iTRAQ-Based Quantitative Proteomic Analysis Reveals Toxicity Mechanisms in *Chlamys farreri* Exposed to Okadaic Acid

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Okadaic acid (OA), produced by dinoflagellates during harmful algal blooms, is a principal diarrhetic shellfish poisoning toxin. This toxin poses a potential threat to bivalves with economic values. To better understand the toxicity mechanism of OA to bivalves, in this study, oxidative stress biomarkers (superoxide dismutase, SOD; catalase, CAT; glutathione S-transferase, GST; malondialdehyde, MDA) and the expression of detoxification genes (heat shock protein 70, HSP70; heat shock protein 90, HSP90; cytochrome P450, CYP450) were assessed in the gills of scallops *Chlamys farreri* after 24 h, 48 h and 96 h exposure to OA. In addition, the digestive glands of scallops exposed to OA for 96 h were dissected for an iTRAQ based quantitative proteomic analysis. The results of OA exposure experiments showed that OA induces oxidative stress and significant enhancement of the expression of detoxification genes in scallops. The proteomics analysis revealed that 159 proteins altered remarkably in OA-treated scallops, and these proteins were involved in phagosomes, regulation of actin cytoskeleton, adherens junction, tight junction, and focal adhesion. Amino acid biosynthesis, carbon metabolism, pentose phosphate pathway, fructose and mannose metabolism in the digestive glands were also significantly impacted. Our data shed new insights on the molecular responses and toxicity mechanisms of *C. farreri* to OA.

**Keywords:** okadaic acid, *Chlamys farreri*, oxidative stress, detoxification genes, iTRAQ

**INTRODUCTION**

In recent years, harmful algal blooms (HABs) have occurred widespread in the ocean, with higher frequency and longer periods than that in the past (Dolah, 2000; Anderson et al., 2012; Gobler et al., 2017). The emergence of HABs poses a major threat to human health, fisheries resources, and marine ecosystems worldwide (Van Dolah and Ramsdell, 2001; Hoagland and Scatasta, 2006; Hallegaard et al., 2021). One main hazard of HABs is the release of a variety of shellfish toxins, including diarrhetic shellfish toxins (DST), neurotoxic shellfish toxins (NST), paralytic shellfish toxins (PST), and forgetful shellfish toxins (AST) (Christian and Luckas, 2008).
Diarrhetic shellfish toxins (DST) is one of the most threatening shellfish toxins in the world. The lipophilicity allows DST to accumulate in animal tissues and to be biomagnified in the food web (Gerssen et al., 2010; Lloyd et al., 2013; Valdiglesias et al., 2013; Liu et al., 2019). Numerous reports have referred to public safety and health incidents caused by diarrheal shellfish poisoning (DSP) around the world (Yasumoto et al., 1978; de Carvalho et al., 2019; Young et al., 2019). Okadaic acid (OA) and its analogs, a typical DST, are responsible for DSP in humans (Garcia et al., 2005). Studies on mammalian or and its analogs, a typical DST, are responsible for DSP in mammals (Carvalho et al., 2019; Young et al., 2019). Okadaic acid (OA) (NRC CRM-OA-d) was obtained from the Celebrated chemical reagent (Sinopharm Chemical Reagent, China). The scallops Chlamys farreri is an important fishery and aquaculture species widely distributed in the subtropical western Pacific, playing an important role in the coastal ecosystem (Smaal et al., 2001). Moreover, C. farreri can accumulate shellfish toxins and therefore is widely used in algal toxin research (Li et al., 2017). As little is known about the toxic effects and the molecular mechanisms underlying the effects of OA on bivalves, it is necessary to carry out studies using omics for more global information. In this study, we combined traditional physiology and iTRAQ proteomics to (1) determine the physiological responses of C. farreri to OA and (2) elucidate the potential mechanisms of OA toxicity on C. farreri from the molecular perspective.

MATERIALS AND METHODS

Reagent

Okadaic acid (OA) (NRC CRM-OA-d) was obtained from the Institute for Marine Biosciences (National Research Centre, Halifax Regional Municipality, NS, Canada), and stored in the dark at 4°C until the experiment. Stock solutions (20 μg/L) were prepared in dimethyl sulfoxide (DMSO, Sinopharm Chemical Reagent, China).

Experimental Conditions

Experimental scallops, C. farreri (shell length: 4–6 cm) were collected from a local farm (Yantai, Shandong, China). After cleaning shells, the scallops were transported to the Muping Coastal Environmental Research Station and acclimated in filter-sterilized seawater (temperature 15.3 ± 0.2°C, pH 8.12 ± 0.07, salinity 31.2 ± 0.5‰, mean ± SD) for 14 days. The scallops were fed daily with commercial algal blends during the acclimation period.

After acclimation, three treatment groups, including blank control (filter-sterilized seawater (FSSW)), solvent control (dimethyl sulfoxide, DMSO, 0.01%, v/v), and exposure groups (OA at nominal concentrations of approximately 10–20 μg OA eq. 100 g−1 shellfish meat) were conducted. The exposure concentration of OA used in this study is close to the European Food Safety Authority (EFSA) regulation limit for human consumption of shellfish (16 μg OA eq.100 g−1 shellfish meat) (Authority, 2008). The experiment was carried out in an 80L aquarium with 40 organisms in each aquarium. Three duplicate aquaria were used in each treatment (a total of 120 individuals in each treatment). In the OA treatment, the scallops were directly injected with 100 μL of 20 μg/L OA. The scallops in the blank control and solvent control were injected with 100 μL FSSW and DMSO, respectively. After injection, scallops were put back into the corresponding aquarium and sampled at 24 h, 48 h, and 96 h. No scallop mortality was observed during the experiment. Sampling time points were set according to the standard methodized proposal by USEPA (2002). It can be proved that Chlamys farreri is a potential indicator organism that can respond quickly in environmental monitoring events.

At each sampled time point, the gills and digestive glands of the scallops were carefully cut off on the ice and immediately frozen in liquid nitrogen and stored at −80°C for subsequent analysis. The gills of the bivalves are more closely exposed to the environment, more susceptible to exogenous substances (Regoli and Principato, 1995; Magara et al., 2018). In addition, the digestive gland is one of the most important tissues for assessing the health status of bivalves and can be associated with functional damage to cells (Teng et al., 2021).

Oxidative Stress Biomarkers

To measure oxidative stress biomarkers, 10-15 individuals (gills) were dissected at each time point and pooled into 5 independent replicates to minimize biological variation. After sampling, the gills were used to prepare a tissue homogenate as follows: the gills were homogenized in phosphate buffer (50 mM potassium dihydrogen phosphate; 50 mM potassium phosphate dibasic; 1 mM EDTA; pH 7.0) using an IKA homogenizer (Ultra Turrax IKA T10 basic, Staufen, Germany). The homogenates were then centrifuged at 10,000 g for 20 min at 4°C to obtain supernatants. To investigate oxidative stress in scallops, superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) activities were determined using the commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Malondialdehyde content was measured also using commercial kits from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. Subsequently, protein concentrations were determined according to the Bradford method using bovine γ-globulin as a standard (Bradford, 1976).
The normality of the data was verified by the Shapiro-Wilk test, error of mean and were analyzed using Graphpad prism 7.0. ± the pathways significantly enriched after OA treatment, protein-hypergeometric test (<p>background of identified proteins were determined via a GO enrichment terms and pathways of DEPs in the Encyclopedia of Genes and Genomes (KEGG) database. Then, (CC). The protein pathway was annotated using the Kyoto process (BP), molecular function (MF), and cellular component (CC). The protein pathway was annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Then, the GO enrichment terms and pathways of DEPs in the background of identified proteins were determined via a hypergeometric test (<p>. To gain a deeper understanding of the pathways significantly enriched after OA treatment, protein-protein network analysis was performed using Cytoscape 3.7.1

**Statistical Analysis**

Experimental data are represented as the means ± standard error of mean and were analyzed using Graphpad prism 7.0. The normality of the data was verified by the Shapiro-Wilk test, and the homogeneity of the variances was analyzed by Levene’s test. One-way ANOVA and Tukey’s post hoc test was performed to analyze whether the differences between each group were significant. <p> was considered significant difference.

**RESULTS**

No significant difference was detected in the physiological indexes between the solvent control group and the control group, thus only experimental results derived from the control group were analyzed and discussed.

**Oxidative Stress Biomarkers**

In the scallops, SOD and CAT activities were significantly stimulated by OA treatment at 96 h, increasing by 1.55-fold and 1.3-fold compared to the control, respectively (Figures 1A,C). Meanwhile, GST activity was enhanced by 1.13-fold after the 48 h exposure to OA (Figure 1B). A significant increase in MDA content was also observed in the OA group in the 48 h and 96 h treatments (Figure 1D).

**Real-Time Quantitative PCR Analyses**

Among OA treatments, a significant increase in mRNA expression of HSP70 was observed in the scallops at 48 h and 96 h compared to those at 24 h (Figure 2A). Unexpectedly, the mRNA expression of HSP70 is significantly suppressed by OA in the 24 h treatment (Figure 2A). Although with no statistical difference, the expression of HSP90 transcripts showed a similar temporal trend to HSP70 after OA injection (Figure 2B). In addition, the expression of CYP450 increased significantly at 96 h in the OA-treated group, which was 4.61 times higher than that at 24 h (Figure 2C).

**Proteome Analysis**

A total of 159 DEPs (Supplementary Materials, Supplementary Table 2) were detected in the digestive glands of the scallops exposed to OA, compared to the solvent controls (fold change > 1.2 as up-regulated or < 0.83 as down-regulated, <p> <p>). In these DEPs, 104 of the 159 DEPs were up-regulated and 55 were down-regulated (Figure 3A). At the same time, all the differences in protein GO enrichment (BP, CC, MF) and pathway KEGG enrichment results and significant (p < 0.05) numbers were summarized, and intuitively display through the column figure (Figure 3B). Specifically, 855 proteins were annotated with biological process (BP), and 239 terms were significantly enriched. In contrast, 156 proteins were annotated with cell component (CC), and 58 terms were significantly enriched; 284 proteins were annotated with molecular function, including 94 terms were significantly enriched. Furthermore, KEGG analysis showed that there are 9 pathways were significantly enriched.

Differentially expressed proteins (DEPs) were classified into various categories based on gene ontology (GO) terms (Figure 4). According to the category of biological process (Figure 4A), the DEPs were mostly annotated to the “single-organism metabolic process,” “organonitrogen compound metabolic process,” and “small molecule metabolic process.” The DEPs covered a wide

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1http://www.cytoscape.org/
range of molecular functions (Figure 4B), mainly including "catalytic activity," "anion binding," "nucleotide binding," and "nucleoside phosphate binding." Based on the category of cellular components (Figure 4C), the DEPs were mainly located in "intracellular," "intracellular part," and "cytoplasm."

To further investigate the main pathways of the scallop's response to OA, KEGG enrichment analysis and protein-protein interaction (PPI) networks were also performed on the DEPs. As shown in Figures 5, 6, OA treatment led to DEPs involved in several cellular processes in the scallops, such as phagosomes, regulation of actin cytoskeleton, adherens junction, tight junction, and focal adhesion. In addition, DEPs were also significantly enriched in pathways of amino acid biosynthesis, carbon metabolism, pentose phosphate pathway, fructose and mannose metabolism.

**DISCUSSION**

**Oxidative Stress Biomarkers**

When exposed to environmental stress, organisms usually produce excessive ROS, which leads to oxidative stress (Lushchak, 2011). Antioxidant enzymes play a central role in the cellular defense mechanism against oxidative stress (Valko et al., 2006; Lushchak, 2011). As the first line of the antioxidant defense system, SOD catalyzes superoxide anion disproportionation to oxygen and hydrogen peroxide, while CAT prevents the formation of excessive H$_2$O$_2$ through decomposing H$_2$O$_2$ once it is formed (Halliwell, 1974). As a phase II detoxification enzyme, GST protects cells and tissues against oxidative stress by catalyzing the conjugation of the reduced form of glutathione to various xenobiotic substrates (Hayes and Strange, 1995). These antioxidant enzymes interact with each other to maintain the balance between ROS production and ROS scavenging in an organism. In this study, all three antioxidant enzymes (SOD, CAT, and GST) were generally stimulated in the gills of scallops, indicating that the antioxidant system was activated in response to the OA exposure, which may have caused oxidative stress to the scallops. Similar to our results, increased antioxidant enzymes activity was also observed in the bivalves *Nodpecten subnodosus* (Campa-Cordova et al., 2009) and *Anomalocardia flexuosa* (Leite et al., 2021), when exposed to *Prorocentrum lima* (a dinoflagellate producing DSP toxins).

Lipid peroxidation (LPO) is a major indicator of cellular oxidative damage level in organisms and is a major contributor to the loss of cell function under OA exposure (Cossu et al., 2000; Alves de Almeida et al., 2007). Lipid peroxidation usually occurs when the production of ROS exceeds the capacity of the antioxidant system (Halliwell, 1996; Lushchak, 2011). The main product of lipid peroxidation is MDA, so generally, the

**FIGURE 1** Activities of SOD (A), GST (B), CAT (C), and MDA content (D) of *C. farreri* exposed to OA (n = 5). Asterisks indicate significant differences between the control and OA-exposed group within each fixed sampling period, and different letters indicate significant differences among different sampling periods within each treatment (p < 0.05).
Alterations in Gene Expression of the Stress-Related Proteins

As molecular chaperones, HSPs help organisms combat environmental stresses and maintain cellular homeostasis by promoting protein folding and preventing aggregation of denatured proteins (Johnston and Kucey, 1988; Liu et al., 2014; Johnston et al., 2018). CYP450 is a monoxygenase that promotes the biotransformation and metabolic detoxification of endogenous and exogenous toxic compounds (Snyder, 2000; Xu et al., 2005). HSPs and CYPs genes can be activated by various forces in aquatic organisms, which are widely used as biomarkers for toxicology research (Chaty et al., 2004; Rewitz et al., 2006; Chae et al., 2009; Liu et al., 2013; Ding et al., 2018). In the present study, the expression of both HSPs and CYP450 genes increased with the OA exposure time, supporting their roles in stress response and detoxification process to OA. The time-dependent changes in genes to OA are also in line with the result of previous studies involving other environmental stresses (Liu et al., 2018; Nguyen et al., 2018; Nie et al., 2020; Zhang et al., 2020). We hypothesize that overexpression of the HSPs gene may counteract the proteins damaged by the ROS after OA exposure, while the enhanced synthesis of CYP450 may contribute to complete the biotransformation of OA. In short, expressional changes of these genes reflect pressures of OA on the scallop tissues from the molecular aspect.

Proteomic Responses of Scallops to Okadaic Acid Exposure

As major organelles in the translation process, ribosomes are essential for protein production in cells (Wimberly et al., 2000; Sonenberg and Hinnebusch, 2009). In the OA-treated...
**FIGURE 4** | GO annotation of DEPs. DEPs were assigned to second-tier GO categories associated with three terms: biological process (A), cellular component (B), and molecular function (C).
scallops of this study, 11 ribosome-associated DEPs exhibited significant over-expression (Supplementary Table 2), while significant enrichment of the amino acid biosynthetic pathway was suggested by the KEGG analysis and PPI network. The up-regulation of these proteins or pathways suggests that protein synthesis is effectively enhanced in the scallops exposed to OA, which possibly compensates for the proteins damaged by oxidative stress (Tomanek, 2010; Dasuri et al., 2013).

In the current study, the KEGG pathway analysis shows that phagosome is one of the important pathways in the scallops’ response to OA (Figure 5). In the invertebrate immune system, phagocytosis is a necessary mechanism for the removal of invading microorganisms, dead cells, and other relatively large particles (Mydlarz et al., 2006). The internalization of phagocytosis of large particles is initiated by the combination of particles with cell surface receptors, followed by the reconstitution and reorganization of the cytoskeleton to form...
FIGURE 6 | DEPs enriched in the protein-protein interaction network in OA-exposed scallops. Proteins are shown as small elliptical nodes (blue nodes represent down-regulated proteins and red nodes represent up-regulated proteins) and are connected with associated KEGG pathways (rectangular Nodes). The size of the line between nodes represents the scoring value of the interaction between the two nodes, and the greater the score, the more reliable.

Our proteomic results showed that a total of 25 cytoskeleton-related proteins changed significantly, and 23 DEPs were up-regulated significantly. KEGG analysis also showed that the regulation of actin cytoskeleton in the OA-treated scallops was changed. In eukaryotic cells, the cytoskeleton is a complex reticular system of protein components such as microtubules, microfilaments, and intermediate fibers (Schliwa and Vanblerkom, 1981), playing a key role in maintaining cell structure, adhesion, migration, differentiation, division, and organelle transport (Hall, 1998). The result of our proteomic study is in accordance with the previous physical and omics studies showing that DST induced extensive exert cytotoxic effects by modulating the cytoskeleton (Espina and Rubiolo, 2008; Hanana et al., 2012; Huang et al., 2015; He et al., 2019). Changes in cytoskeleton-related proteins may also be related to cell connections, such as tight junctions and focal adhesion, thereby affecting the transport of macromolecules between cells and the signal transmission between cells and the extracellular matrix (Schneeberger and Lynch, 1992; Fanning et al., 1998). In this study, OA treatment led to significant alteration in the expression of proteins from tight junction, adherens junction, and focal adhesion pathways. Tight junctions and focal adhesion are two well-known cell connections associated with the cytoskeleton. The former links the plasma membranes of adjacent cells together and maintains normal cell function by acting as a barrier between cells to prevent the diffusion of soluble substances as well as membrane proteins and plasmids (Hartsock and Nelson, 2008). The latter plays a key role in regulating cell migration, proliferation, and apoptosis by mediating mechanical and biochemical signaling between cells and the extracellular matrix (Takeichi, 1995; Gumbiner, 2005;
was well below those tolerated by the scallops, OA still resulted in significant changes in the physiology and proteomics of the scallops. OA not only induced oxidative stress and altered stress gene expression in scallops but also significantly changed biological processes like biosynthesis of amino acids, energy metabolism, cytoskeleton functions, and phagosome in scallops. Our findings provide global information for the potential mechanisms of OA toxicity on bivalves.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**AUTHOR CONTRIBUTIONS**

XW performed part of the experiment, analyzed the data, and drafted the manuscript. DW performed part of the experiment. TZ assisted with data analysis. QZ contributed to the review and editing. JZ did the conceptualization, funding acquisition, and review. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.792050/full#supplementary-material

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