Analysis of Calmodulin Fragments and Preparation of the Peptides Containing Calcium Binding Domains by High Performance Liquid Chromatography

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The degradation process of calmodulin with trypsin in the presence or absence of calcium ions was reinvestigated by the analysis on HPLC. All tryptic peptides and intermediate peptides were mapped on this system. On the basis of these results, the strategy of the preparation of various calmodulin fragments was devised. Twelve peptides which contain calcium binding sites were obtained.

Keywords High performance liquid chromatography, non-ionic macroreticular resin, calmodulin, calcium binding domain, limited proteolysis

Calmodulin is a ubiquitous low molecular weight calcium binding protein in eukaryotes and plays an essential role in the regulation of cell functions by calcium ion.¹ The proteolytic fragmentation of calmodulin has been analyzed in the presence or absence of calcium ions by electrophoretic and chromatographic techniques.²⁻⁷ The isolated fragments of calmodulin are helpful for understanding the interaction of calmodulin with target enzymes or proteins^{3,4,6} and for measuring calcium binding activity.⁸⁻¹⁰

In this study, we reinvestigated in detail the analysis of all of the tryptic fragments of calmodulin obtained under various conditions, and mapped the peptides on HPLC using a non-ionic macroreticular polystyrene resin. Furthermore, we devised an efficient, controlled strategy for the preparation of peptides containing calcium binding domain(s)^{11,12} on the basis of these analyses. Further proteolytic fragmentation was performed on a peptide obtained by HPLC.

Materials and Methods

Calmodulin solution

Calmodulin was prepared by the method of Isobe *et*

Abbreviations. HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid bisodium salt.

*al.*¹³ from bovine brain. The purified calmodulin was dialyzed and lyophilized. The lyophilized calmodulin (21.3 mg) was dissolved in 10.6 ml of 0.2 M *N*-ethylmorpholine-acetate buffer (pH 8.0).

Other reagents

TFA (for amino acid sequence determination), acetonitrile (for liquid chromatography), EDTA, *N*-ethylmorpholine, acetic acid and hydrochloric acid were obtained from Wako Pure Chemical Industries. Calcium chloride dihydrate was obtained from E. Merck. TPCK-trypsin (238 U/mg, 91% protein; 3740 TRTPCK 30A872) was obtained from Millipore Corporation. Protease *S. aureus* V8 was obtained from Miles Laboratories, Ltd.

Preparation of cleaned test tubes

Washed test tubes (12×105 mm) were wrapped with aluminium foil and heated in an electric heater at 500°C for 3 h.

High performance liquid chromatography instruments

A non-ionic macroreticular polystyrene resin, Hitachi Gel #3013 (particle size 5 µm), was packed in a stainless steel column (4.6 mm i.d.×250 mm).¹⁴ The HPLC apparatus (Japan Spectroscopic Co., Ltd.) was composed of a high pressure pump (twinkle), a variable wavelength UV detector (UVIDEC 100-III) and a Rheodyne injector (sample loop 100 µl). The

Fig. 1 Elution position of tryptic peptides of calmodulin (A) and profile of convex gradient (B).

gradient maker was composed of a mechanical stirrer, a mixing chamber and a pump feeding the higher concentration solvent to the mixing chamber.

The convex gradient used here was formed as follows. Thirty ml of 10% acetonitrile in 0.1% TFA was pipetted into the mixing chamber. Fifty % acetonitrile in 0.1% TFA was pumped into the mixing chamber at a flow rate of 0.33 ml/min and mixed with the solvent in the mixing chamber with a mechanical stirrer. The mixed solvent in the mixing chamber was pumped into the column at a flow rate of 0.5 ml/min. The profile of the gradient is shown in Fig. 1B.

Separation of peptides

Each sample was injected to the Hitachi Gel #3013 column and was eluted by the convex gradient with 10 to 50% acetonitrile in 0.1% TFA at a flow rate of 0.5 ml/min at room temperature.¹⁵ The eluate was monitored on the basis of the absorption at 210 nm (0.64 AUFS). Each peak portion was collected in a cleaned test tube (12×105 mm).

Determination of amino acid composition of separated peptides

The collected fraction in a tube was dried up under nitrogen stream with gentle heating at 35–60°C. To the tube, 0.5 ml of 6 M HCl was added and sealed under reduced pressure. Hydrolysis was performed at 110°C for 24 h. The hydrolyzate was dried up in a desiccator under a reduced pressure and 0.25 ml of diluter was added. A 0.1 ml aliquot of the dissolved sample was analyzed with an automatic amino acid analyzer equipped with two column system. A Hitachi

KLA-5 amino acid analyzer was used with some modifications. Shodex ion exchanger HC-125s (Showa Denko Ltd.; particle size 12.5 µm) was packed in a glass column (8 mm i.d.×300 mm) for the separation of acidic and neutral amino acids and in a glass column (9 mm i.d.×40 mm) for basic amino acids. One cycle of analysis was carried out within 128 min.

Results and Discussion

Identification of intact calmodulin and tryptic peptides of calmodulin on the chromatogram

Calmodulin was eluted at 116 min, whether calmodulin was injected to the column with calcium ions or EDTA (Figs. 2A and 3A). Calmodulin probably could not bind calcium ions at the low pH values used here.¹⁶ Berchtold *et al.*¹⁷ reported that calmodulin was totally inactivated at low pH HPLC conditions. However, the purified calmodulin under the HPLC conditions used here retained calcium binding activity.⁸

Twenty two peptides were obtained by the tryptic digestion of calmodulin under various conditions. The peptides eluted from the column were collected and identified by amino acid analysis.⁷ The elution positions of tryptic peptides of calmodulin including intermediate peptides are shown in Fig. 1A. Two peptides 1–13 and 14–30 were eluted at the same position (49 min), while the peptide 1–30 was eluted at 82 min. Although the amino acid compositions of both peaks were identical, these peaks were distinguished by the following: (1) these peptides could be separated partially by slower gradient (Data not shown). (2) The peaks of 1–13 and 14–30 became higher while the peak of 1–30 was lower, depending on digestion period (Fig. 2 C–D). The peptides 38–74 and 78–148 also had the same retention time; both of them, however, were identified because the former was obtained in the absence of calcium ions and the latter was obtained in the presence of calcium ions.

Several workers have reported about the peptide map of calmodulin by HPLC on macroreticular ion-exchange resin^{18,19} or on alkyl bonded silica.²⁰ The peptide mapping described here was performed on a non-ionic macroreticular polystyrene resin. In contrast to the alkyl bonded silica, the non-ionic macroreticular resin is chemically stable and has large loading capacity. The same column used here for analysis could separate up to 2–5 mg of the digest of calmodulin. Since the solvent used was volatile, no desalting process was necessary for subsequent amino acid analysis, the modification of peptides, or the assay of calcium binding activity.

Analysis of tryptic degradation process of calmodulin by HPLC

Figures 2, B–D, show the chromatograms of calcium free calmodulin digested with trypsin for 1.3 min, 5 min

Fig. 2 Chromatograms of degradation process of calcium free calmodulin. Calcium free calmodulin was prepared by the addition of 10 μ l of 0.1 M EDTA to 1 ml of calmodulin solution (2 mg/ml). To this solution, 10 μ l of TPCK-trypsin solution (1 mg/ml) was added. After various incubation times, 100 μ l of this mixture was pipetted into a test tube containing 5 μ l of 6 M HCl. A, calcium free calmodulin; B, digested for 1.3 min; C, digested for 5 min; D, digested for 60 min.

and 60 min incubation, respectively. A 1.3 min digestion of calcium free calmodulin resulted in the peptides 1-106, 107-148, 1-37 and 38-148 (Fig. 2B). The yields of the peptides 1-37 and 38-148 were about 20% of that of the peptides 1-106 and 107-148. This suggests that the initial cleavage of calcium free calmodulin with trypsin occurred at Arg 106 and Arg 37 and that the rate of the cleavage at Arg 37 was about 20% of that at Arg 106.⁷ Walsh *et al.*³ reported that the initial cleavage of calcium free calmodulin occurred at Arg 106. However, they did not observe the cleavage at Arg 37. The yields of the peptides 1-37 and 38-148 were lower and it may be difficult to separate these peptides from the peptides 1-106 and 107-148 by SDS-Urea-Polyacrylamide gel electrophoresis. Newton *et al.*⁴ fractionated calmodulin fragments by HPLC after limited digestion. They obtained the peptides 107-148, 1-90 and 1-106 as three major peaks through the tryptic digestion of calmodulin under calcium free conditions and identified two minor peaks eluted between the peaks of 107-148 and 1-90 as the peptides 38-74 and 78-148 from their retention times. They concluded that the initial cleavage at Lys 77 also occurred under calcium free conditions without the detection of the peptide 1-77. Newton *et al.* also reported that the peptide 1-37 was detected under the tryptic digestion without calcium, but did not accumulate.⁴

At second stage of the degradation, the peptide 1-106 was cleaved at Arg 37, 74 and 90, resulting in the peptides 1-37, 1-90, 38-74, 75-106 and 91-106. The peptide 38-148 also was cleaved at Arg 37, 74 and 106, resulting in the peptides 38-74, 75-106 and 107-148 (Fig. 2C). The cleavage at Lys 30 occurred at a later stage. The peptide bonds of Arg 126 and Lys 13 were very resistant to trypsin attack (Fig. 2D). The peptide bonds of Lys 21 and 94 were not susceptible to trypsin.¹²

The degradation process of calcium bound calmodulin was quite different from that of calcium free calmodulin, as shown in Fig. 3. After 1 h digestion of calcium bound calmodulin at the same enzyme/substrate ratio as the digestion of calcium free calmodulin, calmodulin was degraded to 1-75, 1-74 and 78-148 (Fig. 3B). Upon binding of calcium, the change of initial cleavage site was induced. We could not obtain the peptide 1-77, in contrast to other workers²⁻⁶, possibly because of its low stability. The cleavage of N-terminal half molecule 1-77 at Lys 13, 30 and 77 and Arg 38 and 75 occurred after the cleavage at Lys 77. On the other hand, cleavable sites of C-terminal half molecule 78-148 kept resistant to trypsin attack after the cleavage at Lys 77 (Fig. 3C). Guerini *et al.*⁵ reported that the peptides 75-148 and 76-148 were partially produced by the tryptic digestion of calcium bound calmodulin and that the separation of these

Fig. 3 Chromatograms of degradation process of calcium bound calmodulin. Calcium bound calmodulin was prepared by the addition 100 μ l of 0.1 M CaCl_2 to 1 ml of calmodulin solution (2 mg/ml). Digestion was carried out under the same conditions as in Fig. 1. A, calcium bound calmodulin; B, digested for 60 min; C, digested for 5 days.

peptides, 75-148, 76-148 and 78-148 was achieved by ion-exchange HPLC on Mono-Q column. However, these peptides could not be separated by reverse phase HPLC on alkyl bonded silica or by hydrophobic interaction HPLC.⁵ The amino acid composition of the peak eluted at 95 min was consistent with the composition of 78-148⁷, but this peak may contain the minor contamination by 75-148 and 76-148.

Preparation of peptides containing calcium binding domain[s]

By the tryptic digestion of calcium free or calcium bound calmodulin, 12 peptides that contained the calcium binding domains were obtained. The yields and the digestion conditions of these peptides are summarized in Table 1.

The peptides which contain single calcium binding domain are 1-30, 1-37, 38-74, 91-106, 75-106, 107-148 and 127-148. The peptides 1-37 and 75-106 were obtained with a yield of about 30% by the digestion of

Table 1 Peptides containing calcium binding domain derived from calmodulin

Peptide	Domain	Yield ^a , %	Digestion condition	
			Time/min	Calcium
1-30	I	41 (25)	60	—
1-37	I	26 (28)	5	—
38-74	II	86 (80)	60	—
91-106	III	93 (72)	60	—
75-106	III	30 (25)	5	—
127-148	IV	36 (25)	60	—
107-148	IV	62 (40)	60	—
1-75	I,II	50 (38)	60	+
1-90	I,II	18	5	—
78-148	III,IV	93 (96)	60	+
1-106	I,II,III	42	5 ^b	—
38-148	II, III, IV	10	5 ^c	—

Calmodulin (2 mg/ml) was digested with TPCK-trypsin (enzyme/substrate ratio=1/100) at 37°C.

a. The yield was estimated on the basis of the absorbance at 210 nm considering the number of peptide bond in each fragment.²¹ The molar extinction coefficient at 210 nm for single peptide bond was 804. The figures in parentheses are the yields estimated by amino acid analysis.

b. enzyme/substrate ratio=1/10000.

c. enzyme/substrate ratio=1/1000.

calcium free calmodulin for 5 min at a enzyme/substrate ratio of 1/100. A longer digestion is not recommended to prepare these peptides. The other five peptides containing single domain could be obtained by the digestion of calcium free calmodulin for 60 min at a enzyme/substrate ratio of 1/100 in relatively high yields (Table 1).

The peptides 1-77, 1-90 and 78-148 contain two calcium binding domains. The peptides 78-148 and 1-75 were produced by the digestion of calcium bound calmodulin for 60 min at a enzyme/substrate ratio of 1/100. The peptide 78-148 was stable, but not 1-75. Accordingly, the digestion period of less than 60 min is recommended in order to obtain both fragments at one time. The peptide 1-90 could be obtained by the digestion of calmodulin in the presence of magnesium ions, because the peptide 1-90 was more stable than the peptide 1-106 under such a condition.⁷

The peptides 1-106 and 38-148, which contain three domains (I, II and III or II, III and IV, respectively), were obtained by the limited digestion of calcium free calmodulin. To obtain these labile fragments with a relatively high yield, the effect of enzyme/substrate ratio on the limited digestion was examined (Table 2). The peptide 1-106 was slowly degraded to 1-90 and 91-106 or 1-37, 38-74 and 75-106 (see Fig. 2). In order to obtain the pure peptide of 1-106, the digestion of calmodulin must be controlled so that 1-90 is not produced, because it was eluted near the peak of 1-106. As shown in Table 2, by the digestion at a

Table 2 Effect of trypsin amount

Peptide	Domain	Enzyme/substrate ratio		
		1/10000	1/1000	1/100
		Yield, %		
1-106	I-II-III	42	56	10
38-148	II-III-IV	3	10	0
91-106	III	0	12	31
1-148	I-II-III-IV	78	19	0

TPCK-trypsin was added to calmodulin solution at enzyme/substrate ratio of 1/100, 1/1000 and 1/10000. After 5 min digestion at 37°C, 6 M HCl was added to stop the digestion. These digests were analyzed by HPLC according to the method described in Materials and Methods. The amount of each fragment was estimated on the basis of the absorbance at 210 nm, as described in Table 1.

Fig. 4 Digestion with *S. aureus* V8 protease of the peptide 38-74 obtained by the tryptic digestion. The peptide 38-74 (25 nmole) was dissolved in 0.5 ml of 0.2 M *N*-ethylmorpholine-acetate buffer (pH 8.0). To this solution, 50 μ l of 0.1 M CaCl_2 and 15 μ l of *S. aureus* V8 protease solution (0.2 mg/ml) were added. This mixture was incubated at 37°C for 10 min, and lyophilized after addition of 25 μ l of 6 M HCl. The digest was dissolved in 100 μ l of distilled water and was separated by HPLC under the conditions described in Materials and Methods.

enzyme/substrate ratio of 1/10000 for 5 min, 1-106 was obtained in a relatively high yield, and 91-106 and 1-90 were still not detected. The peptide 38-148 was obtained by the limited digestion of calcium free calmodulin for 5 min at a enzyme/substrate ratio 1/1000 in a yield of 10% (Table 2).

By the combination of enzymatic digestion and HPLC separation, it became possible to prepare various calcium binding peptides of calmodulin. The calcium binding activity of these peptides will be

described elsewhere.

Subsequent enzymatic degradation of a calcium binding peptide

The isolated calcium binding peptide can be modified by the subsequent digestion. Figure 4 shows the partial digestion of the peptide 38-74 by *S. aureus* V8 protease. The resulting peptides were 38-67, 46-74 and 55-74. The yield of these peptides was about 10% each. The peptide 46-74 contains only a helix-loop-helix region.^{11,12}

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