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Short communication

Differential protein expression using proteomics from a crustacean brine shrimp (*Artemia sinica*) under CO_2-driven seawater acidification

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ABSTRACT

Gradually increasing atmospheric CO_2 partial pressure (pCO_2) has caused an imbalance in carbonate chemistry and resulted in decreased seawater pH in marine ecosystems, termed seawater acidification. Anthropogenic seawater acidification is postulated to affect the physiology of many marine calcifying organisms. To understand the possible effects of seawater acidification on the proteomic responses of a marine crustacean brine shrimp (*Artemia sinica*) three groups of cysts were hatched and further raised in seawater at different pH levels (8.2 as control and 7.8 and 7.6 as acidification stress levels according to the predicted levels at the end of this century and next century, respectively) for 1, 7 and 14 days followed by examination of the protein expression changes via two-dimensional gel electrophoresis. Searches of protein databases revealed that 67 differential protein spots were altered due to lower pH level (7.6 and 7.8) stress in comparison to control groups (pH 8.2) by mass spectrometry. Generally, these differentially expressed proteins included the following: 1) metabolic process-related proteins involved in glycolysis and glucogenesis, nucleotide/amino acid/fatty acid metabolism, protein biosynthesis, DNA replication and apoptosis; 2) stress response-related proteins, such as peroxiredoxin, thioredoxin peroxidase, 70-kDa heat shock protein, Na/K ATPase, and ubiquinol-cytochrome c reductase; 3) immune defence-related proteins, such as prophenoloxidase and ferritin; 4) cytoskeletal-related proteins, such as myosin light chain, TCP1 subunit 2, tropomyosin and tubulin alpha chain; and 5) signal transduction-related proteins, such as phospholipase C-like protein, 14-3-3 zeta, translationally controlled tumour protein and RNA binding motif protein. Taken together, these data support the idea that CO_2-driven seawater acidification may affect protein expression in the crustacean *A. sinica* and possibly also in other species that feed on brine shrimp in the ecosystem, particularly marine food webs.

1. Introduction

Atmospheric pCO_2 has increased from ~280 ppm (parts per million) to nearly 400 ppm (parts per million) since the Industrial Revolution in the late 1700s. Compared with the pre-Industrial Revolution time period, seawater pH has declined by 0.1 units because of human activities, such as fossil fuel combustion, cement production and land-use changes [1] [2]. According to the prediction by the Intergovernmental Panel on Climate Change (IPCC), the seawater pH value will be reduced by 0.3–0.4 units by the end of the 21st century, namely, the H^+ concentration in seawater will increase approximately 1–1.5 times, and pH will decrease by 0.7 units by the year 2250 [3]. Since the 1950s, increasing CO_2 concentrations in the atmosphere have called greater attention to greenhouse effects and ocean acidification. Studies have shown that ocean acidification is a huge threat to the chemical and environmental stability required for most marine organisms to exist and to the ecological system, as ocean acidification has a direct impact on marine biological reproduction [4] and on growth and settlement activities [5]. Ocean acidification will negatively impact [6] many marine organisms, but the degree of impact remains to be elucidated.

Multiple experimental studies have shown that the calcification rates of a wide range of marine organisms decline when they are

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reared in experimental seawaters with pH levels corresponding to the predicted levels in the coming centuries due to CO_2-induced ocean acidification. In regard to determining the effects of acidification on animals, previous studies have focused on the early development or calcification of the animals. Some calcifying organisms, such as copepods, molluscs and coral, play important roles in the food chain due to their important roles in marine food webs. On one hand, research has shown that when the pH decreases, the reproductive ability of copepods significantly decreases, and the pH value has detrimental effects on the survival rate, growth rate and reproductive rate [7] [8]. On the other hand, additional studies [9] have shown that the fertilization rate of sea urchins increased with decreasing pH values, the growth rate, larval length and lethal rate were closely related to the concentration of CO_2. Therefore, calcified larvae are always affected by the CO_2 concentration in seawater [10]. In addition, compared to adult animals, the growth status of echinoderm larvae was markedly more vulnerable, exhibiting developmental delays, low survival rates, reduced lengths, and structural deformities [6]. Therefore, calcified organisms are more likely to be easily affected by seawater acidification than non-calcified organisms.

Ocean acidification represents an additional major threat for these calcifying species given its potential effects on growth rates, reproduction and resistance to environmental changes [11]. With increasing pCO_2, reduced calcification rates have been observed for a variety of calcareous organisms even when aragonite or calcite saturation exceeds 1.0 [12]. However, the sensitivity of marine organisms to acidification varies among different taxa, and some species may increase calcification rates with increasing CO_2 levels [13]. However, studies on the response of calcification to ocean acidification have been conducted on a limited number of calcifying species [14]. Laboratory experiments on the effects of increased pCO_2 on calcifying organisms must be interpreted with caution due to the intrinsic limitations [15], but such studies are able to control environmental variables to allow for the ability to assess species-specific responses to increasing CO_2 levels [16].

The brine shrimp (*Artemia sinica*) is a microcrustacean that is found worldwide in natural saltwater lakes and salterns. Due to its rich proteins and its high unsaturated fatty acid content, it has been widely used as an important live feed for different aquaculture species. Juvenile and adult *A. sinica* have become a well-received model animal for studies of stress responses [17]. Moreover, the lethality test of *A. sinica* is usually employed for studying the biological effects of cyanobacteria in coastal environments, such as estuarine, marine and hypersaline ecosystems [18]. Therefore, *A. sinica* has been employed as a useful model organism for the investigation of environmental stress on zooplankton communities. Studies on acid-base regulation and on the ability of different species to counteract pH disturbances have become increasingly important in predicting the effect of future ocean acidification on marine ecosystems. Thus far, the physiological effects of ocean acidification on calcifying organisms, such as marine crustaceans, are unclear.

Protein identification and quantification have become widely utilized with the advent of modern mass spectrometry and are now viewed as a core technology for the verification and validation of candidate protein biomarkers. The separation of proteins in proteomics by two-dimensional gel electrophoresis is the most fundamental experimental technology. To further understand the physiological effects on marine crustaceans associated with the proteomic changes due to seawater acidification, *A. sinica* was studied as a model. Cysts were hatched and further cultured in seawater at three different pH conditions: acidified seawater driven by elevated pCO_2 (7.6 and 7.8) and pH 8.2 employed as a control. After acidification treatment, the differential protein profiles of the brine shrimp raised under acidified seawater at different pH values were characterized by two-dimensional gel electrophoresis followed by mass spectrometry and bioinformatics.

2. Materials and methods

2.1. Animals and samples collection

The brine shrimp cysts were exposed to different pCO_2 concentrations (380 ppm, pH = 8.2; 780 ppm, pH = 7.8; and 1500 ppm, pH = 7.6) at a salinity of 28 at 25 °C in tanks with aerated seawater (control) or CO_2-enriched air. The brine shrimp were collected at different developmental stages post-seawater acidification treatment and were prepared for proteomic analysis based on LC-MS/MS.

2.2. Protein extraction

Total protein extraction was performed according to Lee and Lo with some modifications [19]. For protein extraction, the brine shrimp from each sample was broken by the ultrasonic waves (Digital BRANSON Sonifier, USA) in an ice bath with 1 ml Trizol reagent (Thermo scientific, USA) and centrifuged at 12 000 × *g* for 5 min at 4 °C. The supernatant was then added to 200 μl chloroform, shaken vigorously for 15 s, allowed to rest for 5 min, and then centrifuged at 12 000 × *g* for 15 min at 4 °C. The pellet obtained was added to 750 μl isopropanol, washed with 1 ml ethanol (v/v 95%) and then centrifuged at 14 000 × *g* for 10 min at 4 °C. The protein pellets were solubilized in lysis buffer (7 M urea; 2 M thiourea; 4% m/V CHAPS; 65 mM DDT and 0.2% W/V Bio-lyte buffer) and then incubated for 2 h at room temperature. The homogenate was centrifuged at 20 000 × *g* for 30 min, and the supernatant was subjected to electrophoresis analysis. The obtained total protein samples were preserved in −80 °C, and 2 μl of each was qualified using a Plus One TM2-D Quant Kit (Amersham Biosciences, Sweden). The absorbance was tested at A480 nm.

2.3. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2-DE) was performed according to the method described by O'Farrell. For the first dimension separation, approximately 80 μg of protein from the brine shrimp samples was resuspended in IEF buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.75% (w/v) ampholine 3.5–10.0, 0.25% (w/v) ampholine 4.0–6.0] (GE Healthcare, USA) before loading onto precasted 18-cm-long Immobiline™ DryStrips (GE HealthCare, USA) with a linear wide range pH gradient (pH 4–7). The IEF was conducted at 20 °C with an IPGphor3 (GE Healthcare, USA) system at 50 V for 13 h followed by 100 V for 2 h, 200 V for 2 h, 500 V for1 h, 1000 V for 2 h, 4000 V for 2 h, and 8000 V for 11 h. After the first dimension separation, the IPG strips were subjected to reduction and alkylation through sequential incubation in equilibration buffer I (0.05 M Tris–HCl, pH 8.8; 6 M urea; 30% glycerol; 2% SDS; 1% DTT) and equilibration buffer II (0.05 M Tris–HCl, pH 8.8; 6 M urea; 30% glycerol; 2% SDS; 2.5% iodoacetamide) for 15 min, respectively. After IEF, the IPG strips were equilibrated for 20 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 20 min in buffer 2 (same as buffer 1 but containing 2.5% iodoacetamide instead of DTT). For the second dimension separation, the equilibrated IPG strips were inserted on top of the 12.5% Criterion Tris–HCl gels (Bio-Rad, USA) and sealed with 0.5% w/v agarose. The gels were run at room temperature at 200 V until the bromophenol blue front reached the bottom of the gel.

2.4. Silver staining and image analysis

After electrophoresis, the gels were silver stained according to the method of Mortz and Gharahdaghi [20]. The images were captured using an ImageScanner III (GE HealthCare, USA) and analysed using Progenesis SameSpotst™ version 2.0 (The Beijing Genomics Institute, China). Only protein spots with significant changes of at least 1.5-fold and $p < 0.05$ were accepted as differentially expressed proteins.

2.5. In-gel digestion and protein identification by LC-MS/MS

To further identify differentially expressed proteins, proteins were identified by LC-MS/MS. Protein spots were excised from the gels and destained (50% acetonitrile from Fisher Scientific and 25 mM ammonium bicarbonate) for the digest reaction with 1 μg/μl lysozyme diluted in 25 mM ammonium bicarbonate 15 times followed by addition to dehydrated gel spots overnight at 37 °C. The digestion was terminated by adding 0.1% TFA, and 10 μl samples were tested by mass spectrometry (Q Exactive, Thermo Scientific, USA).

2.6. Data analysis

Each gel with >1000 spots of proteins was analysed using Imagemaster software (GE HealthCare). Each sample was carried out in triplicate. Proteins were successfully identified based on 95% or higher confidence interval of their scores in the MASCOT V2.4 search engine (Matrix Science Ltd., London, U.K.). The following parameters were used in the database search: NCBInr Metazoa (Animals) (2,861,494 sequences) database; trypsin as the digestion enzyme; one missed cleavage site; partial modifications of cysteine carbamidomethylation and methionine oxidization; no fixed modifications; 0.15 Da for precursor ion tolerance and 0.25 Da for fragment ion tolerance. Individual ions scores >40 indicate identity or extensive homology ($p < 0.05$). The MASCOT engine (http://www.matrixscience.com) was used to identify proteins by searching the protein databases (Swiss-Prot and NCBInr). Only proteins whose MASCOT scores greater than the significance threshold were discussed.

3. Results and discussion

3.1. Differential protein expression profile of A. sinica under different seawater acidifications

To gain insight into the protein expression profiles at different pH levels, an LC-MS/MS analysis was employed to identify differentially expressed proteins defined by 2D-electroforesis. Among the 67 differentially expressed proteins, we found 26 homologous proteins, 17 putative novel proteins without homologs, and 24 protein spots that failed to be detected in the mass spectrometry characterization. The protein expression in *A. sinica* showed variations at different pH levels compared to the control (Fig. 1, Table 1). Among these differentially expressed proteins, 11 proteins were identified on day 1, at which time two proteins (Fig. 1 A, spot: 3-1, 5-1) were down-regulated, and nine proteins (Fig. 1 B&C, spot: 1-1, 2-1, 4-1, 6-1, 7-1, 8-1, 9-1, 10-1, 11-1) were up-regulated. Nine proteins were identified on day 7, at which time two proteins (Fig. 1 D, spot: 12-7, 13-7) were increased, and seven proteins (Fig. 1 E&F, spot: 4-7, 14-7, 15-7, 16-7, 17-7, 18-7, 19-7) were decreased. Nine proteins were found differentially expressed on day 14, at which time four proteins (Fig. 1 G, spot: 3-14, 20-14, 21-14, 22-14) were up-regulated, and five proteins (Fig. 1 H&I, spot: 4-14, 23-14, 24-14, 25-14, 26-14) were down-regulated. Here, we focus on a brief interpretation of the diverse functional classes of proteins related to metabolic process-related proteins, stress response-related proteins, immune-related proteins, cytoskeletal-related proteins, and signal transduction-related proteins, which are key putative molecules involved in metabolism and responses to cellular stress caused by CO_2-induced seawater acidification.

3.2. The metabolic process-related proteins

Previous studies have shown that seawater acidification affects metabolism and energetics at early developmental stages of marine invertebrates, such as porcelain crabs [21] and larval sea urchins [22]. In the present study, eleven proteins related to metabolic process were shown to be responsive to the seawater acidification stress, of which small heat-shock/alpha-crystallin protein precursor (spot: 1-1), vitellogenin (spot: 2-1), ribosomal protein S7 (spot: 10-1), and histone H3 (spot: 12-7) were significantly up-regulated, while the remaining seven proteins, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (spot: 5-1), SUMO-1-like protein (spot: 11-1), uridylate kinase (spot: 15-7), zinc metalloproteinase (spot: 17-7), NADH dehydrogenase subunit 1 (spot: 18-7), cytochrome *c* oxidase subunit I (spot: 19-7) and translation elongation factor TEF-1 beta chain (spot: 24-14), were down-regulated.

GAPDH plays a critical role in energy reproduction and anti-apoptosis processes. Spot 5-1 was identified as GAPDH. Spot 15-7 matched uridylate kinase, which participates in nucleotide metabolism. Spot 18-7 was identified as NADH dehydrogenase subunit 1, which is a conserved gene in the energy metabolic process in the electron transport chain. The reduced expression of GAPDH, uridylate kinase and NADH on day 1 or 7 suggested that the acidified condition might negatively impact substance and energy metabolism, resulting in a decreased ability to produce energy, thus affecting the metabolism of *A. sinica*. Moreover, it was reported that cytochrome *c* oxidase (CcO) is the terminal oxidase of the mitochondrial electron transport chain [23]. This enzyme in mammals contains 13 subunits, of which three catalytic subunits are encoded by the mitochondrial genes related to metabolic processes. Meanwhile, the three largest and evolutionarily conserved subunits I, II and III, carry the heme redox centres and form the catalytic core of the enzyme. Considering that reduced CcO function could induce a shift in metabolism towards glycolysis and induce invasive and anchorage-independent growth characteristics [24], the presence of cytochrome *c* oxidase subunit I was decreased distinctly under the acidified condition on day 7, suggesting that the reduced CcO function likely induced a shift in metabolism towards glycolysis, as the rate of growth of the brine shrimp in acidified seawater was lower than that of controls (pH 8.2). A comparison of the proteomes of brine shrimp between two treatments showed increased abundances of ribosomal proteins (ribosomal protein S7) and histone H3 under the low pH conditions on day 1. These results may indicate increases in DNA and chromatin synthesis and thus an increased need for histones, which is consistent with another report that increased histone H3 was related to a higher rate of cell division, requiring more DNA and chromatin synthesis under low pH stress [25]. The presence of vitellogenin (Spot 2-1) following the pH reduction may corroborate this fact: vitellogenins are glycolipoproteins that act as yolk proteins for developing embryos in oviparous species. The transcript and protein abundance of vitellogenin have been shown to decline during the larval development of marine invertebrates, as these maternally provisioned resources are consumed for nourishment. Therefore, increased levels of vitellogenin in the acidified treatment could suggest that these resources are being consumed at a slower rate, which, together with the reduced GAPDH abundance, may point towards

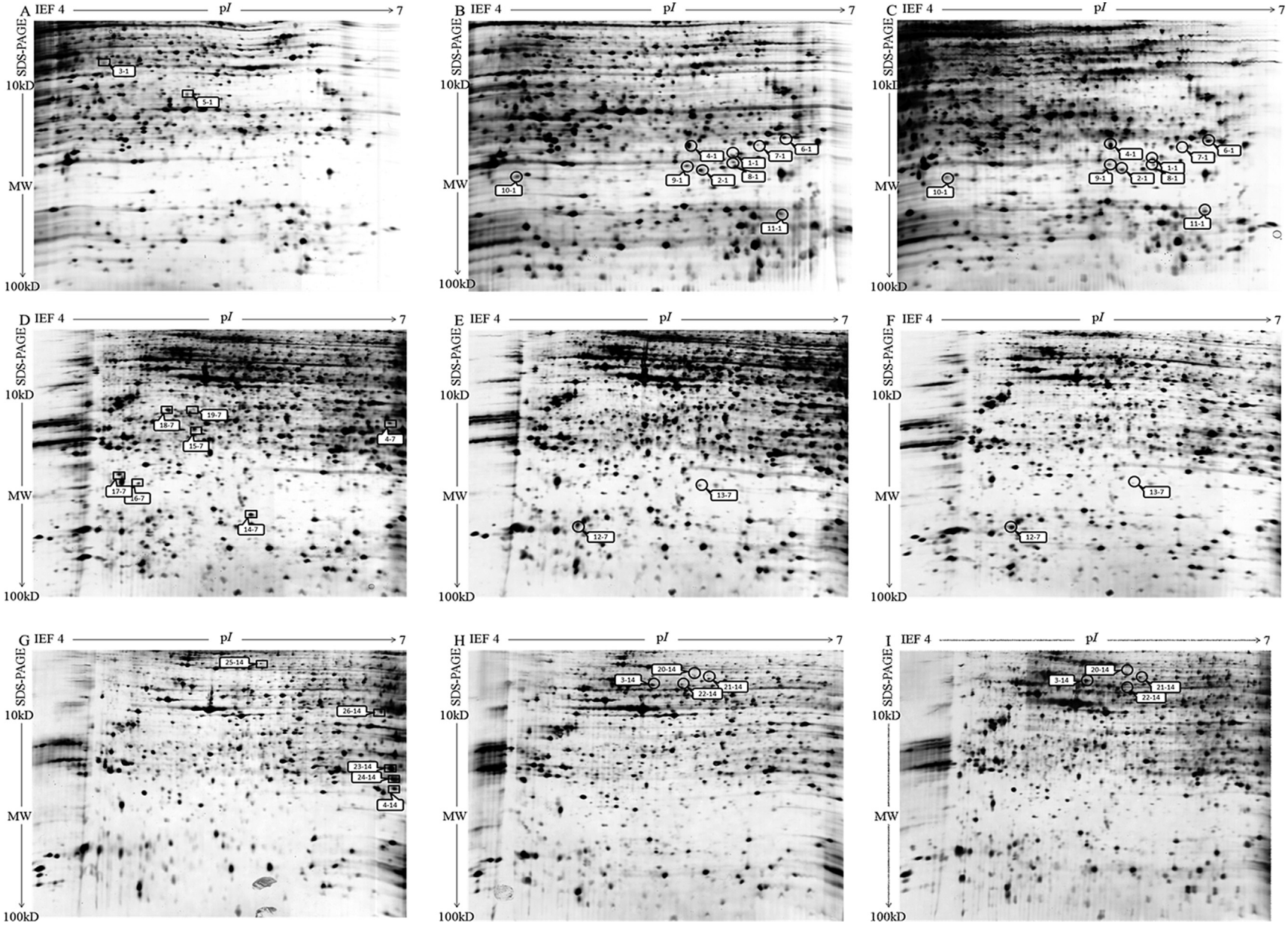

Fig. 1. Differential protein expression according to 2-DE in brine shrimp under seawater acidification at different time points. A: day 1, pH 8.2; B: day 1, pH 7.8; C: day 1, pH 7.6; D: day 7, pH 8.2; E: day 7, pH 7.8; F: day 7, pH 7.6; G: day 14, pH 8.2; H: day 14, pH 7.8; I: day 14, pH 7.6; (Spots in the quadrates: significantly up-regulated proteins in the treatments compared to proteins in the controls. Spots in the circles: significantly down-regulated proteins in the treatments compared to proteins in the controls.

Table 1
Identification of differentially expressed proteins in brine shrimp exposed to acidified seawater driven by CO_2.

Spot No.[a]	Protein name	Accession No.[b]	Score[c]	Fold change pH (8.2/7.8/7.6)[d]	Function[e]
1–1	Small heat shock/alpha-crystallin protein precursor	gi|2655270|	29	1/2.72/2.50	Preventing irreversible protein aggregation; Inhibiting apoptosis.
2–1	vitellogenin-superoxide dismutase fusion protein	gi|315439538|	79	1/2.69/2.06	Role in protecting oocytes and embryonic cells against oxygen toxicity;
3–1	chaperonin-containing TCP1 subunit 2, partial	gi|118429555|	18	1/0.37/0.39	Role in cell cycle progress and cytoskeletal organisation
3–14	chaperonin-containing TCP1 subunit 2, partial	gi|118429555|	576	1/2.80/4.08	the same as 3-1
4–1	peroxiredoxin	gi|194272333|	26	1/2.53/2.04	Role in defending against oxidative damage, eliminating the intracellular H_2O_2, protecting from apoptosis
4–7	peroxiredoxin	gi|194272333|	45	1/0.45/0.47	the same as 4-1
4–14	peroxiredoxin	gi|194272333|	124	1/0.43/0.44	the same as 4-1
5–1	glyceraldehyde 3-phosphate dehydrogenase	gi|41394417|	99	1/0.12/0	Role in energy production; involvement in apoptosis, age-related neurodegenerative disease, prostate cancer and viral pathogenesis
6–1	myosin light chain	gi|152013721|	416	1/2.70/4.18	Change in intracellular calcium levels; role in fungal growth, propagation and physiological processes
7–1	thioredoxin peroxidase	gi|164608828|	14	1/3.60/2.64	Role in detoxification of ROS, balancing the redox environment in the system, resisting oxidative stress
8–1	translationally controlled tumour protein	gi|169657220|	5e+002	1/2.27/2.09	Role in cellular processes, such as cell growth, cell cycle progression, cell survival, anti-apoptosis process, and so on
9–1	tropomyosin	gi|157674645|	4.9e+002	1/3.59/2.51	A component of the Ca^{2+} dependent regulator complex of striated muscle
10–1	ribosomal protein S7	gi|214012053|	94	1/2.26/2.11	Role in anti-apoptosis and inhibition of cell proliferation
11–1	SUMO-1-like protein	gi|146446849|	507	1/2.20/2.17	A small ubiquitin-like modifier protein; Role in conjugating to various intracellular target proteins to alter their cellular distribution, metabolism
12–7	Histone H3	gi|530445204|	61	1/1.86/3.02	A component of innate immunity, possesses antimicrobial activity and represents a defence mechanism
13–7	prophenoloxidase	gi|156622360|	14	1/3.89/1.99	Role in immune reactions, such as the generation of antimicrobial, cytotoxic, opsonic, or encapsulation-promoting activities
14–7	ferritin	gi|18071496|	1105	1/0.49/0.78	Involved in embryo growth
15–7	uridylate kinase	gi|224471449|	57	1/0.27/0.71	Maintains the energy balance in cells
16–7	Putative RNA binding motif protein 8A	gi|218664745|	64	1/0.47/0.17	unknown
17–7	zinc metalloproteinase	gi|157674613|	84	1/0.48/0.50	Involve in regulation of the development in the skeletal
18–7	NADH dehydrogenase subunit 1	gi|11229|	23	1/0.31/0.35	well conserved gene
19–7	cytochrome c oxidase subunit I, partial (mitochondrion)	gi|478437257|	17	1/0.54/0.45	well conserved gene
20–14	phospholipase C-like protein	gi|124494990|	3.8e+002	1/2.49/4.02	Role in inositol phospholipid signalling
21–14	70 kDa heat shock protein	gi|16611913|	187	1/4.63/5.81	Role in fundamental cellular processes, cytoprotective effects, defence against infections agents
22–14	tubulin alpha chain	gi|3348122|	168	1/2.93/2.49	Dynamic role in a dynein-based motility process
23–14	14-3-3 zeta	gi|161898814|	80	1/0.59/0.45	Involved in important cellular processes, such as cell-cycle control, apoptosis, signal transduction, stress responses, etc.
24–14	translation elongation factor TEF-1 beta chain - brine shrimp	gi|84604|	166	1/0.29/0.27	Role in tumourigenesis, signal transduction and apoptosis
25–14	Na/K ATPase	gi|316954001|	14	1/0.35/0.42	Involved in osmotic regulation, the adaptation to the salinity conditions
26–14	ubiquinol-cytochrome c reductase core protein	gi|164608846|	192	1/0.46/0.56	An additional member of the mitochondrial-protein-processing family

[a] Spot No. as assigned in Fig. 1; For example, 1-1: the first number indicates the sequence number of the protein, and the second number indicates the day post seawater acidification treatment.
[b] Database GI numbers in the NCBInr database.
[c] Mascot protein score.
[d] Fold-changes; significant changes (>1.5-fold, $p < 0.05$) were calculated using ImageMaster 2D Platinum 7.0.
[e] GO functional classification for the differentially expressed proteins mapped to their respective GO terms.

developmental retardation. Reduced development or growth as a result of reduced GAPDH abundance could also help to explain the smaller sizes of the animals raised in the acidified conditions compared to the animals raised in the control conditions.

It was reported that small ubiquitin-like modifier (SUMO) plays important roles in protein interactions, the regulation of the activity of transcription factors, and DNA combination [26]. SUMO also has inherent inhibitory effects, as described in previous studies. In this study, the expression of SUMO-1-like protein (spot: 11-1) was significantly increased, which might be due to SOMOylation related to post-translation modifications of many transcription factors and the suppression of gene transcription. In particular, the suppression of SUMO-1-like protein transcription was enhanced, and therefore, the growth of the brine shrimp was affected. This could be a plausible explanation for the decreased growth and development of brine shrimp exposed to seawater acidification [27].

As a core component of the protein synthesis machinery, the eukaryotic elongation factor 1A delivers aminoacylated tRNA to the ribosome, and elongation factor 1B complex catalyses the exchange of GDP to GTP on elongation factor 1A, resulting in the onset of cell transformation. The expression of translation elongation factor

TEF-1 beta chain (spot: 24-14) was also distinctly down-regulated in the acidified seawater on day 14, indicating that acidification might affect the protein synthesis of *A. sinica* by altering GDP to GTP on elongation factor 1A, which requires further investigation.

These findings are in agreement with our previous study, where we found that the growth rate of *A. sinica* was decreased on day 14 under acidified conditions [27]; these results are also consistent with the speculation made by Seibel et al. [28] that seawater acidification leads to metabolic suppression, reduced protein synthesis, respiratory stress, reduced metabolic scope and, ultimately, death. Given the resources needed to sustain a response to an environmental stress, this increase in energetic demand could be detrimental. Therefore, we hypothesize that seawater acidification might inhibit protein synthesis and metabolism. Enhanced mitochondrial energy production after exposure to elevated CO_2 fits a common pattern in responses to environmental stress, which generally require increased energy production to meet the demands of adaptive cellular processes.

3.3. Cellular stress response related proteins

Cellular stress response proteins were also affected by exposure to seawater acidification, perhaps increasing oxidative stress. Three proteins, peroxiredoxin (spot: 4-1, 4-7, 4-14), thioredoxin peroxidase (spot: 7-1), and 70 kDa heat shock protein (HSP70, spot: 21-14), were up-regulated in all treatments compared to controls, whereas the Na/K ATPase (spot: 25-14) and ubiquinol-cytochrome c reductase core protein (spot: 26-14) were markedly down-regulated. Spot 7-1 was increased after exposure to elevated pCO_2, which was identified as thioredoxin peroxidase, a member of a family of proteins that are conserved from yeast to mammals and to which natural killer enhancing factor belongs. Up-regulated thioredoxin peroxidase led to enhanced reactive oxygen species (ROS) activity. The enhanced ROS generation due to seawater acidification may directly lead to oxidative stress through elevated cellular CO_2 and H^+ [29]. The increase in these antioxidant peroxidases after exposure to elevated pCO_2 implies that brine shrimp likely undergo an increase in energy demands. Enhanced metabolism can occur in response to ongoing stress, which is likely to be an important adaptive strategy to counteract the physiological effects of elevated pCO_2 [30].

Spot 4-1 corresponds to peroxiredoxins, which are a group of cysteine-dependent peroxidases that work as a redox sensor to combat endogenous or exogenous peroxide attacks. Previous studies established that up-regulation of some peroxiredoxins are able to protect cells from oxidative damage by reducing H_2O_2, peroxynitrite and scavenging thiyl radicals [49]. Moreover, cells with reduced expression of peroxiredoxins were shown to be more prone to oxidative damage [50]. A similar study that conducted a proteomic analysis also reported the significant variation in peroxiredoxins in the mantle of the eastern oyster (*Crassostrea virginica*) under elevated pCO_2 conditions, indicating the presence of potential oxidative stress [47]. A study on the proteomic changes in adult Pacific oyster (*Crassostrea gigas*) mantle tissue in response to a two-week exposure to low pH stress showed increased abundances of oxidative stress proteins [29]. The changes in oxidative stress proteins were particularly comprehensive, as they included increased abundances of peroxiredoxin on day 1. These changes indicate increased production and detoxification of organic peroxides and hydrogen peroxide or oxidation of peroxiredoxins by nucleoredoxin, while the expression of peroxiredoxins was down-regulated on days 7 and 14. We hypothesized that these findings might be due to (1) lower pH values affecting the activity of the electron transport chain, and (2) the release of transition metals, especially Fe^{2+}, which activates the Fenton reaction and the production of hydroxyl radicals [29].

Spot 21-14 (HSP70) was up-regulated in brine shrimp post exposure to elevated CO_2 on day 14. As a member of the molecular chaperone family, HSP70 plays a key role in modulating the proper folding of other proteins under environmental challenges, such as heat stress. It has been reported that two classes of molecular chaperones, HSP70 and HSP40, are able to recognize and bind to misfolded proteins, thus preventing their toxic biomolecular aggregation and enabling refolding or targeted degradation [31]. A broad-scale increase in the abundance of chaperones and stress response proteins was observed in oysters in response to elevated pCO_2, providing further evidence of increased cellular stress during seawater acidification exposure [32]. It is clear that elevated pCO_2 impacts the cellular stress response likely in a manner that affects the physiology of the organism. This may indicate that HSP70 was up-regulated in this study in a novel manner, as seawater acidification had an inhibitory effect on the growth rate and reproduction of most types of organisms [33]. The exposure to seawater acidification conditions may result in a physiological shift in energy homeostasis, which depends on the strategies that a given species adopts to enhance stress tolerance and promote survival. Thus, brine shrimp adapt to seawater acidification through up-regulation of the expression of peroxiredoxins, thioredoxin peroxidase and HSP70.

3.4. The immune defence-related proteins

Melanization, which is mediated by the proPO (prophenoloxidase)-activating system (proPO system), is one of the key innate immune defence agents against pathogens and damaged tissue in invertebrates. The proPO system is triggered by the presence of minute amounts of compounds of microbial origins, such as beta-1,3-glucans, lipopolysaccharides and peptidoglycans, which ensures that the system is active in the presence of potential pathogens [34]. The activation of the proPO system by specifically recognizing microorganisms via pattern-recognition proteins (PRPs) triggers a serine proteinase cascade. This cascade eventually leads to the cleavage of inactive proPO to active PO, which functions to produce melanin and toxic reactive intermediates against invading pathogens. In the present study, Spot 13-7 was significantly up-regulated and was identified as prophenoloxidase (proPO) in acidified seawater on day 7, implying that the innate immune response, in consideration of the antimicrobial activity via the proPO-activating system, was likely altered by seawater acidification in the brine shrimp.

Ferritin is found in most cell types of humans and other vertebrates and in invertebrates, plants, fungi, and bacteria. The role of ferritin in different cell types includes both specialized functions, such as recycling iron in macrophages and short- and long-term iron storage in the red cells of embryos or hepatocytes of adults, and intracellular housekeeping functions, such as providing a reserve of iron for cytochromes, nitrogenase, ribonucleotide reductase, haemoglobin myoglobin, etc. and possibly detoxifying if excess iron enters the cell. Although all ferritins share structural properties, cell-specific variations in structure, function, and amount indicate the presence of cell-specific features of genetic regulation. One of the more notable features of regulation is translational control of ferritin mRNA by iron in cells specialized for iron storage. Iron is an essential nutrient for nearly all living organisms, including both hosts and invaders. Ferritin regulates iron levels intracellularly. During the event of a pathogenic invasion, the host can use an iron-withholding mechanism to restrict the availability of this essential nutrient to the invading pathogens. In this study, spot 14-7, identified as ferritin, was significantly down-regulated under acidified seawater on day 7. We then speculated

that ferritin in the brine shrimp was likely affected by seawater acidification because iron participates in energy metabolic processes, such as a reserve of iron for cytochromes, nitrogenase, ribonucleotide reductase, and haemoglobin myoglobin, and ferritin even acts as a detoxification agent if excess iron enters the cell. Thus, spot 14-7 was significantly down-regulated under acidified seawater due to the suppression effects of seawater acidification on metabolism, as described before, and possibly also due to its negative impact on the immune system of marine animals based on previous studies [33].

3.5. Cytoskeletal-related proteins

Four cytoskeletal-related proteins were found to be differentially expressed due to seawater acidification stress in the brine shrimp, of which three proteins, including myosin light chain (spot: 6-1), tropomyosin (spot: 9-1) and tubulin alpha chain (spot: 22-14), were clearly up-regulated, while the other protein, TCP1 subunit 2 (spot: 3-1), was markedly decreased in response to seawater acidification. The cytoskeleton is considered an important, complex, and dynamic cell component in mediating cell migration and division, determining cell shape, redistributing plasma membrane components, intracellular movement, organizing organelles, and the forming intercellular junctions [35]. Many cytoskeleton proteins participate in these processes. For example, spot 6-1 was found to be myosin light chain, which is regulated by a calcium-dependent enzyme myosin light chain kinase, and the requirement for calcium is mediated by the calcium-binding protein calmodulin [36]. The cytoplasmic intermediate filaments comprise many distinct members, which are responsible for the spatial organization of the cytoskeleton in most cells. Calponin is an actin-tropomyosin and calmodulin-binding protein that has been suggested to be involved in the Ca^{2+}-dependent regulation of smooth muscle contraction. Calponin may also regulate the pool of free actin available for cytoskeleton organization by anchoring myosin to actin. The motor domain has the ability to undergo all of the functions of myosin (actin binding, ATP hydrolysis, movement and force). This dramatic progress in mechanics, structure, and molecular genetics will ultimately lead to a detailed understanding of myosin motor functions, which will benefit the functional study of myosin as it is affected by seawater acidification.

Spot 9-1 was identified as tropomyosin, a well-characterized regulator of muscle contraction that exists in muscle and non-muscle cells [37]. It has been reported to be associated with troponin complex and functions as a co-regulator of actin–myosin interactions in *C. gigas* [38]. Myosin regulatory light chains also play a crucial role in regulating actin–myosin interactions. The increases in tropomyosin and myosin abundances were previously observed in Sydney rock oysters (*S. glomerata*) exposed to Pb and Cu contamination. Similarly, exposure of *C. virginica* to elevated pCO_2 resulted in variations in the abundance of cytoskeleton-related proteins, such as actin, actin-polymerization factor and calponin 2 in the mantle tissue [39]. A previous study suggested that the proteome of molluscs in response to environmental stress could always provoke alterations in cytoskeleton-related proteins [40]. Moreover, environmental stresses often lead to the induction of adaptive responses in addition to the cellular stress response, which aim to stabilize macromolecular structure and function during adverse, abnormal or pathological conditions and to conserve metabolic energy for homeostatic adaptations. Spot 22-14 was up-regulated under seawater acidification and was characterized as tubulin alpha chain, which is one of the structural proteins known as “universal” cellular stress response proteins that are generally prominent targets in multiple responses to stress. It would be reasonable to suggest that structural protein isoforms can change their expression in response to seawater acidification. Moreover, spot 3-1 was identified as chaperonin-containing T-complex polypeptide-1 (TCP1) subunit 2, a chaperonin class of protein that is ubiquitous in all genera of life and that is involved in cell cycle progress, cytoskeletal organization, and intracellular assembly and folding of various proteins [41]. Chaperonin-containing TCP1 subunit 2 is a specific regulator for mouse mesangial cell contraction, proliferation, and migration affected by glucose in which the alteration of F-actin, particularly for cell contraction, is involved [42]. The amount of TCP1 was down-regulated on day 1 but up-regulated on day 7 post acidification stress. Several new studies have shed light on the regulation of cytoskeletal dynamics in the response of marine taxa to seawater acidification. For example, beta-tubulin was down-regulated in oyster larvae reared under seawater acidification for a short amount of time; in contrast, it was up-regulated under long-term acidification stress [43]. TCP1 functions as a cytosolic chaperone in the biogenesis of tubulin, which might explain why TCP1 was down-regulated on day 1 but up-regulated on day 7 of seawater acidification. Overall, we speculated that the cytoskeleton and cellular conformation of the brine shrimp were likely affected by the seawater acidification, but the mechanism requires further investigation.

3.6. The signal transduction-related proteins

In the present study, two signal transduction-related proteins, including translationally controlled tumour protein (TCTP, Spot: 8-1) and phospholipase C-like protein (Spot: 20-14), were significantly up-regulated under seawater acidification. TCTP is a highly conserved, cyto-protective protein implicated in many physiological and disease processes. TCTP expression is highly regulated both at the transcriptional and translational level by a wide range of extracellular signals. For example, TCTP has been implicated in important cellular processes, such as cell growth, cell cycle progression, malignant transformation and in the protection of cells against various stress conditions and apoptosis. It has been reported that TCTP mRNA translation is regulated by the PI3K/AKT/mTOR pathway [44]. The PI3K/AKT/mTOR pathway is a well-known pathway, which plays important roles in cross-talk pathways. The PI3K/AKT/mTOR network regulates metabolism through multiple kinases, such as glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase 2 (PEPCK), 6-phosphofructo-2- kinase/fructose-2, etc. In addition, PI3K/AKT/mTOR signalling regulates autophagy through beclin1, sequestosome 1 (p62), phosphoinositide-3-kinase and VPS34 (vacuolar sorting protein 34) to enable fast-growing cells to break down cellular organelles, resulting in recycled catabolites that can be used for biosynthesis and energy metabolism [45]. Considering that TCTP protein was remarkably up-regulated in the acidified seawater on day 1, a plausible interpretation is that many cellular processes, such as cell growth and autophagy, are energetically demanding process and, under seawater acidification conditions, there may be a high energy requirement to maintain intracellular pH and to increase the levels of calcification to offset shell corrosion [46].

Spot 23-14 was identified as phospholipase C-like protein, which plays important roles in phosphoinositide turnover by regulating the calcium-protein kinase C signalling pathway [47]. Here, phospholipase C-like protein was significantly up-regulated under acidified seawater on day 14. As calcifying organisms are able to adapt to environmental variables, allowing for species-specific responses to increasing CO_2 levels to be assessed [15], brine shrimp may enhance their activity of calcium-protein kinase C to adapt to seawater acidification.

The RNA binding motif protein (spot: 16-7) and 14-3-3 zeta (spot: 23-14) were markedly down-regulated under the acidified

seawater conditions. Spot 20-14 corresponded to 14-3-3 zeta. The members of the 14-3-3 family of proteins are ubiquitous, and they participate in numerous molecular processes [48]. It was reported that 14-3-3-zeta participates in TLR3-mediated TICAM-1 signal-platform formation. Knockdown of 14-3-3-zeta reduced the production of inflammatory cytokines and the nuclear translocation of IRF3 via the TLR3 (Toll like receptor 3) -TICAM-1 (TRIF) pathway [49]. TLR-mediated signalling depends on adapter molecules that contain TIR domains: MyD88, TIRAP, TICAM-1 (also called TRIF) and TICAM-2 (also called TRAM). TICAM-1 multimerization by ligand stimulation was prohibited by 14-3-3-zeta knockdown. 14-3-3-zeta is involved in the TLR3-TICAM-1 pathway, promoting multimerization of TICAM-1 for the formation of a TICAM-1 signalosome. Recent reports have provided evidence showing that 14-3-3 proteins also play an important role in the regulation of the cytoskeleton [50]. These proteins interact with phosphorylated cofilin and upstream regulators (for LIM kinase and TES kinase), through which they are conserved in phosphorylated and stabilized actin filaments. The 14-3-3 proteins also participate in the phosphorylation of myosin regulatory light chains, which regulate the contractile activity of smooth muscle and non-muscle cells. In the present study, the expression of 14-3-3 zeta was markedly decreased when the brine shrimp were exposed to seawater acidification. As 14-3-3 zeta plays a crucial role in regulating cytoskeletal structures and TGF-β1(The transforming growth factor β1) -induced contraction in TM cells by acting through the RhoA (Ras homolog gene family, member A) signalling pathway [51], we then speculated that seawater acidification might induce a negative effect on the brine shrimp because the 14-3-3 zeta was significantly reduced upon exposure to acidified seawater on day 14, likely via the TLR3 pathway and the RhoA signalling pathway. This is consistent with the fact that TGF-β1 is known to signal through noncanonical pathways, including the Rho GTPase signalling pathways, and is a key regulator of actomyosin contractions. Therefore, these signalling pathways are likely to be regulated upon seawater acidification in brine shrimp, and our data provide evidence for further necessary research on the physiological effects of seawater acidification on marine invertebrates.

4. Conclusion

In summary, the protein expression of proteins related to metabolic processes, cellular stress responses, immunity, cytoskeletal organization and signal transduction were clearly altered by seawater acidification in brine shrimp. This study sheds new light on the proteomic changes underlying the physiological responses of a marine crustacean, brine shrimp, upon CO_2-driven seawater acidification. We conclude that CO_2-driven seawater acidification affects protein expression in *A. sinica* and possibly also in other crustaceans that feed on brine shrimp.

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