum* (Hung et al., 2015b) and GPE from *Gracilaria fisheri* (Boonsri et al., 2017). Marine vibrios are halophilic Gram-negative proteobacteria,

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which occupy a diverse range of ecological niches including sediments, water column, and in association with organisms either as symbionts or pathogens (Tracy et al., 2007). It is an economically important disease of fish, marine invertebrates and is responsible for high mortality rates in aquaculture worldwide (Marhual et al., 2010). Among the Vibrios, *Vibrio alginolyticus* and *V. parahaemolyticus* are quite important, since it causes serious episode to marine fish and shellfish including shrimp (Zorrilla et al., 2003; Marhual et al., 2010). In Vietnam, *V. alginolyticus*, *V. parahaemolyticus* and *V. harveyi* reduced greatly yields of farmed shrimps (FAO, 2013). Thus, the objective of this research was to report on the isolation, biochemical properties, carbohydrate-binding specificity and biological effect including the antibacterial activities against the shrimp pathogenic vibrios of the lectin from marine sponge *S. flexibilis* for future applications.

2. Materials and methods

2.1. Materials

Marine sponge *Stylissa flexibilis* was collected at the VinhHy bay, NinhThuan Province, VietNam, in April 2016, brought to the laboratory, and kept at − 20 °C until used. Prepacked columns used were purchased; Sephacryl S-200 (1.6 × 60 cm) and DEAE Sepharose fast flow ion exchange chromatographic column (1.6 × 20 cm) from GE Healthcare (Sweden). TSKgel ODS-80™ column (4.6 × 150 mm) was obtained from Tosoh Corporation (Japan). Blood from rabbit was obtained from the Institute of Vaccine-NhaTrang, VietNam and human A, B, and O bloods from KhanhHoa General Hospital, VietNam. L-fucose, D-glucose, D-mannose, D-galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, *N*-acetyl-D-galactosamine, transferrin, fetuin, porcine thyroglobulin, and porcine stomach mucin were purchased from Sigma Chemical Co. Yeast mannan and *N*-acetyl-neuraminic acid was from Nakarai Chemical Co. All other chemicals used in this study were of the highest purity available. Three species of shrimp pathogenic Vibrios, *Vibrio alginolyticus*, *V. harveyi* and *V. parahaemolyticus* were obtained from Institute of Aquaculture Research No. 3, NhaTrang, VietNam.

2.2. Extraction and purification of lectin

Specimens were cut into small pieces, ground with a mixer machine and extracted (1:2, *w/v*) with 50 mM Tris–HCl, 150 mM NaCl buffer (pH 7.5) for 6 h at 4 °C. After filtration through a cheese cloth, the filtrate was centrifuged at 3500 × *g* for 30 min at 4 °C. To the supernatant, cold absolute ethanol (− 20 °C) was added to attain a final concentration of 80% and the mixture was kept at 4 °C overnight. The resulting precipitates were collected by centrifugation at 3500 × *g* for 30 min at 4 °C. The pellet was washed three times by cold absolute ethanol (− 20 °C) and centrifuged at 3500 × *g* for 30 min at 4 °C. The pellet was thoroughly dialyzed against 20 mM Tris–HCl buffer (TB), pH 7.5 and examined for hemagglutination activity using erythrocytes from various human and rabbit in a native state or enzyme-treated with trypsin or papain. The non-dialyzable fraction was applied to a DEAE Sepharose fast flow ion exchange chromatographic column (1.6 × 20 cm), equilibrated with the above buffer. Unbound proteins and pigments were eluted with above buffer at a flow rate of 10.0 mL min^{-1} until the column effluents showed absorbance of less than 0.002 at 280 nm, lectin was eluted with 0.5 M NaCl in 20 mM Tris–HCl buffer, pH 7.5; the active fractions were pooled, concentrated by ultrafiltration, and dialyzed against 50 mM Tris–HCl, 150 mM NaCl buffer (pH 7.5). The concentrate was subjected to gel filtration on a Sephacryl S-200 column (1.6 × 60 cm) equilibrated with above buffer. The column was eluted with the same buffer at a flow rate of 0.8 mL min^{-1} and the active fractions were collected. The eluate was monitored at absorbance of 280 nm for protein and for hemagglutination activity with trypsin-treated human A erythrocytes using TBS buffer containing 20 mM $CaCl_2$. Active fractions were pooled and subjected to further analysis.

2.3. Preparation of a 2% suspension of native or enzyme-treated erythrocytes

Each blood sample was washed three to five times with 50 volumes of 150 mM NaCl. After washing, a 2% erythrocyte suspension (*v/v*) was prepared in 150 mM NaCl and used as native erythrocytes. Trypsin- or papain-treated erythrocytes were prepared as follows. One-tenth volume of 0.5% (*w/v*) trypsin or papain solution was added to a 2% native erythrocyte suspension, and the mixture was incubated at 37 °C for 60 min. After incubation, the erythrocytes were washed three to five times with saline and a 2% suspension (*v/v*) of trypsin- or papain-treated erythrocytes was prepared in saline (Hori et al., 1986).

2.4. Hemagglutination assay

Hemagglutination assays were carried out using a microtiter method in a 96-well microtiter V-plate (Hori et al., 1986). First, 25 μL amounts of serially two-fold dilutions of a test solution were prepared in 50 mM Tris–HCl, 150 mM NaCl buffer (pH, 7.5) containing 20 mM $CaCl_2$ on a microtiter V-plate and incubated at room temperature for 1 h. To each well, 25 μL of a 2% erythrocyte suspension was added and the mixtures gently shaken and incubated at room temperature for 2 h. A positive result was indicated by formation of a uniform layer of coagulant over the surface of the well. On the other hand, a negative test result was indicated by the formation of a discrete “button” at the bottom of the well. Hemagglutination activity was expressed as a titer, the reciprocal of the highest two-fold dilution exhibiting positive hemagglutination. The assay was carried out in duplicate for each test solution.

2.5. Hemagglutination-inhibition test

Hemagglutination-inhibition tests were carried according to the method previously described (Hori et al., 1986). All inhibitors were dissolved in 150 mM NaCl at an initial concentration of 100 mM for monosaccharides and 2000 μg mL^{-1} for glycoproteins. First, 25 μL amounts of serially two-fold dilutions of sugar or glycoprotein were prepared in TBS containing 20 mM $CaCl_2$. To each well, an equal volume of a lectin solution (4 doses of agglutination) prepared in TBS containing 20 mM $CaCl_2$ was added, and the plate was mixed gently and allowed to stand at room temperature for 1 h. Finally, 25 μL of a 2% suspension of trypsin-treated human A erythrocytes was added to each well, and the plate was gently shaken and incubated for a further 1 h. Inhibition was observed macroscopically and inhibition activity was expressed as the lowest concentration of sugar or glycoprotein at which complete inhibition of hemagglutination was achieved. The assay was performed in duplicate per sugar and glycoprotein. Asialotransferrin, asialo-fetuin, asialo- porcine thyroglobulin and asialo-porcine stomach mucin were prepared by hydrolyses of their parent sialoglycoproteins with 0.05 M HCl for 1 h at 80 °C followed by dialysis against saline overnight.

2.6. Preparation of trypsin-treated porcine stomach mucin

Porcine stomach mucin (10 mg) was dissolved in 5 mL of 50 mM TBS (pH 7.5). Trypsin (5 mg) was added to the sample and the solution obtained was incubated at 37 °C for 24 h. Treated PSM was heated to 100 °C for 30 min then cooled (final reaction volumes were 10 mL) and further used as inhibitor.

2.7. Effects on hemagglutination activity of divalent cations, pH, and temperature

To examine the effects of divalent cations on hemagglutination