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# Cloning, Characterization, and Expression Patterns of Three Sarco/Endoplasmic Reticulum $Ca^{2+}$-ATPase Isoforms from Pearl Oyster (*Pinctada fucata*)

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**Abstract** A large amount of calcium is required for mollusk biomineralization. Sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) is a well-known protein with the function of sustaining the calcium homeostasis. How does it possibly function in the process of pearl oyster biomineralization? Three SERCA isoforms, namely PSERA, PSERB, and PSERC were cloned from the pearl oyster, *Pinctada fucata*. The cDNAs of the three isoforms were isolated by reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends. PSERA consisted of 3568 bp encoding 1007 amino acids, PSERB included 3953 bp encoding 1024 amino acids, and PSERC comprised of 3450 bp encoding 1000 amino acids. The three isoforms showed high homology (65%–87%) with SERCAs from other species. Consistent with the results from other invertebrates, Southern blot analysis revealed that the three isoforms originated from a single gene that was also related to SERCA1, SERCA2, and SERCA3 of vertebrates. The splicing mechanism of the three isoforms was similar to that of isoforms of vertebrate SERCA3. Semiquantitative RT-PCR was carried out to study the expression patterns of the three isoforms. The results showed that PSERB was ubiquitously expressed in all tested tissues and was a potential "housekeeping" SERCA isoform; PSERA was expressed in the adductor muscle and foot and was likely to be a muscle-specific isoform, and PSERC was expressed in the other tissues except the adductor muscle or foot with the highest expression levels in the gill and mantle, indicating that it was a non-muscle-specific isoform and might be involved in calcium homeostasis during pearl oyster biomineralization.

**Keywords** sarco/endoplasmic reticulum $Ca^{2+}$-ATPase; expression patterns; calcium homeostasis; *Pinctada fucata*

$Ca^{2+}$ is not only a regulatory element in various physiological processes, but also the primary cation used for the biomineralization of shell and nacre in pearl oysters [1]. In the process of biomineralization, various tissues play different roles, including absorption, transport, and storage of calcium. A large amount of $Ca^{2+}$ was absorbed from ambient and transported to mineralization sites by paracelluar or transcellular pathways [2,3]. During transcellular movement, calcium homeostasis is especially important to the oyster cell. In the pearl oyster, the concentration of extracellular $Ca^{2+}$ (approximately 10 mM) is 10,000-fold more than that of intracellular $Ca^{2+}$ (approximately 1 μM). To keep the low intracellular free calcium concentration, $Ca^{2+}$ may be buffered by $Ca^{2+}$-binding proteins or sequestered into vesicles [4]. Sarco/endoplasmic reticulum is a compartment for $Ca^{2+}$ storage [5]. Sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) is a pump which sequesters intracellular calcium into the sarco/endoplasmic reticulum [6].

SERCA belongs to the family of P-type ATPases and it actively transports $Ca^{2+}$ across sarco/endoplasmic reticulum membranes through ATP hydrolysis [7]. SERCA consists of a single polypeptide chain, forming three globular cytoplasmic domains connected by a helical stalk segment to ten transmembrane α helices (M1–M10). Mammalian

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SERCA is encoded by three homologous genes: SERCA1, SERCA2, and SERCA3. By alternatively splicing, the three genes have multiple isoforms [8–12]. In invertebrates, such as *Caenorhabditis elegans*, *Drosophila melanogaster*, *Procambarus clarkia*, and *Patinopecten yessoensis*, many SERCA isoforms have been isolated and characterized [13–16].

This paper intends to isolate and identify the cDNAs of isoforms from the pearl oyster and examines the tissue distribution patterns of different isoforms, with which we may further understand the function of SERCA during oyster biomineralization.

## Materials and Methods

### Animal material

Live adult *Pinctada fucata* were obtained from the Beihai Pearl Farm (Beihai, China). After collection, the oysters were transported to the laboratory and kept in a filtered flow-through system with aerated seawater at 20 ºC for 48 h.

### RNA preparation and cDNA synthesis

Total RNA was extracted from the tissues by using the Trizol reagent (Invitrogen, Carlsbad, USA). The integrity of RNA was determined by the fractionation on 1.2% formaldehyde denatured agarose gel and staining with ethidium bromide. The quantity of RNA was determined by measuring $A_{260\ nm}$ with an Ultrospec 3000 UV-visible spectrophotometer (Amersham, Piscataway, USA). To avoid genomic DNA contamination, total RNA was digested by RNase-free DNase I (Promega, Madison, USA) and purified according to the manufacturer's instructions. Purified total RNA (5 μg) was used to synthesize single-strand cDNA using SuperScript II RNase H$^-$Reverse transcriptase (Invitrogen) and adaptor-oligo(dT)$_{15}$ primer according to the manufacturer's instructions.

### Cloning of pearl oyster SERCA cDNAs

The cDNA fragment F1 of the pearl oyster SERCA from the adductor muscle was amplified by reverse transcription polymerase chain reaction (RT-PCR) using *Taq* DNA polymerase (TaKaRa, Kyoto, Japan). A pair of degenerate oligonucleotide primers was designed based on the conserved regions of SERCA nucleotide sequences. These are the forward primer L1 (5′-CTSACMACMAACCAGATGTC-3′, where S=C/G, M=A/C) corresponding to the sequence from nucleotides 1066 to 1085, and the reverse primer L2 (5′-ACVGCVGTDCCDGADCCCAT-3′, where V=A/C/G, D=A/G/T) corresponding to the sequence from nucleotides 2158 to 2177. PCR was performed on a TGradient thermocycler (Biometra, Gottingen, Germany) with the following settings: denaturation at 95 ºC for 5 min, followed by 33 cycles at 95 ºC for 45 s, 53 ºC for 30 s, and 72 ºC for 75 s, then a final extension at 72 ºC for 10 min. The PCR products were then subjected to electrophoresis, and the bands of the expected size were excised (1112 bp) and purified with the Wizard PCR prep DNA purification system (Promega). The purified PCR products were then subcloned into a pMD18-T vector (TaKaRa) and sequenced.

The full-length sequence of SERCA cDNAs were obtained by using 5′ and 3′ rapid amplification of cDNA ends technique (RACE). Single-stranded cDNA for all RACE reactions was prepared from the above total RNA from the mantle or the adductor muscle using PowerScript reverse transcriptase (Clontech, Palo Alto, USA). 5′-RACE and 3′-RACE were conducted according to the manufacturer's protocol, using the SMART RACE cDNA amplification kit (Clontech) and Advantage 2 DNA polymerase mix (Clontech). The gene-specific primers 5′-TGGTGACGGTGTGAATGATGCCCCG-3′ for 5′-RACE and 5′-TAGCCATCTCCTCAAGTCCAGCATAATC-3′ for 3′-RACE, respectively, were prepared based on the nucleotide sequence of the cDNA fragment F1. The adaptor primers for 5′-RACE and 3′-RACE were both a universal primer mix. The RACE reactions were performed in a thermocycler (Biometra) under the following conditions: 5 cycles of 30 s at 94 ºC, 3 min at 72 ºC; 5 cycles of 30 s at 94 ºC, 30 s at 70 ºC, 3 min at 72 ºC, and 28 cycles of 30 s at 94 ºC, 30 s at 68 ºC, 3 min at 72 ºC, followed by a final extension of 10 min at 72 ºC. All the amplified products were cloned into a pMD18-T vector for sequencing. To confirm cloning and sequencing accuracy, the entire cDNA, including the start codon and stop codon, was re-amplified with high fidelity polymerase (TaKaRa) using three pairs of gene-specific primers: L3 (5′-GCTGCG AAACGGTGGCTGCTCG-3′) and L4 (5′-ATTATACCTTTTACATTCATAAGTG-3′); L3 and L5 (5′-ATTTACGTTTTGATTCCTGTCTG-3′); L3 and L6 (5′-GCGGAATTCGATTTAATCGACTC-3′). The purified PCR products were subcloned into the pGEM-T easy vector (Promega) followed by sequencing three individual clones respectively.

### Sequencing and analysis

All recombinant DNA was sequenced, and sequence similarity searches were performed using the BLAST program in GenBank, National Center for Biotechnology In-

formation (http://www.ncbi.nlm.nih.gov/). Multiple alignments and the phylogenetic tree were created using the Clustal X program [17]. The hydrophobic analyses of the deduced proteins were achieved by the method of Kyte and Doolittle [18] using a window of 13 residues (http://cn.expasy.org/tools/protscale.html).

### Southern blot analysis

The Southern blot analysis was performed to determine whether these isoforms originated from one or multiple genes. Total genomic DNA was extracted from the oyster adductor muscle using the Universal genomic DNA extraction kit (TaKaRa) according to the manufacturer's introductions. The quantity and content of DNA was determined by measuring $A_{260\ nm}$ with an Ultrospec 3000 UV-visible spectrophotometer (Amersham). Genomic DNA (20 μg) was digested with *Eco*RV and *Xho*I, respectively. After digestion for 12 h, the digested DNA was separated on a 0.7% agarose gel, then transferred to a nitrocellulose membrane, and hybridized with a DNA probe. Negative control was performed without adding genomic DNA. The DNA probe labeling and hybridization were completed using DIG high prime DNA labeling and detection starter kit I (Roche, Solna, Sweden) according to the manufacturer's protocol.

### Semiquantitative RT-PCR

Semiquantitative RT-PCR was performed to measure the expression levels of these SERCA isoforms in different oyster tissues, including the mantle, gill, gonad, digestive gland, adductor muscle, and foot. The primers for semiquantitative RT-PCR were designed according to the different 3′ terminals of the three isoforms. The forward primer L7 (5′-CATAGCCCGCAAATTTACTGAC-3′) was common in the three isoforms; the reverse primers were L8 (5′-ATTATACCT TTTACATTCATAAGTG-3′), L9 (5′-ATTTACGTTTTGATTCCTGTCTG-3′), and L10 (5′-GCGGAATTCGATTTAATCGACTC-3′). An equal quantity (2 μg) of total RNA from various tissues was transcribed into the cDNA in 20 μl reaction mixtures using SuperScript II RNase H⁻ reverse transcriptase. Preliminary PCR experiments were carried out to explore the amount of cDNA and the number of cycles needed for semiquantitative PCR. Quality controlled cDNA was then used as the template for the semiquantitative PCR, which was performed in a reaction system with 2.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 U *Ex Taq* DNA polymerase (TaKaRa), and 20 pM of each forward primer and reverse primer, with 26 cycles according to the preliminary PCR study. After amplification, the PCR products were subcloned into a pMD18-T vector and confirmed by sequencing. An equal volume of the PCR products was applied to 1.5% agarose gel stained with ethidium bromide. To avoid sample cross-contamination, a negative RT-PCR control reaction was performed in the absence of the cDNA template. Two gene-specific primers L11 (5′-GCCGAGTATGTGGTAGAATC-3′) and L12 (5′-CACTGTTTTCTGGGTAGCTG-3′) were designed according to the nucleotide sequence of *GAPDH* of *P. fucata* (GenBank accession No. AB205404) to amplify the 252-bp product as the internal control.

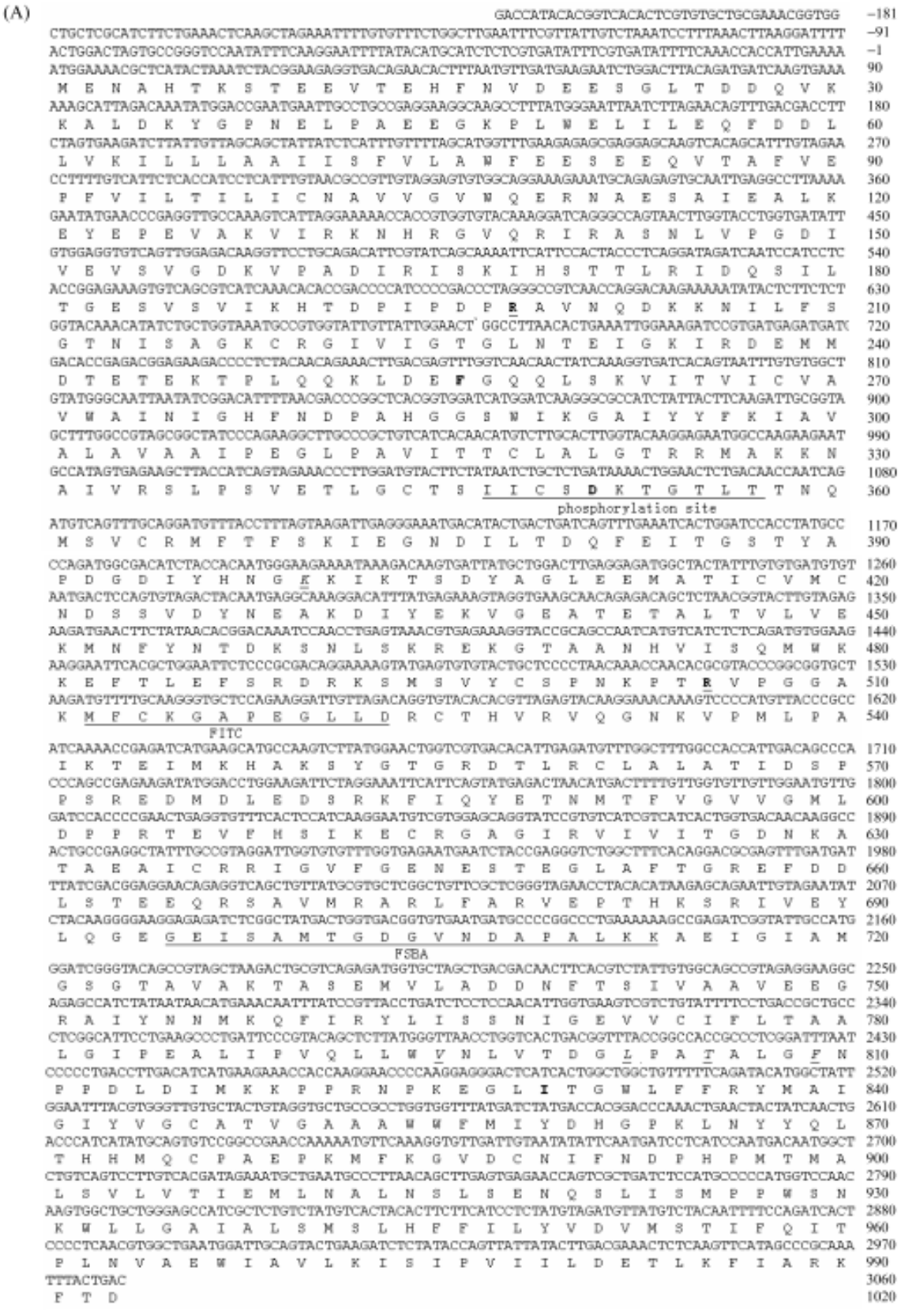
## Results

### Isolation and sequence analysis of cDNA sequence encoding SERCA

A 1112-bp cDNA product F1 was obtained by RT-PCR with two degenerate oligonucleotides (L1 and L2) using total adductor muscle RNA as the template. Based on the sequence, two gene-specific primers were synthesized for 5′-RACE and 3′-RACE, respectively, and were used to amplify the 5′ and 3′ nucleotide sequence of putative SERCA cDNA by touchdown PCR. The product of 5′-RACE was a fragment of 1510 bp, while the products of 3′-RACE contained three fragments with different sizes: 1298 bp, 1680 bp, and 1176 bp. To confirm the sequence obtained by RACE, three pairs of specific primers (L3 and L4, L3 and L5, L3 and L6) corresponding to the 5′ and 3′ untranslated sequences of putative SERCA cDNA were designed and used in RT-PCR with *LA Taq* (TaKaRa). The PCR products were cloned and sequenced, which matched the sequence expected from the results of 5′- and 3′-RACE.

As shown in **Fig. 1**, the cDNA sequences of the three isoforms, namely PSERA, PSERB, and PSERC were 3568 bp, 3953 bp, and 3450 bp in length, respectively. PSERA had a 3021 bp open reading frame (ORF) encoding 1007 amino acids with a calculated molecular mass of approximately 111.2 kDa and a 325 bp 3′ untranslated region; PSERB had a 3072 bp ORF encoding 1024 amino acids with a calculated molecular mass of approximately 112.8 kDa and a 659 bp 3′ untranslated region; PSERC had a 3000 bp ORF encoding 1000 amino acids with a calculated molecular mass of approximately 110.3 kDa and a 228 bp 3′ untranslated region. The three isoforms have a common 219 bp for 5′ untranslated region. These cDNA sequences have been submitted to GenBank with accession No. EF488285, EF488286, and EF488287, respectively.

PSERA had a stop codon TAA and a poly(A) tail, but it had no poly(A) addition signal AATAAA; PSERB had a




(to be continued)

(continued)


**Fig. 1 Nucleotide and deduced amino acid sequences of three *Pinctada fucata* SERCA isoforms**

Three isoforms have been submitted to GenBank with accession No.: PSERA, EF488285; PSERB, EF488286; and PSERC, EF488287. Nucleotides and amino acids are numbered to the right of the sequence. (A) Common sequences of the three isoforms. The phosphorylation site, fluorescein isothiocyanate site (FITC) and the 5´-*p*-fluorosulfonylbenzoyladenosine binding site (FSBA) are underlined and labeled. Two typical tryptic sites R188 and R505 existing in sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA)1 and SERCA2 are marked in bold and underlined. Typical phospholamban-binding motif of SERCA2 is underlined italicized. Putative thapsigargin sites are indicated in bold. (B) Differential 3′ terminals of the three isoforms. Slash in the sequence at nucleotide 2980 is the site at which the three isoforms diverge from each other. Stop codon is indicated by asterisks. Common sequences of PSERA and PSERC are shown in bold and italics.

stop codon TGA and a poly(A) addition signal AATAAA ahead of a poly(A) tail; PSERC had a stop codon TAA and a poly(A) addition signal AATAAA, but no poly (A) tail was found. The three isoforms were almost identical except at their 3′ terminals diverged at nucleotide 2980. It was notable that there was a 156 bp common nucleotide sequence between PSERA and PSERC, locating downstream at the divergence site. The deduced amino acid sequences of PSERA, PSERB, and PSERC all had common domains in other SERCAs, including the phosphorylation region, fluorescein isothiocyanate site, and the 5'-*p*-fluorosulfonylbenzoyladenosine binding site, tryptic sites, phospholamban-binding motif, and thapsigargin sites (**Fig. 1**). Similar to other general SERCAs, PSERA and PSERC showed ten transmembrane domains while PSERB had a potential additional transmembrane domain (**Fig. 2**).

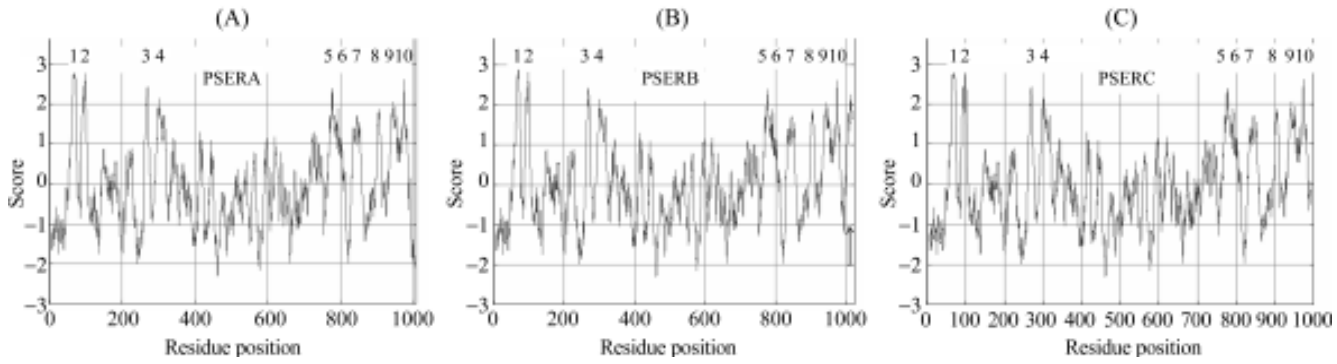



**Fig. 2 Comparison of hydropathy plots of the SERCA isoform PSERA (A) from *Pinctada fucata*, PSERB (B), and PSERC (C)**

Hydrophobicity scores were determined by the method of Kyte and Doolittle [18] using a window of 13 residues (http://cn.expasy.org/tools/protscale.html). Putative transmembrane domains are numbered according to the model of Brandl *et al.* [9]. Arrows indicate the extra potential transmembrane domains.

A homology search was carried out with the BLAST program against all the protein sequences in GenBank. The deduced amino acid sequences of the three isoforms revealed similar homology to each other (97%–99%), and each of them was matched with a large number of SERCAs from various invertebrates and vertebrates. They showed the highest homology with mollusk sea scallop SERCA (84%–87%) and less homology with crayfish (75%–77%). They showed 66%–76% identities with mammalian SERCA1, SERCA2, and SERCA3. The phylogenetic tree revealed a separate branching between invertebrate SERCAs and vertebrate SERCAs. SERCA from *P. fucata* was closer to invertebrate SERCAs than vertebrate SERCAs phylogenetically (**Fig. 3**).

## Southern blot analysis of the SERCA gene copy number

The similarity of the amino acid sequence among the three isoforms indicated that they might originate from the same gene. To investigate this hypothesis, Southern blotting was carried out. The genomic DNA of *P. fucata* was digested by two different restriction enzymes, *Eco*RV and *Xho*I, neither of which cut the three isoforms, and then hybridized with a cDNA probe from nucleotide 2530–2970, a region highly conserved among the three isoforms. The negative control showed no bands (data not shown). The results showed one hybridization band in each DNA

(A)

| | CafATP1 | CafATP2 | HosATP2 | MumATP2 | GagATP2 | GagATP3 | HosATP3 | MumATP3 | MiyATP | PlmATP | PSERA | PSERB | PSERC | PosATP | PrcaATP | PrchATP | GagATP1 | HosATP1 | OrcATP1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CafATP1 | 100 | 85 | 81 | 81 | 81 | 76 | 72 | 73 | 75 | 75 | 75 | 74 | 76 | 74 | 74 | 72 | 90 | 96 | 96 |
| CafATP2 | | 100 | 94 | 94 | 90 | 76 | 74 | 74 | 74 | 74 | 74 | 73 | 75 | 73 | 73 | 71 | 84 | 84 | 84 |
| HosATP2 | | | 100 | 99 | 94 | 76 | 74 | 74 | 70 | 69 | 71 | 71 | 71 | 69 | 69 | 69 | 80 | 80 | 80 |
| MumATP2 | | | | 100 | 94 | 76 | 73 | 74 | 70 | 70 | 71 | 71 | 71 | 69 | 69 | 69 | 80 | 80 | 80 |
| GagATP2 | | | | | 100 | 76 | 74 | 75 | 70 | 70 | 72 | 72 | 72 | 69 | 70 | 70 | 81 | 81 | 80 |
| GagATP3 | | | | | | 100 | 78 | 78 | 68 | 68 | 68 | 68 | 68 | 67 | 67 | 68 | 76 | 76 | 75 |
| HosATP3 | | | | | | | 100 | 93 | 65 | 65 | 66 | 66 | 66 | 66 | 66 | 67 | 73 | 73 | 72 |
| MumATP3 | | | | | | | | 100 | 66 | 66 | 66 | 66 | 66 | 66 | 66 | 66 | 73 | 73 | 72 |
| MiyATP | | | | | | | | | 100 | 96 | 85 | 84 | 86 | 76 | 76 | 75 | 75 | 74 | 74 |
| PlmATP | | | | | | | | | | 100 | 86 | 84 | 87 | 77 | 77 | 75 | 74 | 74 | 74 |
| PSERA | | | | | | | | | | | 100 | 97 | 99 | 77 | 76 | 75 | 75 | 75 | 75 |
| PSERB | | | | | | | | | | | | 100 | 97 | 75 | 75 | 75 | 74 | 73 | 73 |
| PSERC | | | | | | | | | | | | | 100 | 77 | 77 | 75 | 76 | 75 | 75 |
| PosATP | | | | | | | | | | | | | | 100 | 89 | 87 | 73 | 73 | 74 |
| PrcaATP | | | | | | | | | | | | | | | 100 | 97 | 73 | 74 | 74 |
| PrchATP | | | | | | | | | | | | | | | | 100 | 72 | 72 | 72 |
| GagATP1 | | | | | | | | | | | | | | | | | 100 | 91 | 90 |
| HosATP1 | | | | | | | | | | | | | | | | | | 100 | 97 |
| OrcATP1 | | | | | | | | | | | | | | | | | | | 100 |

(B)

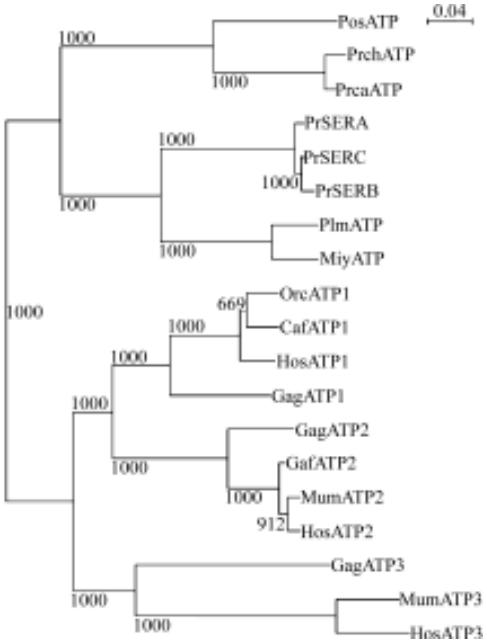



**Fig. 3 Homology comparison and phylogenetic analysis of the three SERCA isoforms from *Pinctada fucata* with Sarco/endoplasmic reticulum $Ca^{2+}$-ATPases (SERCAs) from other species**

(A) Matrix indicating the percentage identities of the aligned SERCAs, as determined by the Clustal X program using the full amino acid sequence. (B) Phylogenetic tree showing the evolutionary relationship of SERCAs from different species according to (A). Values at branch points are bootstrap percentages with 1000 replications. Accession numbers of these sequences found in the GenBank database (National Center for Biotechnology Information) are: HosATP1=*Homo sapiens* SERCA1 (O14983), HosATP2=*Homo sapiens* SERCA2 (NP_733765), HosATP3=*Homo sapiens* SERCA3 (Q93084), OrcATP1=*Oryctolagus cuniculus* SERCA1 (P04191), MumATP2=*Mus musculus* SERCA2 (O55143), MumATP3=*Mus musculus* SERCA3 (Q64518), MiyATP=*Mizuhopecten yessoensis* SERCA (BAA37143), PrcaATP=*Procambarus clarkii* axial muscle SERCA (AAB82291), PrchATP=*Procambarus clarkii* heart muscle SERCA (AAB82290), PlmATP=*Placopecten magellanicus* SERCA (AAC63909), GagATP3=*Gallus gallus* SERCA3 (NP_990222), GagATP2=*Gallus gallus* SERCA2 (B40812), GagATP1=*Gallus gallus* SERCA1 (NP_990850), CafATP2=*Canis familiaris* SERCA2 (NP_001003214), CafATP1=*Canis familiaris* SERCA1 (XP_849777), PosATP=*Porcellio scaber* SERCA (AAN77377), PSERA (EF488285), PSERB (EF488286), and PSERC (EF488287).

lane, suggesting that these isoforms were encoded by an identical gene (**Fig. 4**).

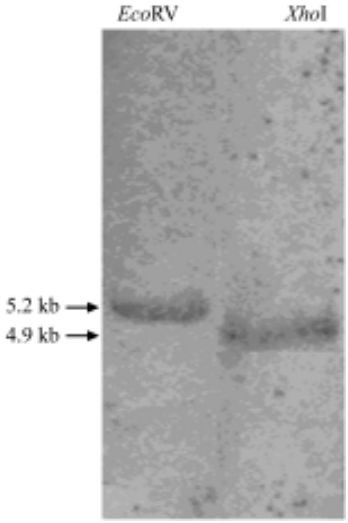



**Fig. 4 Southern blot analysis of the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase gene of the *Pinctada fucata* copy number**

Genomic DNA (20 μg) was digested with *Eco*RV or *Xho*I, respectively, separated on a 0.7 % agarose gel, transfered to a nitrocellulose membrane, and hybridized with a probe from nucleotide 2530–2970 of the isoform PSERA.

### Tissue distribution analysis of isoforms by semiquantitative RT-PCR

To determine the tissue distribution of the three isoforms, semiquantitative RT-PCR was carried out in various tissues of *P. fucata*. The RT-PCR reactions were performed with RNA samples from the mantle, gill, digestive gland, adductor muscle, gonad, and foot. A common forward primer L7 and three reverse primers L8, L9, and L10 specific to every isoform were designed to amplify the three different isoforms. Three RT-PCR products (346 bp, 734 bp, and 271 bp in size, respectively) were obtained with specific primers (L7 and L8, L7 and L9, L7 and L10) and the total RNA of various tissues as the template, while the negative control exhibited no product (data not shown). The PCR products were then inserted into a pMD18-T vector and confirmed by a sequence analysis. As shown in **Fig. 5**, PSERB mRNA was expressed in all the tissues tested; PSERA was expressed in only the adductor muscle and foot; PSERC was expressed in other tissues, except in the adductor muscle or foot. The expression level of PSERB in various tissues was similar. The expression level of PSERA was much higher in the adductor muscle than in the foot, and that of PSERC was higher in the gill than in any other tested tissue. Data from three independent experiments were similar.

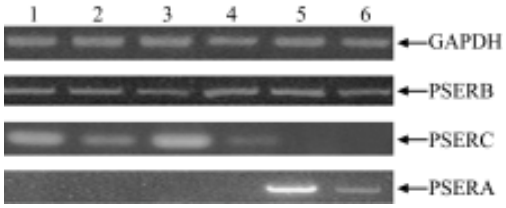



**Fig. 5 Expression analysis of the three sarco/endoplasmic reticulum $Ca^{2+}$-ATPase isoforms in the tissues of *Pinctada fucata***

Semiquantitative RT-PCR was done with RNA samples from the adult tissues of *Pinctada fucata*. PCR products of the PSERA (346 bp), PSERB (734 bp), and PSERC (271 bp) are indicated by the arrows, and the GAPDH (252 bp) mRNA expression level is used as an internal control. 1, mantle; 2, gonad; 3, gill; 4, digestive gland; 5, adductor muscle; 6, foot.

## Discussion

The present research describes the cloning, characterization, and tissue distribution of three SERCA isoforms from oyster *P. fucata*. The three isoforms differed from each other in their C-terminal amino acids, but they were encoded by one gene. Although several SERCA genes have been cloned from the mollusk, including the sea scallop and Yesso scallop, this was the first gene cloned from the pearl oyster which has been used to study biomineralization ubiquitously.

In mammals and birds, the SERCA pumps consist of three genes, SERCA1, SERCA2, and SERCA3 [8]. By alternative splicing, SERCA1 gives rise to two isoforms, SERCA1a and SERCA1b [9,19,20]; SERCA2 brings about SERCA2a, SERCA2b, and SERCA2c isoforms [21–23]; while SERCA3 shows six isoforms, including SERCA3a–f [10,24,25]. In some species of invertebrates, SERCAs have been cloned and characterized and their isoforms have been proven to be encoded by one gene. For example, the crayfish heart muscle and axial muscle isoforms [13,26] and *Artemia* 4.5 kb and 5.2 kb isoforms [27] belong to this case. Our results are consistent with the hypothesis that there is a unique ancestral SERCA gene that gives rise to the three genes in vertebrates and to a single gene in invertebrates [27].

*Artemia* and crayfish SERCA may have a similar splicing mechanism to the vertebrate SERCA2 gene, where the donor splicing site of the penultimate exon can either be recognized and fused to the last exon, giving rise to the mRNA coding for the shorter protein or remains

unrecognized, in which case a poly(A) site is recognized before the last exon of the gene and the mRNA coding for the longer protein is originated [13]. *P. fucata* SERCA gives rise to three isoforms and their splicing mechanism is likely to be similar to that of the vertebrate SERCA3 gene, where the penultimate exon can be alternatively excluded, partially included, or totally included, thus generating, respectively, three isoforms with different C terminals [24]. Among the three isoforms of *P. fucata* SERCA, there is likely to be an optional exon existing downstream of the conserved point of divergence. In PSERA, this exon is partially included; in PSERB this exon is totally included; while in PSERC this exon is excluded. Further research at the genomic level is necessary to disclose the exact splicing mechanism of the three isoforms.

Little is known about the biological significance of the existence of multiple SERCA isoforms in invertebrate. Previous work showed no significant function difference among different isoforms while they had a different tissue distribution pattern [13,28]. The divergent C termini of the three isoforms show poor homology. The C-terminal extension of PSERB has a prominent hydrophobic property and it can potentially form the eleventh transmembrane domain. Semiquantitative RT-PCR showed that PSERB had a ubiquitous expression in various tissues; hence it was hard to connect this extra domain with some particular tissues. Conversely, PSERA and PSERC were expressed in some particular tissues, respectively. The differential 3′ terminal of SERCA might be involved in the location of SERCA in different cell types or different sites of the same cell.

Semiquantitative RT-PCR showed mRNA relative abundance of these isoforms in tested tissues. In mammals, SERCA1 and SERCA3 show a restricted expression while SERCA2 is ubiquitously expressed. SERCA1a is the major isoform of fast-twitch skeletal muscles [29], while the SERCA1b isoform is found in neonatal muscles [9,21]. SERCA2a is expressed mainly in slow-twitch skeletal, cardiac, and smooth muscles [11,22,30]; SERCA2b is ubiquitously expressed and can be considered the housekeeping calcium pump [31,32]. SERCA3 presents in platelets and other blood-forming cells, plus in many endothelial and epithelial cells [33,34]. In addition, SERCA3 isoforms are rarely found alone and are always found to be co-expressed with those of SERCA2 [34]. In the present study, PSERB is ubiquitously expressed in various tissues and it is a potential “housekeeping gene”; its distribution pattern is similar to that of SERCA2b. PSERA is expressed in the adductor muscle and foot and it has a higher expression level in the adductor muscle. PSERC is expressed in other tissues except the adductor muscle or foot and has the highest expression level in the gill and mantle, which is very likely to be a non-muscle-specific isoform. In *P. fucata*, the adductor muscle is responsible for shell opening and closing, and the foot is responsible for movement, both of which consist of muscle cells. Hence, PSERA is very likely to be a muscle-specific isoform. SERCA is an important protein that helps to relax contracted muscles [14,35,36]. Most of the high expression levels of PSERA mRNA in the adductor muscle indicated that PSERA played a vital function in the muscle relaxation of *P. fucata*. In *P. fucata*, the mantle is the key tissue responsible for calcium secretion to the mineralization site with the function of calcium uptake from sea water [36]. The gill is a pivotal tissue for calcium uptake from sea water [37]. They both have active calcium metabolism and calcium homeostasis is especially vital to them. High expression levels of PSERC in the gill and mantle indicates that it may play important roles in calcium homeostasis during oyster biomineralization. Obviously, it is necessary to carry out further studies on the concrete functions of PSERC in the calcium homeostasis of *P. fucata*, with which the mechanism of calcium uptake, deposit, and transport may be understood. Further work regarding the location of PSERC at the mantle and gill of *P. fucata* and the *in vitro* expression of the three isoforms based on their cDNA sequences are ready to be conducted in our laboratory.

In conclusion, the three cloned SERCA isoforms PSERA, PSERB, and PSERC from the pearl oyster, *P. fucata* showed high homology with SERCAs from other species. The splicing mechanism of the three isoforms in *P. fucata* is similar to mammal SERCA3; the three isoforms have different expression patterns in various tissues of *P. fucata*. Isoform PSERC is likely to play a vital role in calcium homeostasis during oyster biomineralization.

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