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Characterization of a lectin from the craysfish *Cherax quadricarinatus* hemolymph and its effect on hemocytes

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Lectins participate in the immune mechanisms of crustaceans. They have been considered as humoral receptors for pathogen-associated molecular patterns; however, some reports suggest that lectins could regulate crustacean cellular functions. In the present study, we purified and characterized a serum lectin (CqL) from the hemolymph of *Cherax quadricarinatus* by affinity chromatography and determined its participation in the regulation of hemocytes' oxidative burst. CqL is a 290-kDa lectin in native form, constituted by 108, 80, and 29-kDa subunits. It is mainly composed of glycine, alanine, and a minor proportion of methionine and histidine. It showed no carbohydrates in its structure. CqL is composed of several isoforms, as determined by 2D-electrophoresis, and shows no homology with any crustacean protein as determined by Lc/Ms mass spectrometry. CqL agglutinated mainly rat and rabbit erythrocytes and showed a broad specificity for monosaccharides such as galactose, glucose, and sialic acid, as well as for glycoproteins, such as porcine stomach and bovine submaxillary mucin and fetuin. It is a Mn^{2+}-dependent lectin. CqL recognized 8% of crayfish granular hemocytes and increased 4.2-fold the production of hemocytes' superoxide anion in vitro assays when compared with non-treated hemocytes. This effect showed the same specificity for carbohydrates as hemagglutination; moreover, superoxide dismutase and diphenyleneiodonium chloride were effective inhibitors of CqL oxidative-activation. The CqL homoreceptor is a 120-kDa glycoprotein identified in the hemocytes lysate. Our results suggest that CqL participates actively in the regulation of the generation of superoxide anions in hemocytes using NADPH-dependent mechanisms.

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1. Introduction

Crustaceans possess different pathways to eliminate foreign particles and it has been determined that hemocytes participate actively in the immune system [1]. These cells seem to possess different mechanisms located on the cell surface, which allow them to recognize and respond to a variety of signals that, in turn, activate processes such a phagocytosis or encapsulation mechanisms [2]. In crustaceans, the hemolymph contains factors or proteins that can discriminate between self and non-self and trigger downstream reactions [3]. Lectins, due to their specificity for carbohydrate structures, participate in the identification of pathogen-associated molecular patterns (PAMP's) through the carbohydrate recognition domain [4].

Lectins from decapod crustaceans show great heterogeneity in structure and sugar specificity [5,6]. There are many reports on the effect of crustacean lectins on defense mechanisms, such as encapsulation and nodulation processes [7], activation of the prophenoloxidase system [8], synthesis of antimicrobial peptides [4], as well as on their participation in antiviral activity [9]. Moreover, it has been reported that lectins from *Procambarus clarkii* and *Litopenaeus setiferus* participate in opsonization and potentiate phagocytosis [10,11]. Hemocytes produce superoxide anion molecules and nitric oxide, and participate in the immune mechanisms [12]; however, there are not many reports suggesting the possible

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effect of lectins in the regulation of hemocytes function, except in the freshwater prawn *Macrobrachium rosenbergii*, where an increase in the production of radical oxygen species (ROS) has been observed after activation of hemocytes with their lectin [2]. In the present report, we have isolated and partially characterized a serum lectin from *Cherax quadricarinatus* (CqL) and determined its participation in defense mechanisms.

2. Material and methods

2.1. Organisms

Adult organisms of *C. quadricarinatus* in intermolt stage were obtained from the "Estanque Don Pablo" fish farm, Puente de Ixtla, state of Morelos. Mexico. Crayfish were acclimated to laboratory conditions for 2 weeks before being used on any experimental study. Organisms were placed in aerated ($O_2 > 5.0$ mg/L) ponds and fed once a day with a commercial diet (Malta Cleyton, SA, Mexico) containing 30% protein. Hemolymph was obtained inserting a 21-gauge needle into the pericardial sinus, at the base of the first abdominal segment, and allowed to rest 24 h at 4 °C. The serum was cleared by centrifugation at 16,000 *g* at 4 °C for 30 min, and then dialyzed against phosphate buffered saline (PBS: 0.01 M sodium phosphate, 0.14 M sodium chloride, pH 7.2) and stored at −20 °C until use.

2.2. Lectin extraction and purification

The lectin (CqL) was purified by affinity chromatography on glutaraldehyde-fixed stroma from Wistar rat erythrocytes. The stroma was obtained by hyposmotic treatment and fixed with 1% glutaraldehyde in PBS (0.01 M Na_2PO_4, 0.15 M NaCl, pH 7.4). The stroma was physically entrapped in a 25 × 1.5-cm column containing Sephadex G-25 (Pharmacia Fine Chem., Uppsala, Sweden) as previously described [5]. The column was equilibrated with PBS at room temperature with a flow rate of 15 mL/h; 1 mL of *C. quadricarinatus* serum (100 mg protein/mL) was applied to the affinity column at a flow rate of 15 mL/h. Unbound material was washed from the column with PBS until absorbance, at 280 nm, of the eluent was <0.01. The lectin was washed with 3% acetic acid and 1.5-mL fractions were collected; the pH was neutralized with 1 M NaOH, and the hemagglutinating activity was tested in presence of rat erythrocytes (2% suspension in PBS). Fractions were then pooled, dialyzed against PBS, and stored at −70 °C until use.

2.3. Analytical methods

Protein concentration was determined by the method of Bradford (Bradford, 1976) with Coomassie blue R-250 (Sigma Chem., St. Louis, MO, USA), using bovine serum albumin as standard. Carbohydrates composition was determined on the heptafluorabutyrate derivate of O-methyl glycosides, obtained after methanolysis in 0.5 M methanol-HCL for 24 h at −80 °C; lysine (Sigma) was used as internal standard, by gas chromatography [13].

Amino acid composition of CqL was determined as follows: 100 μg CqL was hydrolyzed under vacuum with 2 mL of 6 M HCl at 110 °C in sealed tubes for 24, 48, and 72 h. The samples were analyzed with an automatic amino acid analyzer, Durrum 500, using nor-leucine as internal standard [14].

2.4. Determination of molecular mass

Molecular mass and homogeneity of the purified lectin were evaluated in 10% polyacrylamide gel electrophoresis (PAGE) with urea [15]; 1 mg of CqL was mixed with sample buffer (125 mM Tris–HCl, 20% glycerol, 6 M urea, 4% SDS, 0.2 M DTT, 0.02% bromophenol blue; pH 8.8). After electrophoresis, gels were stained with 0.1% Coomassie brilliant blue G-250 (Sigma). The native molecular mass of the purified lectin was determined by gel filtration chromatography on a column (100 × 1.6 cm) containing Sephacryl S-300 HR (Pharmacia, Uppsala, Sweden) in an FPLC system (Amersham). The column was equilibrated with PBS at a flow rate of 0.5 mL/h. Relative molecular mass of the lectin was obtained by comparing the elution profile with molecular weight standards (Sigma). Eluted fractions (1 mL) were analyzed for OD at 280 nm, and their hemagglutinating activity for rat erythrocytes was assessed.

In order to show the homogeneity of the CqL, we used a Blue native electrophoresis (BN-PAGE) [16]. Briefly, 5 μg of CqL were suspended in 50 mM Bis-Tris and 500 mM 6-aminocaproic acid, pH 7.0, and solubilized with digitonin, at detergent/protein ratios of 1:1 (w/w). The sample was loaded on a linear polyacrylamide gradient gel (4–10%) for Blue Native PAGE (BN-PAGE). The molecular weight of CqL was estimated by using the bovine mitochondrial complexes as standard [17].

2.5. 2D-electrophoresis

For 2D-electrophoresis, CqL (40 μg) was placed into the rehydration mixture (8 M urea, 0.5% CHAPS, 0.2% DTT, IPG buffer at pH 3–10, 0.002% bromophenol blue) (Immobiline, Amersham Biosciences, Uppsala, Sweden). The first dimension for the isoelectric focusing system was performed for 16 h, with a continuous current of 200 V–3500 V. For the second dimension, in 10% PAGE, the gels were balanced in an equilibration buffer (50 mM Tris–HCl at pH 8.8, 6 M urea, 30% glycerol v/v, 2% SDS, 0.002% bromophenol blue). The gel was stained with Coomassie brilliant blue G-250.

2.6. Lc/Ms mass spectrometry

Bands were excised from the 2D-electrophoresis gel. After dehydration with acetonitrile for 10 min at room temperature, the gel pieces were vacuum-dried and rehydrated with sequencing-grade trypsin (Promega, Madison, WI, USA) in ammonium bicarbonate (25 mM, pH 8.5) at 37 °C overnight. The in-gel tryptic digested samples were injected into an integrated nano-LC-ESI MS/MS system (quadrupole/time of flight, Ultima API, Micromass, Manchester, UK). The injected samples were first trapped and desalted isocratically on an LC-Packing PepMap C18 μ-pre-column cartridge (Dionex, Sunnyvale, CA, USA). After dissolving with 0.1% formic acid, the samples were loaded onto an analytical C18 capillary column connected online to the mass spectrometer. Instrumental operation, data acquisition, and analysis were performed under the full control of Mass-Lynx 4.0 (Micromass). The acquired peptide ions were analyzed with the Mascot program (www.matrixscience.com) and NCBInr.

2.7. Hemagglutinating activity

Erythrocytes from several animal species were obtained from the animal facilities of the School of Medicine, UNAM, Mexico. Human erythrocytes were obtained from healthy donors. Blood was collected in sterile Alsever's solution (100 mM glucose, 20 mM NaCl, and 30 mM sodium citrate, pH 7.2) and erythrocytes were washed 4-times with PBS by centrifugation (800 *g* for 10 min). Hemagglutinating activity of serum and purified CqL was assayed in microtiter U plates (NUNC, Denmark) by two-fold serial dilution. The agglutinating activity was tested with 2% erythrocyte suspension in PBS and reported as the inverse of the last dilution showing visible agglutinating activity.

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