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Isolation and Characterization of Exogenously Expressed Calmodulin from Endogenous Tobacco Calmodulin by Anion-exchange Fast Protein Liquid Chromatography

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Abstract: A Mono Q HR 5/5 anion-exchange column with a FPLC system was used to separate exogenously expressed calmodulin from endogenous tobacco calmodulins. Transgenic tobacco calmodulins were purified by fractionation with ammonium sulfate, precipitation with sulfuric acid and hydrophobic chromatography on phenyl-Sepharose CL-4B. The purified calmodulins were chromatographed in the FPLC using the column. This method was selected because of the slight differences in the net charge of foreign and endogenous plant calmodulins due to amino acid sequence differences. By this approach, the exogenously expressed calmodulin was isolated from endogenous tobacco calmodulins. The isolated calmodulin was characterized by amino acid composition analysis as well as methylation analysis.
Key words: calmodulin, FPLC, methylation, Mono Q, transgenic plant.

Calmodulin is an acidic (pI≈4.0), highly conserved, calcium binding protein that interacts with a number of enzymes and stimulates their activities (Klee and Vanaman, 1982; Cohen and Klee, 1988; Roberts and Harmon, 1992). N^{ϵ}-trimethyllysine is a posttranslational modification that is found at position 115 in many calmodulins. This position is methylated by a specific calmodulin lysine N-methyltransferase (Rowe *et al.*, 1986; Morino *et al.*, 1987; Oh and Roberts, 1990; Oh *et al.*, 1992; Han *et al.*, 1993). However, the question of the functional significance of calmodulin methylation is unclear. In order to understand the function of calmodulin methylation *in vivo*, a calmodulin mutant (VU-3 calmodulin) that possesses arginine 115 instead of trimethyllysine 115 has been expressed in tobacco plants using transgenic plant technology (Oh *et al.*, 1991; Roberts *et al.*, 1992).

Since VU-3 calmodulin and plant calmodulin differ in their amino acid compositions (5 out of 148 amino acids differ) (Roberts *et al.*, 1992), it is a reasonable approach to use ion-exchange chromatography to isolate the VU-3 calmodulin from endogenous plant calmodulins. This isolation is necessary for further biochemical analyses, for example, i) amino acid composition analysis of isolated VU-3 calmodulin to confirm the foreign gene expression in tobacco cells, ii) methylation state analysis of the isolated calmodulin as a test of the effect of the foreign calmodulin introduction. A similar procedure used for the separation of VU-3 calmodulin from endogenous tobacco calmodulin was described briefly in a recent report (Roberts *et al.*, 1992).

In the present report, we show in detail the procedures used for better isolation and characterization of the foreign VU-3 calmodulin from the endogenous tobacco calmodulin by using a Mono Q HR 5/5 anion-exchange column with a fast protein liquid chromatography (FPLC) system.

Materials and Methods

Chemicals

Bis-Tris was purchased from Sigma. Phenyl-Sepharose CL-4B and Mono Q HR 5/5 columns were from Pharmacia. All other chemicals were of analytical grade.

Buffers

The following buffers were used for FPLC. A, 20 mM Bis-Tris, pH 6.1 containing 0.2 M NaCl, 0.1 mM EDTA, and 0.1 mM DTT; B, 20 mM Bis-Tris, pH 6.1 containing 0.6 M NaCl, 0.1 mM EDTA, and 0.1 mM DTT. The buffers were freshly prepared with nanopure

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deionized water and filtered through 0.2 μm membrane filters and degassed before use.

Sample preparation

37-day old tobacco plants were harvested and ground while cooled with liquid nitrogen. The plant powder (200 g) was used for the purification of tobacco calmodulins. The calmodulins were purified by fractionation with ammonium sulfate, precipitation with sulfuric acid addition near pI point, and hydrophobic chromatography on phenyl-Sepharose CL-4B as described previously (Roberts *et al.*, 1992). The protein fractions of phenyl-Sepharose were pooled and dialyzed against 5 mM NH_4HCO_3. The dialyzed samples were lyophilized and dissolved in the starting buffer (A buffer) and passed through a 0.2 μm filter prior to application to the Mono Q column.

FPLC and protein characterization

The FPLC system (Pharmacia) was used to separate exogenously expressed VU-3 calmodulin from endogenous tobacco calmodulins. The system employed two high pressure pumps and a liquid chromatography controller to form the gradient. A chart recorder with two channels was used to monitor the UV absorbance and the programmed gradient. The Mono Q HR 5/5 (5.0 cm×0.5 cm ID) column was equilibriated with 5 bed volumes of the A buffer at a flow-rate of 1.0 ml/min. The column was operated at 4℃. A 17 μg sample of calmodulins was applied for separation. To inject the sample, a 500 μl injection loop was used. The column was then eluted for 45 min or 50 min at the same flow-rate with a segmented gradient of sodium chloride. For the 45 min elution, the column was eluted with the A buffer containing 0.2 M NaCl for 10 min and then eluted for 20 min with a NaCl gradient from 0.2 M to 0.4 M (0 to 50% buffer B). The NaCl concentration was then increased to 0.6 M to remove the remaining proteins. For the 50 min elution, the column was eluted with the A buffer containing 0.2 M NaCl for 10 min and then eluted for 25 min with a NaCl gradient from 0.2 M to 0.4 M. The NaCl concentration was then increased to 0.6 M. Fractions were analyzed for calmodulins by SDS-PAGE and calmodulin RIA and those containing calmodulins were pooled, dialyzed against 5 mM NH_4HCO_3 and lyophilized. SDS-PAGE was carried out by using the method of Laemmli (1970) in 15% polyacrylamide gel with 1 mM EGTA and the proteins were stained with Coomassie Brilliant Blue as described previously (Roberts *et al.*, 1992). Amino acid composition analysis was done using the general method of Bidlingmeyer *et al.* (1984). For methylation analyses, a radiometric assay (Oh and Roberts, 1990) was used.

Results and Discussion

Calmodulin has been purified from transgenic tobacco plants by fractionation with ammonium sulfate, precipitation with sulfuric acid and hydrophobic chromatography on phenyl-Sepharose. A recombinant DNA-derived VU-3 calmodulin, which differs from plant calmodulin at only 5 residues (Fig. 1), has an electrophoretic mobility on SDS-PAGE similar to that of vertebrate calmodulin (Roberts *et al.*, 1992). However, the numbers of total amino acid residues composing the calmodulins turned out to be identical (Fig. 1) (Lukas *et al.*, 1984). Although a clear explanation for the apparent difference in the electrophoretic mobility is not available, we utilized this mobility difference on SDS-PAGE between VU-3 calmodulin and plant calmodulin in the analysis of transgenic plants (Fig. 2). In order to confirm the VU-3 calmodulin expression further and to test the effect of the foreign calmodulin introduction into tobacco cells, we tried to separate the foreign VU-3 calmodulin of transgenic tobacco plants using a Mono Q anion-exchange column with a FPLC system. The protein fractions of phenyl-Sepharose chromatography (Fig. 2) were pooled and used in FPLC as samples.

Since ion-exchange chromatography is a technique which separates biomolecules according to differences in their charges, we can optimize the selectivity of separation by choosing a suitable buffer pH. Another thing

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          1                  * * * * *  *        39
VU-3      ADQLTDEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSL
Plant           D                    C
          40                 * * * * *  *        75
VU-3      GQNPTEAELQDMINEVDADGNGTIDFPEFLNLMARK
Plant
          76                 * * * * *  *        112
VU-3      MKDTDSEEELKEAFRVFDKDGNGFISAAELRHVMTNL
Plant                              Q
          113                * * * * *  *        148
VU-3      GERLTDEEVDEMIREADVDGDGQVNYEEFVQVMMAK
Plant       K'                       I     K
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Fig. 1. Amino acid sequences of VU-3 and plant calmodulins. The structure of a calmodulin (VU-3) derived from a synthetic gene (Roberts *et al.*, 1985, 1986) is shown. The four calcium binding domains are shown with calcium binding residues (*) indicated and α-helical residues underlined. The entire sequence of VU-3 calmodulin is shown and only the sequence differences are shown for plant (spinach) calmodulin (Lukas *et al.*, 1984). K' at position 115 of plant calmodulin refers to trimethyllysine.

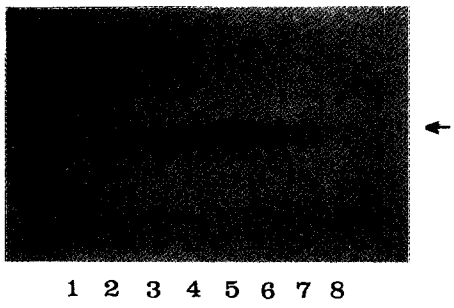

Fig. 2. SDS-PAGE analysis of the samples from phenyl-Sepharose column chromatography (just before the separation using Mono Q column). 12 μl samples out of 0.6 ml fractions were mixed with 3 μl of electrophoresis 5× sample buffer containing 5 mM EGTA and boiled for 3 min. 10 μl of the sample mixtures were loaded to the wells of 15% SDS-polyacrylamide gel containing 1 mM EGTA. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue. Lanes 1 to 8 : calmodulins from VU-3 transgenic tobacco plant. The arrow indicates the position of foreign VU-3 calmodulin.

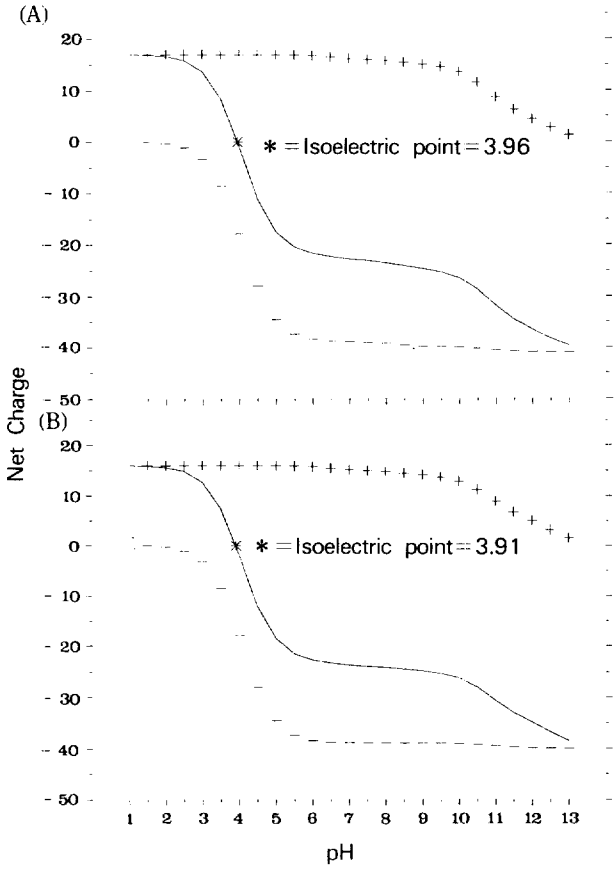

Fig. 3. Comparison of isoelectric plots of plant calmodulin and VU-3 calmodulin. Net charges as a function of pH for the plant calmodulin sequence (Lukas *et al.*, 1984) (A) and VU-3 calmodulin sequence (Roberts *et al.*, 1986) (B) were plotted by using the sequence analysis software package of the Genetic Computer Group (GCG) (University of Wisconsin Biotechnology Center). Plus and minus marks indicate the total positive and negative charges of the calmodulins as a funtion of pH, respectively. The solid lines indicate the net charges of the calmodulins as a function of pH. The asterisks indicate the isoelectric points of the calmodulins.

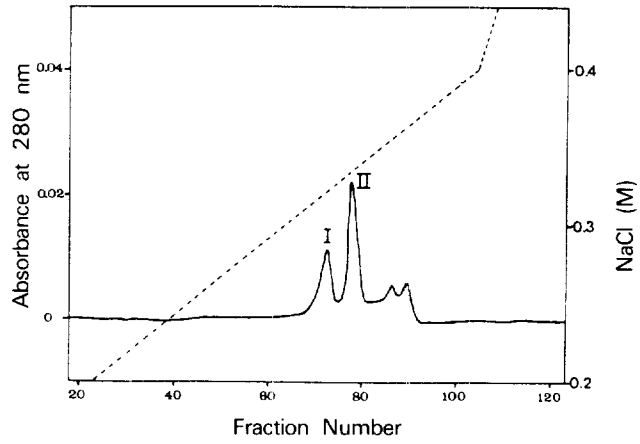

Fig. 4. Elution profile (50 min) of calmodulins on the Mono Q HR 5/5 column after injection of 17 μg of phenyl-Sepharose purified proteins. Flow-rate, 1.0 ml/min; fraction size, 0.3 ml/tube; starting buffer, A buffer containing 0.2 M sodium chloride. The column was eluted for 25 min with sodium chloride gradient from 0.2 M to 0.4 M (0 to 50% buffer B). Then, sodium chloride was increased to 0.6 M to remove the remaining proteins.

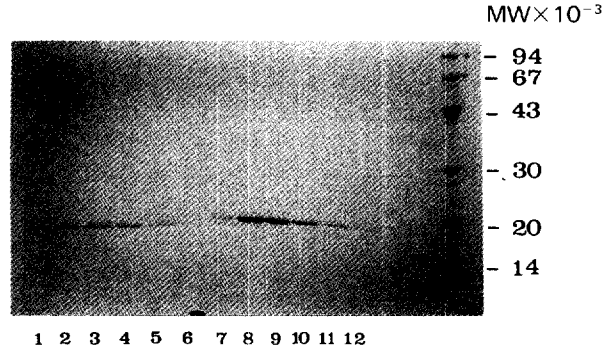

Fig. 5. SDS-PAGE analysis of the fractionated samples from Mono Q column. 12 μl samples out of 0.3 ml fractions (from #70 to #84 of Fig. 4) were mixed with 3 μl of 5× sample buffer and boiled for 3 min. Molecular weight marker proteins were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). Other conditions were the same as described in the legend in Fig. 2. Lanes 1 to 6 : calmodulin fractions (from #71 to #76) of Mono Q column; Lanes 7 to 12 : calmodulin fractions (from #77 to #82) of Mono Q column.

we kept in mind, in the choice of buffer pH, was that the molecule of interest should be stable and active at the pH. With this in mind, we chose Bis-Tris buffer system which has a useful pH range of 5.8~7.2. By examining the isoelectric plots (Fig. 3), we were able to choose a suitable pH for the fractionation using anion-exchange chromatography on a Mono Q column. At the buffer pH we used (pH 6.1), VU-3 calmodulin and plant calmodulin have calculated net charges of −23.1 and −21.9, respectively. Therefore, the charge group on the gel of Mono Q [$-CH_2-N^+-(CH_3)_3$] can selectively recognize the charge differences through

Table 1. Amino acid composition analysis of transgenic VU-3 tobacco calmodulin. Only key amino acid residues are compared. Amino acid composition analysis was done by using the general method of Bidlingmeyer *et al.* (1984). TML, trimethyllysine.

Amino acid	*E. coli* VU-3[a]	Transgenic VU-3[b]	Plant calmodulin[c]
arginine	6.2	6.2	5.0
lysine	8.4	8.2	9.0
glycine	11.1	11.0	10.0
threonine	10.6	10.2	9.0
TML	–	–	1.0

[a]Recombinant DNA-derived VU-3 calmodulin expressed in *E. coli*.
[b]Transgenic VU-3 tobacco calmodulin fractionated from endogenous tobacco calmodulin by Mono Q chromatography.
[c]Composition of plant calmodulin determined by amino acid sequence (Lukas *et al.*, 1984).

Table 2. Analysis of the methylation state of endogenous calmodulin from untransformed tobacco and VU-3 transgenic tobacco plants. The state of methylation was determined as described in Materials and Methods. Transgenic VU-3 and endogenous VU-3 calmodulin (Endo. VU-3) were obtained from the Mono Q chromatography of the purified calmodulin. Control W38, 37-day old untransformed tobacco plants; control VU-1, VU-1 calmodulin expressed in *E. coli*. The values in parenthesis represent the standard error of the mean.

Calmodulins	dpm	mole ^{3}H-methyl groups	mole of TML
Control W38	1,258	0.23 (0.01)	2.77
Endo. VU-3	1,586	0.29 (0.01)	2.71
Transgenic VU-3	–	–	0
Control VU-1	16,153	2.95 (0.10)	0

electrostatic interactions. To improve separation, a relatively shallower gradient was chosen by programming the liquid chromatography controller between 0.2 M and 0.4 M of sodium chloride. This change increased the slat gradient time from 20 min to 25 min (see the Materials and Methods for detail). As shown in Fig. 4, chromatography on Mono Q HR 5/5 column separated the sample proteins. SDS-PAGE analysis confirmed the separation and revealed that the two of them (peak #I and #II of Fig. 4) represent endogenous plant calmodulin and foreign VU-3 calmodulin, respectively (Fig. 5). Further evidence that the peak #II protein is VU-3 calmodulin was established by amino acid composition analysis that showed that this protein is indistinguishable from the recombinant DNA-derived VU-3 calmodulin expressed in *E. coli* (Table 1). Based on the mobility on SDS-PAGE, the two minor peaks seem to represent plant calmodulins or calmodulin isoforms (data not shown). However, the identities of the two minor peak proteins with calmodulins or calmodulin isoforms have to be established by using other approaches.

In order to test the effect of the VU-3 calmodulin introduction into tobacco cells on the plant calmodulin methylation system, the Mono Q fractionated endogenous calmodulin from VU-3 transgenic plants were tested for their ability to accept methyl groups using the radiometric assay (Oh and Roberts, 1990). Since VU-3 calmodulin is a competitive inhibitor for the calmodulin methyltransferase *in vitro*, the levels of methylation of endogenous calmodulin might be reduced in the VU-3 transgenic plants. However, the results show that there were no significant differences in the endogenous methylation compared with that of the calmodulin from normal untransformed plants (Table 2). One of the possible factors in the control of calmodulin methylation is the level of calmodulin methyltransferase present in the cells. At the present time, nothing is known regarding the levels and the properties of plant calmodulin methyltransferase. Future studies with the enzyme in plants may provide insight into what could be the factors controlling the degree of calmodulin methylation.

Overall, the present study suggests that anion-exchange chromatography is a good technique for the separation of transgenic VU-3 calmodulin from endogenous tobacco calmodulin. In addition, this separation facilitates biochemical analyses such as amino acid composition analysis and methylation analysis with the isolated calmodulins.

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