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The protein expression profile in hepatopancreas of scallop *Chlamys farreri* under heat stress and *Vibrio anguillarum* challenge

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ABSTRACT

Heat stress and pathogen infection have been considered as the main causes for mass mortality of cultured scallops during summer. In the present study, the expression profiles of proteins in the hepatopancreas of scallop *Chlamys farreri* were examined to reveal the possible mechanisms of physiological responses of scallop beneath heat stress and bacterial infection. An earlier occurred and higher mortality was observed in the scallops from combination treated group (28 °C and an injection of *Vibrio anguillarum*) in comparison to those in heat stress (28 °C) and bacteria challenge (*V. anguillarum* injection only) group, as well as control (PBS) and blank (untreated) group. The proteins in the hepatopancreas from scallops post 6 h of treatment were analyzed by using 2-D PAGE and ImageMaster 2D Platinum. There were total 1003 spots detected in control group, 1193 spots in heat stress group, 1263 spots in bacteria challenge group, and 1241 spots in the combination group. Fifteen protein spots expressed differentially between the combination treatment group and the bacteria challenge group were successfully identified by mass spectrometry and they were mainly classified as binding and catalytic proteins, such as endoglucanase, methylmalonate-semialdehyde dehydrogenase, xylose isomerase, tryptophanyl-tRNA synthetase, 40s ribosomal protein SA, glutathione S-transferase 4, and Mitochondrial transcription factor A, etc. These results indicated that the mortality of scallops suffered from the combination treatment was probably attributed to the impaired modulation of digestion and metabolism and ruined protein synthesis caused by heat stress together with bacteria infection. These data also provided valuable insights into the possible mechanisms of summer mortality occurrence of scallop at protein level.

1. Introduction

Zhikong scallop *Chlamys farreri*, one of the most important aquaculture bivalve species, has been widely cultured along the north coastline of China [1]. In recent years, cultured scallops had frequently suffered mass mortalities in summer, which might be caused by a combination of several environmental factors such as temperature, salinity, pathogens and overcrowded culture density [2,3].

Temperature and pathogens have been considered as the main causes for the scallop summer mortality. Heat stress can directly result in damage to proteins, DNA, lipids, and other macromolecules [4], and then bring various harmful reactions to organisms, such as ROS burst [5–7], energy metabolism change [4], growth arrest [8,9], immune activity decline [7,10,11], and ultimately lead to homeostasis disorder. *Vibrio*, as the most popular bacteria in the seawater and some of them as the pathogens for aquaculture animals, could cause adductor muscle loose, mantle mucid, hepatopancreas atrophied or tumefacient and other symptoms, and then result in the decrease of feeding rate or the halt of feeding. It have been reported that both heat stress and bacteria challenge could increase the energy consumption, and decrease the cellular energy allocation in scallops [6], therefore many obligatory strategies must be introduced to deal with stressors for their survival.

Both vertebrate and invertebrate have adopted multiple efficient defense strategies to counteract heat stress during evolution. Several universal strategies are applied by both vertebrate and invertebrate against heat stress. The molecular chaperones, such as heat shock proteins (Hsps) [12], are induced to stabilize denaturing proteins, and help refolding denatured proteins [13]. Redox related

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enzymes, e.g. SOD and ACP, are responsible for the elimination of ROS to keep redox balance [6], and the energy generation associated enzymes, such as arginine kinase [14], are involved to regulate the energetic redistribution. Mitochondrial transcription factor proteins, such as TFAM [15,16], are in charge of controlling the mtDNA integrity and transcription. As invertebrates, bivalves mainly rely on innate immunity to defend against pathogen infection [17]. Hemocyte is one of the important components of innate immunity in bivalves, and it can recognize pathogens through pattern recognition receptors [18], phagocytize or encapsulate the pathogens, and subsequently eliminate them via hydrolytic enzymes [19] and reactive oxygen species [20]. Opsonins, antimicrobial peptides and other humoral molecules are induced to mediate various reactions during immune defense process [21]. The information about the protein expression profiles in stress response will be helpful to better understand the mechanism of bivalve defense against the stressors.

Proteomics analysis, aiming at the entire protein pool, can provide information from a whole-scope viewpoint on the processes of protein synthesis, post translation modifications, protein degradation, and the interaction between proteins or other molecules [22]. To date, proteomics analysis has been widely used in microorganisms, plants and animals research, and also been applied to investigate the immune response to bacteria [23] and virus challenge in scallops [24]. However, the proteomics information about the response of scallop against heat stress and/or bacterial challenge is still limited. The hepatopancreas of scallop is not only a digestive tract but also an essential immune organ [23], and it is always selected as a good material to study the internal responses of scallop to exterior stimulations. In the present study, comparative proteomics method was used to characterize the proteins in hepatopancreas from scallops treated with bacteria challenge and heat stress (28 °C) to examine the alternation of protein expression levels. This study could highlight some new molecules for subsequent study about the response of scallop to heat stress and bacteria challenge, and would bring usable clues for scallop culture and breeding to deal with the summer mass mortalities.

2. Material and methods

2.1. Scallops and bacterial strain

Healthy scallops with average wet weight of 15–20 g (without matured gonad) were collected in March, 2012 from a shellfish farm (Qingdao, China) and acclimated in a fiberglass tank at salinity 30 ± 0.1‰, temperature 18 ± 1 °C, dissolved oxygen above 6.0 mg L^{-1} and pH from 7.7 to 8.2. The seawater was changed 100% daily to ensure high water quality.

Gram-negative bacteria *Vibrio anguillarum* was kindly provided by Dr. Zhaolan Mo, and was cultured and harvested by the method of Wang et al. [25]. The pellet was re-suspended in sterilized phosphate buffered saline (PBS, 136.89 mmol L^{-1} NaCl, 2.68 mmol L^{-1} KCl, 8.10 mmol L^{-1} Na_2HPO_4, 1.47 mmol L^{-1} KH_2PO_4, pH 7.4) and adjusted to the final concentration of 2×10^6 CFU mL^{-1}.

2.2. Heat stress and bacteria challenge

After two weeks acclimation at 18 °C, six hundred scallops were employed to survey the cumulative mortality rate (MR) of scallops during 0–24 h exposure to *V. anguillarum* challenge, heat stress, and *V. anguillarum* challenge combined heat stress. Scallops were randomly assigned into 15 buckets with each bucket containing 40 individuals, and every three buckets compose one group. The heat stress was set at 28 °C, which was close to the maximum temperature recorded in near-shore regions in Shandong province in summer [3]. A lower concentration (2×10^6 CFU mL^{-1}) of *V. anguillarum* was chosen to challenge the scallops in the acute infection experiment according to the previous reports [6,25,26]. The scallops in group I received an injection of 50 μl *V. anguillarum* (2×10^6 CFU mL^{-1}, in sterilized PBS) and maintained at 18 °C as bacteria challenge group, and the scallops in group II were maintained at 28 °C after they received an injection of 50 μl PBS (designated as heat stress group). Group III was designated as combination treatment group which received an injection of 50 μl *V. anguillarum* (2×10^6 CFU mL^{-1}, in sterilized PBS) and maintained at 28 °C. The scallops in group IV received an injection of 50 μl PBS and maintained at 18 °C as control group, while the untreated scallops (group V) were maintained at 18 °C as blank group. The numbers of dead scallops in all the five groups were monitored at 0, 3, 6, 12 and 24 h after treatment.

Another 480 scallops were employed to investigate the expression variation of hepatopancreas protein from scallops in the control, bacteria challenge, heat stress and combined treatment groups. The scallops were randomly stocked into 12 buckets with each bucket containing 40 scallops, and every three buckets compose one group. The treatments and the definition of the groups of bacteria challenge, heat stress, combination treatment and control were same as the description above. After 6 h post treatments, six scallops were randomly sampled from each bucket, and the hepatopancreas from six scallops in one bucket were pooled together as one replicate, and three replicates were set for each sampling time point and each group.

2.3. Protein extraction and 2-dimension electrophoresis

Hepatopancreas from six scallops of each group were mixed and used for protein extraction. Total proteins were extracted by using the Total Protein Extraction Kit (Bestbio Company, China), further purified with 2-D Clean-up kit (GE), and finally quantified with Non-Interference Protein Assay Kit (Sangon Biotech Company, China).

For the isoelectric focusing (IEF), 1.0 mg of protein sample was loaded onto the Immobiline Dry Strip Gel (IPG) strip (pH 4–7 L, 24 cm) (GE), and the IEF was carried out on Ettan IPGphor III (GE) at 20 °C using a continuous increase in voltage to 60,000 Vhr. After IEF, the IPG strips were submitted for reduction and alkylation through sequential incubating in equilibration buffer I (2% SDS, 50 mmol L^{-1} Tris–HCl pH 8.8, 6 mol L^{-1} urea, 30% glycerol, 0.002% bromophenol blue, 10 mg mL^{-1} DTT) and equilibration buffer II (2% SDS, 50 mmol L^{-1} Tris–HCl pH 8.8, 6 mol L^{-1} urea, 30% glycerol, 0.002% bromophenol blue, 10 mg mL^{-1} DTT, 25 mg mL^{-1} iodoacetamide) for 15 min, respectively. The 12.5% SDS-PAGE was carried out on Ettan DALT six (GE) at 15 °C for about 6 h, then the gels were stained with Coomassie Brilliant Blue (CBB) R250 staining solution (40% ethanol, 10% acetic acid, 0.15% CBB R250). Three repeats were conducted on protein samples of each group.

2.4. Data analysis

2-DE gels were scanned on ImageScanner III (GE) and analyzed by using ImageMaster 2D Platinum V5.0 (GE). For comparative analysis, the percentage intensity (% vol) of each matched spot was used for comparisons between groups.

2.5. Spots excision and protein identification

Selected protein spots were picked out from the 2-DE gels manually and the proteins were subjected to in-gel digestion with trypsin (10 ng μL^{-1}) at 37 °C overnight. Digested samples were analyzed with ultrafleXtreme MALDI-TOF/TOF (Bruker Daltonics