Molluscan Research 28(3): 158–164
http://www.mapress.com/mr/

ISSN 1323-5818
Magnolia Press

mRNA expression of HSP90 and SOD, and physiological responses to thermal and osmotic stress in the Pacific oyster, *Crassostrea gigas*

PIL GUE JO[1], KWANG WOOK AN[1], MI SEON PARK[2] & CHEOL YOUNG CHOI[1]*
[1]Division of Marine Environment & Bioscience, Korea Maritime University, Busan 606-791, Republic of Korea.
Email: choic@hhu.ac.kr (C.Y. Choi)
[2]Aquaculture Research Department, National Fisheries Research & Development Institute, Busan 619-705, Republic of Korea
* Corresponding author

Abstract

We investigated mRNA expression of heat shock protein 90 (HSP90) and superoxide dismutase (SOD) in gill tissues, and osmolality and hydrogen peroxide (H_2O_2) concentration in the haemolymph of Pacific oysters, *Crassostrea gigas*, exposed to 30ºC and 35 psu (thermal stress) and 20ºC and 52.5 psu (osmotic stress). HSP90 and SOD mRNA expression and H_2O_2 concentration were highest at 6 h, and osmolality was highest at 24 h during thermal stress experiments at 30ºC. HSP90 mRNA expression and H_2O_2 concentration was highest at six days, SOD mRNA expression was highest at nine days, and osmolality maximal at three days in *C. gigas* exposed to osmotic stress at 52.5 psu. These results indicate that high water temperature and high salinity induce changes in osmolality and H_2O_2 concentration, and that SOD and HSP90 play important roles in the effects of temperature and salinity on the gills of oysters. We suggest that the pattern of HSP90 mRNA expression is related to its function as a molecular chaperone contributing to the folding and maintenance of structural integrity, and that SOD mRNA was highly expressed as a defense against reactive oxygen species.

Key words: Heat shock proteins, H_2O_2, HSP90, osmolality, SOD.

Introduction

Among various environmental factors, changes in water temperature and salinity are foremost in causing physiological changes in aquatic organisms. In particular, rapid temperature change acts as a stressor that lowers health and causes disease (Wedemeyer and McLeay 1981). In general, a primary response to stress in fish is the activation of the hypothalamus–pituitary gland–hepatorenal line and the resulting secretion of cortisol into the blood. A secondary reaction is an increase in plasma glucose levels, water–ion imbalance, oxygen consumption, and energy requirements (McDonald and Milligan 1997). However, it is difficult to detect physiological stress in invertebrates such as molluscs, as their endocrine organs are not clearly differentiated.

Changes in salinity, which are closely related to growth, maturity, existence, and metabolism (Navarro *et al*. 2000) in molluscan species, are caused by, among other things, variations in tidal cycles, rainfall, and drainage from adjacent terrestrial sites (Tirard *et al*. 1997). The Pacific oyster, *Crassostrea gigas* (Thunberg, 1793) is an osmo- and thermo-conforming species (Shumway 1977). Consequently, rapid physicochemical changes in environmental factors such as water temperature and salinity negatively affect the physiological state of these oysters (Jo and Choi 2008).

Many natural stresses can directly alter protein conformation and stability. Protein-denaturing stressors such as heat can result in the exposure of hidden hydrophobic domains of proteins and cause nonspecific protein interactions (Hamdoun *et al*. 2003). Under these conditions, several families of proteins known as heat-shock proteins (HSP), or molecular chaperones, perform critical protein-stabilizing functions (Gething and Sambrook 1992). HSPs are divided into three groups based on molecular weight: HSP90 (85–90 kDa), HSP70 (68–73 kDa), and low-molecular HSP (16–47 kDa; (Basu *et al.* 2002). HSP90 makes up 1% ~ 2% of total cellular protein. It also exists in large quantities in cells not affected by stress and has a very important function as a molecular chaperone (Picard 2002). HSPs sensitively react not only to the stresses of water temperature and salinity, but also heavy metals, chemicals, and anoxia (Cruz-Rodríguez and Chu 2002). 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).

When an organism is exposed to stress, reactive oxygen species (ROS), such as the superoxide radical (O^{-2}), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-) and singlet oxygen ($^1O_2$) are generated. ROS, with their strong chemical oxidation activity, bind with other substances, attack the membranes of cells or organs and damage cell functions (Ferraris *et al.* 2002). Large amounts of ROS that promote oxidative stress are induced by many kinds of stress, such as toxicity of heavy metals and pollutants, and abiotic and biotic environmental factors (Stohs *et al.* 2000). Oxidative stress caused by ROS leads to lipid peroxidation, protein denaturation and DNA damage. 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.* 2007). Accordingly, this causes numerous physiological problems such as the promotion of ageing, reduction in disease resistance and lowering of reproductive ability (Kim and Phyllis 1998).

The antioxidant enzyme superoxide dismutase (SOD) is generated inside the body to prevent oxidative damage (Wendel and Feuerstein 1981). SOD is one of the

representative elements in the defence against reactive oxygen species. An important metalloenzyme, it exists in all aerobic organisms. SOD removes superoxide radicals by the process of dismutation into oxygen and hydrogen peroxide ($2O_{2-} + H^+ \rightarrow H_2O_2 + O_2$), (Fridovich 1975).

With the exception of a study by Kim *et al*. (2007) on SOD mRNA expression in abalone exposed to the stress of heavy metals and water temperature, and a study by Ni *et al.* (2007) on SOD mRNA expression in relation to immune response in a scallop, there is almost no research on SOD expression and function in relation to stress in molluscs.

To identify changes in the physiological state of *C. gigas* in relation to water temperature and salinity stress, we investigated changes in the mRNA expression of SOD (antioxidant enzyme) and HSP90 (stress protein) using various tissues of *C. gigas*, and osmolality and H_2O_2 concentrations in the haemolymph.

Materials and Methods

Oysters

We used one year-old Pacific oysters (average shell length: 112 ± 10.7 mm; height: 31.1 ± 5.4 mm; weight: 20.3 ± 3.9 g) obtained from an oyster hatchery on Daebu Island in Goseong (Gyeongnam, Korea). They were acclimated in three 40 L circulating filter tanks in the laboratory at a density of 30 oysters per tank. During the acclimation period of three days, the water temperature and salinity were maintained at 20 ± 1°C and 35 psu, respectively, under a photoperiod of 12 h light/12 h dark. Water was exchanged daily and no food was supplied.

Water temperature and salinity treatment

A control group of oysters was maintained in square circulating filter tanks at 20°C and 35 psu. Experimental group I was directly transferred from 20°C to 30°C and salinity was not changed, remaining at 35 psu (exp. I, thermal stress). Experimental group II was directly transferred from 35 psu to 52.5 psu with water temperature maintained at 20ºC (exp. II, osmotic stress). Dissolved oxygen, temperature and salinity were maintained constant with aeration, 50 W electric heater and daily water exchange. No mortality was observed in the oysters in the experimental period. Five oysters were randomly selected from two experimental groups at the following times: 0, 1, 3, 6, 12, 24, and 48 h of thermal stress; and 0, 1, 3, 6, and 9 days of osmotic stress. Gonad, digestive gland, mantle, gill, and intestine of oysters were dissected and immediately frozen in liquid nitrogen after collection and stored at –80°C until total RNA extraction.

Statistical analyses were performed on data obtained from each experiment. They were tested for significant differences using one-way analysis of variance (Tukey's test or least significant difference test) with the SPSS statistical package (Version 10.0). The significance level was set at $P = 0.05$.

Reverse transcription PCR (RT-PCR)

2.5 µg of total RNA extracted from the gonad, digestive gland, mantle, gill and intestine of oysters under thermal and osmotic stress were reverse transcribed with M-MLV reverse transcriptase (Bioneer) and oligo-d$(T)_{15}$ primer (Promaga). HSP90-specific primers for RT-PCR developed by Choi *et al.* (2008): 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-specific primers for RT-PCR was designed from the published sequence of *Crassostrea gigas* SOD (GenBank accession no. AJ496219) as follows: 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'). Equal amounts of RNA (as determined spectrophotometrically) from the various tissues (gonad, digestive gland, mantle, gill, and intestine) of thermal- and osmotic-treated oysters were used for reverse transcription. RT-PCR was performed using 2x Taq Premix 1 (Solgent, Korea). As a control, 28S ribosomal RNA (GenBank accession no. Z29546) of *C. gigas* was also amplified for each RT reaction product for use as a loading control. The 28S ribosomal RNA primers were 5'-TGC TCT GGA CTG TCC TAG GA-3' for the forward primer and 5'-ACC GAT TCG CCA CTG ACC AT-3' for the reverse primer. PCR products from 30 (28Sr, Hsp90 and SOD) cycles of amplification were visualized on a UV-transilluminator after electrophoresis on a 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).

Quantitative PCR (QPCR)

QPCR was conducted to determine the relative mRNA expression of HSP90 and SOD using total RNA extracted from the gills of the control oysters and those treated with high temperature and hypersalinity. With 2.5 µg of total RNA as a template, cDNA was synthesized using M-MLV reverse transcriptase (Bioneer) according to the manufacturer's instructions. First-strand cDNA synthesis was conducted using oligo-d$(T)_{15}$ primer (Promega) according to the manufacturer's instructions. Primers for QPCR were designed with reference to known gene sequences of *C. gigas* as follows: cgHSP90 forward primer (5'-GGT GAA TGT TAC CAA GGA AGG-3'), cgHSP90 reverse primer (5'-GTT ACG ATA CAG CAA GGA GAT G-3'), cgSOD forward primer (5'-ATG TCA TCT GCT CTG AAG GC-3'), cgSOD reverse primer (5'-TGG TGA TAC CGA TCA CTC CA-3'), cg28Sr forward primer (5'-AAA CAC GGA CCA AGG AGT CT-3'), and cg28Sr reverse primer (5'-AGG CTG CCT TCA CTT TCA TT-3'). QPCR amplification was conducted using a Bio-Rad MiniOpticon™ System (Bio-Rad, Hercules, CA) and iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. QPCR was carried out by denaturation at 95ºC for 5 min, followed by 40 cycles of denaturation at 95ºC for 20 s, annealing at 55ºC for 20 s and extension at 72ºC for 30 s. To ensure that the primers

amplified a specific product, we performed a melt curve (data not shown), and we 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 (data not shown). 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 the analysis software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. mRNA expression levels given below stand for an *n*-fold difference relative to 28Sr as the internal control.

Haemolymph analysis

The haemolymph was withdrawn from the pericardial cavity using a 3-mL syringe (23 gauge). The haemolymph was centrifuged at 10,000 ×*g* at 4°C for 5 min and the supernatant was stored at –80°C until analysis. Haemolymph osmolality was measured with a Vapor Pressure Osmometer (Vapro 5520, Wescor, Logan, UT).

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). Twenty microlitres of whole oyster haemolymph in marine anticoagulant (MAC, 0.1M 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 haemocytes to settle and adhere. A working color reagent was prepared by mixing 100 mL distilled water containing 100 mM sorbitol and 125 µM 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.

Results

Tissue distribution of HSP90 and SOD mRNA by RT-PCR

We investigated HSP90 and SOD mRNA expression in various tissues that had been exposed to high temperature (30°C, exp. I) for 6 h. HSP90 expression in various tissues that had been exposed to hypersalinity (52.5 psu, exp. II) for 6 days and SOD for 9 days, respectively. Expression of HSP90 and SOD mRNA was higher in the gill than the gonad, mantle, and intestine (Figure 1).

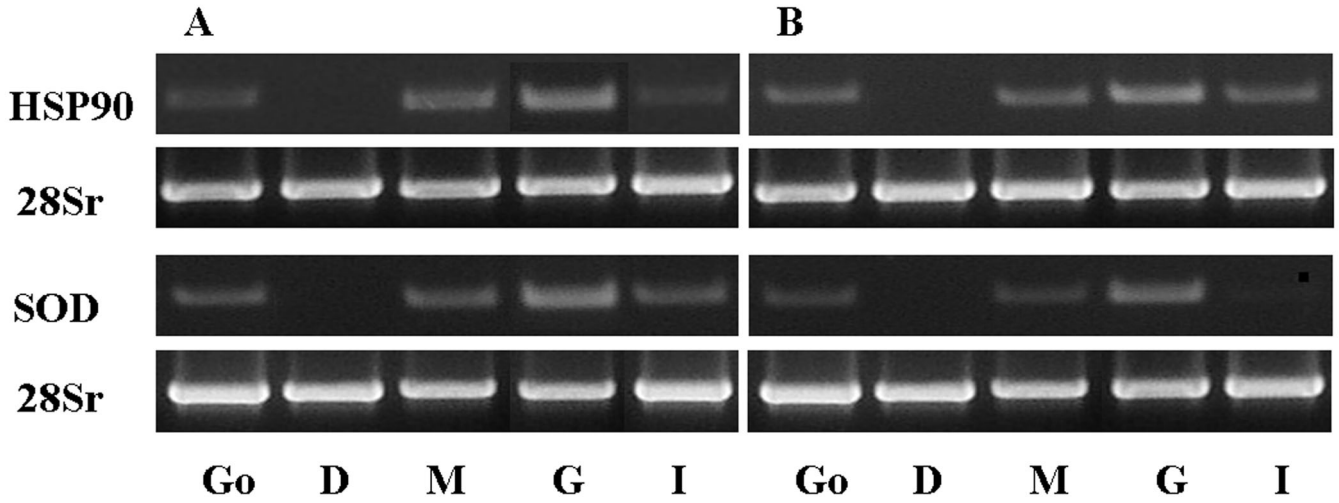

FIGURE 1. Tissue-specific expression of 90-kDa heat shock protein (HSP90) and superoxide dismutase (SOD) mRNA in various tissues of the Pacific oyster, *Crassostrea gigas* by reverse transcription polymerase chain reaction. **A**, exposed to high temperature (30ºC and 35 psu, HSP90 and SOD at 6 h). **B**, exposed to hypersalinity (52.5 psu and 20ºC, HSP90 at 6 days and SOD at 9 days). Amplification of 28S ribosomal RNA (28Sr) was used as an internal control. Go: gonad; D: digestive gland; M: mantle; G: gill; I: intestine.

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 showed significant increases up to 6 h in exp. I (HSP90 by 3.2 times; SOD by 9.1 times) and decreases afterwards (Figure 2A, C). In the gill tissues of oysters exposed to the stress of hypersalinity (exp. II), HSP90 mRNA expression increased significantly for 6 days (by 5.1 times at Day 6) and SOD mRNA for 9 days (3.1 times at Day 9) at 52.5 psu, and then decreased (Figure 2B, D).

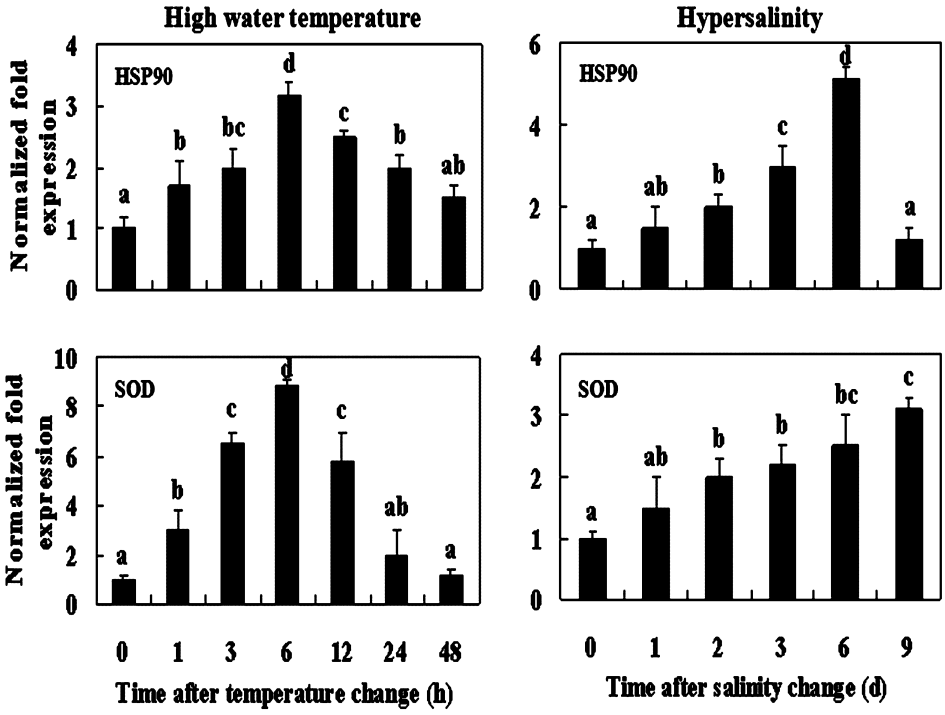

FIGURE 2. Time-related effect on 90-kDa heat shock protein (HSP90) and superoxide dismutase (SOD) mRNA levels in the Pacific oyster gill during high water temperature (from 20°C to 30ºC) and hypersalinity (from 35 psu to 52.5 psu) adaptation as determined by quantitative polymerase chain reaction. Results are expressed as fold change with respect to 28Sr levels for the same sample. Values with dissimilar letters are significantly different (P<0.05) to one another. Values are means±SD (n=5). h: hours; d: days.

Haemolymph osmolality

For oysters exposed to high water temperature (exp. I), haemolymph osmolality significantly increased for 24 h (1,207.7 ± 10.9 mOsm/kg), and then stabilized or even decreased (P<0.05; Figure 3). For oysters exposed to high salinity (exp. II), haemolymph osmolality significantly increased from its baseline (973 ± 9.7 mOsm/kg) after 3 days (1,429.7 ± 5.9 mOsm/kg), after which the concentrations started to decrease (P<0.05; Figure 3). Controls had no significant difference during the experimental periods.

Haemolymph hydrogen peroxide

H_2O_2 concentration significantly increased up to 6 h when it reached 6 ± 0.2 nM/mL from 2.4 ± 0.2 nM/mL at the beginning of the trial in the haemolymph of oysters exposed to high water temperature (exp. I) and then decreased (Figure 4). H_2O_2 concentration in the haemolymph of oysters exposed to hypersalinity (exp. II) increased significantly until 9 days when it reached 3.6 ± 0.1 nM/mL from 2.4 ± 0.2 nM/mL at the beginning of the trial. (Figure 4).

Discussion

In this study, expressions of HSP90 (the stress-response protein) and SOD (the antioxidant enzyme) were compared to identify stress responses of *Crassostrea gigas* to rapid changes in water temperature and salinity. To identify the level of stress, we investigated the amount of hydrogen peroxide and osmolality in the haemolymph.

QPCR indicated that HSP90 and SOD mRNA expression per organ reached their highest levels of expression in the gills of oysters exposed to high temperature and salinity. The high HSP90 and SOD mRNA expression were probably induced because the gill, as the primary interface between the outside environment and the haemolymph or cytoplasm (Hosoi *et al.* 2007) and as the direct absorption route for sea water 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 high temperature (30ºC) and high salinity (52.5 psu) were investigated by using QPCR.

HSP90 mRNA expression significantly increased until 6 h of exposure to water temperatures of 30ºC (Figure 2A). This result corresponds to reports that expression of the HSP family, including HSP90, increased according to increases in water temperature (Piano *et al.* 2004; Farcy *et al.* 2007). In a study on stress from water temperatures of 10ºC to 30ºC on Japanese abalone, *Haliotis discus hannai* (Ino, 1953), HSP mRNA expression increased in the test group up to 6 h at 30ºC and then decreased from 12 h (Kim *et al*. 2006). Kim *et al*. (2006) obtained results for HSP mRNA expression that were very similar to the present results.

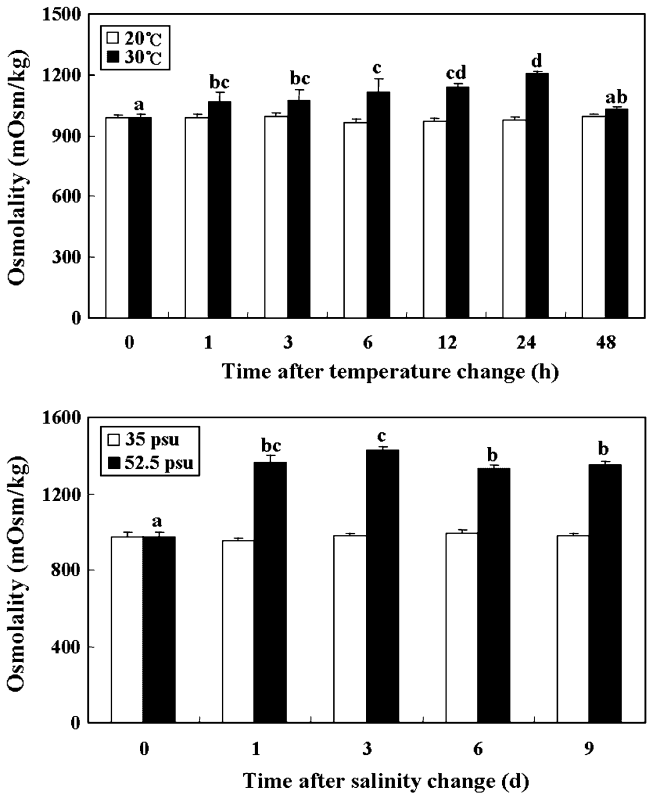

FIGURE 3. Osmorality in the haemolymph of the Pacific oyster, *Crassostrea gigas*, during high water temperature (from 20ºC to 30ºC) and hypersalinity (from 35 psu to 52.5 psu) adaptation.. Values with dissimilar letters are significantly different (P<0.05) from one another. Values are means±SD (n=5). h: hours; d: days.

HSP90 mRNA expression also increased significantly until Day 6 in the oysters exposed to high salinity (52.5 psu; Figure 2B). This suggests that HSP90 acts as a defence mechanism against the stress of not only high water temperature, but also high salinity. As with high temperature, oysters become affected by the stress after tolerating it for a certain period of time. Therefore, it may be hypothesized that HSP90 mRNA expression in the gills of *C. gigas* exposed to high temperature and high salinity is induced so that it may act as a molecular chaperone to maintain structural integrity and binding as well as appropriate control of the cytosolic protein subset (Picard 2002). Similar to the results of the HSP90 experiments, SOD mRNA expression significantly increased until 6 h at high temperature (30ºC) and then decreased (Figure 2C). This result corresponds to that of a study on rapid water temperature stress on Japanese abalone (*H. discus*; Kim *et al.* 2005). Our results are also in line with those of Cho *et al.* (2006), who found that SOD mRNA expression in mudfish, *Misgurnus mizolepis* (Gunther, 1888) exposed to high water temperature increased until Day 3. Although SOD mRNA expression in the gills of *C. gigas* exposed to high salinity significantly increased until Day 9 in our study (Figure 2D), it is necessary to investigate this further because there is as yet no other research on SOD in molluscs exposed to high salinity. SOD mRNA expression parallels the likely amount of cellular function damage due to generation of ROS by the stress of water temperature and salinity and reaches its highest levels at 6 h in the oysters exposed to 30ºC water temperatures and at nine days in the oysters exposed to salinity of 52.5 psu. Accordingly, it can be hypothesized that the greater SOD mRNA expression took place to remove the ROS.

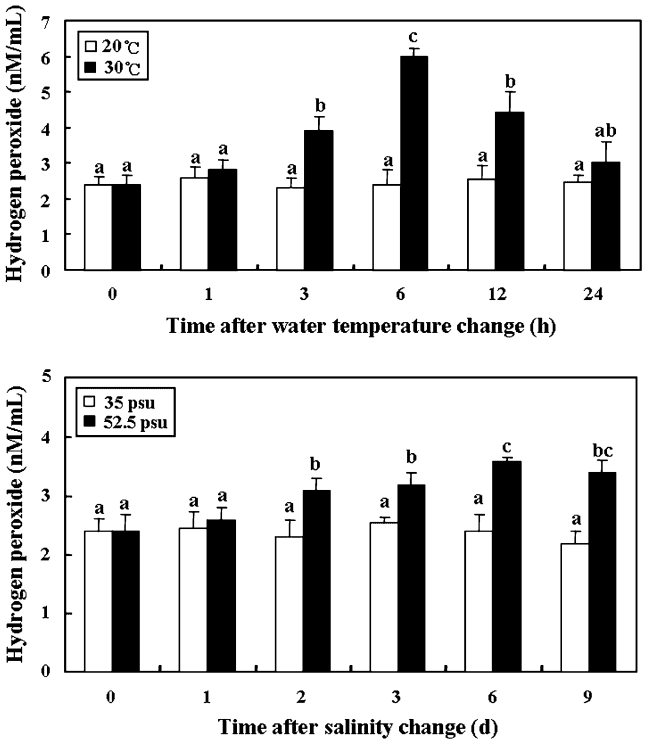

FIGURE 4. Hydrogen peroxide (H_2O_2) concentrations in haemolymph of *Crassostrea gigas* during high temperature (from 20ºC to 30ºC) and hypersalinity (from 35 psu to 52.5 psu) adaptation. Different lowercase letters indicate significant differences (P<0.05) to one another. Values are means±SD (n=5). h: hours; d: days.

In contrast, osmolality in the haemolymph of oysters significantly increased until 24 h in the oysters exposed to 30ºC water temperatures and then stabilized to levels shared by the controls (Figure 3). Franklin *et al*. (1991) reported that as a result of rapidly increasing the water temperature to 10ºC over 10 min, bald rock cod, *Pagothenia borchgrevinki* (Boulenger, 1902), acclimated to a temperature of 0ºC showed significantly increased osmolality, and then osmolality decreased and stabilized. Meincke (1975) reported that osmolality and ion changes in the haemolymph were observed in the foot muscles of snails, *Helix pomatia* (Linnaeus, 1758), when rapidly increasing the temperature from 20ºC to 43ºC. These results are in line with our observed changes in osmolality due to water temperature. Likewise, osmolality in the haemolymph of *C. gigas* immediately after exposure to high salinity significantly increased until the third day and then stabilized to levels shared by the controls (Figure 3). Compared to fish, the *C. gigas* is an environmentally conforming species that is highly influenced by the outside environment – for example

they react directly to rapid changes in salinity (Jo *et al.* 2007). Amado *et al.* (2006) reported that after red crab, *Dilocarcinus pagei* (Stimpson, 1861), acclimated to freshwater and seawater for 10 days each, osmolality and inorganic ion concentrations in the haemolymph rapidly changed and then stabilized to levels similar to external salinity.

H_2O_2 concentrations significantly increased at 6 h in the haemolymph of oysters exposed to thermal stress, and then decreased. In contrast, it increased until six days in response to hypersalinity (Figure 3). This evidence suggests that thermal and osmotic stress induced the formation of ROS in the haemolymph of *C. gigas*. ROS induced by thermal and osmotic stress in organisms are widely known to promote oxidative stress, causing membrane damage, DNA breakage, lipid peroxidation, enzyme inhibition, amino acid oxidation and apoptosis (Choi *et al*. 2007). We suggest that the changes in HSP90 and SOD expression levels are a defence against this stress.

In summary, expression of HSP90 and SOD mRNA was increased in Pacific oysters exposed to high water temperature and hypersalinity. Moreover, osmolality showed a tendency to stabilize after HSP90 expression and H_2O_2 concentrations tended to recover after SOD expression. Therefore, we suggest that HSP90 mRNA may be expressed to protect the cells from both stressors and SOD mRNA was expressed to remove the ROS resulting from them. Given that expression levels stabilized to match those of controls, and that no oysters died due to exposure, stress levels appear to have been reduced through these and possibly other physiological defense mechanisms. Furthermore, the environment used in this study was possibly the minimum water temperature required by Pacific oysters.

Acknowledgements

This work funded by a grant from the National Fisheries Research and Development Institute (RP-2008-AQ-045).

References

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. *Aquatic Toxicology* 79, 1–8.

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.

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

Choi, C.Y., An, K.W., Nelson, E.R. & Habibi, H.R. (2007) Cadmium affects the expression of metallothionein (MT) and glutathione peroxidase (GPx) mRNA in goldfish, *Carassius auratus*. *Comparative Biochemistry and Physiology C* 145, 595–600.

Choi, Y.K., Jo, P.G. & Choi. C.Y. (2008) Cadmium affects the expression of heat shock protein 90 and metallothionein mRNA in the Pacific oyster, *Crassostrea gigas*. *Comparative Biochemistry and Physiology* C 147, 286–292

Cruz-Rodríguez, L.A. & Chu, F.E. (2002) Heat-shock protein (HSP70) response in the eastern oyster, *Crassostrea virginica*, exposed to PAHs sorbed to suspended artificial clay particles and to suspended field contaminated sediments. *Aquatic Toxicology* 60, 157–168.

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. *Comparative Biochemistry and Physiology* B 146, 540–550.

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. *Aquatic Toxicology* 59, 283–296.

Franklin, C.E., Davison, W. & Carey, P.W. (1991) The stress response of an Antarctic teleost to an acute increase in temperature. *Journal of Thermal Biology* 16, 173–177.

Fridovich, I. (1975) Superoxide dismutases. *Annual Review of Biochemistry* 44, 147–159.

Gething, M.J. & Sambrook, J (1992) Protein folding in the cell. *Nature* 355, 33–45.

Hamdoun, A.M., Cheney, D.P. & Cherr, G.N. (2003) Phenotypic plasticity of HSP70 and HSP70 gene expression in the Pacific oyster (*Crassostrea gigas*): Implications for thermal limits and induction of thermal tolerance. *The Biological Bulletin* 205, 160–169.

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. *Fishery Science* 73, 385–394.

Jo, P.G. & Choi, C.Y. (2008) Osmoregulation and mRNA expression of calcitonin-related receptor in the Pacific oyster *Crassostrea gigas*. *Molluscan Research* 28, 137–141.

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. *Journal of Aquaculture* 20, 205–211.

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 and Shellfish Immunology* 23, 1043–1059.

Kim, M.O. & Phyllis, E.B. (1998) Oxidative stress in critical care: is antioxidant supplementation beneficial? *Journal of the American Dietetic Association* 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. *Journal of Aquaculture* 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*). *Journal of Aquaculture* 18, 7–12.

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.

Meincke, K.F. (1975) The influence of extreme temperatures on metabolic substances in hemolymph and foot muscle of *Helix pomatia*. *Comparative Biochemistry and Physiology* A 51, 373–376.

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 and Development* 12, 3788–3796.

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 *Argopectin purpuratus* during reproductive conditioning. *Journal of Experimental Marine Biology and Ecology* 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 and Shellfish Immunology* 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. *Analytical Biochemistry* 220, 403–409.

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

Picard, D. (2002) Heat-shock protein 90, a chaperone for folding and regulation. *Cellular and Molecular Life Sciences* 59, 1640–1648.

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. *Marine Biology* 41, 153–177.

Stohs, S.J., Bagchi, D., Hassoun, E. & Bagchi, M. (2000) Oxidative mechanisms in the toxicity of chromium and cadmium ions. *Journal of Environmental Pathology, Toxicology and Oncology* 19, 201–213.

Tirard, C.T., Grossfeld, R.M., Levine, J.F. & Kennedy-Stoskopf, S. (1997) Effect of osmotic shock on protein synthesis of oyster hemocytes *in vitro*. *Comparative Biochemistry and Physiology A* 116, 43–49.

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. *Biochemical Pharmacology* 30, 2513–2520.

Young, D., Roman, E., Moreno, C., O'Brien, R. & Born, W. (1993) Molecular chaperones and the immune response. *Philosophical Transactions of the Royal Society of London* 339, 363–367.