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Capillary electrophoresis of phosphorylated amino acids with fluorescence detection

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Abstract

A rapid and sensitive capillary electrophoresis (CE) method coupled with fluorescence detection was developed for identification of protein phosphorylation by determination of phosphoamino acids. Naphthalene-2,3-dicarboxaldehyde (NDA), a fluorescence derivatization reagent, was used to label protein hydrolysate. The optimal derivatization reaction was performed with 3.5 mM NDA, 40 mM NaCN and 20 mM borate buffer (pH 10.0) for 15 min. The baseline separation of three phosphorylated amino acids could be obtained in less than 180 s with good repeatability by using 30 mM borate (pH 9.2) containing 2.0 mM β-cyclodextrin (β-CD) as the running buffer. The detection limits for phosphothreonine, phosphotyrosine and phosphoserine were 7.0×10^{-9} M, 5.6×10^{-9} M and 7.2×10^{-9} M, respectively ($S/N = 3$). Also, the interference from other protein amino acids with large molar excess over that of phosphoamino acids was studied. With β-casein as the analysis protein, this method was successfully validated.

Keywords: Phosphoamino acids; β-Cyclodextrin; Capillary electrophoresis; Naphthalene-2,3-dicarboxaldehyde

1. Introduction

Protein phosphorylation, an important type of protein modification, has increasingly become the focus of attention in cell biology and biomedical sciences [1] due to its unique role in many biological phenomena, for example, signal transduction, cell division and cancer formation. A variety of approaches have been developed for the identification of protein phosphorylation, such as radiolabeling [2,3], mass spectrometry [4], antibody recognition [5], thin-layer chromatography [6,7] and high-performance liquid chromatography (HPLC) [8–12]. Yan et al. [1] have given an extensive review of this field with an emphasis on technologies for identification of phosphoamino acids.

Capillary electrophoresis (CE) has been considered as a powerful method with many advantages, such as the high separation speed and efficiency, very low running cost and low sample volume injected. Yoon has successfully applied green fluorescent protein (GFP), CE and laser-induced fluorescence detection to monitor the phosphorylation of the extracellular signal-regulated kinase (ERK2) [13]. And some other reports have demonstrated the usefulness of CE to determine phosphoamino acids with direct [14,15] or indirect ultraviolet detection [16]. Due to their high sensitivity, some fluorogenic derivatization reagents were also applied for the analysis of phosphoamino acids in CE, such as fluorescein isothiocyanate (FITC) [17], 3-(2-furoyl)-quinoline-2-carboxaldehyde (FQ) [18], and 5-(4,6-Dichloro-*s*-triazin-2-ylamino)fluorescein (DTAF) [19].

On the other hand, naphthalene-2, 3-dicarboxaldehyde (NDA) was a frequently used fluorescence derivatization reagent for amino acids and biogenic amines analysis by liquid chromatography [20–24] and CE [25–35]. In comparison with the above-mentioned reagents (FITC, FQ, DTAF), the reaction of NDA with primary amines is simple and quick in the presence of cyanide, and the derivatives are very stable.

In this work, we developed a rapid and sensitive CE method coupled with fluorescence detection for identification of protein phosphorylation by determination of phosphoamino acids. The phosphoamino acids were labeled with NDA/CN^-. The structures of derivatized phosphoamino acids are shown in Fig. 1. The

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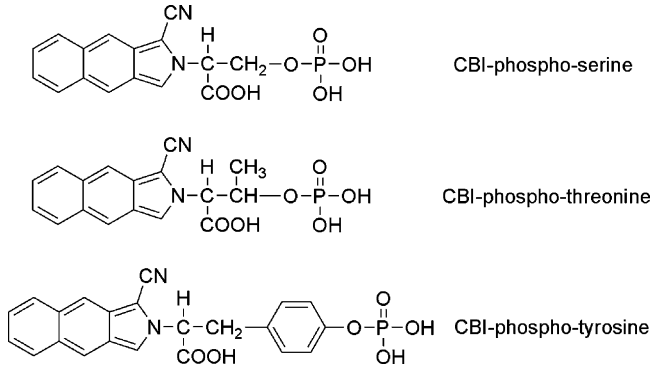

Fig. 1. Structures of derivatized phosphoamino acids.

derivatization and separation conditions were optimized. With the aim to provide the greater sensitivity and separation speed, β-cyclodextrin (β-CD) was used as the additive in the running buffer. And this method was successfully applied to the analysis of the hydrolysate of β-casein, a standard phosphoprotein.

2. Experimental

2.1. Apparatus

CE was carried out on a laboratory-built system based on an upright fluorescence microscope (Olympus, Japan), a photomultiplier tube (PMT), ±30 kV high-voltage dc power supply (Shanghai Institute of Nuclear Research, China), and uncoated fused-silica capillary of 40.5 cm (29.5–30 cm length to the detector window) × 50 μm i.d. × 365 μm o.d. (Yongnian Optical Conductive Fiber Plant, China). A 100-W high-pressure mercury lamp was used as the excitation radiation. The optical subsystem in the microscope consisted of a 40× objective, NIB excitation cube including an excitation filter (EX 400–490 nm), dichroic mirror (DM 510 nm) and barrier filter (BA 515 nm). The signal from the PMT was monitored using photon-counting device (Beijing Bingsong Photon Technological Corporation, China), collected by a computer (Inter PIII550) with photon-counting software, and processed with Origin software packages.

2.2. Chemicals

β-Casein and phosphoamino acids standards, namely, *O*-phospho-L-serine (P-Ser), *O*-phospho-L-tyrosine (P-Tyr) and *O*-phospho-L-threonine (P-Thre) were purchased from Sigma (St. Louis, MO). Other protein amino acids (L) were purchased from China or Japan. β-CD was purchased from Merck. NDA was obtained from Aldrich. Other chemical reagents were of analytical grade and used without further purification. P-Ser, P-Tyr and P-Thre standards were prepared in ultra-pure water at a concentration of 6.0×10^{-3} M, 4.0×10^{-3} M and 5.0×10^{-3} M, respectively, and stored in a refrigerator (−20 °C). Further mixing and dilutions were made with ultra-pure water to required concentrations. 1.0×10^{-2} M NDA stock solution was prepared in methanol, diluted to desired concentration with methanol, and stored in refrigerator. Ultra-pure water purified with milli-Q purified system (Millipore, MA, USA) was used for the preparation of all aqueous solutions.

2.3. Pre-capillary derivatization procedure

To a 500-μl vial, 80 μl of mixed three phosphoamino acids standard solution, 20 μl of 2.0×10^{-2} M sodium tetraborate buffer (pH 10.0), 20 μl of 4.0×10^{-2} M cyanide and 20 μl of 3.5×10^{-3} M NDA solution were added sequentially. The resultant solution was thoroughly mixed, then allowed to stand for 15 min at 65 °C under dark. After cooled to room temperature, the obtained solution was directly injected for CE without dilution.

2.4. Capillary electrophoresis

Thirty millimolar sodium tetraborate buffer (pH 9.2) containing 2.0 mM β-CD was used as the CE running buffer and prepared daily from stock borate solution. Before using the buffer solutions were filtered through 0.22-μm membrane filter. New capillary was pre-treated with 1.0 M NaOH and water for 30 min sequentially. Before using, the capillary was rinsed with 0.1 M NaOH, water for 5 min, respectively and followed by preconditioning with running buffer for 10 min. Separations were carried out at constant voltage of 18–20 kV. Sample injection was performed by hydrodynamic mode with sampling height at 9.5 cm for 45 s.

2.5. Sample preparation

β-Casein from bovine milk was used as a standard phosphoprotein in this study. It is a single polypeptide chain of 209 amino acid residues, five of which are phosphoseryl residues [8]. Hydrolysis of β-Casein was carried out according to that previously reported by Murthy et al. [8]. β-Casein (6.4 mg) was hydrolyzed in 5 ml of 6 M HCl for 4 h at 100 °C. After the hydrolysis, the hydrolysate was dried to remove HCl upon heating at about 70 °C and dissolved in 5 ml of ultra-pure water. The stock hydrolysate was stored at −20 °C refrigerator. Before derivatization, the hydrolysate was diluted to desired concentration with ultra-pure water. The derivatization procedure of hydrolysate was the same as that of phosphoamino acid standards.

3. Results and discussion

3.1. Choice of derivatization conditions

NDA was firstly developed by de Montigny et al. [20] for the derivatization of primary amines to produce high fluorogenic derivatives. The reaction between NDA and amino acid is quick in the presence of cyanide at room temperature. However, we found that the reaction of NDA with phosphoamino acids is slow at room temperature, and the increased temperature could significantly accelerate the reaction rate. As shown in Fig. 2, the relative fluorescence intensity (RFI) dramatically increased with increasing the reaction temperature from 15 to 50 °C, and leveled off while the temperature was between 60–80 °C. Thus,

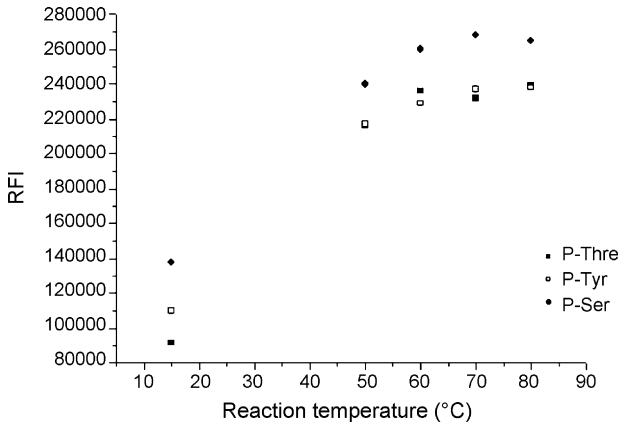

Fig. 2. Effect of reaction temperature on the RFI. CE conditions: capillary, 40.5 cm × 50 μm i.d.; electrophoresis buffer, 30 mM sodium tetraborate with 2.5 mM β-CD pH 9.2; separation voltage, 20 kV; hydrodynamic injection, 9.5 cm (height) for 40 s.

65 °C was selected. The effect of the reaction time varying from 5 to 40 min on RFI was also studied. When the reaction time was 10 min, the RFI could almost reach maximum, and become constant in the range of 10–40 min. So, 15 min was chosen for further experiments.

The pH value of the derivatization buffer plays an important role in controlling the reaction rate. The RFI increased with the increase of the pH value of the derivatization buffer, and leveled off when pH reached 10.0. Thus, pH 10.0 was chosen for the derivatization. The effect of the concentration of the derivatization buffer was also investigated (Fig. 3). The RFI decreased slightly while the concentration increased from 5 to 20 mM, and decreased sharply as the concentration further increased; the peak width at half-height ($W_{1/2}$) increased slowly when the concentration changed from 5 to 20 mM, but increased rapidly when the concentration was 50 or higher than 50 mM. Considering both of sensitivity and buffer capacity, 20 mM borate (pH 10.0) was chosen as the derivatization buffer.

To ensure the complete derivatization reaction, the concentrations of NDA and CN^- should be in large excess over those of analytes [20–22,30]. The investigation of the concentration effect of CN^- revealed that the RFI increased while the concentration of CN^- increased from 1.0 to 30 mM, and then went to flatness. Thus, 40 mM CN^- was selected to ensure the complete derivatization reaction. As the concentration of CN^- was fixed at 40 mM, the effect of the concentration of NDA was tested in the range of 0.5–6.0 mM. The RFI increased rapidly when the concentration of NDA increased in the range of 1.0–3.0 mM, and increased slightly while the concentration further increased. Thus, 3.5 mM NDA was chosen for the rapid derivatization reaction.

3.2. Optimization of CE separation procedure

An important characteristic of cyclodextrins (CDs) is their ability to form inclusion complexes with a wide variety of guest molecules. The frequently used CD is β-CD, which is well known for its potential application to increase the aqueous solubility of low polar compounds [36], and it can be added to CE separation buffers to increase the selectivity and efficiency of CE separations [37–44]. It has been found that adding β-CD, but not α or γ-CDs to CE buffer resulted in a significant enhancement of the fluorescence signal for the NDA-labeled glutamate and aspartate [39]. Thus, according to previous results, β-CD was used in our experiments. It was found that the detection sensitivity and separation speed of NDA-labeled phosphoamino acids can be obviously increased after the addition of β-CD in the running buffer. As shown in Fig. 4A, the RFI increased with the increase of the concentration of β-CD in the running buffer, and the migration time decreased as the concentration of β-CD increased (Fig. 4B). To explore the possible enhancement mechanism of the improved sensitivity, the effect of the concentration of β-CD in matrix on the RFI of derivatives was investigated by the fluorescence spectroscopy. As demonstrated in Fig. 5, after the addition of β-CD to matrix, the RFI increased, and the maximum excitation wavelength of derivatives red-shifted about 3 nm, indicating that the inclusion complexes were formed between β-CD and derivatives. If the concentration of the added β-CD was higher than 2.0 mM, the RFI increased slowly upon further increase of the concentration of β-CD (Fig. 6). The three

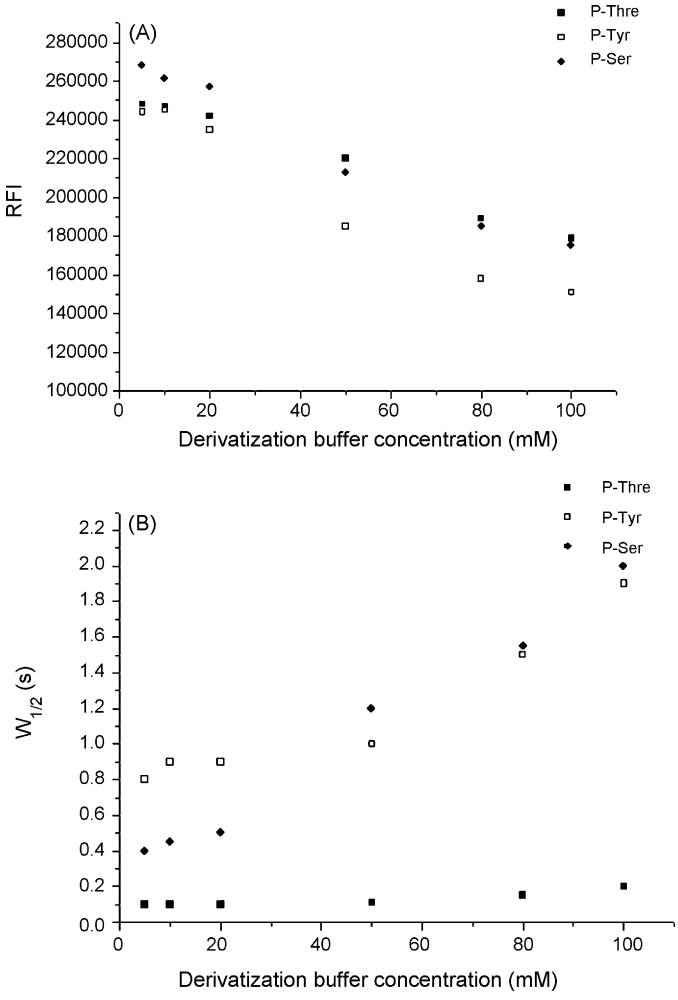

Fig. 3. Effect of derivatization buffer concentration on the RFI (A) and peak width at half-height (B). Other conditions as in Fig. 2.

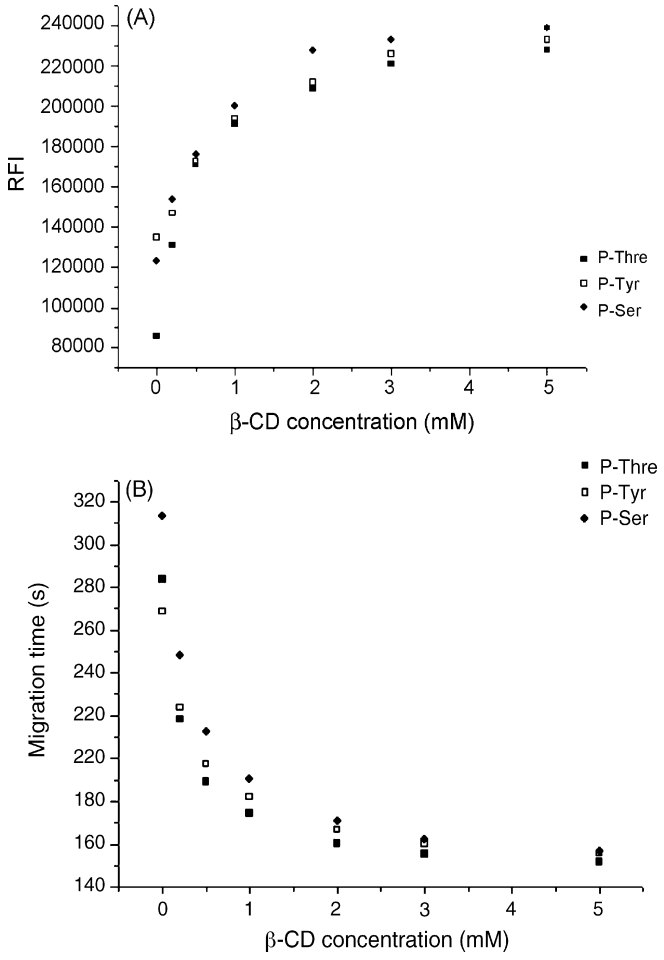

Fig. 4. Effect of β-CD concentration on the RFI (A) and migration time (B). Conditions: electrophoresis buffer, 30 mM sodium tetraborate with β-CD (0–5.0 mM) pH 9.2; other conditions as in Fig. 2.

NDA-labeled phosphoamino acids could be baseline separated with the concentration of β-CD in the range of 0.5–3.0 mM, but when the concentration of β-CD was beyond 3.0 mM, the peak of P-Tyr and P-Ser were overlapped. Thus, 2.0 mM β-CD was used in the running buffer.

The influence of pH of running buffer at the values of 8.0, 9.2 and 10.0 were also examined. The migration time increased while the pH value increased from 8.0 to 10.0, probably due to the increased negative charge of derivatives, which might be caused by the increased dissociation of phosphate of phosphoamino acids with the increased pH value of the buffer. When pH value was 9.2, the RFI was the highest and $W_{1/2}$ was the lowest. Therefore, pH 9.2 is suitable for the separation. The effect of pH value of sample matrix on the RFI of derivatives was also studied by fluorescence spectroscopy. It revealed that the RFI of derivatives was the same at different pH value (8.0, 9.2, 10.0), indicating that the different RFI and $W_{1/2}$ obtained in CE with different running buffer pH were caused by different separation process. The investigation of the concentration effect of the running buffer showed that when a low concentration of borate (10 mM) was used, the peak of P-Thre and P-Tyr were overlapped, and the migration time increased while the buffer concentration increased. Considering both the analysis time and

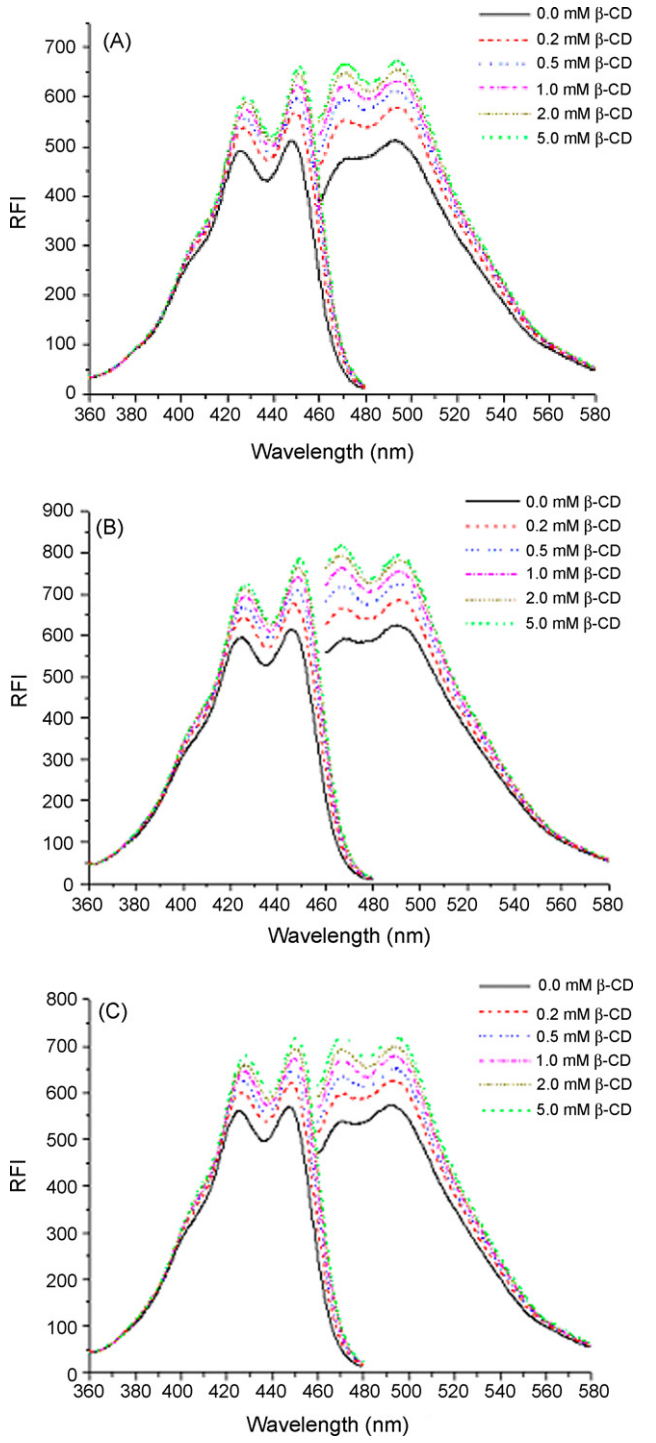

Fig. 5. Excitation and emission spectrum of three NDA-labeled phosphoamino acids with different β-CD concentration (0–5.0 mM). (A) P-Thre, (B) P-Tyr, (C) P-Ser.

the resolution, 30 mM of borate containing 2.0 mM β-CD (pH 9.2) was chosen as the optimum running buffer.

Under the above optimum conditions, the effect of separation voltage (13–22 kV) was investigated. It was found that the migration time decreased and the peak efficiency increased with the increase of voltage, although three NDA-labeled phosphoamino acids could be separated with voltage in the range of 13–22 kV. Since the Joule heating effect would be higher at the

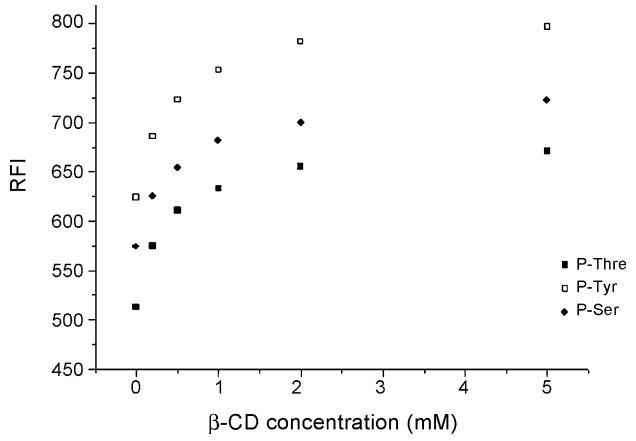

Fig. 6. Effect of β-CD concentration on the RFI of three derivatives at the maximum excitation and emission wavelength according to the data of Fig. 5.

higher voltage, 18–20 kV is suitable. With sampling height at 9.5 cm, the injection time (20–60 s) was also optimized. It was found that the RFI increased rapidly with injection time ranging from 20 to 40 s, and then increased slightly while injection time further increased from 40 to 60 s. When the injection time was 60 s or longer than 60 s, NDA-labeled P-Tyr could not be baseline separated with NDA-labeled P-Ser. Thus, 45 s was used for optimum injection.

3.3. Calibration, reproducibility and detection limit

Fig. 7 shows the separation of NDA-labeled P-Thre, P-Tyr and P-Ser with different concentrations under the optimum conditions. The reproducibility was performed by repeating injection ($n=6$) of the P-Thre, P-Tyr and P-Ser standard solution with concentration fixed at 5.0×10^{-6} M, 4.0×10^{-6} M and 6.0×10^{-6} M, respectively. The relative standard deviations (R.S.D.) of the peak height of P-Thre, P-Tyr and P-Ser were calculated to be 3.2%, 3.3% and 2.6%, respectively, while the R.S.D. of the migration time of P-Thre, P-Tyr and P-Ser were 0.99%, 1.10% and 1.14%, respectively. Using the fluorescence intensity versus sample concentration, the linear calibration curve was obtained for P-Thre in the range of 1.25×10^{-8}–5.0×10^{-6} M with regression coefficient (R) of 0.9998 ($n=7$); the linear calibration curve was obtained for P-Tyr in the range of 1.0×10^{-8}–4.0×10^{-6} M with R of 0.9994 ($n=7$); the linear calibration curve was obtained for P-Ser in the range of 1.5×10^{-8}–6.0×10^{-6} M with R of 0.9994 ($n=7$). From the data shown in Fig. 7B, the detection limits for P-Thre, P-Tyr and P-Ser were calculated to be 7.0×10^{-9} M, 5.6×10^{-9} M, and 7.2×10^{-9} M, respectively ($S/N=3$). It was noted that the concentrations of all the phosphoamino acids or protein amino acids shown in text and figure captions were the concentrations of the corresponding standard solution before derivatization, and it is expected that the sensitivity could be further improved by using laser as the excitation radiation.

3.4. Interference from other protein amino acids

As most of phosphoproteins possess phosphoamino acids with low abundance, it is important to investigate the interference from other protein amino acids with large molar excess over those of phosphoamino acids. In this study, since Cys and Pro could not react with NDA/CN^- to form the fluorescent derivatives [45], 18 protein amino acids were examined. It was found that NDA-labeled phosphoamino acids could be separated from other NDA-labeled protein amino acids. However, other protein amino acids with large excess would affect the derivatization process and the detection of phosphoamino acids. By fixing the concentration of phosphoamino acids, the interference effect is highly dependent on the concentrations of other protein amino acids. When the concentrations of P-Thre, P-Tyr and P-Ser were fixed as low as 1.0×10^{-7} M, 8.0×10^{-8} M and 1.2×10^{-7} M,

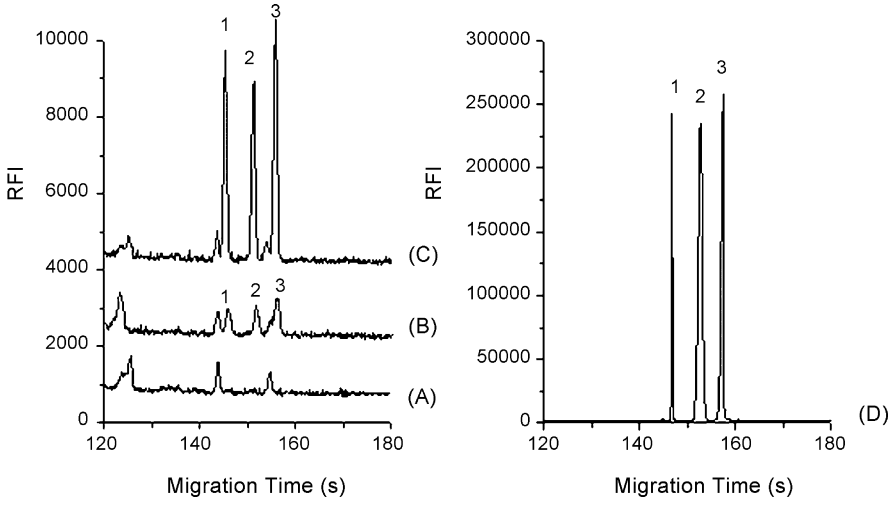

Fig. 7. Electropherograms of three phosphoamino acids with different concentrations under the optimum conditions. CE conditions: capillary, 40.5 cm × 50 μm i.d.; electrophoresis buffer, 30 mM sodium tetraborate with 2.0 mM β-CD pH 9.2; separation voltage, 18 kV; hydrodynamic injection, 9.5 cm (height) for 45 s. The concentration of P-Thre, P-Tyr and P-Ser in (B) were 1.25×10^{-8} M, 1.0×10^{-8} M and 1.5×10^{-8} M, respectively. The concentrations in (C and D) were 8-fold and 400-fold for corresponding phosphoamino acids in (B), respectively. The concentration of P-Thre, P-Tyr and P-Ser in (A) were 0.0 M. Peaks: (1) P-Thre; (2) P-Tyr; (3) P-Ser.

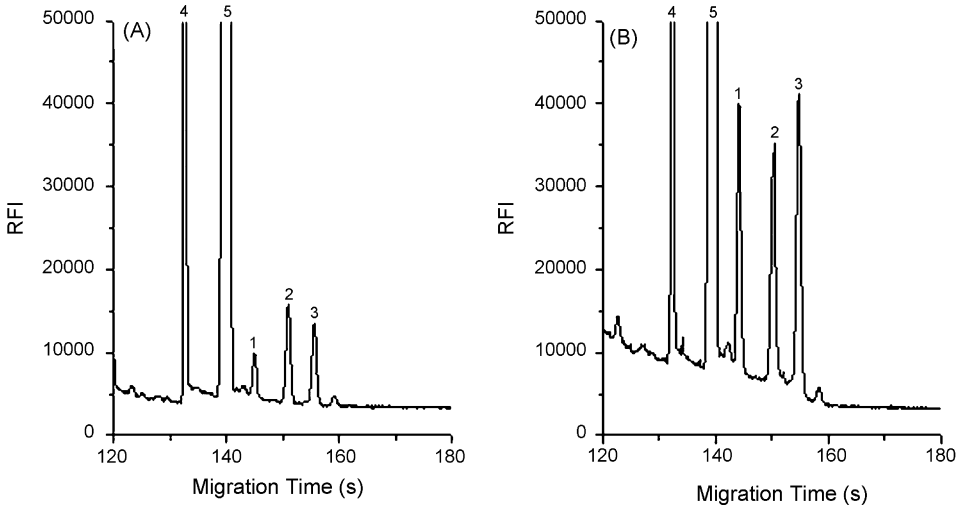

Fig. 8. Interference effect of 100-fold excess of 18 other protein amino acids on the detection of P-Thre, P-Tyr and P-Ser with concentration at 5.0×10^{-7} M, 4.0×10^{-7} M and 6.0×10^{-7} M, respectively, by using different concentration of NDA, (A) 3.5 mM; (B) 25 mM. CE conditions: Concentration of each other protein amino acids in was 5.0×10^{-5} M. Peaks: (1) P-Thre; (2) P-Tyr; (3) P-Ser, (4) Glu; (5) Asp; other conditions as in Fig. 7.

respectively, the derivatization interference from other protein amino acid with a 10-fold or 100-fold excess could be nearly ignored by comparing the peak heights of derivatives, although the 100-fold excess of other protein amino acids would still affect the detection of P-Thre in some degree, because of the uneven baseline. However, when the concentrations of P-Thre, P-Tyr and P-Ser were as high as 5.0×10^{-7} M, 4.0×10^{-7} M and 6.0×10^{-7} M, respectively, or even higher, the interference of other protein amino acids with 100-fold excess was very obvious (Fig. 8A). To decrease the interference from other protein amino acids, high concentration of NDA (25 mM) was used (Fig. 8B). It can be seen that the RFI increased markedly. However, when 25 mM NDA was used, the baseline was uneven, and the noise increased; the derivatized solution is turbid with red precipitates produced. The reason might be that NDA and CN^- can form fluorescent products if they are stored together [33]. According to the above investigation, it can be concluded that (1) high concentration of NDA was not suitable for the detection; (2) the sample of protein hydrolysate should be diluted to a low concentration to decrease the interference from other protein amino acids.

3.5. Phosphoamino acids analysis of protein hydrolysate

β-Casein, a standard phosphoprotein, is a single polypeptide chain of 209 amino acid residues, five of which are phosphoseryl residues [8]. According to the above researches on the interference from the other protein amino acids, it is known that the stock hydrolysate should be diluted to a desired concentration for the detection. But which concentration is suitable? Because of the destruction of the phosphoester bonds of phosphoamino acids during acids hydrolysis of proteins [8], it is

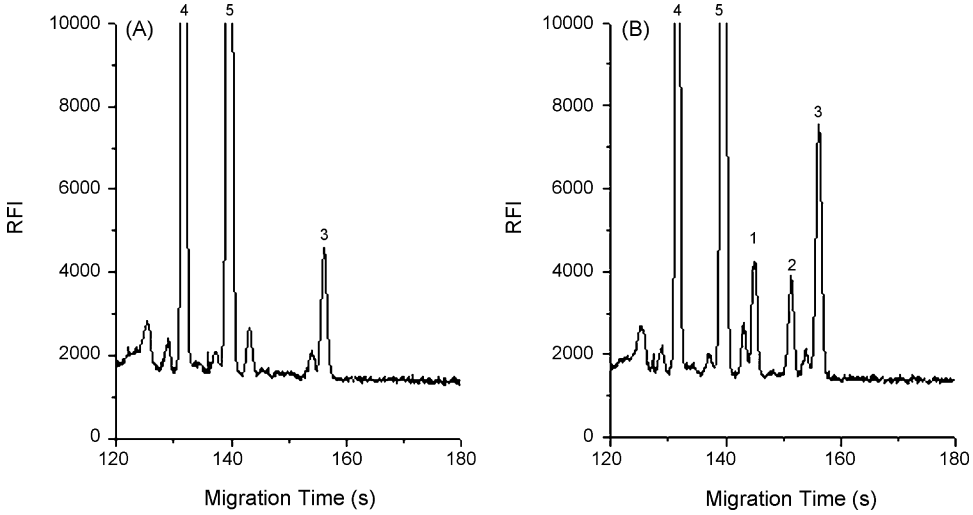

Fig. 9. Electropherograms of phosphoamino acids analysis in hydrolysate of β-casein (5.0×10^{-8} M). (A) sample without addition of standard; (B) sample with addition of P-Thre, P-Tyr and P-Ser standards with final added concentration at 5.0×10^{-8} M, 4.0×10^{-8} M and 6.0×10^{-8} M, respectively, in hydrolysate. Peaks identification was same in Fig. 8. Other conditions as in Fig. 7.

difficult to describe the concentration of stock hydrolysate by using the concentration of phosphoamino acid. So the concentration of β-casein was used as the concentration of protein hydrolysate. The concentration of stock hydrolysate was calculated to be about 5.0×10^{-5} M (FW of β-casein was about 22990; the content of β-casein used in this study was 90% as labeled). Fig. 9A shows the analysis of phosphoamino acids of diluted hydrolysate (5.0×10^{-8} M β-casein). It can be seen that some amount of P-Ser was detected. The peak of P-Ser was confirmed by using the standard addition method (Fig. 9B). The experimental results showed that the RFI of the peak of NDA-labeled P-Ser was linear to the concentration of the diluted hydrolysate in the range of 2.5×10^{-8}–1.0×10^{-6} M, indicating that the most desired concentration of the diluted hydrolysate for the detection was in the range of 2.5×10^{-8}–1.0×10^{-6} M. The recovery of phosphoamino acid was studied by adding the standard to the hydrolysate. The recoveries of P-Thre, P-Tyr and P-Ser were 98%, 93% and 97%, respectively. According to the RFI value obtained in Fig. 9A, the concentration of P-Ser in the diluted hydrolysate (5.0×10^{-8} M β-casein) was calculated to be 6.0×10^{-8} M. From the above data, it can be calculated that only 24% of the actual P-Ser value was detected because P-Ser residues are very sensitive to acid hydrolysis. This result is consistent with that reported previously [8].

4. Conclusion

A rapid and sensitive CE method coupled with fluorescence detection was developed for the identification of protein phosphorylation by determination of phosphoamino acids. NDA was used for the derivatization, and β-CD was added in running buffer to improve the sensitivity and separation speed. Under the optimum analysis conditions, the separation time was within 180 s, and the detection limits of 10^{-9} M were achieved for phosphoamino acids. The investigation of interference from other protein amino acids indicated that sample of protein hydrolysate should be diluted to a low concentration to decrease the interference. This method was successfully applied to the analysis of P-Ser in the hydrolysate of β-casein.

Acknowledgements

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