LVSEP Analysis of Phosphopeptides in Dynamically PVP-Coated Capillaries and Microchannels

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Abstract
To achieve highly sensitive analyses of phosphopeptides with simple experimental procedures in capillary electrophoresis (CE) and microchip electrophoresis (MCE), large-volume sample stacking with an electroosmotic flow pump (LVSEP) was performed in dynamically poly(vinyl pyrrolidone) (PVP)-coated capillaries to suppress the electroosmotic flow. In the analysis of monophosphopeptide (MPP) from bovine β-casein, good enrichments were attained with the sensitive enhancement factor (SEF) of 260 and 400 in CE and MCE, respectively, by using 0.2% PVP in 20 mM HEPES buffer (pH 9.0) in LVSEP.

Keywords: Capillary electrophoresis; Microchip electrophoresis; On-line sample preconcentration; LVSEP; Dynamic coating; Phosphopeptides

1. Introduction
Recently, analytical techniques for phosphopeptides have become important since phosphorylation of proteins controlled by enzymes plays a key role in cellular signal transductions and metabolisms [1-3]. Capillary electrophoresis (CE) is one of the most useful methods to analyze complex peptide mixtures. By applying the CE technique, highly efficient and fast phosphopeptide assay is promising. In spite of high efficiency, however, a low concentration sensitivity is often problematic in CE. One way to overcome this problem is applying an on-line sample preconcentration technique. Several groups reported an online automated system for phosphopeptide analysis using TiO$_2$ (titania)-based preconcentration followed by LC-MS/MS [4,5]. The developed systems were effective for selective enrichment and separation of phosphopeptides but the analysis times was often long. In CE, Yeung group reported an integration of a selective injection with a sample stacking technique used in CE to enrich the sample, followed by electrophoresis to fractionate the components in preparation for MALDI-MS analysis [6-8]. In this study, we investigated the application of large-volume sample stacking with an electroosmotic flow pump (LVSEP) to phosphopeptide analysis. LVSEP has unique characteristics including efficient enrichments by a whole capillary sample-injection, simple experimental procedures based on a voltage application without polarity switching, and high resolutions according to the movement of the stacked analytes zone from the capillary inlet to the outlet [9-19].

In our previous study [15-17], a fused silica capillary was often permanently coated with poly(vinyl alcohol) (PVA) by a thermal passivation method to obtain a specific electroosmotic flow (EOF). In the PVA coating, however, there are several problems, e.g., need of labor-intensive preparations, capillary clogging, and low success yield of the coating. To simplify the experimental procedures, a dynamic coating technique was applied to LVSEP [10]. In the dynamic coating, only the use of a running solution containing polymers alters the zeta potential of the inner surface of the capillary in the conditioning step and during the CE measurements.

Figure 1 shows the principle of LVSEP for anionic analytes. At first, the capillary is coated with neutral polymers such as poly(vinyl pyrrolidone) (PVP) to give weakly and negatively-charged surface (Fig. 1a). A low-ionic strength (I) sample solution containing cationic analytes is...
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fully injected into the capillary as shown in Fig. 1b. When the reversal polarity voltage is applied, the analytes are concentrated by the difference in the $I$ between the sample matrix and the background solution (BGS). Since the low-$I$ sample solution enhances the EOF velocity ($v_{eo}$) in the capillary, the stacked analytes move to the anode. As the BGS is introduced from the outlet vial into the capillary as depicted in Fig. 1c, $v_{eo}$ is gradually decreased and finally suppressed due to the high-$I$ BGS (Fig. 1d). Because the electrophoretic velocity ($v_{ep}$) becomes faster than $v_{eo}$, the stacked analytes zone migrates to the anodic side (Fig. 1e).

Hence, the LVSEP technique requires a faster and slower EOF at the stacking and the separation step, respectively. In this study, LVSEP based on the dynamic coating technique was applied to the analysis of phosphopeptides both in CE and microchip electrophoresis (MCE) formats.

2. Experimental

2.1. Chemicals

A fused-silica capillary of 50 μm i.d. was purchased from Polymicro Technologies (Phoenix, AZ, USA), poly(vinyl pyrrolidone) (PVP, Mw 360000), and polyvinyl alcohol (PVA, Mw 124000, 88% hydrolyzed) from Sigma-Aldrich (Tokyo, Japan), 2-[4-(2-hydroxyethyl)-1-pyperazinyl]ethyl-sulfonic acid (HEPES) from DOJINDO (Kumamoto, Japan), monophosphopeptide (MPP) from bovine $\beta$-casein (H-Phe-Gln-pSer-Glu-Glu-Gln-Thr-Glu-Leu-Glu-Leu-Gln-Asp-Lys-OH, Mw 2063) from Funakoshi (Tokyo, Japan), and fluorescein isothiocyanate (FITC), thiourea and other reagents from FUJIFILM-Wako (Osaka, Japan). For fluorescence labeling, 10 mL of 0.1 mg/mL MPP in a 20 mM borate buffer (pH 9.0) was mixed with 0.25 mL of a 25 mM methanolic FITC solution. The mixture was kept for 24 h at room temperature. The obtained stock solution of FITC-MPP was stored at −20°C. All solutions were prepared with deionized water purified by Auto-Still (WG510, Yamato Scientific, Tokyo, Japan) and Simplicity-UV (Merck Millipore, Billerica, MA, USA) systems, and filtered through a 0.45 μm pore membrane filter prior to use. For the LVSEP analyses, FITC-MPP was dissolved with deionized water, while in conventional CE and MCE analyses with the BGS. In the LVSEP measurements, 0.2 % PVP in 20 mM HEPES buffer (pH 9.0) was mainly used as the BGS.

2.2. CE measurements

CE analyses were performed with a home-made apparatus, which consists of a fluorescence detector (FP-2020, JASCO, Tokyo, Japan) and a high-voltage power supply (HCZE-30PN0.25, Matsusada Precision, Shiga, Japan). The excitation and fluorescence wavelengths were 480 and 520 nm, respectively. Output signals were acquired by using a Chromatopac (C-R8A, Shimadzu, Kyoto, Japan). In the CE analyses, sample injections were performed hydrodynamically by lifting the inlet sample vial 15 cm (0.2 psi) above the outlet BGS vial for 30 s (injection length, 0.6 cm). The separation voltage was applied at −15 kV . In the LVSEP analyses, samples were injected by a syringe pump with 7.5 mL/h for 30 min (whole capillary injection). The value of sensitive enhancement factor (SEF) was calculated by comparing the peak height obtained under the LVSEP condition with that in the conventional CE experiment, while taking into account the dilution factor. In the determination of the electroosmotic mobility ($\mu_{eo}$) in the dynamically PVP-coated capillary, the outlet vial was filled with the BGS, while the inlet vial was filled with 100 ppm thiourea (EOF marker) dissolved in the BGS. The applied voltage and the UV wavelength were set at +15 kV and 200 nm, respectively. The EOF mobility was calculated from the time to move the EOF marker from the inlet to the detector.

2.3. MCE measurements

A PDMS microchannel was fabricated by the conventional soft lithography technique. A straight-channel chip had a single channel (150 μm width × 100 μm depth) with a total separation channel length of 30.0 mm. After producing reservoir wells (2.5 mm diameter) with a piercer, these microchannels were directly bonded onto the slide-glass lids (76.2 × 25.4 mm) via plasma treatment (YHS-R, SAKIGAKE-Semiconductor, Kyoto, Japan). For semi-permanent PVA coating, fabricated PDMS-glass hybrid microchannels were coated with PVA by the vacuum-drying method [20]. Briefly, a 10 mM SDS solution was manually introduced into the whole microchannel by using a microsyringe to hydrophilize the PDMS surface. After
removing the SDS solution, the microchannel was loaded with the coating polymer solution. The microchip was put into a vacuum-chamber under reduced-pressure (< ~1 Torr) with an oil rotary vacuum pump (G-50DA, ULVAC KIKO, Saito, Japan) for 10 min. The bare (no PVA coating) and the PVA-coated channels were washed with water, and finally conditioned with the BGS prior to use.

In the LVSEP analysis on the straight-channel chip, a sample solution was introduced into the entire channel by manually using a syringe. The inlet and outlet reservoirs were filled with 5.0 µL of a BGS. A voltage of –3.0 kV was applied with a high voltage power supply (HCZE-30PN0.25, Matsusada Precision, Kusatsu, Japan) through two platinum electrodes immersed in the two reservoirs. To evaluate the enrichment efficiency in LVSEP, the value of SEF was calculated by comparing the peak height obtained under the LVSEP condition with the fluorescence intensity from the microchannel filled with the sample solution (no preconcentration) taking into account the dilution factor. To determine the EOF rate in the microchannels, current monitoring method [21] was employed. In the EOF experiments, \( \mu_{eo,S,CE} \) and \( \mu_{eo,BGS,MCE} \) were determined from the current-time curve.

3. Results and discussion

To analyze FITC-MPP by LVSEP, PVP was selected as the dynamic coating reagent. Kaneta et al. reported that the addition of PVP to BGS gave effective suppression of EOF in CE [22]. In our previous study, furthermore, we confirmed that PVP was more suitable for LVSEP relative to HPMC and PEO [10]. As shown in Fig. 2, a single peak of MPP was observed both in CZE and LVSEP with the BGS containing 0.2% PVP at pH 7.0~9.0. As can be seen, at pH 7.0 and 8.0, a broader peak of MPP was obtained in LVSEP, whereas at pH 9.0 a relatively sharp peak appeared (Fig. 2). Comparing with the peak height in CZE, the values of SEF obtained with LVSEP were calculated for MPP. As a result, at pH 9.0, the SEF of 260 was apparently higher than those at pH 7.0 and 8.0 (Table 1). This would be caused by a faster migration of MPP since the negative charges of MPP were increased at pH 9.0. At pH 10.0, the reproducibility of the LVSEP analysis of MPP was quite poor (data not shown), which was due to low buffering ability of HEPES. Hence, pH 9.0 buffer was selected in the remaining experiments.

Table 1. Effect of pH on the CE-LVSEP analyses of FITC-MPP

| pH   | migration time / min | SEF       |
|------|---------------------|-----------|
| 7.0  | 19.5 (1.6%)         | 120 (14.8%)|
| 8.0  | 21.6 (0.5%)         | 130 (18.1%)|
| 9.0  | 15.9 (0.7%)         | 260 (7.7%) |

1) BGS, 0.2% PVP in 20 mM HEPES (pH 7.0~9.0).
2) Values in parentheses are %RSDs (n = 3).

Table 2. Effect of the PVP concentration on the CE-LVSEP analyses of FITC-MPP

| PVP   | migration time / min | SEF       |
|-------|---------------------|-----------|
| 0.1%  | 17.0 (3.7%)         | 240 (20.4%)|
| 0.2%  | 15.9 (0.7%)         | 260 (7.7%) |
| 0.5%  | 18.2 (6.6%)         | 200 (17.2%)|

1) BGS, 0.1–0.5% PVP in 20 mM HEPES (pH 9.0).
2) Values in parentheses are %RSDs (n = 3).

Table 3. Mobility data in the present system

|             | \( \mu_{eo,BGS,CE} \) | \( \mu_{eo,S,CE} \) | \( \mu_{eo,BGS,MCE} \) |
|-------------|-----------------------|---------------------|-----------------------|
|             | \( 2.0 \times 10^{-5} \text{ cm}^2\text{V}^{-1}\text{s} \) | \( 9.5 \times 10^{-5} \text{ cm}^2\text{V}^{-1}\text{s} \) | \( 9.0 \times 10^{-5} \text{ cm}^2\text{V}^{-1}\text{s} \) |
| \( \mu_{eo,S,CE} \) | \( 3.5 \times 10^{-5} \text{ cm}^2\text{V}^{-1}\text{s} \) | \( 2.3 \times 10^{-5} \text{ cm}^2\text{V}^{-1}\text{s} \) |                      |

1) BGS, 0.2% PVP in 20 mM HEPES (pH 9.0).
Since the electrophoretic mobility of MPP ($\mu_{eo,MPP}$) exceeded $\mu_{eo,BGS,CE}$, furthermore, they could migrate toward the outlet of the capillary in the separation step. It was confirmed that, therefore, the prepared coating capillary fulfilled the required EOF condition for the LVSEP analysis of anions.

In the MCE analyses by LVSEP, we reported that PVA-coated microchannel prepared by the vacuum-drying method is useful for suppressing the EOF with easy and rapid modification processes [20]. To evaluate the effectiveness of the dynamically PVP-coated channel, the enrichment performance was compared with the semi-permanently PVA-coated microchannel. As shown in Fig. 3, the peak of MPP appeared both in the bare and PVA-coated channels by using 0.2% PVP in HEPES buffer (pH 9.0). However, the peak height in dynamically PVP-coated channel (Fig. 3a) was apparently lower than that in the semi-permanently PVA-coated microchannel (Fig. 3b). As can be seen, the degree of the peak leading of MPP is more significant in Fig. 3a. In the LVSEP technique, the peak leading is often caused by the surface adsorption of the analytes, indicating that the amount of immobilized PVP was insufficient in the dynamic coating technique. As summarized in Table 3, however, PVP was useful to fulfill the required EOF condition for the LVSEP analysis even in MCE, i.e., $\mu_{eo,S,MCE} > \mu_{eo,MPP} > \mu_{eo,BGS,MCE}$. Furthermore, the SEF of 400 was only 20% decrease in the enrichment efficiency compared with PVA-coated microchannel as shown in Table 4. Hence, the proposed dynamic-coating approach can be more effective small and non-biological analytes in MCE since the effect of the surface adsorption is generally negligible for these compounds.

To determine the linearity for the quantitation in CE- and MCE-LVSEP, a calibration curve with five standards of concentrations, i.e., 0.10, 0.15, 0.20, 0.30 and 0.50 ppm of MPP, were prepared. Peak areas obtained with LVSEP were plotted against the concentration. The regression slope, intercept, correlation coefficient and LOD were calculated to be 22700 au·s/ppm, 494 au·s, 0.994, and 0.021 ppm, respectively, in CE, while they were 310 au·s/ppm, 6.6 au·s, 0.994, and 0.013 ppm, respectively, in MCE. Since good linearity were obtained both in CE and MCE, the dynamically PVP-coated capillaries and microchannels are effective for the determination of MPP. For the LVSEP analysis of phosphopeptides in real samples, sample matrix effects on the quantitation ability should be evaluated in the dynamically PVP-coated columns.

4. Conclusions

In this study, the LVSEP technique was combined with the dynamic coating technique in CE and MCE. As the dynamic coating reagent, PVP was useful to fulfill the required EOF condition for the LVSEP analysis of anionic analytes both in the capillary and microchip formats. In the LVSEP analyses of MPP in CE and MCE, the values of SEF were estimated to be 260 and 400, respectively. Since the developed method eliminated the complicated and labor-intensive procedures for the permanent polymer coating, especially for the capillary format, effective preconcentrations by LVSEP can be obtained with simple experimental procedures. As preliminary results, FITC-labeled casein phosphopeptides were well resolved by LVSEP in the dynamically PVP-coated capillaries, so that the proposed method can be applied to the separation of complicated phosphopeptide samples. These results will be reported in a separated paper.

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