Cloning and expression of a pivotal calcium metabolism regulator: calmodulin involved in shell formation from pearl oyster (*Pinctada fucata*)

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Abstract

The shells of bivalves are mainly composed of calcium carbonate, a product of calcium metabolism. In the process of shell formation, the uptake, transport and recruitment of calcium ion are highly regulated and involved in many factors. Among these regulatory factors, calmodulin (CaM), a pivotal multifunction regulator of calcium metabolism in nearly all organisms, is thought to play an important role in the calcium metabolism involved in shell formation. In this study, a full-length CaM cDNA was isolated from the pearl oyster (*Pinctada fucata*). The oyster calmodulin encodes a 16.8 kDa protein which shares high similarity with vertebrate calmodulin. The oyster CaM mRNA shows the highest level of expression in the gill, a key organ involved in calcium uptake in oyster calcium metabolism. In situ hybridization results revealed that oyster CaM mRNA is expressed at the folds and the outer epithelial cells of the dorsal region of the mantle, suggesting that CaM is involved in regulation of calcium transport and secretion. Oyster CaM also showed a typical Ca2+ dependent electrophoretic shift characterization and calcium binding activity. Taken together, we have identified and characterized a pivotal calcium metabolism regulator of the oyster that may play an important role in regulation of calcium uptake, transport and secretion in the process of shell formation.

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1. Introduction

The shells of bivalve animals are all calcium metabolism products containing more than 90% CaCO3 crystals and a few percent of a matrix of biological macromolecules. The shell, especially the nacre (internal lustrous layers of the shell), has drawn much attention for its outstanding mechanical strength and nanoscale microstructure (Addadi and Weiner, 1997). In the past decade, many efforts have been made to elucidate the mechanism of shell formation, and more than 10 matrix proteins have been identified from bivalve mollusks (Zhang et al., 2003). However, previous mechanical studies of the shell formation have concentrated on the purification and characterization of the matrix proteins, while the mechanism of oyster calcium metabolism has been neglected.

Calcium ion is not only a regulatory agent involved in many physiological processes of bivalve animals, but also the primary cation participated in the formation of shell structures (Simkiss and Wilbur, 1989). In the process of shell formation, large amounts of calcium ion were continuously demanded to be deposited onto the framework formed by matrix proteins. The mechanism of Ca2+ uptake, accumulation, transport, incorporation and the particular regulators involved in these processes remains as an attractive field for further study.

Calmodulin (CaM) is a multifunctional calcium-sensor protein, which participates in several cellular processes such as secretion, cyclic nucleotide metabolism, cellular calcium metabolism, muscle contraction and glycogen metabolism (Cheung, 1979; Means and Dedman, 1980). As a pivotal regulator of calcium metabolism, many calcium metabolism related proteins including cyclic nucleotide phosphodiesterase, adenylyl cyclase, Ca2+-ATPase, as well as Ca2+ channel and Ca2+ release channel are regulated by CaM (Cheung, 1979; Means and Dedman, 1980; Eldik and Watterson, 1985; Lee et al., 1999; Zühlke et al., 1999; Ashby and Tepikin, 2002; Saimi and Kung, 2002). It has been demonstrated that the calcium stores in *Drosophila* are regulated by CaM (Arnon et al., 1997). In marine bivalves, calcium is taken up by gill from the external medium and transported to the mantle epithelium. In this process CaM has been thought to
play a regulatory role in the membrane Ca\(^{2+}\)-ATPase system or act as a "calcium sink", and is involved in ciliary arrest of calcium (Stommel et al., 1982; Stommel and Stephens, 1985). Additionally, calcium ion can also be transported by calcium pump directly across the mantle surface into the extrapallial space for shell formation (Richardson et al., 1981). In recent years, L-type Ca\(^{2+}\) channels, which are regulated by CaM, have been suggested to be involved in the calcium transport process for calcification in some marine invertebrates (Marshall, 1996; Zoccola et al., 1999). As an essential subunit of calcium channels and a crucial regulator of Ca\(^{2+}\)-ATPase in nearly all organisms, we propose that CaM plays an important role in the regulation of the uptake and transport of calcium for shell formation.

So far, two CaM proteins and a CaM cDNA have been isolated from mollusk species, Patinopecten, Mytilus edulis and Aplysia, separately (Yazawa et al., 1980; Toda et al., 1981; Mimura et al., 1985; Swanson et al., 1990). To investigate the potential role of CaM in oyster calcium metabolism, we first isolated a full-length CaM cDNA and characterized its expression from Pinctada fucata, a useful animal for marine pearl production. We also expressed, purified the oyster CaM in Escherichia coli, and analyzed its calcium binding characterization. As pearl oyster (P. fucata) contributes greatly to marine pearl production, the results may help to further understand the mechanisms of calcium uptake, accumulation and incorporation into the shell and pearl.

2. Material and methods

2.1. RNA Preparation and cDNA synthesis

Adult specimens of P. fucata were purchased from Guofa Pearl Farm, Guangxi Province, China. Tissues including mantle, gonad, muscle and gill were isolated and kept in RNAlater (Ambion). Total RNA was extracted from the tissues by using TRIzol reagent (Invitrogen). The integrity of RNA was determined by fractionation on 1.2% formaldehyde-denatured agarose gel and staining with ethidium bromide. The quantity of RNA was determined by fractionation on 1.2% formaldehyde-denatured agarose gel and staining with ethidium bromide. The quantity of RNA was determined by measuring OD\(_{260}\) with a UtreeSpec 3000 UV/Visible Spectrophotometer (Amersham). Total RNA (5 \(\mu\)g) extracted from mantle tissue of P. fucata was used to synthesize single-strand cDNA, using SuperScript II RNase H Reverse Transcriptase (Invitrogen).

2.2. Construction of mantle cDNA library

Poly(A\(^+\)) RNA from mantle tissue of P. fucata was isolated using the Poly(A)Tract mRNA Isolation System (Promega). Approximately 5 \(\mu\)g Poly(A\(^+\)) RNA was used to generate double-strand cDNA. The cDNA was subsequently ligated into the Uni-ZAP XR Vector and packaged with the Gigpack III Gold extract (Stratagene).

2.3. Cloning of pearl oyster CaM cDNA

The cDNA fragment CaM1 of the pearl oyster CaM gene from mantle tissue was amplified by RT-PCR using Ex Taq DNA polymerase (TakaRa). The degenerate oligonucleotide primers used for amplification were designed based on the conserved regions of CaM nucleotide sequence: the forward primer F1, 5\(^{\prime}\) -ATY GCW GAR TTY AAR GAR GC-3\(^{\prime}\) (corresponding to the sequence from nt +28 to +47), and the reverse primer R1, 5\(^{\prime}\) -CCR TCW CCA TCA ATR TCH GC-3\(^{\prime}\) (corresponding to the sequence from nt +385 to +404). PCR products of the expected size (377 bp) were excised and purified with the Wizard PCR Prep DNA Purification System (Promega). The purified PCR products were then subcloned into pGEM-T Easy vector (Promega) and sequenced. To obtain the 3\(^{\prime}\) terminal sequence of P. fucata CaM gene, a specific forward primer F2 (5\(^{\prime}\) -GAA TTC TT CTT ACA ATG ATG GC-3\(^{\prime}\)) was designed based on the sequence of fragment CaM1, and the oligo-dT adaptor, 5\(^{\prime}\) -TCG AAT TCG GAT CGC AGC TCT \(V\)-3\(^{\prime}\), was used as reverse primer. A 469 bp PCR product was subcloned as mentioned above and sequenced. To amplify the 5\(^{\prime}\) terminal sequence of P. fucata CaM cDNA, a specific reverse primer R2 (5\(^{\prime}\) -TTT CGA CAT CAT CAT TTT CAC-3\(^{\prime}\)) based on the sequence of the 3\(^{\prime}\) terminal sequence from above and T3 primer (forward primer, corresponding to the T3 promoter on the Uni-ZAP vector) were used to amplify the P. fucata mantle cDNA library, and a 516 bp fragment was produced. PCR products were cloned into the pGEM-T Easy vector for sequencing.

2.4. DNA sequence and analyses

All recombinant plasmid were sequenced using an automated DNA sequencer (Applied Biosystems 377). The nucleotide sequence was blast against GenBank using BlastT algorithm to identify its coding proteins. Multiple alignments were created using the ClustalX program (Thompson et al., 1997).

2.5. Gene expression assay

Expression levels of mRNA of the oyster CaM were examined using semi-quantitative RT-PCR analyses. Total RNA was prepared from tissues including mantle, gonad, muscle and gill as mentioned above. Aliquots (1 \(\mu\)g) of total RNA from different tissues were transcribed into cDNA in 20 \(\mu\)l reaction mixtures using SuperScript II RNase H Reverse Transcriptase (Invitrogen). The generated cDNA was used as a template for PCR, which was performed with 2.5 mM MgCl\(_2\), 200 \(\mu\)M dNTP, 2.5 U Taq DNA polymerase, and 20 pM of each primer G1 (5\(^{\prime}\) -TCA AGG AAG CGT TCA GTT T-3\(^{\prime}\)) and G2 (5\(^{\prime}\) -CGA TCA CCC CCG TCA ATA TCA G-3\(^{\prime}\)). Preliminary experiments showed that a total RNA concentration of 1 \(\mu\)g and 22 cycles were well within linear amplification. After amplification, the PCR products were subcloned into pGEM-T
Easy vector and confirmed by sequence. Equal volumes of the PCR products were applied to 1.5% agarose gel, stained with ethidium bromide and evaluated by band densitometry. To avoid sample cross-contamination, negative control reactions for RT-PCR were performed in absence of cDNA template.

2.6. In situ hybridization

Mantle and gill were separated from adult P. fucata and immediately fixed in 4% paraformaldehyde overnight. In situ hybridization of oyster CaM mRNA was carried out on frozen sections. Digoxigenin-labeled RNA probes were generated from the cDNA clone encoding oyster CaM in plasmid using a DIG RNA Labeling kit (Roche), with T7 and SP6 RNA polymerase for the sense and anti-sense probe, respectively. RNA in situ hybridization was performed as described previously with some modification (Huang et al., 2001). To avoid false positive signals, the hybridization temperature was increased to 58 °C.

2.7. Expression and purification of the oyster CaM in E. coli

The coding region of pearl oyster CaM cDNA was amplified by PCR with Pfu DNA polymerase (TaKaRa). The primers for amplification of oyster CaM cDNA were F3 (5′-GGA TCCATG GCC GAT CAG CTG ACA GAG-3′) containing a NcoI site (underlined), and R3 (5′-CGGATCC CAT TTC GAC ATC ATC ATT TTC AC-3′) containing a BamHI site (underlined). The PCR products were purified with the Wizard PCR Prep DNA Purification System (Promega) and digested with NcoI/BamHI, then inserted into a prokaryotic expression vector pET-15b (Novagen). The recombinant plasmid was named pET-15b/CaM, confirmed by sequencing. The prokaryotic expression vector pET-15b/CaM was fellow transformed into E. coli BL21 (DE3, Novgen). Protein expression was induced with 0.5 mM isopropylthiogalactopyranoside (IPTG) at 37 °C. IPTG was added when the optical density at 600 nm of the culture had reached 1.0. After 2.5 h of induction, bacterial cells were harvested by centrifuging the culture at 9000 rpm for 5 min.

For protein purification, the procedure of Gopalakrishna and Anderson (1982) with slight modification was followed. The bacterial pellet was washed twice with 50 mM Tris–HCl, pH 7.5, then was suspended in 50 mM Tris–HCl buffer, pH 7.5, containing 2 mM EDTA, 0.5 mM DTT and 0.2 mM phenylmethanesulfonyl fluoride (PMSF), and sonicated on ice. The supernatant was collected by centrifuging the lysate at 15,000 rpm for 25 min at 4 °C. After adding 5 mM CaCl2, the supernatant was then loaded at room temperature onto a phenyl-Sepharose FF column (Amersham) previously equilibrated with 50 mM Tris–HCl, pH 7.5, containing 1 mM CaCl2. The column was washed with 50 mM Tris–HCl, pH 7.5, containing 1 mM CaCl2 until absorption at 280 nm

Fig. 1. Nucleotide and deduced amino acid sequence of the P. fucata calmodulin (CaM) cDNA. The stop codon is marked with an asterisk and the possible polyadenylation signal sequence in the 3′-untranslated region is underlined. This cDNA sequence has been submitted to GenBank (accession number: AY341376).
reached the baseline. Then the column was washed with 50 mM Tris–HCl, pH 7.5, containing 1 mM CaCl₂ and 0.5 M NaCl. Finally, CaM was eluted with 50 mM Tris–HCl, pH 7.5, containing 1 mM ethylene glycol-bis(β-amino-ethyl ether) N,N',N'-tetra-acetic acid (EGTA). Fractions with CaM were analysed on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with sample buffer containing 5 mM EGTA, and stained with Coomassie Brilliant Blue R-250. CaM-containing fractions were collected and dialyzed against Mini-Q water and freeze dried. Protein yields were measured by BCA assay kit (Pierce).

2.8. Preparation of recombinant oyster calmodulin polyclonal antibody and Western blot

A rabbit polyclonal antiserum raised against purified recombinant oyster CaM, obtained by injection of 250 μg recombinant protein in complete Freund’s adjuvant at each six dorsal sites, was followed by an equivalent challenge incubation for weeks, bleeding at 2-week intervals. Positive sera were stored at −80 °C.

To verify the expressed protein, Western blot analyses were performed on crude protein extracts from oyster mantle, muscle and gill: 30 mg of tissue were homogenized in 2 vol buffer (0.05 M Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM DTT and 0.5 mM PMSF), then spun at 12,000 rpm for 5 min and the supernatant transferred to 15% SDS-PAGE. Western blot was performed using the Multiphor II Western blotting system (Amersham). Recombinant oyster CaM polyclonal antibody was used at dilutions of 1:1000, and alkaline phosphatase (AP)-labeled anti-rabbit IgG antibody (Amersham) at 1:2000. Other procedures were according to Molecular Cloning: A Laboratory Manual (Sambrook et al., 1989).

2.9. Ca²⁺ dependent electrophoretic migration and ⁴⁵Ca overlay analyses

Ca²⁺ dependent electrophoretic shift assay was carried out according to the method of Burgess et al. (1980). The sample buffer, running buffer and gels all contained 0.1 mM CaCl₂ or EDTA in the presence of SDS. ⁴⁵Ca overlay analysis fol-

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Fig. 2. Alignment of the sequence of oyster calmodulin with other CaMs from principal invertebrates and vertebrates. Apc=(Aplysia californica) CaM, CAA40207; Mes=sea anemone (Metridium senile) CaM, MCXAM; Pat=scallop (Pinctada sp.) CaM, MCSW; Pfa=pearl oyster (P. fucata) CaM, AY34137; Hsa=human (Homo sapiens) CaM, P02593; Bot=bovine (Bos taurus) CaM, MCBO; Rno=rat (Rattus norvegicus) CaM, CAA32119.
allowed the procedure of Maruyama et al. (1984). Purified recombinant oyster CaM was transferred onto nitrocellulose membrane after electrophoresis as described above, then labeled with $^{45}$Ca (Amersham). Autoradiography of the $^{45}$Ca labeled proteins on the nitrocellulose membrane was obtained by a Strom 860 scanner (Amersham).

3. Results and discussion

3.1. Cloning and sequence analyses of P. fucata CaM cDNA

Using two degenerate oligonucleotides (F1 and R1) derived from the conserved regions of CaM nucleotide sequence, a 377 bp cDNA fragment CaM1 was obtained from the mantle of P. fucata. Based on the sequence, a specific primer F2 was synthesized and the 3' terminal sequence of CaM cDNA was obtained by RT-PCR with primer F2 and the oligo-dT adaptor. The 5' terminal sequence of oyster CaM cDNA was amplified the mantle cDNA library by using T3 and a specific primer R2 based on the 3' terminal sequence of CaM cDNA from above.

As shown in Fig. 1, the full length CaM cDNA sequence derived from the mRNA of the pearl oyster was 739 bp, including a 69 base 5' untranslated sequence, an open reading frame consisting of 447 bp, a TGA stop, a 203 base 3' untranslated sequence, and a poly(A) tail of 17 nucleotides. Two putative polyadenylation signals (AATAAA) followed by a stop codon (TGA) and 15 bp upstream of poly(A) were located at the 3' non-coding region of the gene. A Kozak sequence (PuNNATGPu) was found surrounding the methionine initiation codon (AAAATGG) of CaM cDNA. This cDNA sequence has been submitted to GenBank and the accession number is AY341376.

The deduced CaM protein consists of 149 amino acids with a calculated molecular mass of 16.8 kDa and an isoelectric point of 4.14. Like vertebrate and other mollusk CaM proteins, the P. fucata CaM did not contain cysteine and tryptophan residues. From an evolutionary standpoint, CaM is a remarkably conserved protein. Multiple protein sequence alignment analysis (Fig. 2) showed that the CaM sequence of P. fucata shares high similarity with other CaMs isolated from invertebrate and vertebrate animals. Only four residues (Asn$^{61}$, Asp$^{97}$, Lys$^{144}$ and Thr$^{147}$) of P. fucata changed (Asp$^{61}$, Asp$^{97}$, Thr$^{144}$ and Thr$^{147}$) in Patinopecten (Toda et al., 1981), a closer species to P. fucata in evolution. Additionally, alignment analysis also shows that Lys$^{144}$ is conserved in the oyster and sea anemone. As Met$^{147}$ is only found in P. fucata, Met$^{147}$ can be considered as a characteristic residue of the oyster.

3.2. Expression analysis

To investigate the function of oyster CaM in vivo, the tissue-specific expression of oyster CaM mRNA in different tissues was examined by employing semi-quantitative RT-PCR. RT-PCR reactions were performed with RNA samples from mantle, gill, muscle and gonad. A 367 bp RT-PCR product was obtained with specific primers (G1 and G2) and the total RNA of various tissues as template, while the negative control exhibited no product (data not shown). PCR products were then inserted into pGEM-T Easy vector and confirmed by sequence analyses. As shown in Fig. 3, oyster CaM mRNA was expressed in all tissues tested. However, the expression level of CaM mRNA was higher in gill, muscle and mantle than in the gonad despite equal levels of 28S ribosomal RNA in all samples. Data from three independent experiments were similar.

Shell formation needs a large amount of calcium (Wilbur, 1972; Rousseau et al., 2003), and biomineralization implies
the transport and control of calcium ions. The gill is the major organ for calcium uptake from the external medium (Dimtriadis et al., 2003; Rousseau et al., 2003), and CaM has been thought to play a regulatory role in membrane Ca\(^{2+}\)-ATPase system or act as a “calcium sink” in the gill of scallop (Stommel et al., 1982). Additionally, CaM has been demonstrated to be involved in ciliary arrest of calcium (Stommel and Stephens, 1985). In situ hybridization has shown that the oyster CaM mRNA was expressed widely in the epithelial cells of the branchial filaments (data not show). As L-type Ca\(^{2+}\) channels modulated by CaM have been suggested involved in the calcium transport process for calcification in some marine invertebrates (Marshall, 1996; Zoccola et al., 1999), the highest expression level of oyster CaM mRNA in the gill implies that CaM probably plays an important regulatory role in calcium uptake and accumulation in gill.

The mantle of mollusks is another key organ responsible for the metabolism of metal ions, and it participates actively in the secretion of calcium and other ions for mineral growth in the process of shell formation (Wilbur, 1972; Pekkarinen and Valovirta, 1997). To examine the precise expression site of CaM mRNA in the mantle tissue of *P. fucata*, in situ hybridization analysis was performed. Strong hybridization signals were detected at the inner epithelial cells of the outer fold, the outer epithelial cells of the middle fold and the outer epithelial cells of the inner fold of the mantle (Fig. 4A). However, no hybridization signal was detected in the outer epithelial cells of the outer fold of the mantle. Strong hybridization signals were also detected in the basement cells of the outer fold of the mantle (Fig. 4B) and the outer epithelial cells of the dorsal region of the mantle (Fig. 4C), whereas hybridization with the control sense probe did not yield any hybridization signals (data not shown). With these results we predicted that CaM may participate actively in CaM-dependent calcium transport by Ca\(^{2+}\)-ATPase, and be involved in the regulation of calcium secretion in shell formation. The oyster CaM mRNA highly expressed in muscle, which is consistent with the fact CaM is a key modulator of muscle contraction for shell opening or closing. Additionally, oyster CaM mRNA is also expressed in gonad, implying that CaM may relate to oyster procreation.

In conclusion, although we have analyzed oyster CaM mRNA expression levels in different tissues and detected the precise expression site of oyster CaM mRNA in gill and

Fig. 4. In situ hybridization of oyster CaM mRNA in the mantle of pearl oyster (*P. fucata*). Hybridization signals (arrows and arrow heads) were observed in all three folds of the mantle (A). To view the distribution of hybridization signal on the whole tissue, three overlapping pictures of the same section were taken. Strong hybridization signals were presented in the inner epithelial cells (arrow heads) and the basement cells of the outer fold of the mantle (arrow) in (B). Hybridization signals were also shown in the outer epithelial cells of the dorsal region of the mantle (arrows) in (C). OF, outer fold; MF, middle fold; IF, inner fold. Magnifications are 25× (A) and 100× (B and C).
mantle, the detailed mechanisms of the regulation of CaM in calcium uptake, accumulation and release in shell formation still need further study.

3.3. Expression and purification of P. fucata recombinant CaM

The P. fucata CaM cDNA coding region sequence was cloned into pET-15b expression vector and expressed in E. coli. BL21 (DE3). As shown in Fig. 5, the expressed CaM protein detected by SDS-PAGE and the expressed recombinant CaM reached approximately 21% of the total bacteria soluble proteins. After purification by phenyl-Sepharose hydrophobic chromatography, only a single band was observed on 15% SDS-PAGE with the sample buffer containing 5 mM EGTA, and stained by Coomassie Brilliant Blue R-250. The apparent molecular mass of the band is about 17 kDa, which is consistent with the predicted molecular mass of oyster CaM, and the expression level of target protein is 10 mg/l LB culture. Purification of CaM from tissues involves many steps, and the yield is usually very low. On the contrary, expression and purification of CaM in E. coli is relatively simple, and the yield is often higher than that purified from tissues. Human, chicken and rat CaM expression in E. coli have been reported (Rhyner et al., 1992; Putkey et al., 1985; Matsuki et al., 1990; Hayashi et al., 1998), and here, we expressed and purified a mollusk CaM in E. coli, and prepared its antibody. The purified recombinant CaM and the CaMs from oyster tissues of mantle, muscle and gill were detected by Western blot analysis with rabbit polyclonal antibody of the recombinant oyster CaM. Only one visualized immunoreactive band was found at 17 kDa (Fig. 6). This implied that the CaM was expressed in tissues of mantle, muscle and gill, which was in agreement with the results of semi-quantitative RT-PCR. This result may provided a crucial foundation to further study its characterization, expression pattern, distribution and function involvement in shell formation and other important processes in oyster calcium metabolism.

Fig. 5. Expression of recombinant P. fucata CaM in the culture supernatant detected by 15% SDS-PAGE with sample buffer containing 5 mM EGTA, and stained by Coomassie Brilliant Blue R-250. Lane 1: protein molecular mass marker; lane 2: proteins of BL21 (DE3); lane 3: proteins of pET-15b/CaM induced by 0.5 mM IPTG for 2.5 h; lane 4: purified recombinant CaM by phenyl-sepharose column. The molecular mass in kDa is shown on the left of the gel and the purified recombinant CaM is indicated by the arrow on the right.

Fig. 6. Western blot analysis of the expression of CaM in P. fucata. Lane 1: purified recombinant oyster CaM; lanes 2, 3, 4: oyster tissues, mantle, muscle and gill, respectively.

Fig. 7. Ca$^{2+}$-dependent electrophoretic migration of purified recombinant P. fucata CaM. Purified recombinant oyster CaM (lane 2) was run on a 15% SDS-PAGE in the presence of Ca$^{2+}$ (A) or EDTA (B). Sample buffer, running buffer and gels all contained 0.1 mM CaCl$_2$ or 0.1 mM EDTA. Lane 1: protein molecular mass marker; molecular mass in kDa is shown on the left of the gels.

Fig. 8. Identification of calcium binding characterization of oyster CaM on nitrocellulose membrane after SDS electrophoresis. (A) indicates the protein band stained by Amido black after autoradiography. (B) is an autoradiograph of the transferred nitrocellulose membrane.
3.4. Ca\(^{2+}\) dependent electrophoretic shift and \(^{45}\)Ca overlay assay

To further confirm the identification of the purified protein as CaM and its calcium binding activity, Ca\(^{2+}\)-dependent electrophoretic migration and \(^{45}\)Ca overlay analyses were performed. As can be seen in Fig. 7A, when purified recombinant proteins are electrophoresed with the sample buffer, the gels and the running buffer all containing 0.1 mM CaCl\(_2\), a single band with an apparent molecular mass of approximately 14 kDa was observed. However, when CaCl\(_2\) was replaced with EDTA, the band migrated with an apparent molecular mass of 17 kDa (Fig. 7B). Since calcium-induced electrophoretic mobility change is a useful method in identifying CaM (Burgess et al., 1980), this phenomenon confirmed that the purified protein was CaM. Additionally, the \(^{45}\)Ca overlay analysis further demonstrated its calcium binding characterization (Fig. 8). In SDS-PAGE electrophoresis, CaM isolated from vertebrates migrated with an apparent molecular mass of 15 and 21 kDa in the presence or absence of calcium, respectively (Burgess et al., 1980). Amino acid substitutions in the carboxyl terminal residues of \(P.\) fucata CaM may slightly affect its conformation, and lead to a smaller electrophoretic shift than that of vertebrate CaM. The properties of oyster CaM recognition and interaction with target proteins by binding calcium, such as calcium pump, calcium channels and CaM-dependent protein kinase may differ from the vertebrate CaM, based on the presence of calcium induced conformational change, and the detailed mechanisms need further study.

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