Calmodulin from *Pharbitis nil*: purification and characterization

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Abstract

A protein, identifiable as calmodulin (CaM), has been isolated from the seedling tissue of *Pharbitis nil*. The method has been developed to isolate a high quality protein from plant tissue containing the high content of polyphenols. This protein was relatively heat-stable and bound to hydrophobic resin in calcium-dependent manner. It was recognized by the antibody against pea and carrot, but did not bind to antibody against *Dictyostelium discoideum.* This protein had M_r of 15 kDa and 18.5 kDa in the presence and absence of Ca^{2+}, respectively, and was able to stimulate calmodulin-deficient cAMP phosphodiesterase. Based on its migration on SDS-PAGE gels, M_r and binding to anti-CaM antibodies it was deduced that calmodulin from *P. nil* is essentially identical to calmodulin isolated from other plants.

Additional key words: Ca^{2+}, cAMP phosphodiesterase, relative molecular mass, SDS-PAGE.

Introduction

Calcium is very important in the regulation of many plant functions, but the mechanisms by which Ca^{2+} controls these processes are only beginning to be understood. Transient Ca^{2+}, increase in the cytoplasm in response to signals, is sensed by an array of Ca^{2+} sensors, which decode calcium signal (Snedden and Form 2001). Calmodulin (CaM) is a highly conserved and widely distributed multifunctional Ca^{2+}-binding protein commonly found in eukaryotes that fulfils a fundamental role in Ca^{2+}-dependent processes in the cell (Marmé and Dieter 1983, Zielinski 1998, Snedden and Form 2001).

Calmodulin has been isolated and characterized from several higher plants including peanut seeds, barley shoots and roots, pea and spinach leaves (Watterson *et al.* 1980, Dubery and Schabort 1987, Jablonsky *et al.* 1991). These proteins appear functionally similar to calmodulins from animal tissue, but differences have emerged upon comparison of primary structure and physico-chemical properties (Marmé and Dieter 1983).

Some evidence that Ca^{2+} and calmodulin are involved in the control of flowering induction process was published (Friedman *et al.* 1989, Tretyn *et al.* 1994, 1997, Kopcewicz and Tretyn 1998, Jaworski *et al*. 2003). However, the mechanism by which calcium regulates this process has still been poor understood.

As part of a detailed study of the structure and function of calcium-binding protein, we have isolated and characterized CaM from *Pharbitis nil*, a model plant in photoperiodic flower induction studies and compare it to calmodulin from other species. The data reported here will be used for future studies of Ca^{2+} or Ca^{2+}/CaM dependent protein kinases and their endogenous substrates involved in the flowering induction.

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Abbreviations: CaM - calmodulin; cAMP - adenosine 3′5′-cyclic monophosphate; EDTA - ethylenediaminetetraacetic acid; MOPS - 3-morpholinopropanesulphonic acid; PMSF - phenylmethanesulphonyl fluoride.

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Materials and methods

Plants and chemicals: Seeds of *Pharbitis nil* Choisy cv. Violet (*Marutanne Seed Co*., Kyoto, Japan) were stirred with concentrated H_2SO_4 for 50 min, washed thoroughly in running tap water and imbibed overnight in sterile water (25 ºC). The swollen seeds were raised in plastic pots containing vermiculite and sand (2:1) and grown for 5 d in the darkness.

Bovine brain calmodulin, *DEAE-cellulose*, *Phenyl-Sepharose CL-4B*, ATP, bovine serum albumin, phenylmethanesulphonyl fluoride (PMSF), aprotinin, leupeptin, cAMP, deaminase, phosphodiesterase, monoclonal antibodies against CaM from *Dictyostelium discoideum*, anti-mouse Ig-antibodies coupled to alkaline phosphatase, calibration proteins for sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) (SDS6 range 14.3 to 66 kDa) were purchased from *Sigma* (USA). *Sephadex G-25* was from *Serva* (Germany).

Purification: Calmodulin was purified by methods described by Jablonsky *et al.* (1991) and Goplalakrishna and Anderson (1982) with needed modifications. Etiolated plants (1 kg) were homogenized at 4 ºC in 1.5 dm^3 buffer containing 50 mM Tris-HCl (pH 7,2), 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM PMSF, 0.1 M KCl, 5 μg cm^{-3} leupeptin, 5 μg cm^{-3} aprotynin and 14 mM β-merkapto-ethanol. To this crude homogenate 4 % (m/v) polyvinyl-pyrrolidone (PVP) was added and then, it was centrifuged at 6 800 *g* for 30 min. The crude supernatant fraction was brought to 55 % saturation with $(NH_4)_2SO_4$. After centrifugation at 6 800 *g* for 30 min pH of the supernatant was adjusted to 4.2 with acetic acid and stirred for 1 h at 4 ºC. The pellet after 30 min centrifugation at 10 000 *g* was resuspended in TKM buffer (50 mM Tris-HCl, pH 7.5, 0.1 M KCl, 10 mM β-merkaptoethanol) and pH was increased to 7.5 using Tris base. During desalting on a *Sephadex G-25* column the conductivity was measured on *Microcomputer Conductivity Meter* (*CC-315*, *Elmetron*, Poland). Protein fractions were heated to 85 ºC for 3 min, cooled on ice, centrifuged at 10 000 *g* for 15 min and supernatant was applied to *DEAE-Sephacel* column equilibrated in TKM buffer. Protein fractions were eluted with linear gradient of 0.1 - 0.75 M KCl, conductance was measured and proteins were checked by SDS-PAGE. These of them, which contained calmodulin, were collected, adjusted to 6 mM $CaCl_2$ using 1 M $CaCl_2$ and 1 M Tris base to maintain the pH at 7.5. The pool after DEAE was loaded on *Phenyl-Sepharose* column equilibrated with a buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM $CaCl_2$. The column was washed with 3 column volumes of equilibration buffer, followed by one column volume in addition of 0.5 M NaCl. Calmodulin was eluted from this resin with a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EGTA. Fractions with CaM were collected, dialysed against H_2O and lyophilised. Then resuspended in 50 mM Tris-HCl, pH 7.5 and stored at -20 ºC.

Protein concentration was measured by method of Bradford (1976), with bovine serum albumin as a standard.

SDS gel electrophoresis and immunoblotting: Electrophoresis in 15 % acrylamide gel was performed as described by Laemmli (1970). For purification and mobility analysis an electrophoresis was done in the presence of 5 mM $CaCl_2$ or 5 mM EDTA. Immunoblotting was carried out according to method of Jablonsky *et al*. (1991). Proteins were transferred to nitrocellulose by semi-dry system (*BioRad*, USA) using 25 mM Tris, 192 mM glycine and 20 % (v/v) methanol (pH 8.3). Nitrocellulose was blocked with phosphate buffer (PBS) (10 mM sodium phosphate, pH 7.4, 150 mM NaCl) containing 1 % non-fat milk powder, and rinsed 1 h with primary monoclonal antibodies against CaM from pea (1:30), carrot (1:2) and *Dictyostelium discoideum* (1:300) diluted with PBS containing 1 % non-fat milk powder, respectively. After washing three times in PBS membrane was incubated for 1 - 2 h with alkaline-phosphatase-conjugated goat antibodies to mouse immunoglobulin G (IgG), washed and stained for alkaline phosphatase according to the manufacturer's instructions (*Protoblot kit*; *Sigma*). Rinsing the blots in water terminated the reaction.

"*Stains-all*" staining of Ca^{2+}-binding proteins: Staining with cationic carbocyanine dye "*Stains-all*" was carried out as described Campbell *et al*. (1983) with the following modifications. After electrophoresis gel was stained in Coomassie, destained in destaining solution (30 % methanol and 10 % acetic acid) and exhaustively washed in water until pH 7.0. Then the gel was stained in 200 cm^3 solution containing 5 mg powder "*Stains-all*", 50 cm^3 isopropanol and 15 cm^3 formamide. Staining was carried out in the darkness for at least 24 h.

Assay of phosphodiesterase activity: Biological activity of calmodulin samples was estimated by their ability to promote activity of phosphodiesterase (PDE). The activity was monitored by spectrophotometric method of Dedmen and Means (1977) with some modifications. Assay was performed in a total volume of 0.5 cm^3 buffer solution containing 50 mM 3-morpholinopropane-sulphonic acid (MOPS) (pH 7.2), 100 mM KCl, 3 mM Mg-acetate, 10 mM β-merkaptoethanol, 0.1 mM $CaCl_2$, 5 μg 5'AMP deaminase, 0.025 U PDE and 0.675 μg purified calmodulin from *Pharbitis nil* or 0.675 μg CaM from bovine brain. Reaction was initiated by the addition of cAMP to the final concentration 0.2 mM and

conducted during 60 min. Measurement was performed every 5 min in a quartz cuvette at wavelength 265 nm. PDE activity was assessed by the absorbance decrease of hydrolysis cAMP to 5'AMP. Concentration of cAMP was calculated from calibration curve.

Results

CaM from *Pharbitis nil* seedlings was purified using its stability against heat denaturation, standard fractionation and affinity chromatography procedures. Proteins precipitated by ammonium sulphate were desalted.

Table 1. Purification of calmodulin from *Pharbitis nil* seedlings.

Fraction	V [cm^3]	Protein conc. [mg cm^{-3}]	Total protein [mg]
Homogenate	2300	0.5388	1239.25
CH_3COOH	23	2.3020	52.95
Sephadex G-25	40	0.7822	31.30
DEAE-Sephacel	60	0.2234	13.40
Phenyl-Sepharose	24	0.0302	0.725

Steps of CaM preparations were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 1). It shows the electrophoretic behaviour of CaM from *Pharbitis nil* as compared with molecular mass standards. The protein showed high purity and its M_r was approximately 18.5 kDa. All fractions received after purification were also immunoassayed using the monoclonal antibodies against calmodulin from pea (Fig. 2). Those antibodies were able to detect CaM during all steps of protein purification. The pure calmodulin also cross-reacted with monoclonal antibodies against CaM from carrot and pea (Fig. 3) but did not react with antiserum against CaM from *Dictyostelium discoideum* (data not shown).

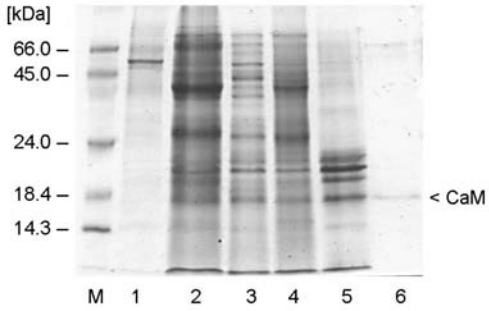

Fig. 1. Analysis of purification pools by SDS-PAGE. Protein from various stage of purification were resolved by electrophoresis in a 15 % polyacrylamide gel in the presence of SDS and stained with Coomassie blue. *Lanes 1 - 6* show the following fractions from each step of the purification procedure: *lane 1* - crude supernatant (6 μg), *lane 2* - CH_3COOH pool (20 μg), *lane 3* - *Sephadex G-25* pools (10 μg), *lane 4* - heated pool (10 μg), *lane 5* - *DEAE-Sephacel* pool (10 μg), *lane 6* - *Phenyl-Sepharose* pool (1 μg). M - molecular mass marker. The *arrow* indicates position of calmodulin.

Proteins fractions were separated on *DEAE-Sephacel* column with a linear salt gradient from 0 to 0.75 M KCl and than protein was eluted from the matrix when salt concentration was 0.1 - 0.3 M. *Phenyl-Sepharose* was used to final CaM purification. The washing step eluted all non-specifically and weakly bound proteins. Proteins specifically bound to this matrix were eluted with EGTA. Purification procedures have allowed to obtain 0.725 mg of CaM. The purity of this protein was approximately

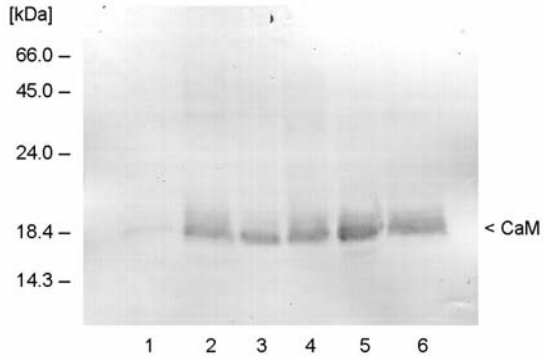

Fig. 2. Analysis of purification pools by Western-blotting. Protein from various stage of purification were resolved by electrophoresis in a 15 % polyacrylamide gel in the presence of SDS and transferred to nitrocellulose, and immmunostained with mixture of monoclonal antibodies specific for CaM from pea. For *lanes 1 - 6* see Fig. 1. The *arrow* indicates position of calmodulin.

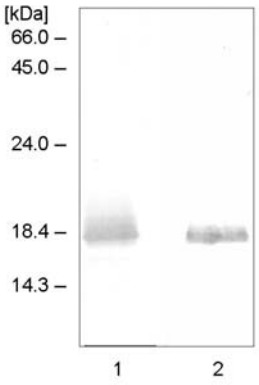

Fig. 3. Immunoblotting of purified calmodulin from *Pharbitis nil*. Calmodulin was resolved by electrophoresis in a 15 % polyacrylamide SDS gel and transferred to nitrocellulose. Immmunostaining was carried out with monoclonal antibodies against CaM from pea (*lane 1*) and from carrot (*lane 2*). Each lane contained 1.5 μg of *Pharbitis nil* calmodulin.

95 % (as determined by gel densitometry). All purification steps are summarized in Table 1.

When CaM from *Pharbitis nil* was analyzed by SDS-PAGE either in presence or absence of Ca^{2+}, a different electrophoretic mobility was found (Fig. 4). The sample analyzed in absence of Ca^{2+} migrated slower, than the sample examined in presence of $CaCl_2$. The calmodulin had an apparent M_r of 15 kDa and 18.5 kDa in the presence and absence of Ca^{2+}, respectively.

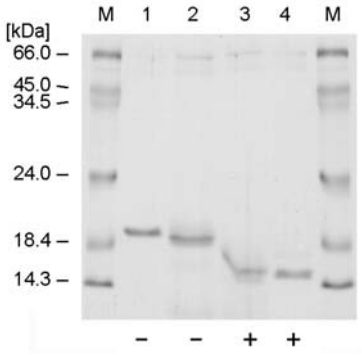

Fig. 4. Electrophoretic analysis of purified calmodulin from *Pharbitis nil* and bovine brain. M - molecular mass marker, *lanes 1, 4* are 3 μg of bovine CaM, *lanes 2, 3* are 1.5 μg of *Pharbitis nil* calmodulin. Samples contained 5 mM $CaCl_2$ (lanes with +) and 5 mM EGTA (lanes with -) and were analyzed by SDS-PAGE in 15 % gel.

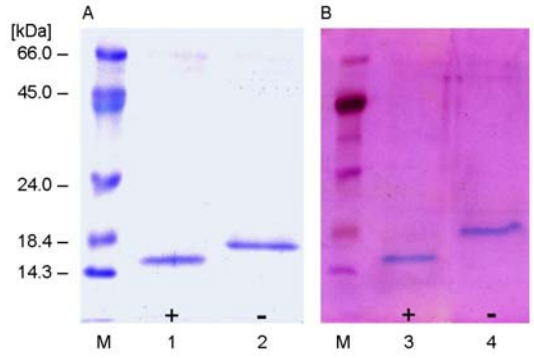

Fig. 5. Coomassie blue and "*Stains-all*" staining of calmodulin. Purified CaM from *Pharbitis nil* was analyzed by SDS-PAGE (15 %) and stained with Coomassie blue (*A*) or "*Stains-all*" (*B*) as described in Materials and methods. Electrophoresis was carried out with 5 mM $CaCl_2$ (*lanes 1* and *3* with +) and 5 mM EGTA (*lanes 2* and *4* with -). Each lane contained 1.5 μg of calmodulin. M - molecular mass marker.

The Ca^{2+}-binding proteins have been shown to stain dark blue or purple with the cationic carbocyanine dye "*Stains-all*", while most proteins stain red or pink (Campbell *et al*. 1983). We have found that purified CaM stains blue with "*Stains-all*" (Fig. 5).

The ability to stimulate calmodulin-dependent cyclic nucleotide phosphodiesterase is one of the properties of CaM (Van Eldik *et al*. 1980). So it was assayed the ability of the *P. nil* calmodulin to stimulate bovin brain phosphodiesterase and compared to that of the homologous bovine brain calmodulin. Calmodulin-affinity PDE showed as much as a 3-fold increase in activity in the presence of saturating amounts of added calmodulin. Essentially no difference was observed between activation of PDE by bovine brain calmodulin or by *Pharbitis nil* calmodulin (Fig. 6).

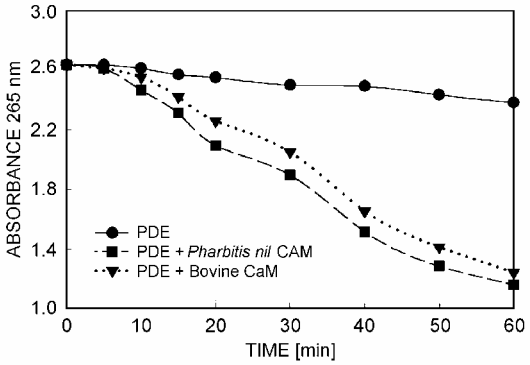

Fig. 6. Activation of cAMP phosphodiesterase by *Pharbitis nil* CaM and bovine brain CaM. PDE assays were conducted for 60 min at 30 ºC. The cAMP hydrolysis was measured in absence and presence of CaM (0.675 μg). When CaM was added the hydrolysis was increased 3-fold compared to control (reaction buffer without CaM). Parameters were determined by averaging two independent assay results.

Discussion

Ca^{2+} ions play fundamental roles in living cells. The major cellular targets of Ca^{2+} in a regulatory role are Ca^{2+}-binding proteins including CaM. Ca^{2+} and some Ca^{2+}-binding proteins have specific regulatory function in light transduction pathway (Neuhaus *et al*. 1993, Millar *et al*. 1994). This is in agreement with the observation of calcium effect on the light-dependent flower induction of short day plant *Pharbitis nil* (Friedman *et al*. 1989, Tretyn *et al*. 1994, Jaworski *et al.* 2003). It has also been shown that some calmodulin inhibitors may affect flower induction (Tretyn *et al*. 1994).

Therefore, purification and biochemical characterization of CaM from *P. nil* seedlings constitute useful starting points to enlarge the knowledge of the regulatory Ca^{2+}-dependent system of the flower induction machinery.

The evidence presented here indicates the presence of the protein species identifiable as calmodulin in *Pharbitis nil* tissue. The procedure of CaM purification is essentially based on its ability to bind to *Phenyl-Sepharose* resin in a Ca^{2+}-dependent manner (Goplalakrishna and Anderson 1982). It has been reported by Gong *et al.* (1993) that after the *Sepharose* affinity column a CaM-binding protein was also collected

in the CaM fractions from *Pinus yunnanensis*. In our CaM preparations we have not identified a similar polypeptide. After last chromatography on *Phenyl-Sepharose* only CaM was observed in gels stained with Coomassie blue so this step was sufficient to complete CaM purification. However, it was found previously that *P. nil* tissues contain highly active proteases and polyphenols (Szmidt-Jaworska *et al.* 2000), which can also rapidly degrade calmodulin during the isolation procedure. Every effort was made to limit this degradation. Isolation procedure was performed quickly at low temperature, and potent protease inhibitors, such as aprotinin and PMSF, and insoluble PVP were included to the isolation media to minimise the activity of proteases.

After purification, *P. nil* CaM was further analyzed by SDS-PAGE. It migrated on SDS-polyacrylamide gels in a fashion identical to calmodulin from other plant species (Anderson *et al*. 1980, Van Eldik *et al*. 1980, Jablonsky *et al*. 1991) and stained blue with "*Stains-all*", like the most of Ca^{2+}-binding proteins (Campbell *et al*. 1983). Only one band was localized on the gel, showing the high purity degree of the isolated protein. Therefore, no CaM isoforms were observed in *P. nil* tissue. Moreover, *P. nil* CaM, like CaM from other plant and animal source, shows a different electrophoretic migration on SDS-PAGE in the presence and absence of Ca^{2+} (Burgess *et al*. 1980).

Using specific antibodies we were allowed to identify CaM in *P. nil* extracts and to test CaM presence during the purification procedure. Antibody specificity was confirmed by experiments performed with bovine brain CaM. The quantitative immunological cross-reactivity of *P. nil* and CaM from other species agrees with previous reports (Chafouleas *et al.* 1979) on the immunological cross-reactivities of calmodulins and calmodulin-like proteins from closely related and distant species.

Furthermore, purified CaM was used for PDE activation assay. The activation of PDE is considered a characteristic property of CaMs (Goplalakrishna and Anderson 1982), therefore we used this assay to compare *P. nil* activity with that of bovine brain CaM. The obtained results allowed us to verify that the protein is physiologically active after the purification procedure.

In conclusion, we believe that the data reported here will help to solute this protein. In addition, purified CaM will be used by us for further studies concerning the role of Ca^{2+} during photoperiodic flower induction of the short day plant.

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