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CALMODULIN FROM *CITRUS SINENSIS*: PURIFICATION AND CHARACTERIZATION

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Abstract—A protein identifiable as calmodulin has been isolated from the fruit tissue of *Citrus sinensis* (cv. Valencia). This protein is relatively heat-stable, binds to hydrophobic gels in a calcium-dependent fashion and to antibody against rat testis calmodulin, and stimulates calmodulin-deficient cAMP phosphodiesterase. The protein has an isoelectric point of 3.7 and M_rs of 14 500 and 17 000 in the presence and absence of Ca^{2+}, respectively. Based on its migration on SDS-PAGE gels, its UV absorption spectrum and its amino acid composition, calmodulin from citrus fruit is essentially identical to calmodulin isolated from other plant tissues, e.g. leaves and seeds.

INTRODUCTION

Calmodulin 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 [1, 2].

It has been isolated and characterized from several higher plant sources including peanut seeds, barley shoots and roots, zucchini hypocotyls and spinach leaves. These proteins appear functionally similar to calmodulins from animal tissues, but differences have emerged upon comparison of primary structures and physico-chemical properties [2].

Several plant enzymes are known to be under the control of Ca^{2+}-calmodulin (for a review, see ref. [3]), and Ca^{2+}-calmodulin dependent metabolic regulation in higher plants appears to be of extreme importance [4].

RESULTS AND DISCUSSION

Calmodulin from the fruit tissue of *Citrus sinensis* L. (cv. Valencia) was purified using its stability against heat denaturation [5] and its calcium-sensitive hydrophobic binding to phenyl-substituted agarose [6]. The purification procedure is summarized in Table 1. The yield of calmodulin was found to be 12.6 μg/g of fruit tissue. This value compares well with the 10 μg/g of wheat germ, *Triticum aestivum*, the best known source of plant calmodulin. The purified calmodulin appeared as a single band on polyacrylamide gels and SDS-polyacrylamide gels when the electrophoresis was performed in the absence of Ca^{2+} (Fig. 1). When SDS-PAGE was performed in the presence of Ca^{2+}, citrus calmodulin showed another band with an apparent higher M_r, similar to the calmodulins from zucchini [2] and bovine brain [7]. The citrus calmodulin also exhibits the characteristic calcium-dependent shift in electrophoretic mobility and has an apparent M_r of 14 500 and 17 000 in the presence and absence of Ca^{2+}, respectively. These values are identical to the 14 500 and 17 000 of zucchini [2], but are lower than the 16 000 and 19 000 reported for bovine brain calmodulin [8].

The amino acid composition of citrus calmodulin is shown in Table 2, and is very similar to the compositions of the calmodulins isolated from peanut, spinach, barley, corn and zucchini [2]. The similarities include the presence of two proline residues, one residue each of trimethyllysine and cysteine, a high content of aspartic and glutamic acid residues and a lack of tryptophan. The high phenylalanine:tyrosine ratio is reflected in the low extinction coefficient ($E^{1\%}_{276}$ of 0.9) and in the UV absorption spectrum (not shown), with multiple peaks at 251, 257, 263, 267, 276 and 280 nm due to the phenylalanine fine structure [9]. The highly acidic nature of citrus calmodulin is consistent with the determined isoelectric point of pH 3.7 (Fig. 1). Identical pIs were also determined for bovine brain and zucchini calmodulins [2].

The ability of the citrus calmodulin to stimulate beef heart cyclic nucleotide phosphodiesterase was assayed as described in the Experimental. The stimulation of the enzyme was found to be qualitively similar to the stimulation by porcine brain calmodulin, although it was slightly less active in this regard (data not shown). This may be due to structural differences resulting in a lower affinity of citrus calmodulin for bovine heart phosphodiesterase or due to the presence of inactive calmodulin species [2]. Identical reactivity in competition radioimmunoassay was observed for the calmodulins from citrus and bovine brain (data not shown). This, however, appears not to be a criterion for structural identity since differences in amino acid composition and peptide maps were shown to occur between the calmodulins from spinach and bovine brain which exhibits quantitative immunological cross-reactivity [10].

The results presented in this study suggest that calmodulin from immature, developing citrus fruit has similar structural and functional properties to those of calmodulins from other plant (and animal) tissues, indicating the high conservation of this protein during evolution [2, 10].

Table 1. Purification of calmodulin from immature *C. sinensis* fruits*

Step	Volume (ml)	Total protein† (mg)	Total calmodulin‡ (mg)	Yield§
1. Crude extract	200	690		
2. pH 7.0 55% $(NH_4)_2SO_4$ supernatant	226	169		
3. pH 4.3 55% $(NH_4)_2SO_4$ precipitate	7.5	101		
4. Heat-supernatant	6.6	26.4		
5. Phenyl-Sepharose chromatography	47	0.752	0.762	12.6

*Data given are from a representative purification starting with 20 g of acetone powder (60 g fr. wt).
†Determined by the dye binding method of Bradford [13].
‡Determined by radioimmunoassay as described in the Experimental.
§Expressed as mg CaM/kg fr. wt.

The recognition of its highly conserved structure across the plant and animal kingdoms [2, 3, 8–10] suggests that it is likely to act in the mediation of the important regulatory role of Ca^{2+} in fruit development, ripening and senescence (for a review, see ref. [11]).

EXPERIMENTAL

Plant material. Young, immature developing citrus fruits (cv. Valencia) with an average mass of 2 g were used for the isolation of calmodulin.

Purification of calmodulin. Me_2CO powders were prepared from the fruit tissue and stored at – 20°. The procedure removed most of the chlorophyll and phenolic compounds which tend to interfere with the purification steps. All isolation procedures were performed at 0–5°. 20 g of the Me_2CO powder was suspended in 200 ml (1:10 m/v) 0.05 M Tris HCl buffer, pH 7.5, containing 1 mM EGTA, 1 mM β-mercaptoethanol, 0.5 mM phenylmethanesulphonyl fluoride and 2 g Polyclar AT. The suspension was homogenized for 2 min, filtered through cheesecloth, and centrifuged for 10 min at 15000 *g*. The pH of the supernatant was adjusted to 7.0 with 6 M HOAc. Powdered $(NH_4)_2SO_4$ was

Fig. 1. Anodic non-denaturating PAGE (A); SDS-PAGE in the absence and presence of Ca^{2+} (B) and thin-layer isoelectric focusing (C), of calmodulin from developing *C. sinensis* fruit. Experimental conditions are as described in the text.

Table 2. Amino acid compositions of calmodulin isolated from *C. sinensis* fruit and other plant tissues

Amino Acid	*C. sinensis* fruit	Peanut seeds [9]	Spinach leaves [10]	Zucchini hypocotyls [2]
Asp	26	27	26	26
Thr	8	9	7	9
Ser	5	5	7	5
Glu	26	28	27	26
Pro	2	2	2	2
Gly	11	11	10	10
Ala	10	11	10	11
Cys	1	1	n.d.	1
Val	8	6	8	7
Met	7	7	8	7
Ile	7	6	7	7
Leu	11	11	11	12
Tyr	1	1	1	1
Phe	8	8	9	9
His	1	1-2	1	1
TM-Lys	1	1	1	1
Lys	8	8	9	10
Trp	0	0	0	0
Arg	5	4	5	5

n.d., not determined.

added slowly with constant stirring to a saturation of 55% (33 g/100 ml). The soln was stirred for 30 min and then centrifuged for 15 min at 15000 *g* to remove the precipitated protein. The supernatant was adjusted to pH 4.0 and stirred for 30 min to allow the isoelectric precipitation of calmodulin. The ppt. was collected by centrifugation for 15 min at 15000 *g* and dissolved in 0.05 M Tris–HCl buffer, pH 7.5, containing 1 mM β-mercaptoethanol and 0.1 mM $CaCl_2$ (buffer 1). The soln was adjusted to pH 7.5 with Tris base and centrifuged. The supernatant was transferred to a thin-walled glass container in a water bath and heated to 80° for 5 min. After rapid cooling in an ice bath, the heat-treated preparation was centrifuged at 15000 *g* for 15 min. The resulting supernatant, containing the calmodulin, was retained.

Ca^{2+}-induced hydrophobic interaction chromatography, as described in ref. [6], was used as the final purification step; the supernatant from the heat step was adjusted to 5 mM Ca^{2+} and adsorbed onto a phenyl-Sepharose column (1.6 × 16 cm), equilibrated with buffer 1. The column was eluted with 10 bed vols. of buffer 1 before the calmodulin was desorbed with a modified buffer 1 (containing 1 mM EGTA in place of 0.2 mM $CaCl_2$).

Assay of calmodulin. (a) Stimulation of phosphodiesterase. Calmodulin-deficient cyclic nucleotide phosphodiesterase from beef heart was obtained from Boehringer-Mannheim and its calmodulin stimulated activity was assayed according to the manufacturer's instructions, based on the method of Sharma and Wang [12].

(b) Radioimmunoassay. Calmodulin radioimmunoassay kits were obtained from New England Nuclear. These assays were of the competitive-binding type employing ^{125}I-labelled calmodulin and affinity purified sheep antibody against rat testis calmodulin. The kits were used according to the enclosed instructions with bovine brain calmodulin as a standard. 100% cross reactivity has been observed with soybean calmodulin.

Protein concns were routinely determined by the dye binding method of Bradford [13], using bovine γ-globulin as a standard.

Amino acid analysis. Amino acid analyses were performed as previously described [14], using a Waters HPLC Amino Acid Analyzer System with post-column derivitization with σ-phthalaldehyde as fluorogenic agent.

Electrophoresis. PAGE was performed in rods under non-denaturating conditions according to a standard procedure described by Gabriel [15] (10% gel, pH 8.3/8.9 Tris–borate/Tris chloride, 5 mA/gel). SDS-PAGE of calmodulin samples was performed according to the method of Laemmli [16] on 15% acrylamide gels containing 1 mM EGTA or 1 mM $CaCl_2$. The following standard proteins were used for the determination of the M_r of calmodulin: lactate dehydrogenase subunit (M_r 36500), carbonic acid anhydrase subunit (M_r 31000), tripsin inhibitor (M_r 20100), porcine brain calmodulin (M_r 16700), lysozyme (M_r 14400) and cytochrome *c* (M_r 12500). Gels were fixed and stained with Coomassie Blue G250 for visualization of the protein [17].

Isoelectric focusing (IEF). Thin-layer IEF of the purified calmodulin was performed with a pH 3–10 gradient (Pharmalyte, Pharmacia; 3000 V, 150 mA, 50 W, 1.5 hr, 10 cm between electrodes) and the isoelectric point was determined from a calibration curve using the Pharmacia IEF calibration kit for isoelectric point determinations of proteins in the range pH 3–10.

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