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Application of high-performance capillary electrophoresis to the analysis of conformation and interaction of metal-binding proteins

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ABSTRACT

A separation method using high-performance capillary electrophoresis was applied to the analysis of calcium- and zinc-binding proteins. Calcium-binding proteins (calmodulin, parvalbumin, thermolysin and proteolytic peptides of calmodulin), zinc-binding proteins (carbonic anhydrase and thermolysin), and internal standard proteins (carbonic anhydrase and lactoglobulin) were separated completely by capillary electrophoresis. Calcium- and zinc-binding proteins were obtained under Ca^{2+} and Zn^{2+}-containing conditions, respectively, cation-chelating conditions for the binding shift assay, and they showed that the binding shift depended on cations in the electrophoresis buffer in capillary zone electrophoresis and micellar electrokinetic chromatography. Two kinds of hydrophobic probes affected the electrophoretic mobility of calmodulin by interaction between its hydrophobic region and the hydrophobic probes under Ca^{2+}-containing conditions.

INTRODUCTION

High-performance capillary electrophoresis (HPCE) is developing rapidly and there are numerous demonstrating highly efficient separations of proteins and peptides [1]. Charged species will migrate electrophoretically towards the appropriate electrode and are separated by differences in their electrophoretic mobilities [2]. The rigorous description of the relationship between electrophoretic mobility and solute and solvent properties is given by the following equations [3,4]:

$$\mu = \frac{V}{E} = \frac{q}{6\pi r\eta}$$

where μ is the electrophoretic mobility, V is the migration velocity, E is the field strength, q is the net charge on the analyte, η is the solvent viscosity and r is the apparent Stokes radius of the analyte. Therefore, the electrophoretic mobility observed in a given solvent depends on the net charge and the apparent Stokes radius of the analyte.

Relatively new electrophoretic separation techniques, capillary zone electro-

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phoresis (CZE) [5] and micellar electrokinetic chromatography (MEKC) [6], are suitable fot the analysis of biological compounds. However, there has been no study concerning the conformation and the interaction of biological molecules such as calcium-binding proteins (CaBPs) using HPCE. It is clear that CaBPs are of importance as physiological regulators [7]. Studies from a number of laboratories have shown that many regulatory activities of Ca^{2+} ions are mediated by a signal CaBPs [8]. These proteins have remained highly conserved in structure and function [9]. Metal ions have an important role in biological reactions, *e.g.*, Zn^{2+} was shown to be essential for the active site of carbonic anhydrase [10] and thermolysin [11]. Herein, we report that the behaviour of CaBPs and zinc-binding proteins (ZnBPs) depends on cations and the interaction between the hydrophobic probes and the hydrophobic region on calmodulin (CaM) in HPCE. A preliminary report of the calcium-binding shift assay of CaBPs has been made [12].

EXPERIMENTAL

Reagents and materials

CaM (bovine testes) was obtained from Polyscience, parvalbumin (PV) (rabbit muscle), carbonic anhydrase (bovine erythrocyte) and lactoglobulin (bovine milk) from Sigma, arginylendopeptidase from Takara Shuzo, thermolysin (*Bacillus thermoproteolyticus* Rokko) from Seikagaku Kogyo, ethylenediaminetetraacetic acid (EDTA) from Dojin Chemical, N-[tris(hydroxymethyl)methyl]glycine (tricine) from Merck and N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7) and 10-[3-(4-methylpiperazin-1-yl)propyl]-2-trifluoromethylphenothiazine (trifluoperazine) from Nacalai Tesque. All other chemicals were purchased from Wako.

Method

The protein runs were carried out in a fused-silica capillary of 50 μm I.D. and total length 122 cm (100 cm to the detector), which was filled with an electrophoresis buffer of 0.1 *M* tris(hydroxymethyl)aminomethane (Tris)–0.1 *M* tricine (pH 8.3) [13] or 50 m*M* Tris–384 m*M* glycine [14] (pH 8.3) with several additives (2 m*M* calcium chloride, zinc chloride and EDTA) for CZE. For MEKC, 0.1% sodium dodecyl sulphate (SDS) was added to 0.1 *M* Tris–0.1 *M* tricine (pH 8.3). Proteins (2.5 mg/ml) were dissolved in the electrophoresis buffer. To study the interaction between the hydrophobic probes (trifluoperazine or W-7) and the hydrophobic region on CaM, 50 μM of hydrophobic probes was added in the electrophoresis buffer. Detection was by UV absorption at 200 nm. A sample was introduced into the capillary by vauum injection for 1.0 s after washing with 1.0 *M* sodium hydroxide solution for 15 min and the electrophoresis buffer for 15 min, and electrophoresis was carried out at 30 kV (Applied Biosystems Model 270A).

RESULTS AND DISCUSSION

Binding shift assay in CZE

To obtain better separation and analytical efficiencies for proteins and peptides, three kinds of electrophoresis buffers, Tris–tricine, Tris–glycine and Tris–borate [15], were used for CZE. Two standard proteins (carbonic anhydrase and lactoglobulin)

and cation-binding proteins were separated well with Tris–tricine and Tris–glycine buffers (Fig. 1). However, Tris–borate buffer showed an unstable separation of proteins in the presence of EDTA in the electrophoresis buffer (data not shown).

The electropherogram for the CZE analysis of the Ca-binding shift assay is shown in Fig. 1. The first five peaks (1–5) and three other peaks (6–8) represented carbonic anhydrase and lactoglobulin, respectively. Although the broadening of lactoglobulin peaks occurred in this system, the accuracies of the migration time of two standard proteins were preserved. The peak of CaM was observed at 32.79 min in the presence of Ca^{2+} ions in a CZE separation, but the peak of CaM was observed at 44.42 min under Ca^{2+}-chelating conditions without any significant change of the

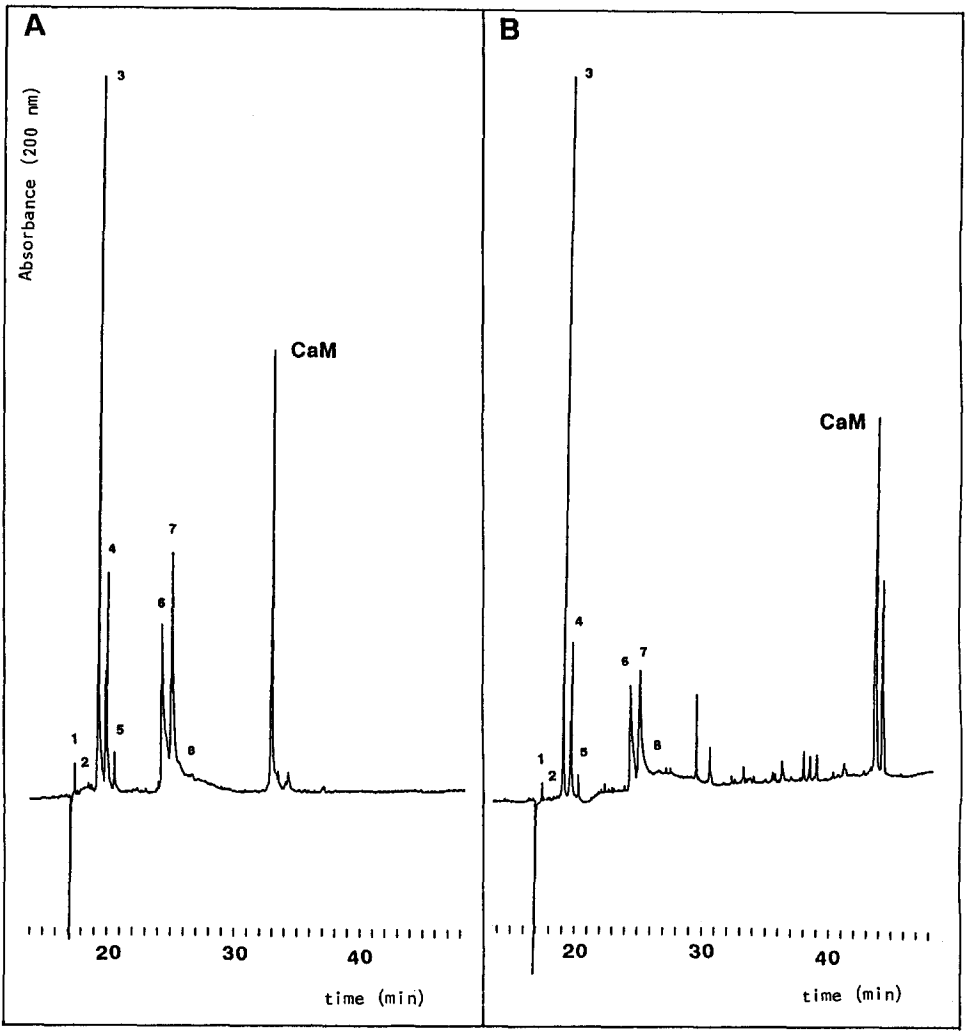

Fig. 1. CZE of carbonic anhydrase (1–5), lactoglobulin (6–8) and CaM for calcium-binding shift assay. Each protein (2.5 mg/ml) was dissolved in Tris–tricine electrophoresis buffer. (A) 2 m*M* calcium chloride in the electrophoresis buffer; (B) 2 m*M* EDTA in the electrophoresis buffer.

migration times of standard proteins. The migration velocity of CaM depended on the Ca^{2+} ions in the electrophoresis buffer, and it increased by Ca^{2+} binding to CaM. The migration velocity of CaM was altered through complexes of calcium-binding sites and Ca^{2+} ions. The complexing altered the net charge on CaM and the Stokes radius of CaM caused by conformational changes. The factor $k(+/-)$, the ratio of the net charge on protein between the Ca^{2+}-containing conditions and the Ca^{2+}-chelating conditions, can be calculated by the following equation:

$$k(+/-) = \frac{q(+)}{q(-)} = \frac{V(+)r(+)}{V(-)r(-)} = \frac{t(-)r(+)}{t(+)r(-)}$$

where + and − represent the presence and absence of Ca^{2+} ions in the electrophoresis buffer, respectively, and t is the migration time of CaM. The Stokes radius of CaM was 2.09 and 2.14 nm in the presence and absence of Ca^{2+} ions, respectively, in CaM [16]. The binding shift of CaM depended mainly on the ratio of the net charge on CaM between the Ca^{2+}-containing conditions and the Ca^{2+}-chelating conditions rather than the ratio of the Stokes radius of CaM between them, because there was only a 2% variance of the Stokes radius between the two sets of conditions. The electroendo-osmotic velocity might not be changed significantly because carbonic anhydrase and lactoglobulin showed same migration times under both conditions. The same phenomenon, a binding shift of CaM in Tris–glycine buffer (data not shown), was also observed. Several experiments on the calcium-binding shift assay of CaM as a function of Ca^{2+} concentration were performed between 2 and 0.2 m*M* calcium chloride, but there was no significant change in behaviour of the calcium-binding shift of CaM (data not shown).

The peak of PV was also shifted on addition of Ca^{2+} ions to the electrophoresis buffer (Fig. 2). The peak of PV was detected between five carbonic anhydrase peaks and three lactoglobulin peaks under Ca^{2+}-chelating conditions, but the peak of PV that contained three Ca^{2+} ions in its binding sites was detected between the peaks 3 and 4 of carbonic anhydrase.

Bovine CaM consists of 148 amino acids and has four specific calcium-binding sites [9]. Two calcium-binding sites (domains 1 and 2) out of the four could be isolated intact by arginylendopeptidase digestion. Four of the starred peaks in Fig. 3 disappeared completely from the electropherogram in the presence of Ca^{2+} ions in the electrophoresis buffer. A possible explanation of this disappearance is that the peptides that bound Ca^{2+} ions changed the net charge or the Stokes radius of the peptide, and therefore the migration velocity or migration direction of the peptide drastically changed. Peptide fragments obtained with trypsin (this protease did not preserve any calcium-binding sites of CaM) did not show the variance in eletropherograms between the two sets of conditions (data not shown).

Fig. 4 shows the Zn^{2+}-dependent variance for carbonic anhydrase. Higher peaks were obtained in the presence of Zn^{2+} ions in the electrophoresis buffer. An increase in the two peaks was not observed on addition of calcium chloride or sodium chloride to the electrophoresis buffer (data not shown). However, carbonic anhydrase did not show the complete binding shift that was observed in the calcium-binding shift assays of CaM and PV. Inactivation of protein by denaturation or a weak affinity of the active site for Zn^{2+} ions of carbonic anhydrase prevented the transition of the peak from a complete binding shift.

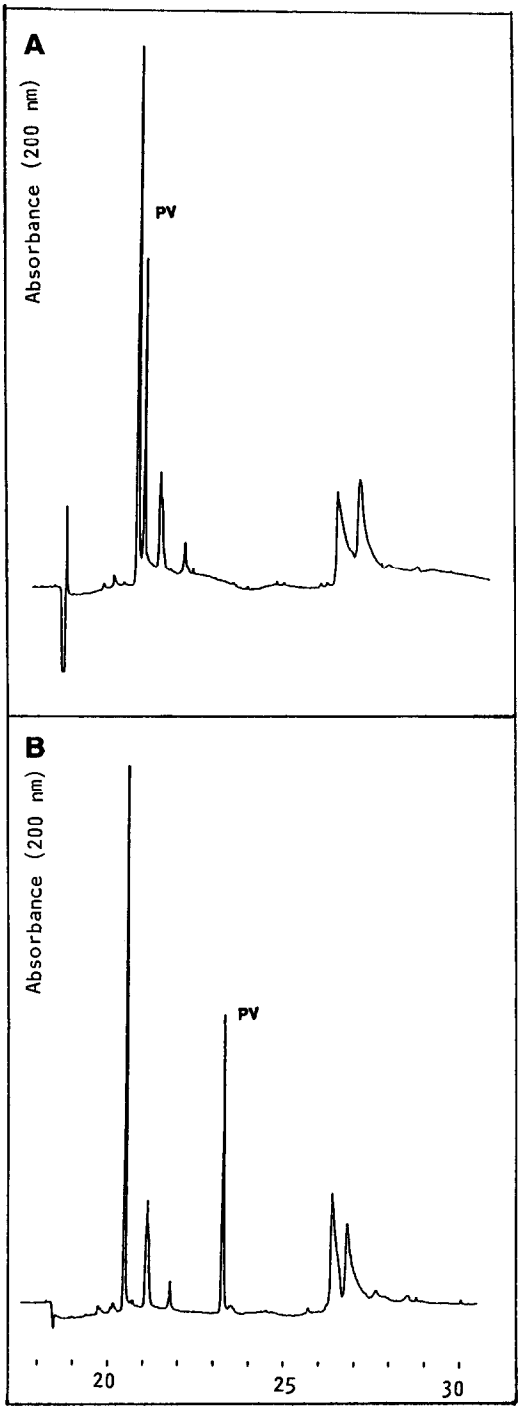

Fig. 2. CZE of carbonic anhydrase, lactoglobulin and PV for calcium-binding shift assay. Each protein (2.5 mg/ml) was dissolved in Tris–tricine electrophoresis buffer. (A) 2 m*M* calcium chloride in the electrophoresis buffer; (B) 2 m*M* EDTA in the electrophoresis buffer.

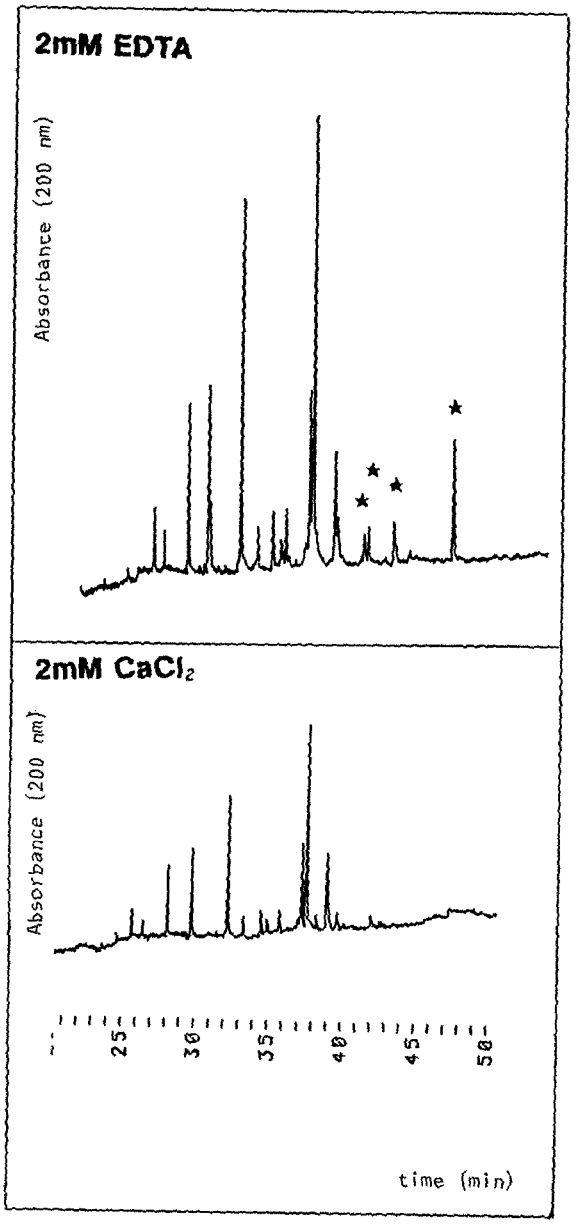

Fig. 3. Peptide mapping of proteolytic fragments of CaM by arginylendopeptidase in CZE. Top, 2 m*M* EDTA; bottom, 2 m*M* calcium chloride.

Thermolysin is a metalloproteinase that binds four Ca^{2+} ions for stability of conformation and one Zn^{2+} ion for the active site [11]. This protease is stable in 1% SDS or 8 *M* urea, and the proteinase activity is preserved. In the presence of EDTA, thermolysin was detected as a broad peak in a Tris–tricine electrophoresis buffer

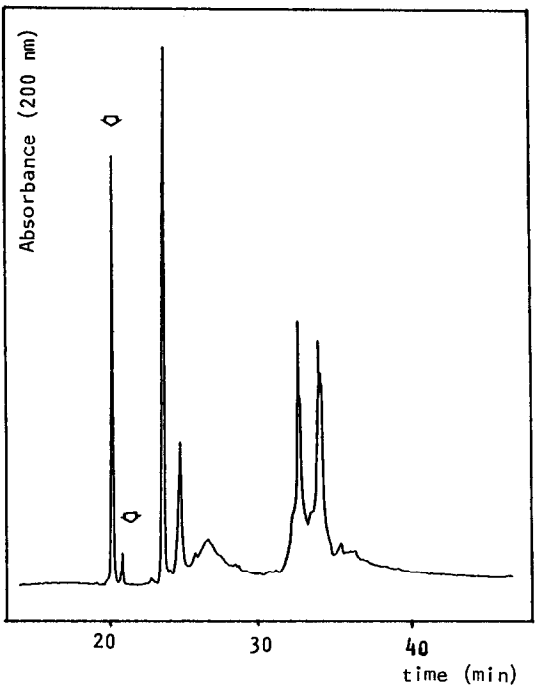

Fig. 4. Partial binding shift of carbonic anhydrase in CZE with 2 m*M* zinc chloride in the electrophoresis buffer. Arrowhead peaks increased in height.

system (Fig. 5). This broadening of the thermolysin peak might reflect the instability of the protein conformation. When 2 m*M* zinc chloride was added to the electrophoresis buffer, the peak of thermolysin varied and relatively sharp peaks were detected. Ca^{2+} ions changed the electropherogram drastically: the peak appeared as a single, sharp peak in the front of the endoosmotic flow peak. In the presence of both cations, the electropherogram did not change significantly and it showed the same electrophoresis pattern as for the addition of Ca^{2+} ions in the electrophoresis buffer. A drastic conformational change occurred because of binding of Ca^{2+} ions and this caused a drastic change in the net charge of thermolysin. These electropherograms for calcium- and zinc-binding shift assays indicate that Ca^{2+} and Zn^{2+} ions were essential for the thermolysin to create an appropriate active site and conformation.

Binding shift assay in MEKC

Variance of the electrophoretic mobility of CaM was also observed in MEKC (Fig. 6). The conformation of CaM remained even in the presence of 0.1% SDS and the migration velocity was greatly changed between the Ca^{2+}-containing conditions and the Ca^{2+}-chelating conditions. This change in electrophoretic mobility might be mainly affected by the hydrophobicity rather than the net charge or the Stokes radius of CaM.

PV also showed the calcium-binding shift in MEKC, although the binding shift

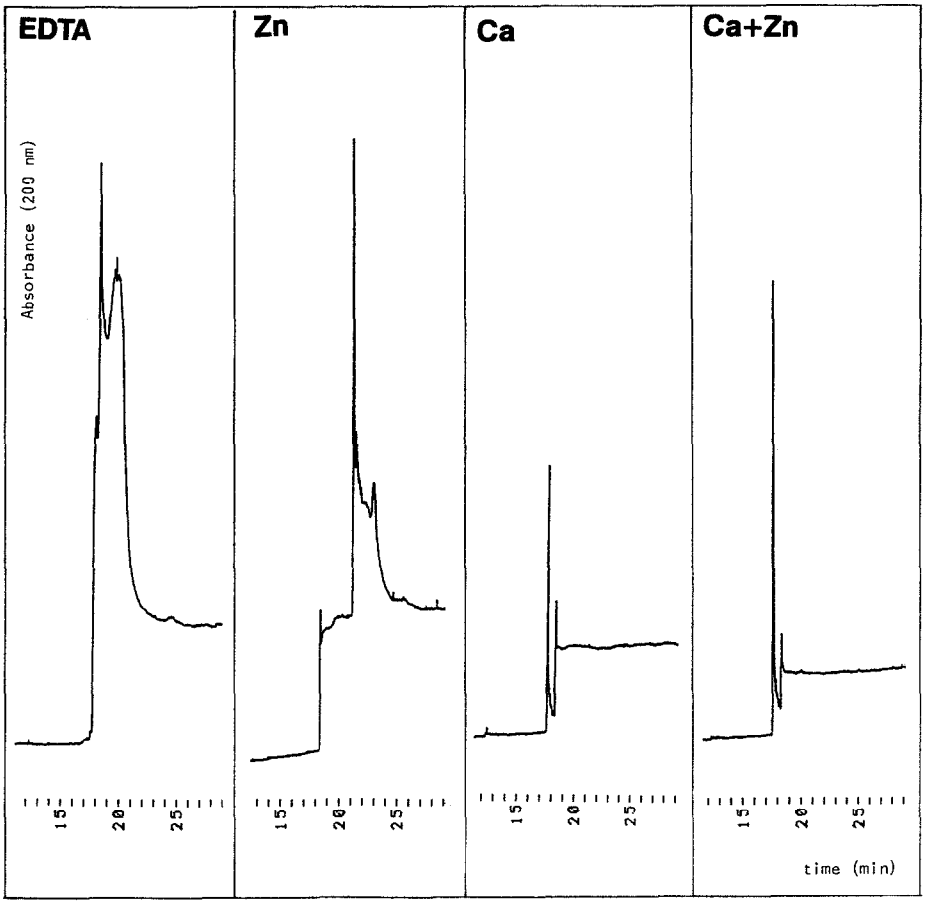

Fig. 5. CZE of thermolysin in the presence of 2 m*M* EDTA, 2 m*M* zinc chloride and 2 m*M* calcium chloride.

of PV in MEKC was less than that of CaM (Fig. 7). These results suggested that the hydrophobicity of PV was not changed so much by Ca^{2+} binding to PV.

A binding shift assay in MEKC was performed for thermolysin (Fig. 8). Under cation chelating conditions, the peak of thermolysin was detected after 80 min in electrophoresis runs. In the presence of Ca^{2+} ions, the migration time of thermolysin drastically changed and the peak of thermolysin was detected at the front of the water peak in MEKC containing 0.1% SDS. The migration velocity of thermolysin was faster than that of the water peak in the presence of 0.1% SDS. When the micellar concentration in the electrophoresis buffer was increased, the peak of thermolysin shifted backwards, depending on the concentration of SDS.

Interaction between hydrophobic probes and the hydrophobic region on CaM

As shown in Fig. 6, the appearance of a hydrophobic region on the surface of CaM by binding of Ca^{2+} ions was suggested by the binding shift assay of CaM in

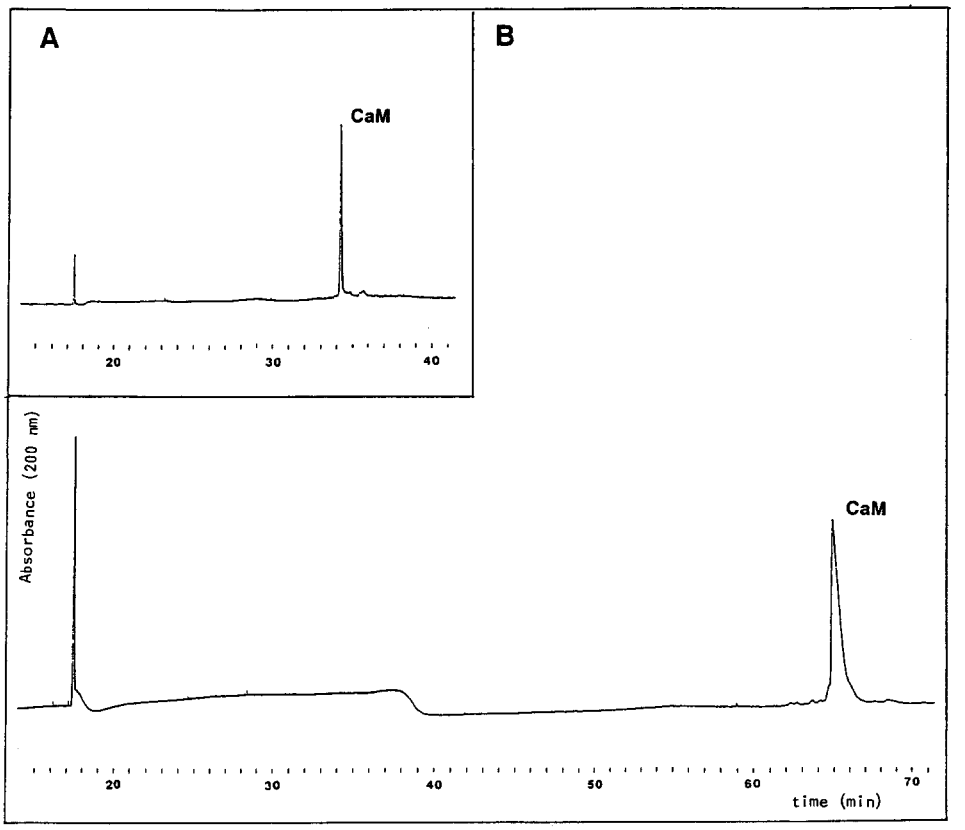

Fig. 6. Binding shift assay of CaM in MEKC. (A) 2 m*M* calcium chloride in the electrophoresis buffer; (B) 2 m*M* EDTA in the electrophoresis buffer.

MEKC. Two kinds of hydrophobic probes were mixed with the electrophoresis buffer (50 μM) and both conditions of Ca^{2+}-containing and Ca^{2+}-chelating conditions were compared (Table I). Only in the presence of Ca^{2+} ions did a hydrophobic region appear on the surface of CaM and the interaction between hydrophobic probes and the hydrophobic region on CaM could be observed. Therefore, a shift of the CaM peak occurred in the presence of Ca^{2+} ions, and the interaction between each standard protein and the hydrophobic probes must be same whether the hydrophobic probes interacted with standard proteins or not. The means of the migration time shown in Table I represent at least five repeated HPCE experiments; two major peaks of carbonic anhydrase (3 and 4) and lactoglobulin (6 and 7) (Fig. 1) were selected for the calculation as reference peaks.

The ratio of the migration time in the presence and absence of trifluoperazine, Ca/Ca(Trif), indicated the shift being caused by hydrophobic probe under Ca^{2+}-containing conditions. Theoretically, the difference in migration time, Ca/Ca(Trif) − EDTA/EDTA(Trif), of carbonic anhydrase and lactoglobulin must be 0% because of the identical interactions between the protein and the hydrophobic probes. The value

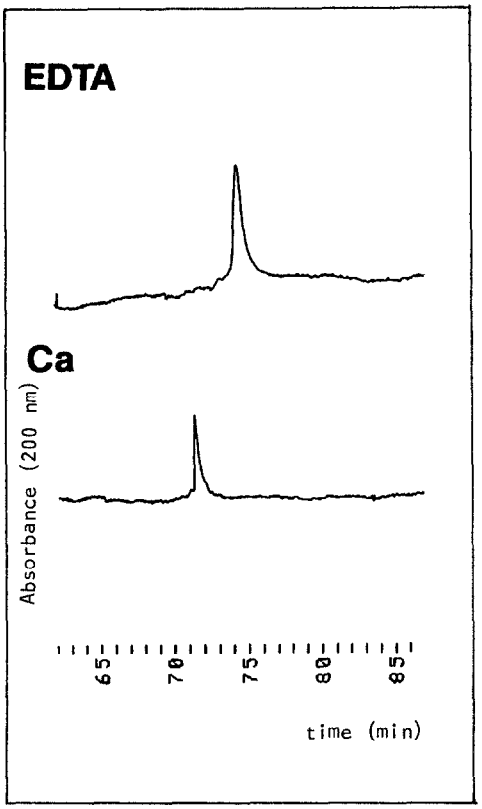

Fig. 7. Calcium-binding shift assay of PV in MEKC. The migration time of PV was 71.34 and 74.34 min in the presence of calcium chloride and EDTA, respectively.

of Ca/Ca(Trif) − EDTA/EDTA(Trif) was, however, −0.64%, 0.47%, 0.72% and 0.42% for the reference peaks, but the value was 1.22% for CaM.

A similar calculation was performed to investigate the interaction between CaM and W-7. The value of Ca/Ca(W-7) − EDTA/EDTA(W-7) was 2.03% for CaM. According to the calculations, a significant interaction between the hydrophobic probes and the hydrophobic region on CaM occurred in HPCE.

CONCLUSIONS

CaBPs (CaM, PV and thermolysin) and ZnBPs (carbonic anhydrase and thermolysin) varied the migration velocity depending on suitable cations in the electrophoresis buffer in CZE and MEKC. An interaction between the hydrophobic region of CaM and the hydrophobic probes was observed in CZE. The binding shift assay in HPCE is a simple and rapid technique for the analysis of conformation and interaction of proteins.

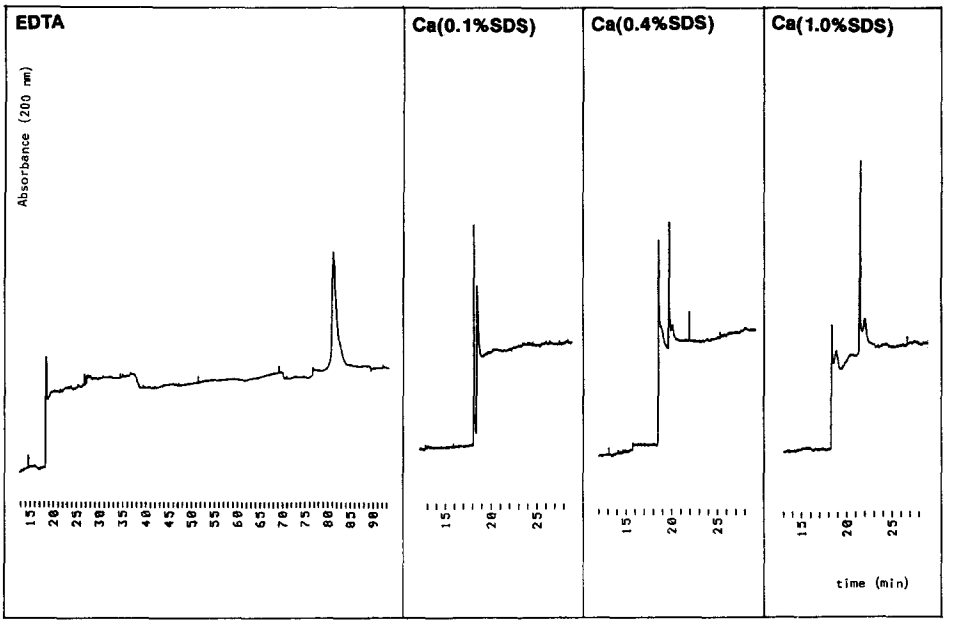

Fig. 8. Cation-binding shift assay of thermolysin in MEKC. EDTA or calcium chloride (2 m*M*) was added to the electrophoresis buffer. The concentration of SDS was 0.1%, 0.4% and 1.0%.

TABLE I

BINDING SHIFT ASSAY OF CaM DEPENDING ON THE INTERACTION BETWEEN THE HYDROPHOBIC PROBES, TRIFLUOPERAZINE (Trif) AND W-7, AND THE HYDROPHOBIC REGION ON CaM

Parameter	Carbonic anhydrase 1	Carbonic anhydrase 2	Lacto-globulin 1	Lacto-globulin 2	Calmodulin
Ca	19.126 min	19.802 min	24.007 min	24.949 min	32.789 min
EDTA	18.891 min	19.544 min	23.958 min	24.994 min	44.415 min
Ca/EDTA	100.58%	100.66%	99.55%	99.17%	73.34%
Ca(Trif)	17.801 min	18.411 min	22.424 min	23.295 min	29.735 min
EDTA(Trif)	18.842 min	19.504 min	24.309 min	25.273 min	43.962 min
Ca(Trif)/EDTA(Trif)	101.23%	101.14%	98.84%	98.75%	72.47%
Ca/Ca(Trif)	100.14%	100.26%	99.79%	99.83%	102.78%
EDTA/EDTA(Trif)	100.78%	100.73%	99.07%	99.41%	101.56%
Ca/Ca(Trif) − EDTA/EDTA(Trif)	−0.64%	0.47%	0.72%	0.42%	1.22%
Ca(W-7)	19.229 min	19.909 min	24.331 min	25.308 min	32.602 min
EDTA(W-7)	19.002 min	19.677 min	24.220 min	25.268 min	45.020 min
Ca(W-7)/EDTA(W-7)	100.44%	100.43%	99.72%	99.41%	71.88%
Ca/Ca(W-7)	100.42%	100.42%	99.63%	99.54%	101.54%
EDTA/EDTA(W-7)	100.28%	100.17%	99.77%	99.77%	99.51%
Ca/Ca(W-7) − EDTA/EDTA(W-7)	0.14%	0.25%	−0.14%	−0.23%	2.03%

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