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Activation of phenoloxidase activity by humoral lectin in hemocytes of freshwater crab *Paratelphusa jacquemontii*

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ABSTRACT

The lectin, Pjlec isolated from the hemolymph of the freshwater crab *Paratelphusa jacquemontii* hemagglutinated (HA) with mice, rabbit and rat erythrocytes. However, the lectin failed to agglutinate neraminidase treated asialylated erythrocytes showing its sialic acid specificity. The poyacyrlamide gel electrophoresis of lectin yielded 310 kDa proteins, on sodium sulphate dodecyl (SDS) gel appeared as a tetramer with subunits of 76 kDa. The observation of *in vitro* phagocytosis in granular hemocytes of lectin opsonized rabbit erythrocyte by Transmission electron microscopy (TEM) showed the release of lytic vesicles by exocytosis prior to engulfment. The Pjlec lectin also showed an ability to oxidize L-3, 4 dihydroxyphenylalanine (L-DOPA) and in hemocyte lysate preparation (HLS) was enhanced on reduction with SDS and on proteolytic cleavage with trypsin. The lectin appeared to have a regulatory role in activation of enzyme activity associated with phagocytosis and melanin formation.

1. Introduction

Pathogenesis in different species of crustacea is difficult to assess as the susceptibility and resistance to microbial infection vary with the immune mechanisms that appear distinct among the species [1,2]. The innate immune system in crustacea comprise of cellular and soluble humoral factors with diverse mechanisms of recognition and elicitation of defensive reactions for elimination of the pathogens [3]. The immune responses appear initiated by recognition of pathogen associated molecular proteins (PAMPs) on non-self or invading microbe by host pathogen recognition receptors, PRR [4].

The molecules of innate immunity, lectins, prophenoloxidase, lysozyme and antimicrobial peptides or proteins elicit cellular immune response in hemocytes such as phagocytosis, nodulation and encapsulation [5]. The lectins are ubiquitous proteins or glycoproteins with binding specificity to sugars or glycosidic linkages at terminal sugars of glycoproteins and glycolipids to recognize and discriminate self from non-self cell surface or infectious microbes [6]. The characteristic ligand binding specificity of the lectin proteins are determined by the combined effect of its primary amino acid sequence and the motif interacting with the glycan structure [7]. The ability of lectins to facilitate phagocytosis as opsonin [8,9] or complement [10], encapsulation [11], and antibacterial activity [12] explains its potent role in microbial challenge.

The activation and inhibition of immune effectors regulate the host defense system in crustacea. The phenoloxidase (PO) a copper containing enzyme catalyzes synthesis of melanin and are related to cellular immune responses of phagocytosis, nodulation and encapsulation [13,14]. The processes of phagocytosis include lytic activity and exocytosis following degranulation [15]. The zymogens of PO the prophenoloxidase (proPO) are activated by prophenoloxidase activating enzyme, a serine proteinase in freshwater crayfish *Pacifastacus leniusculus* [16]. The event of degranulation also appears to release a 76 kD adhesion factor identified as peroxinectin, with peroxidase activity important for interaction of hemocytes with the pathogens concomitant to activation of the proPO system [17]. Also, the antimicrobial peptides or proteins (AMPs) described as small cationic, amphipathic, gene-encoded molecules are highly expressed and upregulated in crustacean hemocytes on microbial challenge [18,19]. Moreover, the cryptic function of immune effectors, such as the proteins of the proPO system and lysosomal degrading enzymes in the hemocyte granules eventually regulate cellular and humoral defense functions [20].

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Previous study on the lectin Pjlec isolated from the serum of freshwater crab *Paratelphusa jacquemontii* has shown that lectin with its ability to interact with sialylated glycoconjugate cell surface serves as a recognition molecule and opsonin [8,21]. The present study concisely elucidates the structure and amino acid composition of the lectin. The induction of phenoloxidase activity and oxidation of di-phenol substrate by lectin has also been studied to understand the role of lectin in cellular immunity. The sialic acid specific lectin from the prostomian crab, requires further elucidation in human viral or bacterial infections and cancer related to adherence or attachment to sialylated cell surface [22–24]. Also with its ability to oxidize L-3, 4 dihydroxyphenylalanine (L-DOPA) need to be investigated with the perspective of neural function in higher organisms.

2. Materials and methods

2.1. Animal and maintenance

The freshwater crab *P. jacquemontii* was collected from the fields in Kanyakumari district of south India (8.15°N and 77.45°E) and maintained in the laboratory in tanks (0.5 × 0.5m) with water to just immerse the crabs and being a tropical place the temperature remained moderate (27–30 °C) throughout the year.

2.2. Preparation of crab sera

The exuded hemolymph from the excised dactyli of the intermoult crabs was collected in a test tube kept on ice and centrifuged at 5000*g*, 30 min at 4 °C and the serum was obtained as supernatant. The protein of the test samples was estimated by Bradford method [25] using bovine serum albumin as the standard.

2.3. Characterization of isolated lectin Pjlec

2.3.1. Electrophoresis

The isolated lectin Pjlec was analysed in discontinuous polyacrylamide gel electrophoresis [26] and with or without β-mercaptoethanol in discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [27]. In the two dimensional (2D) electrophoresis using the immobilized pH gradient (IPG) strip (7 cm length) following the protocol of Bio-Rad system the protein band of the lectin was observed by silver nitrate staining [28].

2.3.2. Amino acid quantification of the lectin, Pjlec

The amino acid composition of the lectin Pjlec was quantitatively analysed on Agilent High Performance Liquid Chromatography, HPLC 1100 series, USA and Agilent TC C18 (24.6 × 150 mm), 5 μ column, with absorbance measured at 254 nm. The flow rate of 1ml\min for run time of 82 minThe coupling reagent (100 μl) of phenyl isothiocyanate (PITC), methanol, triethylamine and filtered MQ and gradient system of buffer A: 10 mM sodium acetate adjusted to pH 6.4 with 6% acetic acid and buffer B: 10 mM sodium acetate 60% acetonitrile (v/v).

2.3.3. Hemagglutination assay (HA) of the lectin, Pjlec

Blood collected from buffalo mice, rabbit and rat in modified alsevier's medium, (sodium citrate 30 mM, NaCl 77 mM, glucose 114 mM, neomycin sulphate 100 μg/ml, chloramphenicol 330 μg/ml,pH 6.1) was washed thrice with TBS (Tris 50 mM, NaCl 100 mM, $CaCl_2$ 10 mM; *p*H 7.5) by centrifugation (5000 x g 10 min, RT) and 1.5% erythrocyte suspension for each erythrocyte type prepared. The serum, HLS and purified lectin Pjlec (250 μg/ml) 25 μl each were serially diluted with equal volume of TBS in V-bottom microtiter plate (Grenier) and 25 μl of erythrocyte suspension added. The hemagglutination titer or HA titre (the unit of agglutinin activity) was determined as the reciprocal of the highest dilution of samples that gave positive agglutination.

The collection of the crab and blood from mammals were in **"Compliance with Ethics Guidelines".** Institutional Animal Ethics Committee-IAEC no.: 05/02/2012: CPCSEA360/01/a dated 19.06.2001.

2.3.4. Binding specificity of Pjlec

The protein on erythrocyte surface were removed by incubation for 1 h at 37 °C in TBS suspension containing trypsin/pronase (5 mg/ml), washed with saline (4000 x g, 5 min at RT) and 1.5% suspension in TBS was used for HA assay. The asialo erythrocytes were prepared by incubation of the erythrocyte types for 3 h at 37 °C as a suspension (50% v/v) of PBS-BSA (NaH_2PO_4/Na_2HPO_4–10 mM, NaCl–100 mM, BSA 1 mM) containing 5 mM $CaCl_2$ and 10 mU of *Vibrio cholera* neuraminidase and *O*-glycosidase (cloned from *Enterococcus faecalis* and expressed in *E. coli*). The treated cells were washed with PBS-BSA three times and pelleted by low speed centrifugation and 1.5% suspension was used for HA activity.

2.4. Hemocytes

The hemolymph collected in pre-chilled iso-osmotic buffer (50 mM Tris, 210 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 1 mM dextrose, pH 7.5, 480 *mOsm*) at dilution ratio of 1:9 and fixed in 3% glutaraldehyde (1 h, 4° C), pelleted (10,000 x g, 5 min at RT) and washed in 0.1 M sodium cacodylate buffer. The fixed hemocytes were post fixed in 1% osmium tetroxide, dehydrated in ascending alcohol graded series (50–100%), cleared by propylene oxide and processed for Transmission electron microscope, TEM (Phillips Technai T12 spirit by Netherland). Ultrathin sections, stained with uranyl acetate were obtained as micrographs and observed for hemocytes

2.4.1. In vitro *phagocytic activity*

Rabbit erythrocyte fixed in glutaraldehyde [29] served as target cell for *in vitro* phagocytosis in hemocyte. The sialic acid specific lectin isolated from the serum of the crab was used for the preparation of opsonized rabbit erythrocyte [8]. The freshly collected hemolymph from the crab in pre-chilled iso-osmotic buffer (dilution 1:9 ratio) was incubated with opsonized and non-opsonized rabbit erythrocyte separately for 20–30 min. After incubation each hemolymph sample with either opsonized or non opsonized erythrocyte were immediately transferred and fixed in 3% glutaraldehyde (1 h at 4° C), pelleted (10,000 x g, 5 min at RT) and washed in 0.1 M sodium cacodylate buffer. The fixed phagocytic hemocytes were processed for TEM (Phillips Technai T12 spirit by Netherland) and TEM micrographs were observed.

2.5. Preparation of Hemocyte lysate supernatant (HLS)

The exuding hemolymph from the excised dactyli of the crabs was collected in pre-chilled iso-osmotic buffer (50 mM Tris, 210 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 1 mM dextrose, pH 7.5, 480 *mOsm*) to dilution of 1:9 and centrifuged at 400 x g, 10 min at 4 °C. The hemocyte sediment was washed and resuspended in 1 ml of 0.1 M sodium cacodylate buffer, homogenized by ultrasonicator (4 pulses of 30 s, 4 °C). The resulting cell homogenate was centrifuged (17000 x g, 15 min, 4 °C) and the HLS was collected as a clear supernatant and stored at −20 °C.

2.5.1. Hemagglutination activity

The methodology followed was same as that given for isolated lectin Pjlec.