A novel 15 kDa Ca^{2+}-binding protein present in the eggs of the sea urchin, *Hemicentrotus pulcherrimus*

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A novel Ca^{2+}-binding protein, different from calmodulin, has been purified to homogeneity from the soluble cytoplasmic protein fraction of the egg of the sea urchin, *Hemicentrotus pulcherrimus*. This protein, designated as 15 kDa protein, shows a Ca^{2+}-dependent mobility shift upon SDS-gel electrophoresis and has Ca^{2+}-binding ability. This protein did not resemble the sea urchin egg calmodulin in either molecular mass or amino acid composition. The 15 kDa protein could not activate cyclic adenosine 3′,5′-monophosphate-dependent phosphodiesterase from bovine brain and did not bind to fluphenazine-Sepharose 6B. Antibodies against the 15 kDa protein did not react with sea urchin egg calmodulin. These results suggest that the 15 kDa protein is a novel Ca^{2+}-binding protein in the sea urchin egg.

Ca^{2+}-binding protein *Ca^{2+}* *(Sea urchin egg)* *Calmodulin*

1. INTRODUCTION

Ca^{2+} plays an important role in the regulation of many activities in eukaryotic cells [1]. In the sea urchin egg, a transient increase in the level of free calcium has been observed after fertilization [2,3], and it has been suggested that this intracellular release of calcium may be the essential factor which causes the dynamic changes in the cytoskeleton of the eggs. It is generally considered that a Ca^{2+}-binding protein, calmodulin, has a key role in Ca^{2+}-dependent intracellular regulation because of its apparent ubiquity, its multifunctions, and its highly conserved structure [4,5]. Calmodulin has also been found in and purified from sea urchin eggs [6]. Thus, it is possible that it has a particularly significant role in the control of various Ca^{2+}-regulated processes during and after fertilization [7–9]. However, since these Ca^{2+}-dependent processes are so complicated, the involvement of another Ca^{2+}-binding mediator should be considered.

To test this hypothesis, we have tried to ascertain whether a second Ca^{2+}-binding protein different from calmodulin is present in sea urchin egg. In this paper, we report the isolation and characterization of a newly identified Ca^{2+}-binding protein, 15 kDa protein, in the egg of sea urchin, *Hemicentrotus pulcherrimus*.

Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; DTT, dithiothreitol

Preliminary accounts of this study have already been published [24,25]

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2. MATERIALS AND METHODS

2.1. *Preparation of egg extracts*

Eggs of the sea urchin, *H. pulcherrimus*, were obtained according to [10]. Egg extracts were prepared as described [10].

2.2. *Purification of the 15 kDa protein*

The egg extracts (800 mg protein) were dialyzed against F-buffer (10 mM Hepes, pH 7.5, 0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.2 mM DTT, 1 μg/ml leupeptin), and aggregates were removed by centrifugation at $10000 \times g$ for 30 min. The supernatant was applied to a DEAE-cellulose (Whatman, DE-52) column (1.6×18 cm) pre-equilibrated with F-buffer. The column was washed with 5–6 column volumes of F-buffer and then eluted with 400 ml of a 0.1–0.5 M KCl linear gradient (prepared in F-buffer). Fractions were analyzed by SDS-PAGE and those which contained the 15 kDa component were combined and applied to a column (1.2×18 cm) of hydroxyapatite (100–300 mesh, Nakarai, Kyoto) pre-equilibrated with F-buffer. The column was washed with P-buffer (10 mM sodium phosphate, pH 7.2, 0.2 mM DTT, 1 μg/ml leupeptin) and then eluted with 300 ml of a 10–400 mM sodium phosphate linear gradient (prepared in P-buffer). Fractions were analyzed by SDS-PAGE and those which contained the 15 kDa component were combined and concentrated. The fraction (mainly composed of 15 kDa protein and actin) was then chromatographed on a column (2.7×98 cm) of Sephadex G-50 (Pharmacia) pre-equilibrated with F-buffer in order to remove actin. Purified 15 kDa protein was stored at -80°C until use.

2.3. *Immunological techniques*

Rabbits were injected subcutaneously with the 15 kDa protein that had been cut out from SDS-polyacrylamide gels and homogenized with Freund's complete adjuvant. Proteins from SDS gels were transferred electrophoretically to nitrocellulose sheets (pore size, 0.45 μm; BIO-RAD) according to Towbin et al. [11]. The nitrocellulose blots were incubated in 10% bovine serum albumin (BSA) in TBS (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl) for 1 h at 37°C. After washing in TBS, the blots were incubated for 1 h at 37°C in the antiserum diluted to 1/50 in TBS containing 1% BSA. They were then washed several times with TBS before incubation for 1 h at 37°C with peroxidase-conjugated goat anti-rabbit IgG (Miles) diluted to 1/1000 in TBS containing 1% BSA. After thorough washing for 1 h, the peroxidase activity was visualized with 2,4 dichloro-1-naphthol. Control experiments were performed using the preimmune serum instead of the antiserum.

2.4. *Fluphenazine-affinity column chromatography*

Fluphenazine was coupled to epoxy-activated Sepharose 6B (Pharmacia) in a buffer solution of 0.1 M sodium carbonate (pH 11), 20% (v/v) dioxane. After coupling for 1 h at 4°C, the gel was blocked according to the Pharmacia technical bulletin instructions. Proteins applied to the fluphenazine-Sepharose 6B column pre-equilibrated with a solution of 20 mM Tris-HCl, pH 7.2, 0.1 M KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 0.4 mM DTT. Unbound materials were washed out extensively with the same buffer solution and then the bound protein was successively eluted with 0.5 M KCl and 2 mM EGTA, both dissolved in the buffer solution.

2.5. *Polyacrylamide gel electrophoresis*

SDS-polyacrylamide (15%) slab gels were prepared according to Laemmli [12]. Protein samples were boiled for 4 min in a sample buffer consisting of 2% SDS, 75 mM Tris-HCl, pH 6.8, 10% glycerol and 5% β-mercaptoethanol. The M_r values of proteins were determined using the following marker proteins: rabbit muscle phosphorylase *b* (M_r 95 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 45 000), calf erythrocyte carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), whale sperm myoglobin (M_r 17 800) and lysozyme (M_r 14 400). Gels were stained with 0.025% Coomassie brilliant blue R-250 dissolved in 25% (v/v) isopropanol and 10% (v/v) acetic acid, and destained in 7% acetic acid.

2.6. *Amino acid analysis*

Purified 15 kDa protein was dialyzed extensively against glass-distilled water, lyophilized and hydrolyzed in 6 N HCl in a sealed tube at 105°C for 24 h. The hydrolyzates were analyzed in a Hitachi 835 automatic amino acid analyzer (Hitachi Co. Ltd.).

2.7. *Other methods*

The detection of Ca^{2+}-binding proteins by $^{45}Ca^{2+}$ autoradiography was performed as described in [13]. Calmodulin was purified from sea urchin egg by the method described in [14]. The measurement of phosphodiesterase activity was carried out as described in [15]. Protein concentration was determined according to Lowry et al. [16] using bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

Two methods were used to survey Ca^{2+}-binding proteins present in the sea urchin egg, SDS-PAGE and autoradiography with $^{45}Ca^{2+}$. Using SDS-PAGE, Ca^{2+}-binding proteins, such as calmodulin, can easily be detected by their Ca^{2+}-dependent mobility shifts [17], while autoradiography enables the Ca^{2+}-binding ability of proteins directly [13]. The unfertilized egg extract was run after addition of either 1 mM EGTA or 1 mM Ca^{2+} to the electrophoretic sample buffer, as described in [17]. It was shown that a low molecular mass protein migrated faster in the presence of Ca^{2+} than in its absence (fig.1A, arrowheads). Autoradiography with $^{45}Ca^{2+}$ (fig.1B) revealed that this protein possesses Ca^{2+}-binding activity. Fig.2 shows SDS-PAGE of the low molecular mass protein fractions during the course of the purification. The yield of this protein was 1.1 mg from 800 mg of the crude egg extract.

The apparent molecular mass of the protein was estimated by SDS-PAGE to be 15 kDa in the absence of Ca^{2+}. Therefore, this protein was designated the 15 kDa protein. However, upon gel filtration on Sephadex G-50, the 15 kDa protein was eluted at an apparent molecular mass of 24 kDa.

In SDS-PAGE, the 15 kDa protein showed the mobility change in the presence or absence of Ca^{2+} similar to calmodulin (fig.3). The electrophoretic mobility of the 15 kDa protein either in the absence or presence of Ca^{2+} as compared with that of egg calmodulin of *H. pulcherrimus*. Fig.3 clearly shows that the mobility of the 15 kDa protein

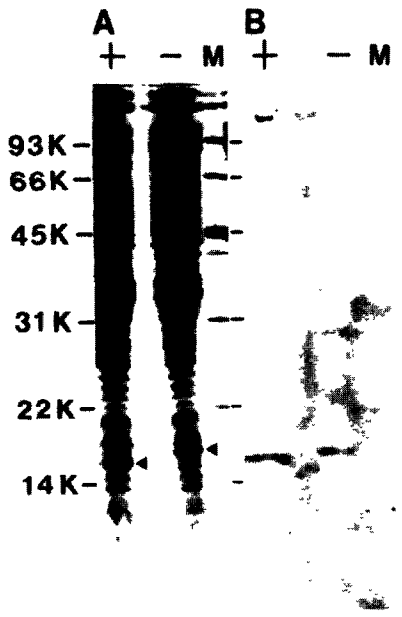


Fig.1. (A) SDS-PAGE (8–17%) of the crude extract. Arrowheads indicate positions of the 15 kDa protein. M, marker proteins; +, in the presence of Ca^{2+}; −, in the absence of Ca^{2+}. (B) Identification of calcium-binding proteins on nitrocellulose membrane after SDS-PAGE. Electrophoresis in a gradient gel was performed as in (A). After gel electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was then exposed to Kodak X-ray film for 18 h. Arrowheads indicate positions of the 15 kDa protein revealed in (A).

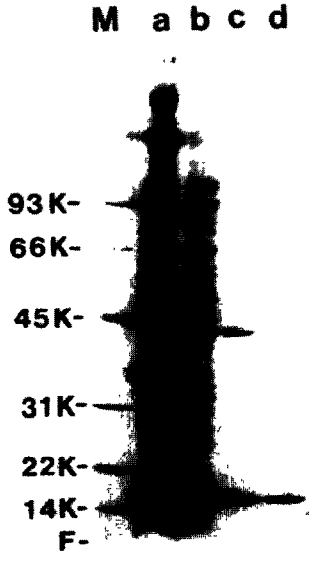


Fig.2. SDS-PAGE of the fractions at each purification step. M, molecular mass markers; a, crude extract; b, DEAE-cellulose fraction; c, hydroxyapatite fraction; d, Sephadex G-50 fraction (purified 15 kDa protein).

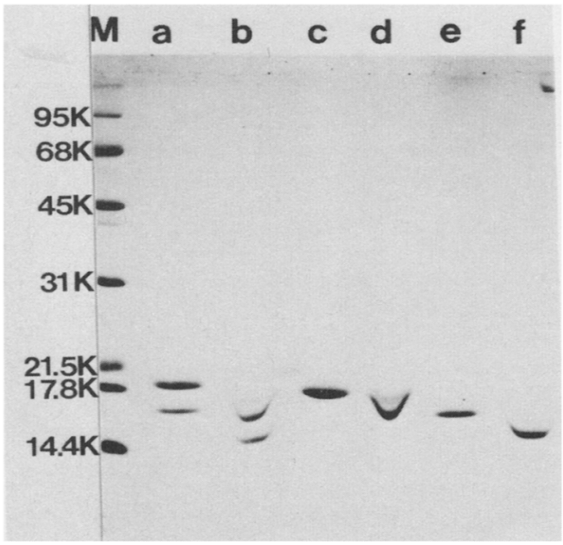
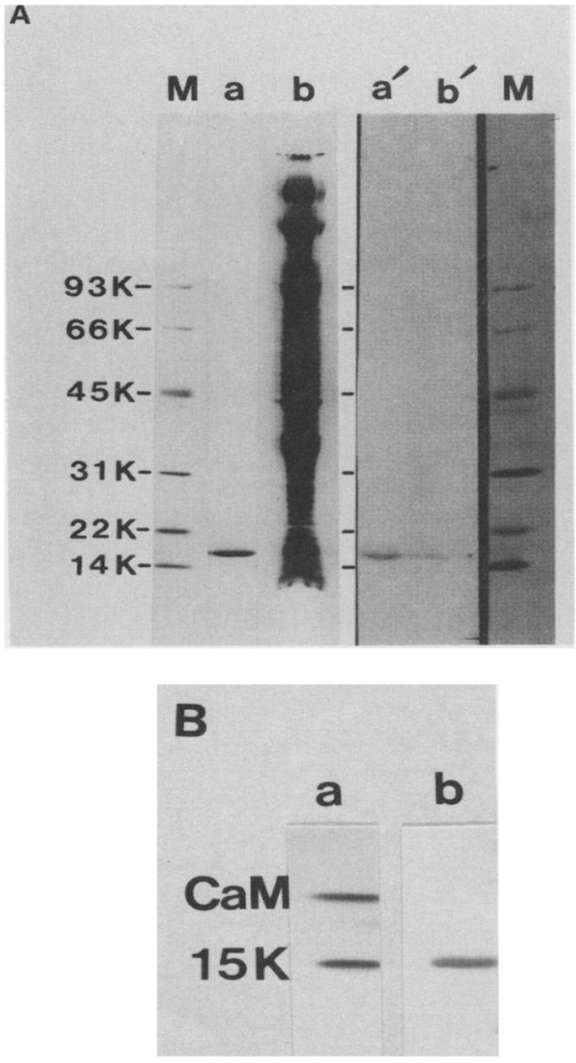


Fig.3. SDS-PAGE of the 15 kDa protein and sea urchin egg calmodulin in the presence or absence of Ca^{2+}. M, molecular mass markers ($\times 10^{-3}$); a and b, 15 kDa protein and calmodulin; c and d, calmodulin; e and f, 15 kDa protein; 1 mM Ca^{2+} was included in the samples a, c and e, while 1 mM EGTA was included in the samples b, d and f. The arrowhead indicates the dye front.

was greater than that of calmodulin both in the absence and presence of Ca^{2+}. Next, antibodies against the 15 kDa protein were raised in rabbit. Egg calmodulin did not react with the antibodies (fig.4), which excluded the possibility that the 15 kDa protein was a degraded fragment of calmodulin.

Fig.4. (A) Immunological reactivity of the 15 kDa protein antiserum to 15 kDa protein (a and a′) and crude extract of sea urchin egg (b and b′) as revealed by immunoblotting. Duplicate samples of each protein were electrophoresed on an 8–17% gradient polyacrylamide gel. One sample of each was stained with Coomassie blue (a and b) and the other sample was electrophoretically transferred to nitrocellulose paper, incubated with 15 kDa protein antiserum, washed and treated with peroxidase-conjugated goat anti-rabbit IgG (a′ and b′). M, marker proteins (stained with Coomassie blue). No reaction was observed when the blotted 15 kDa protein and calmodulin were treated with preimmune serum and 15 kDa protein antiserum, respectively (not shown). (B) Immunological reactivity of the 15 kDa protein antiserum to egg calmodulin. Coomassie blue-stained gel (lane a) contains the sea urchin egg calmodulin and

Purified 15 kDa protein did not activate brain cAMP-phosphodiesterase either in the presence or absence of Ca^{2+} (fig.5). Moreover, unlike egg calmodulin, the 15 kDa protein did not bind to fluphenazine-Sepharose 6B in the presence of Ca^{2+} (not shown). The amino acid composition of the 15 kDa protein is given in table 1 along with the published compositions of calmodulin and other Ca^{2+}-binding proteins. The 15 kDa protein contained less acidic amino acids than other proteins of the calmodulin family (table 1).



15 kDa protein, shown paired with corresponding immunoblot (lane b). The antiserum against 15 kDa protein shows no reactivity to calmodulin.

Table 1

Amino acid composition of 15 kDa protein and other Ca^{2+}-binding proteins

(mol/100 mol)	15 kDa protein	Sea urchin egg calmodulin[a]	TCBP-10[b]	S-100 protein (PAP I-b)[c]
Asx	12.4	14.3	13.9	10.1
Thr	5.2	7.0	7.5	3.4
Ser	7.8	5.8	6.5	5.6
Glx	12.4	16.3	16.1	21.3
Pro	3.3	2.2	5.4	0
Gly	7.3	10.7	8.6	5.0
Ala	7.5	7.9	8.6	5.6
Cys	0.7	0	1.1	2.2
Val	5.6	5.4	5.4	6.7
Met	3.3	4.7	0	3.4
Ile	6.3	4.8	4.3	5.0
Leu	7.7	6.1	5.4	9.0
Tyr	2.1	0.9	2.2	1.1
Phe	4.6	5.0	2.2	7.9
Lys	8.3	4.3	1.1	9.0
His	1.9	1.0	8.6	5.6
Arg	3.6	3.6	2.2	1.1
Trp	—	—	1.1	0

[a] From [6]
[b] From [23]
[c] From [22]

In higher organisms, several Ca^{2+}-binding proteins have been categorized in the calmodulin family [18]. This family includes calmodulin, troponin C, vitamin D-dependent Ca^{2+}-binding protein, S-100 protein (PAP I-b) [19], *Tetrahymena* Ca^{2+}-binding protein (TCBP-10) [20], myosin regulatory light chain, oncomodulin [21], parvalbumin, and rat skin parvalbumin-like Ca^{2+}-binding protein [22].

Carpenter et al. [23] have recently reported the isolation of proteins belonging to the troponin C superfamily present in sea urchin embryos. These proteins are slightly acidic and have molecular masses ranging from 14 to 17 kDa. The amino acid composition of egg 15 kDa protein is different from those of these proteins (see [23] fig.5). It is, however, not yet clear whether the 15 kDa protein is related to these proteins.

In this paper, we demonstrated for the first time that the sea urchin egg contains two kinds of Ca^{2+}-binding proteins, 15 kDa protein and calmodulin, in considerable amounts. The occurrence of the 15 kDa protein might provide an important clue in understanding the mechanism of Ca^{2+}-dependent phenomena in eggs which are difficult to explain simply by the action of calmodulin. We are currently investigating the role of this protein in the egg.

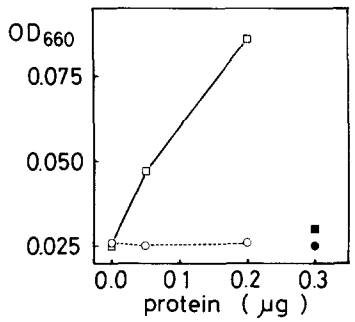


Fig.5. Effects of calmodulin and 15 kDa protein on porcine brain cAMP-phosphodiesterase activity. Brain phosphodiesterase (110 μg) assayed at 15 μg cAMP in the presence (0.1 mM $CaCl_2$; □,■) or absence (0.1 mM EGTA; ○,●) of calcium; (■,●) 15 kDa protein; (□,○) egg calmodulin. The values of the vertical axis are expressed as A_{660}.

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