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Osmoregulation and mRNA expression of calcitonin-related receptor in the Pacific oyster *Crassostrea gigas*

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Abstract

The Pacific oyster *Crassostrea gigas* is extensively cultivated in Korea, constituting an important economic activity in the country. Salinity is highly relevant to the growth, maturation, distribution, food intake, energy activation, and metabolic activities of many types of bivalves, including *C. gigas*. In our experiments, the osmotic concentration of the haemolymph changed immediately after exposure of the oysters to high salinity and the concentrations of osmolality, Na^+, Cl^- and Ca^{2+} peaked at 72 h in the haemolymph. Calcitonin is a key hormone involved in regulation of calcium metabolism. Under conditions of hypersalinity, the expression of the calcitonin-related receptor (CT-R) mRNA in the gill significantly increased for 72 h and then decreased. This pattern was similar to the changes in concentration of osmolality and Na^+, Cl^- and Ca^{2+} concentration. It is likely that CT-R plays an important role in osmoregulation in *C. gigas* through the regulation of calcium metabolism and osmolality.

Key words: CT-R, hyper-osmotic stress, ions, osmolality

Introduction

The Pacific oyster *Crassostrea gigas* (Thunberg, 1793) is an important product of the marine aquaculture industry and comprises about 80% of the world's total oyster output (FAO 2001). Most oyster species inhabit, and are tolerant of, waters of differing salinity (Hosoi *et al*. 2003). Salinity is highly relevant to the growth, maturation, distribution, food intake, energy activation and metabolic activity of many types of bivalves (e.g., Navarro *et al*. 2000). Recent studies of the effects of salinity on *C. gigas* have investigated the soluble amino acid content of haemolymph (Hosoi e*t al*. 2003), hemocyte activity (Gagnaire *et al*. 2006) and the amino acid transporter gene (Toyohara *et al*. 2005). These osmotic adaptations involve accumulation and quantitative regulation of organic solutes ('osmolytes') in the intracellular space to enable cells to respond to the internal variable salinity while maintaining cell volume and low, stable intracellular salinity concentrations (Somero and Bowlus 1983).

In vertebrates, calcitonin (CT) 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 (e.g., Shen *et al*. 2007). CT 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 CT has strong effects on the human body and is used for treatment of human bone diseases. CT 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). CT has been identified so far in five fish species—*Takifugu rubripes* (Temminck and Schlegel, 1850) (GenBank accession no. NM001105219), *Oryzias latipes* (Temminck and Schlegel, 1846) (NM001104924), *Oncorhynchus gorbuscha* (Walbaum, 1792) (X78080), *Danio rerio* (Hamilton, 1822) (BC076343) and *Paralichthys olivaceus* (Temminck and Schlegel, 1846) (AB052782). Also, the calcitonin-related receptor (CT-R) has been cloned so far in several aquatic animals such as the fishes *Oryzias latipes* (BR000372), *Oncorhynchus gorbuscha* (AJ508554), *Danio rerio* (XM690423), *Paralichthys olivaceus* (AB035315), *Takifug obscurus* (Abe, 1949) (AB219840), *T. rubripes* (NM001105219), *Gasterosteus aculeatus* (Linnaeus, 1758) (BR000371), *Tetraodon nigroviridis* (Marion de Proce, 1822) (BR000370), the ascidian *Ciona intestinalis* (Linnaeus, 1767) (AB081313), the sea urchin *Strongylocentrotus purpuratus* (Stimpson, 1857) (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 the physiological changes in *C. gigas* exposed to high salinity, we investigated osmotic pressure, Na^+, Cl^- and Ca^{2+} ion exchange from the haemolymph, and the expression patterns of CT-R mRNA.

Materials and methods

Experimental oyster

We used 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) from the oyster hatchery on Dae-bu Island in

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Goseong (Gyeongnam, Korea) and placed them into 100 L circulation filter tanks in the laboratory, with 50 oysters per tank. During the experimental period, water temperature was kept at 18 ± 0.5ºC and 35 psu[1], and the photoperiod was 12 h light/12 h dark.

Salinity treatment

After the oysters had been acclimated in normal seawater (35 psu) and 40 oysters were transferred to 100 L tank (52.5 psu, 150% of ambient seawater) from different tanks. Samples were then collected from five oysters at each of the following times: 0, 12, 24, 48, 72, 96, and 144 h. No food was supplied during the experimental period.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was conducted (as described below) to determine the relative expression of CT-R and 28S rRNA in *C. gigas* tissues. To optimize the cycle number used for RT-PCR analysis, 2.5 µg of total RNA extracted from the gonad, digestive gland, mantle, gill, intestine and adductor muscle of hypersalinity-treated oysters 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 µg of total RNA was reverse transcripted with M-MLV reverse transcriptase (Bioneer) and oligo-d$(T)_{15}$ primer (Promega). CT-R (AJ551182), and 28S rRNA-specific primers for RT-PCR were designed from the published sequences: cgCT-R forward primer (5'-CTG AAC GCT GTT GCC AGA GA-3') and cgCT-R reverse primer (5'-TCG AAC ACG GTC GTA CTG GT G-3'), cg28Sr forward primer (5'-TGC TCT GGA CTG TCC TAG GA-3') and cg28Sr 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. For CT-R: one cycle of denaturation at 94°C for 5 min, a total of 32 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 1 min, followed by one cycle of 5 min at 72ºC for the final extension. The 28S rRNA was amplified in each PCR reaction as a loading control. Amplification of 28S rRNA (as the control) was as follows: one cycle of denaturation at 94°C for 5 min, a total of 27 cycles of denaturation at 94ºC for 30 s, annealing at 54ºC for 30 s, and extension at 72ºC for 1 min, followed by one cycle of 5 min at 72ºC for the final extension. 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 µg/µL), and the signal intensity was quantified with the Gel-Doc System and Gelpro 3.1 software (KBT, Incheon, Korea).

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was conducted to determine the relative mRNA expression of CT-R using total RNA extracted from gills of control and hypersalinity-treated oysters. Primers for qRT-PCR were designed with reference to known gene sequences of the Pacific oyster as follows: cgCT-R forward primer, 5'-GAC CGA CCA ACA AAC GCT TTC-3'; cgCT-R reverse primer, 5'-GTT GTG TAG AAG GCT GCC ATT G-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'. qRT-PCR amplification was conducted using a Bio-Rad MiniOpticon™ System (Bio-Rad, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. qRT-PCR 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 20 s, and then 95ºC for 1 min and 55ºC for 1 min. To ensure that the primers amplified a specific product, we performed to read every 0.2ºC from 60ºC to 95ºC for a melt curve (data not shown) and analyzed the qRT-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. All analyses were based on the calculated threshold cycle time (TCT) values of the qRT-PCR products. The TCT was defined as the qRT-PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. qRT-PCR data from five replicate samples were analyzed with the Bio-Rad system's analysis software to estimate transcript copy numbers for each sample. mRNA expression levels stood for an *n*-fold difference relative to 28S rRNA as the internal control.

Haemolymph analysis

Haemolymph was withdrawn from the pericardial cavity with a 3 mL syringe and samples were separated by centrifugation (4°C, 10,000×*g*, 5 min) and stored at -80ºC until analysis. Haemolymph osmolality was measured with a Vapor Pressure Osmometer (Vapro 5520, Wescor Co., USA), and Na^+ and Cl^- were measured with an autoanalyzer (Hitachi 7180, Hitachi Co., Japan). Ca^{2+} was measured with an AVL 9180 electrolyte analyzer (AVL, USA).

Statistical analysis

The significance of the differences in the data obtained from each experiment were tested using one-way analysis of variance (ANOVA, Tukey's test or LSD test) with the SPSS statistical package (version 10.0) at a significance level of $p<0.05$.

Results

Osmotic concentration

When *C. gigas* was exposed to high-osmotic concentrations (salinity of 52.5 psu), haemolymph osmolality significantly increased ($p<0.05$) from its baseline (973 ± 9.7 mOsm/kg) after both 48 and 72 h (1429.7 ± 2.6 mOsm/kg), and then started to decrease (Fig. 1) to around 973 ± 9.7 mOsm/kg at the end of the experiment (144 h).

1. Practical salinity unit

Controls had no significant difference in experimental periods.

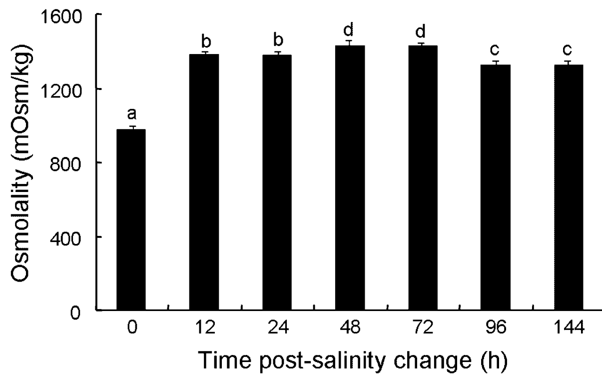

FIGURE 1. Osmolality in the haemolymph of Pacific oysters during adaptation to hypersalinity at 52.5 psu. Haemolymph was separated from oysters and used for osmolality measurements. Values with dissimilar letters are significantly different (p<0.05). Values are means mean square error of three experiments, each with five oysters.

CT-R mRNA expression in tissues

CT-R mRNA expression in tissues of *C. gigas* that had been exposed to high salinity were compared after 72 h. CT-R mRNA was highly expressed in the gill, had less expression in gonad, mantle and adductor muscles, was only slightly expressed in the intestine and not at all in digestive gland tissues (Fig. 2). The expression of CT-R mRNA was very low in various tissues of control (data not shown).

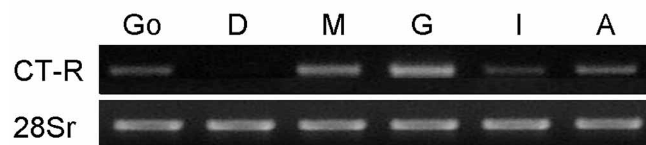

FIGURE 2. Tissue-specific expression of CT-R mRNA in various tissues of Pacific oyster by RT-PCR. Amplification of 28S ribosomal RNA was used as an internal control. Go: gonad; D: digestive gland; M: mantle; G: gill; I: intestine; A: adductor muscle.

CT-R mRNA expression

When the oysters were exposed to high salinity (52.5 psu), the CT-R mRNA expression pattern in gills increased significantly (p<0.05) up to 72 h and then decreased, while controls in normal seawater (35 psu) showed no significant changes in CT-R mRNA expression (Fig. 3).

Na^+, Cl^- and Ca^{2+} concentration

When the oysters were exposed to high salinity (52.5 psu), the concentrations of Na^+, Cl^- and Ca^{2+} in haemolymph increased significantly (p<0.05) for the first 72 h (657.7 ± 3.2, 715.0 ± 1.4, and 172.0 ± 0.9 mmol/L, respectively), then stabilized or even decreased after 96 h of exposure (Table 1). Controls have no significant difference in experimental periods (p<0.05).

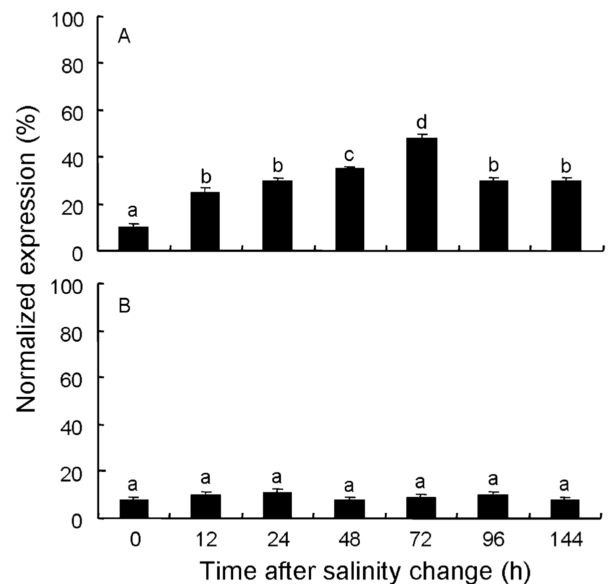

FIGURE 3. Time-related effects on calcitonin-regulated receptor (CT-R) mRNA levels in Pacific oyster gills during hypersalinity (52.5 psu) adaptation (A) and at normal salinity (35 psu) (B). Total RNA was extracted 0, 12, 24, 48, 72, 96 and 144 h after treatment, and 1 L was used for PCR. The values are percentage increases relative to control values. The relative expression level of each sample was normalized with respect to the 28S ribosomal RNA signal. Values with dissimilar letters are significantly different (p<0.05). Values are means mean square error of three experiments, each with five oysters.

TABLE 1. Na^+, Cl^- and Ca^{2+} concentration in haemolymph of Pacific oysters exposed to hyper-salinity (52.5 psu).

Time post-salinity change (h)	Inorganic ions (mmol/L)		
	Na^+	Cl^-	Ca^{2+}
0	$450.3±12.2^a$	$428.3±7.7^a$	$87.0±2.0^a$
12	$600.0±7.2^b$	$643.3±4.9^b$	$144.7±0.9^b$
24	$625.3±8.5^{bc}$	$682.5±6.7^{cd}$	$157.7±1.4^c$
48	$638.3±5.1^{cd}$	$694.7±4.5^{cd}$	$164.3±1.4^d$
72	$657.7±3.2^d$	$715.7±1.4^d$	$172.0±0.9^e$
96	$618.7±16.9^{bc}$	$677.6±6.8^c$	$164.5±1.6^d$
144	$625.3±3.8^{bc}$	$681.3±13.3^{cd}$	$167.7±3.8^{de}$

For hypersalinity (52.5 psu) experiments, haemolymph was withdrawn from the posterior adductor muscle sinus of Pacific oysters. Values (means mean square error of five replication) in each column with no superscript letters in common are significantly different (p<0.05).

Discussion

Salinity is a key factor affecting the physiology and survival of oysters as reported in many studies (*e.g.*, Toyohara *et al.* 2005; Gagnaire *et al.* 2006; Hosoi *et al.* 2007). Most marine invertebrates possess no specific systems to regulate their body functions in response to the external environment, but

they can osmoregulate and adapt at the cellular level by using the osmolyte system for basic osmotic pressure control (Hosoi *et al*. 2007). The system cannot maintain osmotic equilibrium or adapt to osmotic pressure via the haemolymph, but has limited osmoregulatory ability at the cellular level to maintain functional cellular components and volumes (Loosanoff 1953; Burton 1983). Bivalves osmoregulate by opening and closing the 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 been previously shown that *C. gigas* quickly responds to radical changes in salinity (Jo *et al*. 2007), and are thus capable of adapting to different esturarine environments. This was confirmed when the osmotic concentrations of the haemolymph of *C. gigas* changed immediately after exposing these oysters to high salinity (Fig. 1). When oysters were exposed to high salinity, the haemolymph 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 haemolymph 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 (Table 1). In *C. gigas*, the concentrations of Na^+ and Cl^- constituted 70% of the osmotic pressure, as in other marine animals, including fish, indicating that they are the major ions regulating osmotic pressure (Chang *et al*. 2007). Amado *et al*. (2006) acclimated ocean invertebrates like the red crab *Dilocarcinus pagei* (Stimpson, 1861) to freshwater and seawater for 10 days and reported that the osmotic concentrations and mineral ion concentrations of the haemolymph changed sharply and then stabilized at a level close to the external salinity. Their result was similar to ours where the osmotic pressure of the haemolymph in oysters exposed to high salinity, and the concentrations of Na^+, Cl^- and Ca^{2+}, peaked at 72 h.

This study examined the expression of CT-R mRNA in various tissues of Pacific oysters exposed to high salinity using RT-PCR (Fig. 2). Dubos *et al*. (2003) also found high CT-R mRNA expression in the gill. We found that CT-R mRNA was expressed in most tissues, except in the digestive gland, and that the highest expression was in the gill. The gill of marine molluscs constitutes the primary interface between the haemolymph 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 high salinity (52.5 psu) showed the highest expression among all the tissues examined.

Dubos *et al*. (2003) suggested that a significant decrease of 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 where the temporal changes in CT-R mRNA expression were examined using RT-PCR and were shown to increase for 72 h after exposure to high salinity, and then start to decrease (Fig. 3). Our results imply that CT-R mRNA is expressed in response to the salinity change and we postulate 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 concentration increases in high-salinity environments. In addition, the neuropeptide CT increases carbonic anhydrase activity and adenylate cyclase in the gill membranes of the trout *Oncorhynchus masou* (Brevoort, 1856) and the abalone *Haliotis tuberculata* (Linnaeus, 1758) (Arlot-Bonnemains *et al*. 1991; Fouchereau-Peron 2001). Endocrinology and molecular biology studies are needed to examine the relationship of CT, CT-R and Ca^{2+} concentration in the neuroendocrine regulation of molluscs.

In summary, the high 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 the 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.

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