

Purification and Characterization of Calmodulins from *Papaver somniferum* and *Euphorbia lathyris*

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ABSTRACT

Calmodulins (CaM) were isolated and characterized from two well-known latex producing plants, *Papaver somniferum* and *Euphorbia lathyris*. The molecular weights of both were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 17,000 comparable to that of bovine brain CaM. Amino acid compositions also compared similarly with those of known CaMs, with regard to the presence of trimethyllysine and the ratio of phenylalanine to tyrosine. The Cornish-Bowden equation ($S\Delta n$) revealed strong statistical correlations of *P. somniferum* and *E. lathyris* CaM with those of other plants and animals, although their amino acid compositions were not identical. Both plant CaM stimulated CaM dependent cAMP phosphodiesterase: for *Papaver somniferum* the K_a was found to be 1.09 nanomolar and for *Euphorbia lathyris*, 2.01 nanomolar.

A number of researchers have clearly established that CaM¹ is ubiquitous in plant (1, 7, 17, 24, 31) as well as animal systems. Some of the most detailed research concerning plant CaM was conducted by Watterson *et al.* (32) and Van Eldik *et al.* (30) on spinach CaM. The plant CaMs have the ability to activate bovine heart phosphodiesterase albeit at a reduced level to that of animal CaM (10). Further, although plant CaMs differ in amino acid composition from their animal counterparts, the sequence of amino acids is relatively conserved (15, 33). A most significant difference among CaMs from plant and animal sources is that plant CaMs usually contain one residue of cysteine/17 kD. In this study, we report the preparation, characterization and properties of CaM isolated from the leaves of two latex producing plants, *Euphorbia lathyris* and *Papaver somniferum*. Their amino acid compositions are compared with other plant and animal CaMs.

MATERIALS AND METHODS

Materials

Plants of *Euphorbia lathyris* were grown from seed in a greenhouse at 20° to 25°C in clay pots. The photoperiod was 16 h. Leaves were harvested after 6 to 8 months. *Papaver somniferum* was grown from seed in a growth chamber maintained at 20°C and 90% relative humidity with a photoperiod of 4 h; after 1 month the duration of light was extended to 16

¹ Abbreviations: CaM, calmodulin; PDE, phosphodiesterase; TML, ⁶N-trimethyllysine.

h (12). Leaves were harvested just prior to flowering and frozen at -80°C until processed.

Preparation of CaM

Crude CaM from the two plant species was prepared by a modification of the isolation method of Schreiber *et al.* (25). Approximately 500 g of leaves were homogenized in buffer B, pH 6.5, 24 mM NaH₂PO₄, 1 mM EDTA, which had been modified to include 10 mM mercaptoethanol. Following centrifugation at 5°C, the supernatant was heated to 85°C for 5 m, immediately cooled to 4°C, and recentrifuged. The supernatant was chromatographed on DE-52 cellulose, as described by Schreiber *et al.* (25), the CaM fraction was collected, dialyzed salt free and lyophilized. Final purification of CaM was accomplished by hydrophobic interaction chromatography on phenyl Sepharose (Sigma)² according to the procedure of Gopalarkrishna and Anderson (9). Following chromatography, 10 mM CaCl₂ was added to the CaM containing eluate, which was dialyzed salt-free for 4 d and lyophilized.

Amino Acid Analyses

Protein samples were hydrolyzed at 110°C in a forced air oven for 24 h with 4.7 N HCl containing phenol (0.05%) in sealed, evacuated tubes. Analyses were performed in triplicate using a Beckman 119 CL amino acid analyzer. TML was identified by comparison of retention time with a known standard. Similarly, cysteine was identified as half-cystine by comparison of its retention time with a known standard. Data are reported as molar ratios with phenylalanine fixed at seven residues/molecule. Analyses performed by the above method have been described by Thompson *et al.* (28) on bovine CaM preparations.

Protein Analyses

Protein concentrations (micro assay procedure) was determined by the Coomassie method of Bradford (2) using the reagent and protein standard supplied by Bio-Rad.

PDE Activity

PDE assays were performed as described by Butcher (3) except that they were run at room temperature and CaCl₂ (18 μM) and CaM were present. Further, each assay tube was

² Reference to brand or firm name does not constitute endorsement by the United States Department of Agriculture over others of a similar nature not mentioned.

centrifuged to remove turbidity immediately before reading absorbance at 650 nm. Analyses of CaM activation of PDE were carried out by fitting the data with the following equation:

$$R_a = \frac{R_{100}[CaM]}{K_a + [CaM]}$$

where R_a = relative activity of PDE
 R_{100} = activity at 100% activation
 $[CaM]$ = concentration of CaM
 K_a = apparent dissociation constant for one-half activation of PDE

Fitting was done by use of a Gauss-Newton nonlinear regression program, ABCUS, developed by Dr. W. Damert of this center.

PAGE

PAGE and SDS-PAGE were run as described by Thompson *et al.* (27). Molecular weight standards for SDS-PAGE included α -lactalbumin (14,000), β -lactoglobulin (17,000), chymotrypsinogen A (25,000), ovalbumin (43,000) and BSA (67,000).

RESULTS

Figure 1 shows the electrophoretic (SDS-PAGE) behavior of CaMs from *E. lathyris* and *P. somniferum* (lanes 2 and 3) as compared with molecular weight standards. The proteins show high purity, and the mol wt of the plant proteins are approximately 17,000 which is equivalent to that of bovine brain and most CaMs. When SDS-PAGE is performed in the presence of EGTA, each of the proteins undergoes the $Ca^{2+}/$

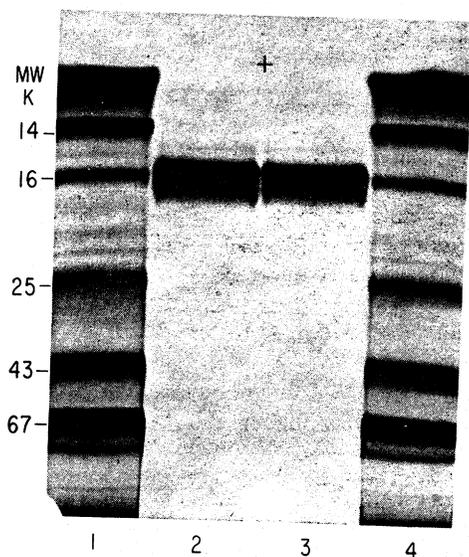


Figure 1. SDS-PAGE of mol wt standards (lanes 1 and 4). Lanes 2 and 3 represent *Euphorbia lathyris* CaM and *Papaver somniferum* CaM, respectively, electrophoresed in the presence of Ca^{2+} . SDS-PAGE was conducted at pH 8.3, Tris-glycine-buffer, 12.5% acrylamide. Protein concentration was approximately 70 μ g/slot in lanes 1 and 4 and 21 μ g/slot in lanes 2 and 3.

EGTA dependent electrophoretic shift which confirms that they bind Ca^{2+} , presumably at the level of 4 g atoms/17 kD (14).

The amino acid compositions of CaM isolated from *P. somniferum* and *E. lathyris* are shown in Table I compared to other plant and animal CaMs. Each of these proteins contains one residue of trimethyllysine and one residue of cysteine as do most plant CaMs. In contrast, bovine brain CaM contains no cysteine. Both *P. somniferum* and *E. lathyris* are higher in proline and lower in methionine and phenylalanine than are their counterparts. However, the ratio of about eight phenylalanine residues to one to two tyrosines, which is quite characteristic of CaMs, is preserved for both proteins. Inspection of the composition of over 300 other proteins (23) showed the ratio of these two residues to be closer to unity in all cases except a hemolysin from *Streptococcus aureus* with a ratio of ten phenylalanines to one tyrosine. In addition, *P. somniferum* is lower in aspartic and glutamic acids than most plant CaMs, but, in that regard, is closer to bovine brain CaM.

We have applied the Cornish-Bowden equation (6) to establish the correlation of *E. lathyris* and *P. somniferum* with other CaMs based upon amino acid composition. If $S\Delta n$ is less than 63.6, based upon the mol wt of 17,000 for CaM, there is a strong correlation between and among the proteins. Although *P. somniferum* and *E. lathyris* show weaker correlations to bovine brain than spinach or *Citrus sinensis* (Table II), they definitely qualify as CaM or CaM-like proteins. Further identification of these CaM with other plant CaMs can only be provided by amino acid sequence data as has been reported for spinach (15) and wheat germ (29).

Since one of the properties of CaM is its ability to activate cAMP phosphodiesterase (4, 5, 11, 30), the apparent K_a (half-maximal activation) was determined for both *P. somniferum* and *E. lathyris* in relation to bovine brain CaM. As shown in Fig. 2, *P. somniferum* CaM strongly activates PDE with a K_a of 1.09 nM. The K_a for bovine brain was 0.60 nM. The activation data of PDE by *E. lathyris* CaM appeared to be best fit by a bimodal curve which yielded two apparent K_a values, one at 0.85 nM and the second at approximately 38.7 nM (Fig. 3). Analysis of the deviation of the data, however, from the fitted curves showed no significant improvement of either the root mean square of the fit or the error of the coefficient (K_a); thus only a single inflection point can be justified. The K_a for *E. lathyris* whole data set was found to be 2.01 ± 0.24 nM. Previous examination of the protein by PAGE and SDS-PAGE (Fig. 1) did not reveal any significant impurities in the CaM preparation. When apo CaM (Ca^{2+} -free) was added at a level of 0.1 μ g/ml, little activation of PDE was observed (Fig. 2 and 3).

Yields of purified CaM were low as expected from plants. *P. somniferum* yielded about 1.2 mg/kg of tissue whereas *E. lathyris* yielded 2.5 mg/kg of tissue.

DISCUSSION

Calmodulin has been clearly demonstrated to be involved in a number of Ca^{2+} -CaM mediated processes in plants (7). For example, it has been shown to be the protein activator of NAD kinase (1, 13, 17) and is strongly implicated in the

Table I. Amino Acid Composition of Calmodulin Isolated from *Euphorbia lathyris* and *Papaver somniferum* as Compared to Calmodulin from Other Plant and Animal Sources

Amino Acid	<i>Papaver somniferum</i>	<i>Euphorbia lathyris</i>	Zucchini (7)	Peanut (1)	Spinach (32)	<i>Citrus sinensis</i> (8)	Bovine Brain (Sequence) (33)
Asp	21	27	26	27	24	26	23
Thr	9	7	9	9	9	8	12
Ser	10	7	5	5	4	5	4
Glu	22	30	26	28	27	26	27
Pro	5	4	2	2	2	2	2
Gly	14	11	10	11	10	11	11
Ala	11	10	11	11	11	10	11
½-Cys	1	1	1	1	1	1	0
Val	8	7	7	6	8	8	7
Met	5	5	7	7	8	7	9
Ile	6	6	7	6	7	7	8
Leu	11	9	12	11	11	11	9
Tyr	2	1-2	1	1	1	1	2
Phe	7	7	9	8	9	8	8
His	2	1	1	1-2	1	1	1
Tml	1	1	1	1	1	1	1
Lys	10	9	10	8	9	8	7
Try	ND	ND	0	0	0	ND	0
Arg	4	4	4	4	5	5	6
Total residues	148	148	147	148	148	146	148

Table II. Statistical Comparisons of Amino Acid Composition of Calmodulins by the $S\Delta n$ Method of Cornish-Bowden^a (6)

	Bovine Brain (Sequence) (33)	Bovine Brain (Commercial) (28)	Bovine Brain (Laboratory) (28)	Bovine Mammary (28)	Rabbit Muscle	Spinach (32)	<i>Papaver somniferum</i>	<i>Euphorbia lathyris</i>
Bovine brain (commercial)	14.1							
Bovine brain (laboratory)	8.8	8.4						
Bovine mammary	4.8	8.0	2.3					
Rabbit muscle	6.4	6.4	5.9	3.8				
Spinach	13.0	23.4	15.7	10.6	12.5			
<i>Papaver somniferum</i>	66.0	39.1	35.5	47.2	41.1	61.2		
<i>Euphorbia lathyris</i>	55.9	69.2	47.0	48.3	51.6	36.4	79.4	
<i>Citrus sinensis</i> (8)	21.7	35.5	24.2	20.3	17.5	6.5	57.3	22.7

^a If $S\Delta n$ is < 63.6, there is a strong correlation; if $S\Delta n$ is > 63.6, but < 141, there is a weak correlation; if $S\Delta n$ is > 141, there is no correlation.

assembly-disassembly of the mitotic apparatus (34). CaM has also been demonstrated to be associated with guard cells (26) and to be a regulator of phospholipase in potato leaves (16). A number of other biological roles for CaM have been described in a recent review by Piazza (19).

In this study we examined CaM isolated from the leaves of *P. somniferum*. No attempt was made to identify and isolate the protein from the capsule latex. However, based upon the research of Nessler et al. (18), it is unlikely that CaM is a constituent of that latex (at least in detectable quantities by SDS-PAGE). Because TML was identified as a constituent of the CaM isolated from the leaves of this plant, we attempted, without success, to identify the presence of CaM in a crude capsule latex protein fraction by analyzing for TML. Overloading the column with a several fold excess of acid hydrolyzed protein (0.5 mg lyophilized latex) failed to reveal a trace

of TML (detection limits: 0.01 nmol TML). Further, we were unable to detect the presence of CaM in the protein fraction of fresh latex (40 μ L) by SDS-PAGE where the detectable limits are 0.02 nmol CaM.

It is noteworthy, however, that Piazza *et al.* (21) observed activity of a CaM-like component in the latex of *E. lathyris* without attempting to concentrate or isolate the protein. Interestingly, the protein could be observed in the PAGE pattern only when the latex extract was treated with urea, mercaptoethanol and EGTA prior to electrophoresis in 4.5 M urea gels. It is not surprising, therefore, that CaM may be involved in triterpene biosynthesis in latex (20) and also appears to inhibit the activity of at least one phosphatase in the latex (22).

Herein we have reported the isolation, characterization and properties of CaM from *E. lathyris* and *P. somniferum*. The

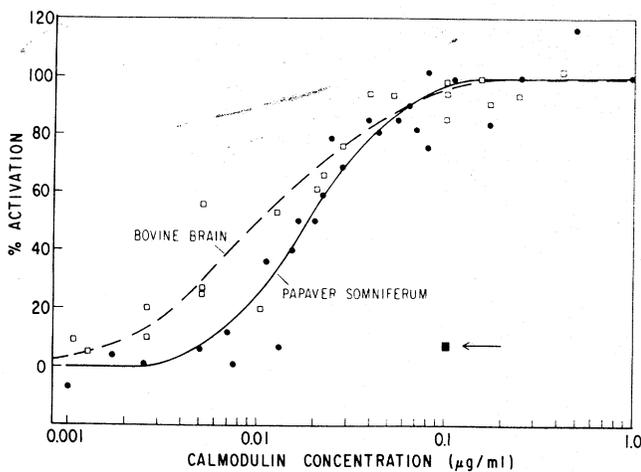


Figure 2. Activation of heart cAMP PDE by *Papaver somniferum* CaM and bovine brain CaM. Phosphodiesterase assays were conducted for 30 min at room temperature. King Cobra venom (0.1 mg) was added in the final 10 min of reaction. The rate of cAMP hydrolysis at a saturating level of CaM was 1.0 $\mu\text{mol/ml}\cdot\text{h}$. When no CaM was added the rate of hydrolysis was reduced 3- to 4-fold. Each data point represents the mean value of three determinations. The lower data point (indicated by arrow) represents the activation of PDE by apo (Ca^{2+} -free) CaM.

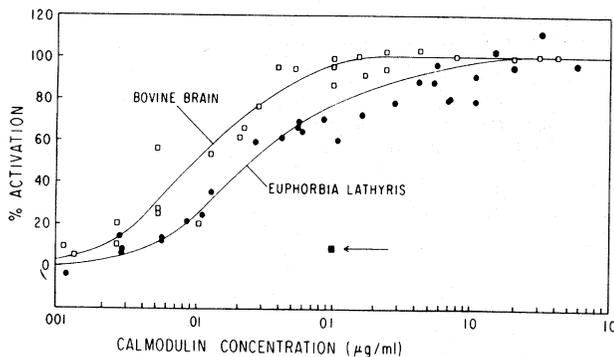


Figure 3. Activation of heart cAMP PDE by *Euphorbia lathyris* CaM and bovine brain CaM. See legend to Figure 2 for details of assay. The lower data point (indicated by arrow) represents the activation of PDE by apo (Ca^{2+} -free) CaM.

isolated proteins are pure and are good activators of PDE. Although their amino acid compositions differ from other characterized plant and animal CaMs, the statistical correlations between them and other CaMs are good. Although a few possible biological role(s) of *E. lathyris* CaM have been reported, the role of CaM in *P. somniferum* biological pathways (for example, alkaloid biosynthesis) is unknown.

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