Korean J. Malacol. 35(4): 347-354 2019
https://doi.org/10.9710/kjm.2019.35.4.347

Effects of domoic acid exposure on the immunological parameters in bay scallop *Argopecten irradians*

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ABSTRACT

Domoic acid (DA) is a neurotoxin produced by the diatom genus *Pseudo-nitzschia*. This toxin is particularly problematic for bivalves that are cultured for human consumption. Scallops have especially low metabolic rates and readily accumulate toxins in their tissues. In this study, bay scallop were exposed to three concentrations of DA (20, 40, and 60 ng/mL) for 48 h, and immunological responses were investigated. Immune parameters were evaluated by estimating the levels of reactive oxygen species (ROS) in the hemolymph, and peptidoglycan recognition proteins (PGRP), fibrinogen-related proteins (FREP), and heat shock protein (HSP) 70 mRNAs in the digestive diverticula of bay scallops. Results showed that not only ROS but also PGRP, FREP, and HSP70 mRNA levels were elevated under exposure to high concentrations of DA. However, mRNA expression levels peaked at different times during exposure, with subsequent declines. Our results suggest that exposure to DA induce protein denaturation and damage, and bay scallops have insufficient defense to repair denatured proteins, owing to the toxicity of DA.

Keywords: *Argopecten irradiance*, Domoic acid, Fibronogen-related protein, Heat shock protein, Immunity, Peptidoglycan recognition protein

INTRODUCTION

Scallops in South Korea comprise more than 20 species, including bay scallop *Argopecten irradians*, yesso scallop *Patinopecten yessoensis*, and farrer's scallop *Chlamys farreri*, among others (Lee and Min, 2002). These scallops are representative of filter feeding species and are very sensitive to the water quality of the marine environment (Hannam *et al*., 2009). They feed on small organisms that are present in the water and as such, bacteria, viruses, and toxins from marine organisms easily accumulate in their tissues. This may lead to bioaccumulation in crustaceans and fish that ingest shellfish contaminated with marine toxins, or cause disease in people who consume them (Perl *et al*., 1990; Hallegraeff, 2010).

The incidence of harmful algae blooms (HABs) is increasing due to eutrophication of the ocean caused by water pollution and global warming (Simões *et al*., 2015). HABs are caused by phytoplankton and have phycotoxins that are harmful to filter feeding organisms (Van Dolah, 2000; Simões *et al*., 2015). Scallops exposed to the toxins in HABs can also cause fatal diseases, such as amnesic shellfish poisoning (ASP), paralytic shellfish poisoning (PSP), and diarrhetic shellfish poisoning (DSP; Zabaglo *et al*., 2016).

In addition, the occurrence of *Pseudo-nitzschia*, a species of marine diatom, has increased in frequency in recent years with the rise in the incidence of HABs. Therefore, domoic acid (DA), which is a toxin produced by *Pseudo-nitzschia*, is also increasingly widely distributed in seawater (Trainer *et al*., 2012; Lelong *et*

Received: November 13, 2019; Revised: November 25, 2019; Accepted: November 28, 2019
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1225-3480/24750

al., 2012). DA accumulates in shellfish ingesting *Pseudo-nitzschia* and acts as a powerful neurotoxin that can cause illness and memory loss, known as ASP, when consumed by humans (Bates *et al*., 1989). DA causes not only toxic stress but also epilepsy in the sea lion, *Zalophus californianus*, off the coast of California (Goldstein *et al*., 2007). In addition, there are many reports that DA has negative effects on the developmental stage of vertebrates, including fish (Adams *et al*., 2007; Van Dolah, 2000; Lelong *et al*., 2012). Although various studies have been conducted on the effects of DA on vertebrates, little research has been conducted on how it affects shellfish, which are filter feeding organisms. As shellfish consumption is rapidly increasing, research on the physiological response of shellfish to algae toxicity is imperative.

In shellfish, exposure to toxic environments produces a large amount of reactive oxygen species (ROS), which are transferred via the hemolymph throughout the body to peroxidate lipids in the tissues and disrupt DNA binding, proteins, and amino acids. As DA has an adverse effect on survival (Chen *et al*., 2007; Hannam *et al*., 2009), shellfish activate various immune mechanisms to protect themselves from the toxic conditions caused by HABs. Marine mollusks, including scallops, lack an adaptive immune system and rely solely on the innate or non-specific immune system for defense (Guo *et al*., 2015). However, mollusks, and some other invertebrates, have greatly expanded sets of innate immune receptors, which may provide improved specificity in immune recognition in the absence of adaptive immunity and its associated autoimmune costs (Guo and Ford, 2016). As a first step in activating this immune system, the immune response plays an important role in the distinction between autologous- and non-self-substances. Immune responses begin when the specialized, soluble or cell-bound pattern recognition receptors (PRRs) recognize the major targets. PRR is highly involved in pathogen recognition and immune regulation (Medzhitov and Janeway, 2002; Akira and Takeda, 2004). Representative PRRs involved in the immune response include peptidoglycan recognition proteins (PGRPs) and fibrinogen-related proteins (FREPs), which are upregulated by pathogens and toxic exposures (Itoh and Takahashi, 2009; Chi *et al*., 2016). In bivalve mollusks, various immune-related substances, including FREP and PGRP, are most highly expressed in the digestive diverticula, which has been reported to be an important tissue for immune responses (Zhang *et al*., 2012).

Heat shock proteins (HSPs) prevent protein denaturation and maintain key proteins by repairing damaged proteins (Csermely *et al*., 1998). In scallops, as in other shellfish, HSP is expressed to protect against the toxicity of foreign substances and pathogens. Their overexpression affects the activity of immunity substance, affecting the immune system of the organism by interfering with the recognition of antigens (Song *et al*., 2015; Giri *et al*., 2016).

To date, studies on *Pseudo-nitzschia*, a causative organism that produces DA toxins causing ASP, as well as various studies on fish and rodents that consume this diatom, have been conducted to confirm its toxicity. Very little research has been conducted on the physiological effects of DA directly on bivalves. Therefore, *A. irradians*, which is known to accumulate toxins in its tissues, was selected as an experimental species because it is a fast growing and profitable shellfish species that is gaining popularity in the food industry in South Korea and China.

This study was performed to investigate the effects and risks of DA on the immune response of *A. irradians*, which has a high economic value and faces potential exposure to DA-producing marine diatoms. To confirm this, we investigated the expression of ROS, expression of immune-related genes (PGRP and FREP), and HSP70 mRNA under various DA concentrations and exposure durations.

MATERIALS AND METHODS

1. Experimental animals

For each experiment, *A. irradians* (shell length, 57.0 ± 10.3 mm) were purchased from a commercial market (Goseong, Korea) and maintained prior to the laboratory experiments in four 400 L circulation filter tanks containing filtered and aerated seawater. The

four experimental treatments were conducted in duplicate and with 50 bay scallops per tank. Half of the total volume of seawater was replaced daily. The scallops were reared with automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Buchoen, Korea), with the water temperature and salinity maintained at 17°C and 30‰, and were allowed a one-week acclimation period.

2. Exposure methods and sample collection

Purified crystalline domoic acid (DA), ≥ 95% (HPLC), was obtained from Millipore Sigma (St. Louis, MO, USA) and stored at −20°C before use. Two hundred and forty *A. irradians* were randomly divided into a control (without DA) and treatment groups (with DA). Each group consisted of 35 scallops and DA treatment groups were treated with one of three concentrations (20, 40, and 60 ng/mL) of DA. Five scallops from each replicate treatment group were randomly collected after exposure to DA for 3, 6, 12, 24, 36, and 48 h. A volume of 1 mL of hemolymph was collected from the adductor muscle using a 1 mL syringe within 3 min of removing scallops from the tank. The digestive diverticula tissues were collected from each scallop for RNA analysis. Hemolymph was separated by centrifugation (4°C, 750 ×g, 3 min), and sampled digestive diverticula and hemolymph were stored at −80°C until analysis.

3. Total RNA extraction and complementary DNA synthesis

Total RNA was extracted from the digestive diverticula using the TRI reagent® (Molecular Research Center, Inc., Ohio, USA) according to the manufacturer's instructions. For the total RNA, the 260:280 ratios, which are an indication of purity, were all between 1.8 and 2.0. Total RNA (2 μg) was reverse-transcribed in a total volume of 20 μL, using an oligo-d$(T)_{15}$ anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. Synthesized complementary DNA (cDNA) was kept at −20°C until use.

4. quantitative PCR (qPCR)

qPCR was conducted to determine the relative expression levels of FREP, PGRP, and RPS18 mRNA using cDNA reverse transcribed from the total RNA extracted from the digestive diverticula. The primers used for qPCR are listed in Table 1. PCR amplification was conducted using the Bio-Rad CFX96™ real-time PCR detection system (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The qPCR cycling conditions were as follows: 5 min at 95°C for first denaturation, followed by 45 cycles of 20 s at 95°C for extension, 20 s at 53°C for annealing and 45 s at 72°C for extension, followed by 10 min at 72°C for the final extension. As internal controls, experiments were duplicated with ribosomal protein S18 (RPS18), and all data were expressed relative to the corresponding RPS18 threshold cycle (ΔCt) levels. The calibrated ΔCt value ($\Delta\Delta$Ct) for each sample and internal controls (RPS18) was calculated using the 2-$\Delta\Delta$Ct method: [$\Delta\Delta$Ct = 2^-(ΔCt_{sample} − $\Delta Ct_{internal\ control}$)].

Table 1. Primers used for QPCR amplification

Genes	Primer	DNA sequences
PGRP (AY437875)	Forward	5′- GAT GCC GAT AGA AGG GTT CA-3′
	Reverse	5′- GCA CAT TGC TGT CAA AAT GG-3′
FREP (EU399719)	Forward	5′- ACG TCG TCG GAT TGG TAC TC-3′
	Reverse	5′- TAC AAG CAA CGC ATC TCC TG-3′
HSP70 (AY485261)	Forward	5′- AGT CGG AGG AGG TCC AAG AT-3′
	Reverse	5′- GGA ATT CCG GTC AGT TCA AA-3′
RPS18 (AF526232)	Forward	5′- GTC TGC AAG AAG GCT GAT GT-3′
	Reverse	5′- GGG TTG GAC ATG ATT GTG AT-3′

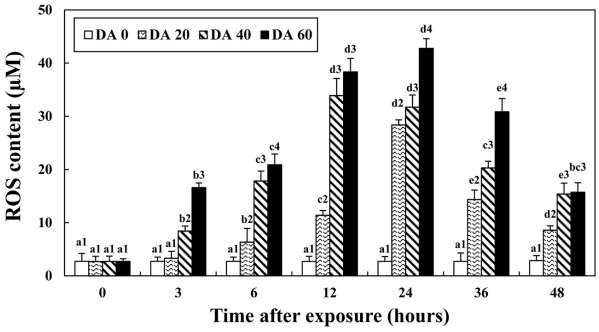

Fig. 1. Contents of ROS in the hemolymph of bay scallop treatment groups exposed to various DA concentrations for 48 h. Significant differences between exposure times within the same treatment are indicated using characters ($P < 0.05$). Significant differences between the treatment groups within the same exposure time are indicated using numbers ($P < 0.05$). All values are means ± SD ($n = 5$).

5. Measurement of reactive oxygen species production

Hemolymph was separated by centrifugation (4°C, 750 ×g, 3 min). The ROS content in hemolymph was measured using assay kits (BO-PER500; BIOMAX Co., Ltd., Korea) according to the manufacturer's instructions.

6. Statistical analysis

All data were analyzed using the SPSS statistical package (version 19.0; SPSS Inc., USA). A one-way ANOVA followed by Tukey's post-hoc test was used to compare differences in the data ($P < 0.05$). The values are expressed as the means ± standard error (SE).

RESULTS

3.1. ROS contents

The ROS content in hemolymph from scallops exposed to DA is shown in Fig. 1. The ROS content was higher in all DA treated groups at all time points, with the highest level of ROS detected after 24 h of exposed to DA at 60 ng/mL, followed by exposure to DA at 40 and 20 ng/mL at any time point.

2. Expression of PGRP mRNA

The expression of PGRP mRNA in the digestive diverticula was significantly lower for all DA concentrations at the initial 3 h time point, compared

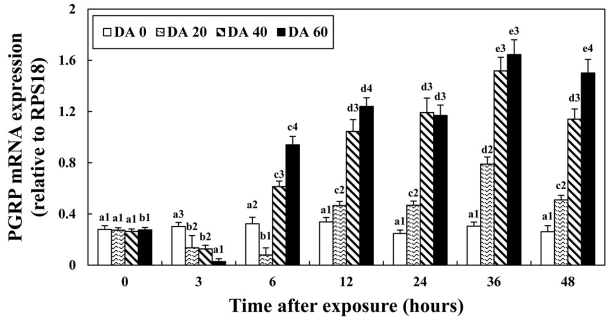

Fig. 2. Changes in the expression levels of PGRP mRNA in the digestive diverticula of bay scallop treatment groups exposed to various DA concentrations for 48 h. Significant differences between exposure times within the same treatment are indicated using characters ($P < 0.05$). Significant differences between the treatment groups within the same exposure time are indicated using numbers ($P < 0.05$). All values are means ± SD ($n = 5$).

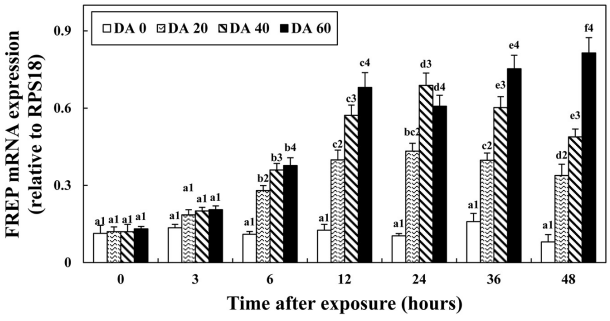

Fig. 3. Changes in the expression levels of FREP mRNA in the digestive diverticula of bay scallop treatment groups exposed to various DA concentrations for 48 h. Significant differences between exposure times within the same treatment are indicated using characters ($P < 0.05$). Significant differences between the treatment groups within the same exposure time are indicated using numbers ($P < 0.05$). All values are means ± SD ($n = 5$).

with the 6-48 h time points, except for DA at 20 ng/mL at 6 h. The highest expression values were observed at 36 h, which then decreased at 48 h but remained elevated when compared with the control (Fig. 2).

3. Expression of FREP mRNA

FREP mRNA expression after exposure to DA were up-regulated at all time points, except at 3 h (Fig. 3). In all treatment groups, no significant increase was observed at 3 h. The mRNA expression in DA at 60

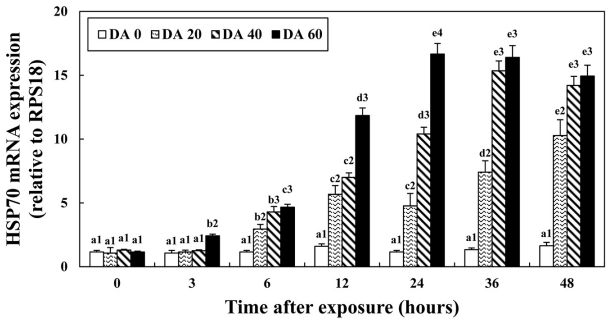

Fig. 4. Changes in the expression levels of HSP70 mRNA in the digestive diverticula of bay scallop treatment groups exposed to various DA concentrations for 48 h. Significant differences between exposure times within the same treatment are indicated using characters (P < 0.05). Significant differences between the treatment groups within the same exposure time are indicated using numbers (P < 0.05). All values are means ± SD (n = 5).

ng/mL gradually increased with the duration of exposure and was significantly up-regulated at 6-48 h, peaking at 48 h, with the exception of a dip in expression at 24 h.

4. Expression of HSP70 mRNA

The expression of HSP70 mRNA treated with three concentrations of DA is shown in Fig. 4. The mRNA expressions in the groups exposed to DA at 40 and 60 ng/mL gradually increased after 6 h, with DA at 60 ng/mL specifically showing significant up-regulation from 3-48 h, compared with the other groups. The highest mRNA expression for DA at 20, 40, and 60 ng/mL was observed at 48, 36, and 24 h, respectively.

DISCUSSION

DA is one of the major marine toxins produced by *Pseudo-nitzschia* that causes strong neuropathic poisoning (ASP), which results in memory loss when consumed by humans (Bates *et al*., 1989). Therefore, there have been various studies on vertebrates that consume DA (Adams *et al*., 2007; Goldstein *et al*., 2007; Van Dolah, 2000; Lelong *et al*., 2012). However, little research has been carried out till date on the effects of DA on shellfish, a filter-feeding organism, before consumption by vertebrates, including humans. In particular, DA is highly likely to act as a toxin to shellfish; thus, further studies are required to elucidate their immune response, which is essential for survival as well as biological defense. Therefore, in this study, to investigate the effect of DA on the immune response of *A. irradians*, we observed the change in immune-related substances (ROS, PGRP, FREP, and HSP70) according to the exposure duration of *A. irradians* to various concentrations of DA (20, 40 and 60 ng/mL).

The production of ROS in bivalves is one of the major mechanisms associated with cellular immunity. Small amounts of ROS can act to defend against pathogens, but excessive ROS can cause severe damage to lipids, proteins, and DNA in cells (Hannam *et al*., 2009). DA causes oxidative stress in the central nervous system and spinal cord of vertebrates (Hiolski *et al*., 2014). In addition, some phycotoxins have been reported to induce oxidative stress when ingested by bivalves (Hégaret *et al*., 2011; Malanga *et al*., 2016). In this study, the ROS concentration in *A. irradians* was higher in the DA treatment groups than in the control, regardless of exposure duration, with higher treatment concentrations resulting in higher ROS concentrations. Similarly, Tian and Zhang (2019) confirmed that ROS formation increased in *Caenorhabditis* elegans when exposed to DA, with ROS formation and the resultant toxicity inducing apoptosis. When scallops are exposed to DA, ROS is excessively generated, causing toxicity, with a higher the concentration of DA resulting in greater amounts of ROS. Additionally, DA may be toxic to *A. irradians*, causing damage to lipids and proteins, which are components of scallops.

In order to investigate the immune response of *A. irradians* to cope with these toxic stresses, the mRNA of the PGRP and FREP in digestive diverticula exposed to various DA concentrations were measured. The expression of PGRP and FREP mRNAs was lower at the initial 3 h time point, than the other time points, with a sharp increase observed thereafter in all treatment groups. In addition, the expression of PGRP mRNA gradually increased depending on the concentration and exposure time, except for the 24 h immediately after DA exposure. First, PGRP

recognizes, binds, and activates the immune response to peptidoglycans, which protect cells and maintain their shape (Song *et al*., 2015). FREP plays an important role in the innate immune system as a pattern recognition receptor for the removal of foreign invaders (Zhang *et al*., 2012). Chi *et al*. (2016) also reported that after exposure of *A. irradians* to the phycotoxin palmitoleic acid for 48 h, the expression of PGRP genes decreased over a short period and then rapidly increased. This suggests that *A. irradians* activates the immune system as soon as it is exposed to DA and then increasing the expression of immune system receptors to counteract cell transformation due to toxicity. A previous study has also reported increased expression of FREP mRNA in *A. irradians* when exposed to the pathogen *Listonella anguillarum* (Zhang *et al*., 2009). This suggests that DA, which is not a pathogen, can also affect the expression of FREP, and that DA can act as a toxin in scallops causing cell death in affected tissues (digestive diverticula).

In addition, HSP plays an important role in preventing irreversible protein denaturation that would promote the destruction of damaged proteins and is regulated by various environmental factors in which influence stress and immune responses (Gestal *et al*., 2008). To confirm the stress and immune activity that occurred when the *A. irradians* was exposed to DA for 48 h, the expression level of HSP70 mRNA was observed. With the exception of the 36 h post DA exposure time point, a significant increase in HSP70 expression was observed with increasing DA concentration and exposure duration. Previous studies have reported that HSP90 expression in the mussel *Mytilus galloprovincialis* gradually increased three days after exposure to okadaic acid, which is a type of phycotoxin (Manfrin *et al*., 2010). Further, Wang *et al*. (2012) reported that when *C. farreri* significantly upregulated the expression of HSP70 24 h after ammonia-N and *Vibrio anguillarum* exposure. In this study, similar trends were observed, suggesting that the expression of HSP is upregulated to promote the repair of proteins damaged by DA. In the case of *A. irradians*, at least 48 h of exposure to DA appears to negatively affect the stress response and immune system, suggesting that even 48 h of exposure to a relatively low concentration of DA (20 ng/mL) is detrimental.

In conclusion, *A. irradians* generates a large amount of toxic ROS when exposed to DA concentrations above 20 ng/mL, which seems to activate the innate immune system to counteract this. In addition, it appears that the expression of HSP is increased to suppress the protein damage that may be caused by excessive ROS. In this study, we present the basic biological results of the immunological response of *A. irradians* induced by phycotoxin DA. However, various phycotoxins in marine ecosystems affects bivalve mollusks grown for human consumption. Therefore, further research is required to examine the long-term or direct effects of algal toxicity on shellfish.

ACKNOWLEDGMENTS

We are grateful to the anonymous reviewers for their thoughtful comments, which substantially improved the research.

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