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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

significant statistical difference ($p < 0.05$) and with 2-fold or greater change in mean volume with respect to the control were considered differentially expressed in the OA treatment. Besides, hierarchial clustering with TMEV heat map showing all the treatments and normalized spot volumes for each spots across the gels was constructed using pearson's correlation coefficient (Saeed et al., 2006).

2.6. Protein identification using MALDI–TOF/TOF mass spectrophotometer

Fifteen abundant spots that were differentially expressed were excised for mass spectrometric identification according to the standard protocol (Shevchenko et al., 1996). The gel pieces (protein spots) were washed twice for 15 min each with water and twice with H_2O/ACN (1:1 v/v) and were then placed in 100% ACN. The gel pieces were dried in a Speed Vac centrifuge before adding 10 μl of 20 ng/μl sequencing grade trypsin (Promega) in 20 mM NH_4HCO_3 buffer. After rehydration with the enzyme solution, the gel pieces were covered with the buffer solution and digestion was allowed to proceed overnight at 37 °C. The peptides were extracted using several volumes of an H_2O/ACN/trifluoroacetic acid mixture (80:20:1), after clean up with ZipTip (Millipore), peptides subjected to mass/charge ratio analysis using MALDI–TOF/TOF mass spectrometer (ABI 4800 Proteomics Analyzer, Applied Biosystems). Combined PMF and MS/MS information obtained from the MS process was subjected to searching against the NCBI non-redundant and EST invertebrate database with entities restricted to other-metazoan sequences using the GPS Explorer algorithm, ver. 3.6 (Applied Biosystems) and in-house MASCOT database ver. 2.2 (Matrix Science). Mass spectrum searches were performed using mass tolerance settings of ±75 ppm for PMF and ±0.2 Da for the MS/MS spectra. MASCOT scores greater than 69 ($p < 0.05$) and ion score of minimum one peptide greater than 50 ($p < 0.05$) or three peptides greater than 20 ($p < 0.05$) were considered significant.

3. Results and discussion

3.1. Oyster larval growth response to OA

To examine the impact of OA on marine organisms, researchers have developed standard protocols to mimic and measure carbonate system parameters in a controlled laboratory conditions (Riebesell et al., 2010). By using these standard protocols, carbonate system variables such as pH, pCO_2, CO_3^{2-} ion concentration, and $CaCO_3$ mineral saturation levels were maintained at significantly different levels between the control and the OA treatment. In this study, when the pH reduced from 7.92 (±0.02, control) to 7.48 (±0.01, OA treatment) by bubbling with high-CO_2 air, the pCO_2 increased 3 times (to 2273 ± 349) and $CaCO_3$ minerals became under saturated ($\Omega_{aragonite} = 0.6 \pm 0.02$; $\Omega_{calcite} = 0.9 \pm 0.03$). There was no significant change in total alkalinity between the control and the OA treatment. Although the pH or pCO_2 levels chosen in this study are expected only in the year 2300 or beyond (Caldeira and Wickett, 2003), this slightly extreme perturbation level is invaluable to understand OA effects at both physiological and molecular levels (Hale et al., 2011; Melzner et al., 2009; Widdicombe and Spicer, 2008). Nevertheless, the perturbated carbonate system in a 450 L OA treatment tanks allowed us (1) to maintain a highly stable carbonate system (compared to small-scale culture containers, authors unpublished data), (2) to perform the OA experiment using typical oyster hatchery procedures such as water change and larval filtration and feeding strategies as described in typical hatchery manual (Breese and Malouf, 1975), (3) to obtain enough larval samples for proteomics analysis and finally, (4) to extrapolate obtained results to commercial oyster hatchery conditions. To our knowledge this is the first study to use such large volume cultures to examine OA effect on oyster larvae (but see (Gazeau et al., 2010). However, this experimental design and scale was not suitable and not aimed to determine the effects of OA on embryo or larval developmental and success rates.

In this study, large proportion of embryos of the Pacific oyster in OA treatment (pH 7.5) developed as fast as control group and attained a statistically similar total shell size on Day 4 from fertilization (Fig. 1). However, there were significant difference in shell size between the control and the OA treatment groups on Day 6. It appears that larval physiology that is required for normal shell growth (e.g. calcification) seem to be hindered by OA only from Day 4. Therefore, the Day 4 larvae with similar shell size or developmental stage between the control and the OA treatment were used for proteomic analysis. This similarly sized sample between the control and treatment groups were necessary for us to determine the potential impact of OA on larval proteome without interference of other co-variables such as delayed growth due to OA effect (Sam Dupont, personal communication). On Day 6, for example, larvae from OA group were significantly smaller in shell size though they were at same age of control group. This issue of larval age and size in molecular OA studies has recently been discussed as "virtual age" (Dupont et al., 2010).

A pioneering study that examined OA impacts on embryo and early larval development of the Pacific oyster, at pH 7.4, has clearly showed a detrimental effect of reduced calcification and early development (Kurihara et al., 2007). It appears that OA effects are highly species-specific in oysters. For instance, a slight drop in pH (0.3 units from ambient level) hindered larval shell growth in the Suminoe oyster (*Crassostrea ariakensis*) but not in the Eastern oyster (*Crassostrea virginica*) (Miller et al., 2009). Among various carbonate system (OA) parameters, only the carbonate ion concentration appears to be detrimental to early larval life stages of oysters (Gazeau et al., 2011). Oyster embryos and early life stages of some species do not appear to have an efficient calcification compensatory mechanism to overcome OA effects especially when the carbonate ion concentration goes below the saturation level. This might explain the observed negative effects of OA on Pacific oyster early life stages. The negative effects of a delayed and/or impaired early larval development under OA conditions on oyster production at hatchery and fishery scale have been highlighted

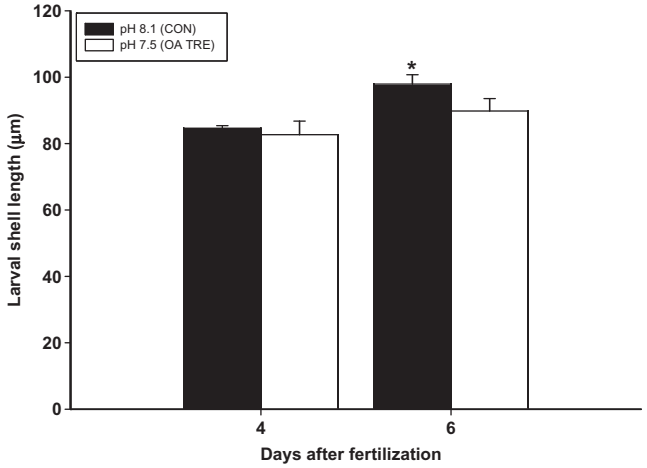

Fig. 1. Shell length of 4 d old (from fertilization) "D-shaped" veliger larvae of *Crassostrea gigas* cultured in 450 L (15–20 larvae per ml) seawater contained ambient CO_2 (pH 8.0, control) or high-CO_2 (pH 7.5, ocean acidification treatment). Each bar represents mean value of three replicate culture tanks. There were 50–100 larval size measurements per replicate tank. *Significantly ($p < 0.05$) different from control.

in various recent literatures (Havenhand and Schlegel, 2009; Miller et al., 2009; Parker et al., 2009; Talmage and Gobler, 2009; Waldbusser et al., 2011; Watson et al., 2009).

3.2. Oyster larval proteome response to OA

In control, there were 379 ± 1 (n = 3) proteins in the proteome of 4 day old "D-shaped" veliger larva (Fig. 2A). To our knowledge, this is the first analysis of oyster larval proteome using 2-DE approach. Generally marine invertebrate larvae tend to have this range of numbers of soluble (in lysis buffer) proteins that can be resolved using conventional 2-DE approach, i.e. there were about 350 in corals (Deboer et al., 2007), 250–500 in polychaete tubeworm (Zhang et al., 2010), 400 in barnacles (Thiyagarajan and Qian, 2008) and 450 in mussels (López et al., 2005). However, larval proteome structure (i.e. distribution of proteins according to their pI and MW) is highly species specific. For instance, majority of proteins in the larvae of tube-worm, *Hydroides elegans*, was visualized in acidic region of the gel (pI 3–6) and were of low-molecular weight (ranged between 50 and 10 kDa) (Zhang et al., 2010). On the other hand, results from the current study showed that oyster larval proteins lie between pI 4–8 and spread between MW 100 and 10 kDa (Fig. 2).

Larval proteome analysis using traditional 2-DE has been a very popular proteomic approach, especially for non-model (organisms without genome sequence data) species, because it can display differentially expressed proteins between a treatment and a control group (López, 2007; Nunn and Timperman, 2007; Thiyagarajan, 2010; Tomanek, 2006, 2011). A similar approach was used in this study to understand the impact of OA on global protein expression pattern in oyster larvae. Additionally, differential expressed proteins that may be responsive to OA stress tolerance or part of OA compensatory response proteins were identified using PD-Quest software and MALDI–TOF/TOF MS analysis.

In contrast to control, there were only 308 ± 1 (n = 3) proteins in OA exposed larvae. Similarities in the global expression pattern of replicate gels in both groups were estimated with a TMEV heat map that resulted from hierarchical clustering analysis of normalized protein spot volume data of all the 6 samples. The proteome or samples were clearly grouped in two well-differentiated clusters, the control and the OA treatment (Fig. 3). Subsequent statistical analysis also revealed dramatic OA dependent changes in several protein spots. Surprisingly, large numbers of low-MW proteins (<40 kDa) completely down-regulated after OA exposure (see Fig. 2B in comparison with Fig. 2A). Such massive down-regulation may be associated with larval metabolic depression in response to OA. There are many strategies that marine organism may employ to tolerate OA stress, and one of which could involve metabolic depression (Hofmann and Todgham, 2010). For instance, oysters tend to reduce their overall metabolic activity and energy metabolism in response to OA stress (Lannig et al., 2010). In larval sea urchin, expression of many genes in the biomineralization, metabolic, cellular stress and apoptotic pathways were down-regulated in response to OA (O'Donnell et al., 2010; Todgham and Hofmann, 2009). Therefore one would expect a down-regulation of a large numbers of proteins in response to OA to conserve energy as part of one of the compensatory responses (Wood et al., 2008). Our results provide preliminary evidence to support the above hypothesis.

Global expression pattern of proteins in response to OA has been analyzed only in couple of instances. In the mantle tissues of an eastern oyster, expressions of only 12% of their total 456 proteins have been altered by OA (Tomanek et al., 2011). Similarly, only 9 of the total 566 proteins in the intertidal barnacle larvae (*Balanus amphitrite*) have been altered by OA (Wong et al., 2011). Thus, in contrast to oyster larval proteome, proteome of oyster mantle tissue and the barnacle larvae appear to be robust to OA stress. Such species specific response to OA at proteome level was not surprising because the sensitivity of OA was not uniform across animal groups (Doney et al., 2009), i.e. some species are robust to OA (e.g. barnacle larvae) than do other species (e.g. oyster larvae) even at pH 7.4 (Whiteley, 2011). Altogether, our global protein expression analysis demonstrates the presence of a compensatory response to OA stress at the level of protein expression.

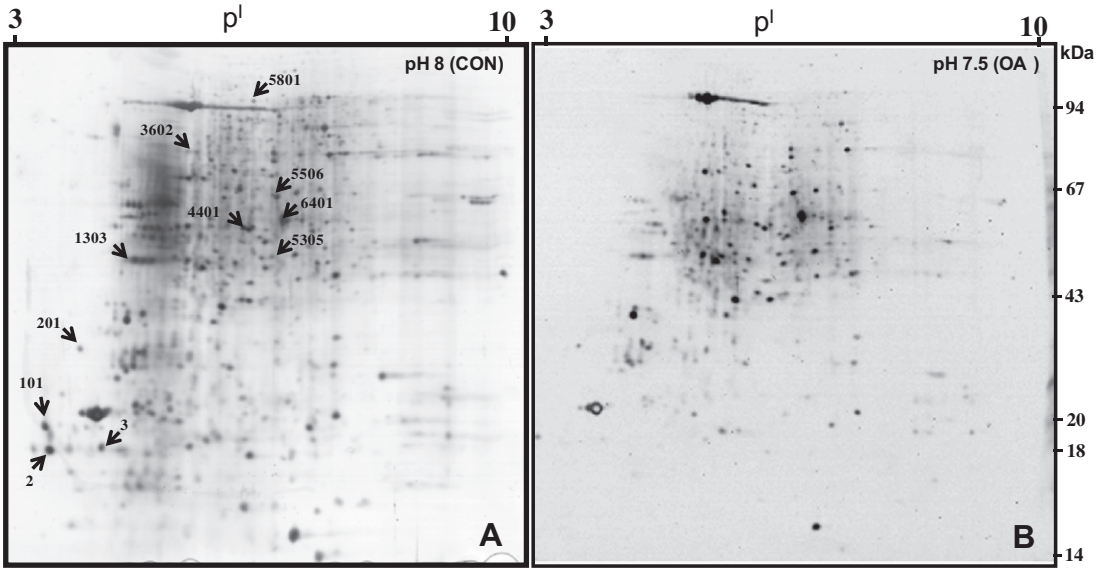

Fig. 2. The Pacific oyster (*Crassostrea gigas*) larval proteome response to high-CO_2 or ocean acidification. (A) 2-DE image of 4 days old larvae cultured in ambient CO_2 conditions (pH 8.0), (B) 2-DE image of the 4 day old larvae cultured in high-CO_2 (pH 7.5). Representative gel image of larval proteome analyzed by 2-DE are shown. There were three biologically independent gels per treatment. The 1st dimension was performed on pH 3–10 linear IPG strips; the 2nd dimension was run on home made 12.5% T acrylamide gel. Gels were stained with silver nitrate solution. Sample amount of protein loaded was 100 μg for each sample. Proteins identified are marked, and spot numbers refer to Table 1.

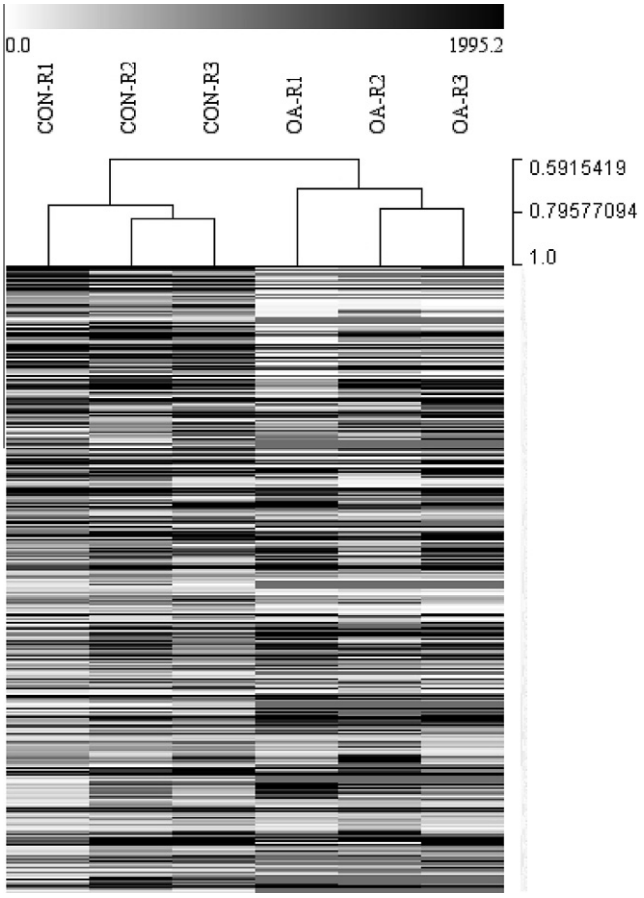

Fig. 3. Hierarchical clustering analysis with the TMEV heat map showing the cluster constructed using Pearson's correlation coefficient for all protein spots in 6 samples (2 treatments × 3 biologically independent replicates). CON, control (ambient CO_2 level, pH 8); OA, ocean acidification (high-CO_2 level, pH 7.5); R, replicates. Each horizontal axis shows the measured normalized spot volumes for each spot from all the treatments. The protein expression volumes are graded based on the color density showing black for higher, grey for medium and white for lower spot expression volumes.

3.3. Differential expression of proteins to OA

In our proteome analysis, 46 proteins were differentially expressed, according to the cut-off value used (2-fold level). Among them, 17 were down-regulated and 29 were up-regulated. Fifteen of these high abundance OA-responsive proteins were excised from the control gels and analyzed through matrix-assisted laser desorption/ionization time of flight (MALDI–TOF/TOF) mass spectrometry (Fig. 4). Among them, 4 were down-regulated and 11 were up-regulated. In accordance with our stringent identification criteria, 11 of the 15 differentially expressed proteins were identified. Proteins that were successfully identified by MS/MS are shown in Table 1. These 11 OA responsive proteins can be treated as part of "protein expression signatures" (PES) for OA in oyster larvae (Bradley et al., 2002). Identity of these proteins has not been validated in this study but they could serve us to understand the molecular mechanisms that evoke compensatory physiological responses to OA in oyster larvae.

Calcification is one of the most vulnerable physiological processes to OA stress (Doney et al., 2009). In this study, 3 calcium metabolism related proteins (two calmodulin and a troponin C protein) have been identified with high confidence using MS/MS evidence alone, which were significantly down-regulated in response to OA stress (Table 1). During $CaCO_3$ shell formation process, the uptake, transport and assimilation of calcium ion are key steps which are governed by action of calmodulin and a potential calcium binding proteins such as troponin C (Yan et al., 2007; Yamamoto et al., 1987). Their down regulation, as observed in this study, is likely to slow down calcification process in OA exposed larvae. It should be noted that, oyster larvae used in this study have started to grow at slower rate in OA treatment soon after the Day 4. Repression of these calcification-related proteins by OA in Day 4 larvae could be a preparatory phase or machinery for subsequent slow calcification growth phase. However, only future investigations will confirm and determine the nature of this hypothetical correlation between low production of calcium metabolism proteins and slow larval shell growth as a compensatory response to OA. Along with these calcification-related proteins, expression of cytoskeleton proteins was decreased substantially in response to OA (Beta-tubulin and tektin in Table 1).

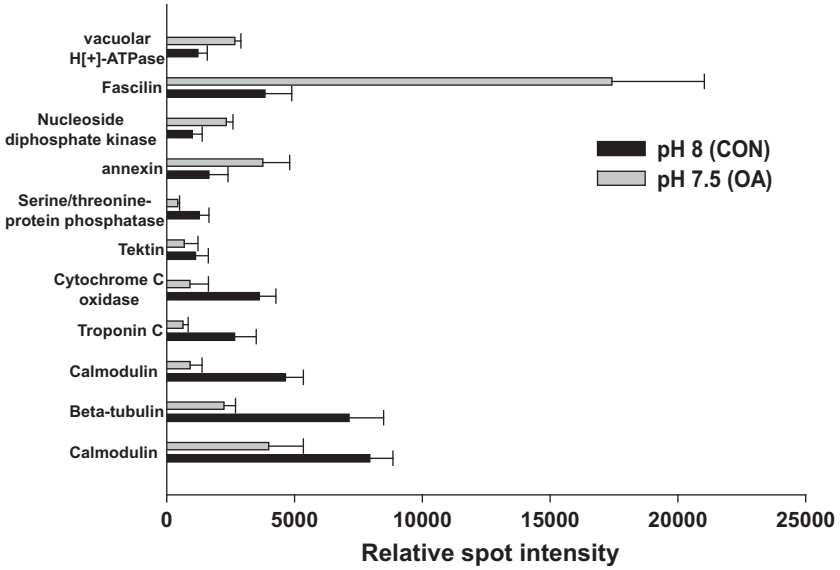

Fig. 4. Relative changes in expression levels of identified proteins that were 2-fold differentially expressed between the ambient CO_2 (pH 8.0, control) and the high-CO_2 (pH 7.5, ocean acidification) treatment. The successfully identified (using MS/MS analysis) spots are listed in Table 1. Each bar represents mean value of three biologically independent replicates.

Table 1
Identification of selected proteins that are abundantly and differentially expressed in the Pacific oyster (*Crassostrea gigas*) veliger larvae in response to high-CO_2 (ocean acidifcation) treatment after 4 days of expsoure from fertilization.

Spot[a]	Putative identification[b]	MASCOT protein score (C.I.)[c]	Peptide count[d]	MASCOT MS/MS score (C.I.)[e]	MASCOT peptides identified by MS/MS[f]	Homology to protein (Genbank)[g]	Theo. Mw/pI[h]	Expt. Mw/pI[i]	Functions
2	Calmodulin	298 (100)	5	75 (100) 71 (100) 110 (100)	DSDSEEELREAFR DGNGFISAAELR VFDKDGNGFISAAELR	156915032 *Crassostrea gigas*	15.7/4.20	18.0/3.50	Calcium binding
101	Calmodulin	359 (100)	6	72 (100) 88 (100) 150 (100)	DGNGFISAAELR DTDSEEEIREAFR VFDKDGNGFISAAELR	2832598 *Branchiostoma lanceolatum*	15.1/4.11	20.0/3.50	Calcium binding
201	Troponin C	222 (100)	3	57 (100) 67 (100) 97 (100)	GTIPVEDLR GNKGTIPVEDLR QLTDAHNTFNLFDK	HS112685*Crassostrea gigas*	29.7/5.16	32.0/4.20	Calcium binding
5506	Annexin	161 (100)	3	45 (100) 75 (100) 41 (100)	AIIDVLAHR DIESDTSGHFKR AVFDAYQK	HS201028*Crassostrea gigas*	26.7/5.47	50.0/6.70	Calcium regulated phospholipid binding
1303	Beta-tubulin	142 (100)	5	87 (100)	INVYYNEATGGK	226471718 *Schistosoma japonicum*	37.4/5.29	48.0/5.00	Cytoskeletal functions
5801	Tektin	169 (100)	10	82 (100)	TLEQTTEQIR	194068377 *Saccostrea kegaki*	42.1/6.27	95.0/6.50	Cytoskeletal functions
5305	Nucleoside diphosphate kinase	83 (100)	2	71 (100) 12 (100)	SEEIEDIILR RVHLTPEQASDFYAEHYGK	HS241176*Crassostrea gigas*	20.8/8.03	59.0/7.10	Cellular metabolism
3602	Serine/ threonine-protein phosphatase	103 (100)	3	19(100) 41 (100) 43 (100)	SPDTNYLFMGDYVDR QITQVYGFYDECLR YSFLQFDPAPR	FQ017914*Spodoptera littoralis*	51.3 /8.97	83.0/5.70	Cellular metabolism
6401	Fascilin	86 (100)	2	38 (100) 48 (100)	LSELGTLVK SVNIVVGQGVK	HS121174*Crassostrea gigas*	26.1/7.96	56.0/6.40	Cell adhesion
4401	Vacuolar $H^{[+]}$-ATPase	108 (100)	2	53 (99.1) 55 (99.8)	IQSSNLLNQSR TILYGANPNR	FQ660948*Crassostrea gigas*	30.2/6.3	53.0/8.80	Cellular transport
3	Cytochrome c oxidase	154 (100)	2	69 (100) 86 (100)	NNYAQFDGWELR NNNDYALATR	FP004182*Crassostrea gigas*	24.3/7.83	83.0/5.70	Cellular respiration

[a] The assigned spot number.
[b] Name of the putatively identified protein.
[c] The in-house MASCOT protein score obtained through searching against the NCBI non-redundant and invertebrate EST database (MASCOT score >69 is considered significant).
[d] Number of matched peptides.
[e] The ion score(s) of the peptide(s) identified by MS/MS; ion score of min. 1 peptide >50 ($p < 0.05$) or 3 peptides >20 ($p < 0.05$) are considered significant.
[f] The peptide(s) identified by MS/MS.
[g] Genbank accession number.
[h] The theoretical MW and pI value of the matched protein.
[i] The experimental MW and pI values estimated from the 2D gel pattern

In addition, energy related protein cytochrome c oxidase was also found to be down-regulated that are responsible for oxidative metabolism. In oyster, oxidative stress occurs in response to OA which in turn suppress cytoskeleton production (O'Donnell et al., 2010; Tomanek et al., 2011). Overall, these results suggest that both calcification and cytoskeleton related proteins were highly suppressed by OA. Vacuolar $H^{[+]}$-ATPase was seen up-regulated indicating that oyster larvae respond to the changes in the acidic environment, by altering its cellular proton transportation at the expense of ATP. Up-regulation of this pump possibly increases the rate of proton exchange maintaining normal intracellular functions (Lopez-Martinez et al., 2009). Other OA studies demonstrated that down-regulation of ion transporters (Todgham and Hofmann, 2009) or unchanged activity in long term exposure (Lannig et al., 2010) and their up-regulation in early stages of exposure (Hu et al., 2011; Stumpp et al., 2011). One of the larval responses to OA stress is the induced expression of a large number of genes whose products (proteins) are assumed to be involved in various adaptive functions under stress conditions such as heat-shock proteins (Cummings et al., 2011). Unfortunately, heat shock proteins (*hsp*) have not been identified that were differentially expressed in OA treatment. Alternatively, annexin – a stress related protein similar to *hsp*'s was found to be increased within such a short exposure period in OA treated larvae (Gerke and Moss, 2002; Rhee et al., 2000). Probably, *hsp*'s might have been differentially expressed but not expressed in abundance to enable us to identify them using our 2-DE approach.

Most studies on molecular plasticity to OA to date have only focused on the changes in gene expression, whereas there is no information available on their functional products, proteins (Hofmann and Todgham, 2010; Tomanek, 2011). Genes exhibiting a plastic expression pattern with respect to OA stress have been identified as candidates with putative roles in OA stress tolerance compensatory mechanisms (Martin et al., 2011). In this study, plasticity of the oyster larval proteome was revealed, and about 18% of the protein spots were affected by OA stress (see Fig. 2). We believe that these differentially expressed proteins or proteome plasticity might allow oyster larval to survive in OA but with delayed shell growth (Fig. 1). However, for firm conclusion and to prove this hypothesis, future studies should confirm functional role of the identified OA stress responsive proteins in stress tolerance using chemical inhibition and gene knock procedures. Rapid advances

in genomic and proteomics studies in non-model marine organisms have just begun to uncover the proteins that are plastic and are responsible for stress tolerance. This study is just a beginning of our global attempt to find molecular mechanisms of action of OA, pollution and environmental stressors in various marine non-model but economically important invertebrate species.

4. Conclusion

Here, we reported the first study where oyster larvae exposed to ambient and high-CO_2 at aquaculture hatchery scale to reveal global expression pattern of proteins that respond to ocean acidification (OA) stress. A simple and a reproducible 2-DE based proteomic approach have been described to examine proteome level changes to high-CO_2. Surprisingly, the expression of 71 out of 379 proteins was either decreased or completely lost or expressed below the detection limit after exposure to OA just for 4 days from fertilization. Importantly, expression of proteins related to calcification and cytoskeleton production appears to be severally suppressed by OA; however, further in vitro tests should be performed to confirm this hypothesis. Nevertheless, demonstrated altered expression pattern of proteins appears to be a part of biochemical compensatory mechanisms (a short-term adaptive response) in oyster larvae to OA stress. Thus, the application of recent advances in mass spectrometers and bioinformatics tool for protein identification, proteome maps generated using conventional 2-DE technique provides new insights into the effects of OA on organisms at molecular level.

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