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Analysis of Pacific oyster larval proteome and its response to high-CO_2

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ABSTRACT

Most calcifying organisms show depressed metabolic, growth and calcification rates as symptoms to high-CO_2 due to ocean acidification (OA) process. Analysis of the global expression pattern of proteins (proteome analysis) represents a powerful tool to examine these physiological symptoms at molecular level, but its applications are inadequate. To address this knowledge gap, 2-DE coupled with mass spectrophotometer was used to compare the global protein expression pattern of oyster larvae exposed to ambient and to high-CO_2. Exposure to OA resulted in marked reduction of global protein expression with a decrease or loss of 71 proteins (18% of the expressed proteins in control), indicating a wide-spread depression of metabolic genes expression in larvae reared under OA. This is, to our knowledge, the first proteome analysis that provides insights into the link between physiological suppression and protein down-regulation under OA in oyster larvae.

1. Introduction

The absorbed one-third of anthropogenic CO_2 by the oceans has started altering seawater carbonate chemistry equilibrium through the process known as "ocean acidification" (OA) (Doney et al., 2009). More data on OA impacts on calcium carbonate ($CaCO_3$) shell forming organisms (called "calcifiers") are urgently needed because OA could exert deleterious effects not only on organism's ability to make their shells but also on their metabolism and physiology (Fabry et al., 2008). Global mean ocean pH has already decreased 0.1 units because of OA, and is predicted to drop by 0.7 units before 2300 under the IPCC's worst case scenario for CO_2 emissions (Zeebe et al., 2008). This excess $H^{[+]}$ combines with carbonate ions to form bicarbonates. The carbonate ions that are in depletion this way concurrently reduces the saturation state of all forms of $CaCO_3$ minerals, which makes marine organisms harder to form their shells and/or even trigger their shells to dissolve (Feely et al., 2009). Due to OA, southern oceans are already corrosive to shells of many invertebrates, making them harder to form their shells or even have their shell dissolved (Fabry et al., 2009). This OA effect is gradually spreading into tropical seas (Kleypas et al., 1999).

The majority of calcifying shellfishes (e.g. edible oysters) have complex life cycles, during which the externally fertilized eggs produce the pelagic larval stage, called "D-shaped" veliger. This newly hatched larva feeds on micro-algae, develops into advanced larval stage, called pediveliger, and finally enters into benthic life by attaching on hard substrates (Collet et al., 1999). Although this pelagic life aids them to disperse and colonize diverse habitats, it is achieved only at an extremely high cost (Thiyagarajan, 2010). Generally, >90% of larvae dies before they reach attachment stage due to predation and environmental stress (Jessopp, 2007). Thus early larval life stages are not only highly susceptible to stressors; their physiological fitness would also determine the success of pre- and post-larval life (Pechenik, 1999). When analyzing the effects of OA on shellfishes, it is thus critical to study their effects not only on adult stage but also on larval stages (Dupont et al., 2008; Gazeau et al., 2010; Kurihara et al., 2007; Talmage and Gobler, 2010). The larvae of oysters are particularly at risk because they use aragonite ($MgCO_3$) in their shell, which is 30 times more sensitive to OA than normal calcite ($CaCO_3$) based adult shells (Medakovié et al., 1989).

Reduced shell calcification rate (and thus growth rate), and metabolic depression are common symptoms of OA in early life stages (Dupont and Thorndyke, 2009; Talmage and Gobler, 2010). These symptoms could most probably be due to the down-regulation of genes responsible for calcification, and energy metabolism (Todgham and Hofmann, 2009). Expression of gene (s) does not always correlate with their product (protein) (s) (Görg et al., 2004). Therefore, knowledge of protein expression pattern is necessary to understand the direct link between OA stress and larval physiological response (Hofmann et al., 2008). However, differential expression of proteins (proteome plasticity), especially in early larval life stages, in response to OA has not yet been well explored (Wong et al., 2011). Recently, two-dimensional electrophoresis

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(2-DE) based proteomics has emerged to be a highly useful tool to study global protein expression pattern in a variety of non-model larval species (Thiyagarajan and Qian, 2008; Wong et al., 2011). Using similar larval proteomic approach, in this study, we have tested the hypothesis that the negative effects of OA on early larval stage (e.g. decreased shell growth) are mediated through differential expression of proteins associated with calcification, metabolism, and stress tolerance. We also explored the role of protein expression variation in acclimation to OA. To accomplish our objectives, (1) embryos of the Pacific oyster (*Crassostrea gigas*) were cultured at high (OA) and ambient CO_2 (control) conditions for 6 days, and (2) larvae of similar physiological age and shell size (on Day 4) from the OA and the control groups were analyzed by 2-dimentional electrophoresis (2-DE), and selected (differentially expressed) proteins were identified by matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI–TOF MS).

2. Materials and methods

2.1. Study organism

The experiment was conducted at the South China Sea Institute of Oceanology (Chinese Academy of Science, China)'s oyster hatchery cum research station located at Zhanjiang (Guangdong province, China) in September 2010. Sperms and eggs were obtained by "strip spawning" from 6 males and 12 females (Rico-Villa et al., 2006) of the wild Pacific oyster, *C. gigas* (Thunberg, 1793). About 4–5 sperms were added per egg for fertilization, and after 30 min prefertilized embryos were used for the following ocean acidification (OA) experiment.

2.2. Experimental design and sample collection

Embryos were cultured in filtered (1 μm) seawater bubbled with ambient air (control) and CO_2-enriched air (OA treatment). Through air and CO_2 gas flow adjustment system, a steady-state carbonate system was reached and maintained, i.e. pH ~8.0 for control and pH ~7.5 for OA treatment using gas flow rotameters (Cole-Parmer, USA). This decreased pH 7.5 and concomitant increase in the partial pressure of CO_2 (pCO_2) ~2275 μatm ultimately resulted in under saturation of aragonite in OA treatment tanks. This high amount of pCO_2 was required in our system to reduce pH from 8.0 to 7.5 because of relatively high total alkalinity (TA) in ambient water (1977 ± 29). Two of the carbonate system parameters, pH (NBS scale) and total alkalinity, were measured according to the standard protocols (Dickson et al., 2007). Titration alkalinity measured data were verified using measurements obtained from seawater reference materials (Batch 103, A.G. Dickson, Scripps Institution of Oceanography). Then, the whole carbonate system was calculated using the program co2sys.xls spreadsheet (Pelletier et al., 2005) by inputting pH, alkalinity, temperature and salinity and by using standard dissociation constants (Dickson and Millero, 1987).

Embryos were divided into 6 randomly assigned tanks of 450 L of volume with three replicates per treatment. As opposed to conventional small scale laboratory cultures (<10 L capacity), a typical oyster hatchery's black polycarbonate larval culture tanks (500 L capacity) were used. Cohorts of embryos were counted and introduced into each of the 6 culture tanks with a density of ~15–20 embryo ml^{-1}. Except seawater pH (a proxy for the changes in seawater carbonate system in response to the increasing pCO_2), both treatment and control culture tanks were maintained at optimal temperature (28 ± 2 °C), salinity (25 ppt), and larval microalgae food concentration (10^5 cells of *Chlorella* sp. ml^{-1}). Embryos were not fed during the first 24 h of their development. Under these culture conditions, >70% of embryos developed into D-shaped veliger larvae in 24 h, after which, water was changed and larvae were cultured for 6 days (including embryo development time). Larval samples with more or less similar physiological age and shell size between the control and the OA treatment were used for comparative proteome analysis using two-dimensional electrophoresis (2-DE). Samples collected on Day 4 fulfilled with those similar larval size and age requirement for proteome analysis. Unfortunately, samples were not collected for proteome analysis before termination of the experiment on Day 6.

2.3. Sample preparation for 2-DE

Actively swimming larvae from each culture tank was filtered (80 μm mesh size) out, washed in double-distilled water and then immediately frozen in liquid nitrogen until further analysis. During analysis, samples were lyzed in 2-DE buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 1X protease inhibitor and 2% Bio-Lyte 3/10 ampholyte (Görg et al., 2004). Larval proteins were solubilized with the aid of a sonicator (Branson Sonifier 150) using setting 3 (6 rounds of 1 min, with 2 min pause-interval) on ice to prevent protein denaturing and centrifuged for 20 min at 14,000 rpm (Thiyagarajan and Qian, 2008). The soluble proteins in the supernatant was quantified by the modified Bradford method (Ramagli, 1999) and used for 2-DE analysis.

2.4. 2-DE analysis

The 2-DE separation of larval proteins was performed according to the optimized larval proteomic protocols (Thiyagarajan and Qian, 2008). 100 μg of proteins dissolved in the rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 40 mM DTT, 0.2% Bio-Lyte, 3/10 ampholyte, and 1% Bromophenol blue) and applied to 17 cm ReadyStrip IPG strips (Bio-rad), pH 3–10 (linear), for overnight active rehydration at 50 V and then subjected to isoelectric focusing (IEF) using a Protean IEF Cell (BIO-RAD Laboratories Inc.). Focusing conditions were as follows: 250 V for 20 min, 1000 V for 2.5 h with a gradient of 10,000 V for a total of 40,000 Vh. After IEF, the IPG strips were equilibrated for 15 min in equilibration buffer 1 (6 M urea, 2% SDS, 0.05 M Tris–HCl (pH 8.8), 50% glycerol, and 2% w/v 1,4-DTT) followed by 15 min in buffer 2 (same as buffer 1 but had 2.5% iodoacetamide instead of DTT). For second dimension, the equilibrated IPG strips were inserted on top of 12.5% SDS–polyacrylamide gels (18.5 cm × 18.5 cm) and sealed with 0.5% w/v agarose. The gel running buffer was the standard Laemmli buffer for SDS–PAGE. The gels were run at 20 °C at a maximum of 24 mA per gel for approximately 6.5 h until the bromophenol blue front reached the bottom of the gel. Gels were then stained using the mass spectrophotometer compatible Vorum silver staining method (Mortz et al., 2001).

2.5. Larval proteome (2-DE gel) analysis

The gels were scanned at an optical resolution of 400 dpi using the GS-800 densitometer (Bio-Rad, Hercules, CA, USA). Then, the gels were compared using the PD Quest software (ver. 8.0; Bio-rad), which models protein spots mathematically as a three-dimensional Gaussian distribution and determines the maximum absorption after correction of the raw image correction and background subtraction. Automatic spot detection in each gel was verified by visual inspection in order to ensure spots were all properly detected. Spot intensities were normalized using total density values, and then spot analysis was performed using both qualitative and quantitative modes using Student's *t*-test. Spots that displayed