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Molecular and Physiological Changes of Pacific Oyster, *Crassostrea gigas* Exposed to Environmental Stresses

Pil Gue Jo

Department of Marine Bioscience and Environment

The Graduate School

Korea Maritime University

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Advisor: Prof. Cheol Young Choi

by

Pil Gue Jo

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Approved as to style and content by :

Mi Seon Park, Ph.D.
Chairman

Ki-Young Lee, Ph.D.
Member

Sung Hwoan Cho, Ph.D.
Member

In-Seok Park, Ph.D.
Member

Cheol Young Choi, Ph.D.
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조 필 규

한국해양대학교 대학원 해양생명환경학과

요 약

1. 고염분 스트레스에 노출된 참굴의 HSP90, SOD, CT-R mRNA 발현 및 헤모림프 생리학적 반응

고염분(52.5 psu) 스트레스에 노출된 참굴의 아가미 조직으로부터 전장의 heat shock protein 90 (HSP90) cDNA (GenBank accession no. EF687776)를 클로닝하였다. Superoxide dismutase (SOD)와 calcitonin-related receptor (CT-R)의 degenerate primer는 이미 보고되어 있는 염기배열을 참고로 하여 설계하였다. 참굴의 HSP90, SOD 및 CT-R mRNA의 발현량은 고염분 스트레스에 노출시킨 후, 72, 96, 192시간째까지 각각 유의하게 증가하였으며, 헤모림프의 삼투질 농도와 Na^+, Cl^- 및 Ca^{2+} 이온 농도는 고염분 스트레스에 노출시킨 후 72시간째에, hydrogen peroxide (H_2O_2) 농도는 192시간째에 각각 최대값을 나타내었다. 이러한 결과로 볼 때, 염분 스트레스에 의하여 체내에서 발생된 활성산소로부터 세포의 구조와 기능을 보호하기 위하여 HSP90 mRNA의 발현이 유도되었으며, 발생된 활성산소를 제거하고 세포를 보호하기 위한 일종의 방어기작으로서 SOD mRNA가 발현·작용한 것이라고 판단된다. 또한, CT-R mRNA는 참굴 체내의 Ca^{2+} 이온 농도 조절을 위하여 발현량이 증가한 것이라고 판단된다. 본 연구에서는 고염분 스트레스에 노출된 참굴의 생리학적 반응을 평가하기 위하여 HSP90, SOD, CT-R mRNA의 발현량의 변화뿐만 아니라 헤모림프의 삼투압과 Na^+, Cl^- 및 Ca^{2+} 이온 농도의 변화를 조사하여 그 상관관계를 파악하였다.

2. 수온 스트레스에 노출된 참굴의 CYP450, HSP90, SOD mRNA 발현 및 헤모림프 생리학적 반응

급격한 수온 변화(30℃, 10℃)에 노출된 참굴의 아가미 조직으로부터 전장의 cytochrome P450 (CYP450) cDNA (EF451959)를 클로닝하였다. 참굴을 급격한 수온 변화에 노출시킨 후, 참굴의 아가미 조직에서 CYP450, HSP90 및 SOD mRNA의 발현량을 시간대별로 조사한 결과, 노출 후 6시간째까지 실험에 사용된 모든 유전자의 발현량이 유의하게 증가한 후 감소하는 경향을 나타내었다. 또한, 참굴을 고수온(30℃) 환경에 노출시킨 후 6시간째에 헤모림프의 H_2O_2 농도와 aspartate aminotransferase (AST)의 활성이 최대값을 나타내었으며, 노출 후 24시간째에는 삼투질 농도가 최대값을 나타내었다. 이와 같은 결과로 볼 때, CYP450 mRNA는 참굴이 수온변화에 적응하는 과정 동안 어떠한 역할을 담당하고 있을 가능성을 시사하고 있다. HSP90 mRNA는 참굴 체내의 세포보호 기작의 하나로써 발현되었을 것이며, 급격한 수온변화에 의하여 생성된 산화 스트레스 요인을 제거하기 위하여 SOD mRNA의 발현량이 증가된 것으로 판단된다. 그러나 고수온 스트레스에 노출시킨 후 참굴 아가미 조직의 변화상을 hematoxylin-eosin (H-E) 이중 염색법과 투과전자현미경(transmission electron microscope, TEM)을 사용하여 관찰한 결과, 실험 종료시까지 대조구와 비교하여 어떠한 조직학적 변화도 관찰되지 않았다. 이와 같은 사실은 수온 스트레스로 인하여 체내에서 다량으로 발생된 활성산소의 제거 및 세포를 보호하기 위한 방어기작으로서 CYP450, HSP90 및 SOD mRNA가 발현된 것으로 사료되나, 수온 스트레스 요인만으로는 단백질 변성 등으로 인한 세포의 조직학적 변화에는 크게 영향을 미치지 않는 것으로 판단된다.

3. 카드뮴에 노출된 참굴의 항산화효소(SOD, CAT 및 GPX) mRNA 발현 및 헤모림프 생리학적 반응

카드뮴에 노출된 참굴의 아가미 조직으로부터 전장의 catalase (CAT) cDNA (EF687775)와 glutathione peroxidase (GPX) cDNA (EF692639)를 클로닝하였다. 카드뮴 0.01과 0.05 ppm 농도에 노출시킨 후, 1, 3, 7, 11일간의 처리시간 경과에 따라 SOD, CAT 및 GPX mRNA 발현량의 변화를 조사한 결과, 모든 항산화 유전자(SOD, CAT 및 GPX)의 발현량이 유의적으로 증가하는 것으로 나타났다. 또한, 카드뮴 0.1 ppm에 노출 후, 3일째까지는 실험에 사용된 모든 항산화 유전자의 발현량이 유의적으로 증가하는 경향이 나타났다. 한편, 노출 7일째에 SOD mRNA의 발현량은 증가하였으나, CAT와 GPX mRNA 발현량은 감소하는 경향을 나타내었다. 이와 동시에 헤모림프에서는 AST, alanine aminotransferase (ALT) 활성과 H_2O_2 농도의 증가가 관찰되었다. 참굴 아가미 조직의 변화상을 H-E 이중 염색법과 TEM을 사용하여 관찰한 결과, H-E 이중염색법으로는 실험 종료시까지 대조구와 비교하여 어떠한 조직학적 변화도

관찰되지 않았으나, TEM을 사용하여 관찰한 결과, 카드뮴 0.05 ppm에 노출시킨 후 11일째와 0.1 ppm에 노출시킨 전 실험구의 아가미 조직에서 미세융모의 팽창 등으로 인한 섬모의 손상이 관찰되었다. 그러나 세포의 변형이나 공포의 발생, 세포내의 핵이나 미토콘드리아 등에 영향을 미치는 극심한 세포 손상까지는 진행되지 않은 것으로 판단된다. 이와 같은 결과로 볼 때, 참굴 체내에서는 고농도의 카드뮴 독성에 의하여 발생된 과도한 활성산소(산화 스트레스)를 제거하기 위하여 SOD mRNA가 발현된 것으로 판단된다. 또한, SOD에 의하여 자연적으로 생성된 H_2O_2는 CAT와 GPX에 의한 2차 항산화 시스템에 의한 해독 능력이 상실(저하)됨으로써 참굴 아가미의 섬모의 손상에 영향을 끼친 것이라고 판단된다. 따라서 항산화 유전자인 SOD, CAT 및 GPX mRNA의 발현은 카드뮴 오염에 따른 참굴의 생리적 지표로서 활용될 수 있는 가능성을 시사하고 있다.

4. 저산소 스트레스에 노출된 참굴의 항산화효소 mRNA 발현 및 헤모림프 생리학적 반응

저산소 스트레스에 노출된 참굴의 산소소비량은 개체당 0.3 mg/L이었으며, 밀폐된 호흡실에서 참굴이 용존산소를 소비함에 따라 저산소 환경이 형성되어졌다. 항산화 효소인 SOD mRNA의 발현량은 저산소 스트레스에 노출시킨 후 1시간째까지는 증가한 후 감소하였으며, CAT와 GPX mRNA의 발현량은 저산소 스트레스에 노출시킨 후 지속적으로 감소하는 경향을 나타내었다. 이러한 결과로 볼 때, 저산소 환경에서는 superoxide radical (O_2^-)을 H_2O_2와 O_2로 전환시키는 SOD의 1차적인 항산화 방어체계까지는 정상적으로 작동되어지나, O_2^-에서 전환된 H_2O_2를 O_2와 H_2O로 분해하는 CAT와 GPX에 의한 2차적인 방어체계가 정상적으로 작동되지 않은 것으로 판단된다. 따라서 참굴 체내에서는 유해한 H_2O_2가 완전히 제거되지 못하게 됨으로써 H_2O_2에 의한 독성이 참굴에 악영향을 끼치게 되는 것으로 판단된다. 그러나 저산소 스트레스에 노출된 참굴 아가미 조직의 변화상을 H-E 이중 염색법과 TEM을 사용하여 관찰한 결과, 실험 종료시까지 대조구와 비교하여 어떠한 조직학적 변화도 관찰되지 않았다.

본 연구에서 제시된 다양한 스트레스 환경요인에 따른 참굴의 생리활성도의 변화를 분자생리학적 측면에서 조사해 본 결과, 다양한 스트레스 환경에서 생존이 가능한 최소한의 서식환경에 따른 bio-marker를 도출해 낼 수 있었다. 또한, 이러한 연구 결과는 다양한 환경변화에 노출된 패류종의 생리적 현상을 파악할 수 있는 환경·생리학적 측면의 bio-marker로서 이용할 수 있는 가능성을 제시하고 있다.

Chapter 1

General Introduction

Oysters belong to the family Ostreidae, the order Pterioda and the class Pelecypoda. Three oyster genera contain more than 100 species distributed throughout the world. Species such as Pacific oyster, *Crassostrea gigas*, Japanese oyster, *C. nippona* and flat oyster, *Ostrea denselamellosa*, are farmed extensively along the Korean coastline. Pacific oyster, in particular, is a species of industrial and economical significance, and contributes greatly to the shellfish farming industry (Torigoe, 1981). This oyster, often referred to as the 'milk of the sea', is a favorite food item in countries renowned for the longevity of their citizens. Nutritionally, Pacific oyster is a good source of glycogen, taurine and 20 amino acids, including eight essential amino acids, as well as vitamins A, B1, B2, B12, C, D, E and F, and minerals such as calcium, phosphorus, iodine, germanium, manganese, potassium and selenium. Thus, Pacific oysters contain an ideal balance of nutrients. As a result, this species is very important to Korea both as a substantial protein supply for the nation and as an economic export.

Korea is surrounded by sea on three sides, which are mainly characterized by long and gentle coastlines and clean waters. This makes Korea ideal for oyster farming. In the 1960s, Pacific oyster farming industry was included in a planned economic development project for Korea, with the specific goal of increasing the income of fishermen, and has been undergoing rapid development ever since. In 2003, Pacific oysters accounted for 9.6% of the gross production of marine products and 28.8% of aquaculture production in Korea. Therefore, the relative importance of oysters among farmed marine products is very high. Currently, Pacific oyster agriculture is mainly being conducted in the waters near Tongyeong and Yeosu in the South Sea, which are designated as clean waters by the Food and Drug Administration of the United States. However, the long-term continuous

cultivation of oyster farms, in addition to aging of the farms and industrial wastewater output, are resulting in frequent occurrence of red tides and deterioration of water quality due to environmental pollution. In addition to a rapid reduction in Pacific oyster production per unit area, these conditions are resulting in the occurrence of black oysters, which are characterized by blackened gills and red spots as well as reduced flesh mass. This has resulted in declining oyster quality, as well as delayed growth and maturity, and frequent occurrences of mass mortality as the oysters readily succumb to viruses, bacteria and parasites. Accordingly, concern about the stability and hygiene of oysters as a food item is increasing. In addition, recent industrial development has resulted in the continuous discharge into aquatic environments of various organic and inorganic materials, chemical substances and heavy metals contained in domestic and industrial sewage. Aquatic organisms are then exposed to these contaminants within the aquatic environment, and even very small amounts of harmful heavy metals such as lead, cadmium (Cd) and mercury can cause toxicity in living bodies. These toxins accumulate within the bodies of aquatic organisms and can also cause adverse effects to humans through the food chain (Kobayashi, 1971; Rainbow, 1989).

Pacific oysters, which are sessile benthic animals, have a strong tolerance for environmental change (Shumway, 1977). However, rapid physicochemical changes in environmental factors, such as water temperature and salinity, negatively affect the physiological state of these oysters and eventually lead to mass mortality, as the oysters more easily succumb to viruses and germs. In addition, Cd, among heavy metals, is released into aquatic environments through industrial sources such as mining, refining of ores and plating processes or from natural sources such as rocks and soils (Henkel and Krebs, 2004; Huang et al., 2004), and is known to be absorbed by marine animals. Cd accumulates within the bodies of organisms and alters and degrades processes of enzyme activation (Sastry and Subhadra, 1982). It also causes imbalances and damage to cells, and can ultimately result in cell death (Benavides et al., 2005). Cd also induces oxidization,

generating reactive oxygen species that promote oxidative stress (Stohs et al., 2000).

To date, however, detection and diagnoses of the health or disease infection status of Pacific oysters have been limited to water quality tests and observations of the external qualities of the flesh and shell parts by breeding managers. Therefore, improvements in breeding environments are required to enhance the physiological performance and to accurately assess the health status of cultivated Pacific oysters.

In this study, advanced molecular biological methods were used to address these issues. Although this type of study has not been widely used in Korea, such methods can provide substantial insight into the metabolic characteristics of a living organism. With respect to aquaculture, genetic studies to date have largely targeted endocrinology, such as the induction of growth, maturity and ovulation by use of growth hormones. However, hormone or endocrinology-related studies in shellfish are still in an early stage of development. Studies of Pacific oysters have been limited to environmental and ecological examinations of shell length, shell height, shell weight and body weight in relation to various environmental factors, such as region, season, water temperature, prey, water depth and heavy metals (Park and Choi, 2002). With respect to reproductive physiology, studies have been conducted on the process of spawning, the reproductive cycle and embryo cryopreservation (Choi and Chang, 1999; Chang et al., 2000; Kim et al., 2003). However, very few endocrinological, physiological or biochemical studies have attempted to understand the health and physiological activities of oysters in greater detail, such as examination of stress responses to environmental factors, osmoregulation or physiological activities.

In this study, stress responses and the impacts of stress experienced by Pacific oysters due to environmental factors such as water temperature, salinity, dissolved oxygen and heavy metals were evaluated. Endocrinological, physiological and biochemical markers were used to predict and assess the overall health and physiological activities of Pacific oysters resulting from environmental changes. From these results, a system will be

developed that can be used to diagnose the health of Pacific oyster populations according to changes in marine environments using an established set of markers.

Chapter 2

Characterization of HSP90, SOD and CT-R mRNA expression and changes in physiological hemolymph responses with hyper-osmotic stress in Pacific oyster, *Crassostrea gigas*

1. Introduction

The coastal habitats of *C. gigas* (Thunberg, 1793), are subjected to frequent and rapid changes in salinity. Salinity is highly relevant to the growth, maturation, distribution, food intake, energy activation and metabolic activities of many types of bivalves, including Pacific oyster (Mills, 2000; Navarro et al., 2000). Fluctuations in salinity induce several osmotic responses in bivalves aimed at preserving cellular volume, such as controlling the accumulation of organic compounds called 'osmolytes' (Somero and Bowlus, 1983). In teleosts, osmotic regulation has also been investigated at the cytological level to clarify the molecular mechanisms involved in responses to changes in salinity (Lee et al., 2006; Chang et al., 2007; Choi et al., 2007b), but few studies have addressed the osmotic responses of bivalves.

Bivalve defense mechanisms involve the circulation of blood cells called hemocytes (Cheng, 1981). In *C. gigas*, two types of hemocyte are differentiated by their morphology: hyalinocytes and granulocytes (Gagnaire et al., 2006). Recent studies on the responses of bivalves to salinity changes have described hemocyte activation (Gagnaire et al., 2006) and the release of amino acids from the hemolymph (Hosoi et al., 2003). However, the responses of osmotic pressure and various ions, including Na^{+}, Cl^{-} and Ca^{2+} in the hemolymph of bivalves, to salinity fluctuations remain unknown.

Similarly, little is known about salinity-induced physiological stress responses (and the

expressions of corresponding stress-related genes) of Pacific oyster. Many organisms synthesize heat shock proteins (HSPs) in response to stressors such as changes in salinity, water temperature and heavy-metal content (Beckmann et al., 1990), and simultaneously exhibit numerous physiological responses (Schlesinger et al., 1992). HSPs play an important role in maintaining homeostasis by protecting cellular structures and functions (Sanders, 1993; Forsyth et al., 1997; Iwama et al., 1998, 1999; Ackerman and Iwama, 2001) and preventing cellular degeneration. HSPs may also limit cell damage by inducing proteolysis or restoring cellular structures (Hartl, 1996; Bukau and Horwich, 1998; Morimoto, 1998). HSPs are divided into several groups based on their molecular masses ($\leq$60, 70, 90 and 110 kDa) and functional aspects (Gao et al., 2007). HSP90 is an abundant member of the 90-kDa HSP family and comprises 1-2% of all cellular proteins. HSP90 occurs in small quantities in normal cells that are not exposed to stress, and binds to protein kinases, steroid receptors, actin, tubulin and other substances in the cells to maintain proteins and deliver signals among cells (Pratt, 1997; Csermely et al., 1998).

In vertebrates, calcitonin is a key hormone involved in the regulation of calcium metabolism and 32 amino acid polypeptides secreted by thyroid gland C cells. It inhibits calcium loss from bones and promotes calcium excretion from the kidney, subsequently lowering calcium in the blood (Shen et al., 2007). Calcitonin also plays a major role in calcium homeostasis by inhibiting osteoclast-mediated bone resorption and stimulating urinary calcium excretion (Purdue et al., 2002). Synthetic salmon calcitonin has a potent effect on the human body and is used for treatment of human bone diseases. Calcitonin also plays an important role in the regulation of internal secretions for bivalve growth by regulating the development of both shells and tissues (Dubos et al., 2003). Shell formation involves ion transport and the secretion of a protein matrix by mantle cells, and the subsequent nucleation of calcium carbonate crystals that grow in association with the organic matrix (Wilburg and Saleuddin, 1983). Calcitonin has been identified to date in five fish species - tiger puffer, *Takifugu rubripes* (NM001105219), Japanese medaka,

Oryzias latipes (NM001104924), pink salmon, *Oncorhynchus gorbuscha* (X78080), zebrafish, *Danio rerio* (BC076343) and bastard halibut, *Paralichthys olivaceus* (AB052782). In addition, the calcitonin-related receptor (CT-R) has been cloned fromseveral aquatic animals, such as the fishes *O. latipes* (BR000372), *O. gorbuscha* (AJ508554), *D. rerio* (XM690423), *P. olivaceus* (AB035315), mefugu, *Takifug obscurus* (Abe, 1949, AB219840), *T. rubripes* (NM001105219), three-spined stickleback, *Gasterosteus aculeatus* (BR000371), spotted green pufferfish, *Tetraodon nigroviridis* (BR000370), the ascidian, *Ciona intestinalis* (AB081313), the sea urchin, *Strongylocentrotus purpuratus* (XM781434) and the bivalve *C. gigas* (AJ551182). However, its function has not yet been thoroughly studied. To examine a possible role for CT-R in physiological changes in *C. gigas* exposed to high salinity, osmotic pressure, Na^+, Cl^- and Ca^{2+} ion exchange from the hemolymph, and the expression patterns of CT-R mRNA were investigated in this study.

Organisms exposed to stress generate reactive oxygen species (ROS), such as superoxide radical (O^2), hydrogen peroxide (H_2O_2), hydroxyl radical (HO) and singlet oxygen ($^1O_2$). ROS are formed by many kinds of stresses, including heavy metal toxins and other abiotic and biotic environmental factors (Stohs et al., 2000). ROS, which have strong chemical oxidation and binding activity, attack the membranes of cells and organs and damage cell functions (Ferraris et al., 2002). ROS-induced oxidative stress leads to lipid peroxidation, protein denaturation and DNA damage. It also changes and inhibits the activities of a variety of enzymes and causes cell damage and an imbalance in cells, resulting in apoptosis (Choi et al., 2007b). This in turn may lead to numerous physiological problems that promote aging, reduce disease resistance and lower reproductive ability (Kim and Phyllis, 1998). The antioxidant enzyme superoxide dismutase (SOD) is generated inside the body to prevent such damage (Wendel and Feuerstein, 1981). An important metalloenzyme, SOD exists in all aerobic organisms. Through dismutation into oxygen and hydrogen peroxide ($2O_2 + H^+ \rightarrow H_2O_2 + O_2$), SOD removes superoxide

radicals (Fridovich, 1975). However, hydrogen peroxide (H_2O_2), a product of this reaction, is also a toxic ROS that has harmful effects on the tissues of marine animals.

In this study, osmoregulation and stress responses of Pacific oysters were investigated by comparing the mRNA expressions of HSP90, SOD and CT-R in the gills of oysters at various salinities. Osmolality and related physiological responses were measured, including changes in Na^+, Cl^- and Ca^{2+} concentrations and H_2O_2 concentrations, and further observations were conducted using hematoxylin-eosin (H-E) stain method and transmission electron microscopy (TEM).

2. Materials and Methods

2.1. Experimental oysters

One-year-old Pacific oysters (average shell length 112±10.7 mm, shell height 31.1±5.4 mm, weight: 20.3±3.9 g), obtained from the oyster hatchery on Dae-bu Island, in Goseong (Gyeongnam, Korea), were and placed them into 100 L circulation filter tanks in the laboratory, at 50 oysters per tank. During the experimental period, water temperature was kept at 18±0.5℃ and 35 psu, and the photoperiod was 12 h light/12 h dark.

2.2. Salinity treatment

After the oysters acclimated to seawater of normal salinity (35 psu) for 48 h, the experimental group was subjected to a salinity of either 52.5 psu (hypersalinity; 150% seawater salinity). Samples were collected from five oysters 0, 12, 24, 48, 72, 96 and 192 h later. Daily water exchanged and no food was supplied during the experiments.

2.3. Rapid amplification of cDNA 5′/3′ ends (RACE)

For RACE reactions, total RNA was extracted using a Trizol (Gibco/BRL, Grand Island, NY, USA) from the gill of Pacific oysters. Reverse transcription was performed using M-MLV reverse transcriptase (Bioneer, Daejeon, Korea). Using 3 μg of total RNA as the template, 5′-RACE-ready complementary DNA (cDNA) and 3′-RACE-ready cDNA were generated using the protocols and reagents provided in the CapFishing Full-length cDNA Premix kit (Seegene, Seoul, Korea). Gene-specific primers were selected from HSP90 partial cDNA (GenBank accession no. CB617443). For 3′-RACE, the 50 μL PCR reaction mixture contained 5 μL of 3′-RACE-ready cDNA, 1 μL of 10 μM 3′ target primer (5′-CTG TGA ATG CTG CGA CTA

CGA T-3′), 1 μL of 10 μM 3′ RACE gene-specific primer of HSP90 (5′-TCC ACA ACA ACT CTG TCT GCA ACC AAG-3′) and 25 μL of SeeAmp Taq Plus Master Mix (Seegene). Polymerase chain reaction (PCR) was carried out for 40 cycles at 94℃ for 45 s for denaturation, 62℃ for 45 s for primer annealing, and 72℃ for 90 s for extension, followed by 5 min at 72℃ for extension. The PCR product was amplified, cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA), and sequenced. For 5′-RACE, the 50 μL PCR reaction mixture contained 5′ -RACE-ready cDNA, 5′ gene-specific primer of HSP90 (5′-CAG GCT GAG ATT GCT CAG TTG ATG AGC-3′), 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), and SeeAmp Taq Plus Master Mix, as for 3′-RACE, under the same PCR conditions. The PCR product was amplified, cloned into pGEM-T Easy Vector, and sequenced. The DNA and deduced amino acid sequences were analyzed using GENETYX-WIN (Software Development, Tokyo, Japan).

2.4. Phylogenetic analysis

Phylogenetic analysis was performed on the amino acid sequences from full-length HSP90 cDNA of *C. gigas*. Amino acid sequence data were aligned using BioEdit Software (Hall, 1999). The amino acid sequence for homology was the following: Zhikong scallop, *Chlamys farreri* (AAR11781); abalone, *Haliotis tuberculata* (AM283515); fruit fly, *Drosophila melanogaster* (CAA27435); mosquito, *Anopheles albimanus* (AAB05639); domestic silkworm, *Bombyx mori* (BAB41209); *D. rerio* (NP 571403, HSP90α O57521, HSP90β); chicken, *Gallus gallus* (P11501, HSP90; CAA49704, HSP90β); human, *Homo sapiens* (NP 005339, HSP90α NP 031381, HSP90β); rat, *Rattus norvegicus* (NP 786937, HSP90α P34058, HSP90β); bovine, *Bos taurus* (NP 001012688, HSP90α BAC82488, HSP90β); barley, *Hordeum vulgare* (AAP87284); rubber tree, *Hevea brasiliensis* (AAQ08597); thale cress, *Arabidopsis thaliana* (BAA00615) and *C. gigas* (EF687776). A phylogenetic

tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) and analyzed using the Mega 3.1 software package (Center for Evolutionary Functional Genomics, Scottsdale, AZ). The degree of support for internal branches was inferred using bootstrapping analysis (1,000 replicates).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was conducted to determine the relative expression of HSP90, SOD, CT-R and 28S ribosomal RNA (28Sr) mRNA in *C. gigas* tissues. To optimize the cycle number used for RT-PCR analysis, 2.5 μg of total RNA extracted from the gonad, gill, digestive gland, intestine and mantle of hypersalinity-treated oysters were using the Trizol method according to the manufacturer's instructions (Gibco/BRL, USA). The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. 3 ug of total RNA was reverse transcripted with M-MLV reverse transcriptase (Bioneer) and oligo-d$(T)_{15}$ primer (Promaga). Specific primer for RT-PCR was designed from the published sequence of HSP90 (EF687776), SOD (AJ496219), CT-R (AJ551182) and 28Sr (Z29546) as follows: HSP90 forward primer (5´-ATG CAG ACG CTT GTG TCT TG-3´) and HSP90 reverse primer (5´-TCT GTC TGC AAC CAA GTA GG-3´), SOD forward primer (5´-GAC CCC ATC CTG TTC CCC AGC-3´) and SOD reverse primer (5´-AGA AGG CGA TCT GTT CCA CCT C-3´), CT-R forward primer (5´-CTG AAC GCT GTT GCC AGA GA-3´) and CT-R reverse primer (5´-TCG AAC ACG GTC GTA CTG GT G-3´), 28Sr forward primer (5´-TGC TCT GGA CTG TCC TAG GA-3´) and 28Sr reverse primer (5´ -ACC GAT TCG CCA CTG ACC AT-3´). PCR amplification was conducted using a 2X Taq Premix 1 (Solgent, Korea) according to the manufacturer's instructions. PCR was carried out as follows: one cycle of denaturation at 94℃ for 5 min, denaturation at 94℃ or 30 s, annealing at 55℃ for 30 s, a total of 30 (28Sr, Hsp90 and SOD) or 32 (CT-R) cycles for 1 min at 72℃ for 30 s, followed by one

cycle of 5 min at 72℃ for the final extension. The 28Sr mRNA was amplified in each PCR reaction as a loading control. The PCR products from different cycles of amplification were visualized on a UV transilluminator after electrophoresis on a 1% agarose gel containing ethidium bromide (0.5 $\mu g/\mu \ell$), and the signal intensity was quantified with the Gel-Doc System and Gelpro 3.1 software (KBT, Incheon, Korea).

2.6. Quantitative real-time PCR (QPCR)

QPCR was conducted to determine the relative mRNA expression of HSP90, SOD, CT-R and 28Sr using total RNA extracted from gills of control and hypersalinity-treated oysters. With 2.5 μg of total RNA as a template, cDNA were synthesized using M-MLV reverse transcriptase (Bioneer). First-strand cDNA synthesis was conducted using oligo-d$(T)_{15}$ primer (Promega). Primers for QPCR were designed with reference to known gene sequences of Pacific oyster as follows: HSP90 forward primer (5´-GGT GAA TGT TAC CAA GGA AGG-3´), HSP90 reverse primer (5´-GTT ACG ATA CAG CAA GGA GAT G-3´), SOD forward primer (5´-ATG TCA TCT GCT CTG AAG GC-3´), SOD reverse primer (5´-TGG TGA TAC CGA TCA CTC CA-3´), CT-R forward primer (5´-GAC CGA CCA ACA AAC GCT TTC-3´), CT-R reverse primer (5´-GTT GTG TAG AAG GCT GCC ATT G-3´), 28Sr forward primer (5´-AAA CAC GGA CCA AGG AGT CT-3´) and 28Sr reverse primer (5´-AGG CTG CCT TCA CTT TCA TT-3´). QPCR amplification was conducted using a Bio-Rad MiniOptico^{TM} System (Bio-Rad, CA, USA) and iQ^{TM} SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. QPCR was carried out by denaturation at 95℃ for 5 min, followed by 40 cycles of denaturation at 95℃ for 20 s and annealing at 55℃ for 20 s. All data were based on the calculated threshold cycle time (Ct). To ensure that the primers amplified a specific product, It was performed a melt curve, as well as

analyzed the PCR product size using capillary electrophoresis (Agilent Technologies, Santa Clara, CA). All primers used were shown to amplify only one size of template, melting at only one temperature. PCR products were also confirmed by sequencing. All analyses were based on the calculated threshold Ct values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR program, QPCR data from five replicate samples were analyzed with analysis software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. mRNA expression levels stood for an *n*-fold difference relative to 28Sr as the internal control.

2.7. Hemolymph osmolality and ion concentrations

Hemolymph was drawn from the pericardial cavity with a 3 mL syringe, then separated by centrifugation (4℃, 10,000 ×*g*, 5 min) and stored at 80℃ until analysis. Hemolymph osmolality was measured with the Vapor Pressure Osmometer (Vapro 5520; Wescor, Logan, UT, USA), Na^+ and Cl^- concentrations were determined with the Biochemistry Autoanalyzer (model 7180; Hitach Corp., Tokyo, Japan) and Ca^{2+} concentrations were assessed with the AVL 9180 (AVL, Roswell, GA, USA).

2.8. Hydrogen peroxide assays

H_2O_2 concentrations were measured using the modified methods of Nouroozzadeh et al. (1994) and a Peroxidetect kit (Sigma-Aldrich, St. Louis, Missouri, USA). 20 ㎕ of whole oyster hemolymph in marine anticoagulant (MAC: 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 50 mM EDTA, 0.45 M sodium chloride, pH 7.5) was added per well to flat bottom 96 well microtitre plates. Plates were left at room temperature for 20 min to allow hemocytes to settle and adhere. A working color reagent was prepared by mixing 100 mL distilled water containing 100 mM sorbitol and 125 uM xylenol orange (Sigma-Aldrich) with 1 mL of 25

mM ferrous ammonium sulphate prepared in 2.5 M sulphuric acid (Sigma-Aldrich). Two hundred microlitres of this reagent was then added to each well and allowed to incubate at room temperature for 1 h. Absorbance was read at 560 nm and concentrations of H_2O_2 were interpolated from a standard curve. Concentrations are expressed as nM/mL.

2.9. Histological analysis

2.9.1. Observation with light microscope

To observe histological changes in the gill tissues of Pacific oyster exposed to hypersalinity, the extracted gill tissues were fixed in 10% neutral formalin solution (100 mL formalin, 6.5 g $Na_2HPO_412H_2O$, 4.5 g KH_2PO_4, 900 mL distilled water) for 1 day. Then, the gill tissues were fixed in bouin solution and a tissue sample was produced according to the series of methods. The fixed sample was cut into serial sections of 5 ㎛ in thickness by paraffin sectioning. Then, the degree of tissue damage was examined with an light microscope after double dyeing with Harris's hematoxylin and 0.5% eosin (Sigma, USA). The degree of damage in gill tissues was photographed by using image analysis system (Axiovision, Zeiss Co., Germany) with a biological microscope (Axioskop, Zeiss, Germany).

2.9.2. Observation with electron microscope

A sample for TEM was produced in order to observe the fine structure of the gill tissues of Pacific oyster exposed to hypersalinity. After extracting gill tissues, primary fixation was carried out for 2 h in 2.5% glutaraldehyde solution (4℃) buffed with 0.1 M phosphate buffer solution (PBS, pH 7.2). The sample completed of fixation was washed for 10 min with PBS and was processed through secondary fixation for 2 h at 4℃ in 1% osmium tetroxide (OsO_4). After fixation was completed, the sample was washed with PBS and was dehydrated for 15 min each in 50-100% ethanol. After dehydration, the sample was placed

in the mixture of propylene oxide and Epon and was polymerized for 1-3 h. Then the sample was embedded in Epon 812. The embedded tissue was semi-thinly sectioned with the thickness of 1.0 ㎛ by using ultramicrotome (Leica, Reichert SuperNova, Sweden). Then, it was dyed with 1% toluidine blue and the part for observation was determined. After the part for observation was determined, the sample was again sectioned with the thickness of 70 mm and was attached to 200 mesh copper grid. The sample completed of ultra-thin sectioning was double-dyed with uranylacetate and lead citrate solution and was observed with a transmission electron microscope (JEM 1200 EX-Ⅱ, 60-80 kv, JEOL, Tokyo, Japan).

2.10. Statistical analysis

Significant differences among the data from each experiment were obtained using a one-way analysis of variance (ANOVA Tukey's test or LSD test) and the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA). The significance level was $P < 0.05$.

3. Results

3.1. Identification of HSP90 cDNA

A PCR-based cloning strategy (RT-PCR followed by 3′ and 5′ RACE) was used to clone cDNA encoding a putative HSP90 from the gill of *C. gigas*. HSP90 full-length cDNA contained 2154 nucleotides, including an open reading frame (ORF), and was predicted to encode a protein of 717 amino acids. The sequence contained the HSP90 protein family signature (NKEIFLRELISN[A/C/S]SDALDKIR, LGTIA[K/R]SGT, IGQFGVGFYSAYLVA[E/D], IKLYVRRVFI, GVVDSEDLPLNISR E) and had the consensus sequence MEEVD at the C terminus (Fig. 1). The cDNA sequence of Pacific oyster HSP90 gene was deposited in national center for biotechnology information (NCBI) GenBank under accession number EF687776. The deduced amino acid sequence of Pacific oyster HSP90 was compared to those deduced from known HSP90 cDNA of other mollusk species (Fig. 1). Pacific oyster HSP90 had an 85 and 84% amino acid sequence similarity with *C. farreri* (AY362761) and *H. asinina* (EF621884), respectively (Fig. 1).

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cgHSP90   1:MPEPE-H-MEEGEVETFAFQAEIAQLMSLIINTFYSNKEIFLRELISNASDALDKIRYES  58
cfHSP90   1:MPEPEGQAMEDGEVETFAFQAGIAQLMSLIINTFYSNKEIFLRELISNCSDALDKIRYES  60
haHSP90   1:MPEPQEAQMDEGEVETFAFQAEIAQLMSLIINTFYSNKEIFLRELISNSSDALDKIRYES  60
            ****    *  ********** ************************* ***********

cgHSP90  59:LTDPSKLDSGKDLEIRIVPDKESKTLTIMDTGIGMTKADLVNNLGTIAKSGTKAFMEALQ 118
cfHSP90  61:LTDPSKLDSGKELEIKIVPNKDDNTLSIMDTGIGMTKADLVNNLGTIARSGTKAFMEALQ 120
haHSP90  61:LTDPSKLDASKDLQIRIVPDKESKTLIIEDSGIGMTKADLVNNLGTIAKSGTKAFMEALQ 120
            ********  * * * *** *   ** * * ***************** ***********

cgHSP90 119:AGADISMIGQFGVGFYSAYLVADRVVVETKHNDDEQYIWESSAGGSFTVKTCSENTIGRG 178
cfHSP90 121:AGADISMIGQFGVGFYSAYLVADRVVVETKNNDDEHYIWESSAGGSFTVRS-GDGSFILG 179
haHSP90 121:AGADISMIGQFGVGFYSAYLVAERVVVESKHNDDEQYIWESSAGGSFTIRSSNDPTLPRG 180
            ********************** ***** * **** ************          *

cgHSP90 179:TKITLFLKEDQTEYLEERRIKEVVKKHSQFIGYPIKLLVEKERDKEVSDDEEEEEKKEED 238
cfHSP90 180:TRITLHMKEDQAEYLEEKKVKEIVKKHSQFIGYPIKLQVEKERDVEVSDDEEEEEKKEED 239
haHSP90 181:TRITLYMKEDQAEYLEERRIKEIVKKHSQFIGYPIKLMVEKERDKEVSDDEEDEKKEDEE 240
            * ***  **** *****   ** ************** ****** ******* * *  *

cgHSP90 239:KAEEK-E-EDKPKVEDLDEDEEDDSKSKD-KKKKKIKEKYTEDEELNKTKPIWTRNPDDI 295
cfHSP90 240:KDAEKSE-DDKPKVEDLDDEDDDEDKSKD-KKKKKIKGKYIEDEELNKTKPIWTRNPDDI 297
haHSP90 241:KKEDEEENEDKPKVEDLD-EDEDEDKSKDKKKKKKIKEKYTEDEELNKTKPLWTRNADDI 299
            *     *  *********    *  **** ******* ** ********** **** ***

cgHSP90 296:TQEEYGEFYKSLTNDWE-----RPFGCEGQLEFRALLFIPRRAPLDLFENKKKKNNIKLY 350
cfHSP90 298:TQEEYGEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFVPRRAPFDLFENKKKKNNIKLY 357
haHSP90 300:TQEEYAEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFLPKRAPFDMFENKKKKNNIKLY 359
            ***** ***********       *  *********** * *** * **************

cgHSP90 351:VRRVFIMDNCEELIPEYLNFARGVVDSEDLPLNISREMLQQSKILKVIRKNLVKKCIELI 410
cfHSP90 358:VRRVFIMDNCNEVIPEYLNFVRGVVDSEDLPLNISREMLQQSKILKVIRKNLVKKCMELF 417
haHSP90 360:VRRVFIMDNCEDLIPEYLNFVRGVVDSEDLPLNISREMLQQSKILKVIRKNLVKKCMELF 419
            **********   ******* ********************************** **

cgHSP90 411:EDLTEDKDNYKKFYEQFAKNLKLGIHEDSTNRKKLADFLRYYSSQSGDEMTSLKDYVSRM 470
cfHSP90 418:DDIAEDKENYKKFYEQFAKNLKLGIHEDTTNRKKIADFLRYHTSQSGDEMTSFKEYVSRM 477
haHSP90 420:EDLTEDKDNFKKFYEQFSKNLKLGIHEDSTNRKKLSELLRYYTSQSGDEMTSLKDYVSRM 479
             *  *** * ******* ********** *****    ***  ********* * *****

cgHSP90 471:KENQKSIYYITGESREVVQSSAFVERVKKRGMEVIYMVDPIDEYAVQQLKEYDGKPLVNV 530
cfHSP90 478:KENQKSIYYITGESREVVQSSAFVENVKKRGIEVIYMVDPIDEYAVQQLKEYEGKTLVSV 537
haHSP90 480:KENQKSIYYITGESRDSVQNSAFVERVKKRGFEVVYMTDPIDEYCVQQLKEYDGKTLVCV 539
      ***************  ** ***** ***** ** ** ****** ******* ** ** *


cgHSP90 531:TKEGLELPEDEEERKRFEEAEAEYEGLCKVMKDILDKKVEKVVVSNRLVTSPCCIVTSQY 590
cfHSP90 538:TKEGLELPEDEEEKKRFEEATAEYEGLCKVVKEILDKKVEKVTVSNRLVTSPCCIVTSQY 597
haHSP90 540:TKEGLELPEDEEEKKKLEEAKAQFEGLCKVMKEILDKKVEKVVVSNRLVTSPCCIVTSQY 599
            ************* *  *** *  ****** * ********* *****************

cgHSP90 591:GWSANMERIMKAQALRDSSTMGYMAAKKHLEINPDHSIIKSLKDKAEADKNDKSVKDLVM 650
cfHSP90 598:GWSANMERIMKAQALRDSSTMGCMAAKKHLEINPDHAIIKSLKEKAGLDKNDKSVKDLVL 657
haHSP90 600:GWSANMERIMKAQALRDTSTMGYMAAKKHLEINPDHPIVKTLKEKADADKNDKAVKDLCM 659
            ***************** **** ************* * * ** **  ***** ****

cgHSP90 651:LLFETSLLASGFSLEEPGTHASRIHRMIKLGLGIDEDE--TPETQEPVTEDMPPLEGDED 708
cfHSP90 658:LLFETSMLASGFSLEEPGTHANRIHRMIKLGLGIDDDDSGAPETSDENVEEPPPLEGDED 717
haHSP90 660:LLFETSLLASGFSLEDPTSHANRIHRMIKLGLGIDEDDIPAESATESGTDEMPPLEGDED 719
            ****** ******** *  ** ************* *               ********
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cgHSP90 709:DASRMEEVD                                                  717
cfHSP90 718:DASRMEEVD                                                  726
haHSP90 720:DASRMEEVD                                                  728
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Fig. 1. Multiple alignment of the HSP90 amino acids of Pacific oyster (*Crassostrea gigas*, HSP90, GenBank accession no. EF687776), Zhikong scallop (*Chlamys farreri*, HSP90, AY362761), and abalone (*Haliotis asinina*, HSP90, EF621884). Shaded regions indicate the five HSP90 family signature sequences and the consensus sequence MEEVD located at the C terminus. Identical amino acids are indicated by asterisks.

3.2. Phylogenetic analysis

The phylogenetic tree for the analysis of *C. gigas* HSP90 amino acid is shown in Fig. 2. HSP90 was categorized into five subgroups according to phylogenetic similarities: Group 1 (human, HSP90α; β, rat, cow, chicken and zebrafish, HSP90 α), Group 2 (rat, cow and chicken, HSP90α), Group 3 (domestic silkworm, mosquito and fruit fly, HSP90), Group 4 (abalone, Zhikong scallop and Pacific oyster, HSP90α), and Group 5 (barley, thale cress and rubber tree, HSP90). In shellfish, HSP90 can be largely divided into type α and β. The oysters used in this study were phylogenetically most similar to Zhikong scallop, a member of the Filibranchia, as is Pacific oyster. The second greatest similarity was with abalone. I also found that types α and β of HSP90 were phylogenetically similar (Fig. 2).

3.3. Tissue distribution of HSP90, SOD and CT-R mRNA by RT-PCR

Investigated HSP90, SOD and CT-R mRNA expression in various tissues that had been exposed to hypersalinity (52.5 psu) for HSP90 at 96 h, SOD at 192 h and CT-R at 72 h, respectively. Expression of HSP90, SOD and CT-R mRNA was higher in the gill than the gonad, mantle and intestine (Fig. 3).

3.4. Levels of HSP90, SOD and CT-R transcripts

HSP90, SOD and CT-R mRNA expression over time in the gill tissues of oysters exposed to the stress of hypersalinity, HSP90 mRNA expression significantly increased for 96 h (5.1 times), SOD mRNA for 192 h (3.1 times) and CT-R mRNA for 72 h (4.8 time), respectively, at 52.5 psu, and then decreased (Fig. 4).

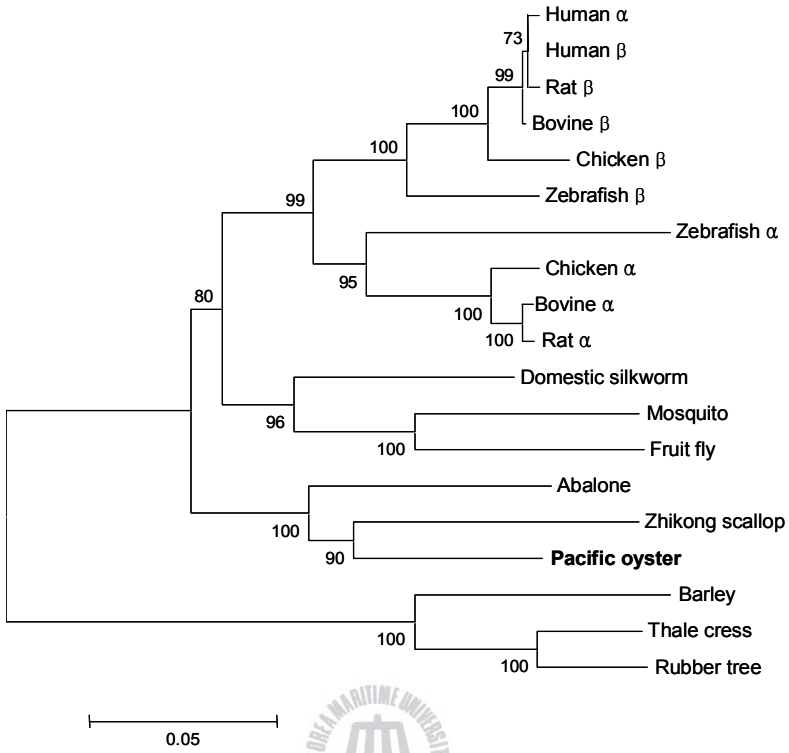

Fig. 2. Phylogenetic tree based on amino acid alignment for 90-kDa heat shock protein in animals. Bootstrap values (%) are indicated (1,000 replicates). The score between two protein sequences, which is a measure of the relative phylogenetic relationship between the two proteins, is represented by horizontal distance in the tree (i.e., the shorter the distance, the more closely the proteins are related). Sequences are Pacific oyster, *Crassostrea gigas* (EF687776), Zhikong scallop, *Chlamys farreri* (AAR11781), abalone, *Haliotis tuberculata* (AM283515), fruit fly, *Drosophila melanogaster* (CAA27435), mosquito, *Anopheles albimanus* (AAB05639), domestic silkworm, *Bombyx mori* (BAB41209), zebrafish, *Danio rerio* (NP 571403, HSP90α O57521, HSP90β), chicken, *Gallus gallus* (P11501, HSP90; CAA49704, HSP90β), human, *Homo sapiens* (NP 005339, HSP90α NP 031381, HSP90β), rat, *Rattus norvegicus* (NP 786937, HSP90α P34058, HSP90β), Bovine, *Bos taurus* (NP 001012688, HSP90α BAC82488, HSP90β), barley, *Hordeum vulgare* (AAP87284) rubber tree, *Hevea brasiliensis* (AAQ08597) and thale cress, *Arabidopsis thaliana* (BAA00615).

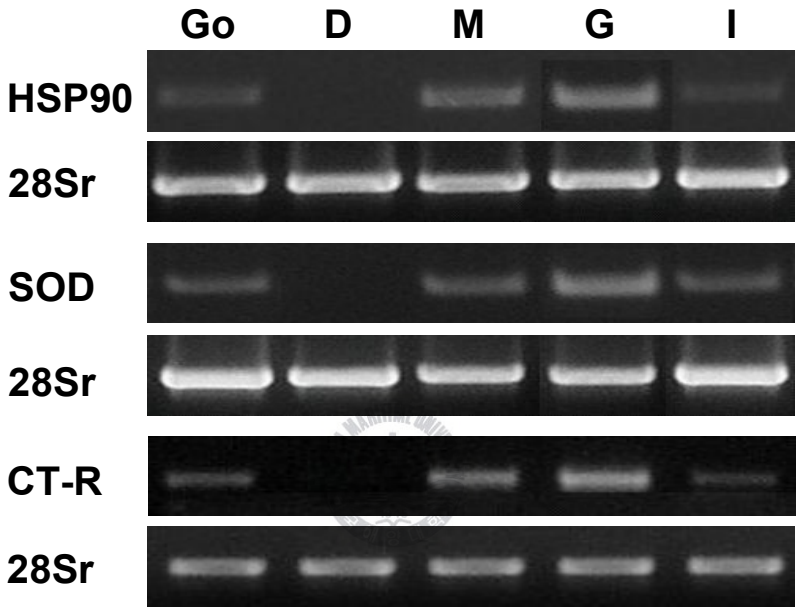

Fig. 3. Tissue-specific expression of 90-kDa heat shock protein (HSP90), superoxide dismutase (SOD) and calcitonin-regulated receptor (CT-R) mRNA in various tissues of Pacific oyster, *Crassostrea gigas* exposed to hypersalinity (52.5 psu) (HSP90 at 96 h, SOD at 192 h and CT-R at 72 h) by reverse transcription polymerase chain reaction. Amplification of 28S ribosomal RNA was used as an internal control. Go: gonad; D: digestive gland; M: mantle; G: gill; I: intestine.

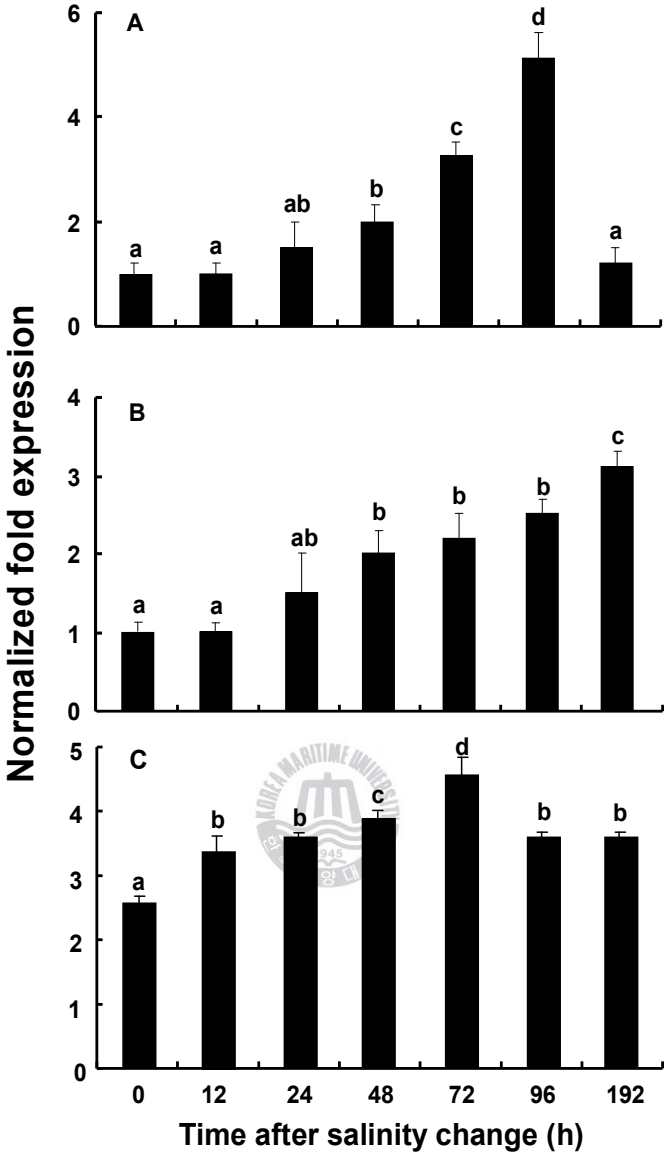

Fig. 4. Time-related effect on 90-kDa heat shock protein (HSP90), superoxide dismutase (SOD) and calcitonin-regulated receptor (CT-R) mRNA levels in Pacific oyster, *Crassostrea gigas* gill during hypersalinity (from 35 psu to 52.5 psu; A: HSP90, B: SOD, C: CT-R) adaptation as determined by quantitative polymerase chain reaction. Results are expressed as fold change with respect to 28Sr levels for the same sample. Values with dissimilar letters are significantly different (P<0.05) from one another. Values are means±SD (n = 5).

3.5. Hemolymph osmolality and ion concentrations

The highest hemolymph osmolality of hypersalinity-treated oysters was 1429.7±5.9 mOsm/kg, which occurred 48 and 72 h after exposure. Thereafter, osmolality significantly decreased and eventually stabilized at 96 and 192 h (Fig. 5). The concentrations of Na^{+}, Cl^{-} and Ca^{2+} ions in the hemolymph of hypersalinity-treated oysters began to significantly increase 12 h after exposure they peaked at 72 h (657.7±5.7, 715±3.1 and 172.3±2.1 mmol/L, respectively), and then gradually decreased (Fig. 6). Controls have no significant difference in experimental periods (P<0.05).

3.6. Hydrogen peroxide assays

H_2O_2 concentrations significantly increased until 192 h as 3.6±0.1 nM/mL from 2.4±0.2 nM/mL at the beginning of the trial in the hemolymph of oysters exposed to hypersalinity (Fig. 7). Controls have no significant difference in experimental periods (P<0.05).

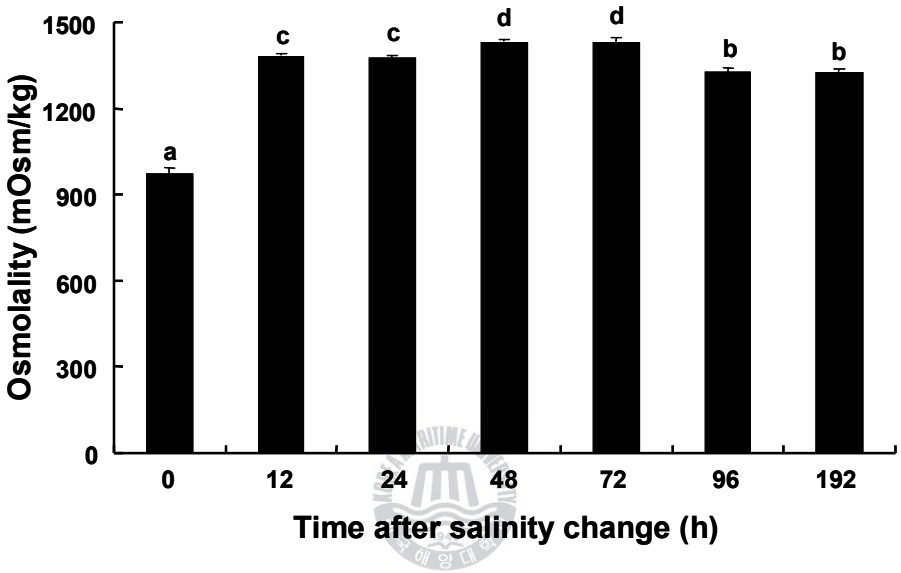

Fig. 5. Hemolymph osmolality during adaptation to hypersalinity conditions of 52.5 psu. Hemolymph was extracted from the oyster and used to measure osmolality. Values with different letters are significantly different (P<0.05) from each other. Values are means±SD (n = 5).

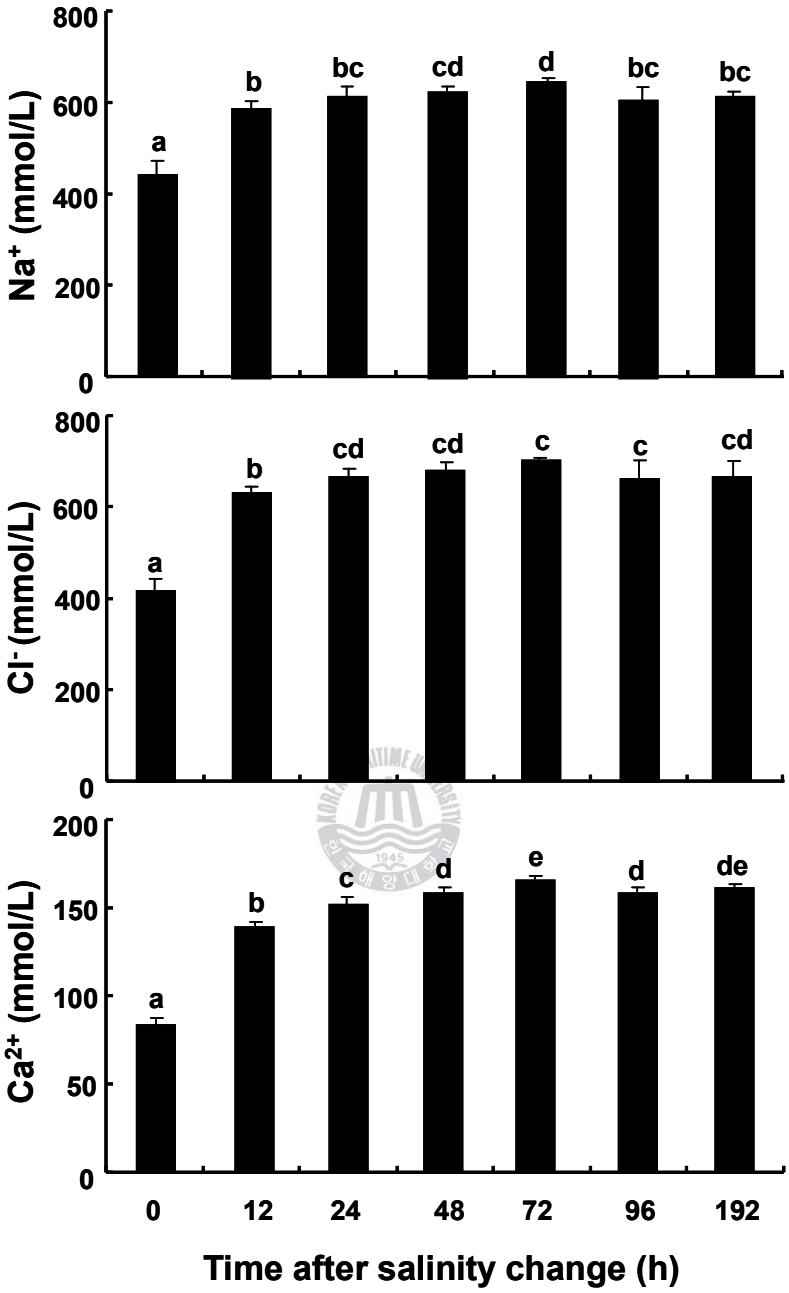

Fig. 6. Changes in hemolymph Na^+, Cl^- and Ca^{2+} concentrations in response to hypersalinity conditions (52.5 psu) for 192 h. Hemolymph was extracted from Pacific oysters and used to measure Na^+, Cl^- and Ca^{2+} concentrations. Values with different letters are significantly different (P<0.05) from each other. Values are means±SD (n = 5).

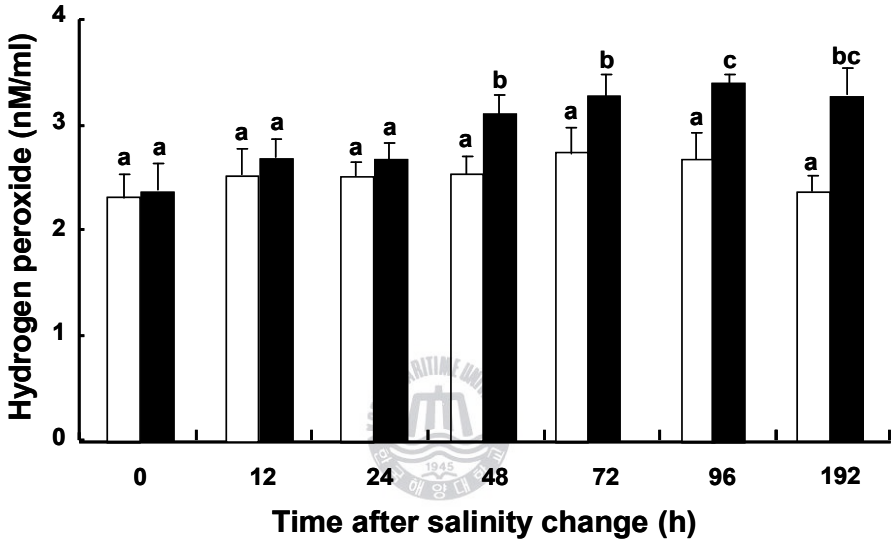

Fig. 7. Hydrogen peroxide (H_2O_2) concentrations in hemolymph of Pacific oyster, *Crassostrea gigas* during hypersalinity from 35 psu (□) to 52.5 psu (■) adaptation. Different lowercase letters indicate significant differences ($P < 0.05$) from one another. Values are means±SD (n = 5).

3.7. Histological analysis

3.7.1. Observation with light microscope

The external shape of the gill tissues in Pacific oyster exposed to hypersalinity was observed with H-E stain method and the result indicated no histological change in the control group and in the gill tissues at the 96 h of exposure to hypersalinity (Fig. 8). Also, by 192 h, the experiment completion, no histological change was observed in the gill tissues of Pacific oyster.

3.7.2. Observation with electron microscope

The internal shape of the gill tissues in Pacific oyster exposed to hypersalinity was observed with TEM and the result indicated no histological change in the control group and in the gill tissues at 96 h after exposure to hypersalinity (Fig. 9). Also, by 192 h, the experiment completion, no histological change was observed in the gill tissues of Pacific oysters.

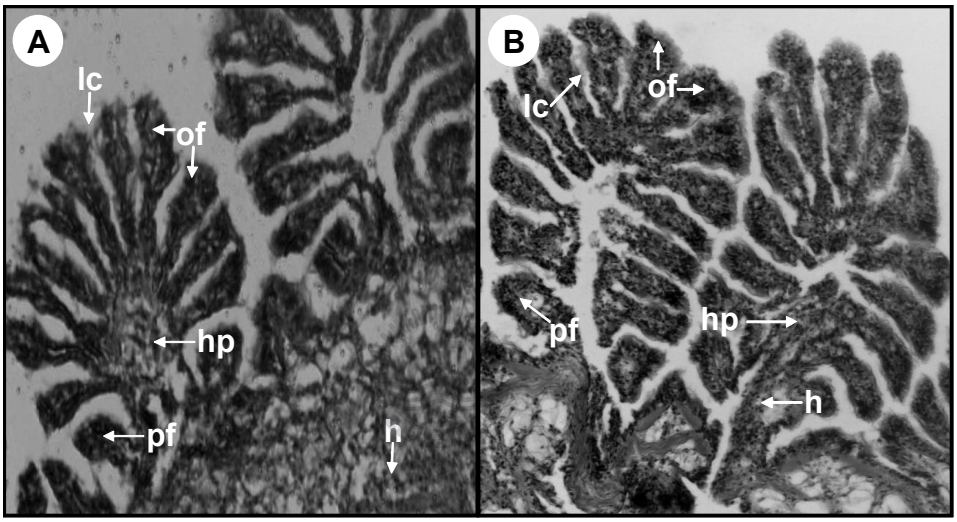

Fig. 8. Photomicrographs of gill filaments and lamellae of Pacific oyster exposed to hypersalinity (52.5 psu). A: control, B: 96 h in hypersalinity, of: ordinary filament, pf: principal filament, lc: lateral cilia, h: hemocytes, hp: hemolymph sinus of plica. X 200.

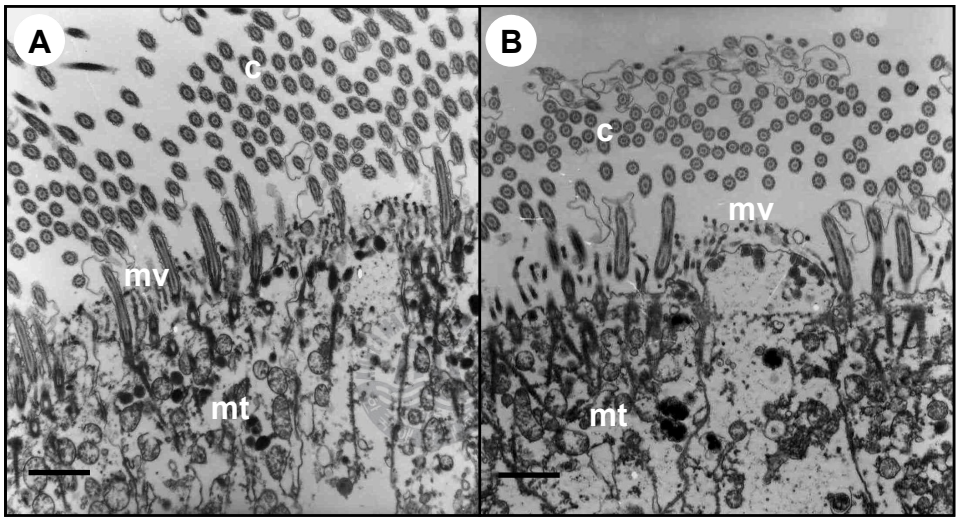

Fig. 9. Electron micrographs of Pacific oyster's gill exposed to hypersalinity (52.5 psu). A: control, B: 96 h in hypersalinity, c: cilia, mv: microvilli, mt: mitochondria. Bars: 1 ㎛. X 5000.

4. Discussion

Pacific oyster is a euryhaline bivalve that is relatively resistant to environmental stresses, such as variations in salinity (Gagnaire et al., 2006). However, localized torrential downpours, for example, may rapidly change salinity, resulting in acute changes in osmotic pressure that disrupt homeostasis. This results in changes in moisture content and intracellular concentrations of nutrients and salts, and in cellular osmotic pressure (Morgan and Iwama, 1991). Changes in the osmotic balance of the heart tissue have adverse effects on heart function and inhibit acetylcholinesterase activity. While these reports described the effects of salinity fluctuations on overall homeostasis and osmoregulation in the oyster, the cellular and molecular events resulting in these effects have not been determined.

Salinity is a key environmental influence on fish and shellfish physiology and survival (Toyohara et al., 2005; Gagnaire et al., 2006; Hosoi et al., 2007). Most ocean invertebrates, including shellfish, do not have specific systems that regulate their body functions in relation to the outside environment, but these organisms can osmoregulate and adapt at the cellular level using osmolytes (Hosoi et al., 2007). However, osmolytes do not function via the hemolymph and have limited ability to maintain functional cellular components and volumes (Loosanoff, 1953; Burton, 1983).

Gagnaire et al. (2006) reported that oysters perished beginning on the third day of exposure to salinities of either 5 or 60 psu, and that mortality was very high after the sixth day. However, in the present study, no individuals perished when exposed to salinities of 52.5 psu. This suggests that euryhaline Pacific oysters can survive at least to a salinity of 52.5 psu.

The full-length HSP90 cDNA contained 2154 nucleotides, including an open reading frame (ORF), and was predicted to encode a protein of 717 amino acids (Fig. 1). It contained five signal peptides that are conserved in the HSP90 family: LGTIA(K/R)SGT, NKEIFLRELISN(A/C/S)SDALDKIR, IGQFGVGFYSAYLVA(E/D), IKLYVRRVFI and

GVVDSEDLPLNISRE (Gupta, 1995; Gao et al., 2007).

The consensus sequence MEEVD located at the 3´terminus (Gupta, 1995; Gao et al., 2007) was identified in HSP90 at amino acid residues 713-717. Scheufler et al. (2000) reported that the amino acid sequence MEEVD located at the 3´ terminus is the most conserved area; moreover, it is involved in the binding of HSP70 and HSP90, and is characteristic of HSP90. In addition, *C. gigas* HSP90 is similar to the HSP90 family in its general features and contains all of the recognized consensus sequences and signal peptides.

The expressions of HSP90 (the stress protein) and SOD (the antioxidant enzyme) were compared to identify the stress responses of Pacific oysters to rapid changes in salinity. RT-PCR indicated that the HSP90 and SOD mRNA expression per organ reached their highest levels in the gills of oysters exposed to salinity. High levels of HSP90 and SOD mRNA expression must have been induced because the gill, which is the primary interface between the outside environment and the hemolymph or cytoplasm (Hosoi et al., 2007) and the direct absorption route for seawater with a wide contact area, was more directly influenced by stress than other organs. Changes in HSP90 and SOD mRNA expression over time in oysters exposed to hypersalinity (52.5 psu) were investigated using QPCR.

HSP90 mRNA expression increased significantly over 96 h in oysters exposed to hypersalinity (52.5 psu, Fig. 4A), indicating that HSP90 mRNA acts as a defense mechanism against high salinity. Therefore, HSP90 mRNA expression in the gills of Pacific oysters exposed to high salinity presumably serves as a molecular chaperone that maintains structural integrity and binding, as well as appropriate control of the cytosolic protein subset (Picard, 2002) to protect the cell.

SOD mRNA expression in the gills of Pacific oysters exposed to high salinity increased significantly over 192 h (Fig. 4B). Further study of this response is necessary because no other research on SOD in shellfish exposed to high salinity has been conducted. Cellular damage due to salinity stress-induced ROS generation reached its highest level after 192 h

exposure to a salinity of 52.5 psu. Therefore, the increased SOD mRNA expression was likely a mechanism for removing ROS.

H_2O_2 concentrations increased significantly over 96 h in oysters exposed to hypersalinity (Fig. 7). Osmotic stress induces the formation of ROS in the hemolymph of *C. gigas*, thereby promoting oxidative stress, which in turn causes membrane damage, DNA breakage, lipid peroxidation, enzyme inhibition, amino acid oxidation and apoptosis (Choi et al., 2007b).

Salinity is a key factor affecting the physiology and survival of oysters as reported in many studies (Toyohara et al., 2005; Gagnaire et al., 2006; Hosoi et al., 2007). Bivalves osmoregulate by opening and closing their valves to control water filtering. When they are exposed to high salinity, they relax the adductor muscles and increase filtering capacity (Galtsoff, 1964).

It has previously been shown that *C. gigas* quickly responds to radical changes in salinity (Jo et al., 2007), and is thus capable of adapting to different estuarine environments. Consistent with the previous results, the osmotic concentrations of the hemolymph of *C. gigas* changed immediately after exposure to hypersalinity (Fig. 5). When Pacific oysters were exposed to hypersalinity, hemolymph osmotic concentrations (52.5 psu) were higher (1429 ± 2.6 mOsm/kg, 72 h) than those in normal seawater (973 ±9.7 mOsm/kg). The increased osmolality of the oyster hemolymph can be attributed to the outflow of water and inflow of salts into the oyster body. The concentrations of Na^+, Cl^- and Ca^{2+} showed a similar pattern (Fig. 6). In *C. gigas*, the concentrations of Na^+ and Cl^- constituted 70% of the osmotic pressure, as in other marine animals including fish, indicating that these are the major ions regulating osmotic pressure (Chang et al., 2007). Amado et al. (2006) acclimated ocean invertebrates such as the red crab, *Dilocarcinus pagei* to freshwater and seawater for 10 days and reported that the osmotic concentrations and mineral ion concentrations of the hemolymph changed sharply and then stabilized at a level close to the external salinity. This previous result was similar to the present results, in

which the osmotic pressure of the hemolymph in oysters exposed to high salinity, and the concentrations of Na^+, Cl^- and Ca^{2+}, peaked at 72 h.

The present study examined the expression of CT-R mRNA in various tissues of Pacific oysters exposed to hypersalinity using RT-PCR (Fig. 3). Dubos et al. (2003) also found high CT-R mRNA expression in the gill. In the present study, CT-R mRNA was expressed in most tissues other than the digestive gland, and the highest expression was in the gill.

The gills of marine molluscs constitute the primary interface between the hemolymph or cytoplasm and the external environment, where the osmolality fluctuates widely (Hosoi et al., 2007). Consequently, the high expression of CT-R mRNA induced by the large surface area that can absorb high salinity water has a great influence on the internal tissue osmotic pressure. In this study, gills exposed to hypersalinity (52.5 psu) showed the highest expression among all the tissues examined.

Dubos et al. (2003) suggested that a significant decrease in receptor transcript levels in the gill as a result of transfer to brackish water indicated a contribution of CT-R to ionic regulation. Their hypothesis is supported by the results of this study, in which the temporal changes in CT-R mRNA expression were examined using QPCR and were shown to increase over 72 h after exposure to hypersalinity, and then decrease (Fig. 4C). The present results imply that CT-R mRNA is expressed in response to the salinity change, which suggests that CT-R is involved in regulating osmolality in *C. gigas*. In addition, CT-R is involved in calcium homeostasis in fish, although the physiological roles of CT and the characteristics of fish CT-R have not been clarified (Nag et al., 2007). CT-R is a major regulator of calcium concentrations and CT-R mRNA expression probably regulates calcium metabolism. Calcium ions flow into the body from the ambient seawater and, in *C. gigas* at least, calcium concentrations increase in high salinity environments. In addition, the neuropeptide CT increases carbonic anhydrase activity and adenylate cyclase in the gill membranes of trout, *O. masou* and *H. tuberculata* (Arlot-Bonnemains et al., 1991; Fouchereau-Peron, 2001). Endocrinology and molecular biology studies are needed to

examine the relationship of calcitonin, CT-R and Ca^{2+} concentration in the neuroendocrine regulation of molluscs.

Changes over time were observed in the gill tissues of Pacific oysters exposed to high salinity. Observation was conducted with an light microscope after dyeing the external tissues of ordinary filaments, the principal filament and the hemolymph sinus of the plica, etc., using the H-E stain method (Fig. 8). The fine structures of the cilia, microvilli, mitochondria and so on, were also observed using TEM (Fig. 9). The structure of the gill tissues in the oysters was consistent with descriptions of other species of bivalve molluscs, including freshwater molluscs, which show a characteristic structure of heterorhabdic plicate gills with differentiated filaments (Beninger and Dufour, 1996). Considering that no histological change was observed in the gill tissues of Pacific oysters exposed to high salinity throughout the experiment, a salinity of 52.5 psu is not considered to damage the gill tissues of Pacific oysters. Based on the above result, it is concluded that HSP90, SOD and CT-R genes are able to protect cells in high salinity environments to at least 52.5 psu.

In summary, the expression of HSP90 and SOD mRNA increased in Pacific oysters exposed to hypersalinity. These results indicate that increases in stress and ROS due to hypersalinity induce these genes, which trigger the production of SOD and HSP90 to remove ROS and protect the cells. Indeed, osmolality tended to stabilize after HSP90 expression, and H_2O_2 concentrations tended to recover to normal after SOD expression. These results, and the fact that no oysters died due to exposure, indicate that stress levels must have been reduced through various physiological defense mechanisms. The water temperatures used in this study were possibly the minimum required by Pacific oysters. These results can be used as a foundation for establishing shellfish farms.

Also, expression of CT-R mRNA paralleled the concentration of inorganic ions, especially Ca^{2+}. Given that the concentrations of Na^{+}, Cl^{-} and Ca^{2+}increased significantly when the expression of CT-R mRNA was highest, it is likely that CT-R mRNA expression takes part in regulating osmotic pressure in the oyster body in response to changes in

salinity. However, further research is needed on the structure and function of the genes and to fully elucidate the osmoregulatory mechanisms involved and the relationships between them.

Chapter 3

Characterization of CYP450, HSP90 and SOD mRNA expression and changes in physiological hemolymph responses with thermal stress in Pacific oyster, *Crassostrea gigas*

1. Introduction

Changes in water temperature and salinity are foremost among the environmental factors that cause physiological changes in aquatic organisms. In particular, rapid temperature change acts as a stressor that reduces health and causes disease (Wedemeyer and McLeay, 1981). In general, the primary response to stress in fish is the activation of the hypothalamus-pituitary-hepatorenal axis and the resulting secretion of cortisol into the blood. Secondary responses include increases in plasma glucose levels, a water-ion imbalance, oxygen consumption and energy requirements (McDonald and Milligan, 1997). However, detecting physiological stress in invertebrates such as shellfish is difficult because their endocrine organs are not clearly differentiated.

Water temperature can alter the physiology of aquatic organisms, including their growth, propagation, metabolism and osmoregulation, and has a major impact on the organisms. As a species inhabiting shallow waters and estuaries, *C. gigas*, is strongly influenced by water temperatures. The optimal water temperature for *C. gigas* is 15-20℃, and outside this range, water temperature acts as a physical stress, adversely affecting physiology, including defense mechanisms (Zhang et al., 2006).

Changes in water temperature and salinity induce the expression of heat shock proteins (HSPs), an important defense mechanism against stress. HSPs are divided into three groups based on molecular weight: HSP90 (85-90 kDa), HSP70 (68-73 kDa) and low

molecular weight HSP (16-47 kDa) (Basu et al., 2002). HSP90, which has the highest molecular weight, constitutes 1-2% of cellular proteins, exists in large quantities in cells not affected by stress and has a very important function as a molecular chaperone (Picard, 2002). HSPs are sensitive to various stresses, not only water temperature and salinity, but also heavy metals, chemicals and anoxia. HSPs maintain the tertiary structure of proteins in the cell, thereby suppressing cell damage through cellular protection and protein hydrolysis (Young et al., 1993; Morimoto, 1998). Many studies have examined HSPs in fish, while few have examined them in shellfish. A recent report identified the cDNAs encoding HSP70 and HSP90 in *H. tuberculata* in response to thermal stress (Farcy et al., 2007).

When an organism is exposed to stress, reactive oxygen species (ROS; O^2, H_2O_2, HO^- and $^1O_2$) are generated. ROS are strong chemical oxidants that bind with other substances they attack the membranes of cells or organs and deleteriously affect cell function (Ferraris et al., 2002). Large amounts of ROS are formed by many kinds of stresses, such as heavy metal toxicity, pollutants and abiotic and biotic environmental factors (Stohs et al., 2000). The oxidative stress caused by ROS leads to lipid peroxidation, protein denaturation and DNA damage to living tissues. This stress also changes and inhibits a variety of enzyme activation effects and causes cell damage and an imbalance in cells resulting in apoptosis (Choi et al., 2007b). Accordingly, numerous physiological problems result, such as the promotion of aging, reduction in disease resistance and lowering of reproductive ability (Kim and Phyllis, 1998). The antioxidant enzyme SOD, a representative component of the defense against ROS, is generated inside the body to prevent such damage (Wendel and Feuerstein, 1981). An important metalloenzyme, SOD exists in all aerobic organisms. In the process of dismutation into oxygen and hydrogen peroxide ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), SOD removes superoxide radicals (Fridovich, 1975). However, hydrogen peroxide (H_2O_2), a product of this reaction, is also a toxic ROS that has harmful effects on the tissues of marine animals.

With the exception of a study by Kim et al. (2007) on SOD mRNA expression in abalone exposed to heavy metal and water temperature stress, and a study by Ni et al. (2007) on SOD mRNA expression and immune response, almost no research has examined SOD expression and function in relation to stresses in molluscs such as Pacific oyster.

The HSP family and a 65-kDA protein related to warm temperature acclimation are used as biological markers for stress factors, such as water temperature change (Choi et al., 2008a). Recently, the cytochrome P450s (CYP450s) have been suggested as biological markers sensitive to water temperature stress (Arukwe and Goksùyr, 2000). CYP450s are enzymes that contain heme (reduced hematin), which colors hemoglobin. Several factors affect the basal level of CYP isozymes, including developmental stage, sex, diet and seasonal changes in steroid levels and temperature (Arukwe and Goksùyr, 2000). CYP450s are heme-dependent oxidases that catalyze a wide variety of reactions, including hydroxylation, epoxidation, N-demethylation, O-dealkylation, deamination, sulfoxidation and oxidative dehalogenation, using NADPH or NADH as electron donors. They comprise a superfamily of hemethiolate enzymes responsible for the oxidative, peroxidative and reductive metabolism of many structurally diverse compounds (Pua et al., 2003).

Diverse varieties of CYP450 are found in plants, animals, bacteria and other organisms, and CYP450s are the subject of active research. The enzyme families forming this large superfamily have various substrates and metabolic products. Isozymes that belong to different subfamilies characteristically form derivatives and have nuclear receptors specific to each group (Yoon et al., 2003). The very diverse CYP450 types are divided into families, subfamilies and polypeptides, and include CYP19A1, CYP11B1, CYP7a1, CYP1A, CYP2d2, CYP4A, CYP739A, CYP513E1 and many others. The CYP450s in group 1A have been cloned from several fish species, including rainbow trout, *Salmo gairdneri* (Berndtson and Chen, 1994), brook trout, *Salvelinus fontinails* and lake trout, *Salmo namaycush* (Rees and Li, 2004), red sea bream, *Sparus aurata* (Mizukami et al., 1994), and sea bass, *Dicentrarchus labrax* (Stien et al., 1998). Temperature compensation

related to the CYP450 enzyme system during acclimation by fishes to environmental changes (*e.g.*, water temperature changes) has been reported for killifish, *Fundulus heteroclitus*, roach, *Rutillus rutillus*, bluegill, *Lepomis macrochirus* and salmonids (Andersson and Koivusaari, 1985; Karr et al., 1985; George et al., 1990). Among shellfish, however, changes in CYP450 enzyme activity and mRNA expression in relation to water temperature change have been studied only in the zebra mussel, *Dreissena polymorpha*, and the mussel, *Mytilus galloprovincialis* (Ricciardi et al., 2006; Bebianno et al., 2007).

Therefore, in this study, the cDNAs of a CYP450-related protein and HSP90 were cloned, and the mRNA expressions of HSP90, SOD and CYP450 were investigated in the gills of Pacific oysters transferred from 20℃ to 30℃ or 10℃. Changes in osmolality, aspartate aminotransferase (AST) activity and H_2O_2 concentrations were also investigated, and further observations were made using hematoxylin-eosin (H-E) stain method and tran smission electron microscopy (TEM) in the hemolymph to identify changes in the physiolo gy of Pacific oysters related to thermal stress.

2. Materials and Methods

2.1. Experimental oysters and water temperature treatment

One-year-old Pacific oyster (average shell length: 112±10.7 mm; height: 31.1±5.4 mm; weight: 20.3±3.9 g) were obtained from the oyster hatchery on Daebu Island in Goseong (Gyeongnam, Korea). These were acclimated in two circulating filter tanks (40 L, 40 oysters per a tank) for a week prior to experiment in the laboratory. Water temperature and photoperiod were maintained at 20℃ and a 12 h light/12 h dark.

The oysters, acclimated at 20℃ (control group) directly, transferred to 30℃ and 10℃ (experimental group), respectively. Gills of five oysters were randomly dissected in the following time period: 0, 1, 3, 6, 12, 24 and 48 h. The tissues immediately were frozen in liquid nitrogen after collection and stored at -80℃ until total RNA extraction.

2.2. Rapid amplification of cDNA 5′/3′ ends (RACE)

For RACE reactions, total RNA was extracted using a Trizol (Gibco/BRL, Grand Island, NY, USA) from the gill of Pacific oysters. Reverse transcription was performed using M-MLV reverse transcriptase (Bioneer, Daejeon, Korea). Using 3 μg of total RNA as the template, 5′-RACE-ready complementary DNA (cDNA) and 3′-RACE-ready cDNA were generated using the protocols and reagents provided in the CapFishing Full-length cDNA Premix kit (Seegene, Seoul, Korea). Gene-specific primers were selected from *C. gigas* CYP450 partial cDNA (GenBank accession no. AF075692). For 3′-RACE, the 50 μL Polymerase chain reaction (PCR) reaction mixture contained 5 μL of 3′-RACE-ready cDNA, 1 μL of 10 μM 3′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μL of 10 μM 3′ RACE gene-specific primer of CYP450 (5′-CTG ACC GGT TCC TGG AGG AGG

GAA AGT-3′), and 25 μL of SeeAmp Taq Plus Master Mix (Seegene). PCR was carried out for 40 cycles at 94℃ for 45 s for denaturation, 62℃ for 45 s for primer annealing, and 72℃ for 90 s for extension, followed by 5 min at 72℃ for extension. The PCR product was amplified, cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA), and sequenced. For 5′-RACE, the 50 μL PCR reaction mixture contained 5′-RACE-ready cDNA, 5′ gene-specific primer of CYP450 (5′-ACT TTC CTT CCT CCA GGA ACC GGT CAG-3′), 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), and SeeAmp Taq Plus Master Mix, as for 3′-RACE, under the same PCR conditions. The PCR product was amplified, cloned into pGEM-T Easy Vector, and sequenced. The DNA and deduced amino acid sequences were analyzed using GENETYX-WIN (Software Development, Tokyo, Japan).

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

2.5 μg of total RNA extracted from the gonad, digestive gland, mantle, gill and intestine of oysters under thermal stress were reverse transcripted with M-MLV reverse transcriptase (Bioneer) and oligo-d$(T)_{15}$ primer (Promaga). RT-PCR was conducted to determine the relative expression of HSP90, SOD, CYP450 mRNA and 28S ribosomal RNA (28Sr) in various tissues of *C. gigas*. To optimize the number of cycles used for RT-PCR, the reactions (1 μL) from the gonad, digestive gland, mantle, gill and intestine of oysters exposed to water-temperature change were used as the template for RT-PCR amplification. HSP90, SOD, CYP450 and 28Sr specific primers for RT-PCR were designed from published sequences: HSP90 forward primer (5′-ATG CAG ACG CTT GTG TCT TG-3′), HSP90 reverse primer (5′ -TCT GTC TGC AAC CAA GTA GG-3′), SOD forward primer (5′-GAC CCC ATC CTG TTC CCC AGC-3′), SOD reverse primer (5′-AGA AGG CGA TCT GTT CCA CCT C-3′), CYP450 forward primer (5′-AGG CGA TAT GAC GAC GAG TT-3′),

CYP450 reverse primer (5´-ACG TGT TCA TCT GTG AGC CA-3´), 28Sr forward primer (5´-TGC TCT GGA CTG TCC TAG GA-3´) and 28Sr reverse primer (5´-ACC GAT TCG CCA CTG ACC AT-3´). The 28Sr was amplified in each PCR reaction as a loading control. The PCR products from 30 cycles of amplification were visualized on a UV-transilluminator after electrophoresis on 1% agarose gel containing ethidium bromide (0.5 μg/μL). The signal intensity was quantified using the Gel-Doc System and Gelpro 3.1 software (KBT, Incheon, Korea).

2.4. Quantitative real-time PCR (QPCR)

QPCR was conducted to determine the relative mRNA expression of CYP450, HSP90 and SOD using total RNA extracted from gills of control and oysters treated water temperature change. With 2.5 μg of total RNA as a template, cDNA were synthesized using M-MLV reverse transcriptase (Bioneer). First-strand cDNA synthesis was conducted using oligo-d$(T)_{15}$ primer (Promega). Primers for QPCR were designed with reference to known gene sequences of Pacific oyster as follows: CYP450 forward primer (5´-GGT GAA TGT TAC CAA GGA AGG-3´), CYP450 reverse primer (5´-GTT ACG ATA CAG CAA GGA GAT G-3´), HSP90 forward primer (5´-GGT GAA TGT TAC CAA GGA AGG-3´), HSP90 reverse primer (5´-GTT ACG ATA CAG CAA GGA GAT G-3´), SOD forward primer (5´-ATG TCA TCT GCT CTG AAG GC-3´), SOD reverse primer (5´-TGG TGA TAC CGA TCA CTC CA-3´), 28Sr forward primer (5´-AAA CACGGA CCA AGG AGT CT-3´) and 28Sr reverse primer (5´-AGG CTG CCT TCA CTT TCA TT-3´). QPCR amplification was conducted using a Bio-Rad MiniOpticon^{TM} System (Bio-Rad, CA, USA) and iQ^{TM} SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. QPCR was carried by denaturation at 95℃ for 5 min, followed by 40 cycles of denaturation at 95℃ for 20 s and annealing at 55℃ for 20 s. The values based on normalization of individual samples to 28Sr and then comparison to

control group. To ensure that the primers amplified a specific product, It was performed a melt curve, as well as analyzed the PCR product size using capillary electrophoresis (Agilent Technologies, Santa Clara, CA). All primers used were shown to amplify only one size of template, melting at only one temperature. PCR products were also confirmed by sequencing. All analyses were based on the calculated threshold cycle time (Ct) values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR program, QPCR data from five replicate samples were analyzed with analysis software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. mRNA expression levels stood for an n-fold difference relative to 28Sr as the internal control.

2.5. Hemolymph osmolality and AST activity

The hemolymph was withdrawn from the pericardial cavity using a 3 mL syringe. Hemolymph samples were then separated by centrifugation (4℃, 10,000 ×*g*, 5 min) and stored at -80℃ until analysis. Hemolymph osmolality was measured with a Vapor Pressure Osmometer (Vapro 5520, Wescor, Logan, UT). The AST activity was measured using Pureauto S AST (Daichi, Tokyo, Japan) kit by biochemistry autoanalyzer (model 7180; Hitachi, Tokyo, Japan), according to the manufacturer's instructions.

2.6. Hydrogen peroxide assays

H_2O_2 concentrations were measured using the modified methods of Nouroozzadeh et al. (1994) and a Peroxidetect kit (Sigma-Aldrich, St. Louis, Missouri, USA). 20 $\mu\ell$ of whole oyster hemolymph in marine anticoagulant (MAC: 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 50 mM EDTA, 0.45 M sodium chloride,

pH 7.5) was added per well to flat bottom 96 well microtitre plates. Plates were left at room temperature for 20 min to allow hemocytes to settle and adhere. A working color reagent was prepared by mixing 100 mL distilled water containing 100 mM sorbitol and 125 uM xylenol orange (Sigma-Aldrich) with 1 mL of 25 mM ferrous ammonium sulphate prepared in 2.5 M sulphuric acid (Sigma-Aldrich). Two hundred microlitres of this reagent was then added to each well and allowed to incubate at room temperature for 1 h. Absorbance was read at 560 nm and concentrations of H_2O_2 were interpolated from a standard curve. Concentrations are expressed as nM/mL.

2.7. Histological analysis

2.7.1. Observation with light microscope

To observe histological changes in the gill tissues of Pacific oyster exposed to water temperature change, the extracted gill tissues were fixed in 10% neutral formalin solution (100 mL formalin, 6.5 g $Na_2HPO_4 12H_2O$, 4.5 g KH_2PO_4, 900 mL distilled water) for 1 day. Then, the gill tissues were fixed in bouin solution and a tissue sample was produced according to the series of methods. The fixed sample was cut into serial sections of 5 ㎛ in thickness by paraffin sectioning. Then, the degree of tissue damage was examined with an light microscope after double dyeing with Harris's hematoxylin and 0.5% eosin (Sigma, USA). The degree of damage in gill tissues was photographed by using image analysis system (Axiovision, Zeiss Co., Germany) with a biological microscope (Axioskop, Zeiss, Germany).

2.7.2. Observation with electron microscope

A sample for TEM was produced in order to observe the fine structure of the gill tissues of Pacific oyster exposed to water temperature change. After extracting gill tissues, primary fixation was carried out for 2 h in 2.5% glutaraldehyde solution (4℃) buffed with

0.1 M phosphate buffer solution (PBS, pH 7.2). The sample completed of fixation was washed for 10 min with PBS and was processed through secondary fixation for 2 h at 4℃ in 1% osmium tetroxide (OsO_4). After fixation was completed, the sample was washed with PBS and was dehydrated for 15 min each in 50-100% ethanol. After dehydration, the sample was placed in the mixture of propylene oxide and Epon and was polymerized for 1-3 h. Then the sample was embedded in Epon 812. The embedded tissue was semi-thinly sectioned with the thickness of 1.0 ㎛ by using ultramicrotome (Leica, Reichert SuperNova, Sweden). Then, it was dyed with 1% toluidine blue and the part for observation was determined. After the part for observation was determined, the sample was again sectioned with the thickness of 70 mm and was attached to 200 mesh copper grid. The sample completed of ultra-thin sectioning was double-dyed with uranylacetate and lead citrate solution and was observed with a transmission electron microscope (JEM 1200 EX-Ⅱ, 60-80 kv, JEOL, Tokyo, Japan).

2.8. Statistical analysis

The existence of significant differences between the data obtained from each experiment was tested using one way ANOVA (Tukey's test or LSD test) with the SPSS statistical package (version 10.0) at a significance level of $P<0.05$.

3. Results

3.1. Identification of CYP450 cDNA

Pacific oysters are bivalves belonging to the family Osteidae, order Tetrabranchia and class Pelycypoda. Classification of Pacific oyster CYP is difficult, and only two studies have been conducted: one on CYP356A1 (EF645271) and the present study on the CYP450-related protein.

An NCBI/GenBank database comparison of Pacific oyster CYP450 cDNA revealed that the deduced amino acid sequence of CYP450 is similar to mouse, *Mus musculus* CYP450 2D9 (AK078880; 29%), rabbit, *Oryctolagus cuniculus* CYP450 2D/II (AB008785; 28%), and marmoset, *Callithrix jacchus* CYP450 2D (AY082602; 28%). It was first cloned Pacific oyster CYP450 and then compared its amino acid sequence to that of other species, families, and subfamilies. It was classified Pacific oyster CYP450 as belonging to family 2, subfamily D.

```
   1 tgtctaccaggcattcgcttcatgggggataggggatacttctttgtgttttctcacact
  61 cctaactgatggttaattgacaaaatgtcctaagaagcggaa**atg**gtctctttaagcact
                                                 M  V  S  L  S  T    6
 121 tttgaagtcatgacatcaaagagaaagaaaaagtccccgccgggaccatgtggcttaccc
      F  E  V  M  T  S  K  R  K  K  K  S  P  P  G  P  C  G  L  P   26
 181 ttccttggttctttttttcgacatcgaccttaaaaatattcacctggattttctgaaatgg
      F  L  G  S  F  F  D  I  D  L  K  N  I  H  L  D  F  L  K  W   46
 241 aaggagcgatatggagatatcgtgtctttcaaaatgaacgggaagaattttcttgtactg
      K  E  R  Y  G  D  I  V  S  F  K  M  N  G  K  N  F  L  V  L   66
 301 aataatattgacattatcaggaaggcattcgagagcgacgaaatcggcgccttaatgagt
      N  N  I  D  I  I  R  K  A  F  E  S  D  E  I  G  A  L  M  S   86
 361 gatcgaccattaaatttcattggagaaaatatatttttggttacaaagacgttctttta
      D  R  P  L  N  F  I  G  E  N  I  F  F  G  Y  K  D  V  L  L  106
 421 aggcgatatgacgacgagtttatgaagatgaagaaactgatgatacgatcgatgaagctc
      R  R  Y  D  D  E  F  M  K  M  K  K  L  M  I  R  S  M  K  L  126
 481 cacgattataactcggacaagtttcaacagctgatgtcagaggagctttcacacatactg
      H  D  Y  N  S  D  K  F  Q  Q  L  M  S  E  E  L  S  H  I  L  146
 541 tctaaattccagaagacagaaggaaagccaacggagcctatggacattttggtgccgtcc
      S  K  F  Q  K  T  E  G  K  P  T  E  P  M  D  I  L  V  P  S  166
 601 ttctgtaacatcatcggaatgctgttcacagggcgccgatgccaggacgaggacaggctc
      F  C  N  I  I  G  M  L  F  T  G  R  R  C  Q  D  E  D  R  L  186
 661 cttaaggtcttggtggacttcgaccgagacggggacaccatgattcagccccaggttcac
      L  K  V  L  V  D  F  D  R  D  G  D  T  M  I  Q  P  Q  V  H  206
 721 gcagtgtacaaactattcccctggatt**cgt**cattctcccggctattacggcggtctgtat
      A  V  Y  K  L  F  P  W  I  **R**  H  S  P  G  Y  Y  G  G  L  Y  226
 781 cgcaatgttattcgggggggaacagaactacacaatctggtccaagatatgaagagtaaa
      R  N  V  I  R  G  G  T  E  L  H  N  L  V  Q  D  M  K  S  K  246
 841 tatgacaaaaccgaggttcaaaacttcatccacgaacttctgggggagcatcaggacttt
      Y  D  K  T  E  V  Q  N  F  I  H  E  L  L  G  E  H  Q  D  F  266
 901 gctgaggacccggataaaggctggctcacagatgaacacgttctaggaatgattatggat
      A  E  D  P  D  K  G  W  L  T  D  E  H  V  L  G  M  I  M  D  286
 961 cttatcaacacgtccgtgttgacaaccaaagctgtgatggcaggtgccctctttctcctc
      L  I  N  T  S  V  L  T  T  K  A  V  M  A  G  A  L  F  L  L  306
1021 tctcactttccggagatacaggaaaagattcgtgaggagatcattaacattgtcggctct
      S  H  F  P  E  I  Q  E  K  I  R  E  E  I  I  N  I  V  G  S  326
1081 cgatctcccacgacagaggacatggcgtcaatgccgtacacggaggcctgtatgatggag
      R  S  P  T  T  E  D  M  A  S  M  P  Y  T  E  A  C  M  M  E  346
1141 atcttacgctatcagtcccatcttcccctcaccgcgccccacgcaaacctaagccaggaa
      I  L  R  Y  Q  S  H  L  P  L  T  A  P  H  A  N  L  S  Q  E  366
1201 gtagaactggagggctacacgatccccaaaggaacggtgattttcgggaacttgtttgcc
      V  E  L  E  G  Y  T  I  P  K  G  T  V  I  F  G  N  L  F  A  386
```

1261 tgccaccacgatgagaccgtgtaccctgacccctgggagttcaaacctgaccggttcctg

C H H D E T V Y P D P W E F K P D R F L 406

1321 gaggaaggaaagttagtcggagccgaccacccagcggtcagaaattttattgga**tttggc**

E E G K L V G A D H P A V R N F I G **F G** 426

1381 **gtcgggaggcggcggtgcgtgggt**cagcaaatggctagaatcagaatgttcctgtacccc

V G R R R C V G Q Q M A R I R M F L Y P 446

1441 acgtgtctcttgcagaaatttaaaatcgaggtcccaaaagatacgtcacttccgtcacat

T C L L Q K F K I E V P K D T S L P S H 466

1501 gacccaagagcgttactttcggagtcccctgtgattttaccgccacctatgcagtattgt

D P R A L L S E S P V I L P P P M Q Y C 486

1561 tctgttgagtgt**tga**acgtgaatgctcgagatctcagtctacagagaccggttgtgaatg

S V E C * 490

1621 aaagttacttgttatgtataatacatggctagtcttttgctttt**attta**ttagagatatg

1681 atgtt**attta**ttgaaatccagaa**aataaa**tgttagaaacatccaaaaaaaaaaaaaaaaa

1741 aaaa

Fig. 10. cDNA and deduced amino acids residue sequence of cytochrome P450 (CYP450) in Pacific oyster, *Crassostrea gigas*. Nucleotide number is shown on the left and the amino acid residue number is shown on the right. The start codon (position 103 bp), putative arginine residue critical to enzymatic function (position 748 bp), stop codon (position 1573 bp) and putative poly-adenylation signals (1665, 1686 and 1704 bp) are underlined and boldfaced. Heme-binding region (position 1375 bp) is boxed and boldfaced. This sequence data is available from the NCBI/GenBank nucleotide sequence databases with the accession number EF451959.

3.2. Tissue distribution of CYP450 mRNA by RT-PCR

This investigated CYP450 mRNA expression in Pacific oyster tissues exposed to 30℃ (high temperature) and 10℃ (low temperature). Although the highest expression was observed in gill tissue, Also observed expression in gonad, mantle and intestine tissues (Fig. 11).

3.3. Tissue distribution of HSP90 and SOD mRNA by RT-PCR

Investigated HSP90 and SOD mRNA expression in various tissues that had been exposed to high temperature (30℃) for 6 h, respectively. Expression of HSP90 and SOD mRNA was higher in the gill than the gonad, mantle and intestine (Fig. 12).

3.4. Levels of CYP450 transcripts

There was no significant difference in CYP450 mRNA expression in the control group at 20℃. However, in the 30℃ and 10℃ test groups, expression levels increased significantly over 6 h, and then decreased (P<0.05, Fig. 13).

3.5. Levels of HSP90 and SOD transcripts

HSP90 and SOD mRNA expression over time in the gill tissues of oysters exposed to the stress of high water temperature indicated significant increases up to 6 h (HSP90 by 3.2 times; SOD by 9.1 times) and decreases afterwards (Fig. 14).

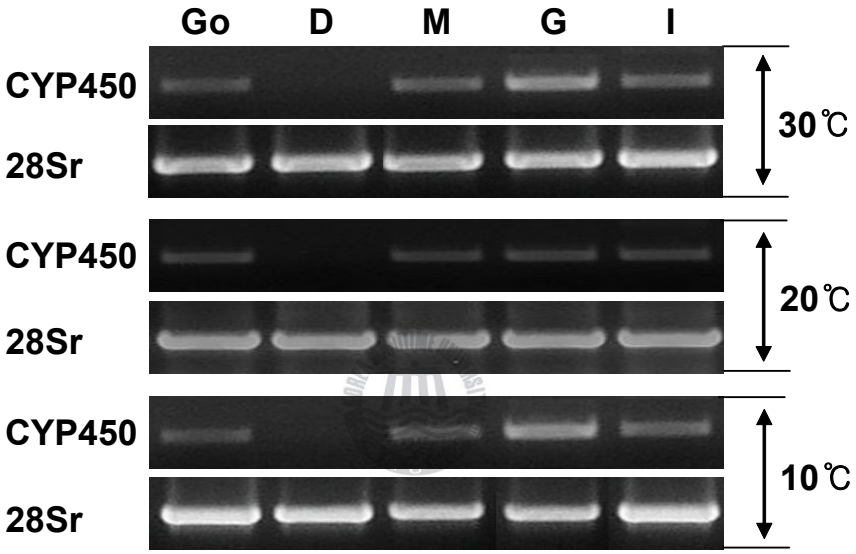

Fig. 11. Tissue-specific expression of cytochrome P450 (CYP450) mRNA in various tissues from Pacific oyster, *Crassostrea gigas* transferred from 20℃ (control) to 30℃ and 10℃ for 6 h by RT-PCR. Amplification of 28S ribosomal RNA was used as an internal control. Go: gonad; D: digestive gland; M: mantle; G: gill; I: intestine.

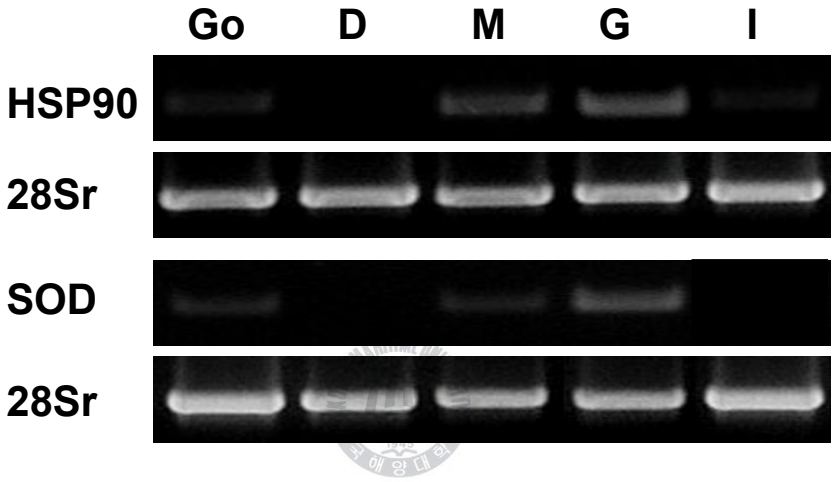

Fig. 12. Tissue-specific expression of 90-kDa heat shock protein (HSP90) and superoxide dismutase (SOD) mRNA in various tissues of Pacific oyster, *Crassostrea gigas*, exposed to high water temperature (30℃, HSP90 and SOD at 6 h) by reverse transcription polymerase chain reaction. Amplification of 28S ribosomal RNA was used as an internal control. Go: gonad; D: digestive gland; M: mantle; G: gill; I: intestine.

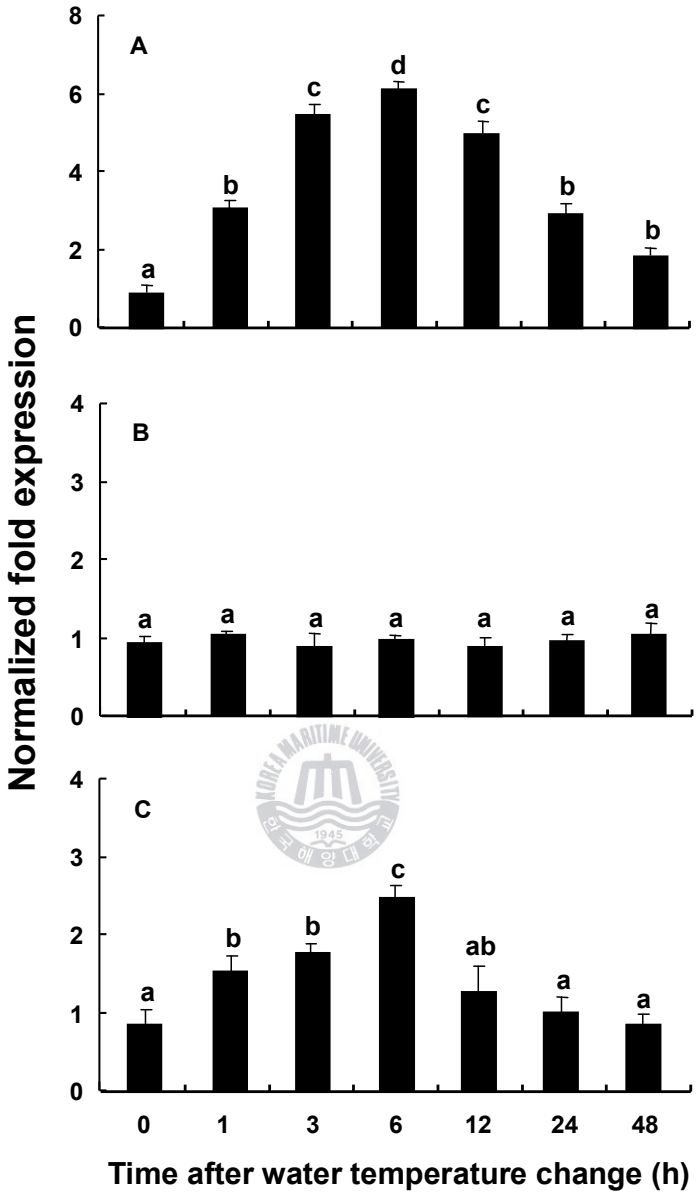

Fig. 13. Time-related effect on cytochrome p450 (CYP450) mRNA levels in the gill of Pacific oyster, *Crassostrea gigas* during water temperature change (A: 30℃, B: 20℃ and C: 10℃) as determined by QPCR. Results are expressed as fold change with respect to levels of 28S ribosomal RNA for the same sample. Values with dissimilar letters are significantly different (P<0.05) from one another. Values are means±SD (n = 5).

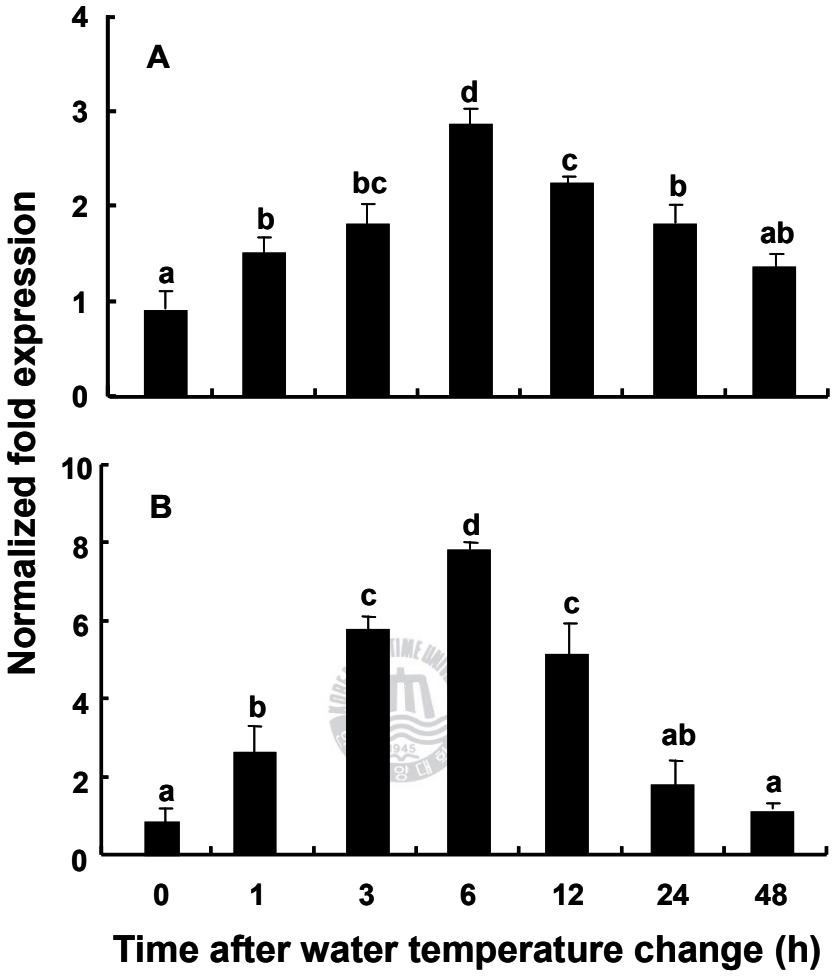

Fig. 14. Time-related effect on 90-kDa heat shock protein (HSP90) and superoxide dismutase (SOD) mRNA levels in Pacific oyster, *Crassostrea gigas* gill during high water temperature (from 20℃ to 30℃ A: HSP90, B: SOD) adaptation as determined by quantitative polymerase chain reaction. Results are expressed as fold change with respect to 28Sr levels for the same sample. Values with dissimilar letters are significantly different (P<0.05) from one another. Values are means±SD (n = 5).

3.6. Hemolymph osmolality and AST activity

For oysters exposed to high water temperature, there were the significant increases in osmotic concentrations in the hemolymph at 24 h (1207.7±10.9 mOsm/kg), after which the concentrations started to decrease (P<0.05 Fig. 15). The levels of AST in the hemolymph exposed to high water temperature (30℃) increased significantly by 6 h and then deceased (P<0.05, Fig. 16).

3.7. Hydrogen peroxide assays

H_2O_2 concentrations significantly increased at 6 h as 6±0.2 nM/mL from 2.4±0.2 nM/mL at the beginning of the trial in the hemolymph of oysters exposed to high water temperature and then decreased (Fig. 17).

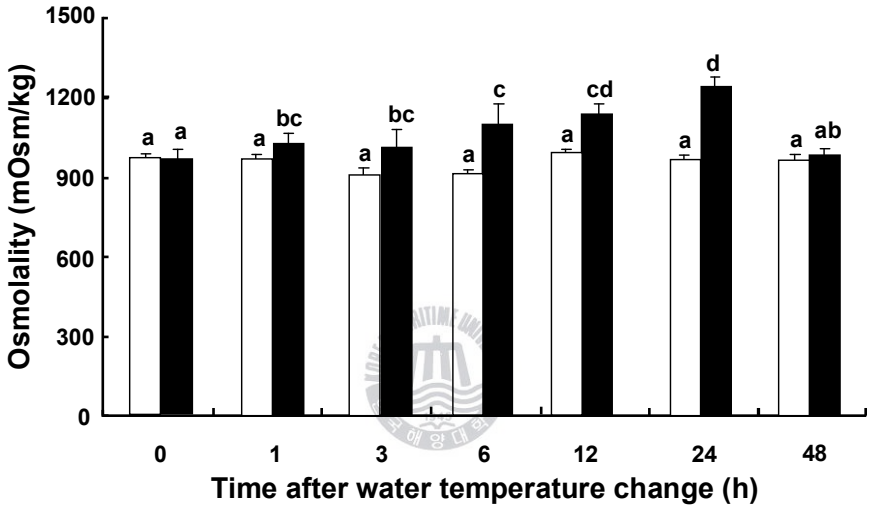

Fig. 15. Osmolality in the hemolymph of Pacific oyster, *Crassostrea gigas* during high water temperature from 20℃(□) to 30℃(■) adaptation. The hemolymph was separated from the oyster and used for osmolality. Values with dissimilar letters are significantly different (P<0.05) from one another. Values are means±SD (n = 5).

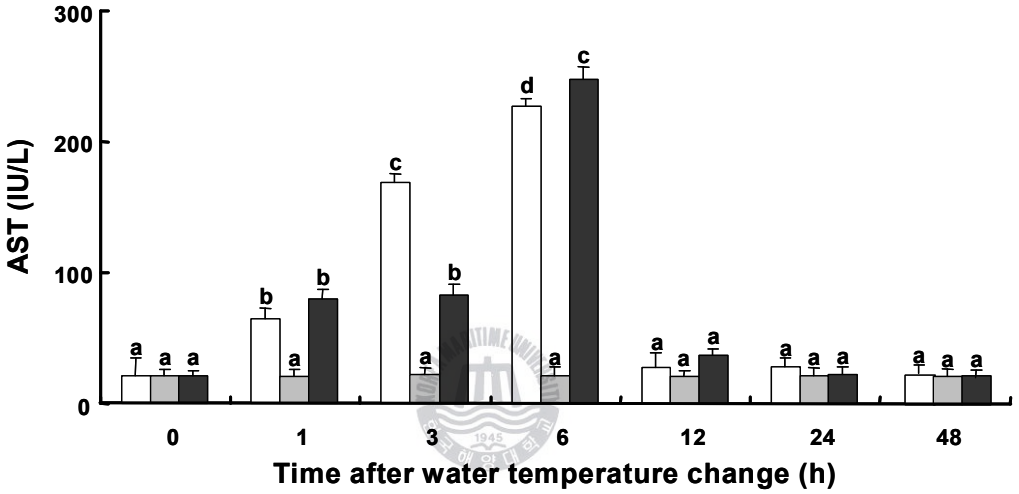

Fig. 16. Change of AST activity in the hemolymph of oyster exposed low temperature of 10℃ (□), normal temperature of 20℃ (■) and high temperature of 30℃ (■) adaptation. Values with dissimilar letters are significantly different (P<0.05) from each other. Values are means±SD (n = 5).

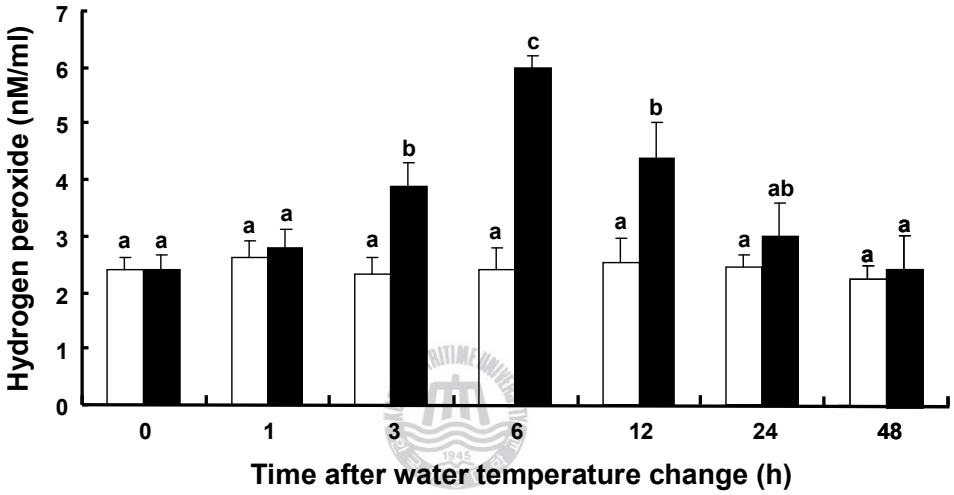

Fig. 17. Hydrogen peroxide (H_2O_2) concentrations in hemolymph of Pacific oyster, *Crassostrea gigas* during high water temperature from 20℃(□) to 30℃(■) adaptation. Different lowercase letters indicate significant differences ($P < 0.05$) from one another. Values are means±SD (n = 5).

3.8. Histological analysis

3.8.1. Observation with light microscope

The external shape of the gill tissues in Pacific oyster exposed to water temperature change was observed with H-E stain method and the result indicated no histological change in the control group and in the gill tissues at the 6 h of exposure to water temperature change (Fig. 18). Also, by 48 h, the experiment completion, no histological change was observed in the gill tissues of Pacific oyster.

3.8.2. Observation with electron microscope

The internal shape of the gill tissues in Pacific oyster exposed to water temperature change was observed with TEM and the result indicated no histological change in the control group and in the gill tissues at 6 h after exposure to water temperature change (Fig. 19). Also, by 48 h, the experiment completion, no histological change was observed in the gill tissues of Pacific oysters.

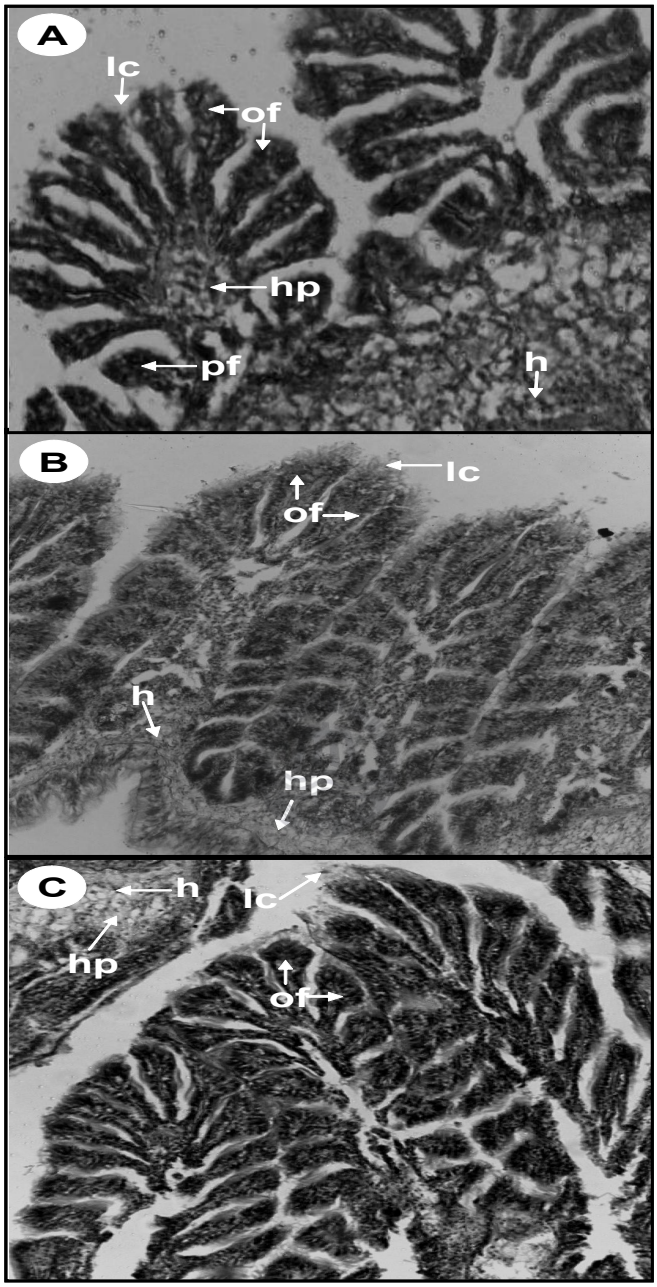

Fig. 18. Photomicrographs of gill filaments and lamellae of Pacific oyster exposed to normal temperature of 20℃ (A), low temperature of 10℃ (B) and high temperature of 30℃ (C) adaptation. of: ordinary filament, pf: principal filament, lc: lateral cilia, h: hemocytes, hp: hemolymph sinus of plica. X 200.

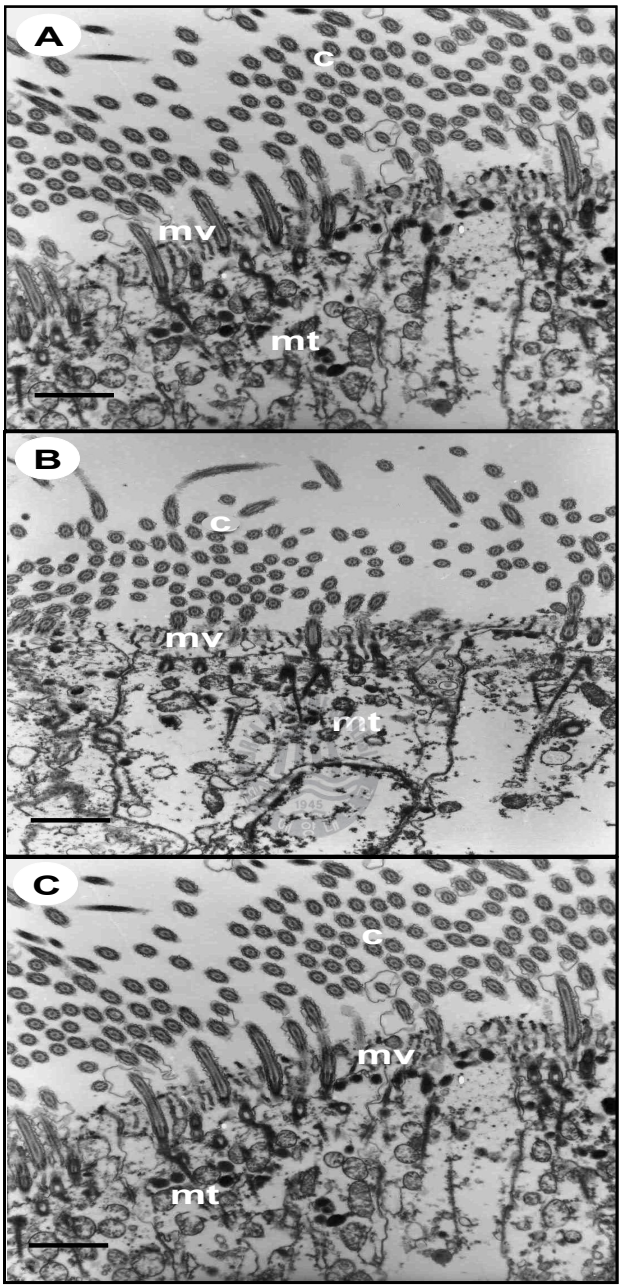

Fig. 19. Electron micrographs of Pacific oyster's gill exposed to normal temperature of 20℃ (A), low temperature of 10℃ (B) and high temperature of 30℃ (C) adaptation. c: cilia, mv: microvilli, mt: mitochondria. Bars: 1 ㎛. X 5000.

4. Discussion

The entire 1744 bp cDNA of CYP450 was isolated from Pacific oyster for the first time and the homology of this cDNA base sequence was compared to the base sequences of the mouse, rabbit and white-tufted-ear marmoset CYP450 in the NCBI database. Low similarities of 29%, 28% and 28% were found, respectively. Pacific oyster CYP450 was cloned and its amino acid sequence was compared to that of other species, families and subfamilies. Pacific oyster CYP450 was classified as belonging to family 2, subfamily D.

CYP450 mRNA expression in the tissues of Pacific oysters exposed to 30℃ (high temperature) and 10℃ (low temperature) was investigated. Although the highest expression was observed in gill tissues, expression was also observed in gonad, mantle and intestine tissues (Fig. 11). Temperature variations trigger greater expression of CYP450 mRNA in the gill than in other tissues. Therefore, RNA extracted from gill tissue was used for an RT-PCR study on changes in CYP450 mRNA expression levels of Pacific oysters after exposure to rapid water temperature changes. No significant differences were found in CYP450 mRNA expression between the 30℃ and 10℃ test groups in both groups, the expression levels increased significantly over 6 h, and then decreased ($P<0.05$). Ricciardi et al. (2006) reported decreased CYP450 activity in *D. polymorpha* acclimated to 20℃ as the water temperature was increased or decreased by 1℃ per day. Bebianno et al. (2007) reported that CYP450 activity in *M. galloprovincialis* differed according to changes in environmental factors such as water temperature and salinity. Yoon et al. (2003) also suggested that physical environmental factors such as water temperature changes affect the expression of CYP450 mRNA. In the present study, CYP450 mRNA was expressed in various tissues of Pacific oysters in response to rapid changes in water temperature. CYP450 mRNA expression increased significantly up to 6 h in all test groups at 30℃ and 10℃, and then decreased, stabilizing at the control levels. It is postulated that this increase in CYP450 mRNA expression resulted from unstable physiological metabolism due to

stress from rapid water temperature change. In addition, the increase in CYP450 mRNA expression over 6 h and subsequent decrease and stabilization may reflect the process of acclimation to high or low water temperatures. Therefore, in Pacific oysters exposed to high or low water temperatures, CYP450 mRNA expression stabilized after 6 h because of the water temperature compensation and metabolism control functions of CYP450. However, other studies have reported both a gradual increase in CYP450 activity in the hepatopancreas of fish relative to a water temperature decrease (Andersson and Förlin, 1992) and decreased CYP450 activity at low water temperatures in an estuarine fish (Stengeman and Hahn, 1994). Therefore, additional studies on the mechanism of CYP450 and water temperature change are needed.

RT-PCR was employed to observe CYP450 mRNA expression levels in various tissues of Pacific oysters, and expression was found to be highest in gill tissue. CYP450 mRNA expression in Pacific oyster gills exposed to rapid water temperature change increased significantly over 6 h and then decreased, indicating that CYP450 mRNA expression occurred as Pacific oysters became acclimated to water temperature changes. This finding can be used as a physiological index for Pacific oysters exposed to water temperature change.

Moreover, the present study compared the expression of HSP90 (the stress protein) and SOD (the antioxidant enzyme) to identify stress reactions of Pacific oysters to rapid changes in water temperature. RT-PCR indicated that HSP90 and SOD mRNA expression reached their highest levels in the gills of oysters exposed to high temperatures. High HSP90 and SOD mRNA expression must be induced because the gill, as the primary interface between the outside environment and the hemolymph or cytoplasm (Hosoi et al., 2007) and as the direct absorption route for seawater with a wide contact area, is influenced more directly by stress than other organs. Changes in HSP90 and SOD mRNA expression over time in oysters exposed to high water temperatures (30℃) were investigated using QPCR.

The expression of HSP90 mRNA increased significantly over 6 h of exposure to a water temperature of 30℃ (Fig. 14A). This concurs with reports that expression of the HSP family, including HSP90, increases with water temperature (Piano et al., 2004; Farcy et al., 2007). In a study of stress associated with water temperatures of 10-30℃ on Japanese abalone, *Haliotis discus hannai*, HSP mRNA expression increased in the test group for up to 6 h at 30℃ and then decreased after12 h (Kim et al., 2006). Kim et al. (2006) obtained results for HSP mRNA expression that were identical to the present study.

Similar to the results of the HSP90 experiments, SOD mRNA expression increased significantly over 6 h at a high water temperature (30℃) and then decreased (Fig. 14B). This concurs with a study of rapid water temperature stress on the *H. d. discus* (Kim et al., 2005). The present results are also in line with those of Cho et al. (2006b), who found that SOD mRNA expression in mudfish, *Misgurnus mizolepis*, exposed to high water temperatures increased through day 3. Regarding SOD mRNA expression, cellular function damage due to the generation of ROS from water temperature stress reached its highest levels at 6 h in oysters exposed to 30℃ water temperatures. Accordingly, it is postulated that SOD mRNA expression increased to remove the ROS.

In contrast, the osmolality in the hemolymph increased significantly over 24 h in the oysters exposed to high water temperatures (30℃) and then stabilized at control levels (Fig. 15). Franklin et al. (1991) reported that as a result of increasing the water temperature to 10℃ over 10 min, osmolality in bald rock cod, *Pagothenia borchgrevinki*, acclimated to a temperature of 0℃ increased significantly, before it decreased and stabilized. Meincke (1975) observed osmolality and ion changes in the hemolymph in the foot muscles of snail, *Helix pomatia*, upon rapidly increasing the temperature from 20℃ to 43℃. These results are consistent with changes in osmolality due to water temperature observed in the present study.

Analysis of the hemolymph of Pacific oysters exposed to water at 30℃ and 10℃ indicated that AST levels increased significantly for up to 6 h of exposure and then

decreased (Fig. 16). In Pacific oyster, various environmental factors, heavy metal contamination and pathogenic agents have been reported to alter hemolymph constituents (His et al., 1996; Xue and Tristan, 2000). Among these changes, an increase in AST activation is generally caused by inflow to the hemolymph of cells separated due to tissue damage by environmental contaminants (Pickwell and Steinert, 1988). Therefore, the significant increase in AST activation in the hemolymph of Pacific oyster over 6 h of exposure to thermal stress was induced as a result of tissue damage caused by the water temperature change.

H_2O_2 concentrations had increased significantly by 6 h in the hemolymph of oysters exposed to thermal stress, and then decreased (Fig. 17). Evidence exists that thermal stress induces the formation of ROS in the hemolymph of *C. gigas*. The ROS induced by thermal stress in organisms is widely known to promote oxidative stress, which causes membrane damage, DNA breakage, lipid peroxidation, enzyme inhibition, amino acid oxidation and apoptosis (Choi et al., 2007a).

The expression of HSP90 and SOD mRNA increased in Pacific oysters exposed to high water temperature. These results indicate that increases in the levels of stress and ROS caused by high water temperatures induced these genes. Moreover, the SOD then removed the ROS and protected the cells via the effects of HSP90. Osmolality and AST activity tended to stabilize after HSP90 expression, H_2O_2 concentrations tended to recover after SOD expression, and histological change was not detected in gill tissues. Given that expression levels stabilized to those of the controls, and no oysters died due to exposure, stress levels must have been reduced through several physiological defense mechanisms. Changes over time in gill tissues of Pacific oysters exposed to water temperature of 30℃ and 10℃ have been observed. Observations were conducted with an light microscope after dyeing the external tissues of the ordinary filaments, principal filament and hemolymph sinus of the plica, etc. using the H-E stain method (Fig. 18). The fine structures of the cilia, microvilli and mitochondria, etc., were also observed using TEM (Fig. 19). Considering

that no histological changes were observed in the gill tissues of Pacific oysters exposed to temperature change throughout the experiment, rapid changes in water temperature at least with the range 10℃ to 30℃ are considered not to cause damage to the gill tissues of Pacific oysters. Based on these results, it is concluded that CYP450, HSP90 and SOD genes are able to protect cells in an environment of rapid water temperature change, at least within the range 10℃ to 30℃.

Furthermore, the conditions examined in this study may represent the minimum water temperature required by Pacific oysters. The results of this study can be used as a foundation for establishing shellfish farming habitat.

Chapter 4

Characterization of antioxidant enzyme mRNA expression and changes in physiological hemolymph responses in Pacific oyster, *Crassostrea gigas*, in response to cadmium exposures

1. Introduction

Recently, heavy metal contamination has become prevalent along the southern coast of Korea, where farming of bivalve molluscs takes place. The contamination is caused by aging farming facilities, aquatic wastes, sediments from farmland and abandoned copper mines. Cadmium (Cd) is a heavy metal pollutant in the aquatic environment that is highly toxic to humans, even at low doses (Benavides et al., 2005). Cd is released to and accumulates not only in the uplands environment, but also in the aquatic environment, through industrial activities such as mining, plating and metal refining (Choi et al., 2007a).

Among aquatic organisms, bivalve molluscs, including oysters, have limited mobility compared to species such as fish and crustaceans, and are therefore directly exposed to Cd concentrations in their habitats. Bivalve molluscs also feed via filtration through the gills, through which a large amount of Cd can be absorbed from the environment and accumulated in the body (Philip, 1995). Excessive Cd accumulation in body tissues induces a variety of oxidative effects. Of these, abundant reactive oxygen species (ROS), e.g., superoxide radical (O^{-2}), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-) and singlet oxygen ($^1O_2$), are formed and promote oxidative stress (Stohs et al., 2000). Oxidative stress caused by ROS leads to lipid peroxidation, protein denaturation and DNA damage to the constituents of a living body. It also changes and inhibits a variety of enzyme activation effects and causes cell damage and an imbalance in cells, resulting in

apoptosis (Choi et al., 2007a). As a result, numerous physiological problems occur, including aging, lowered disease resistance and reduced reproductive ability (Kim and Phyllis, 1998).

Living organisms have antioxidant defense systems for protection against oxidative stress caused by heavy metals and other toxic substances. The antioxidant defense system consists of antioxidant enzymes such as SOD, CAT and GPX. These antioxidant enzymes play an important role in maintaining cellular homeostasis and in antioxidant defense by removing ROS (Rudneva, 1999). SOD is presentative enzyme providing defense against ROS. An important metalloenzyme, it is found in every oxygen-consuming living organism. SOD removes the superoxide radical through the process of dismutation to O_2 and H_2O_2 ($2O_2^- + H^+ \rightarrow H_2O_2 + O_2$) (Fridovich, 1975). The H_2O_2 produced from this process is reduced to H_2O and O_2 by CAT and GPX (Mruk et al., 2002). CAT is an oxidoreductase enzyme that breaks down two molecules of hydrogen peroxide into two molecules of water and oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$), therefore removing hydrogen peroxide and its associated toxicity (Kashiwagi et al., 1997). GPX is another important peroxidase enzyme in the detoxification of hydroperoxides; it catalyzes the reduction of hydroperoxide and hydrogen peroxide to water and oxygen (Swiergosz-Kowalewska et al., 2006). GPX is divided into two types: selenium-dependent GPX (Se-GPX) and selenium-independent GPX (non-Se-GPX). Se-GPX catalyzes the reduction of organic and inorganic peroxides, whereas non-Se-GPX reduces only organic peroxides (Almar et al., 1998).

The enzymatic antioxidant system that includes SOD, CAT and GPX is enhanced as an adaptation or a compensatory reaction to ROS formation or toxic effects (Livingstone et al., 1990). The induction of such an enzymatic antioxidant system is receiving much attention as a biomarker indicating oxidative stress in the environment. Using this system, studies to identify aquatic environmental contamination are being carried out in a variety of species, including bivalve molluscs.

Among such studies are those of the antioxidant system in relation to Cd contamination in a variety of fish species such as tilapia, *Oreochromis mossambicus* (Almeida et al., 2002) and *O. mossambicus* (Siraj and Usha, 2003), brown trout, *Salmo trutta* (Hansen et al., 2007), *D. labrax* (Romeo et al., 2000) and rock bream, *Oplegnathus fasciatus* (Cho et al., 2006a). In bivalve molluscs, studies are limited to mussels such as vent mussel, *Bathymodiolus azoricus* (Company et al., 2004), brown mussel, *Perna perna* (Almeida et al., 2004) and *M. galloprovincialis* (Viarengo, 1990). Thus far, no examination of oysters has been made. Also, most studies of antioxidant enzymes have examined the relationship of stressors with the activation of antioxidant enzymes (Brouwer and Brouwer, 1998; Geret et al., 2002); however, there are insufficient studies of the mRNA expression of antioxidant enzymes at the cellular level.

Therefore, in this study, CAT and GPX cDNA was cloned and sequenced, and changes in enzymatic properties (i.e., aspartate aminotransferase, AST and alanine aminotransferase, ALT) and hydrogen peroxide (H_2O_2) concentrations in the hemolymph were analyzed. Further observations were conducted using hematoxylin-eosin (H-E) stain method and TEM. Changes in the patterns of antioxidant enzyme (SOD, CAT and GPX) mRNA expression were also observed to identify the extent of oxidative stress caused by Cd to Pacific oyster.

2. Materials and Methods

2.1. Experimental oysters

One-year-old Pacific oyster (average shell length: 112±10.7 mm; height: 31.1±5.4 mm; weight: 20.3±3.9 g) were obtained from the oyster hatchery on Daebu Island in Goseong (Gyeongnam, Korea). These were placed in 40 L circulating filter tanks in the laboratory at 30 oysters per tank. During the experimental period, the water temperature and salinity were maintained at 20±1℃ and 35 psu, respectively, under a photoperiod of 12 h light/12 h dark, and no food was supplied.

2.2. $CdCl_2$ treatments and sampling

After acclimatization for 48 h in the tanks, 30 oysters were transferred to 30 L plastic aquaria filled with filtered natural seawater (control) or Cd-treated seawater. For Cd treatments, Cd was added to the water as $CdCl_2 \cdot 2.5H_2O$ (Kanto Chemical Co., Tokyo, Japan) to a dissolved Cd^{2+} concentration of 0.01, 0.05 or 0.1 ppm. Oysters were exposed to treatments for 11 days; the water was changed daily and resupplied with the corresponding concentration of Cd. No mortality was observed in either the Cd treatment or control group during the experimental period. The tissues (i.e., gill, digestive gland, intestine, mantle and adductor muscle) were sampled from randomly selected five oysters after 0, 1, 3, 7 and 11 days of treatments and stored at -80℃ until the extraction of total RNA.

2.3. Identification of CAT and GPX cDNA

Mixed primers for CAT were designed using highly conserved regions of edible mussel, *Mytilus edulis* (GenBank accession no. AY580271), Californianus mussel, *Mytilus californianus* (AY580259) and atlantic nutclam, *Nucula proxima* (AY5802 31) CAT mRNA: CAT forward primer (5´-GAT CCT ATG YTG TTY CCM

AGC-3′) and CAT reverse primer (5′-AGA AGG CKA TYT GTT CYA CYT C-3′). Mixed primers for GPX were designed using freshwater mussel, *Unio tumidus* (DQ830766), western clawed frog, *Xenopus tropicalis* (NM 203630) and black legged tick, *Ixodes scapularis* (DQ066177) GPX mRNA: GPX forward primer (5′-AAY GTR GCS ACM TAC TGA GG-3′) and GPX reverse primer (5′-CAG AAA CTT YTC RAA GTT CCA-3′). Total RNA was extracted from the gill of Pacific oyster using a Trizol kit (Gibco/BRL, Grand Island, NY, USA). With 2.5 ㎍ of total RNA as a template, complementary DNA (cDNA) were synthesized using M-MLV reverse transcriptase (Bioneer, Seoul, Korea). First-strand cDNA synthesis was conducted at 42℃ for 1 h using oligo-$d(T)_{15}$ primer (Promega, Madison, USA). PCR amplification was performed using a 2X Taq Premix I (Solgent, Daejeon, Korea), according to the manufacturer's instructions. The amplified PCR product was processed by electrophoresis on 1% agarose gels. The PCR product was purified and ligated into the pGEM-T Easy Vector (Promega). The colony formed by transformation was cultivated in DH5α, and plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Seoul, Korea) and EcoRI (Fermentas, Hanover, MD, USA). Based on the plasmid DNA, CAT and GPX partial cDNA sequence data were analyzed using an ABI DNA Sequencer (Appleid Biosystems, Foster city, CA, USA).

2.4. Rapid amplification of cDNA 5′/3′ ends (RACE)

For RACE reactions, total RNA was extracted from the gills of Pacific oyster. Using 3 μg of total RNA as template, 5′-RACE-ready cDNA and 3′-RACE-ready cDNA were generated using the protocols and reagents provided in the CapFishing Full-length cDNA Premix kit (Seegene, Seoul, Korea). Gene-specific primers were selected from the CAT and GPX partial cDNA of Pacific oyster obtained by PCR. For 3′-RACE, the 50 μL PCR reaction mixture contained 5 μL of 3′-RACE-ready

cDNA, 1 μL of 10 μM 3′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA-3′), 1 μL of 10 μM 3′ RACE gene-specific primer (3′ RACE CAT primer: 5′-GCG ACC TAC TGA GGT TTC ACC TAC CAG-3′, 3′ RACE GPX primer: 5′-CGA GGT CGG GAA GAT GGT GCT GAA CAG-3′) and 25 μL of SeeAmp Taq Plus Master Mix (Seegene). PCR was carried out for 40 cycles at 94℃ for 45 s for denaturation, 62℃ for 45 s for primer annealing, and 72℃ for 90 s for extension; followed by 5 min at 72℃ for extension. For 5′-RACE, the 50 μL PCR reaction mixture contained 5′-RACE-ready cDNA, 5′ gene-specific primer (5′ RACE CAT primer: 5′-CTG GTA GGT GAA ACC TCA GTA GGT CGC-3′, 5′ RACE GPX primer: 5′-CTA TAA CCG TCC GGT GTG CCT CGG TCT-3′), 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′) and SeeAmp Taq Plus Master Mix at the same volumes as for 3′-RACE under the same PCR conditions. Transformation and sequencing were conducted using the same methods described above. The DNA and deduced amino acid sequences were analyzed using GENETYX-WIN (Software Development, Tokyo, Japan) and the BLAST algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov/blast).

2.5. Phylogenetic analysis

Phylogenetic analysis was performed on the amino acid sequences from full-length cDNA of CAT and GPX in Pacific oyster (CAT and GPX) from various vertebrates and mollusks. Amino acid sequence data were aligned using the BioEdit Software (Hall, 1999). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) and analyzed using Mega 3.1 software package (Center for Evolutionary Functional Genomics, Scottsdale, AZ). The degree of support for internal branches was inferred using bootstrapping (1000 replicates) analysis.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was conducted to determine the relative expression of SOD, CAT and GPX mRNA and 28S ribosomal RNA (28Sr) in various oyster tissues. 2.5 μg of total RNA extracted from the gill, digestive gland, intestine, mantle and adductor muscle of Cd-treated oysters were reverse transcripted with M-MLV reverse transcriptase (Bioneer) and oligo-d$(T)_{15}$ primer (Promaga). Primers for RT-PCR were designed with reference to known SOD (AJ496219), CAT (EF687775), GPX (EF692639) and 28Sr (Z29546) gene sequences of Pacific oyster as follows: SOD forward primer (5′-ATG TCA TCT GCT CTG AAG GC-3′), SOD reverse primer (5′-TGG TGA TAC CGA TCA CTC CA-3′), CAT forward primer (5′-GAC CCC ATC CTG TTC CCC AGC-3′), CAT reverse primer (5′-AGA AGG CGA TCT GTT CCA CCT C-3′), GPX forward primer (5′-AAC GTA GCG ACC TAC TGA GG-3′), GPX reverse primer (5′-AAG AAA CTT CTC GAA GTT CCA-3′), 28Sr forward primer (5′-TGC TCT GGA CTG TCC TAG GA-3′) and 28Sr reverse primer (5′-ACC GAT TCG CCA CTG ACC AT-3′). PCR amplification was performed using a 2X Taq Premix I (Solgent, Daejeon, Korea), according to the manufacturer's instructions. The 28Sr was amplified in each PCR reaction as a loading control. The PCR products from different cycles of amplification were visualized on a UV-transilluminator after electrophoresis on 1% agarose gel containing ethidium bromide (0.5 μg/μL). The signal intensity was quantified using the Gel-Doc System and Gelpro 3.1 software (KBT, Incheon, Korea). The cycle numbers that generate half-maximal amplification were used for subsequent quantitative analysis of gene expression, and they are 30 cycles for SOD, CAT and GPX, 25 cycles for 28Sr.

2.7. Quantitative real-time PCR (QPCR)

QPCR was conducted to determine the relative mRNA expression of antioxidant enzymes (SOD, CAT and GPX) using total RNA extracted from the gills of control and Cd-treated oysters. With 2.5 μg of total RNA as a template, cDNA were synthesized using M-MLV reverse transcriptase (Bioneer). First-strand cDNA synthesis was conducted using oligo-d$(T)_{15}$ primer (Promega). Primers for QPCR were designed with reference to known SOD (AJ496219), CAT (EF687775), GPX (EF692639) and 28Sr (Z29546) gene sequences of Pacific oyster as follows: SOD forward primer (5´-CTC CTG GAA CAC CTG TGA CAT TG-3´), SOD reverse primer (5´-GTG CCT CTC GTG ATC CTC TGG-3´), CAT forward primer (5´-AAC TAC TTC GCT GAG GTG-3´), CAT reverse primer (5´-GGT CTT GGC TTT GTA TGG-3´), GPX forward primer (5´-GAC CGT GGA ACC AAT GGA CAT C-3´), GPX reverse primer (5´-GTT GGA TTC GGA CAC AGA TAG GG-3´), 28Sr forward primer (5´-AAA CAC GGA CCA AGG AGT CT-3´) and 28Sr reverse primer (5´-AGG CTG CCT TCA CTT TCA TT-3´). QPCR amplification was conducted using a Bio-Rad MiniOpticon^{TM} System (Bio-Rad, Hercules, CA, USA) and iQ^{TM} SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. QPCR was undertaken by denaturation at 95℃ for 5 min; followed by 40 cycles of denaturation at 95℃ for 20 s and annealing at 55℃ for 20 s. To ensure that the primers amplified a specific product, It was performed a melt curve, as well as analyzed the PCR product size using capillary electrophoresis (Agilent Technologies, Santa Clara, CA). All primers used were shown to amplify only one size of template, melting at only one temperature. PCR products were also confirmed by sequencing. All analyses were based on the calculated threshold Ct values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR program, QPCR data from five

replicate samples were analyzed with analysis software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. mRNA expression levels stood for an n-fold difference relative to 28Sr as the internal control.

2.8. Hemolymph osmolality, and AST and ALT activity

Hemolymph was withdrawn from the pericardial cavity using a 3 mL syringe. The samples were centrifuged at 10,000 ×g at 4℃ for 5 min, and the supernatant was stored at -80℃ until analysis. The activity of AST and ALT was measured using Pureauto S AST (Daichi, Tokyo, Japan) and Pureauto S ALT (Daichi) kit by biochemistry autoanalyzer (model 7180; Hitachi, Tokyo, Japan), according to the manufacturer's instructions.

2.9. Hydrogen peroxide assays

H_2O_2 concentrations were measured using the modified methods of Nouroozzadeh et al. (1994) and a Peroxidetect kit (Sigma-Aldrich, St. Louis, Missouri, USA). 20 $\mu\ell$ of whole oyster hemolymph in marine anticoagulant (MAC: 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 50 mM EDTA, 0.45 M sodium chloride, pH 7.5) was added per well to flat bottom 96 well microtitre plates. Plates were left at room temperature for 20 min to allow hemocytes to settle and adhere. A working color reagent was prepared by mixing 100 mL distilled water containing 100 mM sorbitol and 125 uM xylenol orange (Sigma-Aldrich) with 1 mL of 25 mM ferrous ammonium sulphate prepared in 2.5 M sulphuric acid (Sigma-Aldrich). Two hundred microlitres of this reagent was then added to each well and allowed to incubate at room temperature for 1 h. Absorbance was read at 560 nm and concentrations of H_2O_2 were interpolated from a standard curve. Concentrations are expressed as nM/mL.

2.10. Histological analysis

2.10.1. Observation with light microscope

To observe histological changes in the gill tissues of Pacific oyster exposed to Cd, the extracted gill tissues were fixed in 10% neutral formalin solution (100 mL formalin, 6.5 g $Na_2HPO_412H_2O$, 4.5 g KH_2PO_4, 900 mL distilled water) for 1 day. Then, the gill tissues were fixed in bouin solution and a tissue sample was produced according to the series of methods. The fixed sample was cut into serial sections of 5 ㎛ in thickness by paraffin sectioning. Then, the degree of tissue damage was examined with an light microscope after double dyeing with Harris's hematoxylin and 0.5% eosin (Sigma, USA). The degree of damage in gill tissues was photographed by using image analysis system (Axiovision, Zeiss Co., Germany) with a biological microscope (Axioskop, Zeiss, Germany).

2.10.2. Observation with electron microscope

A sample for TEM was produced in order to observe the fine structure of the gill tissues of Pacific oyster exposed to Cd. After extracting gill tissues, primary fixation was carried out for 2 h in 2.5% glutaraldehyde solution (4℃) buffed with 0.1 M phosphate buffer solution (PBS, pH 7.2). The sample completed of fixation was washed for 10 min with PBS and was processed through secondary fixation for 2 h at 4℃ in 1% osmium tetroxide (OsO_4). After fixation was completed, the sample was washed with PBS and was dehydrated for 15 min each in 50-100% ethanol. After dehydration, the sample was placed in the mixture of propylene oxide and Epon and was polymerized for 1-3 h. Then the sample was embedded in Epon 812. The embedded tissue was semi-thinly sectioned with the thickness of 1.0 ㎛ by using ultramicrotome (Leica, Reichert SuperNova, Sweden). Then, it was dyed with 1% toluidine blue and the part for observation was determined. After the part for observation was determined, the sample was again sectioned with the thickness of 70 mm and was attached to 200 mesh copper grid. The sample completed of ultra-thin sectioning was double-dyed with uranylacetate and lead citrate solution and was

observed with a transmission electron microscope (JEM 1200 EX-Ⅱ, 60-80 kv, JEOL, Tokyo, Japan).

2.11. Statistical analysis

Treatment differences were tested using one-way ANOVA followed by the Tukey or LSD test, using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA) at a significance level of $P<0.05$.

3. Results

3.1. Identification of CAT cDNA

RT-PCR was used to clone a fragment of CAT using total RNA extracted from *C. gigas* gills, in which tissue the expression was highest. A single PCR product of the expected size (535 bp) was obtained. A PCR-based cloning strategy (RT-PCR followed by 5′ and 3′ RACE) was used to clone a full-length cDNA encoding a putative CAT from the gill of *C. gigas*. CAT full-length cDNA contained 1988 nucleotides, including an open-reading frame of 1548 nucleotides (position 143-1690) that was predicted to encode a protein of 516 amino acids. The cDNA sequence of the CAT gene was deposited in NCBI/GenBank under accession number EF687775. The deduced amino acid sequence of *C. gigas* CAT was compared to those deduced from known CAT cDNA of other vertebrates and mollusks (Fig. 20). The *C. gigas* CAT had high amino acid sequence identity with other mollusks: 74% identity to *C. farreri* CAT (DQ862859) and 72% identity to *H. d. discus* CAT (DQ530211). Also, *C. gigas* CAT was similar to CATs from vertebrates: 69% identity to wrinkled frog, *Rana rugosa* CAT (AB031872), 68% to *H. sapiens* CAT (AY028632), and 65% to *R. norvegicus* (NM 012520), *D. rerio* (NM 130912) and *O. fasciatus* (AY734528) CATs. All of the catalytic amino acids (His72, Asn145 and Tyr355) are conserved in all species. The catalase proximal heme ligand signature sequence RLFSYNDTH (residues 351-359) and the proximal active site signature FDRERIPERVVHAKGAGA (residues 61-68) are also highly conserved for both amino acid composition and location in *C. gigas* CAT (Fig. 20). A phylogenetic tree was constructed to further analyze the evolutionary relationships among various vertebrate and mollusk CAT sequences. The *C. gigas* CAT was found to cluster with the CAT of *C. farreri* and *H. d. discus* (Fig. 21).

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C. gigas          1:M-STRDKATEQLNEFK-LSHA-TPEQCTTGTGAPIGLKTATMTAGPLGPVLVQDFVFNDE 57
C. farreri        1:M-ANRDKATNQLEEF--KKAQSKADVLTTGTGAPVGTKTATLTAGPRGPVLIQDFTFTDE 57
H. d. discus      1:M-ATRDKASEQLNEF--SKGQKKPDVLTTGTGAPVGRKTATMTVGPQGPVLLQDFVFTDE 57
D. rerio          1:MADDREKSTDQMKLWKEGRGSQRPDVLTTGAGVPIGDKLNAMTAGPRGPLLVQDVVFTDE 60
O. fasciatus      1:MADNRGKATDQMKTWKENRSSQRPDTLTTGAGHPVGDKLNLQTAGPRGPLLVQDVVFTDE 60
R. rugosa         1:MADRREKSADQMKLWKESRANQKPDVLTTGGGNPVSDKLNLLTVGPRGPLLVQDVVFTDE 60
R. norvegicu      1:MADSRDPASDQMKQWKEQRAPQKPDVLTTGGGNPIGDKLNIMTAGPRGPLLVQDVVFTDE 60
H. sapiens        1:MADSRDPASDQMQHWKEQRAAQKADVLTTGAGNPVGDKLNVITVGPRGPLLVQNVVFTDE 60
                     *   *     *                 *** * *   *    * ** ** * *   * **

C. gigas         58:MAHFDRERIPERVVHAKGAGAFGYFECTHDISKYTKAKPFESVGKKTPVGVRFSTVGGES 117
C. farreri       58:MAHFNRERIPERVVHAKGGGAFGYFEVTHDITKYCKAKPFEFVGKKTPVGIRFSTVGGES 117
H. d. discus     58:MAHFNRERIPERVVHAKGAGAFGYLEITHDITKYCKAKVFERVGKKTPLAIRFSTVGGEK 117
D. rerio         61:MAHFDRERIPERVVHAKGAGAFGYFEVTHDITRYSKAKVFEHVGKTTPIAVRFSTVAGEA 120
O. fasciatus     61:MAHFDRERIPERVVHAKGAGAFGYFEVTHDITRYCKAKVFEHVGKTTPIAVRFSTVAGES 120
R. rugosa        61:MAHFDRERIPERVVHAKGAGAFGYFEVTHDITRYSKAKVFEFIGKRTPIAVRFSTVAGEA 120
R. norvegicu     61:MAHFDRERIPERVVHAKGAGAFGYFEVTHDITRYSKAKVFEHIGKRTPIAVRFSTVAGES 120
H. sapiens       61:MAHFDRERIPERVVHAKGAGAFGYFEVTHDITKYSKAKVFEHIGKKTPIAVRFSTVAGES 120
                    **** ************* ***** * ****  * *** **  ** **   ***** **

C. gigas        118:GSADTARDPRGFAVKMYTEDGNWDIVGNNTPIFFIRDPILFPSFIHTQKRNPRTHLKDPD 177
C. farreri      118:GSADSARDPRGFAVKFYTEDGNWDVVGNNTPIFFIRDPMLFPNFIHTQKRNPQTHLKDPD 177
H. d. discus    118:GSADTARDPRGFAIKFYTEDGNWDLVGNNTPIFFIRDPMLFPSFIHTQKRNPVTNLKDPD 177
D. rerio        121:GSSDTVRDPRGFAVKFYTDEGNWDLTGNNTPIFFIRDTLLFPSFIHSQKRNPQTHLKDPD 180
O. fasciatus    121:GSADTVRDPRGFAVKFYSEEGNWDLTGNNTPIFFIRDALLFPSFIHSQKRNPQTHMKDPD 180
R. rugosa       121:GSADTVRDPRGFAVKFYTEDGNWDLTGNNTPIFFVRDAMLFPSFIHSQKRNPQTHMKDPD 180
R. norvegicu    121:GSADTVRDPRGFAVKFYTEDGNWDLVGNNTPIFFIRDAMLFPSFIHSQKRNPQTHLKDPD 180
H. sapiens      121:GSADTVRDPRGFAVKFYTEDGNWDLVGNNTPIFFIRDPILFPSFIHSQKRNPQTHLKDPD 180
                    ** *  ******* * *   ****  ******** **  *** *** ***** *  ****

C. gigas        178:MFWDFISLRPETTHQVSFLFSDRGTPDGYRRMNGYGSHTFKLVNKDDKPVFCKFHFKTDQ 237
C. farreri      178:MFWDFISLRPETTHQVSFLFSDRGTPNGFRKMNGYGSHTFKMVNKEGKPVYCKFHFKTDQ 237
H. d. discus    178:MFWDFITLRPETTHQVAFLFSNRGTPDGYRHMNGYGSHTFKMVNAKGECVYCKFHFKTNQ 237
D. rerio        181:MVWDFWSLRPESLHQVSFLFSDRGLPDGYRHMNGYGSHTFKLVNAQGQPVYCKFHYKTNQ 240
O. fasciatus    181:MVWDFWSLRPESLHQVSFLFSDRGLPDGYRHMNGYGSHTFKLVNAAGERFYCKFHFKTDQ 240
R. rugosa       181:MVWDFWALRPESLHQVSFLFSDRGIPDGHRHMNGYGSHTFKLVNAKDEPIYCKFHFKTDQ 240
R. norvegicu    181:MVWDFWSLCPESLHQVTFLFSDRGIPDGHRHMNGYGSHTFKLVNANGEAVYCKFHYKTDQ 240
H. sapiens      181:MVWDFWSLRPESLHQVSFLFSDRGIPDGHRHMNGYGSHTFKLVNANGEAVYCKFHYKTGQ 240
                    * ***  * **  *** **** ** * * * * ********** **       **** ** *

C. gigas        238:GIQNLSAAEANRLSAEDPDYAIRDLYNNIEDGKYPSWTLKIQIMTPEQAEKYKWNPFDVT 297
C. farreri      238:GIKNLMADQAAELSKNDPDYAIRDLFNAISEGDFPSWSLFIQVMTFEEAEKFKYNPFDLT 297
H. d. discus    238:GIKNLTGAQADKLASVDPDYATRDLYNAIAEGKYPSWSLFIQVMNVKDAEKLKWNPFDLT 297
D. rerio        241:GIKNIPVEEADRLAATDPDYSIRDLYNAIANGNFPSWTFYIQVMTFEQAENWKWNPFDLT 300
O. fasciatus    241:GIKNLPVEEADRLASTNPDYAIGDLFNAIANGNCPSWTFYIQIMTFEQAEKFRFNPFDLT 300
R. rugosa       241:GIRNLTVEEANRLSAEDPDYGIHDLYEAIANGNYPSWTFYIQVMTFEQAERYPFNPFDLT 300
R. norvegicu    241:GIKNLPVEEAGRLAQEDPDYGLRDLFNAIASGNYPSWTFYIQVMTFKEAETFPFNPFDLT 300
H. sapiens      241:GIKNLSVEDAARLSQEDPDYGIRDLFNAIATGKDPSWTFYIQVMTFNQAETFPFNPFDLT 300
                    ** *     *  *    ***   **   *  *  ***   ** *    **    **** *
C. gigas        298:KVWSQKDYPLIEVGKMVLNRNPNNYFAEVEQIAFSPAHFIPGVEASPDKMLQGRLFSYSD 357
C. farreri      298:KVWPQGEYPLIPVGRMVLNRNPKNYFAEVEQIAFSPAHMIPGIEASPDKMLQGRLFSYSD 357
H. d. discus    298:KVWPHGEYPLIPVGRMVLDKNPKNYFADVEQIAFSPAHMVTGIEASPDKMLQGRLYSYSD 357
D. rerio        301:KVWSHKEFPLIPVGRFVLNRNPVNYFAEVEQLAFDPSNMPPGIEPSPDKMLQGRLFSYPD 360
O. fasciatus    301:KIWSHKEYPLIPVGKMVLNRNPVNYFAEVEQLAFDPSNMPPGIEPSPDKMLQGRLFSYPD 360
R. rugosa       301:KIWPHKDYPLIPVGKLVLNRNPANYFAEVEQIAFDPSNMPPGIEPSPDKMLQGRLFSYPD 360
R. norvegicu    301:KVWPHKDYPLIPVGKLVLNRNPANYFAEVEQMAFDPSNMPPGIEPSPDKMLQGRLFAYPD 360
H. sapiens      301:RVWPHKDYPLIPVGKLVLNRNPVNYFAEVEQIAFDPSNMPPGIEASPDKMLQGRLFAYPD 360
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C. gigas      358:THRHRLGANYLQIPVNCPYKAKTFHYQRDGPQCVNDNQGGAPNYFPNSFSGPMDNPVG-C 416
C. farreri    358:THRHRLGSNYLQLAVNCPFNTKAKNYQRDGPQCVGDNQGNAPNYFPNSFSGPQDNKQFLE 417
H. d. discus  358:THRHRLGSNYLQLPVNCPYNTRLSNYQRDGPQCVDNNQGGAPNYFPNSFSGPQEESKCME 417
D. rerio      361:THRHRLGANYLQLPVNCPYRTRVANYQRDGPMCMHDNQGGAPNYYPNSFSAPDVQPRFLE 420
O. fasciatus  361:THRHRLGANYLQIPVNCPFRARVTNYQRDGPMCMFDNQGGAPNYYPNSFSAPETQPQFVE 420
R. rugosa     361:THRHRLGANYLQLPVNCPYKARVANYQRDGPMCFSDNQGGAPNYFPNSFSAPENQPAARE 420
R. norvegicu  361:THRHRLGPNYLQIPVNCPYRARVANYQRDGPMCMHDNQGGAPNYYPNSFSAPEQQGSALE 420
H. sapiens    361:THRHRLGPNYLHIPVNCPYRARVANYQRDGPMCMQDNQGGAPNYYPNSFGAPEQQPSALE 420
                   ******* ***   ****       ****** *   *** **** ****  *

C. gigas      417:-ESCPFTTTGECRRYNSVD-ED--NFSQVGIF-WNQV-LKPEERDRL-VENIGNHLINTQ 469
C. farreri    418:-SP--FSITGDVQRYETGD-ED--NFSQVTVF-WNKV-LKPEERQRL-VENIAGHLKNAQ 468
H. d. discus  418:-CP--FKLSGDVARYSTED-ED--NFSQAGIF-WKKV-LPPGERDHL-INNLAGHIINAQ 468
D. rerio      421:SKCKVSPDVARYNSADDDNVTQVRTFFTQVLNEAERERLCQNMAGHLKGAQLFIQKRMVQ 480
O. fasciatus  421:SKFKVSPDVARYNSADEDNVTQVRTFYTQVLNEEERQRLCQNMAGALKGAQLFIQKRMVE 480
R. rugosa     421:SKFRVSADVARYNSSDDDNVSQVRDFYTKVLSEEERKRLCENIAGHLKGAQIFIQKRAVK 480
R. norvegicu  421:HHSQCSADVKRFNSANEDNVTQVRTFYTKVLNEEERKRLCENIANHLKDAQLFIQRKAVK 480
H. sapiens    421:HSIQYSGEVRRFNTANDDNVTQVRAFYVNVLNEEQRKRLCENIAGHLKDAQIFIQKKAVK 480
                                          *           *       *

C. gigas      470:KLIRDRAVKNFGRADPEFGRKLQAHLDSVSNVSKINVVLNGVKMSDK-             516
C. farreri    469:EFIQRRTVHNFTQVHPDFGGGIQKLLNSYKKQSAMSAQL---------             507
H. d. discus  469:EFIQKRAVANFGKADPSSAVAC-RLLST-P------------------             496
D. rerio      481:NLMAVHSDYGNRVQALLDKHNAEGKKN-TVHVYSRGGASAVA-AASKM             526
O. fasciatus  481:NLKAVHPDYGNRVQTLLNKYNAEAQKNTTVHVYSRPGASAIA-ASSKM             527
R. rugosa     481:NFTDVHPDYGNRVQALLDKYNAEGHHKKVIKTYTQHSAHVTANDKANL             528
R. norvegicu  481:NFTDVHPDYGARVQALLDQYN-SQKPKNAIHTYVQAGSHIAAKGKANL             527
H. sapiens    481:NFTEVHPDYGSHIQALLDKYN-AEKPKNAIHTFVRSGSHLVAREKANL             527
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Fig. 20. Multiple alignment of the CAT amino acids of Pacific oyster, *Crassostrea gigas* (GenBank accession no. EF687775), *Chlamys farreri* (DQ862859), *Haliotis discus discus* (DQ530211), *Danio rerio* (NM 130912), *Rana rugosa* (AB031872), *Oplegnathus fasciatus* (AY734528), *Rattus norvegicus* (NM 012520) and *Homo sapiens* (AY028632). The catalase proximal heme ligand signature (RLFSYNDTH) is bold and doublelined. The proximal active site signature (FDRERIPERVVHAKGAGA) is bold and underlined. The conserved catalytic amino acids are bold and boxed. Identical amino acids among the different species are indicated by asterisks.

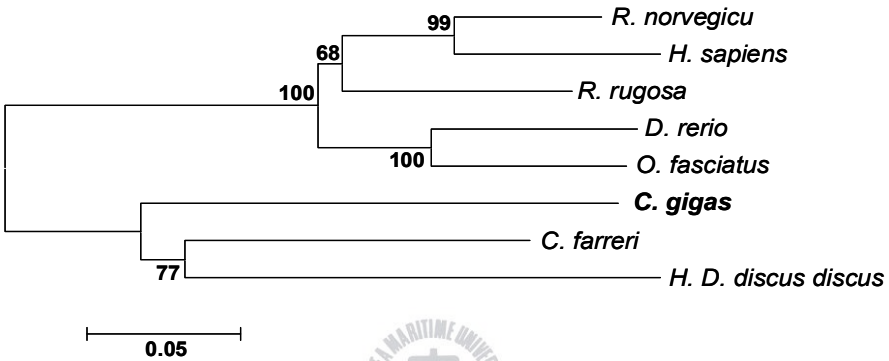

Fig. 21. An unrooted phylogeny showing the most likely relationship between representative CAT amino acids sequences. The number associated with each internal branch is the local bootstrap probability. The CAT sequences are Pacific oyster, *Crassostrea gigas* (GenBank accession no. EF687775), *Chlamys farreri* (DQ862859), *Haliotis discus discus* (DQ530211), *Danio rerio* (NM 130912), *Rana rugosa* (AB031872), *Oplegnathus fasciatus* (AY734528), *Rattus norvegicus* (NM 012520) and *Homo sapiens* (AY028632).

3.2. Identification of GPX cDNA

RT-PCR was used to clone a fragment of GPX using total RNA extracted from *C. gigas* gills, in which tissue the expression was highest. A single PCR product of the expected size (308 bp) was obtained. A PCR-based cloning strategy (RT-PCR followed by 5′ and 3′ RACE) was used to clone a full-length cDNA encoding a putative GPX from the gill of *C. gigas*. GPX full-length cDNA contained 1310 nucleotides, including an open-reading frame of 732 nucleotides (position 22-756) that was predicted to encode a protein of 244 amino acids. The cDNA sequence of the GPX gene was deposited in NCBI/GenBank under accession number EF692639. The sequence shows preservation of the active site containing selenocysteine at the 80th amino acid encoded by the TGA codon, as well as the other two residues of Glu112 and Trp187 in *C. gigas* GPX. The deduced amino acid sequence of GPX was compared to those deduced from known GPX cDNA of other vertebrates and mollusks (Fig. 22). The *C. gigas* GPX had little amino acid identity with other bivalves: 30% identity to *U. tumidus* GPX (DQ830766) and 29% identity to *D. polymorpha* GPX (EF194204). The cgGPX was similar to GPXs from vertebrates: 33% identity to *X. tropicalis* GPX (NM 203630), 27% to *H. sapiens* (M83094) and *R. norvegicus* (S41066), and 20% to *G. gallus* (AF498316). A phylogenetic tree was constructed to further analyze the evolutionary relationships among bivalve GPX sequences (Fig. 23). The *C. gigas* GPX was found to cluster with the GPX of *U. tumidus* and *D. polymorpha*.

Fig. 22. Multiple alignment of the GPX amino acids of Pacific oyster, *Crassostrea gigas* (GenBank accession no. EF692639), *Dreissena polymorpha* (EF194204), *Unio tumidus* (DQ830766), *Xenopus tropicalis* (NM 203630), *Gallus gallus* (AF498316), *Rattus norvegicus* (S41066) and *Homo sapiens* (M83094). The conserved selenocysteine (encoded by TGA codon) are boxed, and catalytic reduces Glu and Trp are shaded. Identical amino acids among the different species are indicated by asterisks.

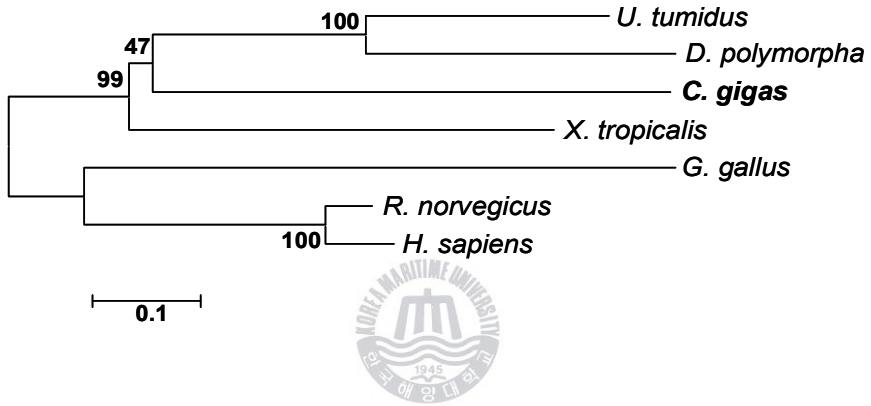

Fig. 23. An unrooted phylogeny showing the most likely relationship between representative GPX amino acids sequences. The number associated with each internal branch is the local bootstrap probability. The GPX sequences are Pacific oyster, *Crassostrea gigas* (GenBank accession no. EF692639), *Dreissena polymorpha* (EF194204), *Unio tumidus* (DQ830766), *Xenopus tropicalis* (NM203630), *Gallus gallus* (AF498316), *Rattus norvegicus* (S41066), and *Homo sapiens* (M83094).

3.3. Tissue distribution of SOD, CAT and GPX mRNA by RT-PCR

The expression of antioxidant enzymes (SOD, CAT and GPX) mRNA in various tissues from Cd-treated oyster measured by RT-PCR (Fig. 24). The antioxidant enzymes mRNA were detected in all tissues tested and was highly expressed in gill. In contrast, digestive gland, intestine, mantle and adductor muscle had low expression.

3.4. Hemolymph osmolality, and AST and ALT activity

The levels of AST and ALT in the hemolymph increased significantly by 7 days at Cd concentrations of 0.05 (AST, 3.7±1.5 IU/L; ALT, 6.3±3.1 IU/L) and 0.1 ppm (AST, 4.3±1.4 IU/L; ALT, 8.0±3.0 IU/L) (Table 1).

H_2O_2 concentrations significantly increased in the hemolymph in a dose- and time-dependent manner of Cd treatment (Fig. 25). H_2O_2 concentrations increased with time and reached the highest level after 11 days with exposure to 0.01 (4.0±0.3 nM/mL), 0.05 (6.0±0.6 nM/mL) and 0.1 ppm Cd (10.5±0.8 nM/mL) from 2.4±0.3 nM/mL at the beginning of the trial. The maximal response of H_2O_2 concentrations was observed at the highest dose of Cd tested (0.1 ppm).

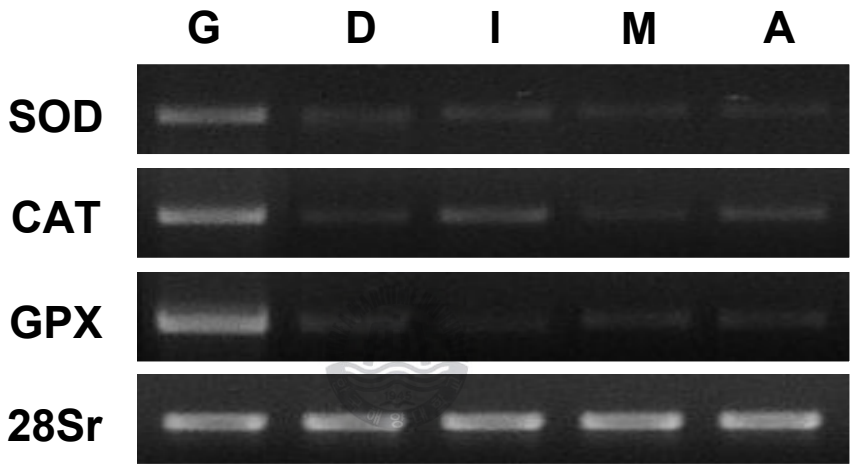

Fig. 24. Tissue-specific expression of SOD, CAT and GPX mRNA in various tissues (gill, G; digestive gland, D; intestine, I; mantle, M; adductor muscle, A) from Pacific oyster, *Crassostrea gigas* by RT-PCR. Amplification of 28S ribosomal RNA was used as an internal control.

Table 1. Changes in AST and ALT in Pacific oyster, *Crassostrea gigas* hemolymph exposed to cadmium for 11 days

Parameter	Cd concentration	Duration of Cd treatment (days)				
		Control	1	3	7	11
AST (IU/L)	0 (control)	1.0±0.4	1.6±0.4	1.5±0.6	1.5±0.6	1.3±0.8
	0.01 ppm	1.2±0.7	1.2±0.7	1.9±0.8	1.1±0.9	1.2±0.7
	0.05 ppm	1.0±0.5	0.9±0.6	1.2±0.3	3.7±1.5*	2.5±1.0
	0.1 ppm	1.2±0.4	1.7±0.6	1.3±1.0	4.3±1.4*	2.2±0.8
ALT (IU/L)	0 (control)	0.6±0.5	0.7±0.3	0.7±0.2	0.7±0.6	0.7±0.6
	0.01 ppm	0.7±0.6	0.7±1.2	2.0±1.0	2.3±1.5	1.3±1.5
	0.05 ppm	0.7±0.6	0.3±0.6	1.0±1.7	6.3±3.1*	3.7±2.5
	0.1 ppm	0.9±0.1	1.7±1.2	2.0±1.0	8.0±3.0*	4.0±3.6

*Asterisk indicates a significant difference from the control (P<0.05).

Values indicate the mean±SD (n = 5)

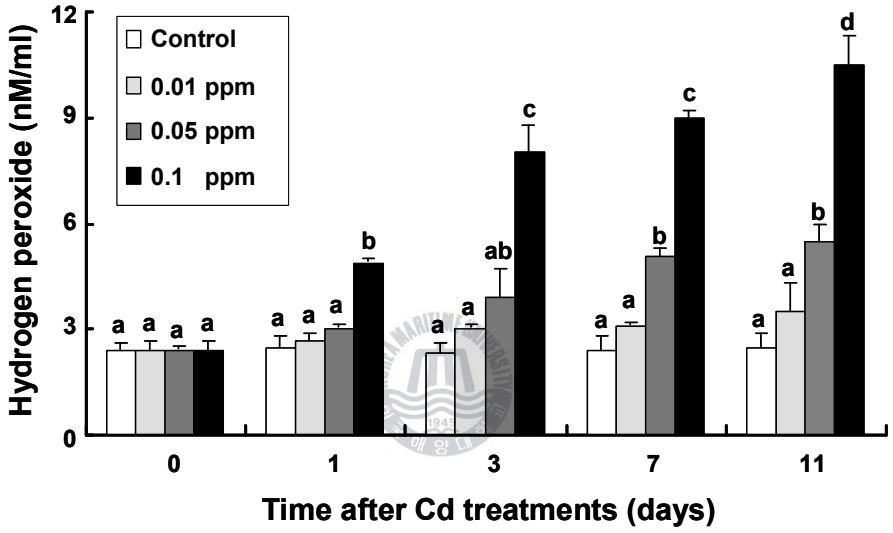

Fig. 25. Effect of Cd exposure on the hydrogen peroxide (H_2O_2) concentrations in hemolymph of Pacific oyster, *Crassostrea gigas* at the different concentration. Oysters were treated with 0 (control), 0.01, 0.05 or 0.1 ppm Cd for 1, 3, 7 or 11 days (mean±SD, n = 5). Different lowercase letters indicate significant differences ($P < 0.05$) among times after treatments within Cd concentrations.

3.5. Levels of SOD, CAT and GPX transcripts

Cd treatment significantly increased antioxidant enzyme mRNA expression in the gill in a dose- and time-dependent manner (Fig. 26). The maximal response was observed at the highest dose of Cd tested (0.1 ppm). SOD mRNA expression increased with time and reached the highest level after 11 days with exposure to 0.01 and 0.05 ppm Cd. It significantly increased until 7 days and then decreased with exposure to 0.1 ppm Cd. The SOD mRNA level was maximal on 7 days (91 times higher than the control; $P<0.05$) for 0.1 ppm Cd. Also, CAT and GPX mRNA expression in 0.01 and 0.05 ppm Cd significantly increased with time and reached the highest level at 11 days, whereas CAT and GPX mRNA increased until 3 days then decreased with exposure to 0.1 ppm Cd. The CAT and GPX mRNA level was maximal on 3 days (CAT, 19 times higher than the control; GPX, 8 times higher than the control; $P<0.05$) for the highest dose of Cd tested (0.1 ppm).

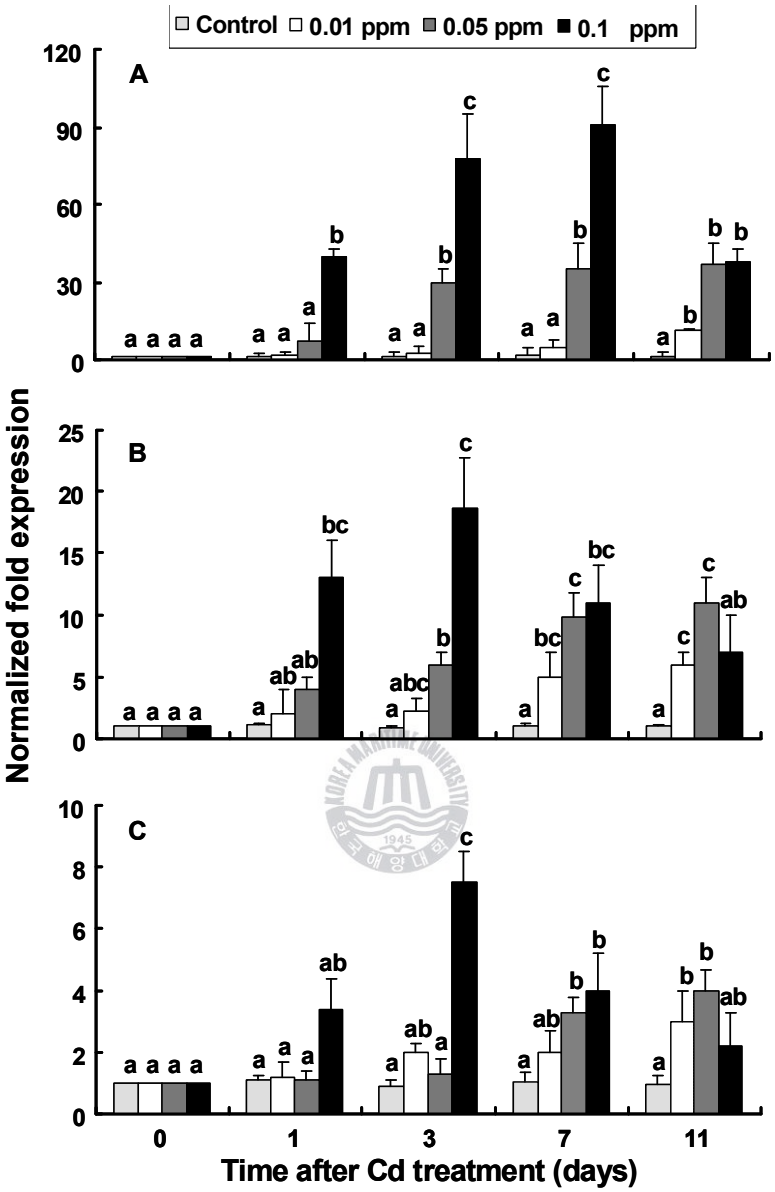

Fig. 26. Expression of antioxidant enzymes mRNA in response to cadmium treatment in Pacific oyster, *Crassostrea gigas* (A) SOD, (B) CAT and (C) GPX. Oysters were treated with 0 (control), 0.01, 0.05 or 0.1 ppm Cd for 1, 3,7 or 11 days. The antioxidant enzymes mRNA expression levels relative to 28S rRNA levels were analyzed using real-time PCR. Different lowercase letters indicate significant differences ($P < 0.05$) among times after treatments within Cd concentrations. Values indicate the mean ± SD (n = 5).

3.6. Histological analysis

3.6.1. Observation with light microscope

The external shapes of the gill tissues in Pacific oysters exposed to Cd by H-E stain method and found no histological change in the control group and the gill tissues of Pacific oysters until day 7 of exposure to 0.1 ppm of Cd was observed (Fig. 27). Also, no histological change was observed in the gill tissues of Pacific oysters exposed to 0.1 ppm of Cd on day 11 of exposure, the completion of experiment, and of Pacific oysters exposed to the low Cd concentrations of 0.01 and 0.05 ppm.

3.6.2. Observation with electron microscope

The internal structures of the gill tissues in Pacific oysters exposed to Cd by using TEM and found no histological change at the concentration of 0.01 ppm in comparison to control group was observed. At the concentration of 0.05 ppm, dilations of microvilli on day 11 of exposure were started observing (Fig. 28). Gill tissues of Pacific oysters exposed to the concentration of 0.1 ppm decreased in the count of cilia over time, while size and count of microvilli dilations increased (Fig. 29). On day 1 of exposure to 0.1 ppm of Cd, cilia count began decreasing. Also, a few areas were found without any cilium and dilations of microvilli in these areas were observed (Fig. 29C). On day 3 of exposure to 0.1 ppm of Cd, a number of areas without cilia were observed. In areas both with and without cilia, dilations of microvilli were observed (Fig. 29D). On day 7 of exposure to 0.1 ppm of Cd, the count of cilia decreased and a large number of microvilli dilations were observed (Fig. 29E). On day 11 of exposure to 0.1 ppm of Cd, the count of cilia rapidly decreased and microvilli dilations were mostly observed (Fig. 29F).

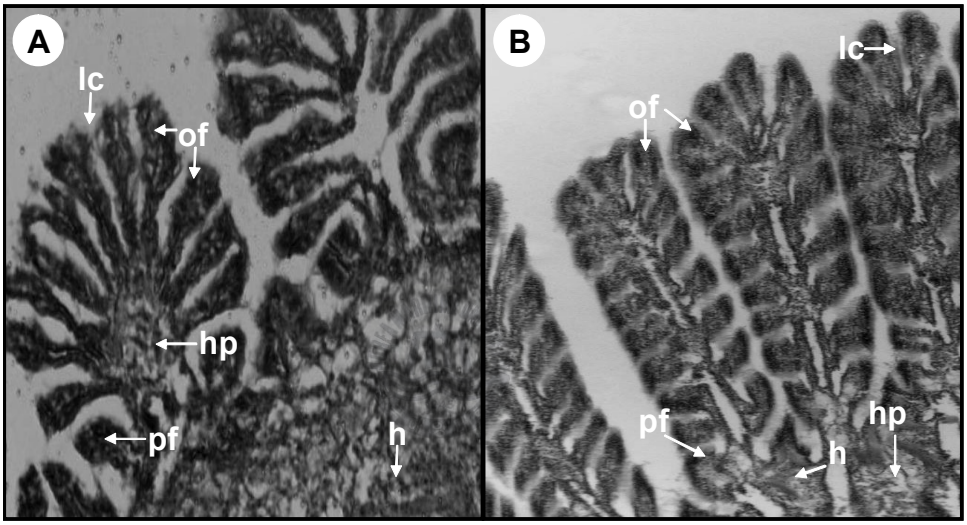

Fig. 27. Photomicrographs of gill filaments and lamellae of Pacific oyster exposed to cadmium (0.1 ppm). A: control, B: 7 days, of: ordinary filament, pf: principal filament, lc: lateral cilia, h: hemocytes, hp: hemolymph sinus of plica. X 200.

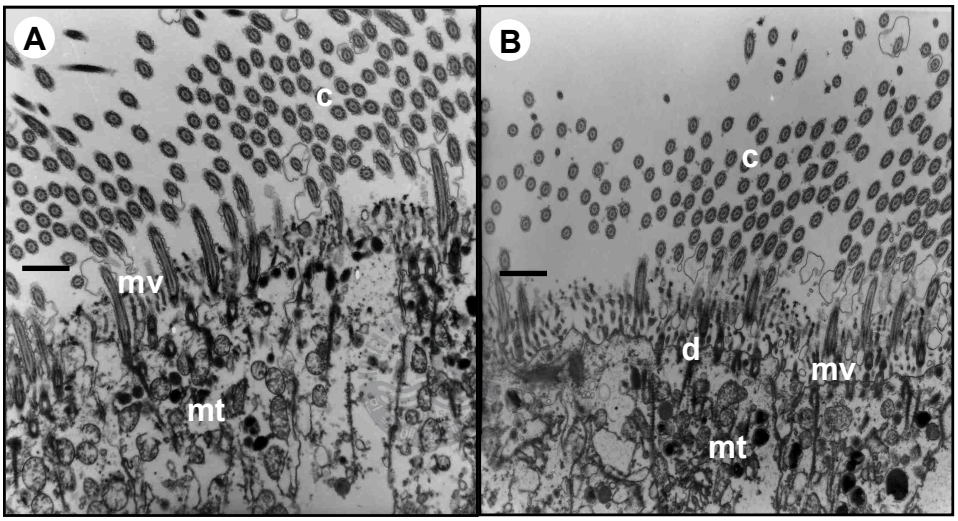

Fig. 28. Electron micrographs of Pacific oyster's gill exposed to cadmium (0.05 ppm). A: control, B: 11 days, c: cilia, mv: microvilli, mt: mitochondria. Bars: 1 ㎛. X 5000.

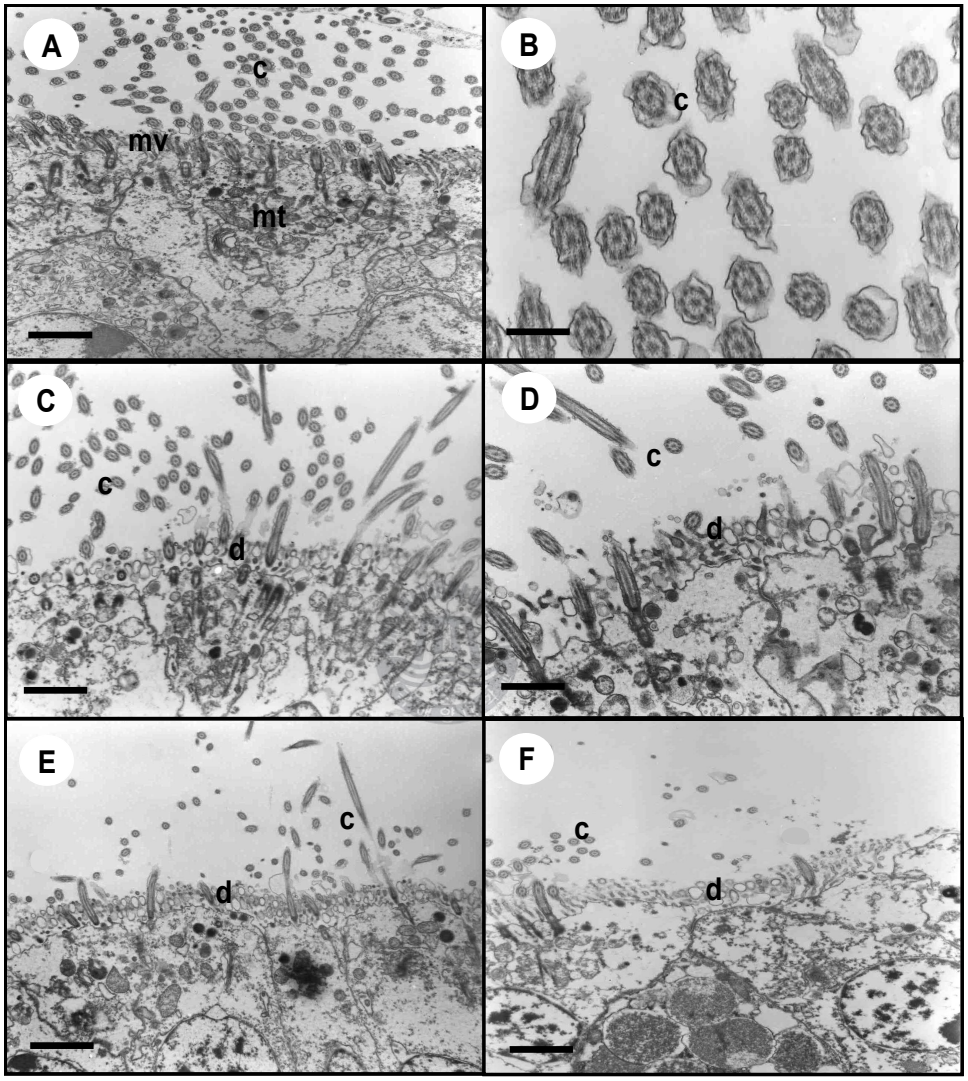

Fig. 29. Electron micrographs of Pacific oyster's gill exposed to cadmium (0.1 ppm). A: control, B: structure of cilia in control, C: 1 day, D: 3 days, E: 7 days, F: 11 days, c: cilia, mv: microvilli, mt: mitochondria. Bars: 1 ㎛. X 5000.

4. Discussion

Industrial development has resulted in the release of Cd to the aquatic environment through a variety of routes and in a variety of forms. Cd accumulates within the body and causes oxidative stress. The living body protects itself against oxidative stress using antioxidant enzymes. Oxidative stress reactions in Pacific oysters exposed to Cd were examined through the analysis of hemolymph properties and the expression of antioxidant enzymes.

The full-length CAT cDNA (EF687775) and GPX cDNA (EF692639) of Pacific oyster were cloned for the first time. The only other full-length CAT cDNA of molluscs belong to the two species *C. farreri* (DQ862859) and *H. d. discus* (DQ530211). The homology of the CAT of Pacific oyster with *C. farreri* and *H. d. discus* was high, at 74% and 72%, respectively. Also, there was homology of ≥65% with the CAT of vertebrates.

From multiple alignments using the cloned CAT, several characteristic elements were identified: His72, Asn145 and Tyr355 are conserved catalytic amino acids of CAT in all species, including Pacific oyster (Tavares-Sanchez et al., 2004; Li et al., 2008). The conserved sites of RLFSYNDTH (residues 351-359), which is the catalase proximal heme ligand signature sequence, and of FDRERIPERVVHAKGAGA (residues 61-68), which is the proximal active site signature sequence, were also identified (Tavares-Sanchez et al., 2004; Li et al., 2008) (Fig. 20). The high homology and two conserved characteristic sites of CAT suggest that the CAT of Pacific oyster is a member of the CAT family.

In the full-length GPX cDNA of Pacific oyster, a characteristic nonsense TGA codon (position 259, 80th amino acid) was identified (Forstrom et al., 1978) (Fig. 22). This codon is a selenocysteine, which was first discovered in mice. This selenocysteine is the activation site of the enzyme (Chambers et al., 1986). Also, in the GPX of Pacific oyster, Glu112 and Trp187, which are catalytic residues that interact with selenocysteine, were conserved (Ursini et al., 1995). These results suggest that the GPX of Pacific oyster is a

Se-GPX gene (Se-GPX). In molluscs, full-length Se-GPX has been reported in only two species: *U. tumidus* (DQ830766) and *D. polymorpha* (EF194204). The comparison of GPX homology between molluscs and vertebrates using the GPX cloned here indicated a low level of homology (≤33%). However, selenocysteine and the catalytic residues Glu and Trp were conserved in the Se-GPX of all species.

The expression of SOD, CAT and GPX mRNA in various tissues of Pacific oyster exposed to Cd was compared using RT-PCR (Fig. 24). SOD, CAT and GPX mRNA expression were observed in all tissues. In particular, high levels of expression were observed in the gill tissues. Gills are the primary and direct absorption route for toxic substances. The highest SOD, CAT and GPX expression occurred in the gills because the gills have a large area of contact with the surrounding environment and are subject to high levels of Cd accumulation (Legeay et al., 2005). No previous study has examined the expression of antioxidant genes among tissues in molluscs; however, higher expression in the liver of fish than in other organs such as gills and kidneys has been found (Cho et al., 2006a), indicating that there are differences among species in terms of expression patterns among tissues.

H_2O_2 concentrations significantly increased in the hemolymph of *C. gigas* as the Cd concentration and exposure time increased (Fig. 25). Many recent studies have reported that exposure to Cd increases the formation of ROS (Wang et al., 2004; Choi et al., 2007a; Murugavel et al., 2007; Soares et al., 2008). ROS induced by Cd toxicity in organisms is widely known to promote oxidative stress, which caused membrane damage, DNA breakage, lipid peroxidation, enzyme inhibition, amino acid oxidation and apoptosis.

SOD, CAT and GPX mRNA expression protects Pacific oyster from oxidative stress caused by Cd exposure. Expression increased as the Cd concentration and exposure time increased (Fig. 26). Funes et al. (2005) reported that CAT, SOD and GPX activation was higher in molluscs (*C. angulata* and *M. galloprovincialis*) from areas that had severe heavy metal contamination than from areas without contamination. Other studies reported the

induction of antioxidant genes in fish exposed to Cd, such as *S. trutta* (Hansen et al., 2007), and *D. labrax* (Romeo et al., 2000). Therefore, the expression of the antioxidant genes SOD, CAT and GPX mRNA through exposure to Cd suggests that ROS were induced by Cd in Pacific oyster, and that the antioxidant system was enhanced to remove the ROS. The present results demonstrated an increasing trend in SOD, CAT and GPX mRNA expression at 0.01 ppm and 0.05 ppm Cd over time (Fig. 26). However, at 0.1 ppm Cd, SOD mRNA expression increased significantly from 3 to 7 days of exposure and then decreased. CAT and GPX mRNA expression increased for 3 days and then decreased. H_2O_2 concentration increased for 11 days. These results suggest that extreme oxidative stress was induced by ROS generation from Cd contamination in Pacific oysters exposed to 0.1 ppm Cd after 3 days. In addition, the decrease in antioxidant enzyme mRNA expression after a specific period of time may have been caused by a lowering of the metabolic capacity of the oyster by the strong toxicity of Cd. Zhang et al. (2004) reported that although defense mechanisms are activated under weak oxidative stress, the body loses appropriate metabolic functions under strong oxidative stress. In the present study, antioxidant gene expression decreased after 3 days of exposure to 0.1 ppm Cd because excess accumulation of Cd beyond a certain level caused strong oxidative stress that dramatically lowered the metabolic capacity, resulting in a decrease in antioxidant enzyme mRNA expression. Also, CAT and GPX mRNA expression rapidly decreased, whereas SOD mRNA expression did not, after 7 days of exposure to 0.1 ppm Cd. This indicates that although SOD converts the superoxide radical to H_2O_2 and O_2, a decrease in CAT and GPX to break down the converted H_2O_2 into O_2 and H_2O leads to a failure to completely metabolize ROS (H_2O_2).

Analysis of the hemolymph of Pacific oysters exposed to Cd indicated no significant differences in the concentrations of AST or ALT at 0.01 ppm Cd from the control. However, at 0.05 ppm and 0.1 ppm, AST and ALT levels significantly increased over 7 days of exposure and then decreased. For Pacific oyster, studies have reported changes in

hemolymph constituents caused by heavy metal contamination, various environmental factors, and pathogenic agents (His et al., 1996; Xue and Tristan, 2000). Among these changes, an increase in AST and ALT activation generally results from an inflow to the hemolymph of cells separated due to tissue damage by environmental contaminants. Choi et al. (2008b) reported an increase in AST and ALT activation in Pacific oysters exposed to Cd. There are reports for several fish species of an increase in AST and ALT activation with Cd concentration over time (e.g., gilthead seabream, *Sparus aurata*, Vaglio and Landriscina, 1999; common carp, *Cyprinus carpio*, de la Torre et al., 2000). Therefore, significant increases in AST and ALT activation in the hemolymph of Pacific oysters from 7 days of exposure to Cd were induced as a result of tissue damage by Cd.

CAT and GPX mRNA expression had significantly decreased together with an increase in AST, ALT and H_2O_2 concentrations within the hemolymph of Pacific oyster after 7 days of exposure to 0.1 ppm Cd. This may have been the result of tissue damage by residual active oxygen if GPX and CAT failed to completely remove the H_2O_2 generated by SOD due to a decrease in the defensive capacity of the antioxidant systems as a result of excess oxidative stress exerted by Cd.

Oysters are useful bioindicators and pollutant vectors in the trophic chain (Bigas et al., 1997). The ultrastructure of the gill epithelium in control oysters revealed three types of columnar epithelial cells with specific abundance and distribution in each area of the filament (Bigas et al., 2001). The gills were the main reservoir of heavy metals, and this behavior may due to their functional activity and relative position to incoming water.

In the present study, changes in gill tissues of Pacific oysters exposed to 0.01, 0.05 and 0.1 ppm of cadmium were evaluated on days 1, 3, 7 and 11 after treatment. Observation was conducted with an light microscope after dyeing the external tissues of the ordinary filaments, principal filament and hemolymph sinus of the plica, etc., using the H-E stain method. The fine structures of the cilia, microvilli and mitochondria, etc., were also observed using TEM. No morphological changes were observed in the external structures

of the gill tissues of Pacific oysters in any experiment group in comparison to the control (Fig. 27).

However, on day 11 of exposure to 0.05 ppm of Cd, dilations were observed using TEM in the microvilli in the internal structure of the gill tissues of Pacific oysters (Fig. 28). Also, after exposure to 0.1 ppm of Cd, the cilia count gradually decreased, while dilations of microvilli displayed a tendency to increase with exposure time (Fig. 29). Based on the above, Cd toxicity decreased the cilia count in gill tissues of Pacific oysters and induced dilations of microvilli, the cellular projections filled with sensory cells that take part in absorption of substances and contraction of tissues. Bigas et al. (2001) reported that an altered plasma membrane of microvilli and cilia is an initial response to stress induced by Hg. Also, condensation or swelling of mitochondria is considered a pathological cellular response to Cd in an insect cell line (Braeckman and Raes, 1999). However, no extreme cellular damage, such as deformation of cells, vacuolation or damage to mitochondria or the nucleus inside a cell was observed within the range of Cd concentrations (0.01, 0.05 and 0.1 ppm) and treatment time (up to 11 days) employed in this study. In this study, mRNA expression of antioxidant enzymatic genes (SOD, CAT and GPX) as well as AST, ALT and H_2O_2 levels stabilized. However, once damaged by toxicity (decrease in the cilia count and dilations of microvilli), the tissue did not recover during the experiment. It is concluded that tissues, once damaged by Cd at 0.1 ppm, cannot recover the functions and roles of antioxidant enzymatic genes.

In conclusion, oxidative stress caused by Cd toxicity accumulated in Pacific oysters exposed to Cd. To counter this stress, antioxidant enzyme mRNA expression increased in the gill tissue. Considering the increase in AST and ALT in the hemolymph, cell damage apparently occurred because of the loss of detoxification ability by the secondary antioxidant system of CAT and GPX in Pacific oyster due to excess oxidative stress caused by the accumulation of Cd beyond a specific tolerance. Therefore, it is possible that the antioxidant genes SOD, CAT and GPX mRNA could be used as physiological indices for

oxidative stress caused by heavy metals such as Cd in Pacific oyster. However, it was found that tissues could not recover their antioxidant gene functions once damaged by Cd toxicity.

Chapter 5

Characterization of antioxidant enzyme mRNA expression and changes in physiological hemolymph responses in Pacific oyster, *Crassostrea gigas*, exposed to a hypoxic environment

1. Introduction

Oxygen (O_2) is a very important element not only for terrestrial animals and plants, but also for aquatic flora and fauna. Hypoxia caused by red tides, high water temperatures and O_2-deficient water masses directly influences the behavioral, biochemical and physiological responses of fish and invertebrates (Wu, 2002). Pacific oysters, sessile organisms that inhabit tidal zones, have developed a control system to maintain themselves in hypoxic environments (David et al., 2005). Specifically, they have the ability to lower their metabolic rate, and are therefore tolerant of hypoxic conditions (Storey, 1993). However, lengthy exposure to a hypoxic environment or the occurrence of severe anoxia acts as a stress factor in Pacific oysters, which leads to mass mortality and outbreaks of diseases due to lowered immunity.

In the course of O_2 metabolism, aerobic organisms produce potentially harmful reactive oxygen species (ROS). ROS, highly oxidative O_2 species that are generated in various metabolic processes as O_2 flows into the living body through respiration, are used in the process of oxidation, but can attack tissues and damage cells. ROS include the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO).

ROS generated in living tissues in a hypoxic environment are highly oxidative and bind with other substances. They attack cells or organ membranes and damage cellular functions (Ferraris et al., 2002). To protect cells from oxidative stress and prevent damage

to cellular functions caused by ROS, living organisms have antioxidant defense systems consisting of antioxidant enzymes, such as SOD, CAT and GPX (Chance et al., 1979; Wendel and Feuerstein, 1981).

These antioxidant enzymes serve an important role in maintaining the homeostasis of cells and cellular antioxidant defenses by removing ROS (Rudneva, 1999). SOD, one of the key defense elements against ROS, is a metalloenzyme found in all living organisms that consume O_2. SOD removes superoxide radicals through the process of dismutation into O_2 and H_2O_2 ($2O_{2\cdot} + H^+ \rightarrow H_2O_2 + O_2$) (Fridovich, 1975), and the H_2O_2 created through this process is broken down into H_2O and O_2 by CAT and GPX (Mruk et al., 2002). CAT, an oxidoreductase, removes toxic H_2O_2 by breaking down two molecules of H_2O_2 into two molecules of H_2O and O_2 ($2H_2O_2 \rightarrow 2H_2O + O_2$) (Kashiwagi et al., 1997). GPX is also an important peroxidase that removes toxic hydroperoxides, breaking them down into H_2O and O_2 (Świergosz-Kowalewska et al., 2006). Furthermore, GPX can largely be divided into selenium-dependent GPX (Se-GPX) and selenium-independent GPX (non-Se-GPX). Se-GPX promotes organic and inorganic peroxide breakdown, while non-Se-GPX promotes only organic peroxide breakdown (Almar et al., 1998).

Studies on hypoxia acclimation by marine invertebrates have been conducted on shore crabs, *Leptograpsus variegatus* (Morris and Butler, 1996), blue crabs, *Callinectes sapidus* (Defur et al., 1990), brown shrimps, *Crangon crangon* (Hagerman and Uglow, 1982), freshwater giant prawn, *Macrobrachium rosenbergii* (Cheng et al., 2003), lobster, *Homarus vulgaris* (McMahon et al., 1978), crayfish, *Orconectes virilis* (McMahon et al., 1974), Asian clams, *Corbicula fluminea* (Byrne et al., 1991) and Taiwan abalone, *Haliotis diversicolor supertexta* (Cheng et al., 2004). However, no comprehensive study has investigated physiological modulation and molecular endocrinology in relation to O_2 consumption by Pacific oysters exposed to hypoxia. Therefore, in this study, O_2 consumption patterns of Pacific oysters were measured and compared to the mRNA expression of SOD, CAT and GPX, as well as changes in osmolality, inorganic ion concentrations, and observations using hematoxylin-eosin (H-E) stain method and TEM.

2. Materials and Methods

2.1. Experimental oyster

One-year-old Pacific oysters (average shell length: 112±10.7 mm; shell height: 31.1±5.4 mm; weight: 20.3±3.9 g), obtained from the oyster hatchery on Dae-bu Island, in Goseong (Gyeongnam, Korea), were placed in 40 L circulation filter tanks at a density of 30 oysters per tank. During the experimental period, the water temperature was kept at 20±0.5℃ and the photoperiod was 12 h light/12 h dark. Five oysters were used for each experimental or control group.

2.2. Measuring O_2 consumption and sampling

Repetitively each experiment was filmed in three different periods by promptly moving stabilized Pacific oysters into a respiration chamber inside a closed O_2 consumption measuring device (OxyGuard 6 OxyGuard International A/S, Birkerd, Denmark). Water temperature was maintained by a constant temperature water bath (JS-WBP-170RP Johnsam Co., Bucheon, Korea). Pacific oysters were not fed during the period of the experiment to minimize changes in respiration and O_2 consumption by metabolizing prey organisms. The structure of the O_2 consumption measuring device is illustrated in Fig. 30. The respiration chamber was constructed of transparent acrylic material to allow observation of shell movement and mortality of the oysters. To measure O_2 consumption, The Oxyguard 6 program, a multichannel dissolved O_2 measuring system was used. In addition, the level of dissolved O_2 was automatically measured every 10 min using a dissolved O_2 sensor attached to an acrylic tank, and data were transferred into a computer. O_2 consumption by Pacific oysters was displayed as the average O_2 consumption per unit weight calculated on the basis of dissolved O_2 automatically measured during the experiment. Prior to commencing the experiment, The gills, digestive glands and

hemolymph of Pacific oysters were collected three times, after 1, 3 and 5 h, from a control group and from the closed acrylic tank. The collected samples were stored in a super low-temperature freezer at -80℃ until RNA extraction.

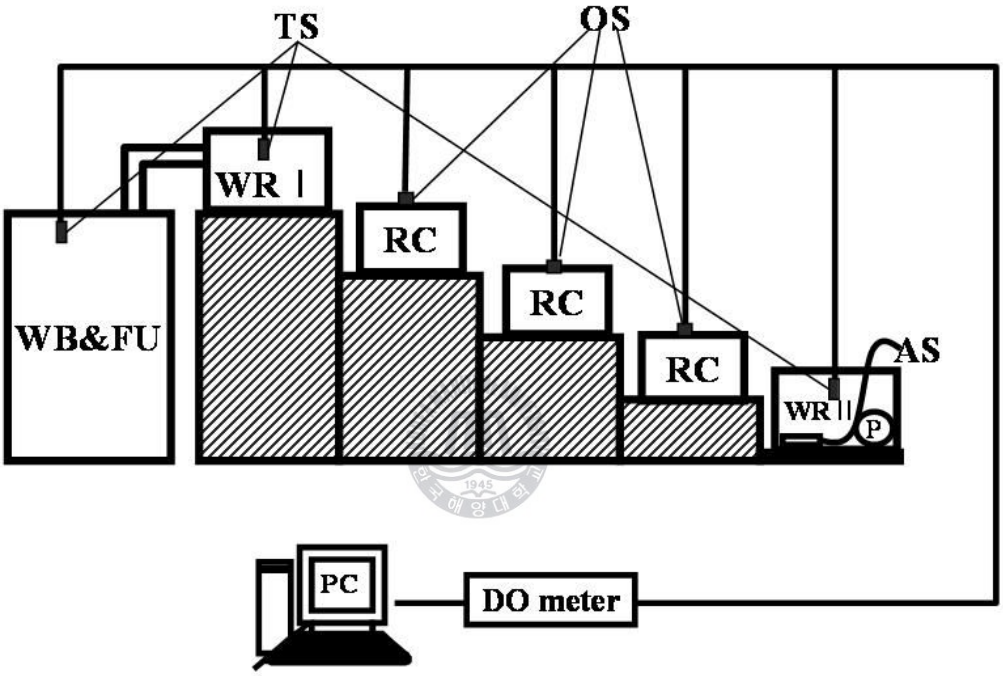

Fig. 30. Schematic diagram of the oxygen (O_2) consumption measuring system used in the present study. AS, air supply; FU, filtering unit; OS, O_2 sensor; P, pump; PC, personal computer; RC, respiratory chamber; TS, temperature sensor; WB, water bath; WR I and II, water reservoirs I and II, respectively.

2.3. Quantitative real-time PCR (QPCR)

QPCR was conducted to determine the relative mRNA expression of antioxidant enzymes (SOD, CAT and GPX) using total RNA extracted from the gills of control and hypoxia-treated oysters. With 2.5 μg of total RNA as a template, complementary DNA (cDNA) was synthesized using M-MLV reverse transcriptase (Bioneer). First-strand cDNA synthesis was conducted using oligo-d$(T)_{15}$ primer (Promega). Primers for QPCR were designed with reference to known SOD (GenBank accession no. AJ496219), CAT (EF687775), GPX (EF692639) and 28S ribosomal RNA (28Sr) (Z29546) gene sequences of Pacific oyster as follows: SOD forward primer (5′-CTC CTG GAA CAC CTG TGA CAT TG-3′), SOD reverse primer (5′-GTG CCT CTC GTG ATC CTC TGG-3′), CAT forward primer (5′-AAC TAC TTC GCT GAG GTG-3′), CAT reverse primer (5′-GGT CTT GGC TTT GTA TGG-3′), GPX forward primer (5′-GAC CGT GGA ACC AAT GGA CAT C-3′), GPX reverse primer (5′-GTT GGA TTC GGA CAC AGA TAG GG-3′), 28Sr forward primer (5′-AAA CAC GGA CCA AGG AGT CT-3′) and 28Sr reverse primer (5′-AGG CTG CCT TCA CTT TCA TT-3′). QPCR amplification was conducted using a Bio-Rad MiniOpticon^{TM} System (Bio-Rad, Hercules, CA, USA) and iQ^{TM} SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. QPCR was undertaken by denaturation at 95℃ for 5 min, followed by 40 cycles of denaturation at 95℃ for 20 s and annealing at 55℃ for 20 s. To ensure that the primers amplified a specific product, It was performed a melt curve, and also analyzed the PCR product size using capillary electrophoresis (Agilent Technologies, Santa Clara, CA, USA). All primers used were shown to amplify only one size of template, melting at only one temperature. PCR products were also confirmed by sequencing. All analyses were based on the calculated threshold Ct values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the

exponential phase of the amplification curve. After the PCR program, QPCR data from five replicate samples were analyzed with analysis software (Bio-Rad) to estimate transcript copy numbers for each sample. mRNA expression levels stood for an n-fold difference relative to 28Sr as the internal control.

2.4. Hemolymph osmolality and ion concentrations

Hemolymph was withdrawn from the pericardial cavity using a 3 mL syringe. The samples were centrifuged at 10,000 ×*g* (MICRO 17TR; Hanil, Seoul, Korea) and 4℃ for 5 min, and the supernatant (after centrifugation) was stored at -80℃ until analysis. Hemolymph osmolality was measured using a vapor pressure osmometer (Vapro 5520; Wescor, Logan, UT, USA). K^{+}, Ca^{2+} and Mg^{2+} were measured using a biochemistry autoanalyzer (model 7180; Hitachi, Tokyo, Japan).

2.5. Histological analysis

2.5.1. Observation with light microscope

To observe histological changes in the gill tissues of Pacific oyster exposed to hypoxia, the extracted gill tissues were fixed in 10% neutral formalin solution (100 mL formalin, 6.5 g $Na_2HPO_4 12H_2O$, 4.5 g KH_2PO_4, 900 mL distilled water) for 1 day. Then, the gill tissues were fixed in bouin solution and a tissue sample was produced according to the series of methods. The fixed sample was cut into serial sections of 5 ㎛ in thickness by paraffin sectioning. Then, the degree of tissue damage was examined with an light microscope after double dyeing with Harris's hematoxylin and 0.5% eosin (Sigma, USA). The degree of damage in the gill tissues was photographed by using image analysis system (Axiovision, Zeiss Co., Germany) with a biological microscope (Axioskop, Zeiss, Germany).

2.5.2. Observation with electron microscope

A sample for TEM was produced in order to observe the fine structure of the gill tissues of Pacific oyster exposed to hypoxia. After extracting gill tissues, primary fixation was carried out for 2 h in 2.5% glutaraldehyde solution (4℃) buffed with 0.1 M phosphate buffer solution (PBS, pH 7.2). The sample completed of fixation was washed for 10 min with PBS and was processed through secondary fixation for 2 h at 4℃ in 1% osmium tetroxide (OsO_4). After fixation was completed, the sample was washed with PBS and was dehydrated for 15 min each in 50-100% ethanol. After dehydration, the sample was placed in the mixture of propylene oxide and Epon and was polymerized for 1-3 h. Then the sample was embedded in Epon 812. The embedded tissue was semi-thinly sectioned with the thickness of 1.0 ㎛ by using ultramicrotome (Leica, Reichert SuperNova, Sweden). Then, it was dyed with 1% toluidine blue and the part for observation was determined. After the part for observation was determined, the sample was again sectioned with the thickness of 70 mm and was attached to 200 mesh copper grid. The sample completed of ultra-thin sectioning was double-dyed with uranylacetate and lead citrate solution and was observed with a transmission electron microscope (JEM 1200 EX-Ⅱ, 60-80 kv, JEOL, Tokyo, Japan).

2.6. Statistical analysis

Treatment differences were tested using one-way ANOVA followed by the Tukey or LSD test using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA) at a significance level of $P<0.05$.

3. Result

3.1. O_2 consumption pattern

At the time of commencing the experiment, the dissolved O_2 level was 5.4 mg/L in the closed respiration chamber. The dissolved O_2 in the closed respiration chamber decreased to 4.3±0.5, 1.8±0.15 and 0.4±0.15 mg/L after 1, 3 and 5 h, respectively. The total length of time until all Pacific oysters consumed all of O_2 was 6 h, with a trend of continuous decrease until approximately 350 min after commencing the experiment (Fig. 31). The hourly dissolved O_2 consumption was 0.9 mg/L and O_2 consumption per hour per individual was 0.3 mg/L. Curve-fitting of the functional relationship between the time elapsed and the level of dissolved O_2 consumption using the least squares method gave a linear relationship. Therefore, The correlation between time were derived and dissolved O_2 through curve-fitting by the least squares method as dissolved O_2 level (y) = 0.1543 x (time) + 4.9675 (Fig. 31).

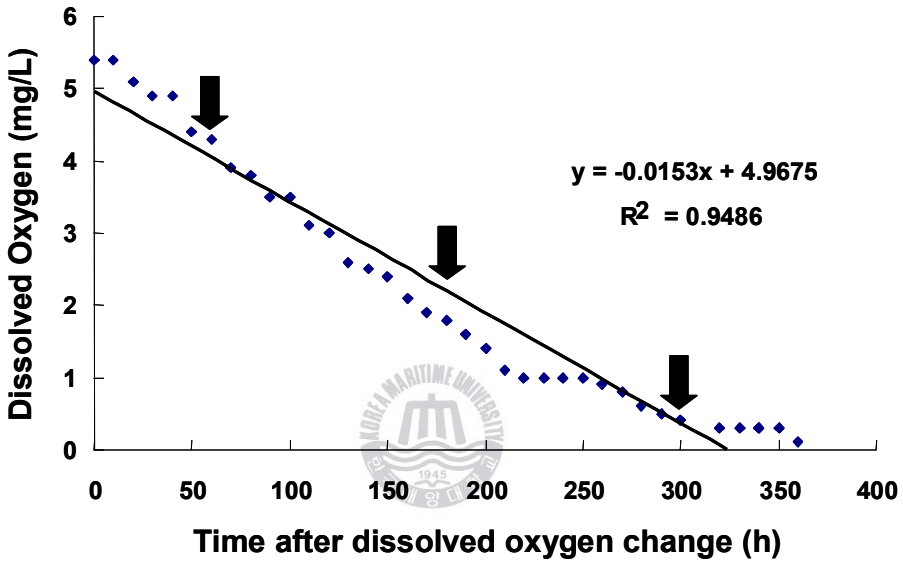

Fig. 31. Linear relationship by least squares curve fitting for oxygen consumption of Pacific oyster, *Crassostrea gigas* vs. elapsed time. Samples were gathered at 1, 3 and 5 h after the start of treatment, and sample points are indicated by arrows.

3.2. Levels of SOD, CAT and GPX transcripts

In terms of SOD, CAT and GPX mRNA expression in the gills and digestive glands of Pacific oysters exposed to hypoxia, SOD mRNA expression displayed a tendency of rapid increase after 1 h, and then decreased. CAT and GPX mRNA expression displayed a significant decrease as time elapsed (P<0.05) (Fig. 32).

3.3. Hemolymph osmolality and ion concentrations

In terms of osmolality as well as K^+, Ca^{2+} and Mg^{2+} ion levels in the hemolymph of Pacific oysters exposed to hypoxia, osmolality decreased from 988±8.86 mOsm/kg at the time of commencing the experiment to 946.3±5.86 mOsm/kg after 5 h, and K^+ decreased from 13.0±1.65 mmol/L to 10.1±0.62 mmol/L. However, Ca^{2+} increased from 75.0±3.0 mmol/L to 95.5±6.06 mmol/L and Mg^{2+} from 55.0±3.77 mmol/L to 81.5±2.29 mmol/L (P<0.05) (Fig. 33).

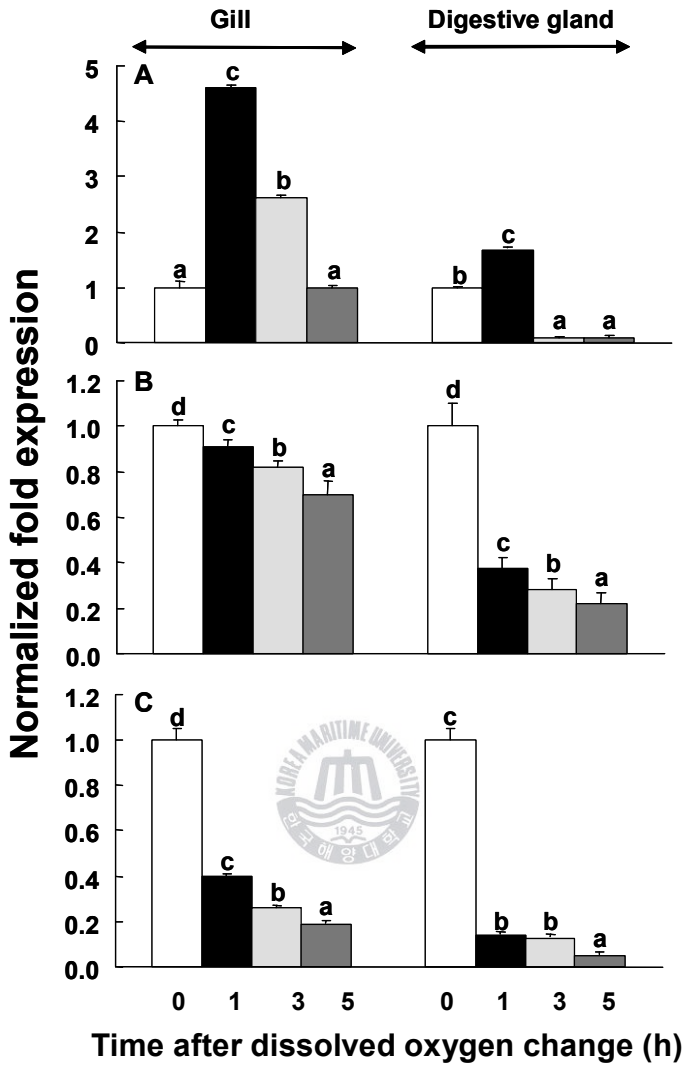

Fig. 32. Time-related effect on superoxide dismutase (SOD, A), catalase (CAT, B) and glutathione peroxidase (GPX, C) mRNA levels in gills of Pacific oyster, *Crassostrea gigas* during hypoxia adaptation. Total RNA was extracted 0, 1, 3 and 5 h after the start of treatment, and 1 μg was used for PCR. The values are percentage increases relative to the control value. The expression level of each sample was normalized with respect to the 28S ribosomal RNA signal, and expressed as a relative expression level. Values with dissimilar letters are significantly different (P<0.05) from each other. Values are means±SD of three experiments. Each experimental group consisted of five oysters.

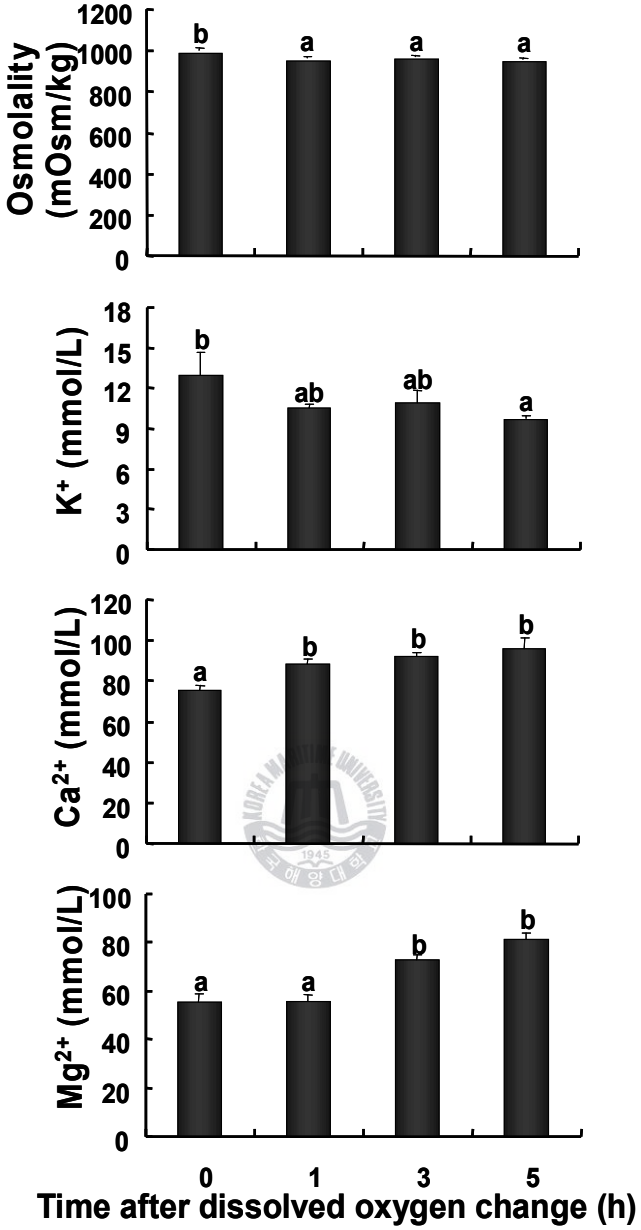

Fig. 33. Osmolality, K^+, Ca^{2+} and Mg^{2+} in the hemolymph of Pacific oyster, *Crassostrea gigas* during hypoxia adaptation. Hemolymph was separated from the oyster and used for osmolality measurement. Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values are means±SD of three experiments. Each experimental group consisted of five oysters.

3.4. Histological analysis

3.4.1. Observation with light microscope

The external shape of the gill and digestive gland tissues in Pacific oyster exposed to hypoxia was observed with H-E stain method and the result indicated no histological change in the control group and in the gill tissues at the 1 h of exposure to hypoxia (Fig. 34 and 35). Also, by 5 h, the experiment completion, no histological change was observed in the gill tissues of Pacific oyster.

3.4.2. Observation with electron microscope

The internal shape of the gill tissues in Pacific oyster exposed to hypoxia was observed with TEM and the result indicated no histological change in the control group and in the gill tissues at 1 h after exposure to hypoxia (Fig. 36). Also, by 5 h, the experiment completion, no histological change was observed in gill tissues of Pacific oysters.

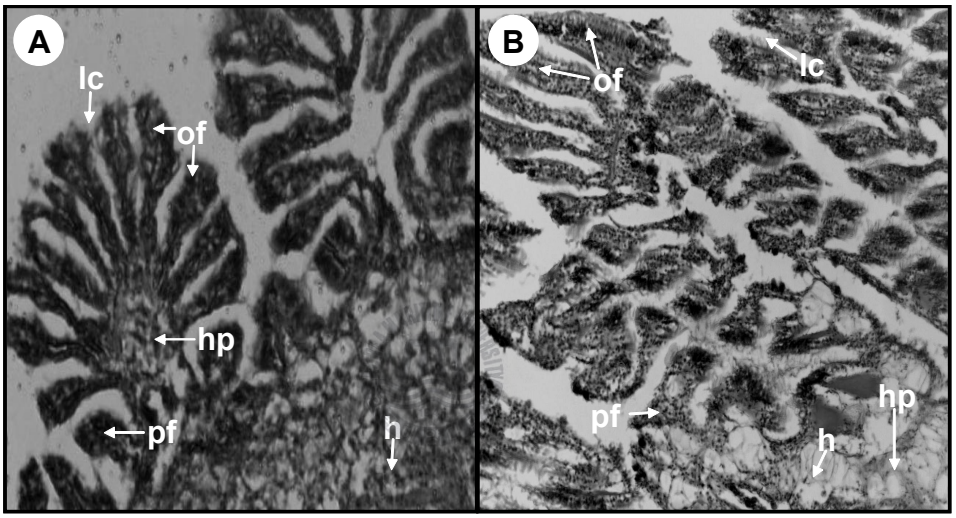

Fig. 34. Photomicrographs of gill filaments and lamellae of Pacific oyster exposed to hypoxia. A: control, B: 1 h in hypoxia, of: ordinary filament, pf: principal filament, lc: lateral cilia, h: hemocytes, hp: hemolymph sinus of plica. X 200.

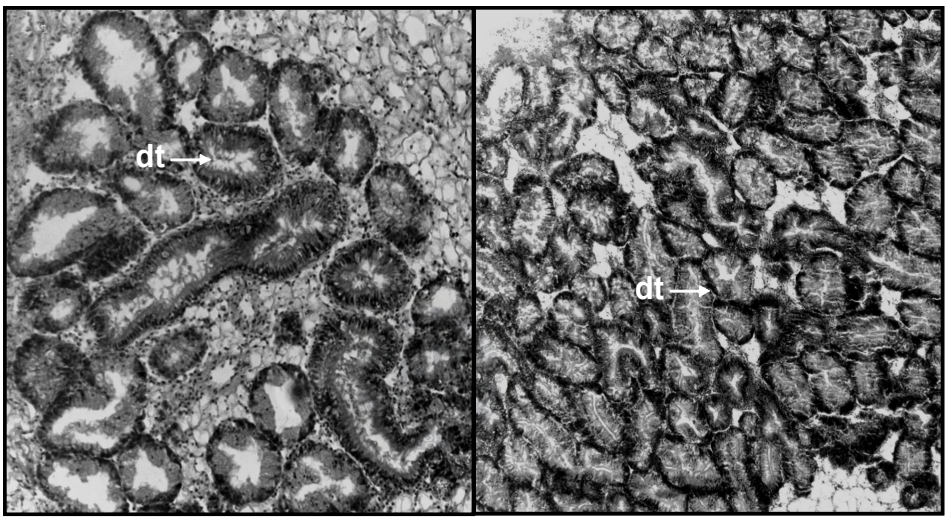

Fig. 35. Photomicrographs of digestive gland of Pacific oyster exposed to hypoxia. A: control, B: 1 h in hypoxia, dt: digestive gland tubules. X 200.

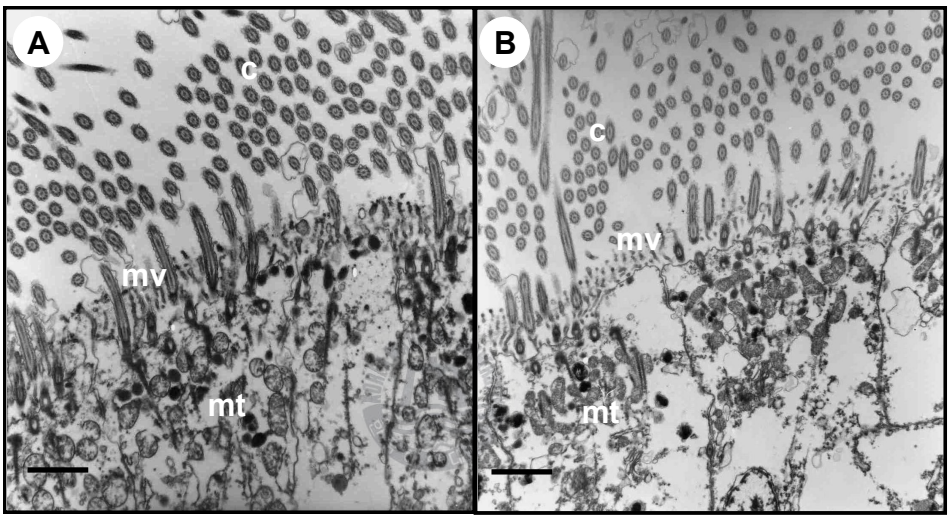

Fig. 36. Electron micrographs of Pacific oyster's gill exposed to hypoxia. A: control, B: 1 h in hypoxia, c: cilia, mv: microvilli, mt: mitochondria. Bars: 1 ㎛. X 5000.

4. Discussion

In this study, hypoxia began to form inside the closed respiration chamber as soon as the oysters began consuming O_2. During the course of hypoxia formation, tissues were sampled from the gills and digestive glands of Pacific oysters over time. Changes in SOD, CAT and GPX mRNA expression in each tissue were investigated using QPCR. The gill tissue of Pacific oysters comprises the primary interface between the hemolymph or cytoplasm and the external environment. As a direct absorption route for seawater, gill tissues have a wide contact area with seawater and are known to be directly influenced by their environment (Hosoi et al., 2007). Tissues of digestive glands, however, take part in energy metabolism such as food digestion, absorption and discharge (David et al., 2005).

During the first hour of exposure to hypoxia, Pacific oysters began consuming O_2 inside the closed respiration chamber (dissolved O_2 level: 4.3 mg/L); SOD mRNA expression in gill tissues increased and then began to decrease. However, CAT and GPX mRNA expression displayed a trend of continuous decrease from the start of the experiment (Fig. 32). This occurred because, while superoxide radicals were transformed into H_2O_2 and O_2 by SOD activity inside Pacific oysters during the first hour following exposure to hypoxia, a decrease in CAT and GPX (which break down the transformed H_2O_2 into O_2 and H_2O) resulted in incomplete removal of H_2O_2 from inside the body. Romero et al. (2007) reported that as a result of exposing stone crabs, *Paralomis granulosa*, to a dry atmosphere of 6℃ for 24 h, SOD mRNA expression rapidly increased in the hemolymph for 3 h and then decreased, but CAT mRNA expression displayed a continuously decreasing trend. Pannunzio and Storey (1998) reported that GPX activity decreased in the hepatopancreas of the gastropod, *Littorina littorea*, exposed to anoxia. In conclusion, a hypoxic environment reduces ROS generation, thereby leading to a decrease in the activities of antioxidant enzymes (Hermes-Lima et al., 2001). The results of Hermes-Lima et al. (2001) correspond with those of this study, that is, the expression of antioxidant genes decreased

in response to exposure to hypoxia once Pacific oysters begin to consume O_2. The pattern of SOD, CAT and GPX mRNA expression in the digestive glands was similar to the pattern displayed in the gill tissues. Larade and Storey (2002) reported that various protein syntheses decreased in the digestive glands 30 min after exposing *L. littorea* to anoxia. These results support the present findings and indicate that a rapid decrease in anti-oxidation-related mRNA expression takes place in the digestive gland tissues of Pacific oysters under hypoxic conditions.

In this study, higher levels of SOD, CAT and GPX mRNA expression were found in gill tissues than in digestive gland tissues when Pacific oysters were exposed to hypoxia. This occurred because gills absorb dissolved O_2 by filtering seawater, and greater levels of oxidative stress following exposure to a hypoxic environment take place in the gills than in digestive glands. To protect cells from damage by anoxia, the antioxidant defense system enzymes are primarily in the gills. Changes in osmolality, K^+, Ca^{2+} and Mg^{2+} ions in the hemolymph were also measured as Pacific oysters were exposed to hypoxia. While osmolality and K^+ concentrations continuously decreased, $Ca^{2+}$and Mg^{2+} concentrations continuously increased (P<0.05) (Fig. 33). Cheng et al. (2003) measured dissolved O_2 with exposure time to hypoxia using the giant prawn, *Macrobrachium rosenbergii*, and found a pattern of decrease in osmolality and K^+ in the hemolymph as the level of dissolved O_2 decreased, similar to the results of the present study. In a hypoxic environment, organisms increase ventilation with seawater, and by enlarging the surface area of the gills to extract O_2 from the seawater, O_2 absorption is enhanced (Cheng et al., 2003). This change in metabolism increased the inflow of water and thereby led to a reduction in osmolality and K^+ concentrations in the present experiment.

The levels of Ca^{2+} and Mg^{2+} in the hemolymph have been reported to increase not only in purple shore crabs, *Leptograpsus variegatus* (Morris and Butler, 1996), but also in Asian freshwater clams, *Corbicula fluminea* (Byrne et al., 1991) exposed to hypoxia, similar to the present results in which Ca^{2+} and Mg^{2+} ions in the hemolymph increased in

Pacific oysters exposed to hypoxia treatment. Byrne et al. (1991) reported that calcium carbonate in the shell was used to supply bicarbonate as a way of buffering acidosis in the body as the hemolymph became acidified under hypoxia due to a decrease in pH. The increase in hemolymph Ca^{2+} in Pacific oysters observed in this study was likely also caused by this mechanism.

Dissolved O_2 in seawater at 20℃ prior to commencement of the experiment was 5.4 mg/L. However, a hypoxic environment was formed as Pacific oysters consumed O_2 within the closed respiration chamber. SOD mRNA expression increased up to 1 h of exposure in this environment and then decreased. CAT and GPX mRNA expression continuously decreased from the time of initial exposure. After 1 h of exposure to hypoxia, the level of dissolved O_2 in the seawater had decreased to 4.3 mg/L. At this time, primary antioxidant defense by SOD to transform superoxide radicals into H_2O_2 and O_2 took place in Pacific oysters. However, the secondary defense by CAT and GPX to break down the transformed H_2O_2 into O_2 and H_2O did not occur therefore, the harmful H_2O_2 was not completely removed, and accordingly, had adverse effects on Pacific oysters. In the hypoxic environment, Pacific oysters increased the inflow of seawater to enhance absorption of O_2 inside the body, leading to a decrease in the osmolality and K^+ ion concentrations in the hemolymph. Moreover, to supply bicarbonate to buffer the acidosis caused by the lowered pH, calcium carbonate in the shell was used to increase Ca^{2+} and Mg^{2+} ion concentrations in the hemolymph.

Changes over time in the gill tissues of Pacific oysters exposed to hypoxic environment were evaluated. Observation was conducted with an light microscope after dyeing the external tissues of the ordinary filaments, principal filament and hemolymph sinus of the plica, etc., using the H-E stain method (Fig. 34). The fine structures of the cilia, microvilli and mitochondria and so on, were also observed using TEM (Fig. 36). In addition, multiple digestive gland tubules were observed using the H-E stain method (Fig. 35). Considering that no histological changes were observed in digestive gland or gill tissues of Pacific

oysters exposed to a hypoxic environment during the experiment, it is concluded that O_2 reductions of at least 0-2 mg/L do not cause damage to the tissues of Pacific oysters.

In this study, the expressions of antioxidant enzymes as well as changes in osmolality and inorganic ions were observed in Pacific oysters exposed to a hypoxic environment. The study results indicate that antioxidant enzymatic genes are able to protect cells from O_2 reductions of at least 0-2 mg/L. These results can be used as a basic marker system to identify physiological responses that occur at the time of exposure to hypoxia not only in Pacific oysters, but also in other shellfish species.

Chapter 6

General Discussion

Pacific oysters inhabit areas with shallow water depth and estuarine regions, which make them particularly susceptible to environmental changes. Such habitats in coastal waters frequently undergo changes in water temperature and salinity due to high water temperatures in the summer, prolonged drought and typhoon seasons, and localized downpours in the rainy season. Water temperature and salinity are closely related to various physiological functions and metabolic activities of Pacific oysters, such as growth, reproduction, metabolism, food intake and energy activity (Mills, 2000; Navarro et al., 2000). Prolonged exposure to hypoxic environments caused by red tides, high water temperatures, and oxygen-deficient water induces mass mortality and diseases by lowering the immunity of Pacific oysters (Gagnaire et al., 2006). Furthermore, while Pacific oyster farming is mainly conducted along the southern coast of Korea, aging farming facilities, heavy metal pollution by marine waste and sediment fallout from the farms, as well as abandoned copper mines and other pollution sources, are becoming increasingly problematic. Cd, a common heavy metal pollutant found in marine environments, can be toxic even at low doses (Benavides et al., 2005). Cd in aquatic environments can accumulate in living tissues, which may result in heavy metal poisoning of humans through food chain exposures. Exposure to rapid changes in environmental factors such as these can negatively affect growth, maturity and general health of oysters, and can lead to mass mortality of Pacific oysters. This has become a significant problem in Korea and throughout the world.

As part of this study, the first full-length cDNA for CYP450 (1744 bp, GenBank accession no. EF451959), HSP90 (2154 bp, EF687776), CAT (1988 bp, EF687775) and GPX (1310 bp, EF692639) was isolated from the gill tissues of Pacific oyster to

understand the physiological responses of *C. gigas* to various environmental stresses. In addition, mRNA expression profile changes related to physiological responses to changes in salinity, water temperature, Cd levels and hypoxia were examined.

RT-PCR indicated that mRNA expression was highest in the gills of oysters exposed to various environment factors (high temperature, salinity, Cd and hypoxia). mRNA expression must have been induced because the gill, as the primary interface between the outside environment and the hemolymph or cytoplasm (Hosoi et al., 2007) and as the direct absorption route for sea water with a wide contact area, is more directly influenced by stress than other organs. Changes in HSP90 and SOD mRNA expression over time in oysters exposed to salinity, water temperature, Cd and hypoxia were investigated using QPCR.

1. The complete HSP90 cDNA was isolated from the gills of Pacific oysters exposed to rapid salinity change. HSP90 has been reported for bivalve species only for *C. farreri* (AY362761) and for *H. asinina* (EF621884). *C .gigas* HSP90 isolated in this study displayed a high homology of 85% with Zhikong scallop HSP90 and 84% with abalone HSP90. Also, it contained 5 signal peptides that are well conserved in the HSP90 family (NKEIFLRELISN [A/C/S] SDALDKIR, LGTIA [K/R] SGT, IGQFGVG FYSAYLVA [E/D], IKLYVRRVFI and GVVDEDLPLNI SRE) and the consensus amino acid sequence (MEEVD) at the 3´ terminus (Gupta, 1995; Gao et al., 2007). In particular, Scheufler et al. (2000) reported that the amino acid sequence of MEEVD at the 3´ terminus was the most well conserved area and the location at which the binding of HSP70 and HSP90 was regulated as a typical characteristic of HSP90. This supports the conclusion that Pacific oyster HSP90 is among the HSP90 families.

In response to rapid salinity change, osmolality, as well as hemolymph Na^{+}, Cl^{-} and Ca^{2+} levels, increased at hypersalinity (52.5 psu), and mRNA expression of HSP90 (the stress-related gene) and CT-R (the osmoregulation-related gene) was significantly elevated in the gill tissues of Pacific oysters when osmolality and inorganic ion levels were highest.

In addition, the H_2O_2 concentration in the hemolymph and the SOD mRNA expression increased significantly over time. When osmolality, Na^+, Cl^- and Ca^{2+} levels returned to normal, mRNA expression levels were also restored to control levels. Given that no deaths occurred during the exposure period, it is concluded that stress was reduced through diverse physiological defense mechanisms. No histological changes were observed compared to the control group throughout the experiment.

These results suggest that HSP90 was induced as part of a defense mechanism to protect cells against increases in salinity stress and that SOD mRNA expression increased as part of an antioxidant mechanism to remove reactive oxygen in the body. Also, CT-R controls high Ca^{2+} levels, thereby reducing osmolality as well as Na^+, Cl^- and Ca^{2+} levels (Dubos et al., 2003).

2. The complete CYP450 cDNA was isolated from the gills of Pacific oysters exposed to rapid water temperature changes. An NCBI/GenBank database comparison of CYP450 genes revealed that the deduced amino acid sequence of cgCYP450 is similar to the *M. musculus* CYP450 2D9 (AK078880; 29%), *O. cuniculus* CYP450 2D/II (AB008785; 28%) and *C. jacchus* CYP450 2D (AY082602; 28%). Pacific oyster CYP450 was cloned and its amino acid sequence was compared to that of other species, families and subfamilies. Pacific oyster CYP450 was classified as belonging to family 2, subfamily D.

The levels of CYP450 mRNA expression were observed in various tissues of Pacific oysters using the previously decided CYP450 cDNA base sequence and the results indicated higher expression in the gills than in other tissues. CYP450 mRNA expression was significantly increased in the gill tissues of Pacific oysters 6 h after exposure to high (30℃) and low (10℃) water temperatures, and then subsequently decreased. Therefore, CYP450 mRNA expression stabilized after the initial temperature change as the oysters acclimated to the new temperatures.

In addition, HSP90 and SOD mRNA expressions increased in Pacific oysters exposed to rapid increases in water temperature, and increased H_2O_2 levels as well as AST activity in

the hemolymph implied damage to the gill cells and other tissues. However, no histological changes were observed in comparison to the control group throughout the experiment. Therefore, stress and active oxygen increased in Pacific oysters due to rapid water temperature increases, resulting in the activation of a defense mechanism involving elevated HSP90 mRNA levels for cell protection and SOD mRNA as part of an antioxidant system to remove reactive oxygen.

3. When Pacific oysters were exposed to increased levels of Cd, oxidative stress occurred due to the toxicity of Cd accumulated within the body. As a result, mRNA expression of the antioxidant enzymes SOD, CAT and GPX was significantly elevated in the gill tissues. The full-length CAT cDNA (EF687775) and GPX cDNA (EF692639) of Pacific oysters exposed to oxidative stress by Cd were cloned. The only other reported full-length CAT cDNA of molluscs belong to the two species *C. farreri* (DQ862859) and *H. d. discus* (DQ530211). The homology of the CAT of Pacific oyster with *C. farreri* and *H. d. discus* was high, at 74% and 72%, respectively. Also, there was homology of ≥65% with the CAT of vertebrates.

Based on multiple alignments using the cloned CAT, several characteristic elements were identified: His^{72}, Asn^{145} and Tyr^{355} are conserved catalytic amino acids of CAT in all species, including Pacific oyster (Tavares-Sanchez et al., 2004; Li et al., 2008). The conserved sites of RLFSYNDTH (residues 351-359), which is the catalase proximal heme ligand signature sequence, and of FDRERIPERVVHAKGAGA (residues 61-68), which is the proximal active site signature, were also identified (Tavares-Sanchez et al., 2004; Li et al., 2008). The high homology and two conserved characteristic sites of CAT suggest that the CAT of Pacific oyster is a member of the CAT family.

In the full-length GPX cDNA of Pacific oyster, a characteristic nonsense TGA codon (position 259, 80^{th} amino acid) was identified (Forstrom et al., 1978). This codon is a selenocysteine, which was first discovered in mice. This selenocysteine is the activation site of the enzyme (Chambers et al., 1986). Also, in the GPX of Pacific oyster, Glu^{112} and

Trp[187], which are catalytic residues that interact with selenocysteine, were conserved (Ursini et al., 1995). These results suggest that the GPX of Pacific oyster is a Se-GPX gene (Se-GPX). In molluscs, full-length Se-GPX has been reported for only two species, *U. tumidus* (DQ830766) and *D. polymorpha* (EF194204). A comparison of GPX homology between molluscs and vertebrates using the GPX cloned here indicated a low level of homology (≤33%). However, selenocysteine and the catalytic residues Glu and Trp were conserved in the Se-GPX of all species.

At 0.1 ppm Cd in the present study, increased AST and ALT activities in the hemolymph indicated that a toxicity-counteracting effect of the secondary antioxidant system of CAT and GPX of Pacific oysters was lost, resulting in damage to cells exposed to excess oxidative stress generated by Cd accumulation above tolerated levels. On the 7th day of exposure to 0.1 ppm of Cd, rapid deterioration in the metabolism of Pacific oysters was observed. Changes in gill tissues of Pacific oysters exposed to 0.01, 0.05 and 0.1 ppm of cadmium were evaluated on days 1, 3, 7 and 11 after treatment. Observation was conducted with an light microscope after dyeing the external tissues of the ordinary filaments, principal filament and hemolymph sinus of the plica, etc., using the H-E stain method. The fine structures of the cilia, microvilli and mitochondria, etc., were also observed with TEM. No morphological changes were observed in external structures of the gill tissues of Pacific oysters in any experimental group compared to the control group.

However, on day 11 of exposure to 0.05 ppm of Cd, dilations were observed through TEM in microvilli in the internal structure of the gill tissues of Pacific oysters. In addition, at 0.1 ppm Cd, the cilia count gradually decreased, while dilations of microvilli increased with exposure time to cadmium. Based on these results, Cd toxicity decreased the cilia count in the gill tissues of Pacific oysters and induced dilations of microvilli, the cellular projections filled with sensory cells that take part in absorption of substances and contraction of tissues. However, no extreme cellular damage, such as deformation of cells, vacuolation or damage to mitochondria or the nucleus inside a cell was observed within the

range of Cd concentrations (0.01, 0.05 and 0.1 ppm) and treatment time (up to 11 days) employed in this study. mRNA expression of the antioxidant enzymatic genes (SOD, CAT and GPX) as well as AST, ALT and H_2O_2 levels stabilized. However, once damaged by toxicity (decrease in the cilia count and dilations of microvilli), the tissue did not recover during the experiment. It is concluded that tissues, once damaged by Cd, cannot recover the functions and roles of antioxidant enzymatic genes. Therefore, it is suggested that increased mRNA expression levels of the antioxidant genes SOD, CAT and GPX may be used as markers for Cd contamination in Pacific oysters.

4. The dissolved oxygen content of the seawater was 5.4 mg/L (20℃) at the onset of the experiment, and oxygen consumption per hour by a single Pacific oyster inside a sealed respiration chamber was 0.3 mg/L. The expression of SOD mRNA in the digestive glands and gills of Pacific oysters increased for 1 h of exposure to the hypoxic environment, then subsequently decreased. Conversely, CAT and GPX mRNA expression decreased throughout the hypoxia treatment. During the first hour of the hypoxia treatment, the primary antioxidant defense for SOD was to transform superoxide radicals into H_2O_2 and O_2. However, the expected secondary defense, during which CAT and GPX break down the transformed H_2O_2 into O_2 and H_2O, did not take place. Therefore, since the harmful H_2O_2 was not completely removed, increased H_2O_2 toxicity negatively impacted the oysters. In addition, the oysters increased their inflow of seawater in an attempt to increase O_2 uptake under hypoxic conditions. Consequentially, hemolymph osmolality and K^+ levels decreased. Finally, hemolymph Ca^{2+} and Mg^{2+} concentrations increased in response to the oysters using calcium carbonate stores in their shells to supply bicarbonate as a way to alleviate acidosis caused by lowered pH. However, no histological changes were observed compared to control group throughout the experiment.

In conclusion, changes in the expression of stress-related and antioxidant enzymatic genes, as well as in osmolality, the levels of various inorganic ions, and AST and ALT activities were observed as Pacific oysters were exposed to various environmental

stressors. It is possible that Pacific oysters can inhabit environments with high salinity, water temperature, heavy metals and low oxygen such as those used in this study. A correlation between environmental conditions and the physiological responses of Pacific oysters was observed. Therefore, the factors outlined above can be used as physiological and stress-response markers for Pacific oysters exposed to environmental changes, and can thus be used to set environmental standards for shellfish farming. In addition, these data may be applied to assess the health of additional shellfish varieties exposed to diverse environments in aquaculture and in the wild.

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References

Ackerman, P.A., Iwama, G.K., 2001. Physiological and cellular stress responses of juvenile rainbow trout to vibriosis. J. Aquat. Anim. Health. 13, 173-180.

Amado, E.M., Freire, C.A., Souza, M.M., 2006. Osmoregulation and tissue water regulation in the freshwater red crab *Dilocarcinus pagei* (Crustacea, Decapoda), and the effect of waterborne inorganic lead. Aquat. Toxicol. 79, 1-8.

Almar, M., Otero, L., Santos, C., Gallego, J.G., 1998. Liver glutathione content and glutathione-dependant enzymes of two species of freshwater fish as bioindicators of chemical pollution. J. Environ. Sci. Heath. 33, 769-783.

Almeida, E.A., Miyamoto, S., Bainy, A.C.D., Medeiros, M.H.G., Mascio, P.D., 2004. Protective effect of phospholipid hydroperoxide glutathione peroxidase (PHGPx) against lipid peroxidation in mussels *Perna perna* exposed to different metals. Mar. Pollut. Bull. 49, 386-392

Almeida, J.A., Diniz, Y.S., Marques, S.F.G., Faine, L.A., Ribas, B.O., Burneiko, R.C,. Novelli, E.L.B., 2002. The use of the oxidative stress responses as biomarkers in Nile tilapia (*Oreochromis niloticus*) exposed to in vivo Cd contamination. Environ. Int. 27, 673-679.

Andersson, T., Förlin, T.L., 1992. Regulation of the cytochrome P450 enzymes system in fish. Aquat. Toxicol. 24, 1-20.

Andersson, T., Koivusaari, U., 1985. Influence of environmental temperature on the induction of xenobiotic metabolism by β-naphthoflavone in rainbow trout, *Salmo gairdneri*. Toxicol. Appl. Pharmacol. 80, 43-50.

Arlot-Bonnemains, Y., Fouchereau-Peron, M., Julienne, A., Milhaud, G., Moukhtar, M.S., 1991. Binding sites of calcitonin gene related peptide (CGRP) to trout tissues. Neuropeptides 20, 181-186.

Arukwe, A., Goksùyr, A., 2000. Strain specific patterns in temperature adaptation of CYP isoenzyme levels in Atlantic salmon (*Salmo salar*). Mar. Environ. Res. 50, 61-81.

Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schelte, P.M., Iwama, K., 2002. Heat shock protein genes and their functional significance in fish. Gene 295, 173-183.

Bebianno, M.J., Lopes, B., Guerra, L., Hoarau, P., Ferreira, A.M., 2007. Glutathione S-transferases and cytochrome P450 activities in *Mytilus galloprovincialisfrom* the South coast of Portugal Effect of abiotic factors. Environ. Int. 33, 550-558.

Beckmann, R.P., Mizzen, L.E., Welch, W.J., 1990. Interaction of HSP70 with newly synthesized proteins: implications for protein folding and assembly. Science 248, 850-854.

Benavides, M.P., Gallego, S.M., Tomaro, M.L., 2005. Cadmium toxicity in plants. Braz. J. Plant Physiol. 17, 21-34.

Beninger, P.G., Dufour, S.C., 1996. Mucocyte distribution and relationship to particle transport on the pseudolamellibranch gill of *Crassostrea virginica* (Bivalvia: Ostreidae). Mar. Ecol. Progr. Ser. 137, 133-138.

Berndtson, A., Chen, T., 1994. Two unique CYP1 genes are expressed in response to 3-methylcholanthrene treatment in rainbow-trout. Arch. Biochem. Biophys. 310, 187-195.

Bigas, M., Amiard-Triquet, C., Durfort, M., Poquet, M., 1997. Sublethal effects of experimental exposure to mercury in European flat oyster, *Ostrea edulis*: cell alterations and quantitative analysis of metal. Bio Metals. 10, 277-284.

Bigas, M., Durfort, M., Poquet, M., 2001. Cytological effects of experimental exposure to Hg on the gill epithelium of the European flat oyster Ostrea edulis: ultrastructural and quantitative changes related to bioaccumulation. Tissue & Cell 33, 178-188.

Braeckman, B., Raes, H., 1999. The ultrastructural effect and subcellular localization of mercuric chloride and metylmercuric in insect cells (Aedes albopictus C6/36). Tissue & Cell 31, 223-232.

Brouwer, M., Brouwer, T.H., 1998. Biochemical defense mechanisms against copper-induced oxidative damage in the blue crab, *Callinectes sapidus*. Arch. Biochem. Biophys. 351, 257-264.

Bukau, B., Horwich, A.L., 1998. The hsp70 and hsp60 chaperone machines. Cell 92, 351-366.

Burton, R.F., 1983. Ionic regulation and water balance. In: Saleuddin, A.S.M., Wilbur,

K.M., (Eds.), The Mollusca, 5. Physiology, Part 2. Academic Press, London, pp. 291-352.

Byrne, R.A., Shipman, B.N., Smatresk, N.J., Dietz, T.H., McMahon, R.F., 1991. Acidbase balance during emergence in the freshwater bivalve *Corbicula fluminea*. Physiol. Zool. 64, 748-766.

Chambers, I., Frampton, J., Goldfarb, P., Affara, N., Mcbain, W., Harrison, P., 1986. The structure of the mouse glutathione peroxidase gene: the selenocysteine in the active site is encoded by the 'termination' codon TGA. EMBO J. 5, 1221-1227.

Chance, B., Sice, H., Boveris, A., 1979. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. 59, 527-605.

Chang, Y.J., Choi, Y.H., Chang, Y.J., Choi, S.W., 2000. Effect of water temperature on the egg development of pearl oyster, *Pinctade fucata martensii* and Pacific oyster, *Crassotrea gigas*. J. Kor. Fish. Soc. 33, 559-564.

Chang, Y.J., Min, B.H., Choi, C.Y., 2007. Black porgy (*Acanthopagrus schlegeli*) prolactin cDNA sequence: mRNA expression and blood physiological responses during freshwater acclimation. Comp. Biochem. Physiol. B, 147, 112-128.

Cheng, T.C., 1981. Bivalves. In: Ratcliffe, N.A., Rowley, A.F., (Eds.), Invertebrate blood cell ? Academic Press, London, pp. 233-299.

Cheng, W., Liu, C.H., Kuo, C.M., 2003. Effect of dissolved oxygen on hemolymph parameters of freshwater giant prawn, *Macrobrachium rosenbergii* (de Man). Aquaculture 220, 843-856.

Cheng, W., Liu, C.H., Cheng, S.Y., Chen, J.C., 2004. Effect of dissolved oxygen on the acid base balance and ion concentration of Taiwan abalone *Haliotis diversicolor supertexta*. Aquaculture 231, 573-586.

Cho, Y.S., Choi, B.N., Kim, K.H., Kim, S.K., Kim, D.S., Bang, I.C., Nam, Y.K., 2006a. Differential expression of Cu/Zn superoxide dismutase mRNA during exposures to heavy metals in rockbream (*Oplegnathus fasciatus*). Aquaculture 253, 667-679.

Cho, Y.S., Lee, S.Y., Bang, I.C., Kim, D.S., Nam, Y.K., 2006b. Transcriptional response of major antioxidant enzyme genes to heat stress in mud loach (*Misgurnus mizolepis*). J. Aquacult. 19, 157-165.

Choi, C.Y., An, K.W., Nelson, E.R., Habibi, H.R., 2007a. Cadmium affects the expression of

metallothionein (MT) and glutathione peroxidase (GPx) mRNA in goldfish, *Carassius auratus*. Comp. Biochem. Physiol. C 145, 595-600.

Choi, C.Y., Min, B.H., Jo, P.G., Chang, Y.J., 2007b. Molecular cloning of PEPCK and stress response of black porgy (*Acanthopagrus schlegeli*) to increased temperature in freshwater and seawater. Gen. Comp. Endocrinol. 152, 47-53.

Choi, Y.H., Chang, Y.J., 1999. Survival rates of trochophores from pearl oyster, *Pinctada fucata martensii* and Pacific oyster, *Crassostrea gigas* immersed in four kinds of cryoprotectant. J. Kor. Fish. Soc. 32, 476-480.

Choi, C.Y., An, K.W., Choi, Y.K., Jo, P.G., Min, B.H., 2008a. Expression of warm temperature acclimation-related protein 65-kDa (Wap65) mRNA, and physiological changes with increasing water temperature in black porgy, *Acanthopagrus schlegeli*. J. Exp. Zool. 309, 206-214.

Choi, Y.K., Jo, P.G., Choi, C.Y., 2008b. Cadmium affects the expression of heat shock protein 90 and metallothionein mRNA in the Pacific oyster, *Crassstrea gigas*. Comp. Biochem. Physiol. C 147, 286-292.

Company, R., Serafim, A., Bebianno, M.J., Cosson, R., Shillito, B., Fiala-Médioni, A., 2004. Effect of cadmium, copper and mercury on antioxidant enzyme activities and lipid peroxidation in the gills of the hydrothermal vent mussel *Bathymodiolus azoricus*. Mar. Environ. Res. 58, 377-381.

Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z., Nardai, G., 1998. The 90-kDa molecular chaperone family: Structure, function, and clinical applications. A comprehensive review. Pharmacol, Ther. 79, 129-168.

David, E., Tanguy, A., Pichavant, K., Moraga, D., 2005. Response of the Pacific oyster *Crassostrea gigas* to hypoxia exposure under experimental conditions. FEBS J. 272, 5635-5652.

Defur, P.L., Mangum, C.P., Reese, J.E., 1990. Respiratory responses of the blue crab *Callinectes sapidus* in long-term hypoxia. Biol. Bull. 178, 46-54.

de la Torre, F.R., Salibian, A., Ferrari, L., 2000. Biomarker assessment in juvenile *Cyprinus carpio* exposed to waterborne cadmium. Environ. Pollution 109, 277-182.

Dubos, M., Badariotti, F., Rodet, F., Lelong, C., Favrel, P., 2003. Molecular and

physiological characterization of an invertebrate homologue of a Calcitonin-related receptor. Biochem. Biophys. Res. Commun. 310, 972-978.

Farcy, E., Serpentini, A., Fiévet, B., Lebel, J.M., 2007. Identification of cDNAs encoding HSP70 and HSP90 in the abalone *Haliotis tuberculata*: Transcriptional induction in response to thermal stress in hemocyte primary culture. Comp. Biochem. Physiol. B 146, 540550.

Ferraris, M., Radice, S., Catalani, P., Francolini, M., Marabini, L., Chiesara, E., 2002. Early oxidative damage in primary cultured trout hepatocytes: a time course study. Aquat. Toxicol. 59, 283-296.

Forstrom, J.W., Zakowski, J.J., Tappel, A.L., 1978. Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. Biochemistry 17, 2639-2644.

Forsyth, R.B., Candido, E.P.M., Babich, S.L., Iwama, G.K., 1997. Stress protein expression in Coho salmon with bacterial kidney disease. J. Aquat. Anim. Health 9, 18-25.

Fouchereau-Peron, M., 2001. Effect of calcitonin gene related peptide on the adenylate cyclase activity in abalone gill membranes. Regul. Pept. 99, 163-168.

Franklin, C.E., Davison, W., Carey, P.W., 1991. The stress response of an Antarctic teleost to an acute increase in temperature. J. Therm. Biol. 16, 173-177.

Fridovich, I., 1975. Superoxide dismutases. Annu. Rev. Biochem. 44, 147-159.

Funes, V., Alhama, J., Navas, J.I., Lopez-Barea, J., Peinado, J., 2005. Ecotoxicological effects of metal pollution in two mollusk species from the Spanish South Atlantic littoral. Environ. Pollution 139, 214-223.

Gagnaire, B., Frouin, H., Moreau, K., Thomas-Guyon, H., Renault, T., 2006. Effect of temperature and salinity on haemocyte activities of the Pacific oyster, *Crassostrea gigas* (Thunberg). Fish Shellfish Immunol. 10, 387-391.

Galtsoff, P.S., 1964. The American oyster *Crassostrea virginica* Gmelin. Fish. Bull. 64, 1-28.

Gao, Q., Song, L., Ni, D., Wu, L., Zhang, H., Chang, Y., 2007. cDNA cloning and mRNA expression of heat shock protein 90 gene in the haemocytes of Zhikong scallop *Chlamys farreri*. Comp. Biochem. Physiol. B 147, 704-715.

George, S., Young, P., Leaver, M., Clarke, D., 1990. Activities of pollutant metabolising

and detoxication systems in the liver of the plaice, *Pleuronectes platessa*: Sex and seasonal variations in non-induced fish. Comp. Biochem. Physiol. C 96, 185-192.

Geret, F., Serafim, A., Barreira, L., Bebianno, M.J., 2002. Response of antioxidant systems to copper in the gills of the clam *Ruditapes decussates*. Mar. Environ. Res. 54, 413-417.

Gupta, R.S., 1995. Phylogenetic analysis of the 90 kD heat shock family of protein sequence and an examination of the relationship among animals, plants and fungi species. Mol. Biol. Evol. 12, 1063-1073.

Hagerman, L., Uglow, R.F., 1982. Effects of hypoxia in osmotic and ionic regulation in the brown shrimp *Crangon crangon* (L.) from brackish water. J. Exp. Mar. Biol. Ecol. 63, 93-104.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41, 95-98.

Hansen, B.H., Romma, S., Garmo, O.A., Pedersen, S.A., Olsvik, P.A., Andersen, R.A., 2007. Induction and activity of oxidative stress-related proteins during waterborne Cd/Zn-exposure in brown trout (*Salmo trutta*). Chemosphere 67, 2241-2249.

Hartl, F.U., 1996. Molecular chaperones in cellular protein folding. Nature 381, 571-580.

Hendershot, L.M., Ting, J., Lee, A.S., 1988. Identity of the Immunoglobulin heavy chain binding protein with the 78,000 dalton glucose-regulated protein and the role of post-transcriptional modifications in its binding function. Mol. Cell. Biol. 8, 4250-4256.

Henkel, G., Krebs, B., 2004. Metallothioneins: zinc, cadmium, mercury, and copper thiolates and selenolates mimicking protein active site features structural aspects and biological implications. Chem. Rev. 104, 801-824.

Hermes-Lima, M., Storey, J.M., Storey, K.B., 2001. Antioxidant defenses and animal adaptation to oxygen availability during environmental stress. In: Storey, K.B., Storey, J.M., (Eds.), Cell and Molecular Responses to Stress. Elsevier, Amsterdam, pp. 263-287.

His, R.B., Seaman, M.N., Pagano, G., Trieff, N.M., 1996. Sublethal and lethal toxicity of aluminum, industry effluents to early developmental stages of the *Crassostrea gigas* oyster. Arch. Environ. Contam. Toxicol. 30, 335-339.

Hosoi, M., Shinzato, C., Takagi, M., Hosoi-tanabe, S., Sawada, H., Terasawa, E., Toyohara, H., 2007. Taurine transporter from the giant Pacific oyster *Crassostrea gigas*: function

and expression in response to hyper-hypo-osmotic stress. Fish. Sci. 73, 385-394.

Hosoi, M., Kubota, S., Toyohara, M., Toyohara, H., Hayashi, I., 2003. Effect of salinity change on free amino acid content in Pacific oyster. Fish. Sci. 69, 395-400.

Huang, H.Q., Cao, T.M., Lin, Q.M., 2004. Characteristics of trapping copper iron with scrolled ferritin reactor in the flowing seawater. Environ. Sci. Technol. 38, 2476-2481.

Iwama, G.K., Thomas, P.T., Forsyth, R.B., Vijayan, M.M., 1998. Heat shock protein expression in fish. Rev. Fish Biol. Fish. 8, 35-56.

Iwama, G.K., Vijayan, M.M., Forsyth, R.B., Ackerman, P.A., 1999. Heat shock proteins and physiological stress in fish. Am. Zool. 39, 901-909.

Jo, P.G., Choi, Y.K., An, K.W., Choi, C.Y., 2007. Osmoregulation and mRNA expression of a heat shock protein 68 and glucose-regulated protein 78 in the Pacific oyster *Crassostrea gigas* in response to changes in salinity. J. Aquacult. 20, 205-211.

Karr, S.W., Reinert, R.E., Wade, A.E., 1985. The effects of temperature on the cytochrome P-450 system of thermally acclimated bluegill. Comp. Biochem. Physiol. C 80, 135-139.

Kashiwagi, A., Kashiwagi, K., Takase, M., Hanada, H., Nakamura, M., 1997. Comparison of catalase in diploid and haploid *Rana rugosa* using heat and chemical inactivation techniques. Comp. Biochem. Physiol. B 118, 499-503.

Kim, J.H., Chang, M.I., Yoo, M.S., 2003. Spermatogenesis and spermatozoan ultrastructure of Pacific oyster (*Crassostrea gigas*). J. Kor. Fish. Soc. 36, 641-645.

Kim, K.Y., Lee, S.Y., Cho, Y.S., Bang, I.C., Kim, K.H., Kim, D.S., Nam, Y.K., 2007. Molecular characterization and mRNA expression during metal exposure and thermal stress of copper/zinc- and manganese-superoxide dismutases in disk abalone, *Haliotis discus discus*. Fish Shellfish Immunol. 23, 1043-1059.

Kim, M.O., Phyllis, E.B., 1998. Oxidative stress in critical care: is antioxidant supplementation beneficial? J. Am. Diet. Assoc. 98, 1001-1008.

Kim, T.H, Kim, K.J., Choe, M.K., Yeo, I.K., 2006. Physiological changes of juvenile abalone, *Haliotis sieboldii*, exposed to acute water-temperature stress. J. Aquacult. 19, 77-83.

Kim, T.H., Yang, M.H., Choe, M.G., Han, S.J., Yeo, I.K., 2005. Physiological studies on acute

water-temperature stress of abalone (*Haliotis discus hannai*). J. Aquacult. 18, 7-12.

Kobayashi, J., 1971. Relation between Itai-Itai disese and the pollution of river water by cadmium from mine, In: Jenkins, S.H. (Ed.), Advances in water pollution research 1970, vol. 1. Pergamon Press, Oxford, pp. 1-25.

Larade, K., Storey, K.B., 2002. Reversible suppression of protein synthesis in concert with polysome disaggregation during anoxia exposure in *Littorina littorea*. Mol. Cell Biochem. 232, 121-127.

Lee, K.M., Kaneko, T., Katoh, F., Aida, K., 2006. Prolactin gene expression and gill chloride cell activity in fugu *Takifugu rubripes* exposed to a hypoosmotic environment. Gen. Comp. Endocrinol. 149, 285-293.

Legeay, A., Achard-Joris, M., Baudrimont, M., Massabuau, J.C., Bourdineaud, J.P., 2005. Impact of cadmium contamination and oxygenation levels on biochemical responses in the Asiatic clam *Corbicula fluminea.* Aquat. Toxicol. 74, 242-253.

Li, C., Ni, D., Song, L., Zhao, J., Zhang, H., Li, L., 2008. Molecular cloning and characterization of a catalase gene from Zhikong scallop *Chlamys farreri*. Fish Shellfish Immunol. 24, 26-34.

Livingstone, D.R., Martinez, P.G., Michel, X., Narbonne, J.F., O`Hara, S., Ribera, C., Winston, G.W., 1990. Oxyradical production as pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis L.*, and other molluscs. Funct. Ecol. 4, 415-424.

Loosanoff, V.L., 1953. Behavior of oyster in water of low salinities. Proc. Natl. Shellfish. Assoc. 43, 135-151.

McDonald, D.G., Milligan, C.L., 1997. Ionic, osmotic and acid base regulation in stress. In: Iwama, G.W., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.) Fish Stress and Health in Aquaculture. Cambridge University Press, London, pp. 119-144.

McMahon, B.R., Burggens, W.W., Taylor, E.W., 1978. Acid-base changes during recovery from disturbance and during long term hypoxic exposure in the lobster, *Homarus vulgaris*. J. Exp. Zool. 205, 361-370.

McMahon, B.R., Burggens, W.W., Wikens, J.L., 1974. Respiratory responses to long term hypoxic stress in the crayfish *Orconectes virilis*. J. Exp. Biol. 60, 195-206.

Meincke, K.F., 1975. The influence of extreme temperatures on metabolic substances in

hemolymph and foot muscle of *Helix pomatia*. Comp. Biochem. Physiol. A 51, 373-376.

Mills, D., 2000. Combined effects of temperature and algal concentration on survival, growth and feeding physiology of *Pinctada maxima* (Jameson) spat. J. Shellfish Res. 19, 159-166.

Mizukami, Y., Okauchi, M., Arizono, K., Ariyosh, I.T., Kito, H., 1994. Isolation and sequence of cDNA-encoding a 3-methylcholanthrene -inducible cytochrome-P450 from wild red-sea bream, *Pagrus major*. Mar. Biol. 120, 343-349.

Morgan, J.D., Iwama, G.K., 1991. Effects of salinity on growth, metabolism, and ion regulation in juvenile rainbow trout and steelhead trout (*Oncorhynchus mykiss*) and fall chinook salmon (*Oncorhynchus kisutch*). Can. J. Fish. Aquat. Sci. 48, 2083-2094.

Morimoto, R.I., 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones and negative regulators. Genes Dev. 12, 3788-3796.

Morris, S., Butler, S.L., 1996. Hemolymph respiratory gas, acid-base, and ion status of the amphibious purple shore crab *Leptograpsus variegatus* (Fabricus) during immersion and environmental hypoxia. J. Crustac. Biol., 16, 253-266.

Mruk, D.D., Silverstrini, B., Mo, M., Chenga, C.Y., 2002. Antioxidant superoxide dismutase -a review: its function, regulation in the testis, and role in male fertility. Contraception 65, 305-311.

Murugavel, P., Pari, L., Sitasawad, S.L., Kumar, S., Kumar, S., 2007. Cadmium induced mitochondrial injury and apoptosis in vero cells: Protective effect of daily tetrasufide from garlic. Int. J. Biochem. Cell. Biol. 39, 161-170.

Nag, K., Kato, A., Sultana, N., Ogoshi, M., Takei, Y., Hirose, S., 2007. Fish calcitonin receptor has novel features. Gen. Comp. Endocrinol. 154, 48-58.

Navarro, J.M., Leiva, G.E., Martinez, G., Anguilera, C., 2000. Interactive effects of diet and temperature on the scope for growth of the scallop *Argopecten purpuratus* during reproductive conditioning. J. Exp. Mar. Biol. Ecol. 147, 67-83.

Ni, D., Song, L., Gao, Q., Wu, L.,Yu, Y., Zhao, J., Qiu, L., Zhang, H., Shi, F., 2007. The cDNA cloning and mRNA expression of cytoplasmic Cu, Zn superoxide dismutase (SOD) gene in

scallop *Chlamys farreri*. Fish Shellfish Immunol. 23, 1032-1042.

Nouroozzadeh, J., Tajaddinisarmadi, J., Wolff, S.P., 1994. Measurement of plasma hydroperoxide concentrations by ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. Anal. Biochem. 220, 403-409.

Pannunzio, T.M., Storey, K.B., 1998. Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod *Littorina littorea*. J. Exp. Mar. Biol. Ecol. 221, 277-292.

Park, Y.C., Choi, K.S., 2002. Growth and carrying capacity of Pacific oyster, *Crassostrea gigas*, in Kamak bay, Korea. Korean J. Environ. Biol. 20, 378-385.

Philip, S.R., 1995. Biomonitoring of heavy metal availability in the marine environment. Mar. Pollut. Bull. 31, 183-192.

Piano, A., Valbonesi, P., Fabbri, E., 2004. Expression of cytoprotective proteins, heat chock protein 70 and metallothioneins in tissues of *Ostrea edulis* exposed to heat and heavy metals. Cell Stress Chaperones 9, 134-142.

Picard, D., 2002. Heat-shock protein 90, a chaperone for folding and regulation. Cell. Mole. Life Sci. 59, 1640-1648.

Pickwell, G.V., Steinert, S.A., 1988. Accumulation and effects of organotin compounds in oysters and mussels: Correlation with serum biochemical and cytological factors and tissue burdens. Mar. Environ. Res. 24, 215-218.

Pratt, W.B., 1997. The role of the hsp90-based chaperone system in signal transduction by nucler receptors and receptors signaling via MAP kinase. Ann. Rev. Pharmacol. Toxicol. 37, 297-326.

Pua, E.C., Lee, Y.C., 2003. Expression of a ripening-related cytochrome P450 cDNA in Cavendish banana (*Musa acuminata* cv. Williams). Gene 305, 133-140.

Purdue, B.W., Tilakaratne, N., Sexton, P.M., 2002. Molecular pharmacology of the calcitonin receptor. Receptors Channels 8, 243-255.

Rainbow, P.S., White, S.L., 1989. Comparative strategies of heavy metal accumulation of Zn, Cu and Cd by crabs and brancles. Estuar. Coast. Shelf Sci. 21, 669-686.

Rees, C.B., Li, W., 2004. Development and application of a realtime quantitative PCR assay

for determining CYP1A transcripts in three genera of salmonids. Aquat. Toxicol. 66, 357-368.

Ricciardi, F., Binelli, A., Provini, A., 2006. Use of two biomarkers (CYP450 and acetylcholinesterase) in zebra mussel for the biomonitoring of Lake Maggiore (northern Italy). Ecotoxicol. Environ. Saf. 63, 406-412.

Romero, M.C., Ansaldo, M., Lovrish, G.A., 2007. Effects of aerial exposure on the antioxidant status in the subantarctic stone crab *Paralomis granulose* (Decapoda: Anomura). Comp. Biochem. Physiol. B 146, 54-59.

Romeo, M., Bennani, N., Gnassia-Barelli, M., Lafaurie, M., Girard, J.P., 2000. Cadmium and copper display different responses towards oxidative stress in the kidney of the sea bass *Dicentrarchus labrax*. Aquat. Toxicol. 48, 185-194.

Rudneva, I.I., 1999. Antioxidant system if Black Sea animals in early development. Comp. Biochem. Physiol. C 112, 265-271.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.

Sanders, B.M., 1993. Stress proteins in aquatic organisms: an environmental perspective. Crit. Rev. Toxicol. 23, 49-75.

Sastry, K.V., Subhadra, K., 1982. Effect of cadmium in some aspects of carbohydrate metabolism in a freshwater catfish, *Heteropneustes fossilis*. Toxicol. Lett. 14, 81-114.

Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F.U., Moarefi, I., 2000. Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. Cell 101, 199-210.

Schlesinger, M.J., Ashburner, M., Tissieres, A., 1992. Heat Shock from Bacteria to Man. Cold Spring Harbor, NY: Cold Spring Harbor Press, pp. 131-137.

Shen, Z., Crotti, T.N., Flannery, M.R., Matsuzaki, K., Goldring, S.R., McHugh, K.P., 2007. A novel promoter regulates calcitonin receptor gene expression in human osteoclasts. Biochim. Biophys. Acta 1769, 659-667.

Shumway, S.E., 1977. Effect of salinity fluctuation on the osmotic pressure and Na^{+}, Ca^{2+} and Mg^{2+} ion concentration in the hemolymph of bivalve molluscs. Mar. Biol. 41, 153-177.

Siraj, B.P., Usha, R.A., 2003. Cadmium-induced antioxidant defense mechanism in freshwater teleost *Oreochromis mossambicus* (Tilapia). Ecotoxicol. Environ. Saf. 56, 218-221.

Soares, S.S., Martins, H., Gutierrez-Merino, C., Aureliano, M., 2008. Vanadium and cadmium *in vivo* effects in teleost cardiac muscle: Metal accumulation and oxidative stress markers. Comp. Biochem. Physiol. C 147, 168-178.

Somero, G.N., Bowlus, R.D., 1983. Osmolytes and metabolic end products of molluscs: the design of compatible solute systems. Biochem. Physiol., 2. In: Hochachka, P.W. (Ed.), Environmental Biochemistry and Physiology, The Mollusca, Vol. 2, London: Academic Press, pp. 77-100.

Stengeman, J.J., Hahn, M.E., 1994. Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions, and regulation of cytochrome p450 in aquatic species. In: Ostrander, G.K., Malins, D. (Eds.), Aquatic toxicology: molecular, biochemical and cellular perspectives. Lexis Publishers, Boca Raton, pp. 87-206.

Stien, X., Amichot, M., Berge, J., Lafaurie, M., 1998. Molecular cloning of a CYP1A cDNA from the teleost fish Dicentrarchus labrax. Comp. Biochem. Physiol. C 121, 241-248.

Stohs, S.J., Bagchi, D., Hassoun, E., Bagchi, M., 2000. Oxidative mechanisms in the toxicity of chromium and cadmium ions. J. Environ. Pathol. Toxicol. Oncol. 19, 201-213.

Storey, K.B., 1993. Molecular mechanism of metabolic arrest in molluscs. In: Hochachka, P.W., Lutz, P.L., Sick, T.J., Osenthal, M.R., Thillart, G. (Eds.), Surviving Hypoxia: Mechanism of Control and Adaptation. CRC Press, Boca Raton, pp. 253-269.

Swiergosz-Kowalewska, R., Bednarska, A., Kafel, A., 2006. Glutathione levels and enzyme activity in the tissue of bank vole *Clethrionomys glareolus* chronically exposed to a mixture of metal contaminants. Chamosphere 65, 963-974.

Tavares-Sanchez, O.L., Gomez-Anduro, G.A., Felipe-Ortega, X., Islas-Osuna, M.A., Sotelo-Mundo, R.R., Barillas-Mury, C.B., Yepiz-Plascencia, G.Y., 2004. Catalase from the white shrimp *Penaeus* (*Litopenaeus*) *vannamei*: molecular cloning and protein detection. Comp. Biochem. Physiol. B 138, 331-337.

Torigoe, K., 1981. Oyster in Japan. Jour. Sci. Hiroshima Univ. Ser. B, Div. Zoology, 29,

292~419.

Toyohara, H., Hosoi, M., 2004. The role of taurine in the osmotic adaptation in the marine mussel *Mytilus galloprovincialis*. Mar. Biotechnol. 6, S511-S516.

Toyohara, H., Ikeda, H., Goto, C., Sawada, H., Hosoi, H., Takeuchi, K., Hayashi, I., Imamura, S., Yamashita, M., 2005. Osomo-responsive expression of oyster amino acid transporter gene and analysis of the regulatory region involved. Fish. Sci. 71, 465-470.

Ursini, F., Maiorino, M., Brigelius-Flohé, R., Aumann, K.D., Roveri, A., Schomburg, D., Flohé, L., 1995. Diversity of glutathione peroxidases. Methods Enzymol. 252, 38-53.

Vaglio, A., Landriscina, C., 1999. Changes in liver enzyme activity in the teleost *Sparus aurata* in response to cadmium intoxication. Ecotoxicol. Environ. Safety 43, 111-116.

Viarengo, A., 1990. Heavy metal effects on lipid peroxidation in the tissues of *Mytilus galloprovincialis* Lam. Comp. Biochem. Physiol. C 97, 3742.

Wang, Y., Fang, J., Leonard, S.S., Rao, K.M.K., 2004. Cadmium inhibits the electron transfer chain and induces Reactive Oxygen Species. Free Radic. Biol. Med. 36, 1434-1443.

Wedemeyer, G.A., McLeay, D.J., 1981. Method for determining the tolerance of fishes to environmental stressors. In: Pickering, A.D. (Ed.), Stress and Fish. Academic Press, London, pp. 247-275.

Wendel, A., Feuerstein, S., 1981. Drug-induced lipid peroxidation in mice-1. Modulation by monoxygenase activity, glutathione and selenium status. Biochem. Pharmacol. 30, 2513-2520

Wilburg, K.M., Saleuddin, A.S.M., 1983. Shell formation. In: Wilburg, K.M. (Ed.), The Mollusca, Vol. 4. New York, London, pp. 235-287.

Wu, R.S.S., 2002. Hypoxia: from molecular responses to ecosystem responses. Mar. Pollut. Bull. 45, 35-45.

Xue, Q., Tristan, R., 2000. Enzymatic Activities in European flat oyster, *Ostrea edulis*, and Pacific oyster, *Crassostrea gigas*, Hemolymph. J. Invertebr. Pathol. 76, 155-163.

Yoon, S.J., Kim, I.C., Yoon, Y.D., Lee, J.S., 2003. Assessment of toxic effect in aquatic environment and the fish cytochrome P450 1A (CYP1A) gene. Korean J. Environ. Biol. 21, 1-7.

Young, D., Roman, E., Moreno, C., O'Brien, R., Born, W., 1993. Molecular chaperones and the

immune response. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 339, 363-367.

Zhang, J.F., Wang, X.R., Guo, H.Y., Wu, J.C., Xue, Y.Q., 2004. Effects of water-soluble fractions of diesel oil on the antioxidant defenses of the goldfish, *Carassius auratus*. Ecoroxicol. Environ. Saf. 58, 110-116.

Zhang, Z., Li, X., Vandepeer, M., Zhao, W., 2006. Effects of water temperature and air exposure on the lysosomal membrane stability of hemocytes in Pacific oyster, *Crassostrea gigas* (Thunberg). Aquaculture 256, 502-509.