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Phosphopeptide-selective Column-switching RP-HPLC with a Titania Precolumn

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A methodology of phosphopeptide-selective analysis coupled with column-switching HPLC utilizing titania as precolumn media is presented. Phosphopeptides were selectively enriched on titania packing within a protein/peptide mixture without any additional procedure, and analyzed by column-switching high-performance liquid chromatography. First, phospho-compounds were separated from complex mixtures by trapping them under acidic conditions on a titania packing, where non-phosphorylated compounds were effused out of the precolumn. Subsequently, phospho-compounds were desorbed from the titania column under a specific condition and analyzed. The behavior of phospho-compounds on a titania surface, especially adsorption/desorption, was precisely examined and optimized. A phosphoric buffer was successively employed for the elution of phosphopeptides on a titania surface by competition with the free phosphate group. From the successes of a selective concentration/analysis of phosphopeptides with column-switching HPLC with a titania precolumn, a novel phosphopeptide-selective RP-HPLC analysis has been shown to have an application possibility as a tool for phosphoproteomics.

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Introduction

The reversible phosphorylation of proteins, especially serine, threonine and tyrosine residues, has been widely recognized as being the major mechanism that regulates a wide range of cellular processes, such as signal transduction, cell division, cell motility, apoptosis, metabolism, differentiation, gene regulation, and carcinogenesis. Peptides representing phosphorylation sites often mimic a partial function of corresponding proteins. The separation of phosphopeptides within their nonphosphorylated form is essential for comparing these two chemical species from the viewpoint of phospho-proteomics. Additionally, since phosphopeptides are apt to be expressed in low abundance, high sensitivity and selectivity have been required for detecting phosphopeptides and mapping protein phosphorylation.

Within the last 10 years, mass spectrometry (MS) has emerged as a key technique in phosphorylated and non-phosphorylated protein/peptide analyses. An effort has been made to apply electrospray ionization mass spectrometry (ESI-MS) to identify the exact site of phosphorylation in peptides. However, since phosphopeptides are only partly ionized in MS analysis due to the electronegativity of phosphoryl groups increasing in a peptide mixture, it was hard to analyze them. Yoshizato *et al*. reported a treatment method using phosphatases to selectively determine phosphorylated protein in 2D-gel electrophoresis before proteolytic digestion.[1,2] However, since it, in the field of proteomics, tends to detect a small amount of protein, including a large amount of other proteins as a matrix within a 2D-gel spot, it is not adequate for the identification/analysis of a significant phosphorylated protein. Selective modifications of phosphopeptides in a digested mixture of proteins for MS analysis were explained by Oda *et al*.[3] and Zhou *et al*.[4] Although these methods were applicable to high-throughput analysis, and were efficient for substantial phosphorylated protein identification in the field of proteomics, it still had difficulties to be widely applied to protein analysis because of complicated reaction steps, for sample preparation.

An attractive method is to remove interfering components by a selective enrichment of phosphopeptides using affinity techniques, such as immobilized metal ion affinity chromatography (IMAC).[5] Concerning the IMAC method, the metal ion for catching phosphopeptide is immobilized so as to make a complex formation of iron with column packing materials by loading the metal-ion solution secondarily. Phosphopeptides are caught by complex formation at the far side of iron. Two steps are required for the selective retention of phosphopeptide; it is of concern that these steps have a possibility to lead to an inadequacy of the reproducibility. Additionally, an alkaline condition is typically necessary for eluting phosphopeptides from IMAC media,[6,7] regardless of their unstableness.[8-10] Thus, a further simplified and suitable method having highly selectivity toward phosphopeptides has been required.

Recently, titanium dioxide (titania, TiO_2) has attracted interest as an alternate support material to silica in the field of HPLC packing[11,12] because of its high chemical stability and sufficient rigidity. Additionally, it has attracted attention concerning its uniquely amphoteric ion-exchange properties. Ikeguchi *et al*.[13] reported the separation of phosphoamino acid, phosphopeptide and nucleotide base with column-switching HPLC equipped with a titania column. Titania was used as the precolumn of an anion-exchange column, where the eluent was H_2O containing TFA and a borate buffer by adding sodium chloride. The reported results indicated that the method was applicable for the selective analysis of phosphopeptides by merely changing the HPLC elution. The results also suggested that organic

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Table 1 Amino acid sequences of phospho- and unphosphorylated peptides

No	Peptide sequence	MW
Pep1	YSK IEK IGE GT(p)Y GVV YKG R	2227.5
Pep2	YSK IEK IGE GTY(p) GVV YKG R	2227.5
Pep3	YSK IEK IGE GTY GVV YKG R	2147.5
Pep4	YPI KPE APG EDA SPE ELN RYY ASL RHY LNL LTR PRY-NH_2	4292.8
Pep5	IKP EAP GED ASP EEL NRY YAS LRH YLN LVT RQR Y-NH_2	4049.5
Pep6	TRD IY(p)E TDY YRK	1702.8
Pep7	TRD IYE TDY YRK	1622.8

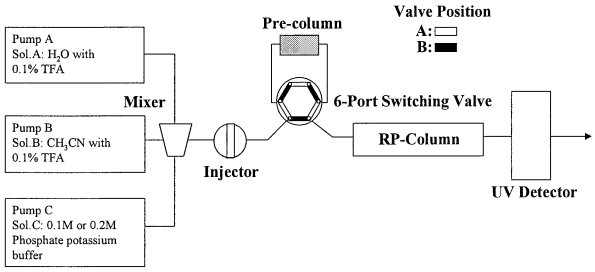


Fig. 1 Schematic diagram of the column-switching HPLC system.

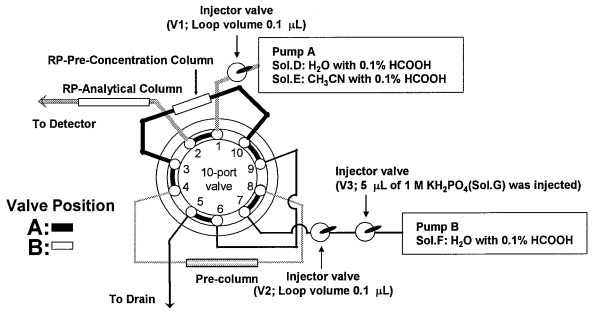


Fig. 2 Schematic diagram of the capillary-sized column-switching HPLC system.

phosphates were effectively absorbed under an acidic condition, and desorbed under an alkaline condition from a titania surface. Further, it was shown that the higher is the salt concentration of the buffer solution, the less is the retention time of organic phosphates on titania. Also, it was not sufficiently suitable for the analysis of phospho-compounds due to its instability to an alkaline condition,[8] and for direct connection to an MS detector due to its containing salts during elution.

In the present study, we precisely optimized the absorption/desorption condition, while utilizing titania as precolumn media for reversed-phase HPLC (RP-HPLC), for a phosphopeptide-selective analysis. Automated column-switching HPLC with UV and capillary-sized column-switching HPLC with MS are also described here.

Experimental

Chemicals and reagents

The phosphorylated and non-phospholylated peptides used in this study are indicated in Table 1. Pep1, Pep2, Pep3, Pep4 and Pep5 were kindly provided by Dr. N. Yumoto (AIST, Osaka, Japan).[14] Pep6 and Pep7 were purchased from Sigma-Aldrich (Tokyo, Japan). Sequencing Grade Modified Trypsin (bovine) was purchased by Promega (Tokyo, Japan). β-Casein was obtained from Sigma-Aldrich. For preparing mobile phase and standard samples, deionized water was prepared with a Milli-Q system (Nihon Millipore Kogyo, Tokyo, Japan). All other reagents were of analytical grade, and purchased from KISHIDA Chemicals (Osaka, Japan).

Sample preparation

For column-switching HPLC analysis, each peptide was dissolved in H_2O at a concentration of 50 μg/mL. For capillary-sized column-switching HPLC analysis, 10 mg β-casein was dissolved in 1 mL of 50 mM NH_4HCO_3, and then 100 μL of that was added to 840 μL of 50 mM NH_4HCO_3. A 100-μL volume of 50 mM NH_4HCO_3 was added to a 1-tube amount of Sequencing Grade Modified Trypsin, and 60 μL of that was added to the β-casein diluted solution. Those solutions were incubated at 37°C for 15 h.

Apparatus

Column-switching HPLC. A column-switching HPLC system consisted of three PU-611 inert pumps (GL Sciences; Tokyo, Japan), an L-7455 UV-VIS Diode Array Detector (HITACHI; Tokyo, Japan), a Model DM-32AP Low-Volume dynamic mixer (GL Sciences) and a Model 8125 injection valve (5 μL of sample loop; Rheodyne, CA, USA). An E1E-010 6-port valve (Senshu Scientific, Tokyo, Japan) was used as the switching valve. The newly developed Titansphere-TiO titania column (GL Sciences) was used as a preconcentration column, and chromatographic separation was performed on an Inertsil WP300C8 column (150 × 4.6 mm i.d.; GL Sciences). Data acquisition and processing were performed using a D-7000 System Manager (HITACHI).

Capillary-sized column-switching HPLC. Capillary-sized column-switching HPLC was performed using an AccuStream-700 automatic capillary HPLC system (GL Sciences) consisting of a MP-711 Micro-Flow Pump and a MV790 10-port auto-switching valve equipped with a JMS-T100LC ESI-TOF-MS detector (JEOL, Tokyo, Japan). Injectors were a Model C4-1004-1 injector valve (sample volume, 0.1 μL; Valco, Schenkon, Switzerland) for sample injection and a Model 8125 injection valve (sample loop, 5 μL; Rheodyne) for introducing the phosphate buffer. Chromatographic separation was performed on a MonoCap for a fast-flow monolithic silica capillary column (150 × 0.1 mm i.d.; through-pore size, 2 μm; meso-pore size, 15 nm; skeleton size, 1 μm; GL Sciences). The column eluent was directed to MS through a Metal Nano Sprayer S that was employed as a metallic nano-ESI tip (50 μm, i.d.; 365 μm, o.d.; 20 μm, tip orifice i.d.; 40 mm, length; GL Sciences). The condition of ESI-MS was as follows: the ionization mode was positive, the needle voltage was 3000 V, the heated nitrogen drying gas temperature and flow rate were 80°C and 1.5 mL/min, respectively. The total ion chromatogram (TIC) was taken in the mass range of 200 – 1600 *m/z*. Mass chromatograms were taken in the mass range of 1031 – 1032 *m/z* and 1483 – 1484 *m/z*. Data acquisition was performed using a JEOL MassCenter (JEOL).

Procedure

Column-switching HPLC. The column-switching HPLC system is depicted schematically in Fig. 1. A precolumn (10 × 4.0 mm i.d.) contained Titansphere-TiO titania packing (particle size, 5

Table 2 Time program for column-switching HPLC analysis

Time/min	Gradient pump, %			Precolumn	Separation column	Valve position
	Sol.A: H_2O with 0.1% TFA	Sol.B: CH_3CN with 0.1% TFA	Sol.C: 0.1 M phosphate potassium buffer (pH 7.0)			
0	100	0	0	TiO_2	RP-column	A
4.9	100	0	0	TiO_2	RP-column	A
5	100	0	0	—	RP-column	B
20	55	45	0	—	RP-column	B
25	0	100	0	—	RP-column	B
30	0	100	0	—	RP-column	B
30.1	100	0	0	—	RP-column	B
39.9	100	0	0	—	RP-column	B
40	0	0	100	TiO_2	RP-column	A
59.90	0	0	100	TiO_2	RP-column	A
60	100	0	0	—	RP-column	B
70	100	45	0	—	RP-column	B
85	55	100	0	—	RP-column	B
90	0	100	0	—	RP-column	B
95	0	100	0	—	RP-column	B
95.1	100	0	0	—	RP-column	B
105	100	0	0	—	RP-column	B

—: Not equipped.

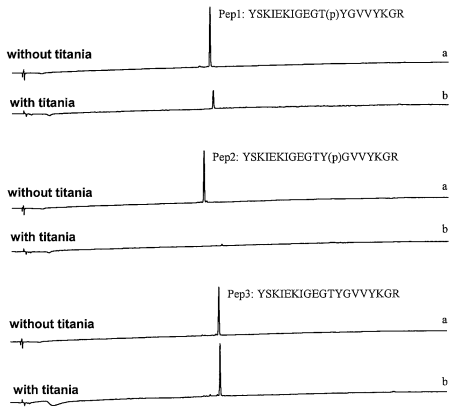


Fig. 3 Chromatograms of three peptides for confirming the adsorption ability of titania. The chromatographic condition was as follows: precolumn, Titansphere® TiO (10 × 4.0 mm i.d.); separation column, Inertsil® WP300C8 (150 × 4.6 mm i.d.); eluent, H_2O (0.1% TFA, v/v)/CH_3CN (0.1% TFA, v/v) = 100/0, after 90 min, 55/45; flow rate, 1.0 mL/min; column temp., ambient; detection, UV 210 nm.

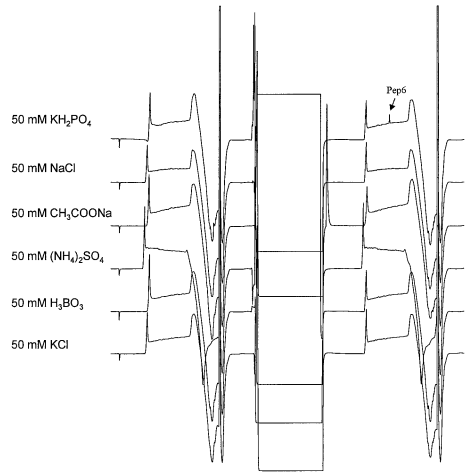


Fig. 4 Evaluation of the influence of the solvent for the elution of phosphopeptides from the titania column.

μm; pore size, 80 Å; GL Sciences). An Inertsil WP300C8 column (150 × 4.6 mm, i.d.; particle size, 5 μm; pore size, 300 Å; GL Sciences) was employed as a separation column. Eluents A, B, and C were H_2O with 0.1% v/v TFA (Sol.A), CH_3CN with 0.1% v/v TFA (Sol.B) and 0.1- or 0.2-M KH_2PO_4–K_2HPO_4 buffer (pH 7.0 or 7.5; Sol.C), respectively. The wavelength for UV detection was 210 nm. The gradient program typically used is shown in Table 2. System operation was generally processed as follows: first, each 4 μL peptide standard was injected to the system under an acidic condition with Sol.A being pumped, where the status of the switching valve was position A. Second, the valve was switched to B to wash out non-phosphopeptides under the following condition of the gradient: 0 min, 0% Sol.B; 0 – 15 min, 0 – 45% Sol.B; 15 – 20 min, 45 – 0% Sol.B; 20 – 25 min, 0% Sol.B. In those steps, phosphorylated peptides were still trapped on the precolumn. Third, the valve position was changed to A and Sol.C (0.1 M KH_2PO_4–K_2HPO_4 buffer) was flowed to the line. Last, after their desalination, the valve position was switched to B once again for gradient analysis.

Capillary-sized column-switching HPLC. Figure 2 presents the configuration of a capillary-sized column-switching HPLC system. A Titansphere-TiO titania column (2 × 0.3 mm i.d., particle size, 5 μm; pore size, 80 Å; GL Sciences) was used as a precolumn. A MonoCap monolithic silica guard column (50 × 0.2 mm, i.d.; through-pore size, >5 μm; meso-pore size, *ca.* 11 nm; skeleton size, 1 μm; GL Sciences) and a MonoCap for a fast-flow capillary column (150 × 0.1 mm, i.d.; through pore size, 2 μm; meso pore size, 15 nm; skeleton size, 1 μm; GL Sciences) were employed as an RP-precolumn and an RP-analytical column, respectively. H_2O containing 0.1% v/v HCOOH (Sol.D), CH_3CN containing 0.1% v/v HCOOH (Sol.E), H_2O containing 0.1% v/v HCOOH (Sol.F) and 1 M KH_2PO_4 (Sol.G) were supplied as eluent. The flow rate of pumps A and B were set at 2 μL/min and 10 μL/min, respectively. Then, 0.1 μL of β-casein tryptic digest was analyzed on the gradient program indicated in Table 5. The

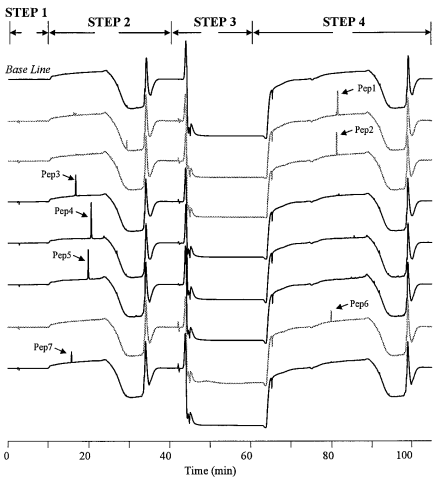


Fig. 5 Chromatograms of seven peptides with the column-switching HPLC system. The system operation consists of the following four steps: Step 1 is a procedure for the selective retention of phosphopeptide on a TiO_2 column; Step 2 is for the RP-gradient analysis of peptides passed through a TiO_2 column; Step 3 is for phosphopeptide elution from the TiO_2 column and its desalination on an RP-column; and Step 4 is for the RP-gradient analysis of phosphopeptide. The flow rate was 1 mL/min. The temperature was ambient. The time program of Table 2 was carried out.

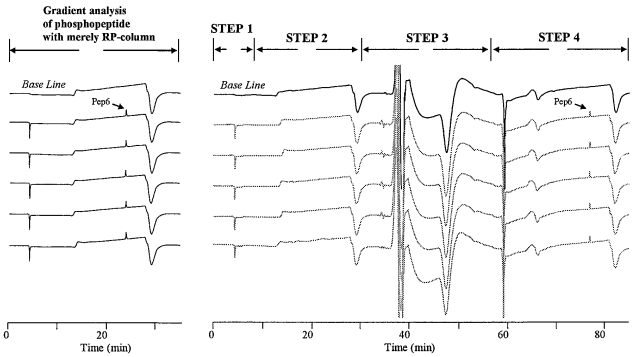


Fig. 6 Chromatograms of phosphopeptide with a column-switching HPLC system for estimating the reproducibility. This is a comparison of chromatograms of merely RP-column (Left) and column-switching HPLC system (Right). System operation consisted of four steps: Step 1 is a procedure for the selective retention of phosphopeptide on a TiO_2 column; Step 2 is for an RP-gradient analysis of peptides passed through the TiO_2 column; Step 3 is for phosphopeptide elution from the TiO_2 column and desalination of it on RP-column; and Step 4 is for an RP-gradient analysis of phosphopeptide. The temperature was 25°C. The time program of Table 3 was carried out.

time program for column-switching HPLC analysis is given in Table 6. A typical procedure for system operation was as follows: a sample was injected through V1 at status A of a 10-port valve, and gradient analysis (0 – 5 min, 0% Sol.E; 5 – 25 min, 0 – 50% Sol.E; 25 – 30 min, 50% Sol.E; 30.1 – 39.9 min, 0% Sol.E) was performed with the RP-analytical column. The sample was then injected through V2 under an acidic condition at status A of the 10-port valve, where the eluent was Sol.F. The valve position was switched to B 5 min later, and a gradient analysis (0 – 20 min, 0 – 50% Sol.E; 20 – 25 min, 50% Sol.E; 25.1 – 34.9 min, 0% Sol.E) was performed with the RP-precolumn and the RP-analytical column. Thereafter, the valve position was changed to A and Sol.G was flowed to the titania precolumn and RP-precolumn. Last, after desalination of the RP-precolumn, the valve position was switched to B, and gradient analysis (0 – 20 min, 0 – 50% Sol.E; 20 – 25 min, 50% Sol.E; 25.1 – 34.9 min, 0% Sol.E) was performed with the RP-precolumn and the RP-analytical column.

Results and Discussion

Property of titania column

The adsorption ability of titania packing toward phospho-compounds is one of the important characteristics for the pre-enrichment process of column-switching HPLC. If the capacity was not sufficient, the analytes would pass, without being trapped, through the precolumn during extraction. First, for evaluating the performance of titania, standard peptides (Pep1, Pep2 and Pep3) were injected at the eluant of an acetonitrile–water solution containing 0.1% TFA. A following gradient analysis was performed: 0 min, 0% acetonitrile; 0 – 90 min, 0 – 45% acetonitrile. Obtained chromatograms are shown as Fig. 3. With titania utilized as a precolumn of the RP-column, the peak height of the phophopeptides decreased compared with the case of the no-titania-column condition, while the peak heights of non-phosphopeptides were the same, regardless of the presence of the titania column. Next, the elution of phosphopeptides from the titania precolumn was examined. The HPLC system is shown in Fig. 1, and the time program for gradient analysis is indicated in Table 2. The influence of the elution of phosphopeptide from titania by the difference of the eluent was evaluated by using six kinds of solvents as Sol.C. A 4-μL volume of 50 μg/mL Pep6 was employed as a test sample. The experimental result is shown in Fig. 4. The peak of phosphorylated peptide appeared in the case of using 50-mM KH_2PO_4 as an elution (Sol.C); meanwhile, no peak was achieved in the case of using another solvent. The obtained results suggested that KH_2PO_4 was suitable for elution in the desorption procedure. The desorbing condition, utilizing titania as precolumn media, for phosphopeptide-selective analysis was precisely optimized with the stability of the phosphorylated amino acid residue being considered. Although borate buffer was employed as the eluant of phosphorylated compounds on titania, the alkaline condition had difficulties of instability. We had strategies to utilize phosphoric buffer to elute the phosphopeptides on the titania surface by competition with a free phosphate group.

Set-up for phosphopeptide-selective column-switching HPLC

To explore the general characteristics of the titania precolumn, column-switching HPLC (described in Fig. 1) was employed for an evaluation. Seven peptide standards were introduced to evaluate the recognition ability of phospho-compounds. Figure 5 shows the obtained chromatogram. Phosphopeptides were retained more strongly than the non-phosphorylated form on the titania column. Thus, non-phosphopeptides were passed through the titania under an acidic condition with H_2O (0.1% TFA, v/v), where phosphopeptides were still trapped. Then, 0.1 M KH_2PO_4–K_2HPO_4 buffer (pH 7.0) eluted the retained phosphopeptides from the titania packing. The obtained results clearly indicated that phospho-compounds were separated within the mixture sample by using the titania precolumn. For examining the reproducibility, Pep6 was injected five times with/without the titania precolumn with full-loop injection (loop volume, 5 μL), where 0.2 M KH_2PO_4–K_2HPO_4 buffer (pH 7.5)

Table 3 Time program for column-switching HPLC analysis for estimating the reproducibility

Time/min	Gradient pump, %			Flow rate/ mL min^{-1}	Precolumn	Separation column	Valve position
	Sol.A: H_2O with 0.1% TFA	Sol.B: CH_3CN with 0.1% TFA	Sol.C: 0.2 M phosphate potassium buffer (pH 7.0)				
0	100	0	0	0.5	TiO_2	RP-column	A
3.9	100	0	0	0.5	TiO_2	RP-column	A
4	100	0	0	1	—	RP-column	B
5	100	0	0	1	—	RP-column	B
25	80	20	0	1	—	RP-column	B
25.1	100	0	0	1	—	RP-column	B
30	100	0	0	1	—	RP-column	B
30.1	0	0	100	0.5	TiO_2	RP-column	A
40	0	0	100	0.5	TiO_2	RP-column	A
40.1	100	0	0	0.5	TiO_2	RP-column	A
56.9	100	0	0	0.5	TiO_2	RP-column	A
57	100	0	0	1	—	RP-column	B
58	100	0	0	1	—	RP-column	B
78	80	20	0	1	—	RP-column	B
78.1	100	0	0	1	—	RP-column	B
85	100	0	0	1	—	RP-column	B

—: Not equipped.

Table 4 Gradient program for RP separation for estimating the reproducibility of column-switching HPLC

Time/ min	Gradient pump, %		Flow rate/ mL min^{-1}
	Sol.A: H_2O with 0.1% TFA	Sol.B: CH_3CN with 0.1% TFA	
0	100	0	0.5
3.9	100	0	0.5
4	100	0	1
5	100	0	1
25	80	20	1
25.1	100	0	1
35	100	0	1

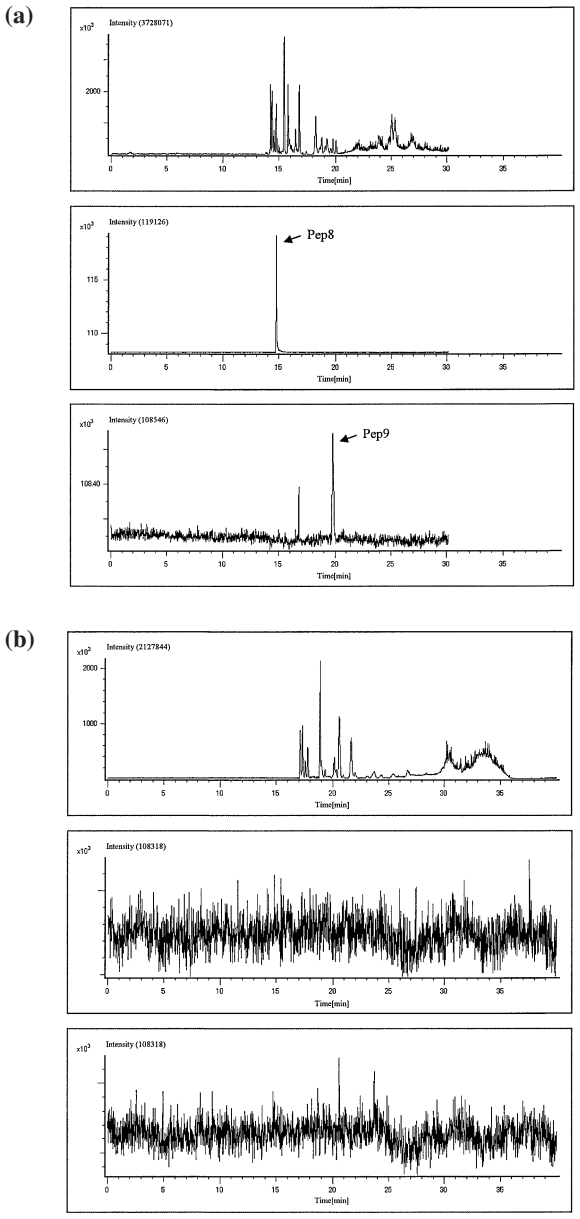
Table 5 Gradient program for the RP separation of capillary-sized column-switching HPLC

Time/ min	Gradient pump, %		Valve position
	Sol.D: H_2O with 0.1% HCOOH	Sol.E: CH_3CN with 0.1% HCOOH	
0	100	0	A
5	100	0	A
25	50	50	A
30	50	50	A
30.1	100	0	A
39.9	100	0	A

was employed as Sol.C, and the time profile for switching was changed to be short, as indicated in Table 3. The C.V. value of merely RP-column analysis (left chromatogram of Fig. 6; the time program in Table 4 was employed) and with titania (the right of Fig. 6) was estimated to be 0.98% and 0.36%, respectively (n = 5). In that case, recovery rate was calculated with 93%. The obtained results suggest the possibility of repeatedly selective analysis of 200-ng phosphopeptide.

Application to capillary-sized column-switching HPLC-MS

Capillary-sized column switching HPLC equipped with a titania precolumn was build up for applying phosphoproteomics research; the effectiveness was evaluated by using *ca*. 100 ng β-casein as a typical sample. The obtained chromatogram is shown in Fig. 7a. The upper part of Fig. 7a shows the TIC of β-casein tryptic digest separated with an RP-analytical column, and the middle and the lower parts of Fig. 7a indicate the mass chromatograms in the mass range of 1031 – 1032 m/z and 1483 – 1484 m/z for the detection of $[M+2H]^{2+}$ of FQS(p)EEQQQTEDELQDK (Pep8; MW: 2061.8) and $[M+2H]^{2+}$ of ELEELNVPGEIVES(p)LS(p)S(p)S(p)EESITR (Pep9; MW: 2966.2), respectively. Figure 7b shows the TIC and the mass chromatograms of β-casein tryptic digest passed through a titania column under an acidic condition, and then separated with an RP-precolumn and an RP-analytical column. Figure 7c shows the TIC and the mass chromatograms of selected peptides in β-casein tryptic digest by a titania precolumn, and separated with an RP-precolumn and an RP-analytical column, where phosphate buffer was employed for elution. In Figs. 7a, 7b, and 7c, the allowed MS peaks were confirmed to be $[M+2H]^{2+}$ of Pep8 and $[M+2H]^{2+}$ of Pep9 by contrasting a mass calculation of a tryptic digest of CASB BOVIN (P02666) using a database of Swiss-Prot and PeptideMass. As shown in Figs. 7a and 7c, those two peaks were clearly detected; meanwhile, both peaks were not detected in Fig. 7b. Those results were indicative of phosphopeptide-selective analysis with the capillary column-switching HPLC equipped with the titania column. It was also confirmed that even though the solvent for the elution of phosphopeptide from the titania column was acidic, the retained phosphopeptide was eluted, owing to the solvent containing phosphoric acid. Considering the stability of phosphopeptide, this condition is preferred when applying elution on the titania column. Additionally, those capillary switching HPLC equipped with a 10-port valve could, without introducing salt into MS, could be used to analyze phosphorylation peptides within a sample mixture by stronger intensity than the conventional method.



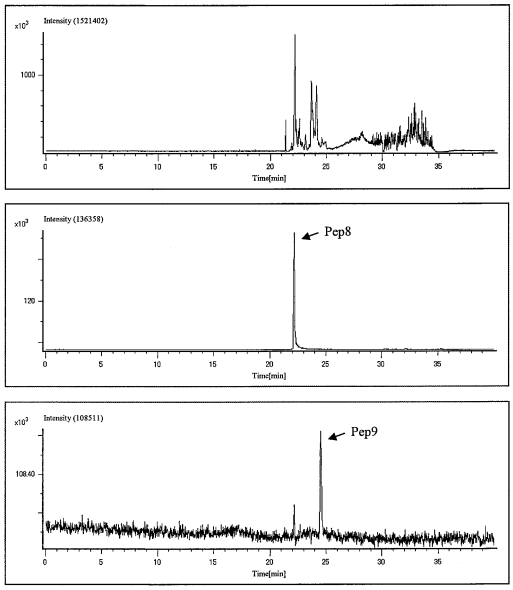


Fig. 7 TIC and mass chromatograms of β-casein tryptic digest obtained by capillary-sized column-switching HPLC analysis. Figure 7a shows the TIC of the β-casein tryptic digest separated with an RP-analytical column (upper chromatogram), mass chromatograms in the mass range of 1031 – 1032 *m*/*z* (the middle) and that of 1483 – 1484 *m*/*z* (the lower). Figure 7b shows the TIC (upper chromatogram) and mass chromatograms in the mass range of 1031 – 1032 *m*/*z* (the middle) and that of 1483 – 1484 *m*/*z* (the lower) of β-casein tryptic digest passed through a titania column under acidic condition, and separated with the RP-precolumn and the RP-analytical column. Figure 7c shows the TIC (upper chromatogram) and mass chromatograms in the mass range of 1031 – 1032 *m*/*z* (the middle) and that of 1483 – 1484 *m*/*z* (the lower) of selected peptides in the β-casein tryptic digest from the titania column, and separated with the RP-precolumn and the RP-analytical column, where phosphate buffer was employed as an eluent.

Conclusion

Phosphopeptide-selective column-switching HPLC within a mixture of peptides utilizing titania as precolumn media was developed. Standard phospho- and unphosphorylated peptides were recognized by a titania column without any sample modification. From the experimental results of the conventional and capillary column-switching HPLC analysis of phosphopeptides/nonphosphopeptides, those methods were shown to be applicable to mono-phosphorylated peptide deriverd from phosphorylated tyrosine (Y), threonine (T), and serine (S) residues. Although some other unphosphorylated peptides were extracted with titania in the capillary column-switching HPLC experiments, the viewpoints are, practically, necessary to be laid on the completeness of capturing phosphopeptides. In this work, phosphorylation peptides within *ca*. 100 ng β-casein tryptic digest could be selectively detected. A further improvement of the detection limit could be expected by the optimization of individual factors, such as the detection sensitivity and an extra-column effect. As future prospects, phosphopeptide-selective analysis in a more complicated mixture would be required. In addition, applications to actual biological samples should be carried out.

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Table 6 Time program of capillary-sized column-switching HPLC

Time/min	Pump A, %		Valve 3	Precolumn	Separation column	Valve position
	Sol.A: H_2O with 0.1% HCOOH	Sol.B: CH_3CN with 0.1% HCOOH	Sol.C: 0.1 M KH_2PO_4			
0	100	0	*	TiO_2	RP-column	A
4.9	100	0	*	TiO_2	RP-column	A
5	100	0	*	—	RP-column	B
25	50	50	*	—	RP-column	B
30	50	50	*	—	RP-column	B
30.1	100	0	*	—	RP-column	B
39.9	100	0	*	—	RP-column	B
40	100	0	Introduced	TiO_2	RP-column	A
59.9	100	0	*	TiO_2	RP-column	A
60	100	0	*	—	RP-column	B
64.9	100	0	*	—	RP-column	B
65	100	0	*	—	RP-column	B
85	50	50	*	—	RP-column	B
90	50	50	*	—	RP-column	B
90.1	100	0	*	—	RP-column	B
100	100	0	*	—	RP-column	B

—: Not equipped. *: Not applied.

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