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RESEARCH ARTICLE

Proteomic approach for acute-phase proteins of hemolymph and muscles in *Scylla serrata* challenged by a pathogenic bacterium

LIU Wenjie, SU Jing, WANG Guizhong, WANG Sanying (✉)
Center of Proteomics, Department of Biology, School of Life Sciences, Xiamen University, Xiamen 361005, China

Abstract Acute-phase response is documented to be a significant mechanism of innate immunity in vertebrates and invertebrates. In this study, proteomic methodologies were applied for different protein expressions in hemolymph of *Scylla serrata* challenged by *Vibrio parahaemolyticus* after immunization, and in muscles of the crabs separately challenged by *V. parahaemolyticus*, *V. anguillarum* and *Aeromonas hydrophila*. Up-regulated cryptocyanin is documented in the hemolymph and up-regulated calexcitin, wingless (fragment) and tachykinin-related peptide in the muscle as acute-phase proteins. All the four altered proteins were responsible for bacterial stress, but cryptocyanin seemed to be a memory response protein against the challenge by a live bacterium after immunization of the live cells. These up-regulated proteins can be indicative of an understanding of immunity of a crab.

Keywords *Scylla serrata*, proteomics, acute phase response, acute phase protein, hemolymph, muscle, differential proteins

1 Introduction

Almost all animals can show acute-phase response (APR) against injury and infections, marking the synthesis of acute-phase proteins (APPs) (Steel and Whitehead, 1994; Armstrong and Quigley, 1999; Wu et al., 2004). These APPs help in resisting invasion from microbes, rehabilitating from ravage, restoring to normal resting status (Peng et al., 2004; Mastaloudis et al., 2006; Weber et al., 2006). APR is documented to be a significant mechanism of innate immunity in mammals including human beings, but this response begins in the lower creatures (Steel and Whitehead 1994; Armstrong and Quigley, 1999). Agnatha and invertebrates mainly depend on innate defense system to protect themselves because they lack the rearrangement of *IgV (D)J* gene segments used by all jawed vertebrates to produce diverse repertoires of T and B lymphocyte antigen receptors (Matsunaga and, 1998; Rahman Laird et al., 2000), which shows the significance of the APR system for them.

Scylla serrata is one of the most valuable and economic breeds of crab in Chinese southeast coastal area. With the rapid development of livestock breeding industry, great economical losses caused by infectious diseases have appeared in this aquaculture (Lo et al., 1996; Andersen and Norton, 2000). An understanding of the immune defense in this crab will be helpful to control infectious diseases . Accumulative data have indicated that the crustaceans mainly depend on innate immunity against infectious diseases (Kurtz and Franz, 2003; Rolff and Siva-Jothy, 2003; Iwanaga and Lee, 2005). Recently, acquired immunity was approached in invertebrates, showing the specific immunity-related phenomenon and molecules in these animals (Kurtz and Franz, 2003; Watson et al., 2005). It is significant, therefore, to study the characteristics of molecules in the immunity of this crab.

Recently, proteomics has emerged as an indispensable methodology for high-accuracy, high-efficiency, and high-throughput protein analyses in the post-genome era. It provides an understanding of the expression of a genotype at the phenotypic level in a target cell in a given stage (Peng et al., 2004; Peng, 2005). Reports have indicated that this methodology is very efficient for the understanding of immunity (Cui et al., 2005; Vierstraete et al., 2005). The result of this study was obtained by the use of proteomic methodologies for the protein expression changes in hemolymph and muscles of *S serrata* infected with pathogenic bacteria. Meanwhile, the innate and acquired immunity-related molecules were characterized by bacterial challenge and the challenge after bacterial immunization, respectively.

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E-mail: wangpeng@xmu.edu.cn

2 Materials and methods

2.1 Bacterial strains, culture and vaccine preparation

Vibrio parahaemolyticus, V. Anguillarum and *Aeromonas hydrophila* strains were available in our laboratory (Xu et al., 2005). The strains were used for vaccine preparation and experimental infection. These bacteria were separately cultured aerobically at 28ºC in the shaker bath overnight as seeds. Fresh overnight cultures were diluted into 1:50 in Luria-Bertani medium and growth continued for 18 hours at 28ºC. The cultures were collected by centrifugation at 6 000 g for 10 min at 4ºC. The gained cultures were re-suspended in 0.65% NaCl for washing and pelleted by centrifugation at 6 000 g for 10 min. The cultures were inactivated with 2% formaldehyde solution for 4 hours at room temperature (25ºC) and washed by centrifugation with 0.65% NaCl. Cells were then suspended in PBS to a final concentration of 2.0×10^7 /mL for *V. parahaemolyticus,* 2.5 $\times 10^8$ /mL for *V. anguillarum* and 3.2×10^7 /mL for *A. hydrophila*. The vaccines were stored at 4ºC until use.

2.2 Vaccination or bacterial challenge

S. serrata were fed in laboratory and randomly divided into two parts separately for analysis on bacterial challenge and the challenge after immunization. The part with bacterial challenge was divided into four groups with five crabs per group. The four groups were separately injected into 0.5 mL of inactivated *V. parahaemolyticus, V. anguillarum*, *A. hydrophila* and sterile saline. After 15 min, a pincer was obtained for muscle samples. The part with the challenge after immunization was divided into five groups with ten crabs per group. These crabs were separately immunized by live (group 1) and inactivated *V. parahaemolyticus* (group 2) and challenged by live (group 3) and inactivated *V. parahaemolyticus* (group 4) , respectively, 7 days later. These bacteria of 0.2 mL (5.0×10^7 /mL) were inoculated into the articular corium of basic pitch in the third pereiopods of *Scylla serrata*. The crabs were injected into sterile saline as a control group (group 5). Hemolymph was collected at 18 hours after injection.

2.3 Sample preparation

Collected hemolymph with 1.5 mL per crab was centrifuged at 10 000 rpm for 20 min and the resulting supernatant was stored at −20ºC for use.

Collected muscles with 0.2 g per crab was mixed with 0.6 mL lysis buffer (8 mol urea, 2 mol thiourea, 0.1 mol DTT, 2% Triton X 100, 40 mmol Tris, 1mmol EDTA, 1 mmol/L PMSF), abraded in the ice and kept in 4ºC overnight. The mixture was centrifuged 18 h later, at 12 000 rpm for 30 min and 4ºC, and the supernatant fluid was extracted and kept at 20ºC. Loading proteins were measured by Bradford method.

2.4 SDS- PAGE

Discontinuous buffer system of Laemmli with 10% resolving gels and 3% stack gels was used to resolve proteins. All samples were heated for 5 min in boiling water and electrophoresed with constant voltage of 120 V until the dye-front reached the bottom of the gel. The protein bands were visualized by Coomassie Brilliant blue-R250 (Merck, Germany) staining.

2.5 Two-dimensional electrophoresis

Two-dimensional electrophoresis (2DE) was performed based on a procedure described by Zhang et al. (2004). Briefly, sample extracts containing 200 μg proteins were dissolved in solution (8 M urea. 2 M thiourea, 4% CHAPS and 80 mM DTT). IEF was carried out using pH 3~9.5 carrier ampholyte for 8 000 Vh. After equilibration for 15 min, the IEF gels were transferred to the 2DE using 10% acrylamide gel. The 2DE gels were stained with Coomassie blue.

2.6 Mass spectrometric analysis

Protein spots were visualized by staining with Coomassie blue. Subsequently, gels were scanned in an AGFA white-light scanner at a resolution of 400 by 200 mm, and the raw images were processed using the 2D software Melanie-4.0. Following background subtraction and spot detection, the gel patterns were matched by visual comparison.

Target protein band in 1DE gels and spots on 2DE gels were excised and dehydrated several times with acetonitrile. After vacuum-drying, the gels were incubated with 10 mM dithiothreitol in 100 mM ammonium bicarbonate at 56ºC for 60 min and subsequently with 55 mM iodoacetamide in 100 mM ammonium bicarbonate at 25ºC for 60 min. After washing with 100 mM ammonium bicarbonate and thereafter with acetonitrile, gels were dried again. Digestion was performed using sequencing grade modified porcine trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate at 37ºC for 15 hours. The gel pieces were extracted with 5% formic acid and acetonitrile alternatively. The extracts were vacuum-dried and re-dissolved in 0.5% trifluoroacetic acid and 50% acetonitrile.

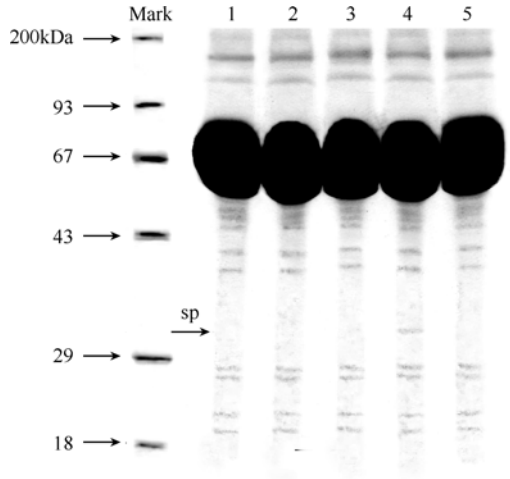
The sample solution (30~100 ppm) with equivalent matrix solution was applied to MALDI TOF-Target and prepared for MALDI-TOF/MS analysis. HCCA was used as the matrix. MALDI-TOF spectra were calibrated using trypsin auto-digestion peptide signals and matrix ion signals. All MALDI analyses were performed by a fuzzy logic feedback control system (Reflex III MALDI-TOF system, Bruker) equipped with delayed ion extraction. Peptide

masses were searched using the program MASCOT (http://www.matrixscience.com/cgi/search_from_select.html) with the condition: taxonomy, metazoa (animals); allowable missed cleavages, 2; variable modification, carbamidomethyl (C); Peptide tol.±, 150 ppm; monoisotopic, being chosen; mass values, MH^+.

3 Results

3.1 APP analysis of hemolymph by SDS–PAGE

Hemolymph was collected from the crabs immunized with live (group 1) and inactivated *V. parahaemolyticus* (group 2), and then challenged by live (group 3) and inactivated *V. parahaemolyticus* (group 4), respectively. These samples were analyzed by SDS–PAGE (Fig. 1). Compared with the control group challenged by sterile saline, groups 1, 2 and 4 showed no significant changes, whereas group 3 showed one more band with ~ 32 kDa, namely SP.



Lane 1: Control; Lane 2: Immunized by live *V. parahaemolyticus*; Lane 3: Immunized by dead *V. parahaemolyticus*;
Lane 4: Challenged by live *V. parahaemolyticus* after immunized by live *V. parahaemolyticus;*
Lane5: Challenged by dead *V. parahaemolyticus* after immunized by dead *V. parahaemolyticus*

Fig. 1 SDS-PAGE analysis of hemolymph of *Scylla serrata*

3.2 2DE maps of muscle proteins of *S. serrata* infected with a bacterium

S. serrata were separately challenged by *V. parahaemolyticus, V. anguillarum*, and *A. hydrophila*. Their muscles were sampled and analyzed by 2DE. Figure 2 showed the comparison of muscle proteome corresponding to the infectious stress between *V. parahaemolyticus* and a sterile saline control. Three differential proteins, namely P1, P2 and P3, were determined, indicating that all of them were only seen in the crabs infected by this bacterium. Meanwhile, P1 and P2 changed in the same manner when the crabs were separately infected with *V. anguillarum* and *A. hydrophila,* but not P3. Figure 3 was partial maps elucidating these altered protein spots.

3.3 Analysis on APPs by PMF

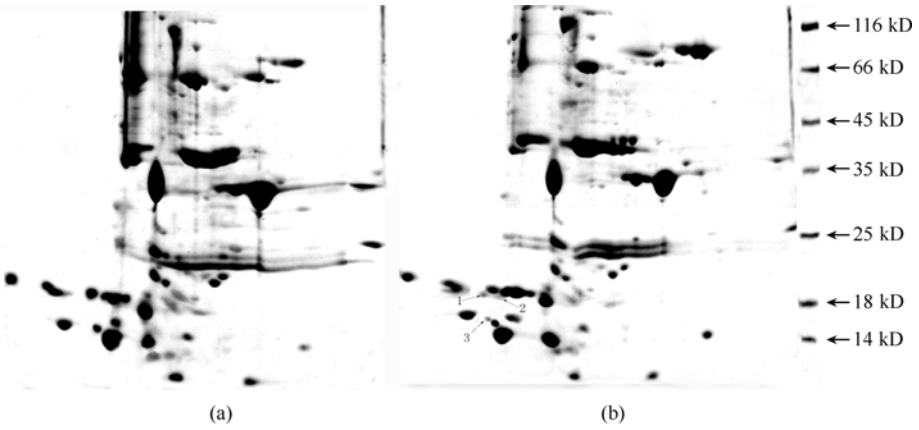
The four proteins, sp in 1DE and P1–3 in 2DE, were subjected to further analysis, and their identities were determined using PMF by MALDI-TOF/MS. Sp, P1, P2 and P3 were identified as cryptocyanin, calexcitin, wingless and tachykinin-related peptide, respectively. Table 1 summarizes the proteins identified by MALDI-TOF/MS and database searching based on the gained PMF data, molecular weight and other relevant parameters. The identified proteins could be assigned to APR-related proteins, although they took part in a wide variety of other cellular processes.

4 Discussion

S. serrata is a large, commercially important portunid that inhabits the coastal waters throughout the southeast of China. High demand and ease of capture have led to overexploitation, so that the culture of this crab has become an economically important marine shellfish species in China. With the rapid development of this crab culture industry, great economical losses are appearing because of the infectious diseases caused by bacteria and viruses (Lo et al., 1996; Andersen et al., 2000). However, little is known about the immunological basis of this crab. In this study, proteomic methodologies were applied to screen APPs of hemolymph and muscles of the crab responding to bacteria. Proteins of hemolymph and muscles were resolved in 1DE and 2DE, respectively. Differential proteins were analyzed by MS. We failed to analyze hemolymph by 2DE because the majority of proteins were hemocyanin in hemolymph of the crab so that the hemolymph could not be measured by routine 2DE.

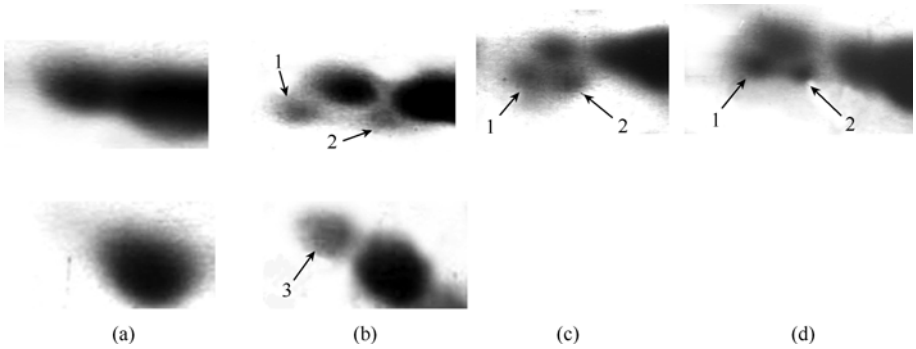
Four APR-related proteins were identified in this study, with one from hemolymph and three from muscles. They were only seen in the experimental crab and identified as cyptocyanin, calexcitin, wingless and tachykinin-related peptide by MS. These proteins were first reported here to be APR-related proteins.

Cryptocyanin, the altered protein from hemolymphs, was upregulated in these crabs challenged by live *V. parahaemolyticus* after immunization with the same live cells, but not in the same condition except for the replacement of live bacterium with dead one. Reports have indicated that cyptocyanin, a copper-free hexameric protein in crab hemolymph, is markedly similar in sequence, size, and structure with hemocyanin, the copper-containing oxygen-transport protein found in many arthropods (Terwilliger et al., 1999). This protein is present in extremely high concentrations in the hemolymph of the crab during the premolt portion of the molt cycle, and plays a major role in forming the new exo-



(a): 0.85% brine; (b): *V. parahaemolyticus*

Fig. 2 Comparison of muscle proteome of *Scylla serrata* responding to stress between *V. parahaemolyticus* and sterile brine. Samples were collected in 15 min when these challenges were performed



(a): Control; (b): *V. parahaemolyticus*; (c): *V. anguillarum*; (d): *A. hydrophila*; 1: Spot 1; 2: Spot 2; 3: spot 3

Fig. 3 Zoom-in map of the proteins of variances in 2-D map

Table 1 Database searching results of differential spots

Protein	Accession	Definition	Taxonomy	Score	Matched peptide	Sequence coverage/%	PI/MW
SP		cryptocyanin(Fragment)	*Dungeness crab*	66	14	32	5.49 /75892
Spot1	gi|3348129	Calexcitin	*Todarode pacificus*	26	4	32	4.98 /22169
Spot 2	Q86S02	Wingless (Fragment)	*Chaetodiopsis meigenii*	24	6	43	9.39 /22100
Spot 3	gi|48105951	Tachykinin-related peptide	*Apis mellifera*	31	3	35	9.56 /13485

skeleton, but its new function should be in assumption (Terwilliger, 1999; Brown-Peterson et al., 2005; Terwilliger et al., 2005). Studies have indicated that hemocyanin shows the ability of non-specific immunity, functioning as defense molecules (Decker et al., 2001; Adachi et al., 2003; Zhang et al., 2004; Zhang et al., 2004). Acquired immunity is not determined in invertebrates, but a report has indicated that a crustacean defense system may be capable of specific memory (Kurtz and Franz, 2003). The result suggests that cryptocyanin may be a characteristic protein responsible for the challenge only by a live bacterium.

Up-regulated P1, P2 and P3 were assigned to calexcitin, wingless and tachykinin-related peptide, respectively, all of which appeared in *V. parahaemolyticus* infection and only in P1 and P2 infected by *V. anguillarum* and *A. hydrophila*. Therefore, the three proteins were responsible to bacterial infection.

Calexcitin (CE) is a low-molecular-weight calcium- and guanosine-triphosphate-binding protein, which is phosphorylated during associative learning through the Ca2+-dependent inhibition of K+ channels and activation of ryanodine receptors in both vertebrates and invertebrates (Gombos et al., 2003; Hatakeyama et al., 2004). This protein has high affinity to protein kinase C. Wingless, a member of Wnt family, has a role in the establishment of different cell fates, working within and between the anterior and posterior compartments of segments. Thus, two distinct roles for wingless in wing morphogenesis have been identified: a primary role in specifying the wing primordium and a subsequent role mediating the patterning activities of the dorso-ventral compartment boundary (Simonnet et al., 2004; Cayuso and Marti, 2005). Tachykinin is one of the most well-known bioactive peptides with multifunctions found in vertebrates, and tachykinin-related peptides have also been isolated from various invertebrate species (Satake et al., 2003; Yasuda-Kamatani and Yasuda, 2004). The three pro-

teins were up-regulated to respond to the challenge by bacterium, suggesting their new function. This not only expands our knowledge on APR-related proteins, but also provides valuable information for strategy of immune prevention in the crab.

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