Purification and Characterization of Tora-mame (Phaseolus vulgaris) Seed Calmodulin

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We purified calmodulin to apparent homogeneity from Tora-mame (Phaseolus vulgaris) seeds, one of the Japanese cultivars of the common bean. Electrophoretically, the purified Tora-mame calmodulin migrated faster than bovine brain calmodulin in the presence or absence of Ca²⁺. The estimated molecular weight of Tora-mame calmodulin was 14,200 in the presence of Ca²⁺ and 18,200 in the absence of Ca²⁺. Like other plant calmodulins, Tora-mame calmodulin lacked tryptophan and contained 1 mol each of tyrosine and half cystine per mol of protein. However, Tora-mame calmodulin has fewer acidic amino acid residues than other plant calmodulins did. Rat brain phosphodiesterase (Ca²⁺-PDE) was stimulated by Tora-mame calmodulin, but the concentration of Tora-mame calmodulin, giving the half-maximal activation of Ca²⁺-PDE, was higher than that of bovine calmodulin, and the maximal activity of Ca²⁺-PDE obtained with Tora-mame calmodulin was lower than that obtained with bovine protein. The Ki value of W-7, a calmodulin antagonist, for Tora-mame calmodulin (17 μM) was larger than that for bovine calmodulin (8 μM). These observations suggest that hydrophobic regions of Tora-mame calmodulin exposed by Ca²⁺-induced conformational changes were slightly different from those of bovine calmodulin.

Calmodulin is a member of a class of calcium-binding proteins and its primary structure is highly conserved through eukaryotes.¹⁻⁵ In plants, enzymes such as NAD kinase and Ca²⁺-ATPase have been shown to be regulated by Ca²⁺ and calmodulin.⁶⁻⁷ Biopharmacological studies, using calmodulin antagonists, have suggested that hydrophobic regions of vertebrate calmodulin that are exposed by Ca²⁺-binding may be important for the functions of this protein to activate the calmodulin-independent enzymes.⁸⁻⁹ However, little is known about characteristics of hydrophobic regions of plant calmodulin.

For this work, we purified calmodulin from Tora-mame seeds (Phaseolus vulgaris), one of the Japanese cultivars of the common bean, and compared the purified protein with mammalian calmodulin with special regard to the effects on rat brain phosphodiesterase, one of the calmodulin-dependent enzymes, and the interaction with a calmodulin antagonist, W-7.

Materials and Methods

Tora-mame seeds were purchased from a commercial source and pulverized finely with a cryo-mill. Phenyl Sepharose 4B and DEAE-cellulose (DE52) were purchased from Pharmacia LKB Biotechnology and Whatman Biochemical Ltd. (Clifton, NJ) respectively. W-7 was purchased from Seikagaku-Kogyo Co., Ltd. (Tokyo). Trypsin inhibitor (soybean) and snake venom (Crotalus atrox) were products of Sigma Chemical Co. (St Louis, MO). Cyclic [8-³H]GMP was purchased from ICN Pharmaceutical. All other reagents were of the highest grade available.

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate; Dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; PDE, phosphodiesterase; TCA, trichloroacetic acid; Mops, 3-(N-Morpholino)propanesulfonic acid; W-7, N-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide.
Bovine brain calmodulin was purified by the method of Yazawa et al.\textsuperscript{10} \textsuperscript{2642} \textsuperscript{2} \textsuperscript{H. Umekawa et al.} Ca\textsuperscript{2+}-PDE was partially purified from rat brain by the method of Kincaid et al.\textsuperscript{11} Dansyl-calmodulin was also prepared by the method of Kincaid et al.\textsuperscript{12}

**Isolation of Tora-mame calmodulin.** Tora-mame calmodulin was isolated from Tora-mame seeds by the TCA precipitation method described by Yazawa et al.\textsuperscript{10} with some modifications. The ground beans (500g) were suspended in 10 volumes of 50mM phosphate buffer (pH 5.7) containing 5mM EDTA and stirred overnight at 4°C. The suspension was centrifuged at 12,000 \times g for 15 min and 100% TCA (w/v) was added to the supernatant to 3% with vigorous stirring for 10 min. Then the suspension was adjusted to pH 5.2 with 6N NaOH, and stirred for 1 hr at 4°C. The precipitate was removed by centrifugation at 12,000 \times g for 15 min and 100% TCA (w/v) was added to the supernatant to 3%. The precipitate was collected by centrifugation at 12,000 \times g for 15 min, dissolved in 1M Tris, and dialyzed against 25mM Tris-HCl buffer (pH 7.5) containing 1mM EDTA. The dialyzed protein solution (275ml) was brought to 50% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} saturation and the precipitate formed was removed by centrifugation at 12,000 \times g for 15 min. The supernatant was dialyzed against 25mM Tris–HCl buffer (pH 7.5) containing 1 mM CaCl\textsubscript{2} and put on a phenyl-Sepharose column (1.5 x 21 cm), which was pre-equilibrated with the same buffer. The column was washed with 25mM Tris–HCl buffer (pH 7.5) containing 0.1mM CaCl\textsubscript{2} until UV absorbance at 280 nm returned to the base-line level. Tora-mame calmodulin was then eluted with 25mM Tris–HCl buffer (pH 7.5) containing 1mM EGTA.

**\textsuperscript{2}Ca\textsuperscript{2+}-dependent phosphodiesterase assay.** Ca\textsuperscript{2+}-PDE was assayed by the method of Hidaka and Asano.\textsuperscript{13} Unless otherwise noted, the reaction mixture (0.5 ml) contained 50mM Tris-HCl (pH 8.0), 5mM MgCl\textsubscript{2}, 0.4 \mu M cyclic [\textsuperscript{8-\textsuperscript{2}}H]GMP (500cpm/pmol), 0.1mM CaCl\textsubscript{2}, an appropriate amount of calmodulin, and 0.34 \mu g of rat brain Ca\textsuperscript{2+}-PDE, and was incubated at 30°C for 15 min. The reaction was stopped by boiling for 5 min, followed by addition of 50 \mu g of snake venom and the mixture was incubated at 30°C for another 10 min. Then, 1 ml of water was added and denatured protein was removed by centrifugation. The clear supernatant was put on a small cation exchange resin column (AG 50W-X4, 200 to 400 mesh, 0.7 x 1.5 cm). The product, \textsuperscript{3}H-guanosine, was eluted with 1.5 ml of 3N ammonium hydroxide after washing the column with 15 ml of water. The product was measured in a liquid scintillation counter (Beckman 1801).

**Dansyl-calmodulin fluorescence spectra.** Dansyl-calmodulin fluorescence spectra were recorded at 25°C with a Hitachi 650-60 spectrofluorometer as described.\textsuperscript{14} The test solution contained 10mM Mops (pH 7.0), 25 \mu M dansyl-calmodulin, and 90mM KCl. Fluorescence intensity was measured with the excitation at 345 nm, and the emission intensity was monitored at 495 nm.

**SDS-polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis was done by the method of Laemmli,\textsuperscript{15} using 5% acrylamide stacking gel and 15% acrylamide resolving gel. The gel was stained with 0.01% Coomassie Brilliant Blue 250.

**Amino acid analysis.** Amino acid analysis of protein hydrolysates were done with a Hitachi amino acid analyzer Model 655A by the method of Spackman et al.\textsuperscript{16} Proteins were hydrolyzed at 110°C for 24 hr in evacuated, sealed tubes.

**Other methods.** Protein was measured by the method of Lowry et al.\textsuperscript{17} with bovine serum albumin as a standard. In some experiments, Ca\textsuperscript{2+}-EGTA buffer\textsuperscript{18} was used to estimate the precise concentration of free Ca\textsuperscript{2+} in a reaction mixture.

**Results**

**Purification of Tora-mame seeds calmodulin.** As shown in Table I, 7mg of purified Tora-mame calmodulin was obtained from 500g of ground Tora-mame seeds and the overall recovery was approximately 17%. Calmodulin accounted for about 0.09% of the soluble protein in the initial Tora-mame seeds extract (Table I). The purified Tora-mame calmodulin was homogeneous when tested with SDS-polyacrylamide gel electrophoresis (Fig. 1). Tora-mame calmodulin migrated slightly faster on the gel than bovine calmodulin both in the presence and absence of Ca\textsuperscript{2+}. The apparent molecular weight of Tora-mame calmodulin was estimated to 14,200 in the presence of Ca\textsuperscript{2+} and 18,200 in the absence of Ca\textsuperscript{2+}.

**Table I. PURIFICATION OF Tora-mame CALMODULIN**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein* (mg)</th>
<th>Total calmodulin* (mg)</th>
<th>Yield (%)</th>
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<tr>
<td>Homogenate</td>
<td>46075</td>
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<td>100</td>
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<tr>
<td>TCA precipitate</td>
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<td>44</td>
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<tr>
<td>50% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}</td>
<td>2362</td>
<td>7</td>
<td>18</td>
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<tr>
<td>Phenyl-Sepharose</td>
<td>7</td>
<td>7</td>
<td>17</td>
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* Measured by the method of Lowry et al.\textsuperscript{17}

* Measured by Ca\textsuperscript{2+}-PDE activator activity as described in Materials and Methods.
## Table II. Amino Acid Composition of Tora-mame Calmodulin

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Tora-mame&lt;sup&gt;a&lt;/sup&gt; mol/mol</th>
<th>Spinach leaf&lt;sup&gt;e&lt;/sup&gt; integer</th>
<th>Wheat germ&lt;sup&gt;f&lt;/sup&gt;</th>
<th>Yeast&lt;sup&gt;g&lt;/sup&gt;</th>
<th>Bovine brain&lt;sup&gt;h&lt;/sup&gt;</th>
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<td>N.D.</td>
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<tr>
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</table>

| Total      | 146                           | 138                              | 149                   | 147            | 148              |

| M.W.       | 16,680<sup>d</sup>            | 16,680                           | 16,900                | 16,800         | 16,800           |

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Ca<sup>2+</sup>. On the other hand, the apparent molecular weights of bovine calmodulin in the presence and absence of Ca<sup>2+</sup> were 16,800 and 20,000, respectively.

### Amino acid composition

The amino acid composition of Tora-mame calmodulin was similar to that of other calmodulins but differed slightly in the following points (Table II). First, Tora-mame calmodulin contained fewer acidic amino acids, such as aspartic acid and glutamic acid residues, than the other calmodulins did. Second, it contained more alanine residues than the other protein did. Third, there was one tyrosine residue in Tora-mame, spinach, and wheat germ calmodulins, but bovine calmodulin contained two tyrosine residues and yeast calmodulin no tyrosine residue. Fourth, Tora-mame calmodulin contained one half-cystine residue per protein like the wheat germ protein.

### Ca<sup>2+</sup>-induced conformational change

The UV absorption spectrum of Tora-mame calmodulin is shown in Fig. 2. From 250 to 275 nm, Tora-mame calmodulin displays a characteristic spectrum due to its high content...
Fig. 1. Electrophoretic Analysis of Tora-mame Calmodulin.
Electrophoresis was done in a 15% polyacrylamide gel containing, 0.1% SDS by the method of Laemmli. Lane a, molecular weight standard; lane b, 3 µg of Tora-mame calmodulin in the presence of 0.1 mM CaCl$_2$; lane c, 2 µg of bovine brain calmodulin in the presence of 0.1 mM CaCl$_2$; lane d, 3 µg of Tora-mame calmodulin in the presence of 1 mM EGTA; lane e, 2 µg of bovine brain calmodulin in the presence of 1 mM of 1 mM EGTA.

Fig. 2. UV Absorption Spectrum of Tora-mame Calmodulin.
The spectra were taken in 0.1 M Tris-HCl (pH 7.5), containing 0.1 mM CaCl$_2$ (--), and in 0.1 M Tris-HCl (pH 7.5) containing 1 mM EGTA (---).

of phenylalanine. The binding of Ca$^{2+}$ to Tora-mame calmodulin dramatically changed the UV spectrum below 280 nm. The calculated extinction coefficient ($e_{280}$) of Tora-mame calmodulin was 1.5 in the presence of Ca$^{2+}$. It has been reported that 5-dimethylamino-naphthalene-1-sulfonyl-calmodulin (dansyl-calmodulin) fluorescence can serve as a probe for the interaction between Ca$^{2+}$ and calmodulin. Thus, we investigated the effects of Ca$^{2+}$ on dansyl-Tora-mame calmodulin fluorescence. The fluorescence emission spectrum of dansyl-Tora-mame calmodulin was dramatically altered by the addition of Ca$^{2+}$ and there was a “blue shift” from 525 to 495 nm and an increase in the intensity (data not shown). The percentages of maximal changes at 495 nm of Tora-mame and bovine calmodulins were plotted as a function of Ca$^{2+}$ concentration, using the Ca$^{2+}$–EGTA buffer system (Fig. 3). Ca$^{2+}$-induced conformational changes of both proteins were observed from 10$^{-6}$ M Ca$^{2+}$. The Ca$^{2+}$-titration curve of dansyl Tora-mame calmodulin was indistinguishable from that of dansyl-bovine calmodulin; the binding constants of both proteins for Ca$^{2+}$ were 1.7 µM.

Activation of rat brain Ca$^{2+}$-PDE
The effects of Tora-mame calmodulin on rat brain Ca$^{2+}$-PDE were examined (Fig. 4). Tora-mame calmodulin was slightly less effective than bovine calmodulin on activating Ca$^{2+}$-PDE. When both calmodulins were assayed under the same conditions, the maximal activity obtained with Tora-mame calmodulin was about 75% of the value obtained with bovine calmodulin, and the half
Phaseolus vulgaris Calmodulin

**Fig. 4.** Calmodulin-dependent Activation of Rat Brain Ca$^{2+}$-PDE.

The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 5 mM MgCl$_2$, 0.4 $\mu$M cyclic [8-3H]GMP, 0.1 mM CaCl$_2$, 0.34 $\mu$M Ca$^{2+}$-PDE, and various concentrations of Tora-mame (○) or bovine (●) calmodulin.

**Fig. 5.** Ca$^{2+}$-dependent Activation of Rat Brain Ca$^{2+}$-PDE by Tora-mame (○) or Bovine (●) Calmodulin.

The reaction mixture contained 50 mM Tris (pH 8.0), 5 mM MgCl$_2$, 0.4 $\mu$M cyclic [8-3H]GMP, 0.34 $\mu$M Ca$^{2+}$-PDE, and 0.4 $\mu$g/ml bovine or 2 $\mu$g/ml Tora-mame calmodulin. Ca$^{2+}$ concentration was controlled with a Ca$^{2+}$-EGTA buffer system.

Maximal activation of Ca$^{2+}$-PDE was obtained at 30 and 68 ng/ml of the Tora-mame and bovine proteins, respectively. The Ca$^{2+}$-dependence of both calmodulins on activation of Ca$^{2+}$-PDE is shown in Fig. 5. The Ca$^{2+}$ concentration for the half maximal activation of Ca$^{2+}$-PDE by Tora-mame calmodulin (1.9 $\mu$M) was slightly higher than that by bovine calmodulin (1.6 $\mu$M).

**Inhibition of the calmodulin stimulation of Ca$^{2+}$-PDE activity by W-7**

The effects of W-7, a calmodulin antagonist, on Ca$^{2+}$-PDE activity stimulated by Tora-mame calmodulin were examined. The addition of W-7 to the reaction mixture above 3 $\mu$M markedly inhibited the activity (data not shown). Figure 6 shows the effects of Tora-mame calmodulin on Ca$^{2+}$-PDE in the absence or presence of 20 $\mu$M and 40 $\mu$M W-7.

**Fig. 6.** Inhibition of Activation with Tora-mame Calmodulin of Rat Brain Ca$^{2+}$-PDE by W-7.

The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 5 mM MgCl$_2$, 0.4 $\mu$M cyclic [8-3H]GMP, 0.34 $\mu$M Ca$^{2+}$-PDE, 0.1 mM CaCl$_2$ and various concentrations of Tora-mame calmodulin; ○, in the absence of W-7 (control); △, in the presence of 20 $\mu$M W-7; ■, in the presence of 40 $\mu$M W-7.

**Fig. 7.** Kinetic Analysis of W-7-Induced Inhibition of Rat Brain Ca$^{2+}$-PDE.

The inhibition by W-7 of stimulation of Ca$^{2+}$-PDE activity by Tora-mame (panel A) or bovine (panel B) calmodulin was analyzed using Dixon plots. The reaction mixture contained 50 mM Tris–HCl (pH 8.0), 5 mM MgCl$_2$, 0.4 $\mu$M cyclic [8-3H]GMP, 0.1 mM CaCl$_2$ and various concentrations of W-7 and bovine or Tora-mame calmodulins. Calmodulin concentrations were 0.01 $\mu$g/ml (○), 0.05 $\mu$g/ml (●), and 0.4 $\mu$g/ml (■).
calmodulin as a calmodulin antagonist. When analyzed by Dixon plots (Fig. 7), the $K_i$ values of W-7 for Tor-mame and bovine calmodulins were 17 and 8 $\mu$m, respectively. W-7 may inhibit Ca$^{2+}$-PDE activity in a competitive fashion with this concentration of Tor-mame calmodulin.

Discussion

The methods described for purifying bovine brain calmodulin$^{10}$ were unsuccessful for Tor-mame seeds, because these seeds contain large amounts of proteins in the TCA-precipitated fraction. Therefore, we used ammonium sulfate fractionation after the TCA precipitation. Tor-mame seed calmodulin differs from bovine calmodulin in electrophoretic mobility (Fig. 1), amino acid composition (Table II), activation of vertebrate phosphodiesterase (Fig. 5), and affinity for calmodulin antagonist (Fig. 7). The amino acid composition of Tor-mame calmodulin was similar to that of wheat germ and spinach leaf calmodulins, but differences were observed in acidic amino acid contents. Since both vertebrate and yeast calmodulins contain no cysteine residue, this amino acid might be specific for plant calmodulin (Table II). Watterson et al. reported that the activation curves of bovine brain Ca$^{2+}$-PDE for spinach calmodulin and bovine brain calmodulin were superimposable.$^{3}$ Recently, Thompson et al. reported that calmodulins from leaves of latex-producing plants stimulated heart Ca$^{2+}$-PDE, and the $K_a$ (half maximal effect) of these plant calmodulins was slightly higher than that of bovine brain calmodulin.$^{2,3}$ However, they did not indicate the maximal activity value obtained with those plant calmodulins. While the Tor-mame calmodulin stimulated rat brain Ca$^{2+}$-PDE, the abilities of Tor-mame protein to stimulate the enzyme were different from that of bovine protein: the maximal activity value of the enzyme, the concentration of calmodulin for half-maximal activation, and the concentration of Ca$^{2+}$ for half-maximal activation (Figs. 4 and 5). These observations suggest that dissimilarities in the primary structure may exist not only between mammalian and plant calmodulins but also among plant proteins. W-7 inhibited the activity of Ca$^{2+}$-PDE in the presence of Ca$^{2+}$ and Tor-mame calmodulin, and addition of excess Tor-mame calmodulin could overcome the inhibition (Fig. 6). However, the apparent $K_i$ value for Tor-mame protein was larger than that for bovine protein (Fig. 7). These kinetic analyses suggest that hydrophobic regions of Tor-mame calmodulin, which may be the functional domain of this protein, were slightly different from those of bovine protein.

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References

18) Y. Ogawa, J. Biochem., 64, 255 (1968).