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Molecular characterization and expression analysis of regucalcin in disk abalone (*Haliotis discus discus*): Intramuscular calcium administration stimulates the regucalcin mRNA expression

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Abstract

Regucalcin is a novel calcium (Ca^{2+}) binding protein and it has been demonstrated to play a multifunctional role in many organisms. Here, we report the molecular cloning of invertebrate regucalcin cDNA from disk abalone *Haliotis discus discus*. The full length cDNA showed 1321 bp of nucleotides with a polyadenylated sequence (AATAAA). Abalone regucalcin (HdReg) open reading frame (ORF) consists of 918 nucleotides encoding 305 amino acids (aa). Estimated molecular mass was 33 kDa and predicted isoelectric point (pI) was 4.9. The HdReg aa sequence did not contain the EF-hand motif as a Ca^{2+} binding domain, suggesting a novel class of Ca^{2+} binding protein. Moreover, it showed 45% identity to chicken and zebrafish, and 44% to rat and mouse regucalcin in deduced aa level. The tissue expression analysis of HdReg mRNA was investigated by RT-PCR and it was expressed in all the tissues tested such as gill, mantle, digestive tract, and abductor muscle. Semi-quantitative RT-PCR results showed that an intramuscular administration of calcium chloride ($CaCl_2$) (0.5 mg $CaCl_2$/g of abalone) could significantly induce regucalcin mRNA in abductor muscle after 30 min of administration and reached maximum after 1 h. Subsequently, the expression level was decreased after 2 h. This indicates that the expression of regucalcin mRNA is constitutive, and specifically up regulated in abalone abductor muscle by Ca^{2+} administration.

Keywords: Calcium binding protein; Calcium chloride; Gene expression; *Haliotis discus discus;* Invertebrate regucalcin

1. Introduction

Calcium (Ca^{2+}) is an important ion for many organisms. It acts as a second messenger in the regulation of many cellular and physiological functions such as muscle contraction, neuronal activation, cell differentiation and cell death. There are many energy dependent Ca^{2+} transporters and Ca^{2+} channels are located in the plasma membrane and membranes of intracellular organs. Intra-cellular Ca^{2+} levels and Ca^{2+} signals are mediated by those energy dependent Ca^{2+} transporters and Ca^{2+} channels (Osterloh et al., 1998). Numbers of Ca^{2+} binding proteins are involved in regulation of Ca^{2+} levels within the cytoplasm. Currently, the largest groups of Ca^{2+} binding proteins are the EF-hand super family genes containing EF-hand motifs (Nakayama and Kretsinger, 1994). It has been reported that most of these proteins such as calmodulin, troponin C, and myosin light chain maintain intracellular Ca^{2+} homeostasis and transmit Ca^{2+} signals to regulate specific target proteins when activated in their Ca^{2+} bound conformation (Osterloh et al., 1998).

Regucalcin is a regulatory protein of Ca^{2+} and also known as senescence marker protein-30 (SMP30). It was discovered in

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1978 as a novel Ca^{2+} binding protein in rat liver cells (Yamaguchi and Yamamoto, 1978; Yamaguchi and Sakurai, 1992). Regucalcin is different from calmodulin and other Ca^{2+} related proteins because it does not contain EF-hand motif as a Ca^{2+} binding domain. Multifunctional roles of regucalcin, related to regulation of cellular functions in rat liver, kidney, and brain neuronal cells have been reported. It regulates intracellular Ca^{2+} homeostasis by enhancing Ca^{2+} pumping activity in the plasma membrane (Kurota and Yamaguchi, 1997; Takahashi and Yamaguchi, 1997) and mitochondria of liver and renal cortex cells through activation of Ca^{2+} dependent ATPases (Yamaguchi, 2005). Regucalcin has a reversible effect on the Ca^{2+} induced activation and inhibition of many liver and renal cortex cell enzymes (Yamaguchi and Sakurai, 1992). It also inhibits various protein kinases (including Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C and tyrosine kinase) and protein phosphatases due to binding of Ca^{2+}, indicating a regulatory role in signal transduction within the cell. Moreover, it has been reported that regucalcin can inhibit synthesis of RNA in the nuclei of normal and regenerating rat liver in vitro (Yamaguchi and Ueoka, 1997).

Regucalcin gene was identified in many vertebrates and regucalcin cDNA was cloned for human (*Homo sapiens*, NP_004674); rat (*Rattus norvegicus*, BAA07490); rabbit (*Oryctolagus cuniculus*, BAA88079); mouse (*Mus musculus*, NP_033086); bovine (*Bos taurus*, BAA88080), and toad (*Xenopus laevis*, BAA90694). The deduced amino acid sequence of anterior fat body protein (8-277aa region) from insect flesh fly (*Sarcophaga peregrina*) exhibited similarity to that of mammalian regucalcin (SMP30) (Nakajima and Natori, 2000). Moreover, on the search of NCBI GenBank including genomic databases, we observed other organisms containing the regucalcin family or a regucalcin homologue, such as bacteria *Bacillus cereus* ATCC 10987 regucalcin family protein, (NP_978918); fungi *Aspergillus fumigatus* Af293 regucalcin putative, (XP_751966); and, invertebrates like *Drosophila melanogaster* regucalcin homologue, (BAA99283). However, their genetic and functional characteristics are not yet clearly understood.

Shimokawa and Yamaguchi (1992) have reported that the expression of regucalcin mRNA is specific in the liver, and that it is increased by the administration of $CaCl_2$ to rats. Ca^{2+} is a regulatory agent involved in many physiological processes of invertebrates such as mollusks and is also the primary cation in the formation of shell structures. It is a product of Ca^{2+} metabolism, which contains more than 90% of $CaCO_3$ crystals (Addadi and Weiner, 1997). In marine bivalves, calcium is taken up by gill from the external medium and transported to the mantle epithelium. L-type Ca^{2+} channels, which are regulated by calmodulin, have been suggested to be involved in calcium transport process for calcification in some marine invertebrates (Zoccola et al., 1999). However, the mechanism of Ca^{2+} uptake, accumulation, transport, incorporation, and the particular regulators involved in Ca^{2+} metabolism remains an interesting field for investigation. Hence, the structural analysis of novel class of Ca^{2+} binding proteins would be helpful to investigate the novel architecture of Ca^{2+} recognition and in understanding the mechanism of intracellular Ca^{2+} signal transduction.

The relationship between vertebrate and invertebrate regucalcin is curious, especially since no reported data on invertebrate regucalcin gene and its functional characterization exist according to our knowledge. To gain insight of the regucalcin gene in mollusks, we established the primary molecular structure of disk abalone, *Haliotis discus discus*, and compared its sequence with known regucalcin genes from other animals. Further, the expression of its mRNA was analyzed with or without intramuscular injection of $CaCl_2$.

2. Materials and methods

2.1. Cloning and sequencing of abalone regucalcin cDNA

H. discus discus regucalcin cDNA was obtained from the normalized cDNA library, which was synthesized using a Creator SMART cDNA library construction kit (Clontech, USA). The cDNA was normalized with Trimmer-Direct cDNA normalization kit according to the manufacturer's protocol (Evrogen, Russia). The plasmid DNA of the putative regucalcin was obtained by Accuprep™ plasmid extraction kit (Bioneer Co., Korea). The full length sequence was obtained by designing the internal primer HdReg-1I (Table 1) from the known sequence of 3′ end and sequenced using termination kit, Big Dye and an ABI 3700 sequencer (Macrogen Co., Korea). After determining the full length, the sequence was compared with other sequences available in the National Center for Biotechnology Information (NCBI) database.

2.2. Abalones

The disk abalones were obtained from Fisheries Resources Research Institute (Jeju, Republic of Korea). Individuals were averaging 80.0±5.0 mm and 60.0±5.0 g in length and body mass, respectively. They were maintained in 40 L tanks with an aerated seawater having a temperature of 20±2 °C and fed with

Table 1
Primers used for HdReg cloning and RT-PCR expression analysis

Name	Target	Orientation	Sequence
HdReg-1I	Internal sequencing	Forward	GATATGGGTTACCCGGATG
HdReg-1F	RT-PCR amplification	Forward	CGCCAATATGTTCAACGACGGCAA
HdReg-1R		Reverse	TTGACAGTACGGATCACCTTGCCA
Ribosomal-2F	RT-PCR positive control	Forward	GGGAAGTGTGGCGTGTCAAATACA
Ribosomal-2R		Reverse	TCCCTTCTTGGCGTTCTTCCTCTT