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Cloning, characterization and immunolocalization of two subunits of calcineurin from pearl oyster (*Pinctada fucata*)

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ABSTRACT

Calcineurin (CN), consisting of catalytic subunit (CN A) and regulatory subunit (CN B), is a multifunctional protein involved in many important physiological processes. Here, we cloned two subunits of CN (*Pf*-CN A and *Pf*-CN B) from pearl oyster *Pinctada fucata* and reported, for the first time, its expression patterns in the developmental stages, its enzymatic activity and immunolocalization in various tissues of adult pearl oyster. The Pf-CN A was extensively localized in all the tested tissues including mantle, gonad, digestive gland, gills, adductor muscle, and foot with strong signals detected in gonad, gills, foot, and mantle. Importantly, Pf-CN A was mainly found in the inner epithelial cells of the basal periostracal groove and lateral surface of the inner mantle fold, in which organic macromolecules used for periostracum formation and shell construction are secreted, respectively. In gill, the strong signals were distributed in the epithelial cells of the branchial filaments and the base of gill filaments. All the results suggested that Pf-CN may participate in the development of the pearl oyster and function in many ways in various physiological activities, especially in the shell formation. Our observations could provide some important clues to further understanding of the functions of CN in the oyster.

1. Introduction

Calcineurin (CN), also called protein phosphatase 2B (PP2B), is the only member of the serine/threonine phosphatase family that can be activated by both Ca^{2+} and calmodulin (CaM) among (Klee et al., 1979, 1998; Stewart et al., 1982). CN is widely distributed in different tissues and in many cell types of various organisms from lower to higher species (Crabtree, 1999), and its roles have been established in T cell activation, vesicular trafficking, cell growth, apoptosis, neuron depotentiation, microtubule assembly, ion channel activity, muscle development, and cardiac valve formation (Zhang et al., 1996; Kayyali et al., 1997; Perrino and Soderling, 1998; Aramburu et al., 2000; Sugiura et al., 2001; Shibasaki et al., 2002; Norris et al., 2005). Most recently, it has been demonstrated that CN plays a key role in the bone formation and absorption (Takayanagi, 2007; Kuroda et al., 2008; Yamanaka et al., 2008). The CN $A\alpha^{-/-}$ mouse showed bone loss for the osteoporosis (Sun et al., 2005). In addition, CN plays a critical role in transducing Ca^{2+} signals into cellular responses (Klee and Haiech, 1980; Klee et al., 1988). Once activated by Ca^{2+} and CaM, CN can directly dephosphorylates various target proteins.

CN is a heterodimeric protein consisting of two subunits: catalytic subunit (calcineurin A, CN A) and regulatory subunit (calcineurin B, CN B) (Klee et al., 1979). From lower eukaryotic species to mammals (Rusnak and Mertz, 2000), CN A, with the molecular mass of 57–71 kDa, contains four distinct functional domains: a catalytic domain, a CN B-binding domain, a CaM-binding domain, and an autoinhibitory domain; while CN B, about 16–19 kDa, has four EF-hand type calcium-binding motifs. Recently, both CN A and CN B genes have been cloned from yeast to mammals and their distribution and functions have been investigated. In mollusks, comparatively little is known about CN functions. Up to now, cDNAs encoding CN A and CN B from scallop testis have been isolated and it was found that CN mRNAs are expressed maximally just before full maturation of the testis (Uryu et al., 2000), and CN A was highly expressed in the scallop gonad (Boutet et al., 2008), while the other information such as the detailed distribution in the tissues and the possible functions of CN in the mollusk largely remain unknown.

In this study, we isolated the cDNAs of two CN subunits (termed *Pf*-CN A and *Pf*-CN B, respectively) from pearl oyster *P. fucata* and reported, for the first time, its expression patterns in the developmental stages, its enzymatic activity and localization in various tissues of adult pearl oyster. All these results will provide some important clues to further understanding of the potential diverse roles of Pf-CN in the pearl oyster.

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2. Materials and methods

2.1. RNA extraction and cDNA synthesis

Live individuals of adult *P. fucata* (with shells of about 5.0–6.0 cm in diameter and 40–50 g in wet weight) were purchased from the Guofa Pearl Farm in Beihai, China and maintained in an aquarium at 20 °C in static tanks containing aerated artificial seawater (ASW) in the laboratory. Total RNA from the mantle, viscera, gill, hemocyte, and adductor muscle tissues from 6 individuals was extracted separately using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) under the manufacturer's instructions. The integrity of RNA was determined by electrophoresis on a 1.2% formaldehyde-denatured agarose gel stained with ethidium bromide. The quantity of RNA was determined by measuring OD_{260} with an Ultrospec 3000 UV/visible Spectrophotometer (Amersham, Piscataway, USA). Total RNA (1 μg) extracted from the mantle tissue of *P. fucata* was used to synthesize single strand cDNA using SuperScript II RNase H-reverse transcriptase (Invitrogen) and an adaptor-oligo(dT) primer in term of the manufacturer's instructions.

2.2. Isolation of Pf-CN A and Pf-CN B cDNA

A cDNA fragment of *P. fucata* CN subunit A from mantle tissue was amplified by RT-PCR using a pair of degenerate oligonucleotide primers APF and APR, respectively, designed based on the conserved amino acid sequences of PVTVCGD and PHPYWLP within CN subunit A (Table 1) and Ex Taq DNA polymerase (Takara, Kyoto, Japan). The PCR was carried out on a T gradient Thermocycler (Biometra, Gottingen, Germany) for 35 cycles of denaturation (94 °C for 30 s), annealing (48 °C for 30 s), and elongation (72 °C for 1 min). PCR products of the expected size (783 bp) were excised and purified with the Wizard PCR Prep DNA Purification system (Promega, Madison, WI, USA). The purified PCR products were then subcloned into a pGEM-T Easy vector (Promega).

The full-length sequence of *Pf*-CN A cDNA was obtained by 5′-RACE and 3′-RACE. To obtain the 3′terminus of *Pf*-CN A cDNA, PCR was conducted with the gene-specific forward primer A3RSP designed based on the sequence of the CN A fragment obtained above and the reverse adaptor primer UP, using the above first-strand cDNA as the template. 5′RACE was performed using a SMART™ RACE amplification kit (Clontech, Mountain View, CA, USA). The first-strand cDNA was synthesized according to the manufacturer's protocol. The reaction was performed with the forward primer UPM and the reverse specific gene primer A5RSP prepared on the basis of the fragment CN A sequence. The reaction was performed in a thermocycler (Biometra) under the following conditions: 5 cycles of 30 s at 94 °C, 3 min at 72 °C; 5 cycles of 30 s at 94 °C, 30 s at 70 °C, 3 min at 72 °C and 25 cycles of 30 s at 94 °C, 30 s at 68 °C, 3 min at 72 °C; followed by a final extension of 10 min at 72 °C. To confirm the nucleotide sequence of *Pf*-CN A cDNA obtained by RACE, PCR was performed using Pyrobest DNA polymerase (Takara), with a pair of specific primers, ACF4 and ACR2, corresponding to the 5′-untranslated and 3′-untranslated regions of *Pf*-CN A mRNA, respectively. The PCR products were subcloned into a pGEM-T Easy vector and five independent clones were sequenced.

For *P. fucata* CN B cloning, two degenerate primers BFP2 and BRP2, based on the conserved sequences of GEVDFKEF and MMVGNNL within CN subunit B (Table 1), respectively, were designed for RT-PCR to amplify the cDNA fragment of the pearl oyster CN subunit B from mantle tissue. PCR products of the expected size (171 bp) were purified and subcloned into a pGEM-T Easy vector. 5′-RACE and 3′-RACE were performed to obtain the full-length sequence of *Pf*-CN B cDNA. Using the above first-strand cDNA as the template, the 3′ terminus of *Pf*-CN B cDNA was obtained with the gene-specific forward primer B3RF2 and the reverse adaptor primer UP; the 5′ terminus of *Pf*-CN B cDNA was obtained with the forward primer UPM and the gene-specific forward primer B5RR2 through a SMART™ RACE amplification kit. To confirm the nucleotide sequence of *Pf*-CN B cDNA obtained by RACE, PCR was performed with a pair of specific primers, BCF2 and BCR2, corresponding to the 5′-untranslated and 3′-untranslated regions of *Pf*-CN B mRNA, respectively. The PCR products were subcloned into a pGEM-T Easy vector and five independent clones were sequenced.

Table 1
Primers sequences used in the paper.

Name	Sequence
APF	5′-CCNGTNACNGTNTGYGGNGA-3′
APR	5′-GGNARCCARTANGGRTGNGG-3′
A3RSP	5′-AGAGCACACGAAGCACAAGATG-3′
A5RSP	5′-CCCCTGTCCACATAATCTCC-3′
ACF4	5′-TGGTTTCATAAAATCTGAAGCCCCA-3′
ACR2	5′-ATGAGCTGGCCACGGAGTC-3′
BFP2	5′-GGNGARGTNGAYTTYAARGARTT-3′
BRP2	5′-ARRTTRTTNCCNACCATCAT-3′
B3RF2	5′-GTGTTGAAAATGATGGTCGG-3′
B5RR2	5′-AGCCGTCCTTGTCCATATCG-3′
BCF2	5′-CTGTTTCACAAATAGTTTTAGCAGC-3′
BCR2	5′-GGTAAGGGCAACTTATCAAAGG-3′
AE5	5′-TGCCGCCCATATGGCAACAACAGATTCTAAG-3′
AE33	5′-CCGGAATTCTCAGTGATGATGATGATGATGGCTATGGGAATTGTTCAATG-3′
ART55-2	5′-ATTATTAGAGCACACGAAGCAC-3′
ART33-2	5′-AGGAGACCATTTGGAGTAAGTC-3′
BRTF1	5′-TAGCAGCAAAAGAAGTAAAAATG-3′
BRTR1	5′-GTTCACATTTGCTGTCATTTCAC-3′
GAPDH5	5′-GCCGAGTATGTGGTAGAATC-3′
GAPDH3	5′-CACTGTTTTCTGGGTAGCTG-3′

2.3. Sequence analysis

All cDNA sequences were sequenced using an automated DNA sequencer (Applied Biosystems 3730XL, Applied Biosystems, CA, USA), and analyzed using the BLAST program available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, USA) was used to identify its encoding protein. The ClustalX (version 1.81, ftp://ftp-igbmc.u-strasbg.fr/pub/clustalx) and the Vector NTI (InforMax, Frederick,USA) were used to perform multiple alignments and to produce the phylogenetic tree.

2.4. Expression and purification of Pf-CN A in E. coli

To express the oyster Pf-CN A in *E. coli*, the pET-25b/ Pf-CN A expression vector was constructed by amplifying the full-length sequence of *Pf*-CN A with primers (Table 1): AE5 containing an Nde I site (underlined), and AE33 containing an EcoR I site (underlined), and then inserting the purified PCR product into the Nde I and EcoR I sites of pET-25b (Novagen, Darmstadt, Germany). The recombinant plasmid, pET-25b/Pf-CN A, confirmed by restriction analysis and DNA sequencing, was then transformed into *E. coli* strain BL21 (DE3, Novagen). Protein expression was induced with 0.8 mM isopropylthiogalactopyranoside (IPTG) at 37 °C. IPTG was added when the optical density of the culture at 600 nm had reached 0.6. After 3 h of induction, bacterial cells were harvested by centrifuging the culture at 8000 g for 5 min.

Harvested cell paste from 1 L of bacterial media was suspended in 50 ml lysis buffer (containing 8 M urea, 0.02 M NaH_2PO_4, pH 7.2, 0.5 M NaCl) and followed by sonication on ice using a Branson sonifier. After centrifugation at 10000 g for 30 min at 4 °C, the supernatant was filtrated through a 0.45 μm filter and applied to a Hi-Trap HP Ni affinity column (Amersham Biosciences, NJ, USA). The column was subsequently washed with binding buffer (containing 8 M urea, 0.02 M Na_3PO_4, 0.5 M NaCl, 0.02 M imidazole, pH 7.5).The column was then eluted with the elution buffer (8 M urea, 0.02 M Na_3PO_4, 0.5 M