Fish & Shellfish Immunology 30 (2011) 241–247

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Characterization of a C-type lectin (PcLec2) as an upstream detector in the prophenoloxidase activating system of red swamp crayfish

Xian-Wei Wang [a,b], Hong-Wei Zhang [a,b], Xiang Li [a,b], Xiao-Fan Zhao [a,b], Jin-Xing Wang [a,b,*]

[a] The Key Laboratory of Plant Cell Engineering and Germplasm Innovation of Ministry of Education, School of Life Sciences, Shandong University, Jinan, Shandong 250100, China
[b] Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, School of Life Sciences, Shandong University, Jinan, Shandong 250100, China

ARTICLE INFO

Article history:
Received 8 June 2010
Received in revised form
6 October 2010
Accepted 17 October 2010
Available online 6 November 2010

Keywords:
C-type lectin
Red swamp crayfish
Prophenoloxidase
Innate immunity

ABSTRACT

C-type lectins are abundantly found in crustaceans. They function in the immune system by recognizing, opsonizing, or agglutinating. Some of them also feature anti-microbial activity. In this study, we identify a hepatopancreas-specific C-type lectin (PcLec2) that responds significantly to immune challenges in red swamp crayfish (*Procambarus clarkii*). Recombinant PcLec2 shows no agglutinating or anti-microbial activity. It can bind to lipopolysaccharides and bacterial cells in the absence of calcium, and its binding to Gram-negative bacteria is stronger than that to Gram-positive ones. Moreover, the protein can stimulate the activation of prophenoloxidase both in vitro and in vivo. We conclude that this C-type lectin may be an upstream detector of the prophenoloxidase activating system in crayfish.

1. Introduction

C-type lectins are a type of key molecule involved in various immune responses in both vertebrates and invertebrates [1]. They are characterized by carbohydrate recognition domains (CRDs), some of which bind sugar ligands in a calcium-dependent (C-type) manner [2,3]. Calcium is directly involved in the interaction of these lectins and carbohydrates [1]. However, the metallic ion is not always the essential factor, and many C-type lectins can still perform their functions in the absence of calcium [4,5].

Many C-type lectins have been reported in crustaceans. These lectins may participate in immune responses generally in two different ways. C-type lectins may first serve as “sensors”. The recognition and binding of conserved microbial components can, on one hand, trigger the following proteolytic cascades, and on the other, promote phagocytosis. In both cases, they function in a manner similar to so-called pattern recognition receptors or opsonins [6,7]. Second, C-type lectins may act as “effectors”. Many of them can agglutinate bacteria, and several even possess direct anti-microbial activity, which is somewhat similar to the effect of anti-microbial peptides [8].

Since crayfish host various bacterial and fungal pathogens [9], research on the immune-related genes of crayfish would be helpful to further understand the innate immune system of invertebrates. A cDNA library was constructed in our laboratory to hunt for the potential candidates involved in the immune system of crayfish. No less than 22 putative C-type lectins were identified from the library, suggesting that they may play crucial roles in protecting crayfish against intruding microorganisms.

In the present study, the C-type lectin PcLec2 with a single carbohydrate recognition domain was chosen to investigate its role in the crayfish immune system. First, the expression pattern of PcLec2 was examined to confirm that it did participate in the immune defense response. Recombinant protein was then produced for the function analysis in vitro. Since the protein could not agglutinate or suppress the growth of bacteria, we focused on its function as a “sensor” to recognize the invading bacteria and then to trigger the following immune actions, especially the prophenoloxidase (proPO) activating cascade. Our results suggested that PcLec2 might be an upstream detector in crayfish anti-bacterial response, and that the successful detection of pathogens would stimulate the activation of proPO.

2. Materials and methods

2.1. Reagents

LPS from *Escherichia coli* 055: B5 was purchased from Sigma (St. Louis, MO, USA). Unizol was obtained from Biostar

* Corresponding author. The Key Laboratory of Plant Cell Engineering and Germplasm Innovation of Ministry of Education/Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, School of Life Sciences, Shandong University, Jinan, Shandong 250100, China. Tel./fax: +86 531 88364620.
E-mail address: jxwang@sdu.edu.cn (J.-X. Wang).

1050-4648/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2010.10.012

(Shanghai, China). RevertAid First Strand cDNA Synthesis Kit was a product of Fermentas (Burlington, Canada). BSA was from SCRC (Shanghai, China) and L-DOPA was from Kayon (Shanghai, China).

2.2. Identification and analysis of PcLec2 cDNA

A cDNA library was constructed from hepatopancreas and gills of red swamp crayfish (*Procambarus clarkii*) and then randomly sequenced. The obtained results were subjected to BLASTx to search the putative C-type lectins. An expressed sequence tag (EST) containing the entire open reading frame and untranslated regions was obtained and resequenced to ensure the correctness. The domain architecture of PcLec2 was predicted by SMART (http://smart.embl-heidelberg.de/). Signal peptide prediction was carried out using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Alignment was performed using MEGA 4.0 and GENEDOC software.

2.3. Cultivation and immune challenge of crayfish

Healthy red swamp crayfish, *P. clarkii*, ranging in weight from 15 to 20 g, were purchased from a local market in Jinan, Shandong, China, and maintained in freshwater at 10 °C. *E. coli* (8099) and *Vibrio anguillarum* kept in our laboratory were cultured overnight in Luria–Bertani (LB) medium and harvested by centrifugation at 6000 rpm for 5 min. After washing with crayfish phosphate buffer solution (CPBS: 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl, 10 mM $CaCl_2$, and 10 mM $MnCl_2$, pH 6.8) [10], the bacteria pellet was resuspended in CPBS to a final concentration of 1×10^9 CFU/mL. One hundred microliters of the microbial-CPBS suspension, LPS-CPBS suspension (0.5 mg/mL), or CPBS was injected into the crayfish via the base of the fourth walking leg. At least three crayfish were treated for each group.

2.4. RNA extraction and cDNA synthesis

Total RNAs were extracted from hemocytes, hepatopancreas, gills, stomach, and intestine of normal crayfish using Unizol following the protocol. At 6 h post-challenge, total RNAs from challenged hepatopancreas were also isolated. The first strand cDNAs were then synthesized and used as templates for the expression profile analysis.

2.5. Expression pattern analysis

For this part of analysis, a pair of primers was designed to produce a short fragment of PcLec2 (PcLec2F1, PcLec2F2, Table 1), and another pair of primers was used to generate a part of the 18S rRNA as the internal control (18S rRNAF, 18S rRNAR, Table 1). Semi-quantitative RT-PCR was performed to analyze the tissue distribution, with 30 cycles for PcLec2 and 25 cycles for 18S rRNA. Quantitative RT-PCR was conducted to detect the immune change of PcLec2 as described previously [7]. The relative expression was calculated using the comparative C_T method with the formula $2^{-\Delta\Delta C_T}$ ($\Delta\Delta C_T = \Delta C_{TPcLec2} - \Delta C_{T18S\ rRNA}$).

Table 1
Sequences of the primers used in this study.

Primers	Sequence (5′–3′)
PcLecF1	GGAACGACGCCAGGGATTACT
PcLecR1	CAGGATACCAAAAGGGACAGCC
PcLecF2	TACTCAGAATTCTGCAACAGTCCCTTCATCCC
PcLecR2	TACTCACTCGAGTCAATTGAGGTCGAGGGATG
18S rRNAF	TCTTCTTAGAGGGATTAGCGG
18S rRNAR	AAGGGGATTGAACGGGTTA

The underlined nucleotides indicate the locations of restricted endonucleases (*Eco*RI or *Xho*I).

Statistical analysis was performed using an unpaired sample *t*-test, and significant differences were considered at $P < 0.05$.

2.6. Preparation of recombinant PcLec2 and antiserum

Based on the full length cDNA of PcLec2, a pair of primers was designed for recombinant expression of the mature peptide (PcLec2F2, PcLec2R2, Table 1). The amplified fragment was digested using *Eco*RI and *Xho*I, and then ligated into the pET30a(+) vector (the vector will add a 5.7 kDa tag to the recombinant protein) or pGEX 4T-1 vector (adding a 27 kDa GST tag to target protein). The recombinant plasmid was introduced into *E. coli* BL21 (DE3) or BL21 component cells for expression under the induction of IPTG. The recombinant protein from pET30a(+) vector was expressed as inclusion body, and the soluble renatured protein was obtained following the described method [11]. Recombinant protein was injected into a New Zealand white rabbit to prepare the antiserum following a published method [11]. The protein from pGEX 4T-1 vector was soluble and purified by affinity-chromatography.

2.7. Agglutinating/hemagglutinating assay

Mid-logarithmic phase bacteria cells, as well as human and rabbit erythrocytes, were harvested and resuspended in TBS. Fifty microliters of the resuspension (2×10^8 CFU/mL for bacteria and 2% v/v for erythrocytes) was mixed with an equal volume of PcLec2 solution (400 μg/mL). $CaCl_2$ was added to a final concentration of 10 mM. After incubation for 1 h at room temperature, the mixture was observed under a microscope. FcLec4 was used as positive control and BSA or GST as negative controls [7].

2.8. Anti-microbial activity test of PcLec2

E. coli and *V. anguillarum* were used in this part. For the anti-bacterial assay, about 200 bacteria cells (the number was determined using a hemocytometer and following serial dilution) were treated with PcLec2 (final concentration at 100 μg/mL) with or without $CaCl_2$ (10 mM), ampicilin plus kanamycin (100 μg/mL each), or BSA (100 μg/mL) in TBS at 37 °C for 0.5 h. The suspension was then plated onto LB agar plates to determine the number of surviving cells.

For the bacteria-growth-suppression assay, 1 μL of an overnight culture (about 10^6 CFU) was inoculated in 3 mL LB medium. PcLec2 (100 μg/mL) with or without $CaCl_2$ (10 mM) was added, and then the tubes were shaken for 200 rpm at 37 °C. The OD at 630 nm (OD_{630}) was measured every 1 h. Ampicilin plus kanamycin (100 μg/mL each) served as the positive control; BSA (100 μg/mL) was used as the negative control.

2.9. Binding activity assay of PcLec2

For the bacteria binding assay, Gram-negative bacteria *E. coli* and *V. anguillarum*, and Gram-positive bacteria *Staphyloccocus aureus* and *Micrococcus luteus* were used. About 2×10^8 microbes, pre-coated with 3% BSA first, were incubated for 1 h with 5 μg PcLec2 in 1 mL TBS at 28 °C by gentle rotation. The microbes were pelleted and washed thrice with TBS by vigorous shaking. The pellets were then subjected to SDS-PAGE, and the binding was determined by Western blot. $CaCl_2$ (10 mM) or EDTA (10 mM) was added in some treatments. Bacteria cells without treatment were used as control.

For LPS binding, different amounts of LPS were incubated for 1 h with 5 μg PcLec2 at 28 °C. Excess *E. coli* cells were then added to absorb the residual protein. After gentle rotation for 1 h at 28 °C, the mixture was centrifuged and the supernatant was analyzed by Western blot. For the treatments with $CaCl_2$ (10 mM), EDTA (10 mM) was added after the first incubation.