Short Communication

Sensitivity Enhancement by Sweeping via Solid Phase Extraction Using Titania Nanoparticles in Capillary Electrophoretic Analysis of Phosphopeptides

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Abstract
This paper reports an on-line sample preconcentration by a sweeping technique based on the surface complexation between TiO₂ (titania) nanoparticles and phosphate groups in the capillary electrophoresis (CE) analysis of phosphopeptides. It is well-known that titania particles can trap phosphopeptides under an acidic condition, while under an alkaline condition they are eluted from the titania surfaces. In the sweeping via solid phase extraction using the titania nanoparticles, a phosphopeptide solution is injected as a long plug into a capillary filled with an acidic background solution. Due to the complexation onto the titania surfaces, the analytes in a long sample zone are swept by the titania particles to a narrow zone. This preconcentration technique was applied to the analysis of monophosphopeptide from β-casein. When a 1% titania dispersion and 100 ppm phosphopeptide solution was successively introduced into the capillary at the injection time of 30 s and 60 s, respectively, a sharp peak of the phosphopeptide was obtained by UV detection without any optical interferences of the titania. Comparing with a conventional CE analysis, a 22-fold sensitivity increase was achieved by the preconcentration effect.

Keywords: Capillary electrophoresis; On-line sample preconcentration; Titania nanoparticles; 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]. In the analysis of phosphopeptides, selective and sensitive methods should be introduced due to minor amounts of phosphopeptides, i.e., signals from a larger amount of non-phosphopeptides interfere with the detection of phosphopeptides [4]. In the case of MS detection, furthermore, the sensitivity of phosphopeptides is generally lower than that of non-phosphopeptides. Hence, it is necessary to develop a sensitive technique that can separate phosphopeptides from others.

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 on-line automated system for phosphopeptide analysis using TiO₂ (titania)-based preconcentration followed by LC-MS/MS [5-7]. 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 concentration, followed by electrophoresis to fractionate the components in preparation for MALDI-MS analysis [8-10] or to combine directly with ESI-MS. To our knowledge, an on-line coupling of the titania enrichment with CE was not found for the phosphopeptide analysis.

It is well known that phosphate species are absorbed onto TiO$_2$ only under acidic conditions. By utilizing this characteristic, phosphopeptides can be enriched by solid phase extraction (SPE) on a titania column [11]. After applying a sample solution containing peptides on the titania column, an acidic eluent is passed through the column. Only phosphopeptides are adsorbed onto the titania adsorbents and others are eluted by the acidic solution. Finally, phosphopeptides are eluted by an alkaline solution. Hence, titania has recently become a common tool of separating selectively phosphopeptides from others by SPE method.

In this study, a new on-line preconcentration CE technique was developed by using titania nanoparticles, which have a high surface-to-volume ratio. In our previous report, the on-line preconcentration of glycoproteins was attained via the complexation between the analytes and borate ions in CE [12]. In this technique, an anionic glycoprotein solution containing no borate ions is injected as a long plug into a capillary filled with a borate buffer. Due to the complexation between glycoproteins and borate ions, the analytes in a long sample zone are swept by borate ions to a narrow zone, resulting in the 30~40-fold increases in the sensitivity. Since borate ions exhibit almost no absorption in the UV region, the UV detection of glycoproteins is not interfered. However, the titania nanoparticles have strong UV absorption. Hence, we modified the sweeping technique, i.e., the titania dispersion is partially injected in front of a long sample zone.

Fig. 1 shows the schematics of the sweeping via SPE using the titania nanoparticles. In the proposed on-line sample preconcentration technique, at first, a bare fused-silica capillary is filled with an alkaline background solution (BGS, pH 11.1). In the capillary, a faster electroosmotic flow (EOF) occurs by preconditioning the alkaline BGS. Next, the titania nanoparticles dispersed in an alkaline solution (pH 10.1) is partially injected as a long plug. A large volume of a phosphopeptide sample which is dissolved in an acidic solution (pH 2.3) is subsequently injected into the capillary (Fig. 1a). When the voltage is applied, the titania particles having negative charges due to the basicity of the dispersion matrix invade the sample zone. Since the acidity of the sample matrix (pH 2.3) was stronger than the basicity of the titania dispersion matrix (pH 10.1), the pH around the sample/TiO$_2$ boundary is kept low, resulting in the retention of phosphate analytes onto the titania surface. Assuming that the electrophoretic mobility of the titania particles is higher than that of phosphopeptides owing to their multiple charging and higher surface/volume ratio of the TiO$_2$ nanoparticles, the apparent mobility toward the cathode of the free analytes in the sample matrix exceeds that of the titania particles holding phosphates (Fig. 1b). Furthermore, the titania nanoparticles become positive in the acidic sample matrix. As a result, the titania particles holding the analytes are concentrated because of the changes in the surface charges. After the concentration of the analytes and the titania is completed, hydroxide ions in the BGS vial arrive at the sample/TiO$_2$ boundary. When the pH around the concentration boundary becomes higher, the analytes are eluted from the titania surface (Fig. 1c). Finally, phosphate analytes are separated by capillary zone electrophoresis (CZE) as shown in Fig. 1d. In this study, we investigated the applicability of the sweeping via SPE using titania nanoparticles to the enrichment of phosphopeptides in CE.

2. Experimental

2.1. Chemicals

Adenosine triphosphate (ATP) and trifluoroacetic acid (TFA) were obtained from Nacalai Tesque (Kyoto, Japan), titanium (IV) oxide nanopowder (anatase, 25 nm) from SIGMA-ALDRICH (Tokyo, Japan), monophosphopeptide (from β-casein bovine, H-Phe-Gln-pSer-Glu-Glu-Gln-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys-OH) from Funakoshi (Tokyo, Japan), and 25% ammonia solution from Wako (Osaka, Japan). All reagents were of analytical or HPLC grade. All solutions were prepared with deionized water purified by using a Direct-Q System (Nihon Millipore,
Japan), and filtered through a 0.45 μm pore membrane filter prior to use.

2.2. CE measurements

All CE measurements were performed on a P/ACE MDQ system (Beckman Instruments, Fullerton, CA, USA) equipped with a diode-array UV detector. Separations were carried out on fused-silica capillaries (50 μm i.d., Polymicro Technologies, Phoenix, AZ, USA) of 30.0 cm effective length. BGS) used in the CE analysis of ATP and the monophosphopeptide were 10 mM phosphate buffer (pH 7.0) or 0.1% NH₃ (pH 11.1). Titania nanoparticles were dispersed to give the concentration of 0.25~1.0% in 0.01% TFA (pH 2.3) before and after adding 0.05 ppm TiO₂ nanoparticles. As can be seen, the absorption of ATP apparently disappeared after the addition of titania nanoparticles. The observed UV spectra clearly indicates that ATP and phosphate compounds can be efficiently trapped on the surface of the titania. It was reported that rutile-form titania, which is prepared by the calcination at 800 °C, can trap phosphopeptides more efficiently than anatase-form [6]. On the basis of the UV-absorption measurement, however, we consider that the anatase titania nanoparticles have a sufficient ability to sweep the phosphate analytes. Hence, the titania particles were used without the calcination.

In the sweeping via SPE, a titania dispersion and a sample solution containing phosphate analytes are injected subsequently into the capillary filled with a BGS consisting of ammonia as shown in Fig. 1. First, the effect of the titania nanoparticles was investigated. When a long plug of the ammonia solution (pH 10.1) devoid of the titania nanoparticles was introduced for 30 s into the capillary filled with the basic BGS (pH 11.1), and then a sample solution containing ATP (pH 2.3) was injected as a short plug (the injection time of 5 s), the migration time and the plate number of ATP were 2.23 min and 9900, respectively. On the other hand, the first injection of 0.25% titania dispersion (pH 10.1) in place of the ammonia solution gave the ATP peak with the migration time of 3.32 min and the plate number of 6200 (data not shown). The slower migration time obtained in the presence of the titania indicated the retention of the phosphate analytes by the nanoparticles. The lower efficiency under the sweeping condition would be due to the heterogeneity of the surface charges of the nanoparticles retaining the analytes, resulting in the band broadening. Furthermore, the pH selections of the BGS, the dispersion of TiO₂ and the sample matrix were also important. As a typical example, when the pH of the partially injected plug in front of the sample solution was same with that of the BGS (pH 11.1), the migration time and the plate number of ATP were almost unchanged both in the presence and absence of the titania nanoparticles, indicating a lower retention by the titania. Higher basicity of the titania dispersion (pH 11.1) should cause a quick neutralization of the sample solution compared to the lower pH condition (pH 10.1). Since phosphate analytes can be retained only under acidic conditions, the lower retention of ATP by the titania nanoparticles was observed at pH 11.1. Considering these factors, the pH values of the BGS, TiO₂ dispersion and the sample matrix were set at 11.1, 10.1 and 2.3, respectively.

3. Results and discussion

In the sweeping technique, the retention by pseudostationary phase strongly affects the preconcentration performance, i.e., higher retention factor gives higher enrichment efficiency [13]. Prior to the sweeping experiments, thus, the retention feature of the titania nanoparticles was studied by using ATP as a standard phosphate analyte. Fig. 2 shows the UV absorption spectra of 10 ppm ATP in 0.05% TFA (pH 2.3) before and after adding 0.05 ppm TiO₂ nanoparticles. As can be seen, the absorption of ATP apparently disappeared after the addition of titania nanoparticles. The observed UV spectra clearly indicates that ATP and phosphate compounds can be efficiently trapped on the surface of the titania. It was reported that rutile-form titania, which is prepared by the calcination at 800 °C, can trap phosphopeptides more efficiently than anatase-form [6]. On the basis of the UV-absorption measurement, however, we consider that the anatase titania nanoparticles have a sufficient ability to sweep the phosphate analytes. Hence, the titania particles were used without the calcination.

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Fig. 3 shows the electropherograms of ATP obtained with
a conventional CZE and the sweeping via SPE. Under the sweeping condition with a longer injection time of the sample (120 s), an 18-fold higher peak was obtained relative to the normal CZE due to the preconcentration effect. Broader and multiple peaks appeared from 1.2 to 2.2 min were assigned to the TiO2 nanoparticles. Although the higher ATP peak was observed in Fig. 3b, the shape was step-like. This may be due to a slower and lower elution from the titania surfaces (Fig. 1c). As a corroborating evidence, the peak shape of “mono”-phosphopeptide from β-casein bovine was almost symmetry (shown later), not step-like. Hence, “tri”-phosphate of ATP might cause a stronger adsorption onto the titania and a slower elution from the surface. To investigate the effect of the amount of the pseudostationary phase on the sweeping, the injection time of the titania dispersion was varied. At the injection time of 30, 60, 120 and 180 s, the SEFs were calculated to be 16, 18, 17 and 19, respectively, and all the peak shapes remained step-like. These constant SEFs suggested that 30 s-injection of 0.25% TiO2 nanoparticles gave a sufficient amount of the pseudostationary phase for the sweeping. In the ATP analysis, the relative standard deviations of the migration time and the peak height were calculated to be 2.6% and 8.7%, respectively, which were adequate repeatabilities in the on-line sample preconcentration CE experiments.

Under the optimized sweeping condition, monophosphopeptide from β-casein was analyzed, but a poor preconcentration was obtained. As mentioned above, the retention of the “mono”-phosphopeptide by the titania particles would be weaker than ATP consisting of three phosphate groups. Lower retention of the analytes by the titania nanoparticles reduced the sweeping efficiency. To enhance the SEF of the monophosphopeptide, the concentration of the TiO2 nanoparticles was increased from 0.25 to 1.0%, which can enhance the retention factor [14,15]. When 1.0% titania dispersion (pH 10.1) was introduced for 30 s into the capillary, and then 100 ppm monophosphopeptide (pH 2.3) was injected for 120 s, the analytes were successfully concentrated to a sharp peak as shown in Fig. 4, giving the SEF of 20. Comparing with the ATP analysis (Fig. 3), the longer migration time was observed. This was mainly attributed not to an effective retention of the monophosphopeptide but to a slower migration of the analyte in the alkaline BGS. Since the monophosphopeptide consists of one phosphate group and sixteen amino acids, which are charged to strongly anionic at pH 11.1, the apparent mobility at the CZE separation step (Fig. 1d) is quite low. Generally, a slower migration of the analytes at the separation step causes a broader peak due to the band broadening. The obtained sharp and almost symmetric peak of the monophosphopeptide in spite of the long migration time suggested the efficient and rapid enrichment and elution processes under the sweeping condition.

Fig. 5 shows the effect of the sample injection time on the SEFs of the monophosphopeptide and ATP. The SEFs of the monophosphopeptide were increased upon increasing the injection time from 15 to 60 s, while almost constant SEFs were obtained above 60 s. Since longer injection
times gave broader peaks, the sample injection time of 60 s was optimal in the analysis of the monophosphopeptide. On the other hand, the SEFs of ATP were proportionally enhanced from 8 to 25 upon increasing the sample injection time from 60 to 180 s. Hence, the longer sample injection time was effective for improving the SEF of ATP relative to the monophosphopeptide. This would be due to a higher retention factor of ATP, which allowed the enrichment of the analytes in the long sample zone [13]. Therefore, the obtained results demonstrated that the sweeping via SPE using the titania nanoparticles was effective for the preconcentration of phosphate analytes.

4. Conclusions
The sweeping via SPE using the titania nanoparticles was successfully applied to the preconcentration of phosphopeptides. By partially injecting the titania dispersion in front of a long sample plug, the maximum SEF of 22 for the monophosphopeptide from β-casein bovine was achieved relative to a conventional CZE. The developed sweeping technique is expected to realize the selective preconcentration and the separation of phosphopeptides from non-phosphorylated peptides, and works along the line is now in progress in our laboratory.

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References