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Sialic acid-specific lectin participates in an immune response and ovarian development of the banana shrimp *Fenneropenaeus merguiensis*

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ABSTRACT

A sialic acid-specific lectin was purified from the hemolymph of *Fenneropenaeus merguiensis* by repetitive affinity fetuin-agarose column chromatography. The purified *F. merguiensis* lectin (called FmL) consisted of two distinct 30.9 and 32 kDa subunits with identical N-terminal amino acid sequences of ten residues. FmL was also composed of sugar moieties; glucosamine, glucose, mannose and *N*-acetyl neuraminic acid but not N-glycolyl neuraminic acid. It was postulated to be a glycoprotein as it was positively stained by glycoprotein staining kit and detected by some bionylated plant lectins. Deglycosylation by either peptide N-glycosidase F or trifluoromethanesulfonic acid turned both types of FmL subunits to 28 kDa peptides. The internal peptide sequence of FmL was similar to a fibrinogen-related domain of human ficolin and the horseshoe crab lectin. Determination of the lectin concentrations in the hemolymph was performed by ELISA while its hemaglutinating activity (HA) was tested by hemagglutination. Both specific lectin concentrations and HA increased as shrimp developed ovarian maturation stages 2 to 4. Their constitutive levels were found in pre-vitellogenic females and higher than those of males. Both specific lectin concentrations and HA of FmL were inducible to the highest levels at 12 h after *F. merguiensis* was challenged by pathogenic *Vibrio harveyi*. The FmL-induced agglutination of *V. harveyi* was specifically abolished by sialic acid, fetuin and bacterial cell wall components. These findings might indicate the implication in an immune response of FmL to protect the shrimp themselves or their spawning eggs towards pathogenic bacteria in surrounding environment.

1. Introduction

Invertebrates including crustaceans lack an adaptive immune system, but they develop efficient immune defenses capable of protecting them against invading microorganisms. Crustacean innate immunity is initially triggered by pattern recognition proteins (PRPs), which can recognize conserved molecules on the microbial cell surface. The specific binding of PRPs to these molecules leads to activation of humoral and cellular responses (Janeway and Medzhitov, 2002; Beutler, 2004). The hemolymph of invertebrates was found to contain internal defense factors against potential pathogens and humoral factors or PRPs such as lectins that contribute to such defense responses by promoting an agglutination of invading bacteria. They are present in the invertebrate hemolymph and actively participate in cellular and humoral immune recognition mechanisms (Cooper et al., 1992). Decapod lectins seem to share the capacity to interact with *N*-acetylated sugar residues (Ratanapo and Chulavatnatol, 1990; Rittidach et al., 2007). A lectin in the hemolymp of *Fenneropenaeus merguiensis* was reported to be specific for *N*-acetylated sugar espectially *N*-acetyl neuraminic acid. This sialic acid-specific lectin could selectively agglutinate shrimp pathogenic but not non-pathogenic bacteria (Rittidach et al., 2007). In some crustacean species, lectins are stored in hemocyte cytoplasmic granules and released to the hemolymph upon infectious challenge (Vasta, 1992; Sierra et al., 1999; Jayasree, 2001). Monodin was reported to increase in the hemolymph of *Penaeus monodon* after infection with pathogenic *Vibrio vulnificus* (Ratanapo and Chulavatnatol, 1992). Investigaion of lectin genes in relation with their roles is a sensitive approach. Recently, many shrimp lectins have been cloned and charaterized for their roles in the immune responses (Wang et al., 2009; Rattanaporn and Utarabhand, 2011; Thepnarong et al., 2015). There is no other report concerning sialic acid-specific lectin gene from decapods including shrimp. It may need more information at the protein level of this lectin. In this study, we reported another approach to purify the same sialic acid-specific lectin from *F. merguiensis* which gained higher yield and purity and its chemical and biological properties were also presented.

Several roles have been noted for invertebrate lectins, including participitation in defense response, phagocytosis, cell aggregation, fertilization, embryonic development, and transport of complex sugars (Vasta, 1992; Sierra et al., 1999; Rattanaporn and Utarabhand, 2011). The elevation of the acorn banacle (*Megabalanus rosa*) lectin levels occurred along with ovarian development (Muramoto et al., 1991). In

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oviparous animals including shrimp, the most important sources of nutrients for the development of oocytes are yolk proteins which are mainly lipoglycoproteins named vitellin. During vitellogenesis or yolk synthesis, vitellin is accumulated in oocytes during ovarian maturation. Otherwise, another yolk component is lipoprotein 1 (LP1) which is only found in the hemolymp of female *Penaeus vannamei* and *Penaeus californiensis* (Yepiz-Plascencia et al., 1998). LP1 is identical to β-1,3-glucan binding protein, which is involed in triggering the immune response (Cerenius et al., 1994). By challenge of *Penaeus indicus* females with the pathogenic *Vibrio penaeicida*, the higher mortality occurred earlier in pre-vitellogenic shrimp than in vitellogenic females (Avarre et al., 2003). *F. merguiensis* s is one of the economically important species in Thailand. They face up to a disease caused by the violent pathogenic bacterium, *Vibrio harveyi*. Considering the possible relation between vitellogenesis and immune response in crustaceans, this report, performed on the shrimp, *F. merguiensis*, addresses the question: does the hemolymph lectin participate in a defense response towards pathogenic bacterial challenge and ovarian maturation?

2. Materials and methods

2.1. Animals and serum preparation

F. merguiensis shrimp (15–30 g in body weight) were collected from Nakhon Si Thammarat province, Thailand. They were maintained in aerated tanks and fed with food pellets four times a day. Hemolymph was drawn from the pericardial sinus and left to clot at 4 °C overnight. After centrifugation at 2500 ×*g* at 4 °C for 20 min, the serum was immediately used or stored at −20 °C for further analysis.

For *V. harveyi* challenge experiment, male shrimp of 15–20 g in body weight were used. In addition, lectin content and hemagglutinating activity in the hemolymph of adult shrimp in related to sex or ovarian maturation were also evaluated. Ovarian development in female *F. merguiensis* was classified into four stages on the basis of the colour and size of the ovary observed through the external carapace as previously described in Auttarat et al. (2006). Here, we defined shrimp at different stages of ovarian development as follows: stage 1 as previtellogenic; stage 2 as early vitellogenic; and stage 3–4 as vitellogenic. Thus, females with each stage of ovarian development and adult males were collected and determined for lectin contents or their activities in the hemolymph.

2.2. Purification of sialic acid-specific lectin

Sialic acid-specific lectin was isolated from *F. merguiensis* serum by affinity chromatography following the modified method of Rittidach et al. (2007). Briefly, 5 ml of the serum was applied onto a fetuin-agarose column (Sigma, St. Louis, MO, USA) previously equilibrated with TB-NaCa (50 mM Tris–HCl, pH 7.5–0.3 M NaCl-0.1 M $CaCl_2$). Unbound material was washed from the column with equilibrating buffer at a 15 ml/h flow rate. Fractions (1 ml) were collected and measured for the absorbance at 280 nm. After washing until base-line absorbance was reached, bound lectin was desorbed with 0.1 M GlcNAc (*N*-acetyl glucosamine) in the same buffer. Dialysis of the eluate fractions was monitored against 50 mM Tris-HCl, pH 7.5 at 4 °C overnight and then they were assayed for hemagglutinating activity (HA). Fractions with high HA were pooled, concentrated and reloaded onto a new fetuin-agarose column and treated in the same manner as the first one except fractions of 0.6 ml were collected. The bound fractions containing high HA were pooled, concentrated, dialysed and analyzed for purity of the lectin by 4–10% native polyacrylamide gel electrophoresis (native PAGE) (Davis, 1964). Protein content was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

2.3. SDS-PAGE

SDS-PAGE (Laemmli, 1970) was carried out using a slab 6–18% gel in the presence or absence of 1% β-mercaptoethanol at a constant voltage of 250 V for 2 h. The molecular mass of the subunits of purified lectin was estimated by measuring its relative mobility in

SDS-PAGE compared to those of the molecular weight standards (Amersham Pharmacia Biotech, Uppsala, Sweden). Protein bands were stained with Coomassie blue (0.02% Coomassie brilliant blue R-250 in 50% methanol-7.5% acetic acid) or by a silver staining kit (Bio—Rad, California, USA). For glycoprotein staining, SDS-PAGE of purified lectin samples and glycoprotein standards ($\propto_2$-macroglobulin, 180 kDa; glucose oxidase, 97 kDa; $\propto_1$-acid glycoprotein, 42 kDa and avidin, 18 kDa) were run on a 10% slab gel. Thereafter, the gel was stained with Pro-Q Emerald 300 glycoprotein gel staining kit (Molecular Probes, Eugene, OR, USA) following the manufacturer's protocol. Glycoprotein bands were visualized using a 300 nm UV transilluminator.

2.4. Peptide sequence analysis

For N-terminal amino acid sequence analysis, purified lectin was separated in SDS-PAGE and transferred to a polyvinyl difluoride (PVDF) membrane in 10 mM 3-(cyclohexylamino)-1-propane-sulfonic acid containing 1% methanol at a constant current of 500 mA for 1.5 h. After Coomassie blue staining and extensive rinsing in distilled water, the 30.9 kDa and 32.3 kDa bands were cut out separately and subjected to N-terminal sequencing by Edman degradation (Edman, 1950) in a protein/peptide sequencer.

For peptide analysis by MALDI-TOF-TOF MS, the protein bands (30.9 and 32.3 kDa) on SDS-polyacrylamide gel of purified lectin were excised and subjected to in-gel tryptic digestion. Mass spectra of these two samples were obtained on a matrix assisted laser desorption and ionization/time-of-flight mass spectrometer (MALDI-TOF MS), TOF/TOF 4700 Proteomics analyzer (Applied Biosystems, Perkin-Elmer, CA, USA). MALDI spectra were calibrated using a peptide mixture provided by the manufacturer (Applied Biosystems). Peptide identification was realized by the method of peptide mass fingerprinting (PMF) and database searches.

2.5. Quantification of neutral amino sugars by HPAEC-PAD

Purified lectin was hydrolyzed with 2 M trifluoroacetate (TFA) at 100 °C for 4 h to cleave all glycosidic linkages. After drying, the hydrolysate was dissolved in water and determined by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a CarboPac PA-1 column eluted with 16 mM NaOH. Common monosaccharide standards found in invertebrates (mannose, galactose, glucose, *N*-acetyl glucosamine, *N*-acetyl galactosamine, fructose) were treated in parallel and used for calibrating the HPAEC-PAD response. The printout of the results shows the profile and individual monosaccharide content expressed in nanomoles present in the volume injected. The aforementioned monosaccharides were identified by elution position. Blanks representing background from the method used for sample preparation were required for optimum quantitative results.

2.6. Determination of sialic acid content

Sialic acid content was determined as DMB (1,2-diamino-4,5-methylenedioxybenzene) derivatives by reverse-phase HPLC with fluorescent detection. Purified lectin was dissolved in a final concentration of 2 M acetic acid and heated to 80 °C for 3 h to release sialic acids. The released materials or filtrates were collected by ultra-filtration through a 3000 MWCO filter, derivatized with DMB and then analyzed by reverse-phase HPLC with a fluorescence detection. Identification

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