Volume 234, number 2, 431-434 FEB 06094 July 1988

Purification and partial characterization of a calmodulin-like protein from cell suspension cultures of *Catharanthus roseus*

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Received 24 May 1988

A calmodulin-like protein was isolated from suspension cultured cells of *Catharanthus roseus* and purified by a combination of Ca^{2+}-facilitated phenyl-Sepharose affinity chromatography and reverse-phase HPLC. The HPLC-purified protein was analysed using SDS-PAGE and found to be a homogeneous 19.5 kDa band in gels containing 1 mM EGTA unlike a higher plant calmodulin from spinach which migrated as a 17.38 kDa band. Despite this apparent difference in molecular mass, the purified protein showed a similar increase in electrophoretic mobility (4010 Da) to spinach calmodulin (4200 Da) in gels containing 1 mM Ca^{2+} and had a plant calmodulin-like UV spectrum and phosphodiesterase-activation profile.

Calmodulin; Phosphodiesterase; Enzyme activator; Protein purification; Cell suspension; (*Cantharanthus roseus*)

1. INTRODUCTION

Calmodulin is now considered to be a ubiquitous Ca^{2+} modulator protein in all higher plant and algal cells. However, there are no published accounts describing its purification from cultured, undifferentiated plant cells and subsequent identification in vitro by its Ca^{2+}-induced shift in electrophoretic mobility [1] in combination with its ability to activate cAMP phosphodiesterase (EC 3.1.4.17) and other known calmodulin-dependent enzymes according to Cheung's original definition of the protein [2,3]. These strict criteria must be applied to establish the presence of calmodulin in cultured plant cells not only due to their physiologically de-differentiated state, but also due to the recent discovery of calmodulin-like proteins in higher plants and algae; these migrate with similar molecular masses to plant and animal calmodulins in SDS gels and can cross-react with calmodulin antibodies but cannot stimulate the activity of brain cAMP phosphodiesterase [4,5].

In cell suspensions of *Catharanthus roseus*, calmidazolium has been shown to inhibit the functioning of the plasma membrane redox pump system, suggesting a role for Ca^{2+}-calmodulin in regulating the membrane potential [6]. We have found that application of the Ca^{2+} channel blocker verapamil and the calmodulin antagonist pimozide can stimulate secondary metabolism in *C. roseus* cell suspensions as detected by increased alkaloid accumulation (unpublished), suggesting that calmodulin-like proteins whose functioning can be perturbed are present in these cultures.

We report here on the isolation and purification of a calmodulin-like cAMP phosphodiesterase activator protein from extracts of *C. roseus* cell suspension cultures. The purified protein exhibited plant calmodulin-like characteristics but had a higher molecular mass, as determined by SDS-

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Abbreviations: K_I, half-inhibition constant; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulphonyl fluoride; PVPP, polyvinylpolypyrollidone; nkat, nanokatal; PDE, phosphodiesterase; HPLC, high-performance liquid chromatography

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

PAGE, than a plant calmodulin from spinach used as a standard.

2. MATERIALS AND METHODS

2.1. *Equipment and biochemicals*

All chemicals were of analytical grade. Purified spinach calmodulin, activator-deficient cAMP phosphodiesterase and phenyl-Sepharose CL-4B were purchased from Sigma.

2.2. *Cell culture*

Undifferentiated cell suspensions of *C. roseus* (cell line LBE-1, derived from anther tissue) were grown using inorganic salts and B-complex vitamins according to Murashige and Skoog [7], 100 mg/l glycine, 100 mg/l *myo*-inositol, 30 g/l sucrose, 2.8 μM α-NAA and 1 μM kinetin (120 rpm, 27°C).

2.3. *Calmodulin-like protein isolation*

C. roseus cells were homogenized by sonic disruption in 100 mM Tris-HCl (pH 8.0), 2.5 mM EDTA, 2.5 mM EGTA, 0.2 mM DTT, 1 M KCl, 0.5 mM PMSF (2 ml/g fresh wt) with 5% (w/v) PVPP. The acidic proteins were precipitated from this homogenate with ammonium sulphate and heat treated according to [8]. The heat-stable proteins (HSPs) were dialyzed vs 20 mM Tris-HCl (pH 7.5), 0.2 mM DTT.

2.4. *Calmodulin-like protein purification*

The HSP fraction was made 2 mM with respect to $CaCl_2$ and applied to a 14 ml bed volume column of phenyl-Sepharose CL-4B. The column was washed with 10 bed volumes of 25 mM Tris-HCl (pH 7.5), 0.2 mM DTT, 2 mM $CaCl_2$ followed by 10 bed volumes of buffer containing 0.5 M NaCl instead of $CaCl_2$. The calmodulin-like protein peak was then eluted with 25 mM Tris-HCl, 0.2 mM DTT, 4 mM EGTA. The calmodulin-containing fractions were identified using their ability to activate cAMP phosphodiesterase, dialyzed vs distilled water, 0.2 mM DTT and freeze-dried.

Final purification was achieved using reverse-phase HPLC with a 0.46×22 cm RP-8 Spheri-5 C_{18} column (Brownlee, Santa Clara, CA). The proteins were applied with 10 mM sodium phosphate buffer (pH 6.2), 2 mM EGTA and eluted with a linear gradient of 5–50% acetonitrile in the same buffer at a flow rate of 1 ml/min using a Hewlett Packard 1090 liquid chromatograph. Absorbance (at 230 and 215 nm) and UV spectra were monitored with a diode-array spectrophotometer.

2.5. *Protein electrophoresis*

Discontinuous SDS-PAGE [9] was performed according to Burgess et al. [1] in a 14% resolving gel with a 7% stacking gel containing 1 mM $CaCl_2$ or 1 mM EGTA. The fractions and a spinach calmodulin standard were electrophoresed at 5 mA/gel. Proteins were stained with silver nitrate according to [10].

2.6. *Phosphodiesterase assays*

The assay solution (1 ml) consisted of 0.02 U activator-deficient bovine brain cAMP phosphodiesterase, 0.1 mg/ml ovalbumin, 0.2 mM $CaCl_2$, 4 mM $MgSO_4$, 0.2 mM DTT, 2 mM cAMP and various amounts of the protein fractions in 40 mM Tris-HCl (pH 7.5) buffer as in [11]. P_i was assayed as described by Fiske and SubbaRow [12].

2.7. *Protein determination*

Protein concentrations were determined according to Bradford [13] with ovalbumin as standard and with HPLC using known amounts of spinach calmodulin.

3. RESULTS AND DISCUSSION

3.1. *Detection of calmodulin-like activity*

The calmodulin-like protein from *C. roseus* cell

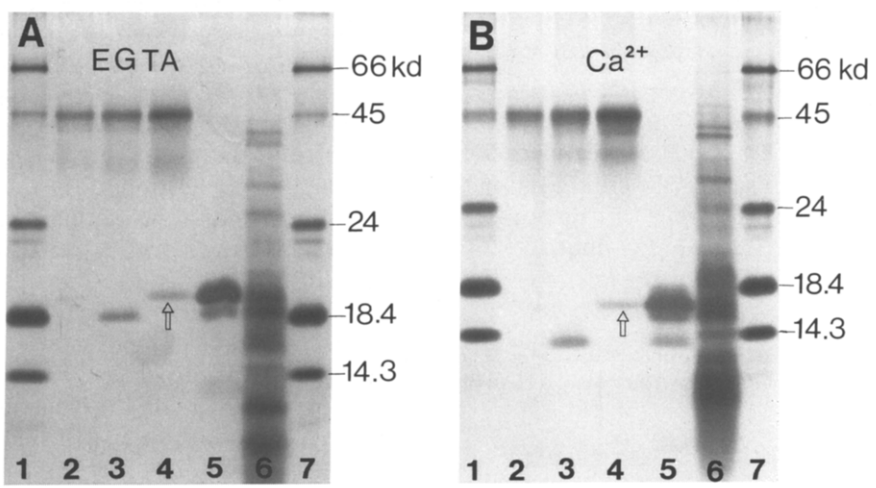

Fig.1. SDS-PAGE of protein fractions prepared from *C. roseus* cell suspension cultures in the presence of 1 mM EGTA (A) or 1 mM $CaCl_2$ (B) in 14% gels. Lanes: (1,7) molecular mass markers as indicated (250 ng each); (2) ovalbumin alone; (3,4) HPLC-purified spinach calmodulin (500 ng) and HPLC-purified calmodulin-like protein from *C. roseus* cells (500 ng), respectively, both spiked with ovalbumin; (5) phenyl-Sepharose column EGTA eluted peak (1000 ng load); (6) original HSP fractions (5000 ng load).

suspension cultures was first detected in the heat-stable fraction (HSP) with SDS-PAGE (fig.1) as a protein band exhibiting enhanced mobility in the Ca^{2+}-containing gels. The HSP fraction was found to enhance bovine brain cAMP phosphodiesterase activity 4-fold relative to the basal Ca^{2+}-dependent level at saturation. This is typical of calmodulin from all animal and most plant sources [14]. The presence of a calmodulin-like protein was further confirmed by the inhibitory effects of trifluoperazine and EGTA on activator-dependent enzyme activity (not shown). The decrease in enzyme activity with trifluoperazine exhibited log-dose-dependent inhibition with an apparent K_i of 3 μM. The drug had no effect on the Ca^{2+}-dependent, calmodulin-independent basal activity of the enzyme. The K_i found for EGTA (0.2 mM) corresponded to the original unbuffered Ca^{2+} concentration present in the assay solution and established the Ca^{2+} dependence of the calmodulin-like activator fraction.

3.2. *Purification of the calmodulin-like protein*

The calmodulin-like protein was purified from the HSP fraction using phenyl-Sepharose chromatography followed by reverse-phase HPLC.

Calmodulin undergoes a marked increase in hydrophobicity after binding Ca^{2+} and this has been utilized for the purification of the protein from semi-purified extracts by first adsorbing it to phenyl-Sepharose with buffer containing Ca^{2+} and then eluting it with EGTA [15]. In our case, the phenyl-Sepharose chromatography step (fig.2) co-purified a single dominant calmodulin-like band with a lower molecular mass band after SDS-PAGE (fig.1). The densely stained upper band migrated as a band of 19500 Da with EGTA and showed altered mobility in the presence of Ca^{2+} (15490 Da) as originally detected in the HSP fraction. This accounted for an increase in mobility of 4010 Da, similar to the value of 4200 Da for the increase for spinach calmodulin (fig.1) and is in agreement with Ca^{2+}-induced mobility shifts found with other higher plant calmodulins [8,16]. The apparent molecular mass of the protein in the presence or absence of Ca^{2+} was higher than what is typically found for plant calmodulins such as the spinach calmodulin used here. Native gel electrophoresis, amino acid analysis, Ca^{2+}-binding and CD-spectral studies will confirm whether the purified protein is indeed a higher molecular mass form of plant calmodulin.

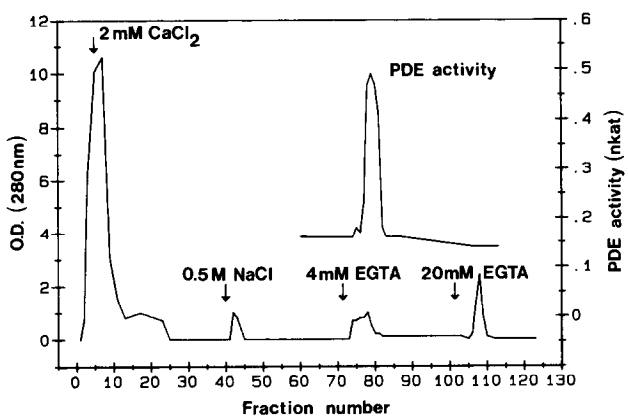

Fig.2. Ca^{2+}-facilitated hydrophobic-interaction chromatography of the HSP fraction using phenyl-Sepharose. 17 mg HSP fraction was loaded and the column treated with successive buffer washes as indicated. Absorbance is expressed in relative units.

Final purification was achieved by reverse-phase HPLC to remove other lower molecular mass contaminants detected after electrophoresis of loads greater than 1 μg. Spinach calmodulin was also further purified by HPLC using the same column and elution conditions.

The calmodulin-like protein had a lower retention time than spinach calmodulin and was eluted with 36% acetonitrile. The HPLC peak fractions were dialyzed vs 10 mM Tris-HCl (pH 7.5) and freeze-dried. Only a small amount of calmodulin-like protein (45 μg) was recovered after HPLC, since various gradient rate trials were required before the one yielding maximal resolution was found. As a result, aliquots of freeze-dried purified protein were purposely spiked with ovalbumin (0.1 mg/ml) after dissolution to minimize loss from adsorption to container walls. The appearance of a 45 kDa band in the spinach and *C. roseus* cell suspension culture calmodulin lanes (fig.1) is the result of this treatment and also demonstrated that the increased mobility of the calmodulin-like protein in Ca^{2+}-containing gels was not due to differences in gel consistency in those lanes.

The final purified protein from *C. roseus* cell suspension cultures was found to have a plant calmodulin-like UV spectrum (not shown) and

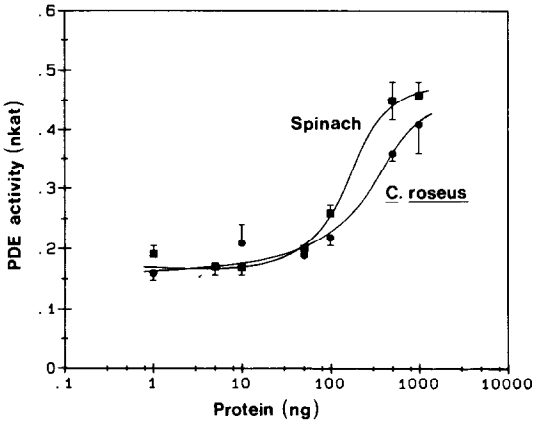

Fig.3. Stimulation of activator-deficient cAMP phosphodiesterase by various amounts of HPLC-purified spinach calmodulin and calmodulin-like protein from *C. roseus* cell suspensions. Points are means of triplicate enzyme assays; bars indicate SD. Standard deviations are within the dot radius for some points.

phosphodiesterase-activation profile (fig.3). Its UV spectrum in the presence of 2 mM EGTA monitored during HPLC was very similar to that of spinach calmodulin under these elution conditions as well as other higher plant calmodulins in other buffer systems [17].

In summary, suspension cultured cells of *C. roseus* maintained in an undifferentiated state contain a calmodulin-like protein which may be a higher molecular mass form of plant calmodulin with a plant calmodulin-like UV spectrum and Ca^{2+}-dependent phosphodiesterase stimulatory activity in vitro. Further characterization of the protein, including determination of whether it is able to activate NAD^+ kinase, is presently being carried out in our laboratory.

Acknowledgements: We thank Dr Hiroyuki Aoki for his help and advice during the HPLC purification work. This work was supported by a Natural Science and Engineering Research Council of Canada operating grant given to F. DiC.

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