Expression and applications of recombinant crustacean hyperglycemic hormone from eyestalks of white shrimp (Litopenaeus vannamei) against bacterial infection

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ABSTRACT

Crustacean hyperglycemic hormone (CHH) has many functions to regulate carbohydrate metabolism, ecdysis and reproduction including ion transport in crustaceans. The cDNA encoding CHH peptides containing 369 bp open reading frame encoding 122 amino acids was cloned from eyestalk of white shrimp (Litopenaeus vannamei) and was produced by a bacterial expression system. The biological activity of recombinant L. vannamei crustacean hyperglycemic hormone (rLV-CHH) was tested. The hemolymph glucose level of shrimp increased two-fold at 1 h after the rLV-CHH injection and then returned to normal after 3 h. In addition to the effect of rLV-CHH administration (25 μg/shrimp) on immunological responses of white shrimp against pathogenic bacteria, Vibrio harveyi was studied. Results showed that the blood parameters of shrimp injected with rLV-CHH; the THC, PO activity, serum protein level and clearance ability to diisulphide bonds [8]. The sequence of CHH hormone from Carcinus maenas was first reported by Kegel and coworkers [9]. Subsequently, CHH peptides sequences have been described and characterized in lobster [10], crabs [11], crayfishes [12], prawns [13,14] and shrimp [8,15]. CHH functions mainly in the regulation of glucose levels, as well as in the metabolism of carbohydrates and lipids [16]. In addition, it has a significant role in the processes of molting [17], reproduction [18], and osmoregulation [19,20]. However, there is no report about its role on the activation of the immune response in crustacean. The objective of the present study is therefore to elucidate the characterization of the cDNA encoding CHH peptides of white shrimp (Litopenaeus vannamei) and to investigate the effect of the rLV-CHH protein on the induction of shrimp immunity against luminous bacteria, Vibrio harveyi by intramuscular injection.

1. Introduction

Physiological processes in crustaceans are mainly regulated by neuroendocrines of natural peptides, of which approximately fifty neuroendocrines have been described, although the functions of some hormones are still not well defined [1]. Crustacean hyperglycemic hormone (CHH), the most abundant neuroendocrine, is synthesized by X-organ (XO) and stored in the sinus gland (SG) prior to being released directly into the hemolymph [2]. The X-organ and sinus gland (XO-SG) are located in the optic ganglia in the eyestalk which synthesizes and controls neuropeptide hormones essential for the regulation of the physiology and metabolism of crustaceans. The major hormone family produced from XO-SG is composed of crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH), gonad-inhibiting hormone (GIH) or vitellogenesis-inhibiting hormone (VIH) and mandibular organ-inhibiting hormone (MOIH) [3–7]. These hormones are known as the CHH-family of peptides on account of the extensive similarities in their sequences between individuals in a species or among different species. The peptides of the CHH-family consist of 72–78 residues and contain six conserved Cys residues that form three disulfide bonds [8]. The sequence of CHH hormone from Carcinus maenas was first reported by Kegel and coworkers [9]. Subsequently, CHH peptides sequences have been described and characterized in lobster [10], crabs [11], crayfishes [12], prawns [13,14] and shrimp [8,15]. CHH functions mainly in the regulation of glucose levels, as well as in the metabolism of carbohydrates and lipids [16]. In addition, it has a significant role in the processes of molting [17], reproduction [18], and osmoregulation [19,20]. However, there is no report about its role on the activation of the immune response in crustacean. The objective of the present study is therefore to elucidate the characterization of the cDNA encoding CHH peptides of white shrimp (Litopenaeus vannamei) and to investigate the effect of the rLV-CHH protein on the induction of shrimp immunity against luminous bacteria, Vibrio harveyi by intramuscular injection.
2. Materials and methods

2.1. Animals

Subadult shrimp, L. vannamei (size 10−15 g) were obtained from a local farm in Satun province, Thailand. The shrimp were transported to the laboratory in aerated plastic tanks and maintained for two weeks in seawater (15 ppt) at an ambient temperature (28 ± 2 °C). During the acclimation period, the shrimp were fed on a commercial diet 4 times daily at 7:00 am, 12:00 am, 4:00 pm and 11:00 pm. After acclimatization, intermolt shrimp were collected for use in each treatment and feeding was stopped for 24 h before starting the experiment.

2.2. Bacterial strains

V. harveyi, a luminous bacterium, was isolated from hepatopancreas of infected-juvenile L. vannamei cultured in an earthen pond in Satun Province, Southern Thailand in 2005. Prior to the study, the bacterium was re-challenged for increasing virulence in L. vannamei by intramuscular injection and re-isolated on thiosulfate citrate bile salt sucrose agar (TCBS) as a Vibrio − selective media. Purified bacteria were cultured on tryptic soy agar (TSA) with 1.5% NaCl and incubated at 37 °C for 18 h. One loopful of bacteria was transferred into a sterile saline solution (1.5% NaCl). Turbidity was adjusted to an optical density of 640 nm to 0.09−0.10 and counted on TSA agar with 1.5% NaCl. A bacterial suspension of 1.5 × 10^8 CFU/ml was used as the stock solution for the challenge and clearance ability test. A mean lethal dose (LD50) of bacterial suspension, 1.5 ml/C2 was induced by the addition of 0.1 mM isopropyl-β-D-thio-galactopyranoside (IPTG) with gentle shaking at room temperature for 5 h. After induction, the bacterial cells were harvested by centrifugation at 10,000g (4 °C) for 15 min and washed with phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4). The pellet was collected by centrifugation at 8000g (4 °C) for 10 min and resuspended in lysis buffer (100 mM NaH2PO4, 10 mM Tris-Cl, pH 8.0, 8 M urea). The cells were lyzed by sonication on ice, freezing and thawing treatment according to Bangrak et al. [21]. The lysate was centrifuged at 18,000g for 30 min at 4 °C. The pellet and supernatant were stored at −80 °C until analysis.

2.3. Isolation of total RNA and RT-PCR

Total RNA was extracted from the eyestalk using TRIzol reagent (Invitrogen Corporation, USA) according to the manufacturer's protocol. A partial sequence of the CHH gene of L. vannamei (GenBank accession no. AV434016) was used to amplify a 783 bp segment of CHH cDNA sequence. The gene-specific oligonucleotide primers were as follows: forward 5'-TCACAAAAACCCCTCCTTC-3' and reverse 5'-TGATGCCCAGTGTTATTC-3'. Primers were synthesized by Invitrogen Corporation (Carlsbad, CA, USA); RT-PCR product was carried out with total RNA (500 ng) by the Qiagen (Invitrogen Corporation, USA) according to the manufacturer's protocol. The initial reaction started at 50 °C for 30 min and 95 °C for 15 min. The PCR reactions were performed as follows: 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The RT-PCR product was analyzed on 1.8% agarose gel electrophoresis and visualized by ethidium bromide staining under ultraviolet light. The identity of PCR products was confirmed by cloning into pGem®-T Easy vector (Promega Corporation, USA) and the sequence was analyzed using the ABI prism 377 apparatus. The data on the nucleotide sequence of CHH was used to design specific primers for cloning and expression.

2.4. Construction of the recombinant LV-CHH plasmid

CHH gene was amplified by specific oligonucleotide primers using RT-PCR technique. For forward primer, nucleotide sequences CACC were added for coupling with the site of T-overhang, located in pET-TOPO vector (Invitrogen Corporation, USA) as an expression vector. Oligonucleotide primers were as follows: forward primer 5'-CACCGATCCAAAGGCTCGCTCTCTC-3' and reverse primer 5'-TTTCCGACCATCTGGACAGCTAG-3'. The amplified PCR product (size 369 bp) was cloned into the pET-TOPO vector, forming a sequence encoding a fusion protein with an NH2-terminal hexa-histidine (6xHis) tag. The recombinant plasmid was termed LV-CHH-pET. The cloned fragment was sequenced using the ABI prism 377 apparatus to ensure authenticity of the cloned nucleotide sequence. Then, the LV-CHH-pET was transformed into competent Escherichia coli BL21 (DE3) using Champion™ pET-101 Directional TOPO expression kit (Invitrogen Corporation, USA).

2.5. Expression of recombinant LV-CHH in E. coli

The E. coli BL21 (DE3) harboring LV-CHH-pET was grown in LB medium containing 100 µg/ml ampicillin with vigorous shaking at 37 °C until the OD600 reached 0.6−0.8. The recombinant protein was induced by the addition of 0.1 mM isopropyl-β-D-thio-galactopyranoside (IPTG) with gentle shaking at room temperature for 5 h. After induction, the bacterial cells were harvested by centrifugation at 10,000g (4 °C) for 15 min and washed with phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4). The pellet was collected by centrifugation at 8000g (4 °C) for 10 min and resuspended in lysis buffer (100 mM NaH2PO4, 10 mM Tris-Cl, pH 8.0, 8 M urea). The cells were lyzed by sonication on ice, freezing and thawing treatment according to Bangrak et al. [21]. The lysate was centrifuged at 18,000g for 30 min at 4 °C. The pellet and supernatant were stored at −80 °C until analysis.

2.6. Purification of recombinant LV-CHH (rLV-CHH) protein using Ni2+-column

The rLV-CHH was purified using Ni-NTA column. Briefly, the supernatant was mixed gently with a slurry of Ni2+-resin on a rocking shaker for 1 h at room temperature. The mixture was packed into the column and washed with binding buffer (20 mM NaH2PO4, 500 mM NaCl, 5 mM imidazole, pH 7.4) before elution. The 6xHis tagged-fusion protein was eluted by elution buffer (20 mM NaH2PO4, 500 mM NaCl, 500 mM imidazole, pH 7.4). The fractions of eluted protein were analyzed by 12% SDS-PAGE gel. Authenticity of the rLV-CHH was confirmed by Western blot analysis using an anti-V5 antibody as primary antibody (Invitrogen Corporation, USA) and anti-mouse IgG conjugated to horse peroxidase serum as secondary antibody (GE Healthcare, UK). The total protein yield after purification was measured by the modified Lowry's method [22]. The rLV-CHH protein was stored at −70 °C until used. The bacterial protein from E. coli was used as a negative control, namely Neg-protein, according to the same method as mentioned above.

2.7. Effect of rLV-CHH on physiological parameters in L. vannamei

One hundred and sixty eight of the white shrimp (10−15 g) were divided into three groups including rLV-CHH, Neg-protein and control group. Individual shrimp in each treatment group were injected with 25 µg of rLV-CHH or Neg-protein through the abdominal muscle. PBS buffer was used to inject the control group. After the injection at 0, 0.5, 1, 3, 6, 12 and 24 h, hemolymph samples of eight individual shrimp were withdrawn from the ventral sinus at the 3rd segment of walking leg using a 1-ml syringe with a 25 G needle and then transferred into a 1.5-ml microtube for physiological parameters analysis as follows.

2.7.1. Hyperglycemic activity

One hundred microliters of hemolymph from eight individual shrimp in each time point of each treatment were separately well mixed with 900 µl of 3% trichloroacetic acid (TCA) as in
deproteinization. The hyperglycemic activity assay was performed by the modified method of Dubowski [23]. Briefly, 300 µl of supernatant after deproteinization with TCA was mixed with 2.7 ml of color reagent (0.6% o-Toluidine with 1.5% Thiourea in glacial acetic acid) and then tubes were immersed in boiling water for 8 min. Distilled water and glucose solution were used as a blank and a standard, respectively. All tubes were removed and rapidly cooled on ice. The optical density at 630 nm was measured by a spectrophotometer (Shimadzu UV-1201, Japan) and the glucose concentration was calculated from the calibration curve of the standard. Glucose was reported in milligrams per 100 ml of blood sample (mg/dL).

2.7.2. Total hemocytes count
After withdrawal, 50 µl of hemolymph from each shrimp was immediately diluted with 450 µl of 0.5% trypan blue in 2.6% NaCl. Hemocytes were counted using a haemocytometer under a microscope [24] and calculated as the number of total hemocytes (cell/ml) following the equation below:

\[
\text{Total hemocyte count (cell/ml)} = \frac{(A + B)}{2} \times \frac{\text{dilution}}{0.1 \text{ mm}^3} \times 10^3 \text{ mm}^3 \times 10^3 \text{ mm}^3
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whereas \( A, B \) = number of hemocyte cells in each side of haemocytometer.

2.8. Administration of rLV-CHH in shrimp against \( V. \) harveyi
Forty five shrimp (10–15 g) were divided into three groups, and injected with either rLV-CHH protein or Neg-protein or PBS (control group). \( V. \) harveyi were used as a pathogenic organism. Three hours after the injection, shrimp were challenged with 0.1 ml of bacterial suspension (1.5 \( \times \) 10^6 CFU/ml) resulting in 1.5 \( \times \) 10^5 CFU/shrimp. Hemolymph from each shrimp was collected by using a 1-ml syringe at the ventral sinus at 3 h after bacterial infection. Each sample was measured for immune parameters analysis by the following methods.

2.8.1. Total hemocyte count (THC)
After withdrawal, 50 µl of hemolymph from each shrimp was immediately diluted with 450 µl of 0.5% trypan blue in 2.6% NaCl. The hemocytes were counted using a haemocytometer [24] and calculated as the number of total hemocytes (cell/ml) following the same equation as above.

2.8.2. Protein concentration in serum
The total protein concentration of the shrimp serum was determined using the modified Lowry’s method [22]. Bovine serum albumin served as a protein standard. Briefly, hemolymph in each sample was clotted on ice and then homogenized with a hand homogenizer. Then, samples were centrifuged at 10,000 g for 15 min at 4 °C. Forty microliters of the supernatant was used to react with 120 µl of Folin reagent (1:15 in distilled water) to produce an unstable product that reduced molybdenum/tungsten blue under alkaline conditions and the optical density of 640 nm was read using a microplate reader (BioTek PowerWaveX, US).

2.8.3. Phenoloxidase activity in serum
Fifteen shrimp from each experimental group were used for PO activities determination. Phenoloxidase activity was measured by recording the formation of the red pigment dopachrome from L-dihydrophenylalanine (L-DOPA) using a microplate reader [25]. Briefly, 100 µl of hemolymph from individual shrimp was mixed with cadoxyle-citrate (CAC) buffer (0.01 M sodium cacodylate, 0.45 M NaCl, 0.1 M sodium citrate; pH 7.0) and kept immediately in liquid nitrogen before assays. Hemolymph samples were homogenized and centrifuged at 6000 g at 4 °C for 10 min. The supernatant was diluted (1:6) with chilled CAC buffer and 25 µl of this solution was pre-incubated with an equal volume of 1 mg/ml of trypsin solution for 5 min at 25 °C in 96-microwell plate. For blank assays, hemolymph was replaced by CAC buffer. After incubation, 50 µl of L-DOPA (4 mg/ml in CAC buffer) was added to the wells as a substrate and the enzyme activity was measured the optical density at the wavelength of 490 nm every 2 min for 10 min. The protein content in hemolymph was determined by Lowry’s method.

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**Fig. 1.** Nucleotide and amino acid sequence of CHH gene from \( L. \) vannamei. The stop codon is indicated by asterisk. The position corresponding to the sequences of the primers (forward, reverse) used in the RT-PCR are underlined. The nucleotide sequence has been submitted in GenBank (accession no. FJ598023).
Fig. 2. Multiple alignments of nucleotide (A) and amino acid sequence (B) of eyestalk CHH gene from *L. vannamei* (CHH4: accession no. FJ59823) with that of various CHHs: 
using bovine serum albumin as a standard. One unit of PO activity was defined as an increase in absorbance of 0.001 min⁻¹ mg protein⁻¹ [26].

2.8.4. Bacterial clearance ability

The ability of shrimp to remove foreign particles from the blood circulation system was measured by the modified method of Martin and coworkers [27]. Fifteen shrimp from each group were tested. Hemolymph of challenged-shrimp with 0.1 ml of _V. harveyi_ for 3 h was collected from each shrimp without using an anticoagulant. Twenty microliters of whole blood (without dilution) was dropped on TCBS agar. A two-fold dilution of whole blood was made using a sterile 1.5% NaCl solution. The number of bacteria was counted on the TCBS as above. The bacterial cells in the hemolymph of each shrimp were reported as CFU/mL.

2.9. Challenge test

The challenge test was conducted in triplicate with 15 shrimp per replicate in each treatment. Individual shrimp was injected with rLV-CHH protein or Neg-protein or PBS (control) on the sixth segment of the abdominal muscle for 3 h and then re-injected with 0.1 ml of bacterial suspension (1.5 × 10⁶ CFU/ml) resulting in 1.5 × 10⁵ CFU/shrimp. After the injection, shrimp were kept in a separate 80-L glass aquarium. Shrimp mortality was recorded every day for a period of 14 days.

2.10. Statistical analysis

Data was analyzed by one-way ANOVA. When significant differences existed among groups, multiple comparisons among means were tested by the Tukey’s test. The *P* ≤ 0.05 was considered statistically significant.

3. Results

3.1. CHH gene of _L. vannamei_

The CHH segment was amplified from total RNA derived from eyestalk of _L. vannamei_, and cloned into a pET-TOPO vector. The nucleotide sequences of CHH (Fig. 1) were submitted to GenBank (accession no. FJ598023). The partial cDNA of CHH contains an open reading frame (ORF) of 369 bp encoding a putative protein of 122 amino acid residue. A putative peptide was identified using MLC workbench 5.7 with a calculated molecular mass of 14.72 kDa and a predicted isoelectric point (pI) of 8.64. Multiple alignments of LV-CHH with other CHH homolog using ClustalW program are shown in Fig. 2. The percentages of nucleotide sequence identities of CHH had a similarity with _Penaeus monodon_ (GQ221085), _L. vannamei_ (AY434016) and _P. vannamei_ (X99731) at 94%, 99% and 100%, respectively.

3.2. Expression and purification of rLV-CHH

The rLV-CHH was expressed in _E. coli_ strain BL21 (DE3). The protein was purified in a Ni-NTA affinity column and separated on 12% SDS-PAGE (Fig. 3A). The purified protein was further analyzed by Western blot analysis using V5-epitope monoclonal antibody. A specific blot band was detected at the corresponding position as shown in Fig. 3B. The predicted molecular mass of the rLV-CHH protein was 14.72 kDa with additional 6xHis-tag and V5-epitope in the N-terminal of the expressed fusion protein, the extra amino acids increased the molecular mass of the expressed target protein by ~3.81 kDa. The apparent molecular mass of the purified protein was approximately 18.53 kDa. The final yield of the total protein was 1.3 mg/ml after a single Ni²⁺ affinity chromatographic step.

3.3. Biological activity in white shrimp ( _L. vannamei_ ) on rLV-CHH administration

After an injection of rLV-CHH or Neg-protein or PBS, the hemolymph glucose level showed an increase at 0.5 h post injection (Fig. 4A). In the group that injected with rLV-CHH, the glucose level increased to a maximum level at 1 h after the injection. However, the glucose level in both the PBS and Neg-protein group was changed rapidly at 0.5 h and then decreased toward the resting state level within 6 h after the injection (Fig. 4A). These results show that rLV-CHH can enhance glucose levels in shrimp hemolymph.

The total hemocyte count in shrimp after injection with rLV-CHH or Neg-protein or PBS shows the same trend as the glucose level in hemolymph. The THC of _L. vannamei_ that injected with rLV-CHH was significantly higher than those of shrimp that injected with Neg-protein and PBS (control group) at 1 h after injection (Fig. 4B). The THC of shrimp injected with rLV-CHH has the highest value of 22.08 ± 1.24 cell/ml at 3 h post injection. On the other hand, shrimp injected with Neg-protein and PBS has a value of 16.38 ± 1.46 cell/ml and 13.70 ± 0.92 cell/ml, respectively.

![Fig. 3. The rLV-CHH profile was performed by SDS-PAGE and Western immunoblot technique. (A) Coomassie blue stained SDS-PAGE of the rLV-CHH protein after purification by Ni⁺⁺-column; Lane M: molecular weight marker, lane 1: Neg-protein, lane 2: rLV-CHH at first elution, lane 3: rLV-CHH at second elution and lane 4: rLV-CHH at fourth elution. (B) The recombinant LV-CHH protein was analyzed by Western immunoblot technique using anti-V5 epitope (Lane M: molecular, lane 1: Neg-protein, lane 2: rLV-CHH protein).](image-url)
3.4. The effect of rLV-CHH on immune response of L. vannamei against V. harveyi infection

The immune response in L. vannamei injected with rLV-CHH or Neg-protein or PBS was checked at 3 h after challenging with V. harveyi. The total hemocyte count of shrimp that injected with rLV-CHH was significantly higher \( (P < 0.05) \) than those of Neg-protein and PBS groups (Fig. 5A). In the rLV-CHH group, the THC increased significantly by \( 12.33 \pm 1.27 \times 10^6 \text{ cell/ml} \) after 3 h post bacterial challenging. No significant difference in THC was observed among the shrimp that received Neg-protein \( (8.45 \pm 2.19 \times 10^6 \text{ cell/ml}) \) and PBS \( (9.01 \pm 1.53 \times 10^6 \text{ cell/ml}) \). In addition, a similar trend in serum protein levels was recorded where it was significantly higher \( (P < 0.05) \) in rLV-CHH group \( (169.30 \pm 12.08 \text{ mg/ml}) \) compared to Neg-protein \( (137 \pm 10.91 \text{ mg/ml}) \) and PBS \( (121.80 \pm 15.36 \text{ mg/ml}) \) as the control group (Fig. 5B). For the PO activity test, rLV-CHH injected shrimp showed a significant \( (P < 0.05) \) increase at \( 4.20 \pm 0.50 \text{ unit/min/mg protein} \) in comparison with the Neg-protein group \( (2.10 \pm 0.74 \text{ unit/min/mg protein}) \) and PBS group \( (1.93 \pm 0.50 \text{ unit/min/mg protein}) \), respectively (Fig. 5C).

To study the clearance ability to remove foreign particles in shrimp hemolymph after injection with V. harveyi \( (1.5 \times 10^5 \text{ CFU/shrimp}) \) for 3 h. Bacterial cells in shrimp injected with rLV-CHH decreased significantly by \( 45.50 \pm 4.26 \times 10^2 \text{ cell/ml} \) when compared to Neg-protein \( (130.69 \pm 21.66 \times 10^2 \text{ cell/ml}) \) and PBS group \( (142.17 \pm 28.97 \times 10^2 \text{ cell/ml}) \). No significant difference in clearance ability was observed between the Neg-protein and PBS group (Fig. 6).

3.5. The effect of rLV-CHH on the resistance of L. vannamei to V. harveyi

The death of shrimp occurred after 18 h post bacterial infection and no shrimp mortality occurred after 7–14 days in the treatments of rLV-CHH or Neg-protein or PBS. At 14 days post bacterial infection, survival rate of the rLV-CHH group was significantly highest.
4. Discussion

Crustacean hyperglycemic hormone was identified in the eyestalk of *L. vannamei*. Its mRNA was present at a high level in crustacean eyestalks. In previous work, the expression of the CHH gene in *P. monodon* was not only restricted to the eyestalk but also detectable in the heart and in the gill [14]. In crustacean, neuroendocrine centers are located in different structures of the nervous system and one of these structures, the eyestalk of X-organ-sinus gland complex (XO-SG), is traditionally considered as the major endocrine system of decapods [6,28]. The XO-SG complex produced several neuropeptides including the CHH/MIH/GHH family [6,29–31]. The CHH/MIH/GHH family consists of two kinds of neuropeptides, the CHH hormone, and the molt-inhibiting hormone (MIH), the gonad-inhibiting hormone (GHH) or the vitellogenin-inhibiting hormone (VIIH) and the mandibular organ-inhibiting hormone (MIOH) [6,9,32,33]. Although the biochemical and molecular study of the CHH-family of neuropeptides has been performed on a large number of crustacean species, including shrimp, crab, crayfish and lobster, the expression pattern and the evolutionary relationship of the peptides have not been completely resolved. CHH peptides belonging to CHH/MIH/GHH family are polymorphic due to changes in the amino acid sequence, and to the isomerization of one single amino acid residue from L- to D-configuration [6]. The different peptides of the CHH/MIH/GHH family are expressed in single cells [4,34]. Some studies have revealed that these peptides express in different neurons [35,36]. However, in this study we expressed rLV-CHH from eyestalk in *E. coli* and characterized the hyperglycemic activity of the protein in *L. vannamei*. After purification, we found a band of non-target protein contaminate in the protein solution, indicating that the result of rLV-CHH purification in this study has a little contamination of residual bacterial protein. Therefore, Neg-protein was used as a control in the experiments.

In this study, the rLV-CHH protein demonstrated a function in stimulating the hyperglycemic activity in shrimp by intramuscular injection. The THC was also increased to maximum level at 3 h after rLV-CHH administration. It may be possible that the hemolymph in this period is composed of glucose or other proteins at a high concentration which is stimulated by rLV-CHH. However, THC increased to a maximum level at 3 h and then decreased to a normal level within 12 h when compared to the control group. For the short term response from rLV-CHH administration, excess glucose will not cause organ damage. It is possible that rLV-CHH can cause excessive urination to remove the excess blood sugar in the body system. However, hyperglycemia in crustacean is still lacking in information about the effects of a high blood sugar on the physiological response and regulating excess blood sugar in the circulating system. The ability to elevate the glucose level in hemolymph of rLV-CHH protein indicated that rLV-CHH function as crustacean hyperglycemic hormone (CHH) in *L. vannamei*. Even though the purified target protein was contaminated by the residual protein from *E. coli*, it had no effect on the hyperglycemia property of the rLV-CHH. Neg-protein is expressed proteins from *E. coli* which is transformed with an expression vector without CHH gene. Neg-protein was used in this study to confirm that contaminated protein in *E. coli* does not affect shrimp immunity.

In fact, crustacean need to temporarily regulate their blood glucose concentration in hemolymph to ensure that adequate glucose levels are available for all cells and that excess glucose can be stored as glycogen in the hepatopancreas [37]. D-glucose is the major component of hemolymph carbohydrate [38,39] and is controlled within a strict range. The balanced secretion of several hormones is an essential part of the controlled glucose homeostasis. In particular, CHH is the important signaling molecule that played a role in the regulation of glycemia by increasing the glucose level in hemolymph via mobilization from the hepatopancreas and muscle glycogen stores [6,37]. The effects of hormones other than CHH on glucose levels in crustaceans are not well understood. Although, the major function of CHH is to regulate glucose metabolism in crustaceans, several studies have indicated that it may also regulate the reproductive and the molting cycles [15,40]. For example, the hemolymph CHH varies during molting cycles in *Astacus leptodactylus* [41] and *Carcinus maenas* [42]. The functional specificities of CHH hormone and other related hormones are not clearly defined because of the overlapping of their biological activities [2].

In addition, the possibility of using rLV-CHH to induce shrimp immune responses was tested. Immunostimulants have been administered to marine fish and shellfish through oral, immersion and injection methods [43]. In this study, the rLV-CHH was performed by intramuscular injection to ensure the amount of injected
protein per shrimp. THC has been reported to be a potential indicator of immune responses in crustacean especially in shrimp [44] and a higher-than-normal number of circulating hemocytes in crustaceans correlates with an increased resistance to pathogens [45]. The role in melatonigenia related to the PO activity system, is to stimulate several cellular defense reactions, including phagocytosis, nodule formation, encapsulation and hemocyte migration [46]. The activation of the PO system has been used as an indicator to measure immunostimulation in shrimp [47]. The experiment showed that the injecting of shrimp with rLV-CHH resulted in an increase in their ability to survive after being infected with *V. harveyi*, in comparison with Neg-protein and PBS as the control group. Besides, injecting rLV-CHH in shrimp significantly increased the blood parameters such as THC, PO activity and serum protein, including clearance ability than those of Neg-protein and PBS-injected shrimp. The clearance ability and PO activity are important humoral defense mechanisms against pathogens in crustaceans [48,49]. The PO activity results from the activation of the pro-PO enzyme. The pro-PO activating system has been well studied in crustaceans such as crayfish, shrimp, crab and lobster [26,44,50,51].

The cumulative effect of elevated THC, serum protein and PO activity coupled with the rapid clearance ability of bacteria in hemolymph reflects a higher survival rate in the rLV-CHH injected group compared to that in the Neg-protein and PBS-injected group, which suggests the possibility that rLV-CHH could enhance the immunity in *L. vannamei* against luminous bacteria, *V. harveyi*. In order to get more information of the mechanism on the immune system of shrimp by rLV-CHH administration, more experiments and specific parameters (such as lysozyme activity, complement, antioxidant enzymes activity) are required to understand how recombinant CHH can stimulate shrimp immune against pathogens.

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