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CALMODULIN-BINDING PROTEINS FROM *ZEA MAYS* GERMS

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Key Word Index—*Zea mays*; Gramineae; germ; seed germination; calmodulin-binding proteins; ATPase; superoxide dismutase.

Abstract—Using calmodulin–Sepharose 4B affinity chromatography, five to six calmodulin-binding proteins were isolated from *Zea mays* germs germinated for 0, 12 and 24 hr. These proteins could inhibit calmodulin-activated phosphodiesterase activity, but this inhibition could be eliminated by adding an excess of maize germ calmodulin. The assay of enzyme activity indicated that there were calmodulin-dependent ATPase and superoxide dismutase activities, but no phospholipase D, peroxidase and catalase activities in the calmodulin-binding proteins of maize germs. Electrophoretic analysis showed a pattern change of the calmodulin-binding proteins and the appearance of new calmodulin-binding proteins in germinating maize germs, suggesting that the Ca^{2+}-calmodulin system takes part in the activation and re-establishment of metabolism in the early phase of maize seed germination.

INTRODUCTION

Ca^{2+} acts as a second messenger in the coupling of many extracellular signals and intracellular biochemical and physiological responses, and as a major metabolic and developmental regulator involved in the regulation of many diverse physiological processes in plants through the mediation of calmodulin (CaM) [1–4]. However, it is also well known that CaM has no enzymic activity of its own, and can play its important roles only when it is bound to its potential target proteins/enzymes (CaM-binding proteins) and activates them to trigger biochemical and physiological events [5, 6]. The investigation of CaM-binding proteins will therefore be helpful in understanding the function of CaM.

The embryo of seeds is metabolically active when seeds are germinating. The CaM level in radish embryos increases rapidly during early seed germination, which suggests a relationship between CaM level and the reactivation of seed growth and metabolism [7, 8]. In the present study, using maize germs as research material, the aim was to isolate CaM-binding proteins, identify the enzymological properties of these proteins, and investigate the pattern change of these proteins during early seed germination.

RESULTS

It is known that CaM-binding proteins show a Ca^{2+}-dependent and reversible association with CaM immobilized on Sepharose 4B [9]. As shown in Fig. 1, when the extraction solution of maize germs was loaded on a CaM–Sepharose 4B column, a fraction containing CaM-binding proteins was eluted from the column by the buffer containing EGTA. A larger amount of eluted CaM-binding proteins could be observed from the extract of maize germs germinated for 24 hr as compared with the maize germs germinated for 0 or 12 hr (data not shown). So the CaM-binding proteins extracted from the maize germs germinated for 24 hr was used in the following inhibition and recovery experiments.

To further identify the EGTA-eluted protein fraction as CaM-binding proteins, it was tested for its ability to inhibit reversibly the activity of CaM-activated phosphodiesterase (PDE). According to the procedure of Ref. [10], when 0.5 $\mu g\,ml^{-1}$ maize germ CaM was present in the reaction mixture, bovine brain cAMP PDE was activated to its maximum activity; however, when the EGTA-eluted proteins were added to the PDE reaction mixture, the activity of CaM-activated PDE decreased with an increasing amount of these proteins, and even disappeared after 15 μg EGTA-eluted proteins was added to the PDE reaction mixture (Fig. 2).

On the other hand, the inhibition of CaM-activated PDE activity by the EGTA-eluted proteins could be eliminated by an excess addition of maize germ CaM to the PDE reaction mixture. As shown in Fig. 3, when 15 μg EGTA-eluted proteins was added to the PDE reaction mixture containing 0.5 $\mu g\,ml^{-1}$ CaM, no CaM-activated PDE activity was detected. However, when maize germ CaM was re-added to the PDE reaction mixture containing the same amount of CaM and EGTA-eluted proteins, the activity of CaM-activated PDE was recovered gradually with an increase of the

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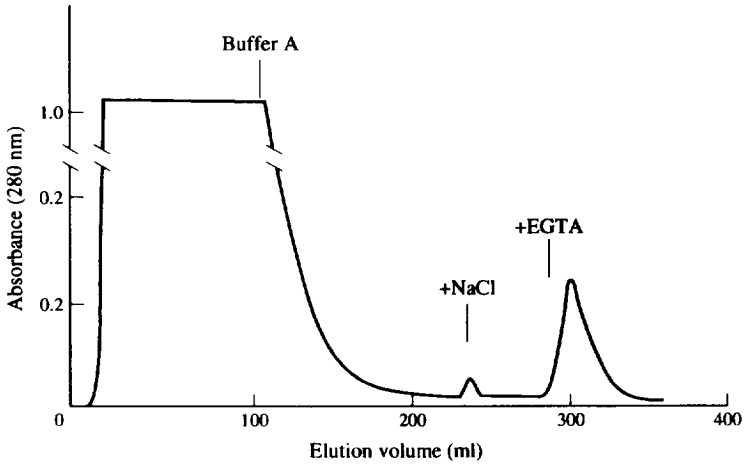

Fig. 1. CaM-Sepharose 4B affinity chromatography of CaM-binding proteins from maize germs germinated for 24 hr. ' + NaCl' refers to buffer A containing 200 mM NaCl, and ' + EGTA' refers to buffer A without $CaCl_2$, but containing 2 mM EGTA.

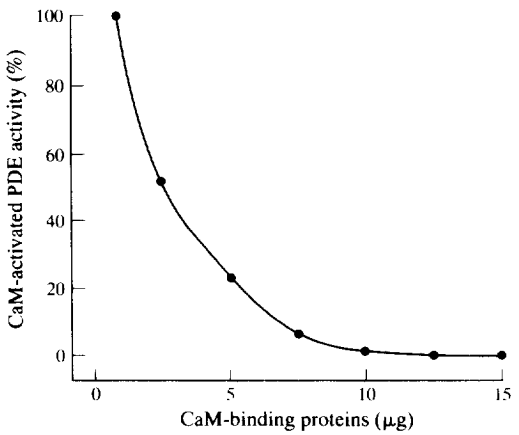

Fig. 2. Inhibition of CaM-activated PDE activity by the CaM-binding proteins from maize germs germinated for 24 hr. The maximum activity of PDE in the presence of a saturating level (0.5 $\mu g\,ml^{-1}$) of maize germ calmodulin, 23.6 μmol cAMP hydrolysed mg^{-1} protein min^{-1}, was taken as 100%; the basal activity of PDE in the absence of added maize germ calmodulin, 1.7 μmol cAMP hydrolysed mg^{-1} protein min^{-1}, was taken as 0%. The EGTA in the solution of CaM-binding proteins eluted from the calmodulin affinity column was dialysed out prior to adding these proteins to the PDE reaction mixture.

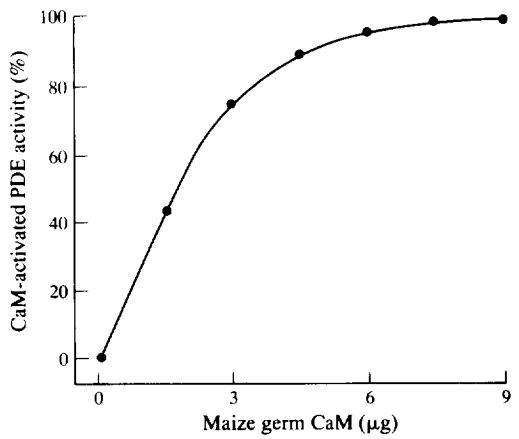

Fig. 3. Recovering effects of maize germ calmodulin on CaM-activated PDE activity after inhibition by maize germ CaM-binding protein. See the caption to Fig. 2 for details.

amount of CaM added, and finally to the full extent. All these results indicated that the EGTA-eluted proteins could competitively bind CaM with PDE, consistent with their being CaM-binding proteins.

Electrophoretic analysis of the CaM-binding proteins on PAGE showed about five to six bands, implying that there were at least five to six CaM-binding proteins in maize germs. In addition, a distinguishing feature of the PAGE electrophoretogram was that the CaM-binding proteins from maize germs germinated for different times showed different protein patterns; for example, the maize germs germinated for 24 hr demonstrated about two new CaM-binding proteins in near anode as compared with the maize germs germinated for 0 hr (Fig. 4, lanes 5 and 6), and this feature was more apparent in SDS-PAGE. As shown in Fig. 5, a M_r 57200 CaM-binding protein appeared clearly in maize germs germinated for 12 and 24 hr (Fig. 5, lanes 4–7), but was not present in the maize germs germinated for 0 hr (Fig. 5, lanes 2 and 3). On the contrary, a M_r 38100 CaM-binding protein was evident in the maize germs germinated for 0 hr (Fig. 5, lanes 2 and 3), but was not apparent in the maize germs germinated for 12 and 24 hr (Fig. 5, lanes 4–7). These results could imply a relationship between pattern change of CaM-binding proteins and metabolic activation during the early stage of seed germination.

To further study the properties of the CaM-binding proteins from maize germs, activities of several enzymes were detected in the extraction solution (prior to CaM-Sepharose 4B affinity chromatography) and the CaM-

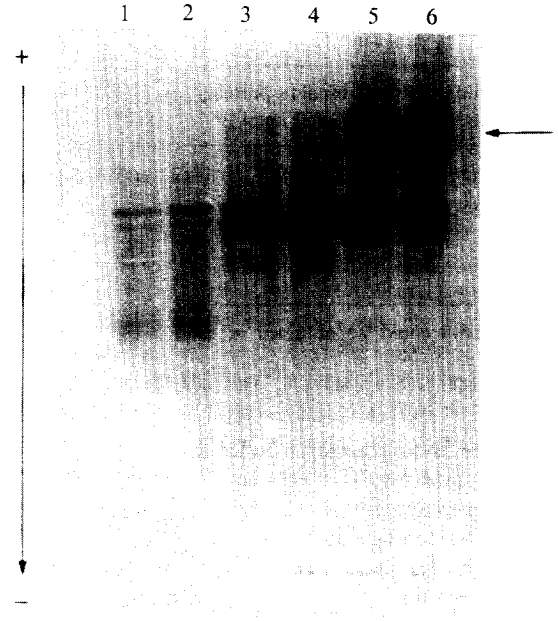

Fig. 4. PAGE of the CaM-binding proteins from maize germs germinated for different time on a 12% gel. About 35 μg of the proteins was added to each lane. Lanes 1 and 2: germinated for 0 hr; lanes 3 and 4: germinated for 12 hr; and lanes 5 and 6: germinated for 24 hr. Left arrow shows the direction of migration, and right arrow shows the appearance of new CaM-binding proteins.

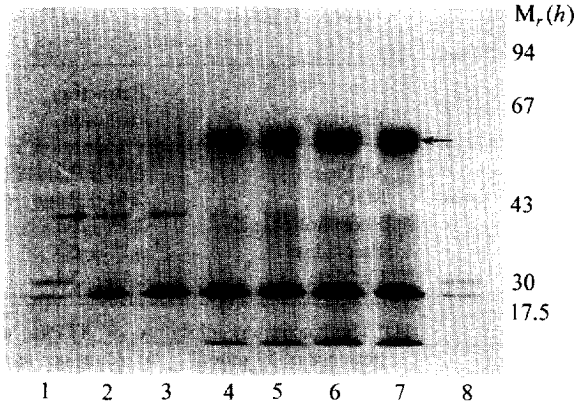

Fig. 5. SDS-PAGE of the CaM-binding proteins from maize germs germinated for different time on a 15% gel. About 50 μg of the proteins was added to lanes 2–7. Lanes 1 and 8 contained molecular weight markers (15 μg per lane, Dong Feng Co., People's Republic of China) including phosphorylase *b* (94 000), albumin (67 000), actin (43 000), carbonic anhydrase (30 000) and TMV coat protein (17 500). Lanes 2 and 3: germinated for 0 hr; lanes 4 and 5: germinated for 12 hr; and lanes 6 and 7: germinated for 24 hr. Left arrow shows the M_r 38100 CaM-binding protein, and right arrow shows the M_r 57200 CaM-binding protein.

binding proteins of maize germs germinated for 24 hr. The results showed that there were obvious activities of ATPase, superoxide dismutase (SOD), phospholipase D, peroxidase and catalase in the extraction solution of maize germs; however, after the extraction solution was passed through a CaM-Sepharose affinity column and a CaM-binding proteins fraction was eluted, only ATPase and SOD activities could be detected in the CaM-binding proteins, but they showed higher activities in the CaM-binding proteins than in the extraction solution of maize germs (Table 1). In addition, after the CaM antagonist W_7 was added to the CaM-binding proteins, ATPase and SOD activity was inhibited severely, indicating the presence of CaM-dependent ATPase and SOD in maize germs germinated for 24 hr.

DISCUSSION

Some CaM-binding proteins were found and isolated from spinach and pea chloroplasts [11], wheat etiolated coleoptiles [12] and *Pinus yunnanensis* pollen [13]. The appearance and distribution of certain CaM-binding proteins in gametes and embryos of fucoid algae was shown to be developmentally regulated by using ^{125}I-calmodulin gel overlay technique [14]. Recently, it was found that a new M_r 64000 CaM-binding proteins was induced with 2,4-D during carrot callus formation by using a biotinylated calmodulin probe [15]. All these results could imply that the appearance and pattern of CaM-binding proteins are under developmental regulation, and may reflect the different roles of calmodulin at various developmental stages.

The early stage of seed germination is a process of initiation, activation and re-establishment of metabolism [16], which had been found to be related with the involvement of CaM [7, 8]. The present results showed that a pattern change of CaM-binding proteins happened and several new CaM-binding proteins appeared in germinating maize germs during the early stage of seed germination (Figs 4 and 5), suggesting that the Ca^{2+}-CaM system takes part in the activation and re-establishment of metabolism in early germination phase of maize seeds. In addition, it is well known that a large amount of proteolysis could cause the change of protein patterns in germinating seeds [16]. In the present experiment, however, a protease inhibitor (PMSF) was used in the extraction and chromatography processes. Also, the proteins analysed on PAGE and SDS-PAGE which were eluted from a CaM affinity column showed a reversible inhibition on PDE activity (Figs 2 and 3). These facts suggest that Figs 4 and 5 are a real reflection of the changes of CaM-binding proteins during early seed germination.

It is well known that ATPase plays multiple roles in cellular physiological and biochemical processes [17], and several ATPases including H^+-ATPase, $Ca^{2+} \cdot Mg^{2+}$-ATPase and Ca^{2+}-ATPase were found to be Ca^{2+}/CaM-dependent [4, 6]. We isolated a Ca^{2+}/CaM-dependent ATPase from pine pollen by CaM-Sepharose 4B affinity chromatography [13], the present results also indicated the presence of a CaM-dependent ATPase in maize germs.

Table 1. Enzyme activity in the extraction solution and CaM-binding proteins from maize germs germinated for 24 hr (U mg^{-1} protein min^{-1})

Enzymes	Extraction solution	CaM-binding proteins	CaM-binding proteins + W_7 (50 μmol/l)*
ATPase	35.6 ± 3.2	64.7 ± 2.5	3.2 ± 0.3
SOD	2.64 ± 0.1	5.13 ± 0.2	0.4 ± 0.02
Phospholipase D	1.95 ± 0.1	0	—
Peroxidase	8.37 ± 0.8	0	—
Catalase	54.6 ± 3.1	0	—

*The maximum inhibition of ATPase and SOD activity could be obtained in the presence of 50 μmol l^{-1} W_7 (data not shown).

SOD has been known to play a very important role in the defence of active oxygen stress in plant cells [18, 19]. Recently, Li and Zhang [20], and Chong *et al.* [21] found that SOD from bovine blood and cucumber cotyledons was inhibited severely by CaM antagonist chlorpromazine and was activated by exogenous CaM. Our present research further shows that SOD from maize germs could reversibly bind with CaM immobilized on Sepharose in a Ca^{2+}-dependent manner, and also be severely inhibited by CaM antagonist W_7, indicating that SOD in maize germs is a CaM-dependent enzyme. In addition, phospholipase D was reported to be regulated by Ca^{2+} [22, 23], and the secretion and activation of peroxidase was also found to be a Ca^{2+}/CaM-dependent process [24, 25]. However, the activity of these enzymes and of catalase could not be detected in the CaM-binding proteins from maize germs.

EXPERIMENTAL

Plant material. Seeds of *Zea mays* (Qing No. 3) were purchased commercially and its germination percentage was about 94%. The seeds were sterilized in 0.1% $HgCl_2$ for 10 min, and soaked in H_2O for 2 hr. The soaked seeds were sowed in moist filter papers for germination at 25° for 0, 12 or 24 hr (no germination was observed in 24 hr). The maize germs were removed manually for the extraction of CaM-binding proteins.

Preparation of CaM–Sepharose 4B affinity column. Maize germ calmodulin was extracted and purified according to the method of Refs [26, 27]. Sepharose 4B was activated with cyanogen bromide [28], and conjugation of activated Sepharose 4B with calmodulin was performed as described in Ref. [9]. The CaM–Sepharose 4B was then loaded into a glass column (15 × 65 mm), and the column was equilibrated with buffer A (50 mM Tris–HCl, pH 7.2, 1 mM imidazole, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 2 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulphonyl fluoride (PMSF)), then re-equilibrated with buffer A containing 1 mg ml^{-1} BSA, and was finally washed with buffer A to the baseline level of UV absorption.

Extraction and affinity chromatography of CaM-binding proteins. 20 g of maize germs germinated for 0, 12 or 24 hr were homogenized in buffer B (50 mM Tris–HCl, pH 7.2, 1 mM $MgCl_2$, 1 mM imidazole, 1 mM EGTA, 5 mM 2-mercaptoethanol, 0.5 mM PMSF, 0.4% Triton X-100). The homogenate was centrifuged at 11 000 *g* for 30 min, and the pellet re-extracted once with buffer B. The supernatant was passed through a DEAE-cellulose column (20 × 80 mm) to remove endogenous CaM as described in Ref. [9]. After DEAE-cellulose column chromatography, the eluate was adjusted to a Ca^{2+} concn of 5 mM, then stirred for 1 hr, and centrifuged at 11 000*g* for 1 hr. The supernatant was applied to the CaM–Sepharose 4B affinity column, and the CaM-binding protein fraction was eluted according to Ref. [9]. The eluted protein soln was dialysed overnight against buffer A and then concd. The concd soln of CaM-binding proteins was used for enzyme assay and electrophoresis. Polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE of CaM-binding proteins were performed as described in Ref. [29]. Protein was determined by the method of Ref. [30].

Enzyme assay. Bovine brain cAMP PDE activity was determined as described in Ref. [10]. ATPase was assayed according to Ref. [31], and one unit of ATPase activity was defined as 1 nmol ATP hydrolysed mg^{-1} protein min^{-1}. SOD was determined as described in Ref. [32], and one unit was defined as the amount causing 50% inhibition of the photoreduction of nitroblue tetrazolium (NBT) min^{-1}. Phospholipase D was determined as described in Ref. [22], and one unit was defined as the amount hydrolysing 1 μmol choline min^{-1}. Peroxidase was monitored according to Ref. [24], and one unit was defined as the increase of 0.01 in *A* at 436 nm min^{-1}. Catalase was assayed as described in Ref. [33], and one unit was defined as 1 μmol H_2O_2 decomposed min^{-1}. All the above-mentioned enzyme reaction mixtures contained 0.2 mM $CaCl_2$ and 0.5 μg ml^{-1} maize germ calmodulin.

Chemicals. EGTA, cAMP, bovine brain PDE, snake venom, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide hydrochloride (W_7) and NBT were purchased from Sigma, Sepharose 4B was from Pharmacia, and PMSF and cyanogen bromide were from Merck.

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