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Alterations of protein expression in response to crowding in the Chinese shrimp (*Fenneropenaeus chinensis*)

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ABSTRACT

Crowding, as a result of high stocking densities in aquaculture, can affect the physiological state and flesh quality of aquatic animals. To better understand the mechanism of response to crowding stress in Chinese shrimp (*Fenneropenaeus chinensis*), two-dimensional gel electrophoresis (2-DE) coupled with matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry (MS) identification were used to analyze differentially expressed muscle proteins between shrimp under crowded and non-crowded conditions. Spots corresponding to 34 differentially expressed proteins were found. Further MALDI-TOF/TOF analysis indicated that three of the enhanced proteins, 14-3-3-like protein, actin T_2 and pyruvate kinase 3, were involved in myogenic signaling, muscle cytoskeleton and carbohydrate catabolism, respectively. Moreover, quantitative RT-PCR showed that mRNA levels of 14-3-3-like protein and pyruvate kinase 3 were up-regulated after a 2-h crowding stress, whereas actin T_2 showed down-regulation. The alterations of protein expression observed in the muscle may cause flesh quality decline of shrimp after crowding stress.

1. Introduction

The Chinese shrimp, *Fenneropenaeus chinensis*, is one of the most commercially important species in aquaculture due to the high demand for consumption. However, in recent years, with the continuous expansion and over-intensification of aquaculture, productivity has been steadily decreasing, and serious infectious diseases have been emerging and increasing (Beveridge et al., 1997; Kautsky et al., 2000). Increased stocking density can influence growth and survival of shrimp due to the stress response induced by crowding (Allan and Maguire, 1992; Mena-Herrera et al., 2006).

Stress has been defined as a physiological cascade of events that takes place when an individual attempts to re-establish homeostatic norms in the face of a perceived threat (Schreck et al., 2001). Studies have shown that stress could reduce shrimp resistance to pathogenic diseases, resulting in decreased growth rate and mass mortality (Burgents et al., 2005; Moullac and Haffner, 2000; Moullac et al., 1998). Costas et al. (2008) also found that crowding stress could affect amino acid metabolism in Senegalese sole, *Solea senegalensis* juveniles. In addition, crowding stress influences muscle glycogen content, rigor evolution and several post-mortem muscle properties of fish, which affect the firmness of muscle (Skjervold et al., 2001). Lowered muscle pH and delayed *rigor mortis* of gilthead seabream (*Sparus aurata*) also resulted from crowding stress in the harvest and slaughter conditions; due to the stress, collagen and sulfated glycosaminoglycan content of the samples decreased markedly through storage time (Matos et al., 2010). Another recent study showed that significant negative effects were mostly seen due to long-term stress (LS) at early stages *post-mortem*, but also short-term stress (SS) had significant negative impacts on muscle quality of *pre-* and *post-rigor* filets of farmed Atlantic salmon (*Salmo salar* L.) (Bahuaud et al., 2010). Stress further accelerated the incidence of myofiber–myofiber detachments, increased the percentage of myofiber–myocommata detachments over the storage period and increased the percentage of myofiber breakages and contracted myofibers 96 h *post-mortem*. In addition, significant correlations were observed between muscle pH and cathepsin B + L activity, muscle texture and muscle degradation parameters, and cathepsin B activity was correlated with muscle degradation and *cathepsin L* gene expression with muscle degradation and texture (Bahuaud et al., 2010).

The behavioral and physiological responses of crustaceans to crowding have been well studied (Saroglia and Liu, 2012) in order to search for stress biomarkers. Reduced survivorship and growth rate of shrimp post-larvae may imply negative effects of stress under high stocking densities. Significantly increased blood plasma levels and osmolality of cortisol, glucose and lactate also could be indicators of crowding response. However, the reliability of these traditional stress

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biomarkers remains controversial, since their application in some circumstances is complicated by the modulation of stress responses by several intrinsic and extrinsic factors in aquatic animals (Barton, 2002; Barton and Iwama, 1991). Therefore, changes at the cellular and molecular levels of organization, especially in genes and proteins, are increasingly used to supplement more traditional biomarkers (Gui and Zhu, 2012). The search for appropriate candidate-gene indicators of stress response can be approached by genomic analysis using microarray or mRNA differential display (DD) techniques. One of the previous studies (Ribas et al., 2004) identified one differentially expressed sequence, enolase gene homologue (S-enolase), as a potential molecular biomarker for stress diagnosis in gilthead sea bream (*S. aurata*). In addition, current proteomics also provides a high-throughput approach to study sophisticated molecular networks in signaling transduction, transcription, protein synthesis/turnover and cross-tolerance to different stress conditions (Pandey and Mann, 2000; Wetie et al., 2014). Mass spectrometry (MS)-based proteomic profiling and protein identification have become powerful tools for the discovery of new disease biomarkers. Among the MS platforms, matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) MS offers high sample throughput and the flexibility to couple with different off-line sample fractionation techniques (Leung and Pitts, 2008).

In the present work, differentially expressed proteins of Chinese shrimp muscle involved in response to crowding stress were investigated by a proteomic approach. Moreover, the transcriptional levels of genes of the proteins identified were quantified by real-time PCR. The aim of our study was to find potential molecular mechanisms linking effects of crowding stress on flesh quality of the shrimp, as well as possible molecular biomarkers for stress response.

2. Materials and methods

2.1. Shrimp culture and crowding stress

Healthy Chinese shrimp (*F. chinensis*), approximately 16–20 g body weight and 11–13 cm length, were purchased from a local shrimp farm. They were reared in oxygenated seawater with 25‰ salinity at 25 °C, and acclimated to laboratory conditions for 7 days. Fed with commercial feed at 5% body weight per day, vigorous shrimp were chosen for the experiment.

Thirty shrimp designated as crowding-stressed group were kept in an aquarium (50 × 20 × 22 cm) containing 3 L of flow-through sea water, while 30 control shrimp were kept in an aquarium (100 × 50 × 55 cm) containing 30 L of flow-through sea water. Both crowding-stressed and control groups were in triplicate (3 subgroups for each group and 30 shrimp in each subgroup). They were placed in vessels of sea water at shrimp densities of 10/L and 1/L for 2 h, respectively. After 2 h, three shrimp were randomly selected from each subgroup, and slaughtered by placing in ice for 20 min. The muscles were excised from individual shrimp, and then preserved in liquid nitrogen for protein and RNA extraction.

2.2. Extraction of muscle protein

The frozen muscles of individual shrimp from each group were ground into fine powder in liquid nitrogen. The powders of individual shrimp (n = 3 for each subgroup) were separately suspended in a lysis buffer containing 8 M urea, 2 M thiourea, 4% (w/v) 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% (v/v) ampholyte and 65 mM DTT. The lysates were incubated at 4 °C for 2 h and centrifuged at 12,000 *g* for 1 h at 4 °C. Protein concentrations were measured by the Bradford method using bovine serum albumin (BSA) to produce the standard curve (Bradford, 1976).

2.3. Two-dimensional gel electrophoresis (2-DE)

The first dimension of 2-DE was conducted using the IPGphor isoelectric focusing (IEF) system (Bio-Rad). Approximately 120 μg of total proteins to a final volume of were 300 μL per sample were loaded on each Readystrip IPG strip (17 cm, pH 4–7, linear). Rehydration of samples was performed for 12 h in rehydration buffer (8 M urea, 2 M thiourea, 4% (w/v) 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% (v/v) ampholyte and 65 mM DTT) up to 300 μL. IEF was conducted at 50 V for 1 h, 500 V for 1 h, 1000 V for 0.5 h, 4000 V for 0.5 h, 8000 V for 1.5 h, and 8000 V to reach a total of approximately 80 kVh. After the IEF run was completed, the strips were equilibrated by gentle shaking in two steps for 10 min each in equilibration buffer I [1% DL-dithiothreitol (DTT), 50 mM Tris–HCl (pH 6.8), 6 M urea, 30% glycerol and 2% sodium dodecyl sulfate (SDS)] and equilibration buffer II [2.5% iodacetamide, 50 mM Tris–HCl (pH 6.8), 6 M urea, 30% glycerol and 2% SDS]. Afterwards, the IPG strips were loaded onto 12.5% acrylamide gels for electrophoresis in the second dimension at 30 mA for 3 h until the bromophenol blue front reached the bottom of the gel. Separated proteins were visualized by silver diamine staining as described by Gottlieb and Chavko (1987).

2.4. Image analysis

Silver-stained 2-DE gels were scanned using a calibrated densitometer (GS-800, Bio-Rad). Spot detection, spot matching, and quantitative intensity analysis were performed using PDQuest 2-D analysis software (Bio-Rad). The gel images were normalized according to the total quantity of protein in the analysis set. Statistical testing of spot intensity between crowding-stressed and control groups was performed using the Student's *t* test. Expression intensity ratios of stress/control values greater than 2.0 ($p \leq 0.05$) or less than 0.5 ($p \leq 0.05$) were set as the thresholds to indicate significant changes.

2.5. Destaining and in-gel tryptic digestion

The protein spots of interest were excised manually from the silver-stained gels and destained with 100 mM NH_4HCO_3 in 30% acetonitrile (ACN). After removing the destaining buffer, we lyophilized and rehydrated gel pieces in 30 μL of 50 mM NH_4HCO_3 containing 50 ng trypsin (Sigma). After digestion at 37 °C overnight, the peptides were extracted three times with 5% trifluoroacetic acid (TFA) in 50% ACN. Resultant lyophilized tryptic peptides were kept at −80 °C until mass spectrometric analysis. A protein-free gel piece was treated as described above, and then used as a control to identify autoproteolytic products derived from trypsin.

2.6. MALDI-TOF/TOF MS analysis and database searching

The peptide mixtures were re-dissolved in a 0.8-μL volume of 10 mg/mL matrix solution (α-cyano-4-hydroxycinnamic acid (Sigma) in 0.05% trifluoroacetic acid, 50% ACN), and then spotted on the MALDI plate. Samples were allowed to air-dry and analyzed by a 4800 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems). Parent mass peaks with a mass range of 900–4000 Da and minimum signal-to-noise ratio of 10 were chosen for tandem TOF/TOF analysis. Combined mass spectrometry (MS) and MS/MS spectra were subjected to analysis using MASCOT (Version 2.1, Matrix Science) by GPS Explorer software (version 3.6, Applied Biosystems) and searched with the following parameters: National Center for Biotechnology information non-redundant (NCBInr) and EST databases (release date, June 30, 2012), trypsin digest with one missing cleavage, carbamidomethylation as the fixed modification, possible oxidation of methionine, peptide mass tolerance of 100 ppm, and fragment tolerance of 0.3 Da. Known contaminant ions (tryptic autodigest peptides) were excluded. Any individual MS/MS spectrum with a statistically significant (confidence