Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1043 (2004) 19–25
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

# Development of a monolithic silica extraction tip for the analysis of proteins

Shota Miyazaki [a,*], Kei Morisato [b], Norio Ishizuka [b], Hiroyoshi Minakuchi [b], Yukihiro Shintani [a], Masahiro Furuno [a], Kazuki Nakanishi [c]

[a] *GL Sciences Inc., 237-2 Sayamagahara, Iruma, Saitama 358-0032, Japan*
[b] *Kyoto Monotech Co., 13 Shibanomiya-cho, Shimotsubayashi, Nishikyo-ku, Kyoto 615-8035, Japan*
[c] *Department of Material Chemistry, Graduate School of Engineering, Kyoto University, Katsura Campus, Nishikyo-ku, Kyoto 615-8510, Japan*

Available online 14 April 2004

**Abstract**

In proteomics, pre-treatment of sample is the most important procedure to remove the matrix for interfacing with mass spectrometry (MS). Additionally, for the samples with low concentration, the process of pre-concentration is required before MS analysis. We have newly developed solid-phase extraction (SPE) tool with pipette-tip shape for purification of bio-samples of various characteristics, utilizing monolithic silica gel as medium. The monolithic silica surface was modified with a $C_{18}$ phase or coated with titania phase. A $C_{18}$-bonded tip and a non-modified tip were used for sample concentration, desaltination and removal of detergents from sample. A titania-coated tip was also applied for purification and concentration of phosphorylated peptides. This novel pre-treatment method using monolithic silica extraction tip is much effective and suitable for protein analysis.


*Keywords:* Proteomics; Solid-phase extraction; Monolithic silica; Proteins; Phosphopeptides

## 1. Introduction

Proteomics has recently been recognized as the comprehensive identification and the analysis of proteins within cells. Analyzing/identification of proteins lead the possibility of establishment of the technology which artificially controls mechanisms of the proliferation differentiation, growth and aging. Additionally, the necessary information for diagnosis and dosing was also acquired by the recognition of the change in the proteins by stresses such as the disease, the environments and medicines. The more detailed information can be obtained with the analysis of protein compared to the analysis of deoxyribonucleotide acid (DNA) sequence because it is possible to identify post-translational modifications [1]. Especially, protein phosphorylation known as the most common modification of protein is found in nearly all cellular processes, thus the analysis of phosphorylated peptides and proteins are highly demanded. The characterization methodology of choice is no longer Edman-based sequencing, but rather the more rapid and sensitive approach. Mass spectrometry (MS) has been extensively used for the analysis of peptides, proteins and their digests [1]. On the other hand, the pre-treatment of sample is the most important procedure in proteomics to remove the matrix such as salts, buffers and detergents for interface with MS. Reversed-phase packing or filtration was employed as desalination. Additionally, for the samples with low concentration, the process of pre-concentration is required before LC–MS analysis.

Today, solid-phase extraction (SPE) is the most popular sample preparation method for the extraction and pre-concentration of samples, as well as clean up matrix interferences and undesired compounds from analytical samples [2–4]. Most sorption materials for SPE are in the bead shape. Typically, these beads are packed in a cartridge or column [5]. However, because these media pass sample solution by the high-pressure, extra instruments such as pumping or vacuuming are inevitably needed, and the intrinsic problem of all particulate media is their inability to completely fill the available space. In addition, the channeling between particles reduces the extraction efficiency. Also, development of two-dimensional gel electrophoresis, with agarose gels in the first dimension (ca. 1.5 mg) it is

* Corresponding author. Fax: +81-4-2934-3412.
*E-mail address:* s-miyazaki@gls.co.jp (S. Miyazaki).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.03.025

possible to introduce sample amount more than conventional method [6], led the necessity of pre-enrichment media having enough sample capacity. Recently, to solve the problem pointed out with the particle material, double-pore structure (macro-pore and meso-pore) silica gel monoliths were developed by Nakanishi and Soga, which is manufactured by combining the sol–gel reaction with phase separation and a subsequent solvent exchange treatment [7]. By controlling the composition of the starting mixture or changing the concentrations of alkoxysilane and poly(ethylene glycol) (PEG), it is possible to control the size of silica skeletons and through-pores to produce monolithic silica having (through-pore size)/(skeleton size) ratios much greater than those found in a particle-packed column [8]. Cabrera et al. [9] and Ishizuka et al. [10] applied the monolithic silica to the packings for conventional/capillary-sized HPLC, and proved its unique features, including low pressure-drop and total porosity higher than particle column. Shintani et al. [11] indicated that the monolithic silica had the application possibility as a solid-phase microextraction media.

We have developed a novel pre-enrichment medium suitable for purification of peptides and proteins, which enables treatment with relatively low pressure. Monolithic silica (2.8 mm i.d. × 1 mm), consisting of continuous mesoporous (∼20 nm pore size) silica skeletons of ∼10 μm size and through-pores of ∼20 μm, was fixed into the 200 μl pipette tip. Monolithic silica surface was modified with a $C_{18}$ phase or coated with titania phase. Titania has attracted interest as an alternative support material to silica in the field of HPLC packing because of its high chemical stability and enough rigidity [12,13]. Additionally, its unique amphoteric ion-exchange properties have been paid attention to. In column switching HPLC equipped with $TiO_2$ column, phosphoamino acid, phosphopeptide and nucleotide base were separated [14]. Those results indicated the possibility of its application in the selective analysis of phosphopeptides by optimizing the eluent condition. $C_{18}$-bonded and non-modified tips were used for sample concentration, desalting and removal of detergents from sample. Titania-coated tip was also applied for purification and concentration of phosphorylated peptides.

## 2. Experimental

### 2.1. Chemicals and reagents

Phosphorylated and unmodified synthetic peptides, Tyr–Ser–Lys–Ile–Glu–Lys–Ile–Gly–Glu–Asp–Thr[$PO_3H_2$]–Tyr–Gly–Val–Val–Tyr–Lys–Gly–Arg (Pep1; $M_r$: 2227.5), Tyr–Ser–Lys–Ile–Glu–Lys–Ile–Gly–Glu–Asp–Thr–Tyr[$PO_3H_2$]–Gly–Val–Val–Tyr–Lys–Gly–Arg (Pep2; $M_r$: 2227.5), Tyr–Ser–Lys–Ile–Glu–Lys–Ile–Gly–Glu–Asp–Thr[$PO_3H_2$]–Tyr[$PO_3H_2$]–Gly–Val–Val–Tyr–Lys–Gly–Arg (Pep3; $M_r$: 2307.5), Tyr–Ser–Lys–Ile–Glu–Lys–Ile–Gly–Glu–Asp–Thr–Tyr–Gly–Val–Val–Tyr–Lys–Gly–Arg (Pep4; $M_r$: 2147.5) were kindly provided by Dr. N. Yumoto (AIST) [15]. Standard peptides, Thr–Arg–Asp–Ile–Tyr[$PO_3H_2$]–Glu–Thy–Asp–Tyr–Tyr–Arg–Lys (Pep5; $M_r$: 1702.8) and Thr–Arg–Asp–Ile–Tyr–Glu–Thy–Asp–Tyr–Tyr–Arg–Lys (Pep6; $M_r$: 1622.8), were obtained from Sigma (USA). HPLC-grade acetonitrile (Kishida Chemicals, Japan) and water (Milli-Q system; Millipore, USA) were used in the preparation of the mobile phase. Trifluoroacetic acid (TFA) and formic acid were of HPLC grade, and purchased from Wako (Japan). Bradykinin, angiotensin II, insulin, cytochrome c, β-lactoglobulin, bovine serum albumin (BSA) and catalase were provided by Sigma. Tris buffer was prepared from Trizma base (Sigma), and phosphate buffer was prepared from sodium dihydrogenphosphate, 2-hydrate and disodium hydrogenphosphate, anhydrous (Kishida Chemicals). All other reagents were of analytical grade, and purchased from Kishida Chemicals.

### 2.2. Instrumentation

#### 2.2.1. HPLC analysis

The HPLC system consisted of PU610 pumps (GL Sciences, Japan), an L-7400 UV detector (HITACHI, Japan), a model 8125 sample injector (Rheodyne, USA), a model DM-22AP low-volume dynamic mixer (GL Sciences) and a model 554 LC column oven (GL Sciences). Data acquisition and processing were performed using a V-station chromatography manager (GL Sciences). The reversed-phase analytical column was Inertsil WP300 $C_{18}$ (5 μm, 150 mm × 2.1 mm i.d., GL Sciences). Eluents A and B were acetonitrile and water, respectively, both containing 0.1% TFA. The flow-rate was typically 0.3 ml/min. In quantification of peptides and proteins, samples were dissolved in buffer solution, and analyzed by reversed-phase chromatography.

#### 2.2.2. HPLC–MS analysis

A MonoCap for peptide column (150 mm × 0.1 mm i.d., through-pore 2 μm, meso-pore 15 nm; GL Sciences) was used for the HPLC–MS system consisting of PU711 microflow pumps (GL Sciences) and internal sample injector model C4 (0.1 μl; VICI Valco, USA). MS detection was performed on an Accu-TOF instrument (JEOL, Japan) equipped with a metal nano-sprayer (GL Sciences) as metallic nano-ESI tip (50 μm i.d., 365 μm o.d., 20 μm tip orifice i.d., 4 cm length). Mass center software (JEOL) was used for data acquisition and data analysis. Eluents A and B were acetonitrile and water, respectively, both containing 0.05% trifluoroacetic acid. Typical flow-rate was 0.5 μl/min (split flow). Elution of peptides was separated/analyzed with following linear gradient: 0 min, 20% B; 0–10 min, 20–60% B. Sample injection volume was 0.1 μl. Electrospray ionization was performed on the positive ion mode with nitrogen as drying gas. The extract source conditions were as follows: needle voltage, 2500 V; ring voltage, 10 V; orifice 1 voltage, 70 V; orifice 2 voltage, 5 V.

### 2.3. Sampling procedure

In case of the performance examination of $C_{18}$-bonded, non-modified and titania-coated tips, each Pep1–6 standard sample was dissolved in 50 mM Tris–HCl (pH 7.0) containing 10 mM NaCl, 20 mM ammonium hydrogen carbonate buffer (pH 8.0) and 50 mM Tris–HCl (pH 7.0) respectively. Trypsin digestion was prepared according to the following process: samples in 50 mM ammonium hydrogen carbonate were added with 1:100 (v/v) modified sequencing grade trypsin (Promega, USA) and incubated overnight at 37 °C. Samples were stored at 20 °C before analysis.

### 2.4. Preparation of monolithic silica material

Tetraethoxysilane (TEOS; Shin-Etsu Chemical, Japan) was added to 1 M aqueous solution of nitric acid (in the presence of polyethylene oxide (PEO; Aldrich, USA) with an average molecular mass of 100 000) and D-glucitol (Nacalai Tesque, Japan) [7]. The mixture was stirred for 15 min at room temperature. The solution was kept at 40 °C for gelation and aging over 15 h. The aged gel was immersed in 1.5 M aqueous urea solution at 110 °C for 20 h. After drying at 40 °C for 24 h, the gel was heat-treated at 600 °C for 5 h. The silica rod of 2.8 mm diameter and 1 mm thickness was cut. The size of meso-pore and surface was analyzed by a Autosurb-3B nitrogen absorption system (Quantachrome, USA). The surface of monolithic silica was chemically modified to $C_{18}$ [16] for applying to the reversed solid phase, which was employed as desalination of protein/peptide sample for MS analysis. Carbon content was ca. 12%, which was determined with thermogravimetric analysis by WS002 (MAC Science Co., Japan). Furthermore, titania gel was coated with monolithic silica by immersing the silica gel disc in titanium tetraisopropoxide diluted in 2-propanol. Monolithic silica gel was fixed into 200 μl pipette tip by supersonic adhesion.

## 3. Result and discussion

### 3.1. $C_{18}$-bonded monolithic silica extraction tip

Conditioning the tip was performed with the wetting and equilibration solutions to ensure optimum binding to analytes. During sample application, the peptides and proteins have a strong affinity with the hydrophobic monolithic silica surface. Salts, detergents and other hydrophilic contaminants present in samples unretained the $C_{18}$-phased monolithic silica. Residual contaminants which were weakly bound to the monolith silica were washed from the tip with a slightly acidic water rinse. The target peptides and proteins were concentrated and purified on the monolithic surface, and recovered with a slightly acidic aqueous–organic solvent. The experimental procedure is shown in Table 1. Fig. 1 illustrates the obtained chromatograms of 60 μg cytochrome c which was pre-treated by $C_{18}$-bonded tip. Fig. 1A is the result of an untreated sample, while Fig. 1B that of a treated sample. The recovery was 94%. Necessary sensitivity in analyzing the recent proteins/peptides analysis with MS is assumed to be in the order of fmol–pmol. Since the sample capacity of $C_{18}$-bonded tip was in the nmol order, those have enough performance for the analysis of proteins. Fig. 2 is the analytical results of the five-fold concentration

Table 1
Purification profile of proteins/peptides on $C_{18}$-bonded tip

| | |
|---|---|
| Pre-treat sample | Adjust sample to 0.1% trifluoroacetic acid (0.1% TFA) |
| Wet tip | Aspirate 200 μl acetonitrile (0.1% TFA) solution and discard solvent |
| Equilibrate | Aspirate 200 μl acetonitrile and discard solvent |
| Apply sample | Aspirate up to sample into tip and dispense, six to eight times repetition |
| Rinse tip | Aspirate 200 μl acetonitrile (0.1% TFA) solution and discard solvent |
| Elute sample | Aspirate 100 μl 60% acetonitrile (0.1% TFA) solution and dispense into tube, five times repetition |

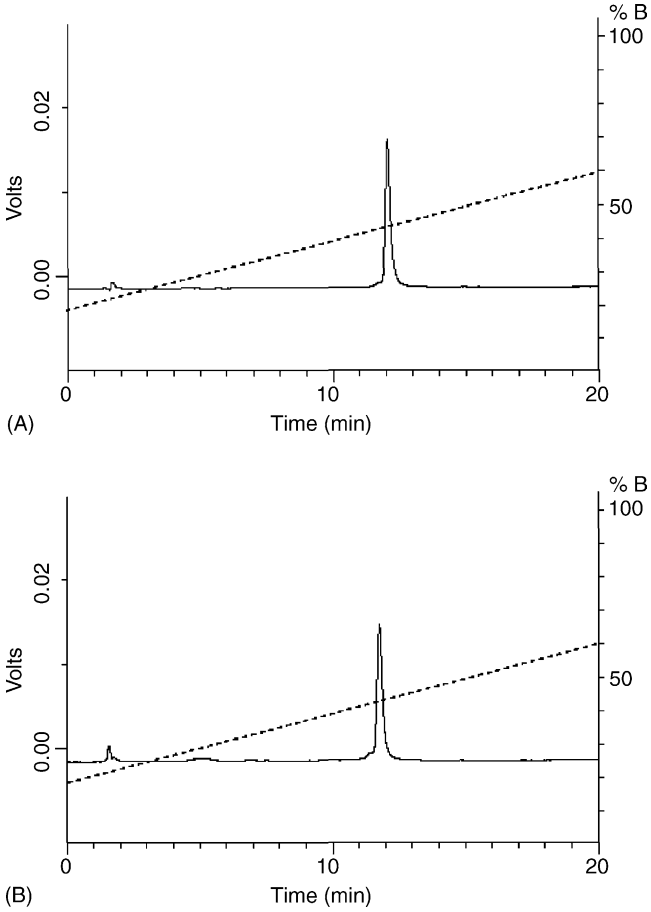


Fig. 1. The comparison of cytochrome c, with/without purification by $C_{18}$-bonded tip: (A) without and (B) with purification. Sample: 60 μg cytochrome c containing 10 mM NaCl in Tris–HCl (pH 7.6). Gradient: A–B (80:20) in 20 min to A–B (40:60). Detection: UV at 280 nm. Sample volume: 10 μl.

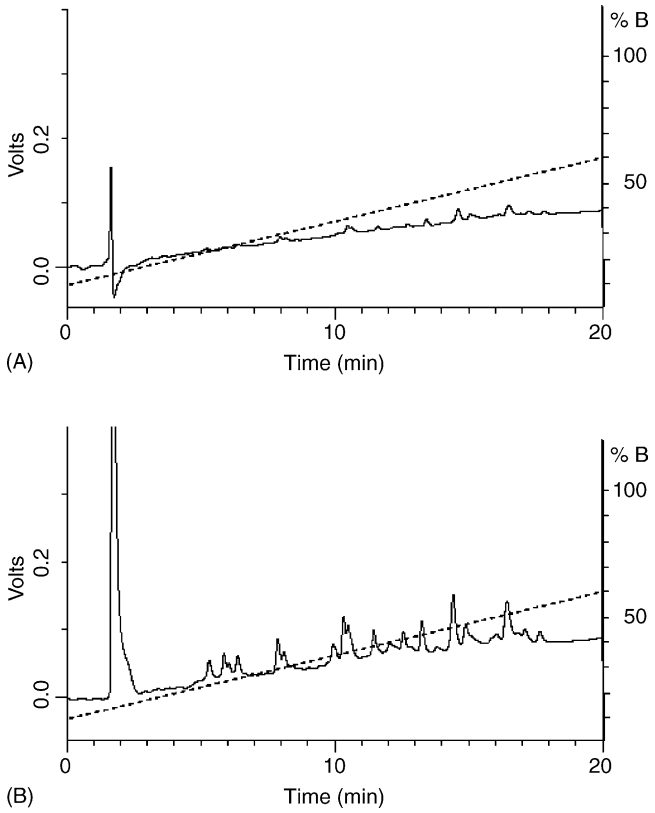


Fig. 2. The purification/enrichment of tryptic digest of β-lactoglobulin with $C_{18}$-bonded tip. (A) Chromatogram of sample without purification. (B) Chromatogram of sample with purification. Sample: tryptic digest of 10 μg β-lactoglobulin. Gradient: A–B (90:10) in 20 min to A–B (40:60). Detection: UV at 210 nm. Sample volume: 10 μl.

of tryptic digests of β-lactoglobulin (10 μg). One hundred microliters tryptic digests of β-lactoglobulin were concentrated on the $C_{18}$ surface, and then extracted by 20 μl eluent. Fig. 2B is the result of concentrated analysis, and Fig. 2A the direct analysis of digests. The substances which were not confirmed by direct injection of the sample were clearly detected by using $C_{18}$-bonded tip. The recovery rate of protein standards was shown in Table 2. Results indicated the fact that the proteins having large molecular mass had some difficulties in enough infiltration in the interior of the meso-pore because the meso-pore size of monolithic silica used was 20 nm and $C_{18}$ had high hydrophobic properties. Conventional procedure of protein identification is as follows: firstly, the proteins are separated by two-dimensional electrophoresis; secondly, targeted gel spots are in-gel digested; and finally, peptides are analyzed/identified by MS. Since the identification of peptides is the main aim of protein analysis, it is reasonable to assume that the newly developed $C_{18}$-bonded tip, which has an adsorption ability of proteins of $M_r$ till ca. 40 000, has enough performance.

Table 2
The recovery of protein/peptide purified with $C_{18}$-bonded tip ($n = 6$)

| Sample | $M_r$ | Average recovery (%) | R.S.D. (%) ($n = 6$) |
|---|---|---|---|
| Angiotensin II | 1 046 | 91 | 3.5 |
| Bradykinin | 1 060 | 90 | 1.7 |
| Insulin | 6 000 | 92 | 2.4 |
| Cytochrome c | 12 500 | 94 | 2.1 |
| β-Lactoglobulin | 360 000 | 80 | 3.0 |
| BSA | 640 000 | 24 | 11 |
| Catalase | 2 000 000 | 9.0 | 15 |

The recovery rate is calculated by the following formula:

$$\text{recovery (\%)} = (I_{elute}/I_{total}) \times 100$$

where $I_{elute}$ represents the amount eluted from the tip, and $I_{total}$ the amount introduced to the tip. Gradient: A–B (90:10) in (20 min) to A–B (40:60). Detection: UV at 280 nm. Sample volume: 10 μl.

Table 3
Purification profile of protein/peptides on non-modified tip

| | |
|---|---|
| Rinse tip | Aspirate 200 μl acetonitrile and discard solvent |
| Equilibrate | Aspirate 200 μl 20 mM hydrogen carbonate buffer (pH 8.0) |
| Apply sample | Aspirate up to sample into tip and dispense, six to eight times repetition |
| Rinse tip | Aspirate 200 μl hydrogen carbonate buffer (pH 8.0) |
| Elute sample | Aspirate 100 μl 20% acetonitrile (0.1% TFA) solution and dispense into tube, five times repetition |

### 3.2. Non-modified monolithic extraction tip

In reversed-phase chromatography, silanol is known as tailing factor. The silanol group dissociates at pH ∼5. It is known that a characteristic of silanol group is the adsorption of basic material. We investigated whether dissociation was utilized in peptide and protein purification. Conditioning the tip was employed with the wetting and equilibration solutions to ensure optimum binding. Sample was absorbed to solid phase (non-modified), and acid material and negative-charged material passed though the tip and were washed out from the tip with basic buffer. The target peptides and proteins were retained/concentrated and purified with a acidic aqueous–organic solvent because silanol group was inhibited by acidic condition. The procedure is shown in Table 3. Recovery of some proteins is shown in Table 4. The

Table 4
The recovery of protein purified with non-modified tip ($n = 5$)

| Sample | p*I* | Average recovery (%) | R.S.D. (%) ($n = 5$) |
|---|---|---|---|
| TI | 4.5 | 15 | – |
| BSA | 4.7 | 23 | – |
| β-Lactoglobulin | 5.2 | 16 | 13 |
| Ribonuclease A | 8.7 | 69 | 4.1 |
| Cytochrome c | 9.0 | 91 | 2.1 |
| Lysozyme | 9.3 | 90 | 2.3 |

TI: trypsin inhibitor; p*I*: isoelectric point.
The recovery rate is calculated by the following formula:

$$\text{recovery (\%)} = (I_{elute}/I_{total}) \times 100$$

where $I_{elute}$ represents the amount eluted from the tip, and $I_{total}$ the amount introduced to the tip. Sample: protein standards dissolved in 20 mM ammonium hydrogen carbonate buffer (pH 8.0). Detection: 280 nm.

Table 5
The pH effect of buffer solution dissolved ($n = 3$) on recovery of protein purified with non-modified tip

| pH | Average recovery (%) | R.S.D. (%) ($n = 3$) |
|---|---|---|
| 5.8 | 82 | 2.1 |
| 7.0 | 87 | 1.7 |
| 8.0 | 91 | 1.6 |
| 9.2 | 92 | 1.0 |

Sample: cytochrome c dissolved in phosphate buffer (pH 5.8–8.0) and Tris–HCl (pH 9.2). Detection: 280 nm.

higher the isoelectric point of protein was, the more excellent recovery rate was obtained. The pH influence of buffer solution was examined by cytochrome c which obtained high recovery rate by experiment (Table 5). Obtained results suggested that a better recovery rate was acquired by higher pH. Dissociation of silanol influenced the adsorption of proteins. Reversed phase has deficient performance in the ability to trap high hydrophilic substances, where non-modified tip has interaction with. Therefore, non-modified tip has the possibility of being an alternative to reversed phase.

### 3.3. Titania-coated monolithic silica extraction tip

The precise site of phosphorylation is identified by MS. A common problem with such analysis is that the amount of protein which has undergone phosphorylation tend to be small compared to the unphosphorylated form. That led to the fact that the amount of phosphorylated material is, after purification of a protein, insufficient for analysis. In addition, in such analysis the signal from phosphorylated material must be measured against a high level of back-ground signal that arises from unphosphorylated protein. To overcome difficulties, we had a strategy of coating titania on silica to apply to the purification of phosphopeptides. For coating the surface of monolithic silica, the

Table 6
Purification profile of phosphorylated peptides on titania-coated tip

| | |
|---|---|
| Rinse tip | Adjust sample to 200 μl 0.1% formic acid and discard solvent |
| Wet tip | Aspirate 200 μl 0.1% formic acid solution |
| Apply sample | Aspirate up to sample into tip and dispense, 20 times repetition |
| Rinse tip | Aspirate 200 μl 0.1% trifluoroacetic acid solution 10 cycle |
| Elute sample | Aspirate 100 μl 200 mM phosphate buffer (pH 7.6) and dispense into tube, 10 times repetition |

The recovery rate is calculated by the following formula:

$$\text{recovery (\%)} = (I_{\text{elute}}/I_{\text{total}}) \times 100$$

where $I_{\text{elute}}$ represents the amount eluted from the tip, and $I_{\text{total}}$ the amount introduced to the tip. Gradient: A–B (90:10) in (8 min) to A–B (60:40). Detection: UV at 210 nm. Sample volume: 10 μl.

Table 7
The recovery of protein/peptide purified with titania-coated tip ($n = 5$)

| | Average recovery (%) | R.S.D. (%) ($n = 5$) |
|---|---|---|
| Pep1 | 58 | 10 |
| Pep2 | 60 | 8.6 |
| Pep3 | 70 | 6.5 |
| Pep4 | 14 | 20 |
| Pep5 | 67 | 4.9 |
| Pep6 | 6.0 | 33 |

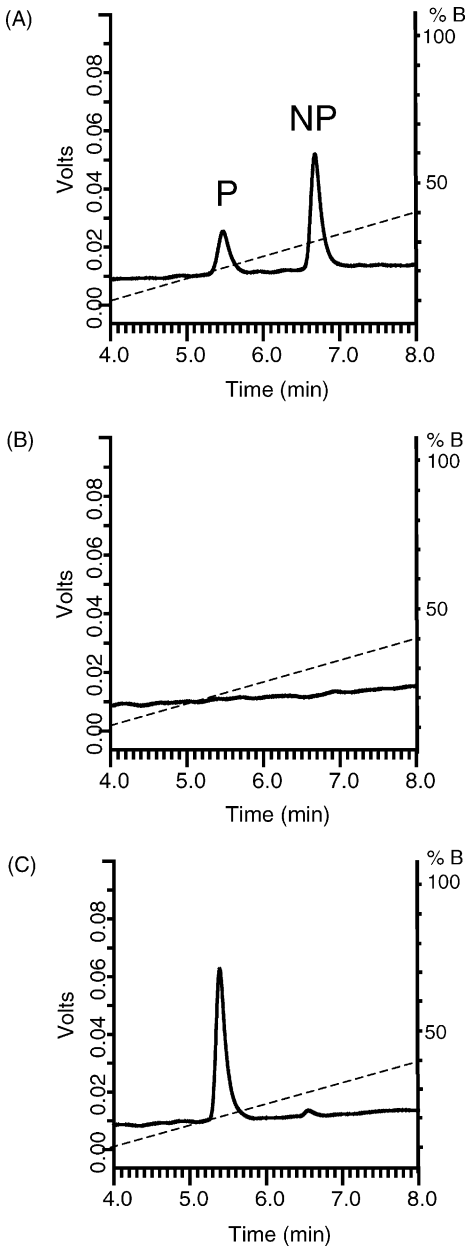


Fig. 3. Chromatogram of the mixture of phosphorylated and non-phosphorylated peptides with/without purification by $C_{18}$-bonded tip. (A) Sample without purification; P and NP indicate phosphorylated peptide and non-phosphopeptide, respectively. (B) Sample with purification by non-modified tip. (C) Sample with purification by titania-coated tip. Sample: Standard peptides dissolved 50 mM Tris–HCl (pH 7.6), 1 μg phosphopeptide (0.01 mg/ml), 5 μg non-phosphopeptide (0.05 mg/ml). Gradient: A–B (90:10) in 20 min to A–B (60:40). Detection: UV at 210 nm. Sample volume: 10 μl.

procedure was carried out [7]. For conditioning the tip, the equilibration solutions were passed through to ensure optimum binding. Phosphopeptides were retained more strongly than non-phosphorylated form on the titania-coated packing in the tip, thus non-phosphopeptides were possibly passed through the titania on a slightly acidic condition, where phosphopeptides were still trapped, and they were eluted with phosphate buffer by the competition with the free phosphate group. The procedure is shown in Table 6.

Titania-coated silica was investigated for the ability of peculiar recognition of the phosphopeptides. One microgram of Pep5 and 10 μg of non-phosphopeptide Pep6 were dissolved with Tris–HCl (pH 7.6) and 0.1% formic acid. Fig. 3A is the result of untreated sample. This sample was purified with non-modified monolithic silica tip and titania-coated monolithic silica. In non-modified tip, neither of the peptides were detected (Fig. 3B). However, titania-coated tip had the ability to retain selectively concentrated phosphopeptide. This result indicated that titania-coated tip retained specifically phosphopeptides, and selectively concentrated phosphopeptide (Fig. 3C). The recovery rate of phosphopeptides and non-phosphopeptide standards was shown in Table 7. Experimental data indicate that titania-coated tip retained and eluted single- or multiple-phosphopeptides, respectively.

### 3.4. Detection of phosphopeptide from β-casein tryptic digestion

Tryptic digest of 50 μg β-casein purified with titania-coated monolithic extraction tip was introduced to HPLC. No peaks were detected in the refined sample at UV 210 nm (Fig. 4B), while two peaks were observed at the unrefinement (Fig. 4A). Target peptides were dissolved in the phosphate buffer that had the possibility of decrease of sensitivity in LC–MS analysis. Thus, phosphate buffer was changed to water or water containing organic solvent suitable for LC–MS analysis, and introduced to nano-LC–MS. Fig. 5A was the analytical result of total ion chromatogram of the sample. Since the ionization of second peak having large mass was not enough, only one peak was recognized. The selective ion mass spectrum of this peak is shown in Fig. 5B. The experimentally obtained 1032 $[M + 2H]^{2+}$ and 688 $[M + 3H]^{3+}$ peaks agreed with the single-phosphorylated peptide representing the amino acid sequence from residues 48 to 63 (Phe–Gln–Ser[$PO_3H_2$]–Glu–Glu–Gln–Gln–Gln–Thr–Glu–Asp–Glu–Leu–Gln–Asp–Lys, $M_r$: 2062). The result indicates that it has possibility of simplifying the isolation and identification of phosphopeptides from protein digests through specific interaction of phosphate groups. Specific phosphorylation of serine, threonine and tyrosine residues is the most common mechanism for the regulation of

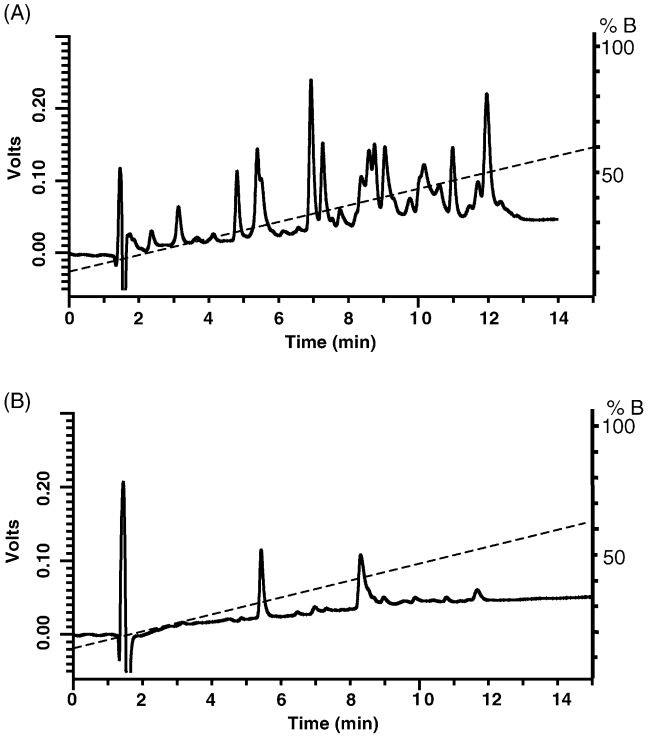


Fig. 4. Purification of tryptic digest of β-casein by titania-coated tip. Chromatogram of sample without purification. Chromatogram of sample with purification. Sample: 50 μg tryptic digest of 0.5 mg/ml β-casein. Gradient: A–B (90:10) in 15 min to A–B (40:60). Detection: UV at 210 nm. Sample volume: 10 μl.

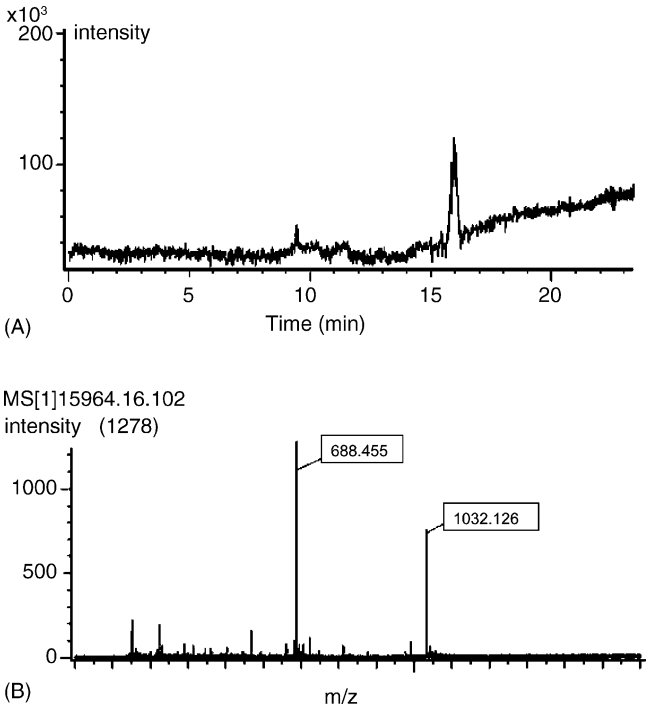


Fig. 5. Result of phosphorylated peptide derived from β-casein purified with titania-coated tip. Total ion chromatogram of sample. SIM of single-phosphopeptides (FQSpEEQQQTEDELQDK); 1032 and 688 peaks indicate the single-phosphorylated peptide representing the amino acid sequence from residues 48 to 63.

cellular protein activity. Fig. 5 and Table 7 indicate that titania-coated monolithic silica tip had the retention ability of the phosphopeptides.

## 4. Conclusion

A common platform of pre-treatment tip, which correspond to bio-samples of various characteristics (hydrophilic, hydrophobic, phosphorylated form and so on), was developed by chemically changing the surface of monolithic silica. Those have enough sample capacity and minimum dead volume for protein/peptide analysis. Additionally, the tip-shape has an advantage of easy applicability. Non-modified and $C_{18}$-bonded tips were employed for the desalination and exclusion of impurities, where their ion-exchange or hydrophobic property was functional. Also, titania-coated tip, which has the characteristics of peculiar recognition of the phosphorylated substances, was applied to the isolation of phosphopeptides from non-phosphorylated form. Titania-coated tip simplifies the isolation and identification of phosphopeptides from protein digests through specific interaction of phosphate groups. It is an ideal tool for enrichment of phosphorylated peptides from enzymatic digests to MS.

Newly developed pipette-tip tool has the suitability for combining automated system, which lead to the shortening of analytical time. A further kind of chemical modification of the surface of monolithic silica would also be required for coping with the analysis of biological samples having wide variety of properties.

## Acknowledgements

The authors are grateful to Drs. N. Yumono and Y. Tatsu (National Institute of Advanced Industrial Science and Technology) for providing phosphopeptide standards. We also wish to thank Dr. Y. Kodera (Kitasato University) for his useful comments on $C_{18}$-bonded monolithic silica extraction tip.

## References

[1] P.R. Graves, T.A.J. Haystead, MMBR 66 (2002) 39.
[2] M.C. Hennion, J. Chromatogr. A 856 (1999) 3.
[3] I. Liska, J. Chromatogr. A 885 (2000) 3.
[4] C.W. Huck, G.K. Bonn, J. Chromatogr. A 855 (2000) 51.
[5] R.E. Majors, D.E. Raynie, LC–GC 15 (1997) 1106.
[6] M. Ohishi, M. Satoh, T. Maeda, Electrophoresis 21 (2000) 1653.
[7] K. Nakanishi, J. Porous Mater. 4 (1997) 67.
[8] N. Tanaka, H. Kobayashi, K. Nakanishi, H. Minakuchi, N. Ishizuka, Anal. Chem. 73 (2001) 420A.
[9] K. Cabrera, D. Lubda, H.-M. Eggenweiler, H. Minakuchi, K. Nakanishi, J. High Resolut. Chromatogr. 23 (2000) 93.
[10] N. Ishizuka, H. Minakuchi, K. Nakanishi, N. Soga, H. Nagayama, K. Hosoya, N. Tanaka, Anal. Chem. 72 (2000) 1275.
[11] Y. Shintani, X. Zhou, M. Furuno, H. Minakuchi, K. Nakanishi, J. Chromatogr. A 985 (2003) 351.
[12] K. Tani, M. Ozawa, J. Liq. Chromatogr. Relat. Technol. 22 (1999) 843.
[13] H. Matsuda, H. Nakamura, T. Nakajima, Anal. Sci. 6 (1990) 911.
[14] Y. Ikeguchi, H. Nakamura, Anal. Sci. 13 (1997) 479.
[15] S. Deshimaru, Y. Miyake, T. Ohmiya, Y. Tatsu, Y. Endo, N. Yumoto, T. Toraya, J. Biochem. 131 (2002) 705.
[16] N. Tanaka, H. Kinoshita, M. Araki, J. Chromatogr. 332 (1985) 57.