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Short communication

Haemocyanin content of shrimp (*Fenneropenaeus chinensis*) associated with white spot syndrome virus and *Vibrio harveyi* infection process

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ABSTRACT

Haemocyanin (Hc) is frequently reported to vary significantly by physiological status and environmental stress in Crustaceans. In this paper, the shrimp *Fenneropenaeus chinensis* was infected with different concentrations of white spot syndrome virus (WSSV) and *Vibrio harveyi*. Then, the variation of Hc and total protein content of the haemolymph (TPCH) were investigated using the established double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) and Coomassie brilliant blue method, respectively. The results showed that the Hc content peaked at 12 h post-infection (PI) in the 10^{-2}, 10^{-4} and 10^{-6} viral supernatant (VS) groups, and the maximum was 93.03 ± 2.55 mg ml^{-1}, 77.57 ± 6.02 mg ml^{-1} and 70.25 ± 3.96 mg ml^{-1}, respectively. TPCH reached the maximum of 108.18 ± 1.36 mg ml^{-1} and 103.49 ± 1.33 mg ml^{-1} at 12 h PI in the 10^{-2} and 10^{-4} VS groups, respectively. The maximum was 96.94 ± 1.06 mg ml^{-1} at 24 h PI in the 10^{-6} VS group. In the *V. harveyi* infection groups, the Hc content reached a maximum of 87.97 ± 4.39 mg ml^{-1} at 36 h PI in the 10^6 CFU ml^{-1} group, 73.74 ± 4.38 mg ml^{-1} and 72.47 ± 2.09 mg ml^{-1} at 12 h PI in the 10^7 and 10^8 CFU ml^{-1} groups, respectively. TPCH reached a maximum of 111.16 ± 0.86 mg ml^{-1} at 36 h PI in the 10^6 CFU ml^{-1} group, 100.41 ± 0.51 mg ml^{-1} and 101.94 ± 0.47 mg ml^{-1} at 12 h PI in the 10^7 and 10^8 CFU ml^{-1} groups, respectively. These data showed that both Hc content and TPCH varied as the same extent after infection. The up-regulation of the Hc content at 6–36 h PI might be a reference threshold for shrimp infection.

1. Introduction

Haemocyanin (Hc) is one of the most abundant copper-containing proteins in shrimp haemolymph. Aside from its primary function as a respiratory protein, Hc was proven to participate in hormone transportation, ontogeny, protein storage and osmoregulation, as well as agglutination and phenoloxidase-like activity [1–3]. Moreover, studies documented that the Hc content varied with manganese ion exposure, dissolved oxygen, temperature, salinity, and moulting [4–6]. The Hc content increased in the shrimps *Penaeus indicus* and *Penaeus monodon* after white spot syndrome virus (WSSV) and *Vibrio harveyi* infection, respectively, as shown using a spectrometric method and two-dimensional electrophoresis [7,8]. In this paper, a double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) was developed using a monoclonal antibody (mAb) against *Fenneropenaeus chinensis* Hc, which was initially produced in our laboratory [9]. Then, the Hc content variation was investigated using DAS-ELISA, and the total protein content was determined using the Coomassie brilliant blue method in haemolymph of WSSV- or *V. harveyi*-infected *F. chinensis*. These findings may facilitate to know that Hc content was associated with the WSSV and *V. harveyi* infection, and then the role Hc plays in shrimp physiological process.

2. Materials and methods

2.1. Shrimp

Shrimp, *F. chinesis* (15–18 cm size), were collected in Qingdao Harbor, China. Gill tissues were randomly sampled and tested for WSSV by a two-step polymerase chain reaction (PCR). Then, shrimp were maintained at 23 °C in tanks containing aerated filtered seawater (31 ppt), with 50% renewed daily. After acclimatisation for 7 days, the shrimp were used for Hc purification and experimental infection.

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2.2. Hc purification

Hc in the haemolymph of shrimp was purified as follows. Haemolymph was extracted from the heart of shrimp using a sterile syringe with 1:1 (v/v) anticoagulant (26 mM Na citrate, 30 mM citric acid, 140 mM NaCl, 100 mM glucose, 10 mM EDTA, pH 7.2, 843 mOsm/kg). After centrifugation at 800× g for 15 min at 4 °C, the supernatant was applied to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and then bands of 75 kDa and 73 kDa which were previously proved to be subunits of Hc [9] were excised and electroeluted for 8 h at 120 V in electrode buffer (0.05 M ammonium bicarbonate containing 0.1% SDS). Finally, the eluted proteins were dialysed in phosphate buffered saline (PBS, 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). The protein concentration was determined by the Coomassie brilliant blue (CBB) method, followed by the Bradford assay [10]. The purified Hc was serially diluted in PBS and detected by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA).

2.3. Monoclonal antibody and polyclonal antibody

The mAb against Hc was previously produced by our laboratory [9]. In this study, the mAb was used as the detection antibody in DAS-ELISA.

Next, New Zealand white rabbits were immunized with 1 mg ml^{-1} purified Hc by a modification of the procedure of Ihara et al. [11]. After three booster injections, serum was collected, the titre of the polyclonal antibody from the rabbit (Pab-R) was determined by ELISA, and the reaction of Pab-R with the haemolymph of shrimp was tested by western blotting according to the procedure of Tang [9]. Then the Pab-R was used as the capture antibody in the DAS-ELISA.

2.4. DAS-ELISA

One hundred microlitres of the capture antibody was coated in 96-well microplates and incubated overnight at 4 °C. After three washes with PBS containing 0.05% Tween-20 (PBST), 200 μl of 4% bovine serum albumin was added to each well and incubated for 1 h at 37 °C. After three washes with PBST, 100 μl serially purified Hc or shrimp haemolymph, which was pre-incubated with 50 μl of 0.5 M EDTA to eliminate endoenzyme activity [12], was added and incubated for 1 h at 37 °C and then washed with PBST three times. After that, 100 μl of the detection antibody was added and incubated for 1 h at 37 °C, washed again, and 100 μl of the goat-anti-mouse IgG-alkaline phosphatase (1:5000 in PBS, Sigma) was added and incubated at 37 °C for 45 min. Finally, 100 μl of 0.1% *p*NPP (Sigma) in *p*NPP buffer (0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, 0.5 mM $MgCl_2$, pH 9.8) was added to each well, and the reaction was stopped by the addition of 50 μl of 2 M NaOH. The absorbance values were measured at 405 nm with an automatic ELISA reader (Molecular Devices).

2.5. Hc standard curve

The results of the DAS-ELISA for the serial dilutions of purified Hc were analysed by the Lavenberge-Marquardt method [13]. A four-parameter fit $Y = \{(A-D)/[1+(X/C)^B]\}+D$ was applied, where X is the logarithm of purified Hc concentration; Y is the absorbance at 405 nm; A is the Y value corresponding to the asymptote at the low values of the X-axis; and D is the Y value corresponding to the asymptote at high values of the X-axis. The coefficient C is the X-value corresponding to the midpoint between A and D, and the coefficient B describes how rapidly the curve makes its transition from the asymptote in the centre of the curve. A standard curve was calculated according to this curve. The equation $Y = E + F \times X$ was obtained, and the concentration range of serial dilutions of Hc was determined.

2.6. WSSV infection

The gill tissue (1 g) from heavily WSSV-infected shrimp stored at −80 °C in the laboratory was homogenized in 10 ml PBS. Then, the homogenate was centrifuged at 600× g for 20 min at 4 °C and the supernatant was filtered using 450 nm membrane, the resulting filtrate was centrifuged at 55,000× g for 1.5 h at 4 °C, the deposit was resuspended with PBS, and the WSSV copies was determined using quantitative real time PCR according to the procedure of Durand and Lightner [14]. Finally, WSSV concentration was adjusted to 10^7 copies μl^{-1} in the viral supernatant (VS) which was used for the experimental infection.

The VS was diluted to 10^{-2}, 10^{-4} and 10^{-6} with PBS, and the acclimatized shrimp were divided into four groups with 50 individuals in each group. The shrimp were injected in the muscle with 50 μl of VS dilutions. The control group was injected with 50 μl of PBS. Any dead individuals were counted daily. The survival curve was drawn with post-infection (PI) time (day) on the X-axis and the percent survivorship (PS) (%) on the Y-axis.

The survival curve proved that the three VS dilutions would ensure both a successful challenge and enough individuals for sampling.

Therefore, the acclimatized shrimp were divided into four groups again, with 100 shrimp in each group, with each shrimp receiving either a 50 μl injection of a 10^{-2}, 10^{-4}, or 10^{-6} VS dilution or PBS. Then, six shrimp were sampled randomly at 0 h, 6 h, l2 h, 24 h, 36 h, 48 h and 72 h PI, and their haemolymph was collected. The total protein content in haemolymph (TPCH) and the Hc content were determined using the CBB method and DAS-ELISA, respectively.

2.7. V. harveyi infection

V. harveyi stored at −80 °C in the laboratory was cultured in liquid 2216E medium (0.5% tryptone, 0.1% yeast extract, 3.4% NaCl and 0.01% $FePO_4$, pH 7.6–7.8) at 28 °C for 24 h. Then, the cultured medium was centrifuged at 2000× g. The pellets were resuspended in PBS and were measured with Densicheck (BIOMÉRIEUX), with 10^6 CFU ml^{-1}, 10^7 CFU ml^{-1} or 10^8 CFU ml^{-1} used as the infection dilution. The acclimatized shrimp were divided into four groups with 50 shrimp in each group. The shrimp were injected with 50 μl of *V. harveyi* dilutions. The control group was injected with 50 μl of PBS. After infection, any dead individuals were counted daily.

The survival curve proved that the *V. harveyi* dilutions of 10^6 CFU ml^{-1}, 10^7 CFU ml^{-1} or 10^8 CFU ml^{-1} would ensure a successful challenge; therefore, the acclimatized shrimp were divided into four groups again, with 100 shrimp in each group, with each shrimp receiving a 50 μl injection of 10^6 CFU ml^{-1}, 10^7 CFU ml^{-1}, or 10^8 CFU ml^{-1} *V. harveyi* dilution or PBS. Then, six shrimp were sampled randomly at 0 h, 6 h, l2 h, 24 h, 36 h, 48 h and 72 h PI, and their haemolymph was collected. The TPCH and Hc content were determined using the CBB method and DAS-ELISA, respectively.

2.8. Statistical analysis

All of the data are expressed as the mean ± standard deviation, with each measurement performed three times. The statistical analysis of PS, TPCH and Hc content was performed by One-way analysis of variance (ANOVA) using SPSS 19.0 software. The level of significance was defined as $p < 0.05$.