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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

seaweeds for 7 days to acclimatize to laboratory conditions before $CaCl_2$ injection.

2.3. $CaCl_2$ administration and tissue collection

$CaCl_2$ was dissolved in sterile distilled water and abalones were injected with 0.5 mg $CaCl_2$/g of abalone intramuscularly. Untreated abalones were kept as controls for the experiment. Gill, mantle, digestive tract, and abductor muscle tissues were collected separately from uninjected and injected abalones at 30 min, 1 h, and 2 h after injection. Three ($n=3$) abalones were used at each time point of $CaCl_2$ injection and the control. Tissues were immediately frozen in liquid nitrogen and stored at −70 °C until RNA isolation. Three independent experiments were conducted for the study.

2.4. RNA extraction and cDNA construction

Total RNAs of the gill, mantle, digestive tract and abductor muscle tissues (100 mg each) were isolated using Tri Reagent™ (Sigma, USA) according to the manufacturer's protocol. The first strand cDNA synthesis was carried out based on SuperScript III First Strand cDNA synthesis kit (Invitrogen, USA). In brief, 1 μL of 50 μM oligo$(dT)_{20}$ and 1 μL of 10 mM dNTP were incubated with isolated 2.5 μg of RNA for 5 min at 65 °C. After incubation, 2 μL of 10× cDNA synthesis buffer, 4 μL of 25 mM $MgCl_2$, 2 μL of dithiothreitol (DTT, 0.1 M), 1 μL of RNAsaseOUT™ (40 U/μL), and 1 μL of SuperScript III reverse transcriptase (15 U/μL) were added and incubated for 1 h at 50 °C. The PCR reaction was terminated by adjusting the temperature to 85 °C for 5 min and the resulting cDNA was stored at −20 °C for further experiments.

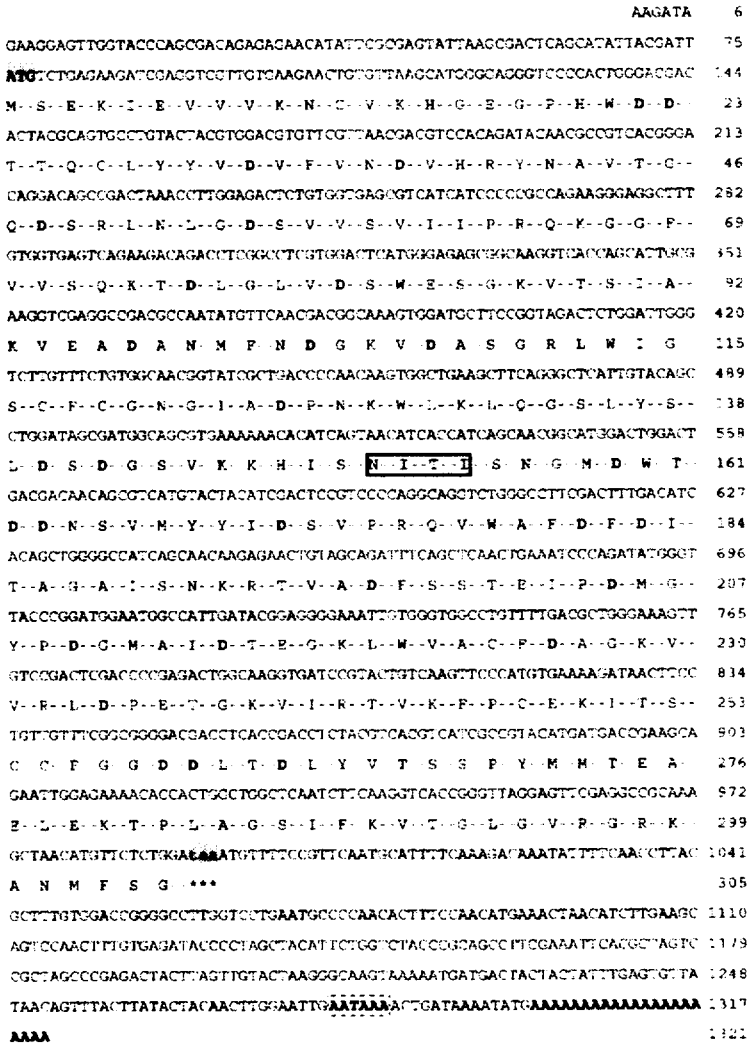

Fig. 1. The complete nucleotide and deduced amino acid sequences of HdReg. The predicted amino acid sequence was presented below the nucleotide sequence. Position one of the amino acid sequence represents methionine (Met) residue. The termination codon (TAA) was indicated by ***. The putative polyadenylation signal AATAAA is boxed with dash lines and *N*-glycosylation site is boxed. The poly A tail is at the end marked in bold letters. The aspartic acid residues were marked in bold. The abalone regucalcin cDNA sequence has been submitted to GenBank under the accession number **EF103358**.

Table 2
Pairwise CLUSTALW analysis and comparison of the deduced amino acid sequence of HdReg protein with other vertebrate regucalcin

Species	NCBI accession no.	Identity (%)	Amino acids
Gallus gallus	BAA90693	45	299
Danio rerio	NP_991309	45	295
Rattus norvegicus	BAA07490	44	299
Mus musculus	NP_033086	44	299
Sus scrofa	NP_001070688	43	299
Xenopus laevis	BAA90694	43	299
Oryctolagus cuniculus	BAA88079	42	299
Bos taurus	BAA88080	42	299
Homo sapiens	NP_004674	42	299

2.5. Semi-quantitative RT-PCR analysis

RT-PCR was performed to study the regucalcin mRNA expression in disk abalone tissues including gill, mantle, abductor muscle, and digestive tract. Furthermore, tissue specific relative mRNA expression among different tissues and relative mRNA expression levels of $CaCl_2$ injected tissues were analyzed using semi-quantitative RT-PCR. For comparison of relative regucalcin mRNA levels, statistical analysis was performed with one way ANOVA and mean comparisons were performed by Duncan multiple range tests using SPSS 11.5. Significant P values were obtained by Duncan multiple range test. Results are shown as mean ± SE of three animals per group. A pair of regucalcin gene specific primers HdReg-1F and HdReg-1R was used to amplify a 444 bp fragment of HdReg. As an internal control, 420 bp disk abalone ribosomal protein cDNA (GenBank: EF103427) was amplified using ribosomal 2F and ribosomal 2R primers (Table 1).

RT-PCR was carried out in a TaKaRa PCR thermal cycler in 25 μL reaction volume containing 2 μL of cDNA from each tissue, 2.5 μL of 10× NEB buffer, 2.0 μL of 2.5 mM dNTP mix, 1.0 μL of each primer (20 pmol/μL, including regucalcin and ribosomal protein forward and reverse primer), and 0.13 μL (5 U/μL) of NEB *Taq*™ DNA polymerase (NEB, USA). The PCR temperature conditions for regucalcin were 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. For ribosomal protein, it was 94 °C for 2 min followed by 23 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. After the final cycle, samples were incubated for a further 5 min for 72 °C, and then held at 4 °C prior to analysis. The PCR products were analyzed by 1.5% agarose gel electrophoresis containing ethidium bromide and 100 bp molecular marker (TaKaRa, Japan). Electrophoretic images and the optical densities of amplified bands were analyzed using the Scion Image software (Release alpha 4.0.3.2).

2.6. Sequence analysis

Sequence similarity analysis was performed with BLAST algorithm at the National Center for Biotechnology Information (NCBI) BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The HdReg deduced amino acid sequence was analyzed for signal sequence and motif prediction using ExPASy (http://www.au.expasy.org). Multiple alignment of the HdReg was performed using ClustalW multiple alignment program (http://www.ebi.ac.uk/clustalw/). MEGA 3.1 (Kumar et al., 2004) was used to produce the phylogenetic tree using the neighbor joining (NJ) method.

3. Results

3.1. Sequence characterization of regucalcin

We characterized the primary molecular structure of *H. discus discus* regucalcin gene. The regucalcin cDNA was

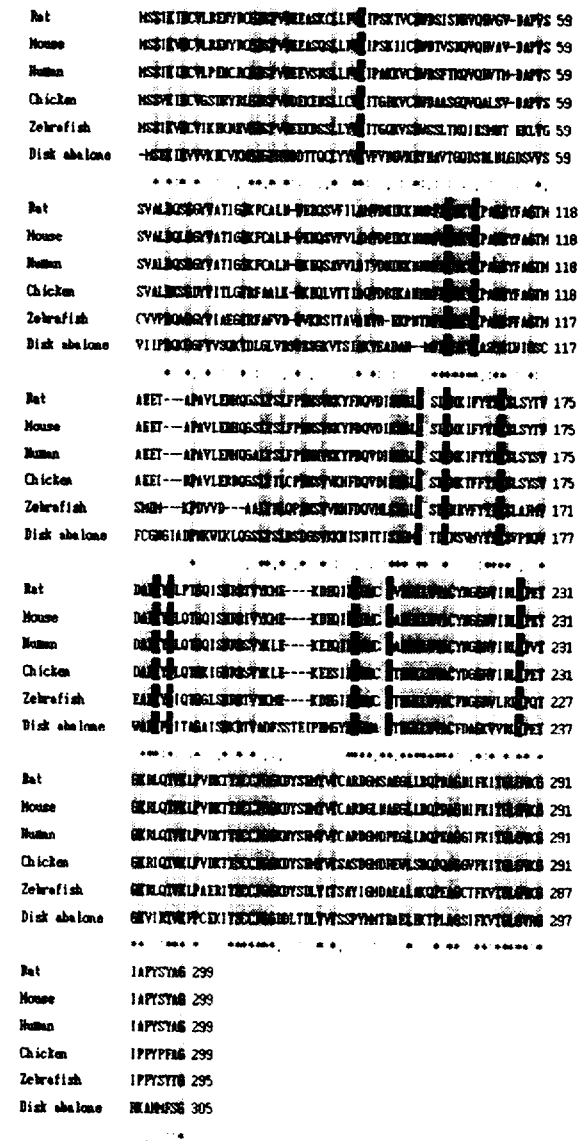

Fig. 2. ClustalW multiple sequence alignment of HdReg with known regucalcin. The GenBank accession numbers for the vertebrate regucalcin sequences are same as the numbers used in Fig. 4. Asterisk marks indicate identical amino acids and the numbers to the right indicate the amino acid position of regucalcin in the corresponding species. Conserved aspartic acid residues are highlighted in black and conserved glutamic acid residues are in bold type among other vertebrate regucalcin aligned.

obtained from the disk abalone normalized cDNA library and it showed similarity to known sequences of regucalcin by BLAST analysis. The full length sequence was obtained using terminator reaction kit, Big Dye, and ABI 3700 sequencer (Macrogen Co., Korea). The full length HdReg cDNA consisted of 1321 bp with a 5′ untranslated region (UTR) of 75 bases followed by an ORF of 918 bp and a 3′ UTR of 329 bp (Fig. 1). It contained a polyadenylation signal with AATAAA sequence and a poly (A) tail. The ORF can encode a polypeptide of 305 amino acids. The estimated molecular mass and predicted isoelectric point were 33 kDa and 4.9, respectively. A search for potential *N*-glycosylation sites using the PROSITE program (http://kr.expasy.org/prosite/) showed *N*-glycosylation site at the aa positions 151–154 (NITI). The amino acid composition of regucalcin shows a relatively high content of valine (10%), aspartic acid (9%), glycine (9%), and serine (8%). The pairwise CLUSTALW analysis of deduced amino acid sequences was performed to show identity percentage (%) of HdReg with different species of regucalcin (Table 2). It showed 45% identity with chicken and zebrafish and 44% to rat and mouse regucalcin. Moreover, it showed 42% identity to human regucalcin. *S. peregrine* anterior fat body protein and *D. melanogaster* regucalcin homologue were 33 and 32% identical to HdReg, respectively. ClustalW multiple sequence alignment of HdReg with other regucalcin species in database is shown in Fig. 2. The overall identity of HdReg with those selected vertebrate species was 34%. Of 29 aspartic acid residues in HdReg, 11 residues (38%) were conserved with vertebrate species. However, only 3 of 12 (25%) glutamic acid residues were conserved with all other vertebrate regucalcin aligned in this study. Moreover, using the Kyte and Doolittle (1982) method, we analyzed the hydrophilicity profile of regucalcin and hydrophilic as well as hydrophobic residues in both N-terminal and C-terminal regions of the regucalcin molecule were detected. It showed 66% of hydrophilic residues at positions between 100 and 200 of the amino acid sequence and as a molecule, regucalcin could be considered a hydrophilic molecule.

Phylogenetic analysis was carried out to find out the evolutionary position of HdReg. The analysis was carried out using MEGA 3.1 software with Neighbor-joining (NJ) method and boot strap values were taken from 1000 replicates. The tree was constructed using 15 organisms. Analysis results showed that the deduced amino acid sequence of HdReg was clearly placed in a single cluster and all the other vertebrate and invertebrate regucalcin were placed in distinct separate clusters (Fig. 3). Two insects, *S. peregrine* anterior fat body protein and *D. melanogaster* regucalcin homologues, were placed in one sub-cluster. The two bacterial regucalcins *Xanthomonas axonopodis* pv. citri str. 306 (AAM36628) regucalcin and *X. oryzae* pv. oryzae MAFF 311018 (BAE69525) regucalcin were used as an outgroup for the analysis.

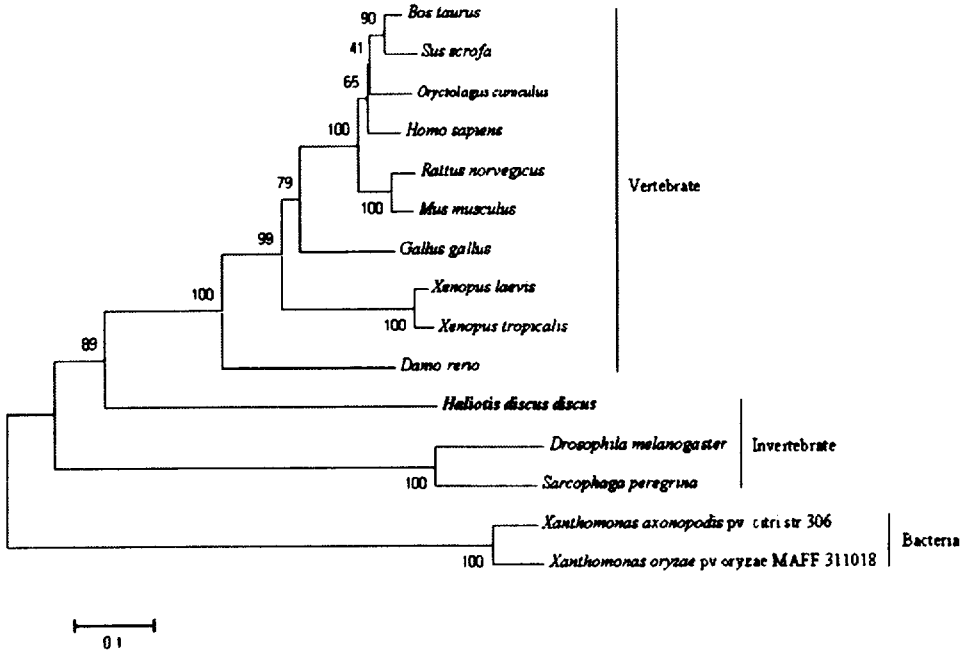

Fig. 3. Phylogenetic tree of 15 regucalcins, including regucalcin from disk abalone (*H. discus discus*), GenBank accession nos. **EF103358**; chicken (*Gallus gallus*), **BAA90693**; bovine (*Bos taurus*), **BAA88080**; zebrafish (*Danio rerio*), **NP_991309**; rat (*Rattus norvegicus*), **BAA07490**; mouse (*Mus musculus*), **NP_033086**; human (*Homo sapiens*), **NP_004674**; pig (*Sus scrofa*), **NP_001070688**; rabbit (*Oryctolagus cuniculus*), **BAA88079**; African clawed frog (*Xenopus laevis*), **BAA90694**; frog (*Sirulana tropicalis*), **NP_001006822**; *Xanthomonas axonopodis* pv.citri str. 306, **AAM36628**; *Xanthomonas oryzae* pv. oryzae MAFF 311018, **BAE69525**; anterior fat body protein of flesh fly (*Sarcophaga peregrina*), **BAA99282** and *Drosophila melanogaster* regucalcin homologue, **BAA99283**. A phylogenetic tree was constructed by the Neighbor-Joining method with MEGA 3.1. Numbers at the nodes are bootstrap values representing their robustness (1000 replications)

3.2. Expression of regucalcin in different tissues

The tissue specific HdReg expression was analyzed *in vivo* by amplifying 444 bp regucalcin cDNA fragment. A constitutive expression gene, 420 bp abalone ribosomal protein was used as a housekeeping gene. RT-PCR results showed that regucalcin mRNA was constitutively expressed in all the tissues tested such as gill, mantle, digestive tract, and abductor muscle. The tissue distribution of regucalcin mRNA in abalone is shown in Fig. 4A. In all the tissues, a single mRNA expression band was observed on 1.5% agarose gel. Moreover, tissue specific variation was observed and significantly higher and lower ($P<0.05$) mRNA expression levels were detected in abductor muscle and mantle, respectively, than the other tissues tested (Fig. 4B).

3.3. Response of intramuscular $CaCl_2$ injection

The effect of calcium administration on HdReg mRNA expression was detected by RT-PCR analysis. A solution of $CaCl_2$ (0.5 mg/1 g of abalone) was administered to abalones intramuscularly, and the tissues were collected at 30 min, 1 h, and 2 h after the administration. Even though constitutive expression

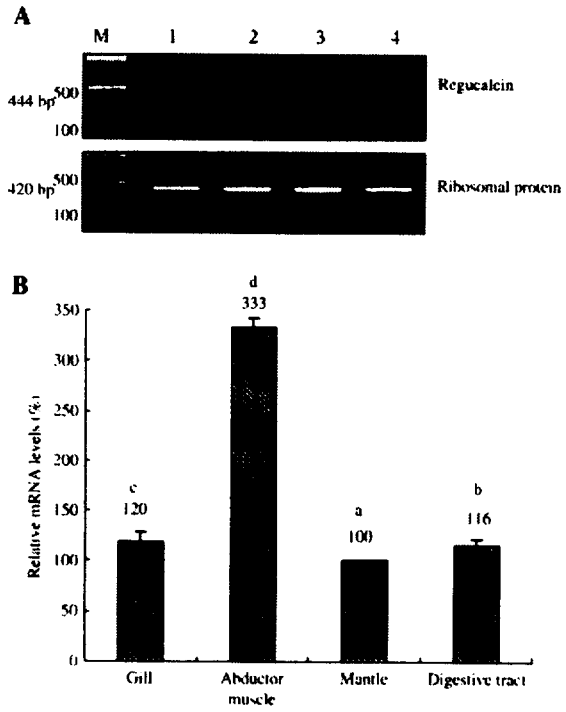

Fig. 4. Tissue specific expression of HdReg in different tissues. A: RT-PCR analysis of HdReg in different tissues. Lane 1- Gill, 2- Abductor muscle, 3- Mantle, 4- Digestive tract and M- 100 bp marker. Ribosomal protein fragment was used as a housekeeping gene. B: Relative mRNA levels of Regucalcin in *H. discus discus* tissues. The levels of HdReg mRNA are the means of three assays ($n=3$), which are calculated relative to that of the expression recorded for the mantle (shown as 100%). Bars represent the means ± SD. Significance of the differences ($P<0.05$) are illustrated as a, b, c, and d.

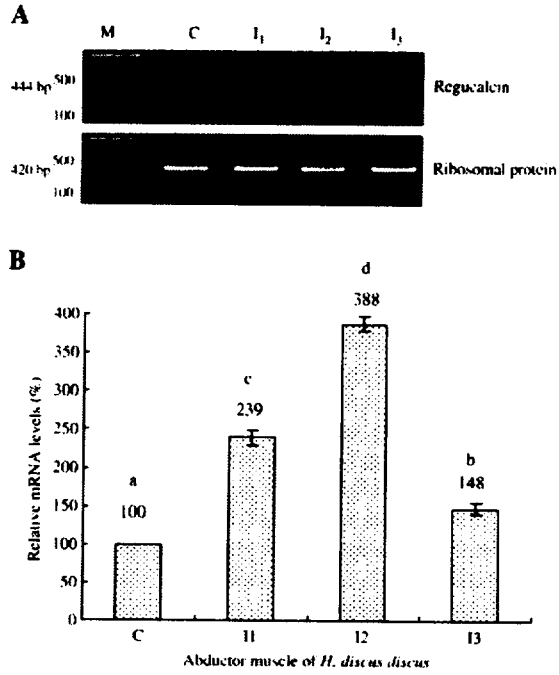

Fig. 5. Expression of HdReg mRNA after $CaCl_2$ administration. A: RT-PCR analysis of *H. discus discus* abductor muscle regucalcin gene expression at different time points after $CaCl_2$ administration. Lane C: control, I_1: 30 min, I_2: 1 h, I_3: 2 h after administration. M: 100 bp marker. Abalone ribosomal protein fragment was used as a house keeping gene. B: Relative mRNA levels of HdReg after $CaCl_2$ administration. C-control, I1-30 min, I2-1 h and I3-2 h after administration. The levels of HdReg mRNA are means of three assays ($n=3$), which are calculated relative to that of the expression recorded for the control (shown as 100%). Bars represent the means ± SD. Significance of the differences ($P<0.05$) are shown as a, b, c, and d.

was observed in HdReg in gill, mantle and digestive tract, clear induction was observed only in abductor muscle after $CaCl_2$ administration. Therefore, only the abductor muscle RT-PCR expression results and respective semi-quantitative analysis are shown in Fig. 5A and B, respectively. The results showed that the abalone abductor muscle regucalcin mRNA expression was induced significantly ($P<0.05$) at 30 min, 1 h, and 2 h after $CaCl_2$ administration when compared to uninjected abalones (Fig. 5B). Induction was significantly higher (3.88-fold) after 1 h of $CaCl_2$ administrated abalones than uninjected abalones. Moreover, the relative mRNA level was significantly decreased after 2 h of $CaCl_2$ administration.

4. Discussion

In this study, we studied the primary structure of regucalcin gene from *H. discus discus* (Phylum mollusca, class Gastropoda). We isolated, sequenced, and characterized the full length cDNA of *H. discus discus* regucalcin. Tissue specific mRNA expression was also investigated. This is the first report describing gene characterization of mollusk regucalcin.

Regucalcin, novel calcium binding protein that differs from calmodulin and other calcium binding proteins, is mainly

distributed in hepatic cytosol of rats and may play a physiological role in the cell different from that of calmodulin, which can amplify the Ca^{2+} effect on liver metabolism (Yamaguchi and Sakurai, 1992). Among the various regucalcins that have been cloned and described from vertebrates, the closest amino acid sequence identity for abalone was chicken (45%) and zebrafish (45%). It was 44% identical to rat and mouse and 42% identical to human regucalcin. The maximum identity of deduced amino acid sequence of anterior fat body protein (8–277 aa region) from flesh fly and rat SMP30 was 33% (Nakajima and Natori, 2000). Although the insects belong to invertebrates, a low identity was (33%) observed between HdReg and flesh fly anterior fat body protein. The HdReg contained 305 amino acid residues and molecular mass was calculated to be 33 kDa. It has been reported that estimated molecular mass of rat regucalcin was 33 kDa and was composed of 299 aa residues. Most of the coding sequences reported in vertebrate regucalcin were 299 aa such as chicken, rat, mouse, and human. Some of the regucalcin family proteins deposited in NCBI data base showed varying length of amino acids such as *B. cereus* ATCC 10987 (300 aa), *A. Fumigatus* (281 aa), and *D. melanogaster* (303 aa). Furthermore, the amino acid sequences of regucalcin did not show significant homology, when compared with the GenBank databases containing several other Ca^{2+} binding proteins such as calmodulin, S-100beta, and calbindin-D28k. HdReg does not contain the typical structure of EF-hand motif (helix-loop-helix domain) as a Ca^{2+} binding domain. The EF-hand loop consists of 12 amino acids and 5 of them have a carboxyl group (or hydroxyl group) in their side chain, precisely spaced so as to coordinate the Ca^{2+} (Yamaguchi, 2000). Therefore, the regucalcin was considered as a novel class of calcium binding protein, which differs from calmodulin and other calcium binding proteins (Yamaguchi and Sakurai, 1992).

The amino acid composition of HdReg shows relatively high content of valine (10%), aspartic acid (9%), glycine (9%), and serine (8%). It has been reported that aspartic acid (8%) and glutamic acid (5%) present in rat regucalcin could be related to Ca^{2+} binding (Yamaguchi, 2000). Similar to rat regucalcin, 9% aspartic acid and 3% glutamic acid were observed in HdReg, and these residues could be important in HdReg. Moreover, in previous studies the hydrophilic region could be a functional domain, which is related to the binding of Ca^{2+} (Shimokawa and Yamaguchi, 1993). HdReg as a molecule, showed hydrophilic character and at positions between 100 and 200 showed comparatively higher hydrophilic region (66%). Interestingly, a similar result was observed for rat liver regucalcin (Shimokawa and Yamaguchi, 1993).

Even though a potential *N*-glycosylation site at the position of 151–154 aa (NITI) was observed in HdReg, whether it is necessary for the regucalcin activity is unknown. Therefore, further investigations of its functional characteristics are required. In mouse and human regucalcin, *N*-glycosylation sites were found at positions of ^{164}NQS^{166} and ^{254}NYS^{256} in their sequences. However, no potential N-linked glycosylation site (Asn-X-Ser (or Thr) in rat regucalcin sequence (Shimokawa and Yamaguchi, 1993) was present. Moreover, a signal peptide was absent in all the known regucalcin sequences and HdReg. SOSUI prediction program of trans-membrane region analysis (Nagoya University, Japan (http://bp.nuap.nagoya-u.ac.jp/sosui/)) showed that HdReg and other known regucalcin sequences were cytoplasmic proteins and not trans-membrane proteins.

Comparison analysis of regucalcin nucleotide sequences from seven vertebrate species (human, rat, mouse, rabbit, bovine, chicken, and toad livers) showed that they were highly conserved in their coding region. Conservation of regucalcin genes throughout the evolution is 69.9–91.3% identity (Misawa and Yamaguchi, 2000; Yamaguchi, 2000). In this study, the overall identity of HdReg with those selected vertebrate species was 34%. The structural features suggest that the HdReg is partially similar to vertebrate regucalcin, but its functional characteristics may not completely similar to vertebrates as suggested by Nakajima and Natori (2000). Further, a constructed phylogenetic tree shows that the abalone is placed in a single cluster separated from a vertebrate main cluster. Moreover, it was separated from the invertebrate sub-cluster. This shows that there is a phylogenetic distance between vertebrate and invertebrate as well as among vertebrates and invertebrates even though they were derived from a common ancestor.

Abalone gill, mantle, digestive tract, and abductor muscle were used for the expression analysis and HdReg was expressed in all the tissues tested. This suggests that HdReg is not confined to the specific tissue and may function in all tissues to some degree. However, expression level was higher in abductor muscle compared to other tissues tested and data from three independent experiments were similar. Shimokawa and Yamaguchi (1992) have suggested that specific expression of rat regucalcin is mainly in liver and very limited in kidney. Further investigation showed that the regucalcin mRNA expression was observed in rat heart muscle and it was present in the cytoplasm of heart muscle cells (Yamaguchi and Nakajima 2002). Ca^{2+} is an important element for many cellular processes in all the organisms including mollusks. The abalone mRNA was highly expressed in abductor muscle, showing that regucalcin may be involved in muscle contraction and relaxation for locomotion, attaching to substrates. Although we have analyzed the tissue specific HdReg mRNA expression levels in different tissues, the detailed mechanism or functional role of regucalcin in Ca^{2+} uptake, accumulation and release remains unelucidated.

HdReg mRNA expression was significantly up regulated after 30 min of calcium administration and expression was higher at 1 h of calcium administration than 30 min and 2 h. However, other tissues tested did not show any significant induction. These results suggest that the expression of regucalcin mRNA rapidly responds in abductor muscle after Ca^{2+} administration and a regulatory system may suppress over-expressed regucalcin in abductor muscle or the injected Ca^{2+} may diffuse away to normal concentration. However, whether the dose of Ca^{2+} administration is sufficient to increase the Ca^{2+} content in other tested tissues is to be explored. Regucalcin mRNA expression (Shimokawa and Yamaguchi, 1992) and its protein content analysis (Yamaguchi and Isogai, 1993) were studied in liver and kidney cortex and expression of regucalcin in liver and kidney cortex has been shown to be stimulated by the administration of $CaCl_2$ to rats *in vivo* (Shimokawa and Yamaguchi, 1992; Shimokawa and Yamaguchi, 1993; Yamaguchi and Kurota, 1995). Moreover,

Shimokawa and Yamaguchi (1992) reported that several steps may be related to the regulation of regucalcin mRNA levels during the process of transcription, mRNA stability, translation, and posttranslational events.

In conclusion, we have cloned a novel regucalcin gene from *H. discus discus* as a first reported regucalcin gene in invertebrate mollusks. Then, we characterized the primary structure of regucalcin and observed common characteristics related to regucalcin. Regucalcin mRNA expression was observed in all the tissues tested and it was clearly increased by intramuscular calcium administration in the abductor muscle. However, the exact function of the HdReg and mechanism of its regulation needs to be investigated.

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