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Using capillary electrophoresis with laser-induced fluorescence to study the interaction of green fluorescent protein-labeled calmodulin with Ca^{2+}- and calmodulin-binding protein

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Abstract

A separation using capillary electrophoresis with laser-induced fluorescence (CE-LIF) was applied to the study of green fluorescent protein tagged calmoldulin (GFP-CaM) that was expressed from *Escherichia coli* and purified with Ni^{2+}-nitrilotriacetate (Ni-NTA) resin column. It was found that GFP-CaM not only has good fluorescence properties under various conditions similar to GFP, but also retains its calcium-binding ability as the native CaM. GFP-CaM was separated and detected by CE-LIF within 10 min with a limit-of-detection (LOD) of 2×10^{-10} M for an injection volume of 3 nl, higher than that of common chemical fluorescent-tagged protein method. The results indicated that, as a fluorescence probe, GFP could overcome the drawback of inefficient derivatization of chemical fluorescence probes. The interaction between the GFP-CaM and Ca^{2+} was studied in detail using affinity capillary electrophoresis with laser-induced fluorescence and the dissociation constant (K_d) between GFP-CaM and Ca^{2+} was determined to be 1.2×10^{-5} M, which is in good agreement with the literature values of untagged CaM (10^{-6} to 10^{-5} M) obtained by conventional method. As a preliminary application, the interaction between GFP-CaM and OsCBK was also investigated. The method makes it possible to screen the trace amounts of target proteins in crude extracts interacting with CaM under physiological conditions.

Keywords: Calmodulin; Proteins

1. Introduction

Ca^{2+} serves as a second messenger in the transduction of environmental stimuli in a variety of organisms. Ca^{2+}-dependent modulation of cellular processes occurs via intracellular Ca^{2+}-binding proteins, of which calmodulin (CaM) is one of the best characterized. CaM binds four calcium ions with high affinity and acts as an intracellular calcium sensor that translates the Ca^{2+} signal into a variety of cellular processes [1–3]. Ca^{2+}-CaM binds to a short peptide within target proteins, thereby altering their activities in response to changes in cytosolic Ca^{2+} concentration. Calcium binding induces a conformational change that enables Ca^{2+}-CaM to recognize and bind target proteins with high affinity ($K_d = 10^{-11}$ to 10^{-7}) [2], so it is crucial to understand the Ca^{2+}-CaM signal pathways to identify the proteins interacting with Ca^{2+}-CaM. The traditional methods used to demonstrate CaM target proteins include activity assay, binding assay, cloning via ligand binding and surface plasmon resonance (SPR) analysis [1,4]. Although the traditional methods have been well established, those techniques typically are laborious, time-consuming, and difficult-to-automate. In addition, the activity assay requires radioactive materials. These drawbacks may be avoided by the use of capillary electrophoresis.

Compared with those conventional methods, the features of capillary electrophoresis (CE), such as small sample volume, rapid analysis time, high resolving power and direct injection of biological samples, make it an attractive technique. Affinity capillary electrophoresis (ACE) [5] is generally performed in a homogeneous solution without stabilizing agents and with a more or less physiological composition so that proteins can maintain their native and functional forms. Hence, ACE has been successfully extended to study biomolecular interactions such as protein–protein [5–7], protein–DNA [8], protein–drug [9] and protein–lipid interactions [10]. ACE is applicable to both tight and weak-binding systems and is a useful tool

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for the quantitative measurement of binding constants, estimation of kinetic rate constants, and determination of stoichiometries of protein interactions [11,12].

Furthermore, CE-LIF, as a highly sensitive technique, could greatly increase the detection limits [13,14]. Although fluorescence derivation can allow for the low concentration analysis of proteins by CE, major problems in the fluorescent derivatization of proteins are inefficient chemistry and multiple derivatives. If the analytes of interest contain more than one reactive site, multiple products are produced differing in the number and spatial orientation of the covalently bound probe [15]. Another disadvantage of chemical fluorescence probes is that labeling of analytes might change the interaction behavior of the solutes under investigation and influence the binding site of the protein. These occurrences may influence sensitive detection in ACE. Thus it is essential for an ideal system in which components are detected in their native form without a chemical tagging process that may disturb the cellular environments. Recently a group of natively fluorescent proteins have been applied in molecular and cellular studies [16–20] and can be detected by CE-LIF [21–24]. One of them, green fluorescent protein (GFP) could be used for this purpose.

Since GFP was firstly isolated from the jellyfish *Aequorea victoria*, it has become one of the most widely studied and exploited proteins in biochemistry and cell biology [16–20]. Its amazing ability to generate a highly visible, efficiently emitting internal fluorophore is both intrinsically fascinating and tremendously valuable. Because exogenous substrates and cofactors are not required for its fluorescence, GFP has become well established as a marker of gene expression and tagging in living cells and organisms [16]. There are many reviews on the application of GFP, namely its use in protein tagging and in monitoring gene expression as well as its potential in a variety of biological screening techniques [17–20]. Another advantage of GFP is that its excitation spectrum within the visible range has an excitation line compatible with the frequently used argon ion laser, so it could be used as a fluorescent probe in CE. Analysis of GFP by CE-LIF has been reported [21–24].

Here we report our study on the fluorescent and electrophoretic properties of GFP-CaM by CE-LIF. The quantitation of GFP fusion protein was first done by CE-LIF and the interaction between GFP-CaM and Ca^{2+} was also studied in detail. As a preliminary application, the interaction between GFP-CaM and OsCBK (a CaM-binding protein) was also investigated.

2. Materials and methods

2.1. Chemicals and samples

All chemicals used were of analytical grade and all solutions were prepared with autoclaved water (Millipore-Q, Millipore, Bedford, MA, USA). Sodium dodecyl sulfate (SDS) was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and used at the concentration of 0.2 M. The OsCBK gene was isolated from rice and its protein was obtained from our Lab [4].

2.2. Cloning

The coding sequence of GFP in pET32a was amplified by PCR from the plasmid pBin35S-mGFP4 with 5′ primer (5′-CG GGA TCC AAG GAG ATA TAA CAA TG-3′) and 3′ primer (5′-CG GGA TCC TT GTA TAG TTC ATC CAT GGA-3′). The PCR products were digested by *Bam*H I and cloned into the expression vector pET32a as pGFP. The coding sequence of CaM was amplified by PCR with 5′primer (5′-CCC AAG CTT CTC GCT CTC TTC CTC GCT ATG GCG GAT CAG CTC ACC-3′) and 3′primer (5′-CCC AAG CTT CTT GGC CAT CAT CAT GAC CT-3′), digested with *Hin*d III, and cloned into pGFP as pGFP-CaM, resulting in the fusion protein of GFP-CaM expressed in this construct.

2.3. Expression and purification of GFP-CaM and GFP

The expression and purification of proteins (GFP and GFP-CaM) were performed as described previously [4]. Briefly, the constructs (pGFP and pGFP-CaM) were introduced into *Escherichia coli* BL21 (DE3) and both GFP and GFP-CaM were purified with Ni-NTA affinity chromatography. After dialyzation against 25 mM Tris–HCl, pH 7.5, both GFP and GFP-CaM were separated by SDS–PAGE (12% gels). The concentrations of GFP and GFP-CaM were determined by the Bradford method [25].

2.4. Ca^{2+}-binding assay of GFP-CaM and CaM

For Ca^{2+}-dependent electrophoretic mobility shift assay of GFP-CaM and CaM, the proteins in non-denaturing sample buffer (0.20 M Tris–HCl, 1 M Sucrose, 0.1% bromophenol blue, pH 6.8) in the presence of 1 mM $CaCl_2$ or 5 mM EGTA or 1 mM $MgCl_2$ were directly loaded onto gels and analyzed by SDS–PAGE as described by Rhyner [26]. The fluorescence in gels was detected on Biorad Geldoc 2000 with a CCD camera (BioRad, USA) before proteins were stained with Coomassie Brilliant Blue R-250.

2.5. Capillary electrophoresis

All CE experiments were performed on an Agilent 3D capillary electrophoresis instrument (Palo Alto, CA, USA) equipped with a ZETALIF laser-induced fluorescence detector (Picometrics, Ramonville, France). Detection was accomplished using a single-wavelength (488 nm) argon ion laser for excitation with emission detection at 510 nm. Data collection and peak analysis were performed on a HP Chemstation (Palo Alto, CA, USA). An uncoated fused-silica capillary of 65 cm (total length) × 50 cm (effective length)

× 75 μm i.d. × 375 μm o.d. (Yongnian Optic Fiber Inc., He Bei, China) was used for the analyses. Electrophoresis buffer solutions were filtered through a 0.22 μm membrane filter. New capillaries were pre-treated with 1 M NaOH for 60 min followed by deionized water for 60 min at room temperature. Prior to use, the capillary was washed with 0.1 mol l^{-1} NaOH, pure water and electrophoresis buffer (100 mM Tris–100 mM tricine, pH 8.3) for 5 min respectively, followed by preconditioning with electrophoresis buffer for 10 min. The above flushing cycle was repeated to ensure the separation reproducibility for each injection. Injection of samples was performed with applying a pressure (50 mbar) unless otherwise specified. The temperature of the CE system was 25 °C and the operating voltage was 25 kV. Each experiment was repeated three times.

The Ca^{2+}-binding assay experiments for CaM and GFP-CaM were performed in electrophoresis buffer (100 mM Tris–100 mM tricine, pH 8.3) with variable concentrations of Ca^{2+} using GFP as an internal standard. In the following experiments, the concentration of GFP-CaM is 0.1 μM, and the concentration of GFP is 0.02 μM. The binding constant for Ca^{2+}/GFP-CaM was determined by Scatchard analysis as follows [27,28]: the equation $(\delta\Delta t/\delta\,\Delta t_{max})(1/[L]) = (1/K_d) - (1/K_d)(\delta\Delta t/\delta\Delta t_{max})$, for Scatchard analysis to determine the K_d between the GFP-CaM and Ca^{2+} was used. Here, $\Delta t_{[L]}$ is the difference between the migration time of the protein of interest (GFP-CaM) and the reference protein (GFP) at concentration $[L]$ of the Ca^{2+}, $\delta\Delta t = \Delta t_{[L]} - \Delta t_{[L]=0}$, and $\delta\Delta t_{max}$ is the value of $\delta\Delta t_{[L]}$ at the saturating concentration of L. K_d is the dissociation constants between GFP-CaM and Ca^{2+}.To explore the interactions between GFP-CaM and OsCBK by CE, GFP-CaM and OsCBK in Tris–saline buffer (TBS; 50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 0.5 mM $CaCl_2$, and 50 mM $MgCl_2$) were incubated for 3 h at 4 °C. CaM was also added to compete with GFP-CaM for binding specific assay.

3. Results and discussion

3.1. Calcium-binding shift assays of GFP-CaM by gel electrophoresis

To analyze GFP-CaM protein by CE, both GFP and GFP-CaM were expressed in *E. coli* and purified with Ni-NTA affinity chromatography. The purified proteins were separated by SDS–PAGE as single bands (Fig. 1). Then these proteins were further used for calcium-binding assays by SDS–PAGE. To do so, the proteins in the presence of calcium or EGTA or Mg^{2+} were loaded onto a polyacrylamide gel and analyzed by SDS–PAGE. As expected, while GFP mobility was not affected by either Ca^{2+} or EGTA or Mg^{2+} (Fig. 2A), CaM ran faster in the presence of Ca^{2+} than EGTA or Mg^{2+}, indicating its calcium-binding (Fig 2C). Like CaM, the presence of Ca^{2+} also made GFP-CaM

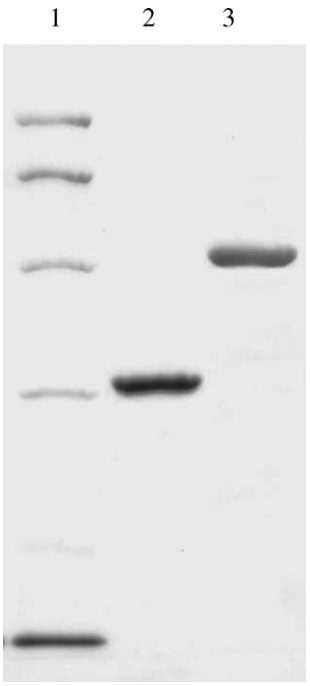

Fig. 1. SDS–PAGE of GFP and GFP-CAM. (1) Protein marker; (2) GFP; (3) GFP-CaM.

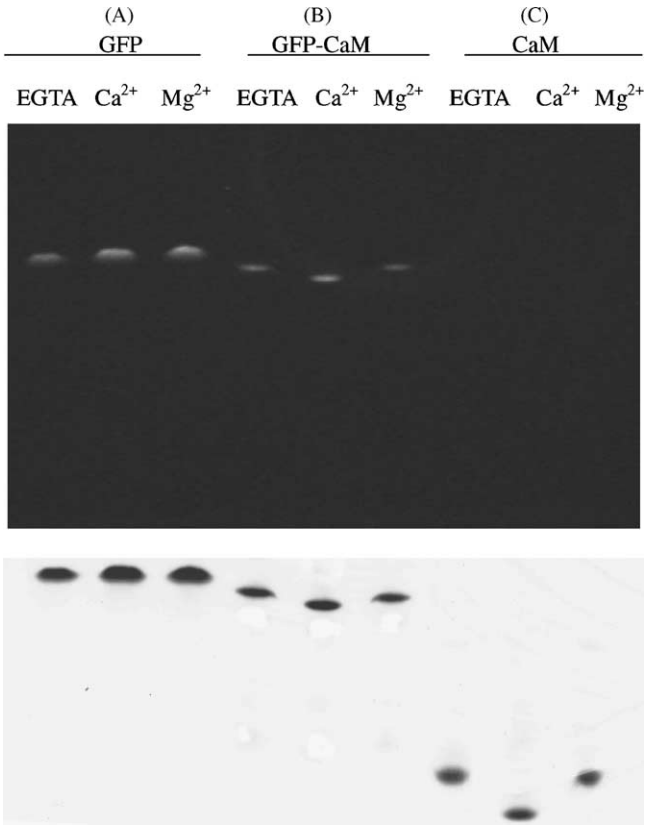

Fig. 2. Calcium-binding assays of GFP-CaM by SDS–PAGE. Proteins (A, GFP; B, GFP-CaM; C, CaM) in the presence of either 5 mM EGTA or 1 mM $CaCl_2$ or 1 mM $MgCl_2$ were separated by SDS–PAGE. Then the gels were used for fluorescence detection with Biorad Geldoc 2000 with CCD camera before they were stained with Coomassie Brilliant Blue R-250.

move faster than the presence of EGTA, suggesting that GFP-CaM still had calcium-binding ability (Fig. 2B). These results indicated that this GFP-CaM could be used for assay of calcium binding of CaM. Furthermore, both GFP-CaM and GFP were stable in the presence of SDS because they could maintain their fluorescence when subjected to analyses by SDS–PAGE (Fig. 2A,B). It was noted in Fig. 2 that the increase in migration time of CaM in the presence of Ca^{2+} was larger than the increase in migration time of GFP-CaM under the same condition. This could mainly be because the molecular weight of GFP-CaM ($37K_d$) is much larger than CaM ($16K_d$).

3.2. Capillary electrophoresis of GFP-CaM and GFP

To examine parameters that could affect the fluorescence intensity of GFP-CaM and GFP, different buffer concentrations, sample pHs, voltages and surface additives were analyzed in our experiments done with Tris–tricine buffer. Tris–tricine buffer was selected because it showed a stable separation of protein in the presence of EGTA or EDTA in the electrophoresis buffer as mentioned in another article [29]. As shown in Fig. 3A, the influence of buffer concentrations was examined since the ionic strength of the running buffer has been demonstrated to be an important factor for CE separation. While the buffer concentration had very little influence on the fluorescence intensity of GFP-CaM and GFP, different concentrations of buffer affected the migration rates of both GFP and GFP-CaM. It can be seen that the migration time increased as the buffer concentration increased, which should be attributed to the effect of buffer concentration (ionic strength) on electroosmotic flow (EOF) rates. Although lower concentrations of electrophoresis buffer shortened the separation time of GFP and GFP-CaM, reproducibility became poorer than for higher buffer concentrations. However, higher buffer buffer concentrations resulted in longer separation time. Thus, 100 mM Tris–100 mM tricine buffer system (pH 8.3) was selected for our experiments. When different buffer pHs, from 2 to 13, were examined for this impact on the fluorescence intensity of both GFP-CaM and GFP, the results showed that GFP-CaM and GFP were stable over a relatively broad pH range (Fig. 3B). However, the fluorescence intensity was dramatically decreased in extreme pH conditions (beyond pH 12 or below pH 5). From the Fig. 3B, it can be seen that the fluorescence intensity of GFP-CaM and GFP is relatively stable during the pH 7 and 9. pH 8.3 was chosen in the following experiments. Different voltages also had great

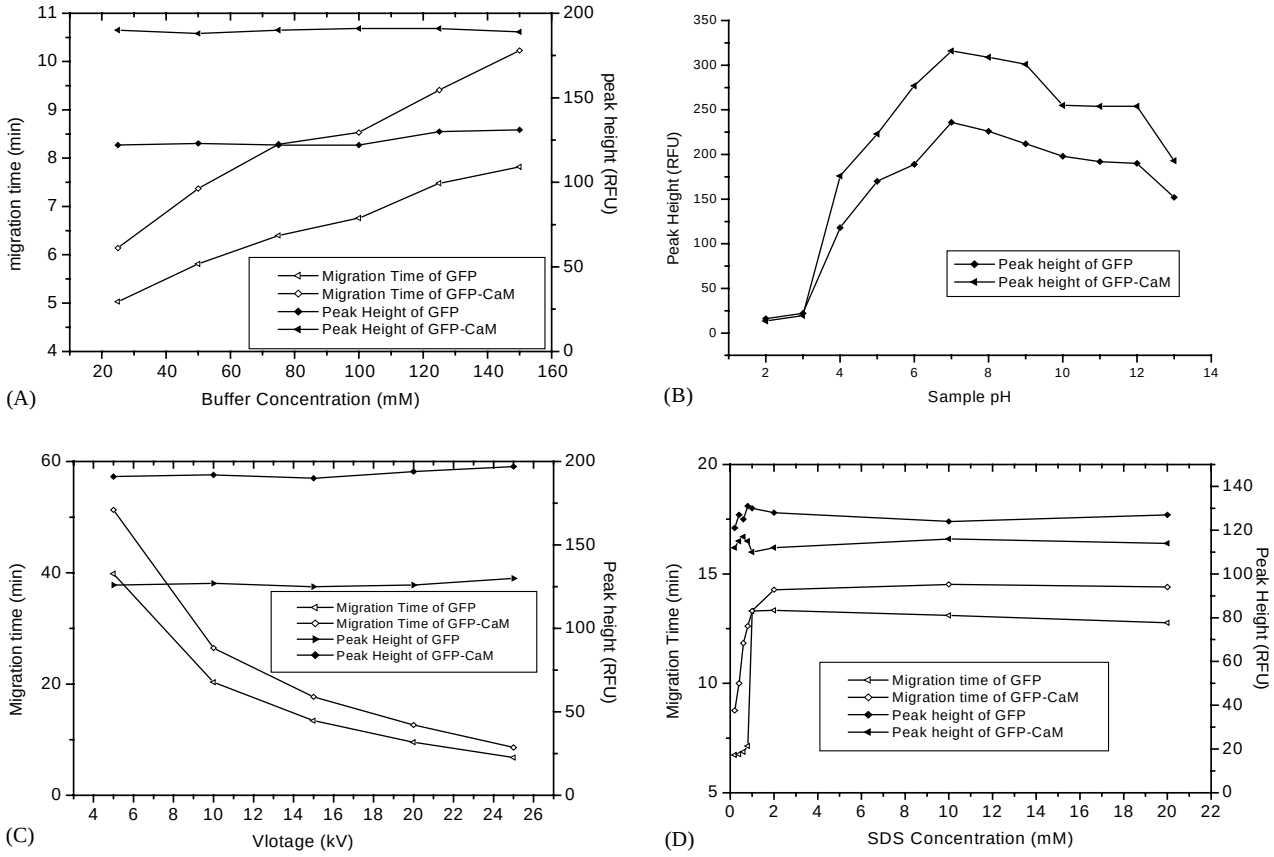

Fig. 3. The impacts of different parameters on the migration and fluorescence intensity of both GFP-CaM and GFP. The experiments were performed under the conditions—capillary: 65 cm (50 cm effective length) × 75 μm i.d.; injection: 50 mbar × 3 s; running buffer: 100 mmol l^{-1} Tris–100 mmol l^{-1} tricine (pH 8.3); separation voltage: 25 kV and temperature: 25 °C, with the changes of single factors as follows—(A) running buffer concentrations; (B) pH; (C) voltage; and (D) SDS concentration.

influences on the electrophoretic mobility, but they had no influence on the fluorescent intensity of GFP and its fusion protein (Fig. 3C). Because of the long time, low voltages led to the broadening of peaks, so 25 kV was used in the further experiments. SDS, as the most popular surface-active additive, was also investigated for its role on the separation of these fluorescent proteins by CE. It was observed that when the concentration of SDS was above 2 mM, the migration time of GFP-CaM and GFP was not significantly changed (Fig. 3D). These results also indicated that the GFP fusion protein and GFP were stable under various conditions. Following established conditions as described above, both GFP-CaM and GFP were successfully separated using a 100 mM Tris–100 mM tricine buffer system (pH 8.3) (Fig. 4).

As previously reported, GFP was previously quantified by capillary electrophoresis [23], and the limit-of-detection (LOD) was 3.0×10^{-12} M for an injection volume of 17 nl. In our work, LODs of both GFP and GFP fused protein (GFP-CaM) were also studied by serial dilution of the samples until the analyte peak was no longer visible above the baseline noise (Fig. 5). The LOD of GFP was 1.25×10^{-10} M for an injection volume of 3 nl (about 0.1 fg) and that of GFP-CaM was 2×10^{-10} M for an injection of 3 nl (about 0.27 fg). These LODs are more sensitive than by the common chemical fluorescent-tagged protein methods and are more sensitive than with detection by UV. From the LOD of GFP fusion protein and GFP, it can be seen that the target protein had little influence on the fluorescence intensity of GFP and that CaM can be quantitated with high sensitivity using the help of GFP and CE-LIF. These results also show that GFP can avoid inefficient derivatization by chemical fluorescence probes.

Previous research work showed that GFP was stable in 1% SDS, 6 M guanidine chloride, temperatures up to 65 °C, pH from 2 to 11, and that it resists digestion by most proteases for many hours [30]. With the use of GFP, some disadvantages of chemical fluorescence probes, such as multiple derivatives, inefficient chemical tagging, and the need for non-physiological conditions can be avoided [15]. It is known that an ideal fluorescence probe would be stable over long periods of time and would allow simple, sensitive, and direct detection. Our results above have indicated that GFP-CaM was stable and sensitive, suggesting that GFP could be a good fluorescent probe to label proteins for protein analyses.

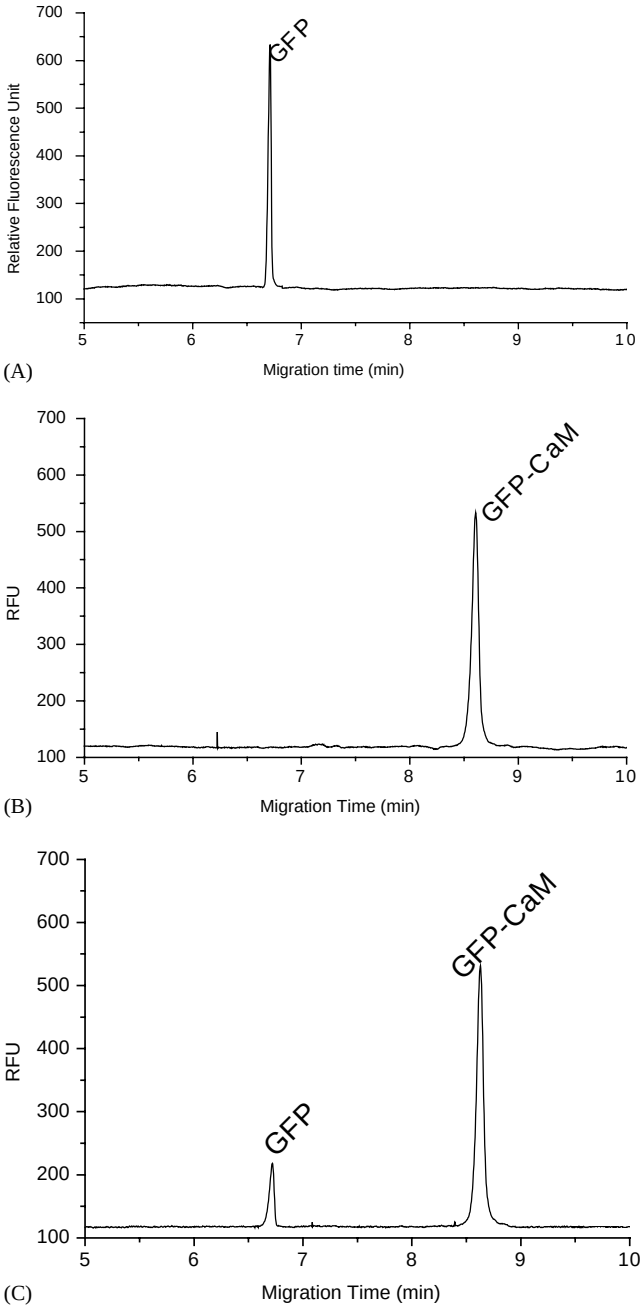

Fig. 4. Assays of GFP and GFP-CaM by CE-LIF. The experiments were done under the following conditions—running buffer: 100 mmol l^{-1} Tris–100 mmol l^{-1} tricine (pH 8.3); capillary: 65 cm (50 cm effective length) × 75 μm i.d.; injection: 50 mbar × 3 s; separation voltage: 25 kV; temperature: 25 °C. (A) GFP; (B) GFP-CaM; (C) GFP and GFP-CaM.

3.3. Calcium-binding shifts assays GFP-CaM by ACE

Besides detecting the interactions between biomacromolecules [7–14,31], ACE can be also used to study the interaction between protein and metal ions [29,32,33]. Here, ACE was employed for the calcium-binding shift assay of GFP-CaM. The electropherogram for the analysis of Ca^{2+}-binding shift assay is shown in Fig. 6A. While the electrophoretic mobility of GFP was not affected if the electrophoretic buffer contained Ca^{2+} or EGTA or Mg^{2+}, Ca^{2+} dramatically changed the mobility of GFP-CaM. The result is similar to that obtained by gel electrophoresis (Fig. 2). Compared to the migration time of GFP-CaM (8.57 ± 0.08 min) with 5 mM EGTA in the electrophoretic buffer, the migration time of GFP-CaM was 8.05 ± 0.05 min with 1.0 mM Ca^{2+} in the electrophoretic buffer. Furthermore, the migration time (8.57 ± 0.08 min) of GFP-CaM

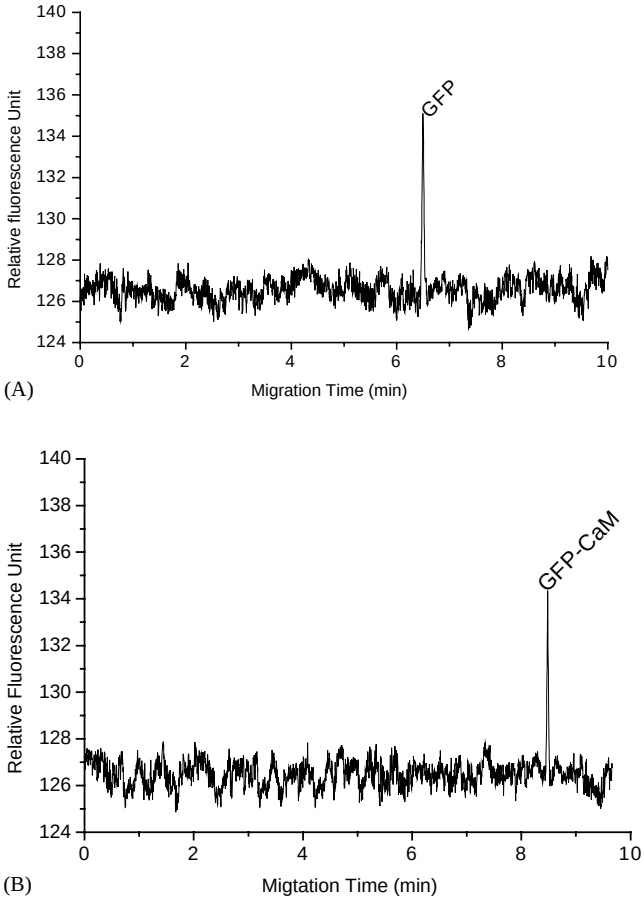

Fig. 5. Assays for the LOD of GFP and GFP-CaM. The conditions were identical to Fig. 4: (A) LOD of GFP; (B) LOD of GFP-CaM.

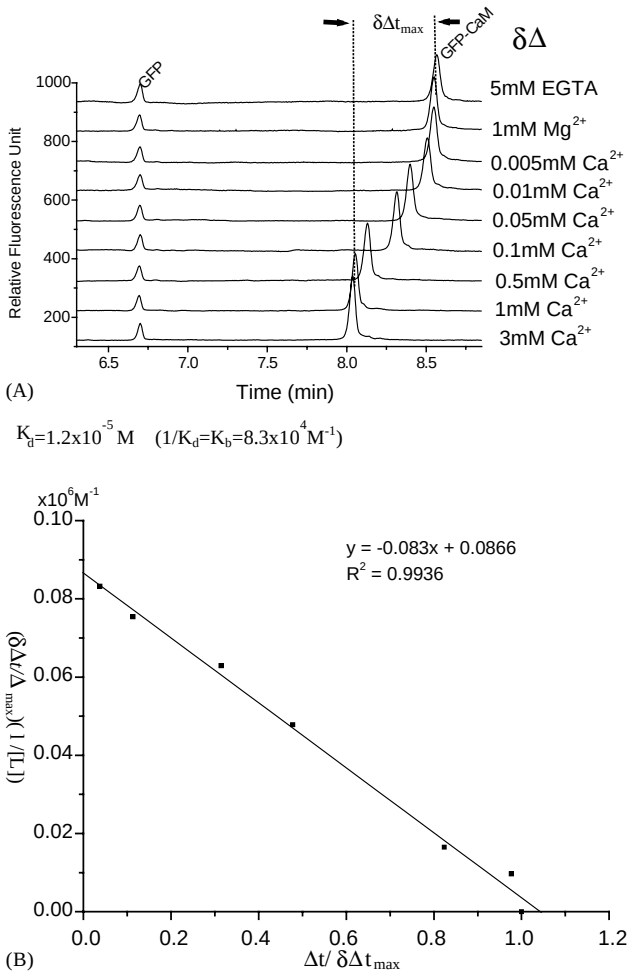

Fig. 6. CE-LIF assay for Ca^{2+} binding by GFP-CaM. (A) Ca^{2+}-binding assays of GFP-CaM. GFP-CaM (0.1 μM) and GFP (0.02 μM) were analyzed by CE-LIF under the conditions identical to Fig. 4 except with various concentrations of Ca^{2+}; (B) Scatchard analysis.

was almost not affected by the presence of 1.0 mM Mg^{2+} in the electrophoretic buffer, indicating that Ca^{2+} should selectively cause the changes in migration time of GFP-CaM.

In the experiments with increasing amounts of Ca^{2+} in the buffer, the migration time of GFP-CaM decreased until a plateau was reached at a Ca^{2+} concentration of approximately 1 mM (Fig. 6A). Above 1 mM Ca^{2+}, the GFP-CaM migration times remained constant. This indicated that an equilibrium type of binding took place throughout the course of the electrophoretic run when increasing amounts of Ca^{2+} were present. Also, too high concentrations of Ca^{2+} (above 5 mM) in the buffer greatly changed the current and the migration time of both GFP-CaM and GFP (data not shown). Based on the migration time change of GFP-CaM, a Scatchard analysis was done to determine the K_d of Ca^{2+} and GFP-CaM (Fig. 6B). Analysis indicated $K_d = 1.2 \times 10^{-5}$ M ($K_b = 8.3 \times 10^4\ M^{-1}$), which was coincident with the literature values from equilibrium dialysis analysis ($K_d = 10^{-5}$ to 10^{-6} M [2]; $K_b = 9.31 \times 10^4\ M^{-1}$ [34]) and CE analysis ($K_b = 0.47 \times 10^6\ M^{-1}$ [36]) for CaM. These results showed that GFP did not alter the Ca^{2+}-binding character of CaM and GFP-CaM could still bind Ca^{2+} with similar high affinity.

As mentioned above, the migration time of GFP-CaM depended on the concentration of Ca^{2+} in the electrophoresis buffer. With increasing amounts of Ca^{2+} in the buffer, the migration time of GFP-CaM was decreased until a plateau was reached. The migration time of GFP-CaM was altered because GFP-CaM obtained a more positive charge by binding Ca^{2+} and moved faster towards the cathode than its free form without Ca^{2+} binding. Besides this, conformational changes of CaM could also occur when Ca^{2+} was bound to CaM [34,35]. Since the mobility in free solution capillary electrophoresis is a complicated function of charge, molecular size, shape, hydrophobicity, and solvent properties, conformational changes may also contribute to the altered mobility of GFP-CaM in the presence of Ca^{2+} [32]. It can be concluded that there were at least two different parameters (charge and conformational changes) involved in the change of migration behavior. Therefore, we cannot calculate directly the number of binding site on GFP-CaM

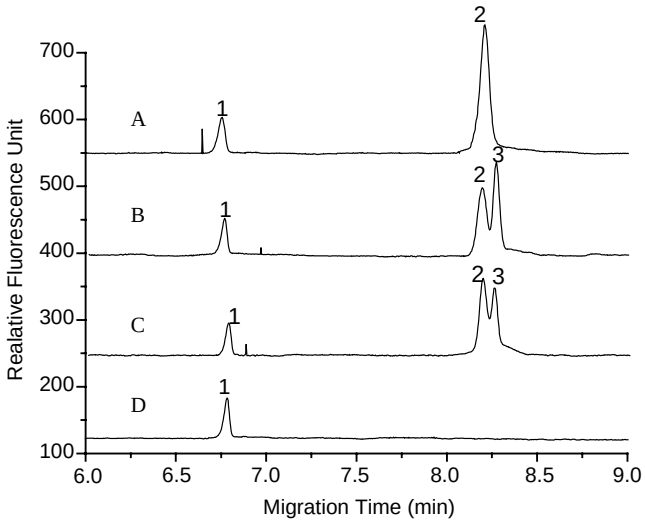

Fig. 7. Assays for the interaction between GFP-CaM and OsCBK by CE-LIF. CE conditions were identical to Fig. 4. The 0.1 μM GFP-CaM, 0.02 μM GFP with various concentrations of OsCBK and CaM were used in each assay. (A) GFP-CaM and GFP without OsCBK; (B) GFP-CaM and GFP with 0.2 μM OsCBK; (C) GFP-CaM and GFP with 0.2 μM OsCBK and 0.05 μM CaM; (D) GFP with 0.2 μM OsCBK. (1) The peak of GFP; (2) The peak of GFP-CaM; (3) the peak of GFP-CaM/OsCBK.

for Ca^{2+} by estimating the mobility of the complex from the saturation value.

3.4. Detection of the interaction between GFP-CaM and OsCBK by CE

On the basis of the binding shift assay for GFP-CaM and Ca^{2+} performed by capillary electrophoresis, the interaction between Ca^{2+}/GFP-CaM and OsCBK was investigated. OsCBK has been well documented to be a CaM-binding protein kinase with high affinity [4]. The electropherograms revealed here that with addition of OsCBK to the sample containing GFP-CaM and GFP, a new peak with mobility slightly slower than that of GFP-CaM appeared and the peak of GFP-CaM became smaller (Fig. 7A,B) indicating this new peak was the complex of OsCBK and GFP-CaM. This was further evidenced by the experiments with additional unlabeled CaM. When the additional unlabeled CaM was added into the system, the new peak became smaller because the complex of OsCBK and CaM was not detectable by CE-LIF (Fig. 7C). It was noted there were slight differences of GFP peaks in Fig. 7A–C. This was probably due to very slightly difference of sample loadings from time to time in our experiments. When only GFP was incubated with OsCBK, no new peak appeared (Fig. 7D), suggesting that GFP did not bind OsCBK. These results showed that the GFP-CaM fusion protein still retained the binding ability of its target protein and ACE is a useful tool to study the interaction between the CaM and its target protein.

4. Conclusion

In this article, the fluorescent and electrophoretic properties of GFP and GFP-CaM were investigated by CE-LIF. The results showed that the fused protein did not change the fluorescent properties of GFP and that the GFP did not change the characteristics of the labeled CaM, such as binding ability to calcium and its target protein. The GFP fused protein can be quantitatively assayed with high sensitivity by CE-LIF (almost at femtogram levels). As a preliminary study, the interaction between GFP-CaM and OsCBK was also investigated using ACE with LIF. These results demonstrated that GFP could be an ideal fluorescence probe for protein anaylses under relatively native conditions. As a fluorescence probe, GFP could overcome some shortcomings of common chemical fluorescence probes and may provide a way to quickly screen for CaM target proteins in crude extracts under native conditions.

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