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Identification of two LGBPs (isoform1 and isoform2) and their function in AMP expression and PO activation in male hepatopancreas

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ABSTRACT

Two lipopolysaccharides (LPS) and β-1, 3-glucan binding protein (LGBP), designated as *PcLGBP* isoform1 and *PcLGBP* isoform2, respectively, were identified from *Procambarus clarkii* in this study. The full-length cDNA of *PcLGBP* isoform1 was 1308 bp containing an open reading frame (ORF) of 1113 bp encoding a protein of 370 amino acids. The full-length cDNA of *PcLGBP* isoform2 was 1440 bp containing an ORF of 1245 bp encoding a protein of 414 amino acids. Predicted PcLGBP isoform1 and PcLGBP isoform 2 proteins contained a signal peptide, a glycoside hydrolase domain, and a low-complexity region. The difference between the two LGBP isoforms was that PcLGBP isoform2 had 44 more amino acids behind the signal peptide than the PcLGBP isoform1. The *PcLGBP* isoform1 and *PcLGBP* isoform2 transcripts mainly expressed in the hepatopancreas in female and male crayfish. Moreover, the expression levels of the two genes in the hepatopancreas were higher in male than that in female crayfish. Upon being challenged with *Vibrio parahaemolyticus* or LPS, the expression levels of *PcLGBP* isoform1 and *PcLGBP* isoform2 in the hepatopancreas of female and male crayfish were most significantly up-regulated at different time points. The transcripts of anti-lipopolysaccharide factors (ALF5, ALF6, ALF8, and ALF9) and crustins (CRU1, CRU2, CRU3, and CRU4) were evidently down-regulated in the hepatopancreas of *V. parahaemolyticus*-challenged total *PcLGBP* (including *PcLGBP* isoform1 and *PcLGBP* isoform2)-silenced male crayfish. In addition, the phenoloxidase (PO) activity in the hepatopancreas of male crayfish was evidently higher than that of female crayfish. *PcLGBP* knock down could significantly decrease the PO activity in the hepatopancreas lysate (HLS) in male crayfish. The PO activity of male crayfish HLS was significantly increased when incubated with a mixture of recombinant LGBP protein and LPS or β-1, 3 glucan. We conclude that LGBP isoforms from *P. clarkii* function as a pattern recognition protein for recognizing and binding LPS and β-1, 3 glucan, and thus regulate the synthesis of antimicrobial peptides and activate the prophenoloxidase system.

1. Introduction

Procambarus clarkii is an important freshwater species in China, and brings huge economic benefits every year. However, farming of this species has always been threatened by many kinds of pathogenic microorganism infections, including bacteria, viruses, and parasites under deteriorated culture environments [1,2]. To date, no effective methods are available for disease control in *P. clarkii* aquaculture. Therefore, expanding the limited knowledge about the immune defense mechanisms of *P. clarkii* is the current research focus for disease prevention.

Unlike vertebrates, invertebrates lack a true adaptive immune system and only rely on the innate immune system composed of cellular immunity and humoral immunity to resist the invasion of pathogens [3–5]. “Self” and “non-self” recognition, which is mediated by pattern recognition receptors (PRRs), is the first and most vital step of the innate immune system in invertebrates. The PRRs can specifically recognize and bind pathogen-related molecular patterns (PRMPs) existing on the surface of pathogenic microorganisms, such as lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycan (PGN) from Gram-positive and negative bacteria, and β-1,3-glucans from

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fungi, and then activate various immune responses [6,7]. These immune responses including phagocytosis, encapsulation, nodule formation, clotting cascade, induced synthesis of antimicrobial peptides (AMPs), and the activation of prophenoloxidase (proPO) system, have been characterized in invertebrates [8–12].

In crustaceans, several types of PRRs, such as Gram-negative binding proteins, PGN recognition protein, LPS and β-1,3-glucan binding protein (LGBP), β-1,3-glucan binding protein, C-type lectin, tachylectin, galactoside-binding lectins (galectins), masquerade-like protein, and fibrinogen-like domain immunolectins, have been reported [13,14]. LGBP was originally purified from the silk worm *Bombyx mori* with specific affinities for LPS and β-1, 3-glucan [15]. After recognizing and binding the specific PRMPs by LGBP, a series of immune responses is activated in crustaceans. For example, LGBP from *Pacifastacus leniusculus* was involved in the activation of the proPO activating system [16]. In addition, binding of the LGBP from *Marsupenaeus japonicus* and LPS complex could activate the innate immune system [17]. *Penaeus monodon* LGBP (PmLGBP) functioned as a PRR for LPS and β-1, 3-glucan in the shrimp proPO activating system [18]. The LGBP transcript in hemocyte of *Litopenaeus vannamei* was regulated by *Vibrio alginolyticus* infection [19]. A significant enhancement of *Fenneropenaeus chinensis* LGBP transcription was observed in response to *Vibrio anguillarum* and *Staphylococcus aureus* infection [20]. However, less is known about the roles of LGBP in the innate immunity of *P. clarkii*. The pattern recognition function of LGBP and the triggered immune responses remain to be examined.

In this study, we identified two LGBPs (designated as *PcLGBP* isoform1 and *PcLGBP* isoform2) from *P. clarkii*. The tissue distribution and expression patterns of the two LGBP isoforms after *Vibrio parahaemolyticus* or LPS stimulation in female and male crayfish were investigated. Double stranded RNA (dsRNA)-mediated RNA interference (RNAi) analysis was conducted to study the relationship between *PcLGBP* and the synthesis of AMPs or the activation of proPO system. In addition, the recombinant PcLGBP protein (rPcLGBP) and polysaccharides were incubated together to detect their effects on the PO activity. Our results suggest that two LGBP isoforms of *P. clarkii* may act as PRR involved in the innate immunity, and mainly participate in regulating the synthesis of AMPs and activating the proPO system.

2. Materials and methods

2.1. Animals and materials

The *P. clarkii* (approximately 20 g/each crayfish) were purchased from an aquaculture market in Nanjing City, Jiangsu Province, China. The *P. clarkii* were placed in tanks with air-pumped freshwater for temporary cultivation (25 °C–26 °C) at least 7 days before processing. *Escherichia coli* Trans1-T1 and *E. coli* BL21 (DE3) competent cells were purchased from Transgen Biotech (Beijing, China). LPS from *E. coli* 055: B5, PGN from *S. aureus* and β-1, 3-glucan (GLU) were obtained from Sigma (St. Louis, MO, USA).

2.2. Cloning of PcLGBP isoforms full-length cDNA

The hepatopancreas from healthy *P. clarkii* was acquired for total RNA extraction using a high-purity total RNA rapid extraction kit (Spin-Column, BioTek, Beijing, China) following to the instructions of manufacturer. RNA quality was assessed by electrophoresis for 15 min on 1.0% agarose gel, and the total RNA concentration was determined by measuring the absorbance at wavelengths of 260:280 nm. Then, 5 μg of DNA-free total RNA was used to synthesize the 3′- and 5′-RACE-Ready cDNA samples by using SMARTer ® RACE 5′/3′ Kit (Clontech) in accordance with the instructions of manufacturer. On the basis of unigenes obtained from *P. clarkii* transcriptome data, the specific primers (*PcLGBP*-isoform1-R, *PcLGBP*-isoform1-F, and *PcLGBP*-isoform2-F) (Table 1) were designed to clone the 5′ and 3′ fragments of *PcLGBP* isoform1 and *PcLGBP* isoform2 with a Universal Primer A Mix. Polymerase chain reaction (PCR) was conducted in accordance with the protocol of the Advantage 2 PCR Kit (Clontech). The PCR program was run under the following conditions: 5 cycles of 94 °C for 30 s and 72 °C for 3 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min; and 25 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min. The full-length cDNA of *PcLGBP* was obtained by overlapping the expressed sequence tag (EST) sequences and the 5'and 3'fragments.

2.3. Sequencing and phylogenetic analysis

BLAST (http://www.ncbi.nlm.nih.gov/blast) online was used to obtain the homologous sequences of PcLGBP isoform1 and PcLGBP isoform2. Expert Protein Analysis System (http://www.expasy.org) was utilized to analyze the nucleotide sequence and deduced amino acid sequence of PcLGBP. The theoretical isoelectric point (pI) and molecular weight (Mw) of PcLGBP were predicted through ExPASy-compute pI/Mw tool (http://web.expasy.org/compute_pi/). The possible signal peptide and domain were predicted by SMART (http://smart.embl-heidelberg.de/). In addition, the phylogenetic tree of PcLGBP isoform1 and PcLGBP isoform2 was obtained using MEGA 6.0 software. The sequences alignment between PcLGBP isoform1 and PcLGBP isoform2 was conducted by DNAMAN software.

2.4. Immune stimulation of P. clarkii and cDNA synthesis

In this part, *V. parahaemolyticus* and LPS were used to stimulate *P. clarkii*. In details, *V. parahaemolyticus* was cultured in sterile Luria-Bertani (LB) liquid culture medium at 37 °C (200 rpm) overnight. Then the bacteria were suspended with 1 mL physiological saline (PBS, 0.01 M, pH 7.2–7.4). In the bacterial challenge group, crayfish (female and male) were injected 50 μL of PBS containing *V. parahaemolyticus* (approximately 3.0×10^6 colony-forming units (CFUs) per crayfish). In the LPS stimulation group, crayfish (female and male) were injected 50 μL of LPS (0.5 μg/μL). At 2, 6, 12, and 24 h after *V. parahaemolyticus* or LPS injection, the hepatopancreas of female and male *P. clarkii* was separately collected for total RNA extraction. In addition, the hemolymph of healthy *P. clarkii* was collected using a 1 mL sterile syringe preloaded with an equal volume of improved anticoagulant buffer (ACD-B, glucose, 1.47 g; citric acid, 0.48 g; trisodium citrate, 1.32 g; prepared in double-distilled water and brought to 100 mL, pH 7.3) and then centrifuged immediately at 800 × *g* for 10 min at 4 °C to isolate the hemocytes. Other tissues, including the heart, hepatopancreas, gills, stomach, and intestine were also collected from untreated female and male *P. clarkii* for RNA extraction and tissue distribution studies.

Total RNA was extracted from all the tissues mentioned above by using a high-purity total RNA rapid extraction kit (Spin-Column, BioTeke, Beijing, China) following the instructions of the manufacturer. The integrity of total RNA was routinely checked with 1% agarose gel. The first-strand cDNA for quantitative real-time PCR (qRT-PCR) analysis was synthesized using the TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) (Transgen Biotech, Beijing, China). The mixture was incubated at 42 °C for 15 min, terminated by heating at 85 °C for 5 s, and subsequently stored at - 20 °C.

2.5. qRT-PCR analysis

The gene-specific primers (*PcLGBP*-qRT-F and *PcLGBP*-qRT-R, *PcLGBP*-isoform1-qRT-F and *PcLGBP*-isoform1-qRT-R, and *PcLGBP*-isoform2-qRT-F and *PcLGBP*-isoform2-qRT-R) were designed for qRT-PCR analysis. The synthesized first-strand cDNAs were used as templates. In the qRT-PCR experiment, the *TansStart* Top Green qPCR SuperMix kit (Transgen Biotech, Beijing, China) was used in accordance with the instructions of the manufacturer. The qRT-PCR was carried out in a total volume of 10 μL, which contained 5 μL of 2 × *TansStart*® Top

Table 1
Sequences of the primers used in this study.

Primers name	Sequence (5′-3′)
PcLGBP-isoform1-F	CCAGGGGCAACGATGACTACTCCAACC
PcLGBP-isoform1-R	GCCGAATAGTCAGCGTGGGTCCTCTCA
PcLGBP-isoform2-F	GGCTGAACAACACAGAGACAACACCGC
PcLGBP-qRT-F	GCGTCTGGGGCGCTGGCGGCCG
PcLGBP-qRT-R	TCCTGAGCCTGGCGCTGATCACG
PcLGBP-isoform1-qRT-F	CGCTGGCGGCCGATGTGGTG
PcLGBP-isoform1-qRT-R	GATCTCGTGCTCCCAGACCTC
PcLGBP-isoform2-qRT-F	CGTCAGTCTGCTGCAGCTACG
PcLGBP-isoform2-qRT-R	CAGCCGAGCCAATCGCTGAGT
Pc-CRU1-qRT-F	CAACTACCCTAACCACCTCAAC
Pc-CRU1-qRT-R	CCTCAGAGCTACGACAAATGAG
Pc-CRU2-qRT-F	TTCTGTCCTCCTCCTCTCAA
Pc-CRU2-qRT-R	CAGGTGTCAAAGCAACACTTC
Pc-CRU3-qRT-F	TACGTCTTGCCCTCGTCTTA
Pc-CRU3-qRT-R	CAGCGTCCTCCTCTTTGTAATC
Pc-CRU4-qRT-F	CTCTGACTGCCAGGTGTTT
Pc-CRU4-qRT-R	TGCGAGCTGTGATGGTTAG
Pc-ALF5-qRT-F	ATGGGGAGGTGAGGCTACT
Pc-ALF5-qRT-R	CCTTCCTGCTCGGTGATGA
Pc-ALF6-qRT-F	ACAAATGAACACAAGCCACCC
Pc-ALF6-qRT-R	TGATAAACCTGTCCTCCCAAC
Pc-ALF8-qRT-F	GCGGAACGGTGAGGTGGAG
Pc-ALF8-qRT-R	TGATGAGGCCGGCTTGGAA
Pc-ALF9-qRT-F	AGTGGCGTCATACAGGAAGGGG
Pc-ALF9-qRT-R	CCAAAGGATGGCGAGAAATAGT
Pc-18S rRNA-qRT-F	ACCGATTGAATGATTTAGTGAG
Pc-18S rRNA-qRT-R	TACGGAAACCTTGTTACGAC
PcLGBP-6P-2-ex-F	GGATCCCCAGGAATTCCCGCCGATGTGGTGGCCCCTGAGG
PcLGBP-6P-2-ex-R	GATGCGGCCGCTCGAGTTACTGCTCCACACTCTCCATCTTC
PcLGBP-iF	GCGTAATACGACTCACTATAGGCATCAACCCCGTGATCAGC
PcLGBP-iR	GCGTAATACGACTCACTATAGGGTTGTCCAGGGAGTTGTCG
GFP-iF	GCGTAATACGACTCACTATAGGTGGTCCCAATTCTCGTGGAAC
GFP-iR	GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC

Green qPCR SuperMix, 0.2 μL of forward primer (10 μM), 0.2 μL of reverse primer (10 μM), 1 μL cDNA, and 3.6 μL nuclease-free water. The primers Pc-18S rRNA-qRT-F and Pc-18S rRNA-qRT-R were used to amplify the 18S rRNA for internal standardization. The qRT-PCR was programmed at 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, and 60 °C for 30 s. All samples were examined in triplicate, and the data were calculated using the $2^{-\Delta\Delta CT}$ method [21]. A Student's *t*-test was conducted, and statistically significant differences were accepted when $P < 0.05$. The primers used for qRT-PCR are presented in Table 1.

2.6. RNAi assay

The specific primers, *PcLGBP*-iF, *PcLGBP*-iR, *GFP*-iF, and *GFP*-iR were designed to synthesize the total *PcLGBP* (including *PcLGBP* isoform1 and *PcLGBP* isoform2) and *GFP* cDNA fragments. The resulting cDNAs were used as templates to synthesize *PcLGBP*-dsRNA and *GFP*-dsRNA with MEGAscript RNAi Kit (Thermo, USA) following the instructions of the manufacturer. Healthy male crayfish were divided into three groups: the unchallenged normal group, the *GFP*-dsRNA injection group, and the *PcLGBP*-dsRNA injection group. For the dsRNA injection groups, 30 μg of *PcLGBP*-dsRNA or *GFP*-dsRNA (as control) was injected into each crayfish. After 48 h injection, the total RNA in hepatopancreas of male crayfish was extracted to synthesize the cDNAs. The RNAi efficiency of the *PcLGBP* gene was detected by qRT-PCR. After 48 h post dsRNA (*PcLGBP*-dsRNA or *GFP*-dsRNA) injection, the male crayfish were stimulated by *V. parahaemolyticus* (approximately 3.0×10^6 CFU, each crayfish). The healthy crayfish only challenged by the same amount of *V. parahaemolyticus* was referred as the control group. The hepatopancreas of male crayfish post 24 h *V. parahaemolyticus* challenge was collected for RNA extraction and cDNA synthesis. The *PcLGBP* gene expression in each experimental group was detected by qRT-PCR. The gene-specific primers including *PcLGBP*-qRT-F, *PcLGBP*-qRT-R, Pc-18S rRNA-qRT-F, and Pc-18S rRNA-qRT-R (Table 1) were used for qRT-PCR. 18S rRNA was amplified for internal standardization.

2.7. Detection of the expression levels of AMPs and PO activity after PcLGBP silencing

On the one hand, the expression levels of *PcLGBP* were first detected in each experimental group (including the normal, *V. parahaemolyticus* only, *GFP*-dsRNA + *V. parahaemolyticus*, and *PcLGBP*-dsRNA + *V. parahaemolyticus* groups) by qRT-PCR. Then, the anti-LPS factors (including ALF5, ALF6, ALF8, and ALF9) and crustins (including CRU1, CRU2, CRU3, and CRU4) were also detected in each experimental group mentioned above by qRT-PCR. The experiment was repeated three times, and the primers of AMPs for qRT-PCR analysis are shown in Table 1. On the other hand, the PO activity was detected in the healthy and *PcLGBP*-silenced crayfish. First, the hepatopancreas, gills, and stomach from female and male crayfish were collected for PO activity analysis. In addition, the PO activity in hepatopancreas lysate (HLS) of male crayfish in different experimental groups (normal, *GFP*-dsRNA injection, and *PcLGBP*-dsRNA injection) was measured. For PO activity assay, $_L$-3, 4-dihydroxy-phenylalanine ($_L$-DOPA) was used as substrate following the existing methods [18,22]. The PO activity was monitored by spectrophotometry at 490 nm and defined as ΔA490/mg total protein/min × 10^{-2}.

2.8. Expression and purification of recombinant PcLGBP protein

The primers PcLGBP-6P-2-ex-F and PcLGBP-6P-2-ex-R (Table 1) that contained the restriction enzyme sites *Eco*R I and *Xho* I were designed to amplify the cDNA fragment. After PCR amplification, enzyme digestion and fragment connection, the recombinant plasmid pGEX-6P-2-PcLGBP was transformed into *E. coli* BL21 (DE3) cells for isopropyl *β*-D-thiogalactoside (IPTG)-induced recombinant expression (final IPTG concentration of 0.5 mM). The recombinant protein (rPcLGBP) was purified

(A)

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cgtcagtctgctgcagctacggatgacgatgagagcggcgctgttgttcctgttgctggcg
                       M  T  M  R  A  A  L  L  F  L  L  L  A
tctggggcgctggcggccgatgtggtggcccctgaggactgcaccggcttcccctgcctc
 S  G  A  L  A  A  D  V  V  A  P  E  D  C  T  G  F  P  C  L
atcttcaatgatgagttcgacttcctggatcatgaggtctgggagcacgagatcaccatg
 I  F  N  D  E  F  D  F  L  D  H  E  V  W  E  H  E  I  T  M
tccggtggagggaactgggagttccagatgtacattaataccgcagcatcagttacact
 S  G  G  G  N  W  E  F  Q  M  Y  I  N  N  R  S  I  S  Y  T
cgtgactcaacactattcatcagaccggacctgacgtccaactggcagactacggacttc
 R  D  S  T  L  F  I  R  P  D  L  T  S  N  W  Q  T  T  D  F
ctgagcagtggggacgtgaacctgtggggcatgaacgggcgcggggacgtgtgcacaggc
 L  S  S  G  D  V  N  L  W  G  M  N  G  R  G  D  V  C  T  G
aactcctactacgggtgtgagcgcgtgggcaacccggtcaacatcatcaaccccgtgatc
 N  S  Y  Y  G  C  E  R  V  G  N  P  V  N  I  I  N  P  V  I
agcgccaggctcaggacgctcgagaacttcgccttcaaatatggacgtattgaggtccgg
 S  A  R  L  R  T  L  E  N  F  A  F  K  Y  G  R  I  E  V  R
gcgaagctgccccgcggggactggttgtggccagcaatatggctcctgcccaggtactgg
 A  K  L  P  R  G  D  W  L  W  P  A  I  W  L  L  P  R  Y  W
ccctacggcccttggcccgcaagtggcgagattgacattatggaatccaggggcaacgat
 P  Y  G  P  W  P  A  S  G  E  I  D  I  M  E  S  R  G  N  D
gactactccaacctgggcaaccagtacgctgcaaccaccttccactggggacccaactgg
 D  Y  S  N  L  G  N  Q  Y  A  A  T  T  F  H  W  G  P  N  W
cagactaacttgtatgagaggacccacgctgactattcggccaatgatggatcctacgcc
 Q  T  N  L  Y  E  R  T  H  A  D  Y  S  A  N  D  G  S  Y  A
aacagcttccacacctggaggatggactggactaaggacaacatacaggtgttcgtggac
 N  S  F  H  T  W  R  M  D  W  T  K  D  N  I  Q  V  F  V  D
gaccagctccagataaccgtggacccgggcaccaacttctgggagttcggaggcttcgac
 D  Q  L  Q  I  T  V  D  P  G  T  N  F  W  E  F  G  G  F  D
aactccctggacaacccctggaaggctggcagcaagatggctcccttcgaccagaagttc
 N  S  L  D  N  P  W  K  A  G  S  K  M  A  P  F  D  Q  K  F
tacgtggtgctgaacgttgctgtgggaggcgtgaacggctacttcccggacggcgtcccc
 Y  V  V  L  N  V  A  V  G  G  V  N  G  Y  F  P  D  G  V  P
agcaacccggccaagccttggagcaacacctcgccccaggccttcctcgacttctggaac
 S  N  P  A  K  P  W  S  N  T  S  P  Q  A  F  L  D  F  W  N
gctcgggactcttggctgcccagctgggagaatggtgagggccgtgtgagtgagaacgcc
 A  R  D  S  W  L  P  S  W  E  N  G  E  G  R  V  S  E  N  A
gccctgcaggtcgactacgtgaaggtgtggaagatggagagtgtggagcagtaggtccac
 A  L  Q  V  D  Y  V  K  V  W  K  M  E  S  V  E  Q  -
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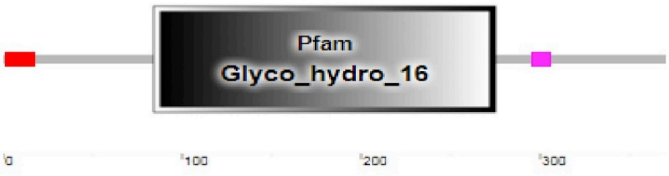

Fig. 1. Nucleotides, deduced amino acid sequence, and protein structure of PcLGBP isoform1 (A) and PcLGBP isoform2 (B) from *P. clarkii*. The signal peptide sequences were both underlined. The glyco-hydro-16 domain was both marked in gray shade. The low complexity region sequences were both double underlined. The amino acid sequences existing in PcLGBP isoform2 but not in PcLGBP isoform1 were marked in blue. The initiation codon (atg) and stop codon (tag) were both marked in red. In the diagram of the protein structure, the red block (■) represented the signal peptide sequence. The glyco-hydro-16 pfam was the feature of glycoside hydrolase family 16. A pink block (■) presented a low complexity region sequence. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(B)

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cgtcagtctgctgcagctacggatgacgatgagagcggcgctgttgttcctgttgctggcg
                      M  T  M  R  A  A  L  L  F  L  L  L  A
tctggggcgctggcggccgcctatctggtgaggtgtatagtgggaggtgggcgagaggta
 S  G  A  L  A  A  A  Y  L  V  R  C  I  V  G  G  G  R  E  V
gcgcctggcagcgggctgaacaacacagagacaacaccgcgccagaacacatctagtcac
 A  P  G  S  G  L  N  N  T  E  T  T  P  R  Q  N  T  S  S  H
cttgttgtgcactcagcgattggctcggctgatgtggtggcccctgaggactgcaccggc
 L  V  V  H  S  A  I  G  S  A  D  V  V  A  P  E  D  C  T  G
ttccccctgcctcatcttcaatgatgagttcgacttcctggatcatgaggtctgggagcac
 F  P  C  L  I  F  N  D  E  F  D  F  L  D  H  E  V  W  E  H
gagatcaccatgtccggtggagggaactgggagttccagatgtacattaataaccgcagc
 E  I  T  M  S  G  G  G  N  W  E  F  Q  M  Y  I  N  N  R  S
atcagttacactcgtgactcaacactattcatcagaccggacctgacgtccaactggcag
 I  S  Y  T  R  D  S  T  L  F  I  R  P  D  L  T  S  N  W  Q
actacggacttcctgagcagtggggacgtgaacctgtggggcatgaacgggcgcgggggac
 T  T  D  F  L  S  S  G  D  V  N  L  W  G  M  N  G  R  G  D
gtgtgcacaggcaactcctactacgggtgtgagcgcgtgggcaacccggtcaacatcatc
 V  C  T  G  N  S  Y  Y  G  C  E  R  V  G  N  P  V  N  I  I
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 N  P  V  I  S  A  R  L  R  T  L  E  N  F  A  F  K  Y  G  R
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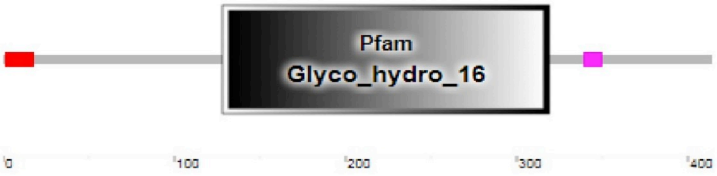

Fig. 1. (*continued*)

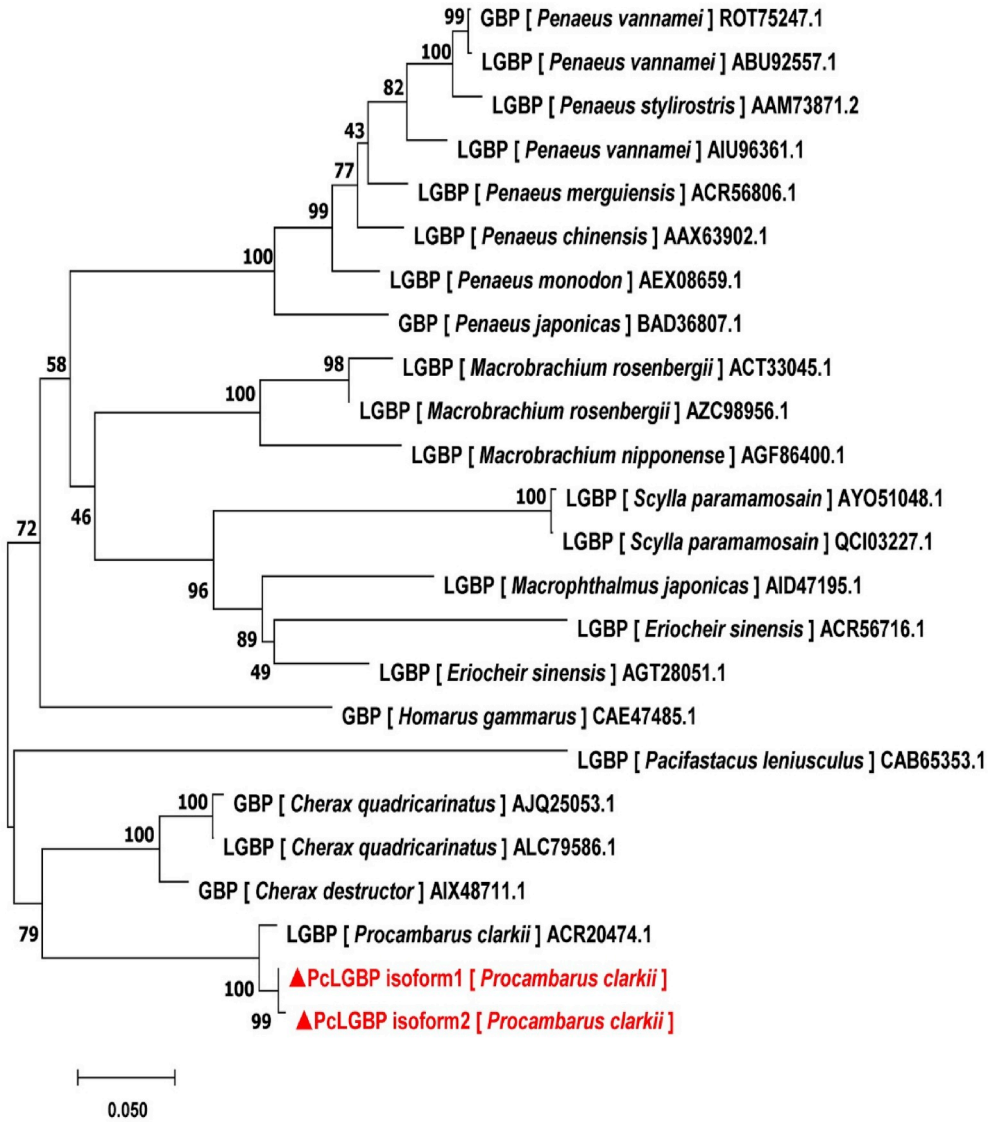

Fig. 2. Phylogenetic tree analysis of LGBPs (PcLGBP isoform1 and PcLGBP isoform2) and other members of LGBP family from crustacean. Red triangle symbol was set in front of PcLGBP isoform1 and PcLGBP isoform2. The phylogenetic tree was produced by MEGA 6.0 software. The protein name, species sources, and the GenBank accession number were shown in the evolutionary tree. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

through affinity chromatography with Glutathione Sepharose 4 Fast Flow (GE Health Care, USA) in accordance with instructions of the manufacturer. The purified protein was detected through 12.5% SDS-polyacrylamide gel electrophoresis. The rPcLGBP concentration was quantified with the Bradford Protein Assay Kit PA102 (Tiangen, China).

2.9. Involvement of purified rPcLGBP and polysaccharides in proPO activation

The HLS of male crayfish was prepared. HLS of 200 μL was mixed with LPS (0.1 μg/mL), GLU (0.1 μg/mL), or PGN (0.1 μg/mL) solution, followed by adding rPcLGBP of 4 μM, and then incubated at room temperature for 30 min. The mixture of HLS and polysaccharide was prepared by the same method mentioned above without rPcLGBP and also incubated at room temperature for 30 min. Subsequently, the pre-cooled $_{L}$-DOPA (0.01 M/L) of 350 μL was added into the mixture at room temperature for 30 min. Thereafter, the absorbance was measured at 490 nm every minute. All assays were carried out in triplicate.

3. Results

3.1. Cloning and sequence analysis of PcLGBP isoforms

Except for the open reading frame (ORF) that has been reported, we acquired the full length cDNA of *PcLGBP* isoform1. The full length cDNA of *PcLGBP* isoform1 was 1308 bp with an ORF of 1113 bp encoding a protein of 370 amino acid residues (Fig. 1A). The full-length cDNA of *PcLGBP* isoform2 was 1440 bp containing an ORF of 1245 bp encoding a protein of 414 amino acids (Fig. 1B). The predicted protein structure by SMART showed that the two LGBP isoforms had a signal peptide of 18 amino acid residues, a glyco-hydro-16 domain of 194 amino acid residues, and a low complexity domain of 12 amino acid residues. The difference between the two LGBP isoforms was that PcLGBP isoform2 had 44 more amino acids behind the signal peptide than the PcLGBP isoform1 (Fig. 1B).

The BLAST results indicated that the homologous sequences of LGBP isoforms (PcLGBP isoform1 and PcLGBP isoform2) nearly came from crustaceans selected for building the evolutionary tree. The phylogenetic analysis showed that the two LGBP isoforms and other LGBP

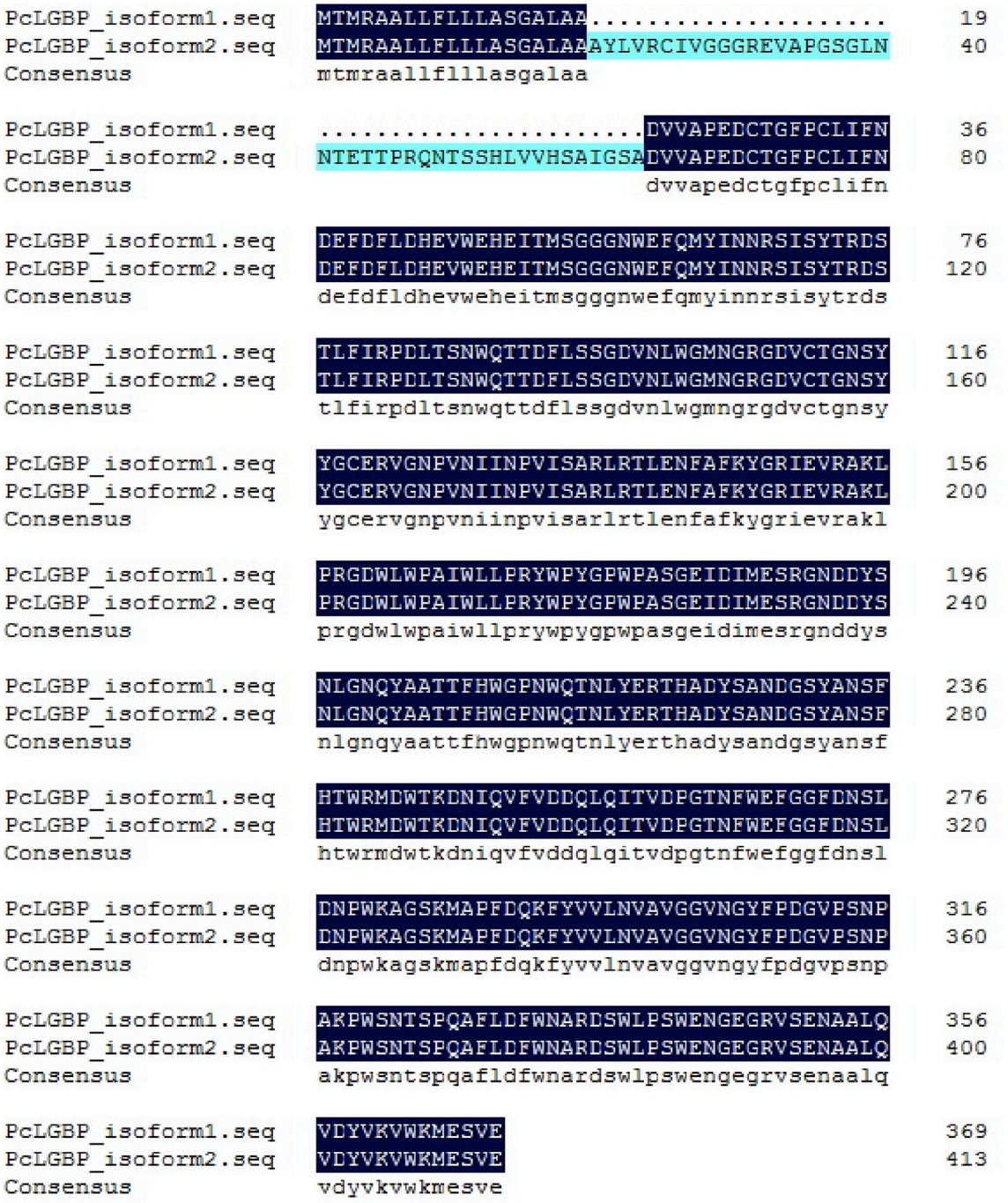

Fig. 3. Amino acid sequence alignment of PcLGBP isoform1 and PcLGBP isoform2 by DNAMAN software. The amino acid sequence difference between the PcLGBP isoform1 and PcLGBP isoform2 was marked in blue shade. The black shade represented the exact same sequence. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

proteins from crustaceans were evolutionary close to each other (Fig. 2). Moreover, the results of amino acid sequence alignment revealed that PcLGBP isoform2 had 44 more amino acids than the PcLGBP isoform1 (Fig. 3). The protein domains of PcLGBP isoform1 and PcLGBP isoform2 were the same.

3.2. Tissue distributions of LGBP isoforms

The expression levels of *PcLGBP* isoform1 and *PcLGBP* isoform2 in different tissues of female and male crayfish were detected by qRT-PCR. The results showed that *PcLGBP* isoform1 (Fig. 4A) and *PcLGBP* isoform2 (Fig. 4B) mainly expressed in the hepatopancreas of female and male crayfish. Moreover, the expression levels of the two *LGBP* isoforms in the male hepatopancreas were evidently higher than that of female (Fig. 4).

3.3. Expression patterns analysis of LGBP isoforms

The expression patterns of *PcLGBP* isoform1 and *PcLGBP* isoform2 in the hepatopancreas of *P. clarkii* (female and male) were analyzed after stimulated by *V. parahaemolyticus* or LPS. In female hepatopancreas, the expression levels of *PcLGBP* isoform1 was significantly up-regulated at 2, 6, and 24 h post *V. parahaemolyticus* challenge (Fig. 5A). *PcLGBP* isoform1 in the hepatopancreas of male crayfish transcripted

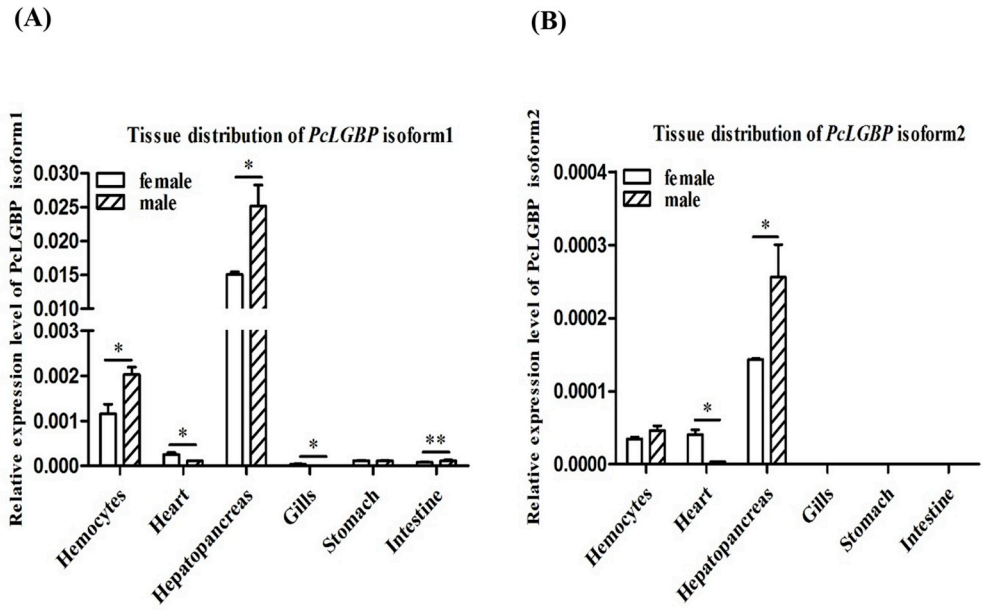

Fig. 4. Tissue distribution of *PcLGBP* isoform1 (A) and *PcLGBP* isoform2 (B) in the hemocytes, heart, hepatopancreas, gills, stomach, and intestine of *P. clarkii* (females and males). Asterisks indicated significant differences (*$P < 0.05$, **$P < 0.01$) between female and male.

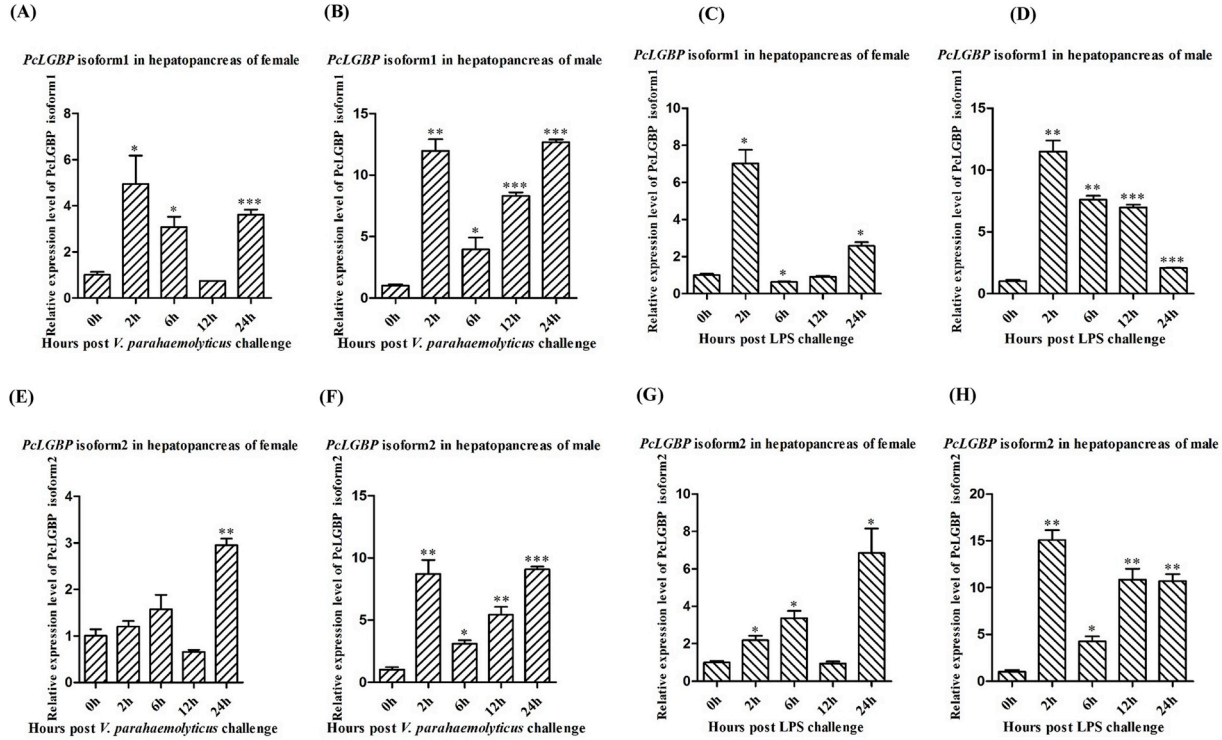

Fig. 5. Expression profiles of *PcLGBP* isoform1 and *PcLGBP* isoform2 in the hepatopancreas of female and male crayfish after *V. parahaemolyticus* or LPS challenge. (A) Expression levels of *PcLGBP* isoform1 in female hepatopancreas post *V. parahaemolyticus* injection; (B) Expression levels of *PcLGBP* isoform1 in male hepatopancreas post *V. parahaemolyticus* injection; (C) Transcripts of *PcLGBP* isoform1 in female hepatopancreas after LPS stimulation; (D) Transcripts of *PcLGBP* isoform1 in male hepatopancreas after LPS stimulation; (E) Expression levels of *PcLGBP* isoform2 in female hepatopancreas post *V. parahaemolyticus* injection; (F) Expression levels of *PcLGBP* isoform2 in male hepatopancreas post *V. parahaemolyticus* injection; (G) Transcripts of *PcLGBP* isoform2 in female hepatopancreas after LPS challenge; (H) Transcripts of *PcLGBP* isoform2 in male hepatopancreas after LPS challenge. Each column represented the mean ± S.D. of three independent PCR amplifications and quantifications. The asterisks indicated significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) compared with the unchallenged group (0 h).

rapidly up-regulated at 2 h and then down-regulated at 6 h, followed by being up-regulated at 12 and 24 h after *V. parahaemolyticus* injection (Fig. 5B). In the LPS injection group, *PcLGBP* isoform1 expression in the hepatopancreas of female crayfish was quickly up-regulated at 2 h and then down-regulated at 6 and 24 h post LPS challenge (Fig. 5C). Meanwhile, that in male was first up-regulated at 2 h and then down-regulated at 6, 12, and 24 h after LPS injection (Fig. 5D). The same analysis was processed for *PcLGBP* isoform2. The *PcLGBP* isoform2 expression was only up-regulated at 24 h in the hepatopancreas of female crayfish (Fig. 5E) post *V. parahaemolyticus* stimulation. In the hepatopancreas of male crayfish, *PcLGBP* isoform2 transcript was up-regulated from 2 h to 24 h after *V. parahaemolyticus* challenge (Fig. 5F).

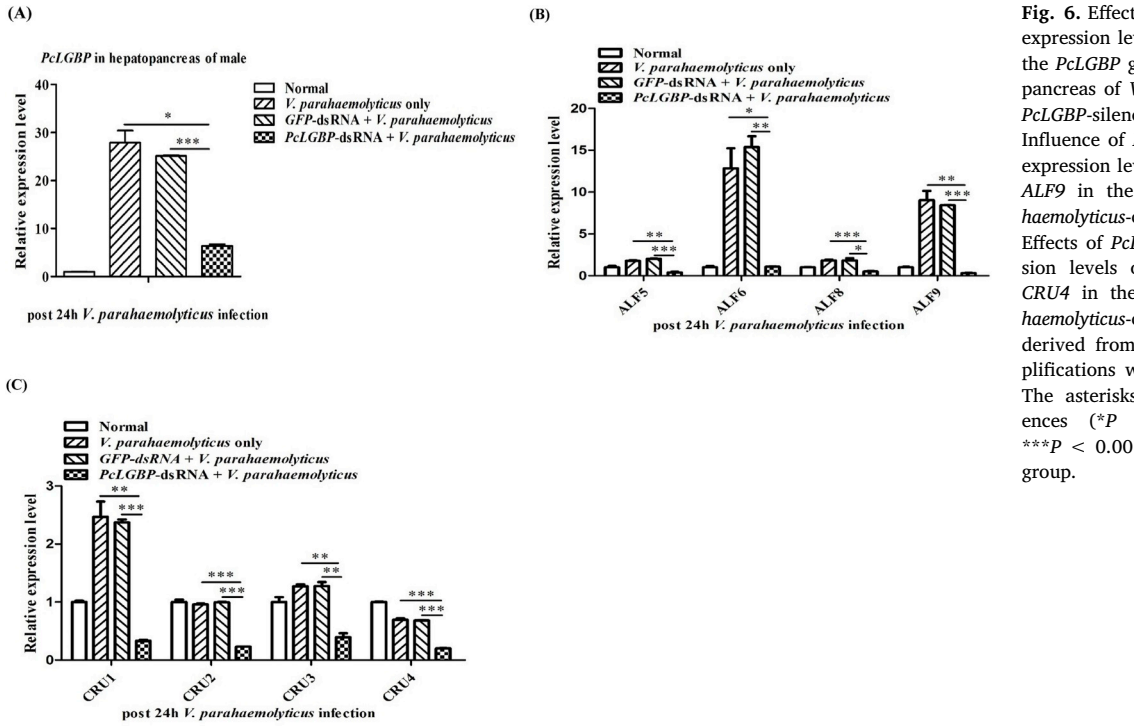

Fig. 6. Effects of *PcLGBP* silencing on the expression levels of AMPs. (A) Silencing of the *PcLGBP* gene expression in the hepatopancreas of *V. parahaemolyticus*-challenged *PcLGBP*-silenced male crayfish. (B) Influence of *PcLGBP* gene silencing on the expression levels of *ALF5*, *ALF6*, *ALF8*, and *ALF9* in the hepatopancreas of *V. parahaemolyticus*-challenged male crayfish. (C) Effects of *PcLGBP* silencing on the expression levels of *CRU1*, *CRU2*, *CRU3*, and *CRU4* in the hepatopancreas of *V. parahaemolyticus*-challenged male crayfish. Data derived from three independent PCR amplifications were shown as mean ± S.D. The asterisks indicated significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) compared with control group.

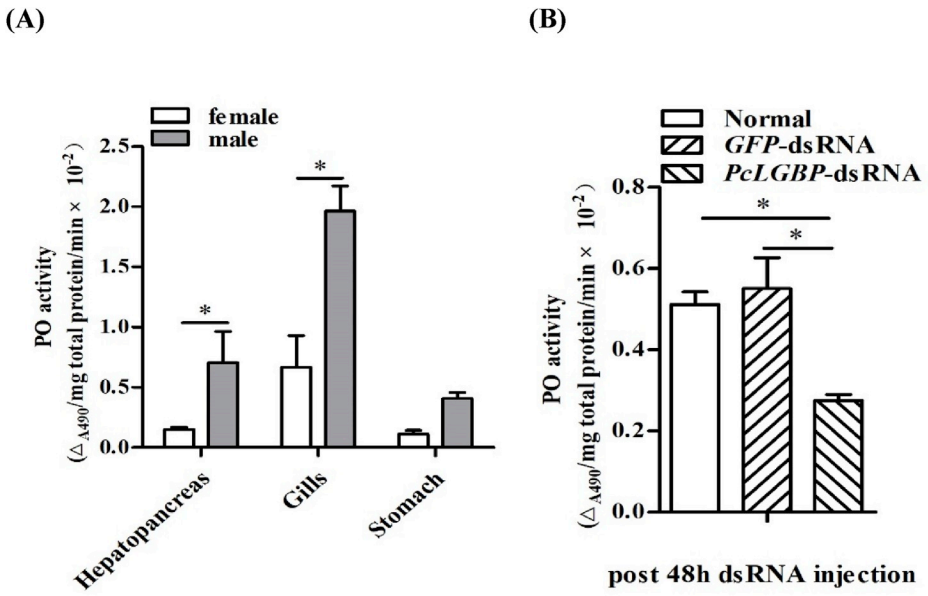

Fig. 7. Detection of PO activity in healthy and *PcLGBP-silenced* crayfish. (A) PO activity in the hepatopancreas, gills, and stomach of healthy female and male crayfish were detected. (B) Analyzing the PO activity in male hepatopancreas in the three experimental groups, namely, the normal, the *GFP*-dsRNA, and the *PcLGBP*-dsRNA injection groups. Data derived from three PCR repetitions were shown as mean ± S.D. The asterisks indicated significant differences (*$P < 0.05$) compared with control group.

After LPS challenge, *PcLGBP* expressed in the female hepatopancreas up-regulated at 2, 6, and 24 h (Fig. 5G) but up-regulated from 2 h to 24 h in the hepatopancreas of male crayfish compared with the unchallenged *P. clarkii* (Fig. 5H).

3.4. Effects of PcLGBP silencing on the expression levels of AMPs

PcLGBP-dsRNA was injected to male crayfish to silence the *PcLGBP* expression. After 48 h dsRNA injection, the crayfish were injected with *V. parahaemolyticus*. After 24 h *V. parahaemolyticus* challenge, the *PcLGBP* expression was detected in every experimental group. As shown in Fig. 6A, the up-regulated *PcLGBP* expression in the *V. parahaemolyticus* only or the *GFP*-dsRNA + *V. parahaemolyticus* group was evidently inhibited in the *PcLGBP*-dsRNA + *V. parahaemolyticus* group. The expression levels of all the tested AMPs except CRU2 and CRU4 were up-regulated after *V. parahaemolyticus* infection. Moreover, *PcLGBP* silencing significantly inhibited the expression of ALFs (Fig. 6B) and CRUs (Fig. 6C) in the hepatopancreas of *V. parahaemolyticus* challenged male crayfish.

3.5. Determination of PO activity in healthy and PcLGBP-silenced crayfish

In healthy female and male crayfish, the PO activity in the hepatopancreas, gills, and stomach was detected. The results showed that the PO activity in the hepatopancreas and gills of male crayfish was evidently higher than that of female crayfish (Fig. 7A). In addition, the PO activity in the hepatopancreas of male crayfish was significantly decreased in the *PcLGBP*-dsRNA injection group compared with the control groups (the *GFP*-dsRNA and normal groups) (Fig. 7B).

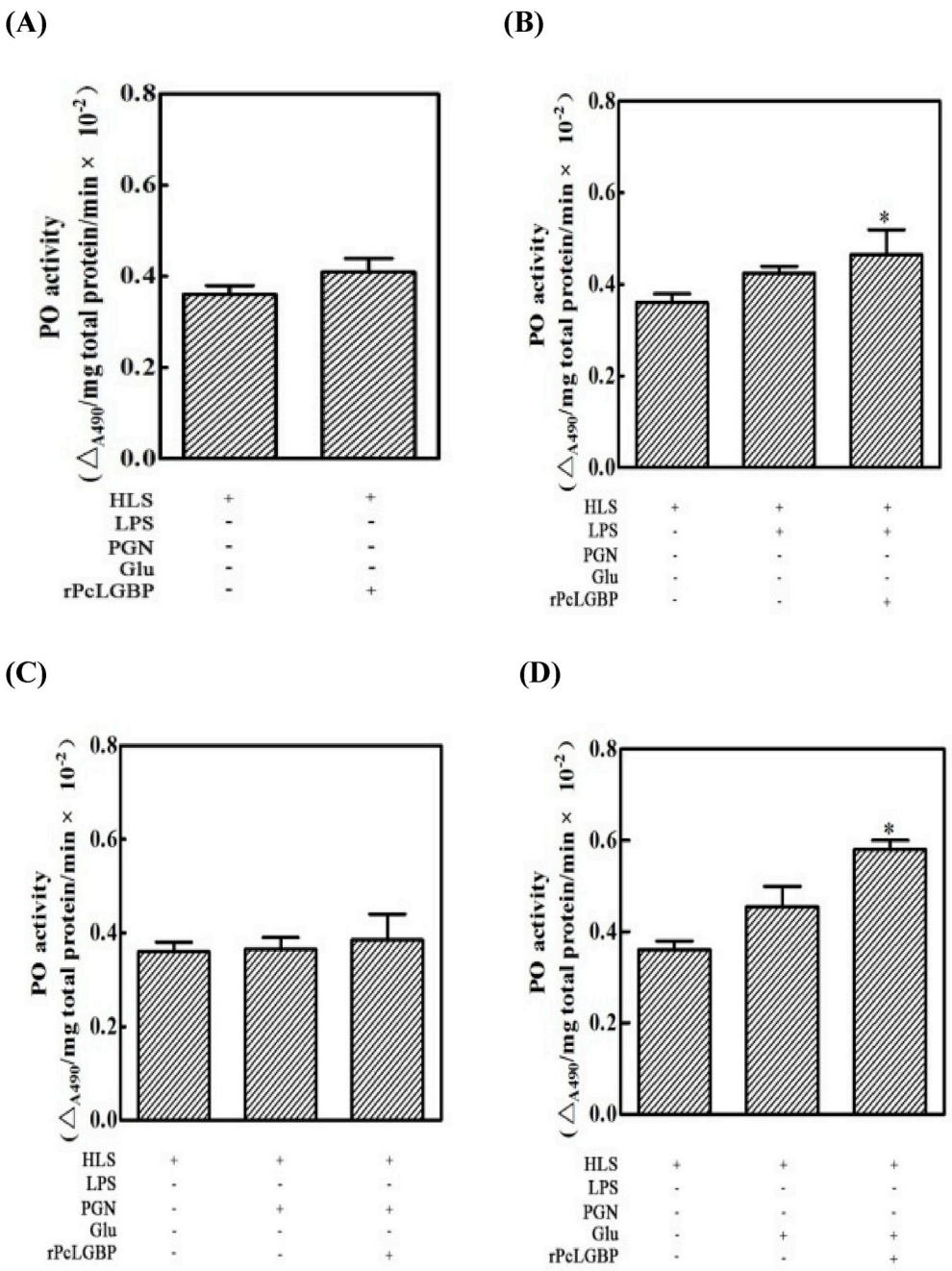

Fig. 8. Effects of rPcLGBP and polysaccharides (LPS, PGN, and GLU) on the PO activity in the hepatopancreas of male crayfish. (A) Effect of rPcLGBP (4 μM) on the PO activity. (B) PO activity was detected by adding LPS (0.1 μg/mL) and rPcLGBP (4 μM). (C) PO activity was detected by adding PGN (0.1 μg/mL) and rPcLGBP (4 μM). (D) PO activity was detected by adding GLU (0.1 μg/mL) and rPcLGBP (4 μM). The PO enzymatic activity was measured with L–DOPA as substrate and monitored by spectrophotometry at 490 nm. The PO activity was defined as ΔA490/mg total protein/min × 10^{-2}. Each sample was composed of three crayfish. Data were analyzed by Student's *t*-test, and the results derived from three independent experiments were expressed as the mean ± S.D.

3.6. Effects of rPcLGBP and polysaccharides on the PO activity

The purified recombinant PcLGBP protein (rPcLGBP) was successfully obtained *in vitro*. As shown in Fig. 8A, the PO activity in HLS did not change after adding rPcLGBP alone. Similarly, the PO activity in HLS did not evidently change when added LPS, PGN, or Glu alone. However, when either LPS (Fig. 8B) or Glu (Fig. 8D) and rPcLGBP were simultaneously added into HLS, the PO activity was significantly increased. Compared with the LPS or Glu, the mixture of PGN and rPcLGBP did not affect on the PO activity in HLS (Fig. 8C). These results indicated that the polysaccharides (LPS and Glu) and rPcLGBP were together involved in activating the proPO system.

4. Discussion

We identified two *LGBP* isoforms from *P. clarkii*, namely, *PcLGBP* isoform1 and *PcLGBP* isoform2. Molecular evolution analysis showed that the two LGBP isoforms were close to other LGBPs in crustaceans in terms of genetic distance. Moreover, the protein domains of *PcLGBP* isoform1 and *PcLGBP* isoform2 were the same, that is, both contained the conservative glycoside hydrolase domain. In crustaceans, the hepatopancreas was known as a key tissue in innate immune response and many immune defense molecule transcripts in this organ [23,24]. In female and male crayfish, *PcLGBP* isoform1 and *PcLGBP* isoform2 were mainly expressed in the hepatopancreas, which was consistent with that reported previously for *LGBP* in *L. vannamei* [25] and *LGBP* in *Fenneropenaeus indicus* [26]. Interestingly, the expression levels of the two LGBP isoforms in male hepatopancreas were evidently higher than that in female crayfish. These results suggested that the gender differences of *P. clarkii* might affect the body's innate immune responses to pathogen invasion. The high expression of LGBP in the male hepatopancreas of *P. clarkii* due to multiple reasons needs to further study.

V. parahaemolyticus is ubiquitous in estuaries and coastal waters throughout the world, and causes many humans and aquatic animal

diseases [27,28]. The expression patterns of *PcLGBP* isoform1 and *PcLGBP* isoform2 were observed in the hepatopancreas of *V. parahaemolyticus* or LPS-challenged female and male crayfish. The *PcLGBP* isoform1 transcripts in the hepatopancreas were evidently up-regulated at 2, 6, and 24 h after *V. parahaemolyticus* injection in female and male crayfish. The expression of *PcLGBP* isoform2 was only up-regulated at 24 h in the hepatopancreas of female crayfish, whereas its transcript in the hepatopancreas of male crayfish was significantly up-regulated from 2 h to 24 h post *V. parahaemolyticus* challenge. The expression levels of some other *LGBP* genes in shrimp, such as *F. chinensis* LGBP [20], *M. japonicus* LGBP [17], *P. monodon* LGBP [18], and *Macrobrachium nipponense* LGBP [29], were also regulated by *Vibrio*. LPS was the main component of Gram-negative bacterial cell wall. The expression of *PcLGBP* isoform1 and *PcLGBP* isoform2 in the hepatopancreas of female crayfish were both significantly up-regulated at 2 and 24 h after LPS stimulation, while that in the hepatopancreas of male crayfish were evidently up-regulated from 2 to 24 h post LPS challenge. These results suggested that PcLGBP transcript was critical to recognize and eliminate the invading bacteria.

The proPO system activation and AMP synthesis are the important components of humoral immunity in invertebrates. In crayfish, LGBP has been verified to be involved in the activation of the proPO system [16,30]. In this study, *PcLGBP* silencing could significantly inhibit the PO activity in male crayfish, which indicated that *PcLGBP* played a vital role in the proPO system activation. In addition, the transcriptional levels of some AMPs were analyzed in *V. parahaemolyticus*-challenged *PcLGBP*-silenced male crayfish. The results showed the knock down of *PcLGBP* could obviously inhibit the up-regulated expression levels of some AMPs after *V. parahaemolyticus* infection. *P. clarkii* and *Drosophila* were known as arthropods, and the LGBP in *Drosophila* was reported to be involved in inducing the synthesis of AMPs in *Drosophila* immunocompetent cells [31]. However, few studies on the relationship between the LGBP and the synthesis of AMPs in crustaceans have been reported. Thus, further studies are needed to understand the connection between the *PcLGBP* and immune signaling pathways that regulate the AMP expression.

A purified rPcLGBP protein was acquired to study the role of the interaction between the PcLGBP and polysaccharides in the activation of proPO system. In addition, the complex of rPcLGBP-LPS, rPcLGBP-GLU, or rPcLGBP-PGN was incubated with HLS in male crayfish. The results showed that the complex of rPcLGBP-LPS and rPcLGBP-GLU could significantly enhance the PO activity of the HLS, whereas the individual rPcLGBP and the mixture of rPcLGBP-PGN could not activate PO activity. PmLGBP from *P. monodon* also could enhance the PO activity of hemocyte suspensions in the presence of LPS or GLU [18].

In conclusion, two *LGBP* isoforms from *P. clarkii*, namely, *PcLGBP* isoform1 and *PcLGBP* isoform2, was identified. *PcLGBP* isoform1 and *PcLGBP* isoform2, which were important PRRs, were involved in responding the *V. parahaemolyticus* and LPS stimulation. The AMP synthesis and the PO production were regulated by *PcLGBP* in male crayfish. In addition, only the complex of rPcLGBP-LPS and rPcLGBP-β-1, 3-glucan could activate the PO activity. These results clearly demonstrated that PcLGBP isoform1 and PcLGBP isoform2 functioned as PRR by mainly activating the crayfish proPO system through recognizing and binding the LPS and β-1, 3-glucan.

Acknowledgements

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