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Characterization of phosphorylation of a novel protein kinase in rice cells by capillary electrophoresis

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Abstract

It was demonstrated that a separation of 20 amino acids constituting a protein and three phosphoamino acids that mostly frequently occur in eukaryotes was achieved within 15 min by capillary electrophoresis coupled with lamp-induced fluorescence detection. Fluorescein isothiocyanate was employed as the fluorescence label to facilitate the fluorescence detection of the 23 amino acid species. The fluorescent derivatization conditions and separation parameters including concentration of electrolyte, surfactant in buffer, applied voltage and sample injection were investigated in detail and optimized. The influence of buffer additives such as methanol, acetone and polyvinylpyrrolidone on separation selectivity and sensitivity were discussed. We showed that addition of 2% polyvinylpyrrolidone into the running buffer could dramatically enhance the separation selectivity of amino acids at the expense of a decrease of sensitivity of phosphoamino acids. Under the optimized conditions, the detection limits ($S/N=2$) ranged from $1.90\cdot10^{-8}$ M to $5.66\cdot10^{-8}$ M with an average efficiency of 620 000/m. The method was applied to characterization of the phosphorylation of a novel protein kinase RCaMBP (calcium/calmodulin-binding protein kinase) encoded by a cDNA newly isolated and cloned from rice. We verified that RCaMBP belonged to a type of Ser/Thr kinase, providing insight into its function in signal transduction.

Keywords: Proteins; Enzymes; Phosphoamino acids; Organophosphorous compounds; Amino acids

1. Introduction

With the completion of the human genome project (HGP), proteomics [1,2] promises to provide a holographic picture of every protein in cells, and characterization of protein function has been recognized as a post-genomic challenge to understand gene function [3]. At present it is widely accepted that a separation tool combining two-dimensional gel electrophoresis (2DGE) with an identification strategy using mass spectrometry (MS) and bioinformatics is the proteomic solution of practical choice [4], although these methods are still in their infancy. To provide a deeper insight into proteomics, attention should be paid to elucidate the function of the proteins that form the basis of life.

Protein phosphorylation [5] is an important post-transcriptional modification of proteins. Protein phosphorylation/dephosphorylation [5] plays a crucial role as a regulatory mechanism in many aspects of cell biology, such as cell differentiation and proliferation, apoptosis, gene expression, signal

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transduction and metabolism. For instance [6,7], in the calcium signaling pathway, CDPK, a calcium-dependent but calmodulin-independent protein kinase, transduces its up-stream signal from the second messenger calcium to its down-stream substrate by phosphorylation/dephosphorylation. Consequently, it is essential for the comprehension of the function of such proteins to characterize their phosphorylation mechanism.

Many analytical methods have been exploited to characterize protein phosphorylation, such as radioactive labeling [8], the use of specific antibody probes [9], MS [10], high-performance liquid chromatography (HPLC) [11] and thin-layer chromatography (TLC) [12]. Yan et al. [13] have given an extensive review of this field with an emphasis on technologies involved in identifying phosphoamino acids.

Capillary electrophoresis (CE) is emerging as a powerful method in the field of analytical biochemistry to analyze bio-macromolecules [14] such as DNA, proteins and sugars, as well as low-mass organic molecules [15] such as amino acids and hormones. Due to its high performance, high speed and low sample consumption, CE is now considered as a competitive alternative to other methods in proteomics research [16]. However, limited attention has been given to CE to characterize protein phosphorylation. Crowder et al. [17] and Che et al. [18] demonstrated the usefulness of CE as a method to identify the phosphoamino acids in proteins coupled with indirect and direct ultraviolet detection, respectively. The separation of three phosphoamino acids was investigated in their papers with no respect to amino acids. Because of the low sensitivity of their photometric methods, relatively large amounts of the protein were required, posing a problem for the analysis of proteins of low abundance or those that are difficult to purify. Ideally, fluorescence detection, particularly laser-induced fluorescence detection that allows detection at the single molecules level [19] is a more rational choice for CE when combined with a fluorescent label derivatization scheme. However, no fluorescent label is available for specific phosphoamino acids. In general fluorescent labels can react with both amino and phosphoamino acids. This means that high selectivity of the separation method is needed, which is the strength of CE.

In this article, we demonstrated that 20 amino acids constituting natural proteins and the three phosphoamino acids found most frequently in eukaryotic cells could be separated by CE coupled with lamp-induced fluorescence detection. Fluorescein isothiocyanate (FITC) was used as the fluorescent label for amino and phosphoamino acids. The method we established was successfully applied to characterizing the phosphorylation of a novel protein kinase encoded by a cDNA isolated and cloned from rice cells in our laboratory. Only sub-microgram amounts of protein were needed with this method, and the sensitivity of the method could be further reduced to low picogram amounts if a laser was utilized instead of the lamp for fluorescent detection.

2. Experimental

2.1. Chemicals

Amino acids (DL) and phosphoamino acids (L) purchased form Sigma (USA) were prepared at a concentration of $5 \cdot 10^{-2}$ *M* in pure water, and then were diluted to the desired concentration. The stock solution of $1 \cdot 10^{-3}$ *M* FITC (Sigma) was prepared by dissolving it in acetone containing 0.05% pyridine. The stock solution of sodium dodecyl sulfate (SDS, Sigma) and polyvinylpyrrolidone (PVP, Sigma) were used at the concentration of 0.2 *M* and 10% (w/v), respectively. Water purified by a Millipore-Q system (Millipore, USA) was used for the preparation of all solutions. All chemicals were of analytical grade. Carrier electrolyte for capillary electrophoresis was prepared daily, and then filtered through a 0.45-μm membrane prior to use.

2.2. Protein preparation

The calcium/calmodulin-binding protein kinase (RCaMBP) was produced by the Gibco Bac to Bac baculovirus expression system [20] (Gibco, USA). The detailed protocol was depicted in Fig. 1. A cDNA clone encoding RCaMBP was isolated by screening a λ ZAPII cDNA expression library constructed from rice (IR62266) using MCK1 (maize

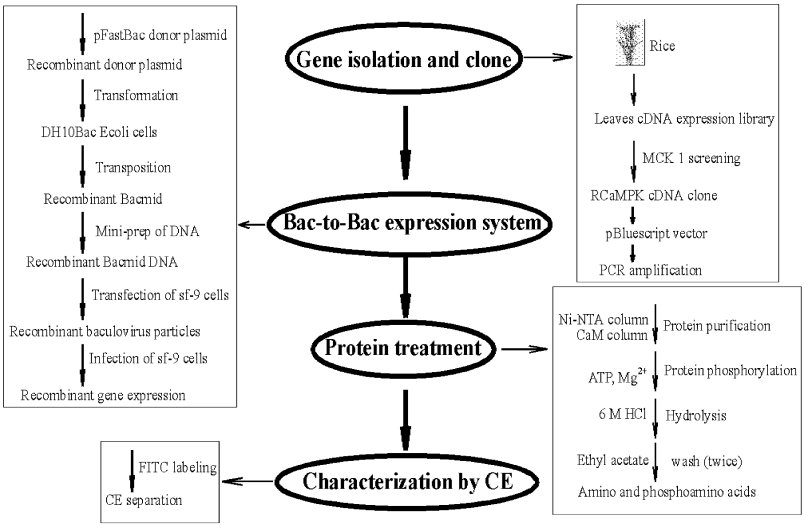

Fig. 1. Protocol of protein preparation and phosphorylation characterization.

homolog of mammalian calmodulin kinase II) [21] as the probe. The full open reading frame of the gene was then cloned into a pFASTBAC HTB expression vector. The recombinant cDNA was expressed in sf-9 cells using the baculovirus expression system. The recombinant protein extracted from sf-9 cells was purified by a Ni–NTA resin. The recombinant protein was expressed with 6×histamine residues at its amino terminus, having a strong affinity with Ni–NTA resin, allowing it to be easily purified using an elution buffer of 50 m*M* KH_2PO_4–K_2HPO_4 (pH 6.0) containing 300 m*M* KCl, 200 m*M* imidazole and 10% (v/v) glycerol. The eluted protein was further purified by calmodulin (CaM) affinity chromatography. The purified protein could be stored for 2 months at −70°C in 25 m*M* Tris buffer (pH 7.5) containing 30% glycerol with no loss of enzymatic activity.

2.3. Protein phosphorylation

Purified RCaMBP (2.5 μg) was auto-phosphorylated by incubating with ATP and Mg^{2+}, in 25 m*M* Tris buffer (pH 7.5) containing 0.5 m*M* dithiothreitol, 10 m*M* $Mg(Ac)_2$, 100 μ*M* cold ATP, 1 μ*M* CaM and 1 m*M* $CaCl_2$ for about 30 min at 30°C.

2.4. Hydrolysis of phosphorylated and unphosphorylated proteins

Phosphorylated and unphosphorylated RCaMBP were hydrolyzed in 6 *M* HCl for 12 h at 110°C. Aliquots were collected, dried and dissolved in pure water. The hydrolyzed product was then washed by ethyl acetate and further dried. The residue was finally dissolved in 20 μl 10 m*M* borate buffer (pH 10.0).

2.5. Derivatization procedure

Amino acids and phosphoamino acids were fluorescently tagged by adding 2 μl mixed standard solution which contained each amino acid and phosphoamino acid at a concentration of $5 \cdot 10^{-4}$ *M*, 46 μl FITC stock solution, 100 μl 20 m*M* borate buffer (pH 10.0), and 52 μl water in a 750 μl PTFE tube. The mixture was then oscillated for a few seconds and placed in a dark room for 12 h at room temperature.

Ten microlitres FITC was directly mixed with 10 μl solution of hydrolyzed product in a 750-μl PTFE tube. Using procedures were the same as for the standard sample mentioned above.

2.6. Capillary electrophoresis

Analyses were carried out on a home-made capillary electrophoresis system based on an upright fluorescence microscope (Olympus BX60, Japan), a cooled charge-coupled device (CCD, Diagnostic Instrument, USA) camera as the detector, a high-voltage power supply (Shanghai Nuclear Research Institute, China) and an uncoated fused-silica capillary of 60 cm (44 cm length to detector window)×50 μm I.D.×365 μm O.D. (Yongnian Optic Fiber, China). A 100-W high-pressure mercury lamp was used as the excitation radiation. The optical subsystem in the microscope consisted of a 20× objective (UPlanApo, NA 0.70), a NIB excitation cube including an excitation filter (EX 470–490 nm), a dichroic mirror (DM 510 nm) and a barrier filter (BA 515 nm). The main working preferences of the CCD camera are as follows: pixel depth: 8 bpp; gain: 4; binning: 4×4. Data were collected by a computer (Intel PIII 550) with Spot Advanced software, and further processed with Scion Image and Origin software packages.

A new capillary was pre-treated with 1 *M* NaOH and water for 60 min at room temperature prior to use. After every five injections, the capillary was washed with 0.1 *M* NaOH and water for 15 min, then preconditioned with running buffer for 20 min at room temperature to ensure reproducibility.

3. Results and discussion

The goal of this work was to develop a CE method for the characterization of protein phosphorylation. To achieve such a purpose, a thorough separation of phosphoamino acids from 20 amino acids that constitute a protein must be accomplished.

Protein phosphorylation occurs at specific amino acid residues of peptide chains catalyzed by enzymes referred as to protein kinases. In eukaryotes the hydro-amino acids serine (Ser), threonine (Thr) and tyrosine (Tyr) are phosphorylated by protein kinase, while in prokaryotes like bacteria histidine (His), aspartic acid (Asp) and glutamic acid (Glu) are the preferred substrates. Although some other amino acids residues such as lysine (Lys), arginine (Arg) and cysteine (Cys) have been identified occasionally as the phosphorylation site in eukaryotes and in prokaryotes, they appear to be reaction intermediates. Furthermore, these phosphoamino acids are very unstable under the conditions of standard protein purification and currently, no analytical techniques are suitable for their analysis except for radioactive labeling or an enzyme-based approach. As a consequence, we focussed our attention on three phosphoamino acids including phospho-Ser (P-Ser), phospho-Thr (P-Thr) and phospho-Tyr (P-Tyr) in our experiment. Many papers describing the separation of amino acids by CE [22–26] and microchip CE [27–29] coupled with fluorescence detection have been published. FITC has been used as the most common reagent for fluorescent derivatization due in part to its good reactivity with both primary amino acids and secondary amino acids. Moreover, the effective excitation wavelength range (about 488–495 nm) of FITC matches well with the emission wavelength (488 nm) of the argon ion laser. Ultra-high sensitivity can be achieved compared with the use of other fluorescent labeling agent such as 4-fluoro-7-nitroben-2-oxa-1,3-diazole (NBD-F) [30]. Thus, we also favor the use of FITC as the fluorescent label of the amino acids and the phosphoamino acids.

3.1. Choice of derivatization conditions

Amino acids are zwitterions, and in neutral buffers the α-amino group of the amino acid is protonated. Protonation of α-amino group can inhibit the reaction of amino acid with FITC. Therefore, derivatization is often performed in a basic medium where the amino group is readily de-protonated [31]. Based on the previous success of others [23,25], 10 m*M* borate buffer (pH 10.0) was chosen as the derivatization medium in this work. Under these conditions, the α-amino group of all the amino acids and phosphoamino acids was free from protons, and could be tagged with FITC with high efficiency. It was reported that excessive amounts of FITC presented in sample solution were beneficial to derivatization efficiency. When the concentration ratio of FITC to the amino acid was over 30, good quantification could be achieved, however, it could lead to difficult separation, because many by-products of the derivatization reaction were formed. At

the same time, because of the hydrophobic nature of FITC, it was easily adsorbed onto the hydrophobic sites distributed on the surface of the inner capillary wall. This further decreased the signal-to-noise ratio, which in turn decreased the sensitivity of the detection. Consequently, a concentration ratio of FITC to the amino acid of 2 was chosen.

We compared the effect of temperature and time on derivatization efficiency. It was found that the derivatization reaction could be controlled at 50°C for a time of 5 h or 40°C for 7 h as discussed by others [23–26], but the derivatives were not very stable and many by-products were formed. In this work the derivatization reaction was conducted at room temperature for 12 h in a dark room and the derivatization products were stable for at least 96 h with fewer by-products.

3.2. Optimization of CE separation parameters

Because FITC and FITC-tagged amino acids are hydrophobic, a micellar electrokinetic capillary chromatography (MECC) separation mode was recommended by several literatures [25–28], although separation could be also achieved by capillary zone electrophoresis (CZE) mode in the presence of organic additives. In MECC, a stable electroosmotic flow (EOF) with high speed was required as the separation engine. In theory, a high quality separation could be achieved using high concentration of running buffer due to the sample stacking effect, however, Joule heating should be also taken into account. Higher electrolyte ionic strength results in higher current in the capillary, which produces more heat. When the amount of the heat produced in the capillary exceeds the dissipated heat by capillary, band broadening occurred. This required the concentration of buffer to be optimized to taking these opposing effects into account. In our work, 40 m*M* borate buffer (pH 9.35) was selected for further investigation.

In MECC separation, the concentration of surfactant added in the running buffer plays a central role, taking into account the separation selectivity that is accomplished by adjusting the partition of analytes between SDS phase and aqueous phase, when SDS was used as the surfactant at a concentration above its critical micelle concentration (CMC). Fig. 2

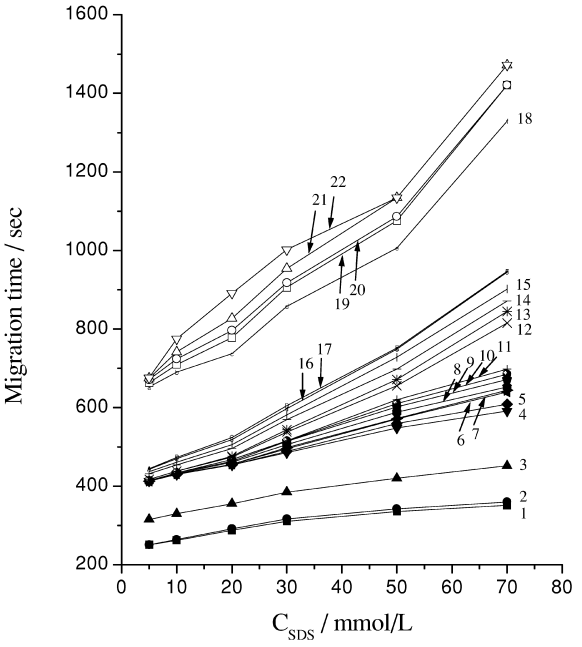

Fig. 2. Effect of concentration of SDS on separation. Conditions: electrophoresis buffer: 40 m*M* borate buffer containing SDS varied as in figure, pH 9.35; Capillary: 60 cm (44 cm effective length)×50 μm I.D.; hydrodynamic injection: 9 cm (height) for 5 s; separation voltage: +22 kV; temperature: 25°C. 1=Lys; 2=Arg; 3=His; 4=Trp; 5=Tyr; 6=Gln; 7=Pro; 8=Leu/Ile; 9=Met; 10=Val; 11=Asn; 12=Thr; 13=Phe; 14=Ser; 15=Ala; 16=Cys; 17=Gly; 18=Glu; 19=Asp; 20=P-Tyr; 21=P-Thr; 22=P-Ser.

described the influence of the concentration of SDS in electrophoretic buffer on the separation of 20 amino acids and three phosphoamino acids. As indicated in Fig. 2, with the increase of concentration of SDS, the separation was apparently improved. Unfortunately, the peaks of glutamine (Gln)/proline (Pro), leucine (Leu)/isoleucine (Ile) were poorly resolved. The latter peak-pair proved to be resolved only in the presence of a chiral additive [32] such as cyclodextrin (CD) or crown derivatives. Furthermore, the selectivity of peak-pair of glycine (Gly)/Cys, Asp/P-Tyr and P-Thr/P-Ser decreased. Surprisingly, the detection sensitivity of Asp and the three phosphoamino acids was lost if the concentration of SDS was over 20 m*M*.

Some other separation parameters such as separation voltage and sample injection were further optimized. Increasing voltage could speed up CE separation and ameliorate separation selectivity until

it reached 22 kV. But further increase in voltage reduced separation selectivity. Separation efficiency, counted as the average theoretical plate number of all the peaks in electropherogram began to decrease as indicated in Fig. 3, due to the Joule heating effect that broadened the electrophoretic peak, although the separation time was dramatically shortened. As for sample injection, the electrokinetic mode gave better resolution of separation than hydrodynamic mode. But sample discrimination occurred when electrokinetic sampling mode was employed. The detection of the three phosphoamino acids was seriously challenged due to their slow mobility. Thus, gravity introduction of sample was chosen. With this mode, the amount of injected sample was determined by the height and the time of sampling. When the sampling height was fixed at 9 cm, an injection time of 5 s could achieve good sampling reproducibility.

Based on the optimization mentioned above, the separation of FITC and the derivatized amino acids and phosphoamino acids were accomplished within 15 min as shown in Fig. 4. Although the 20 derivatized amino acids were not thoroughly resolved, these amino acids did not interfere with the high sensitive detection of the three phosphoamino acids. Clearly, the conditions employed in Fig. 4 could satisfy the requirements for the characterization of phosphoamino acids during protein phosphorylation. Table 1 gives the detection limits of the amino acid 23 species with this method, calculated based on a peak height of twice the background noise, as well as the separation efficiency and reproducibility. From the data in Table 1, it could be estimated that only sub-microgram or low femtomole amounts of protein were required to obtain a signal of 10 times the detection limits, provided that the molecular mass of a protein was 100 000 and the recovery of phosphoamino amino in hydrolyzed product was 10%. The sensitivity could be further increased by three to four orders of the magnitude if an argon ion laser was used instead of the mercury lamp.

Other approaches were attempted for further optimizing the separation of amino acids and phosphoamino acids in a single electrophoretic run by employing buffer additives. It was found that addi-

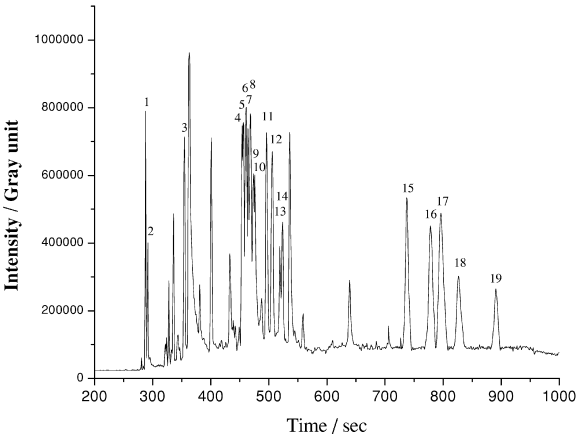

Fig. 4. Electropherograms of FITC-labeled amino acids and phosphorylated amino acids. Conditions: electrophoresis buffer: 40 m*M* borate buffer containing 20 m*M* SDS, pH 9.35. Other conditions as in Fig. 2. 1=Lys; 2=Arg; 3, His; 4, Trp/Tyr; 5=Gln/Pro; 6=Leu/Ile; 7=Met; 8=Val/Asn; 9=Thr; 10=Phe; 11=Ser; 12=Ala; 13=Cys; 14=Gly; 15=Glu; 16=Asp; 17=P-Tyr; 18=P-Thr; 19=P-Ser. The concentration of all the analytes is ca. $5 \cdot 10^{-6}$ *M*.

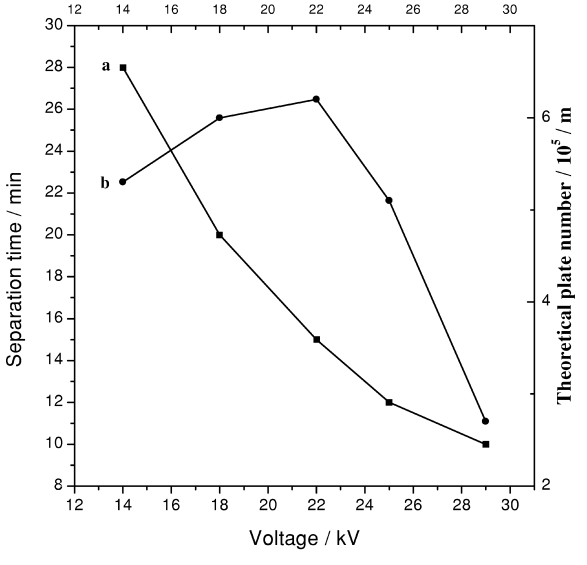

Fig. 3. Influence of applied voltage on separation. Conditions: electrophoresis buffer: 40 m*M* borate buffer containing 20 m*M* SDS, pH 9.35; Separation voltage are varied as indicated in figure; Other conditions as in Fig. 2. Curve identification: a=separation time; b=separation efficiency.

Table 1
Data of separation performance, reproducibility and detection sensitivity

Species	Detection limits ($S/N=2$, 10^{-8} M)	Efficiency (10^5/m)	Reproducibility of migration time (R.S.D., %, $n=7$)
Lys	1.90	7.9	0.42
Arg	3.74	4.0	0.42
His	2.10	7.1	0.35
Trp	4.00	7.5	0.36
Tyr	4.00	7.5	0.36
Gln	3.96	7.6	0.45
Pro	3.96	7.6	0.45
Leu	3.74	8.0	0.45
Ile	3.74	8.0	0.45
Met	2.03	7.3	0.63
Val	3.84	7.8	0.62
Asn	3.84	7.8	0.62
Thr	2.48	6.1	0.58
Phe	2.50	6.0	0.59
Ser	2.07	7.3	0.44
Ala	2.24	6.7	0.63
Cys	3.84	3.9	0.85
Gly	3.25	4.6	0.67
Glu	2.81	5.3	0.65
Asp	3.33	4.5	0.72
P-Tyr	3.08	4.9	0.52
P-Thr	4.97	3.0	0.64
P-Ser	5.66	2.6	0.66

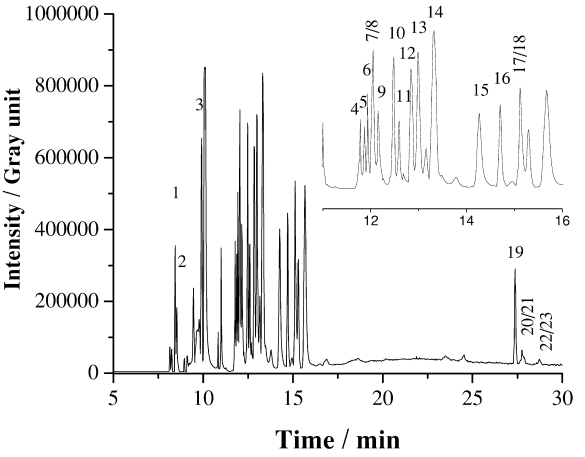

Fig. 5. Electropherogram of FITC-labeled amino acids and phosphorylated amino acids. Conditions: electrophoresis buffer: 40 mM borate buffer containing 20 mM SDS and 2% PVP, pH 9.35. Other conditions as in Fig. 2. 1=Lys; 2=Arg; 3=His; 4=Trp; 5=Tyr; 6=Gln; 7=Leu; 8=Ile; 9=Pro; 10=Met; 11=Val; 12=Asn; 13=Thr; 14=Phe; 15=Ser; 16=Ala; 17=Cys; 18=Gly; 19=Glu; 20=Asp; 21=P-Tyr; 22=P-Thr; 23=P-Ser. The concentration of all the analytes is ca. $5\cdot10^{-6}$ M.

tion of 5% (v/v) methanol to the running buffer could result in an increase of the selectivity of tryptophane (Trp)/Tyr, while 5% (v/v) acetone could partly resolve the peak-pair Gln/Pro. No improvement on the selectivity of other amino or phosphoamino acids was found. Meanwhile, the detection sensitivity of all the species unexpectedly decreased with methanol and acetone. It might be associated with the solvent-dependent nature of fluorescence. It was reported that PVP [33] could be an effective modulator of EOF by chemically dynamic modification of the inner capillary wall to improve the selectivity of the separation. When 2% (v/v) PVP was added into the buffer, the separation was enhanced with the accompanying loss of the separation speed. Eighteen amino acids could be resolved in a single run as shown in Fig. 5, which is the largest reported number of amino acids in a protein resolved by CE to date. Nevertheless, with PVP the detectability of three phosphoamino acids was impaired. Furthermore the peaks of Asp and P-Tyr overlapped, while the peaks of P-Thr and P-Ser were also co-eluted.

3.3. Charaterization of RCaMBP phosphorylation

The RCaMBP encoded by a newly isolated and cloned cDNA is a novel enzyme of 597 amino acids with a calculated molecular mass of 65 700 ca. and a predicted isolectric point (pI) of 8.5. Its biological functions and biochemical properties are under the characterization. It proves now that RCaMBP contains a calmodulin-binding domain in the molecular structure, but the enzymatic activity is quite free from the regulation of calcium and calmodulin (unpublished data).

To characterize the phosphorylated form of RCaMBP, the phosphorylated enzyme was hydrolyzed into a mixture of the amino acids and phosphoamino acids that were fluorescently tagged with FITC for assaying by CE. Fig. 6A described the

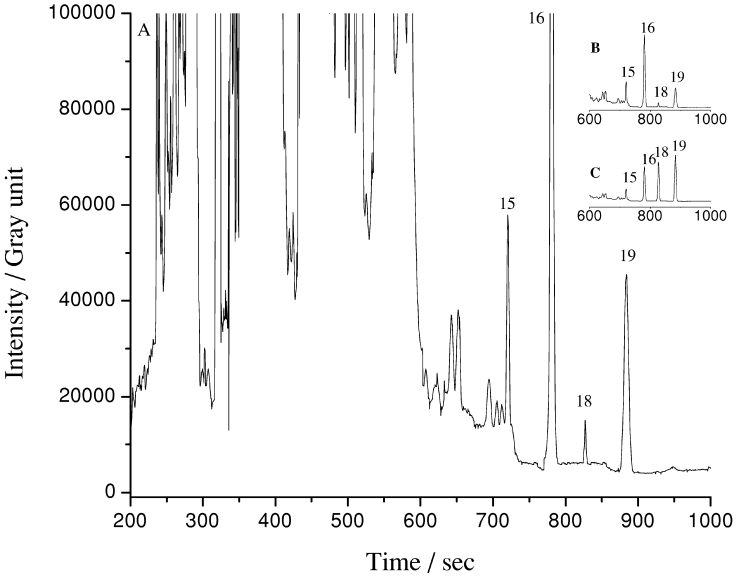

Fig. 6. Electropherograms of FITC-labeled hydrolyzed products of phosphorylated RCaMBP. Conditions as in Fig. 4. (A): Whole electropherogram; (B): electropherogram intercepted from (A); (C): electropherogram obtained by a standard addition approach; Peak identities as in Fig. 4.

separation of hydrolyzed products of phosphorylated RCaMBP using the CE method in Fig. 4. Fig. 6B exhibited an electropherogram intercepted from the corresponding part of Fig. 6A showing clearly that the P-Thr and P-Ser were detected. To further identify the peaks of the P-Thr and P-Ser, a standard addition approach has been employed as shown in Fig. 6C. It was confirmed from Fig. 6C that the RCaMBP belonged to a type of Ser/Thr kinase, which would help understand deeply the role and the function of RCaMBP in signal transduction pathway. To obtain a convincing and comparable result, the separation of hydrolyzed products of unphosphorylated RCaMBP was also conducted as shown in Fig. 7, and as predicted no phosphoamino acid was found. For identifying the factual site of phosphorylation in the protein sequence, a site mutation analysis or an MS–MS technique etc were needed, which undoubtedly was also important and was under our further consideration.

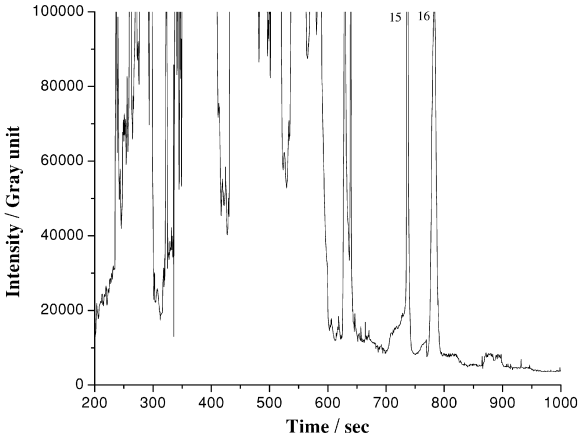

Fig. 7. Electropherogram of FITC-labeled hydrolyzed products of unphosphorylated RCaMBP. Conditions as in Fig. 6; Peak identities as in Fig. 4.

4. Conclusion

It was demonstrated that CE is compatible for the characterization of protein phosphorylation by identifying specific phosphoamino acids. A novel calcium/calmodulin-binding protein kinase (RCaMBP) found in rice was successfully shown to be a Ser/Thr type protein kinase by the CE method. Coupled with fluorescence detection, the amount of protein required for analysis was reduced by nearly two to

three orders of magnitude compared with UV detection. If a laser was employed, it could be further reduced to sub-pgram or low-amole amount, which was very important in the field of bio-analysis.

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