Extracellular calmodulin-binding proteins in plants: purification of a 21-kDa calmodulin-binding protein

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Abstract. A 21-kDa calmodulin (CaM)-binding protein and a 19-kDa calmodulin-binding protein were detected in 0.1 M CaC\textsubscript{2} extracts of Angelica dahurica L. suspension-cultured cells and carrot (Daucus carota L.) suspension-cultured cells, respectively, using a biotinylated cauliflower CaM gel-overlay technique in the presence of 1 mM Ca\textsuperscript{2+}. No bands, or very weak bands, were shown on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels overlayed with biotinylated cauliflower CaM when 1 mM Ca\textsuperscript{2+} was replaced by 5 mM EGTA, indicating that the binding of these two CaM-binding proteins to CaM was dependent on Ca\textsuperscript{2+}. Less 21-kDa CaM-binding protein was found in culture medium of Angelica dahurica suspension cells; however, a 21-kDa protein was abundant in the cell wall. We believe that the 21-kDa CaM-binding protein is mainly in the cell wall of Angelica dahurica. Based on its reaction with periodic acid-Schiff (PAS) reagent, this 21-kDa protein would appear to be a glycoprotein. The 21-kDa CaM-binding protein was purified by a procedure including Sephadex G-100 gel filtration and CM-Sepharose cation-exchange column chromatography. The purity reached 91% according to gel scanning. The purified 21-kDa CaM-binding protein inhibited the activity of CaM-dependent NAD kinase and the degree of inhibition increased with augmentation of the 21-kDa protein, which appeared to be the typical characteristic of CaM-binding protein.

Key words: Angelica – Calmodulin-binding protein – Extracellular glycoprotein

Introduction

In recent years, calmodulin (CaM), an important constituent of cellular signal- transduction systems, has also been found extracellularly and may therefore have some function outside the cell. In plant systems, Biro et al. (1984) first detected CaM in oat coleoptile cell walls by radioimmunoassay. In our laboratories, a series of experiments also indicated the existence of extracellular CaM; these included purification of extracellular CaM from wheat coleoptile cell walls (Ye et al. 1988, 1989), observation of CaM-antibody gold particles in the cell wall of corn root tips by electron microscopy (Li et al. 1993), and detection of CaM in the culture medium of Angelica dahurica suspension-cultured cells and that of their protoplasts (Sun et al. 1994, 1995). Recently, further work in our laboratories indicated that CaM could extracellularly stimulate the proliferation of several kinds of plant suspension-cultured cells and their protoplasts, e.g. Angelica dahurica (Li et al. 1992; Sun et al. 1994, 1995) Tenmisumy typhoides and Setaria italica (Sun et al. 1995), which indicated that extracellular CaM had some biological significance. In animal systems, Boynton et al. (1980) first reported that exogenous CaM promoted the proliferation of non-neoplastic rat liver cells, and subsequent reports (Gorbachevskaya et al. 1983; MacNeil et al. 1984; Crocker et al. 1988) also showed similar results in various experimental systems in which Crocker et al. (1988) first suggested CaM functioned extracellularly. In addition, MacNeil et al. (1984, 1988) proved the presence of extracellular CaM in normal human body fluids by the method of phosphodiesterase assay and radioimmunoassay. Although the existence of extracellular CaM and its effect on cell proliferation has been verified both in plants and animals, compared with intracellular CaM, little is known about its role. Since intracellular CaM is known to regulate various target proteins or enzymes, detecting and investigating proteins which bind to extracellular CaM could help in understanding its role and the underlying mechanism.

In this study, extracellular CaM-binding proteins were detected in suspension-cultured cells of Angelica dahurica and Daucus carota. A major extracellular CaM-binding protein of A. dahurica was purified and partially characterized.
Materials and methods

Plant materials and culture conditions. A suspension culture of *Angelica dahurica* L. cells, obtained from calli which were donated by the biology department of Shandong University, was cultured in MS liquid medium (Murashige and Skoog 1962) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 1 mg·1⁻¹) and N⁶-benzyladenine (0.5 mg·1⁻¹). A suspension culture of *Daucus carota* L. maintained in the same conditions as the *A. dahurica* suspension culture, was obtained from white, vigorously dividing callus which had been formed by culturing small round sections of carrot root, obtained locally, on MS agar medium containing 2,4-D (1 mg·1⁻¹) followed by several periods of subculture and selection.

Protein preparation. Suspension-cultured cells, harvested at the phase of logarithmic growth, were centrifuged at 500·g. The culture medium was recovered by passing the supernatant successively through 5-μm qualitative filter paper and 0.22-μM microporous filter membrane. The extracellular salt-extracted solution was prepared from sedimented living cells by the method of Van Engelen et al. (1991) with little change. After washing twice with distilled water, sedimented living cells were incubated in 0.1 M CaCl₂ on ice for 20 min, and then centrifuged. Extracellular salt-extracted solution was obtained by passage through the same filter paper and membrane as above. To the culture medium and extracellular salt-extracted solution, 2.5 volumes of ethanol were added and the mixture allowed to stand overnight at 4°C (De-Vries et al. 1988). The resulting protein precipitate was collected by centrifugation at 10000·g, at 4°C for 30 min, and then stored at -20°C or dissolved in a buffer for immediate use. In order to determine the degree of breakage of living cells, the activity of glucose-6-phosphate dehydrogenase, a cytoplasmic marker enzyme, was assayed by the method of Edward et al. (1984).

For the isolation of cell wall proteins from *A. dahurica* suspension cells, the cell walls, which had been isolated as described by Stuart and Varner (1980), were extracted with 0.2 M CaCl₂ at 4°C for 16 h. The extracts were centrifuged at 500·g for 5 min, then precipitated by the addition of 2.5 volumes of ethanol. The resulting cell wall proteins were collected by centrifugation at 10000·g for 30 min, and resuspended in 50 mM Tris-HCl buffer. Total cell extracts were prepared by homogenizing suspension cells which had been treated by freezing and thawing in extraction buffer containing 1 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride, pH 8.0. The homogenate was then centrifuged at 10000·g, at 4°C for 30 min. The supernatants were collected.

The protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as the standard.

Biotinylation of CaM. Homogeneous CaM was prepared from cauliflower according to the procedure as described by Biro et al. (1984) and biotinylated according to the procedure of Billingsley et al. (1985) with small modifications. Briefly, purified cauliflower CaM was dialyzed against 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. Biotinamidocaproate N-hydroxysuccinimide ester (2.8 mg; Sigma Chemical Co., St. Louis, Mo., USA) was dissolved in 90 l of N,N-dimethylformamide, added to 6 ml of CaM solution and made up to a final concentration of 1 mM CaM with 1 mM CaCl₂; the molar ratio between the biotinylating agent and CaM was kept at 19:1. The reaction was carried out at 4°C for 2 h with constant stirring.

Electrophoresis and biotinylated-CaM gel overlay. Proteins were separated by SDS-PAGE according to the method of Laemmli (1970). Protein bands were visualized by Coomassie blue staining when there was sufficient protein applied, otherwise by silver staining as described by Blum et al. (1987). Molecular markers (from Dong Feng Biochemical Company, China) were: capsid of Tobacco Mosaic Virus (17500), carbonic anhydrase (30000), actin (43000), albumin (67000) and phosphorylase b (94000).

For the biotinylated-CaM gel-overlay assay, proteins fractionated by SDS-PAGE were electrophoreted onto nitrocellulose (NC) membrane (0.2 μm pore size) at 190 mA for 2 h (Li et al. 1994). After blotting, the NC membrane was incubated in blocking buffer solution (5% nonfat dry milk in buffer A which contained 50 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl₂, pH 7.4) for 30 min followed by washing with buffer A, and then overlayed with biotinylated CaM, which was also dissolved in buffer A, for 1 h and washed with buffer A again. In the control, buffer A was changed to buffer B (1 mM CaCl₂ in buffer A was replaced by 5 mM EGTA) in the above steps. After a further wash with buffer A, the membrane (whether treatment or control) was incubated with avidin-peroxidase (Sigma) at 1:500, and the colour developed in 3,3'-diaminobenzidine (Sigma) solution (0.5 mg·ml⁻¹ in buffer A containing 0.03% H₂O₂).

Detection of glycoproteins and heat treatment of proteins. Glycoproteins on an electrophoresis gel were detected by an improved periodic acid-Schiff (PAS) reaction (Konat et al. 1984).

For detection of heat resistance of proteins, the protein preparation was first heated at 100°C for 5 min, and denatured proteins were removed by centrifugation. The remaining non-denatured proteins were separated by SDS-PAGE.

Purification of 21-kDa CaM-binding protein. About 500 ml of living cells of *A. dahurica*, without medium, were extracted by 0.1 M CaCl₂ at 4°C for 20 min. The supernatant collected was passed through 5-μm qualitative filter paper and mixed with 2.5 volumes of ethanol at 4°C overnight. The resulting precipitate was collected by centrifugation at 10000·g for 20 min, resuspended in 2.5 ml of 50 mM malonate buffer (pH 5.7), dialyzed against the same buffer overnight, and centrifuged again at 15000·g for 30 min. The resulting supernatant was applied to a Sephadex G-100 (Pharmacia, Uppsala, Sweden) gel-filtration column (1.6 cm diameter, 76 cm long) pre-equilibrated with 50 mM malonate buffer (pH 5.7). The column was washed with equilibration buffer, and eluted with the same buffer containing, successively, 0.02 M NaCl, 0.04 M NaCl and 1.0 M NaCl. The 21-kDa protein was eluted by the buffer containing 0.04 M NaCl. Fractions in peak regions were pooled and concentrated. The CaM-binding character of the purified 21-kDa protein was again detected by biotinylated-CaM gel overlay.

Effect of the 21-kDa CaM-binding protein on the activity of CaM-dependent NAD kinase (NADK). The activity of CaM-dependent NADK was detected as described by Wang and Wu (1992). The effect of 21-kDa CaM-binding protein on the reaction was studied by adding various amounts of 21-kDa CaM-binding protein to the reaction mixture in which CaM-dependent NADK was present in excess; CaM was kept at a concentration of 100 ng·ml⁻¹.

Results

Detection of extracellular CaM-binding proteins in suspension cultures of *A. dahurica* and carrot. The CaM-binding proteins were assayed in extracellular protein preparations of *A. dahurica* suspension cells, including preparations of culture medium, extracellular salt-extracts and cell wall extracts, using a biotinylated-CaM gel-overlay method with or without Ca²⁺. The results (Fig. 1) showed that a CaM-binding protein with a relative molecular mass (Mr) of 21-kDa was present in extracellular salt extracts and cell wall extracts prepared from each batch of suspension-cultured cells; little was found in the culture