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A fully automated phosphopeptide purification system for large-scale phosphoproteome analysis

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For large-scale phosphoproteome analysis based on mass spectrometry, a fully automated phosphopeptide purification system is essential to obtain reproducible results. An automated system involving pre-cleaning of a sample with a polymer-based reversed-phase column, phosphopeptide purification with a titania column and analysis of the phosphopeptide fraction with a reversed-phase column was developed, and then the analytical conditions for a complex peptide mixture were optimized. A lower flow rate for application of samples to the titania column was essential to obtain high recoveries of phosphopeptides from complex protein digests. Washing with 1 M NaCl and 2-propanol, and two cycles of washing with four solvents for the titania column were necessary to minimize non-phosphorylated peptides in the phosphopeptide fraction. Using this system under the optimized conditions, a peptide fraction including >90% phosphopeptides could be obtained highly reproducibly from a tryptic digest of a complex protein mixture, *i.e.* a *Xenopus* egg cytosol fraction, without any pre-treatment.

Keywords: phosphopeptide/phosphoproteome/phosphorylation/proteomics/titania.

Abbreviations: IMAC, immobilized metal ion affinity chromatography; LC-MS/MS, nano-flow liquid chromatography-ion trap-tandem mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight-mass spectrometry; MS, mass spectrometry; ODS, octadecylsilica; TFA, trifluoroacetic acid.

Protein phosphorylation is one of the most important post-translational modifications and is related to regulation systems mediated by protein kinases and phosphatases. To understand complicated systems, large-scale phosphoproteome analyses based on mass spectrometry (MS) are essential. To analyse phosphorylated proteins comprehensively by MS, crude protein mixtures are digested with a proteinase and then the generated phosphopeptides have to be highly purified from many other non-phosphorylated peptides prior MS to minimize suppression of ionization of the phosphopeptides. Therefore, such a purification procedure is one of the key processes for large-scale phosphoproteome analysis by MS.

Various methods including immobilized metal ion affinity chromatography (IMAC), titania affinity chromatography and others have been developed for phosphopeptide purification (*1*–*3*). The IMAC method was established first and used for large-scale phosphoproteome analysis (*4*, *5*). However, it is known that an IMAC column bed is not stable enough for repeated use without recharging of metal ions (*6*, *7*). A titania column is stable and binds phosphopeptides most strongly, although it binds non-phosphorylated peptides too (*8*, *9*). Therefore, a titania column is more suited for the automated purification of phosphopeptides, although optimization of the washing conditions for the column is necessary (*8*, *9*).

On the other hand, most reported methods for phosphopeptide purification involve offline pre-treatment or manual procedures. Offline pre-treatment for desalting with a reversed-phase cartridge (*5*) and manual purification of phosphopeptides with an titania tip (*8*–*10*) may involve some contamination by impurities and some loss of phosphopeptides, and thereby give lowered reproducibility as a result. Therefore, an automated system for phosphopeptide purification is necessary to obtain reproducible results and to reduce tedious tasks. The development of a fully automated system is also essential for carrying out large-scale phosphoproteome analysis on a routine basis.

Here, we developed a fully automated phosphopeptide purification system involving a pre-cleaning column, a titania column and a separation column, and optimized the analytical conditions. This system gave highly reproducible results because it automatically precleaned and purified phosphopeptides directly from the supernatant of a tryptic digest of a complex protein mixture without any other pre-treatment. The thus purified phosphopeptide fraction could be used directly for MS.

Experimental Procedures

Solvents for chromatography

Sol.A1, 0.1% trifluoroacetic acid (TFA); Sol.A2, 750 mM TFA in 80% acetonitrile; Sol.A3, 1 M NaCl in 0.1% TFA; Sol.A4, 2-propanol; Sol.B1, 0.1% TFA; Sol.B2, 0.1% TFA in acetonitrile;

Sol.B3, 0.5 M sodium phosphate buffer (pH 8.0); and Sol.B4, 3% formic acid.

Preparation of Xenopus egg cytosol fractions

Synthetic (S)-phase and mitotic (M)-phase *Xenopus* egg cytosol fractions were prepared as described previously (*11*).

[^{32}P]-Labelling of Xenopus egg cytosol proteins

To examine the efficiency of a titania or an IMAC column, [^{32}P]-labelled digests of *Xenopus* egg cytosol fractions were prepared. An S-phase *Xenopus* egg cytosol fraction (80 µl, 2 mg protein) was first passed twice through a 1 ml Sephadex G25 (Pharmacia, Uppsala, Sweden) column equilibrated with 50 mM Tris–HCl buffer (pH 7.5), 5 mM $MgCl_2$ and 0.1 mM $CaCl_2$ by centrifugation to remove low molecular weight compounds. Then the flow-through fraction was collected, mixed with cAMP (final 10 µM) and 0.9 U protein phosphatase 1, and incubated at 23°C for 20 min. Okadaic acid (final 5 µM) and [γ-^{32}P]-ATP (1.5 MBq, final 0.13 µM) were added to the sample on ice, followed by incubation at 23°C for 10 min. The labelled proteins were isolated from the remaining [γ-^{32}P]-ATP after the addition of cold ATP (final 9 mM) using a 1 ml gel filtration column equilibrated with 10 mM ATP. The medium was changed to 50 mM NH_4HCO_3 by passage through another 1 ml gel filtration column. The sample was denatured by heating at 100°C for 10 min, and then digested with trypsin and desalted as described below.

Tryptic digestion of proteins

An M-phase cytosol fraction of *Xenopus* eggs (4 mg protein/160 µl) was added to 16 µl of a protein phosphatase inhibitor cocktail (final concentrations: 2 mM EDTA, 2 mM NaF, 4 mM Na_3VO_4, 2 mM Na_2MoO_4, and 1.2 µM okadaic acid) and then diluted with 80 µl of 50 mM Tris–HCl buffer (pH 8.6). Then, urea crystals were added to the solution, the concentration being brought to 8 M to denature proteins. After treatment with 100 mM DTT at 37°C for 1 h, 300 mM (final concentration) acrylamide was added to the solution, followed by incubation for a further 1 h. After dilution with 3 volumes of water and the addition of 5 mM $CaCl_2$ (final concentration), 40 µg of trypsin (Sigma) was added to the solution, followed by digestion at 37°C for 6 h with further addition of 40 µg trypsin at 12 h. TFA was added to the reaction mixture to lower the pH to 2.5, followed by centrifugation at 10,000*g* for 10 min. The thus obtained supernatant was designated as the digest of an M-phase *Xenopus* egg cytosol and stored at −80°C until further analysis. Purified proteins were digested as above.

Preparation of a tryptic peptide mixture of purified proteins

Tryptic digests of phosphoproteins, *i.e.* α-casein, β-casein and ovalbumin, 20 µg each, and of non-phosphorylated proteins, *i.e.* bovine serum albumin, β-lactoglobulin and carbonic anhydrase, 1 mg each, were mixed and centrifuged at 10,000*g* for 10 min before use.

Quantitative analysis of phosphopeptides

[^{32}P]-Phosphopeptides retained on and eluted from a titania column were quantitated with a liquid scintillation counter. A radio-labelled digest of an M-phase *Xenopus* egg cytosol (4 mg in solution) was applied to a polymer-based reversed-phase column of Oligo R3 (4.6 mm i.d. × 100 mm, 30 µm; Applied Biosystems), which was washed with Sol.A1 at 1 ml/min for 1 h and then eluted with 80% Sol.B2. The thus eluted peptide fraction was dried *in vacuo* and then dissolved in Sol.A1. A titania column was equilibrated with Sol.A1. Then the sample was loaded onto the column at various flow rates and washed with Sol.A1. The flow-through and wash fractions were combined and the radioactivity was determined. After washing the column, the retained fraction was eluted with Sol.B3. The eluate was collected and the radioactivity was determined. The percentage recovery of phosphopeptides is expressed as (flow-through or eluted fraction count/applied sample count) × 100.

Preparation of an IMAC column

Fe (III)-NTA-IMAC beads were prepared as previously described (*12*). The Ni (II) contained in Ni-NTA Sepharose beads was removed by washing with 100 mM EDTA, 50 mM Tris–HCl (pH 8.0), and water, and then the beads were equilibrated with 100 mM acetic acid. Fe (III) was loaded onto the NTA Sepharose beads by adding 100 mM Fe (III) chloride in 100 mM acetic acid, and then the beads were washed with 100 mM acetic acid. A 4.0 mm i.d. × 10 mm column was filled with the Fe (III)-NTA beads, inserted into a high-performance liquid chromatography (HPLC) apparatus, and equilibrated with five column volumes of 0.1 M acetic acid. For comparison with a titania column, phosphopeptide separation was performed under the same conditions as for the titania column.

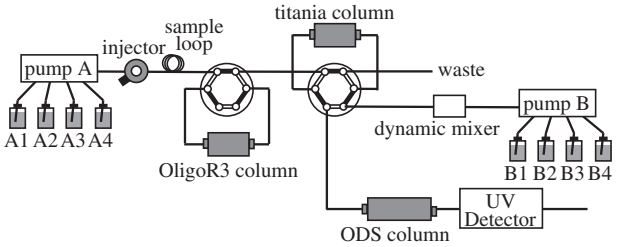

Fig. 1 A fully automated phosphopeptide purification system, a 2-pump system, involving a titania column and two reversed-phase columns. Black and white lines within valves show positions 1 and 2, respectively.

Construction of a fully automated phosphopeptide purification system

The system comprised two L-2100 pumps (Hitachi, Tokyo, Japan), an injector (9725*i*, Rheodyne), and an L-2400 UV detector (Hitachi, Tokyo, Japan) (Fig. 1). All tubing following the injector, except the octadecylsilica (ODS) column, was made by PEEK. A dynamic mixer (Hitachi, Tokyo, Japan) was used to obtain a flat baseline through complete mixing of the solvents. Column switching was achieved using two 2-position switching valves (GL Sciences, Tokyo, Japan). A reversed-phase column (Oligo R3, 4.0 mm i.d. × 100 mm, 30 µm; Applied Biosystems), a titania column (Titansphere TiO, 4.0 mm i.d. × 10 mm, 5 µm; GL Sciences, Tokyo, Japan) and an ODS column (Capcell Pak MGII, 4.6 mm i.d. × 100 mm, 3 µm; Shiseido, Tokyo, Japan) were connected to valves 1, 2 and 2, respectively. Operation of the system was automatically carried out with a program made with EZChrom Elite software (Hitachi, Tokyo, Japan), and the same software was used for analysis of the chromatographic data.

A typical method for purification of phosphopeptides from a complex peptide mixture

After centrifugation to remove insoluble peptides, a tryptic digest of *Xenopus* egg cytosol proteins (4 mg) was directly applied at 1 ml/min to an Oligo R3 column, which was washed with Sol.A1 at 1 ml/min for 1 h for clarification through removal of salts and urea, and then the bound peptides were eluted with Sol.A2 and passed through a titania column at 0.1 ml/min for 30 min. The lower flow rate was critical for a higher recovery of phosphopeptides. The titania column was exhaustively washed with Sol.A2, Sol.A3 and Sol.A4 at 0.5 ml/min for 10 min each. The washing was further repeated for one cycle. Then, phosphopeptides on the titania column were eluted with Sol.B3 at 0.5 ml/min for 30 min and the eluate was applied to an ODS column online. The elution with Sol.B3 was followed by that with Sol.B4 at 0.5 ml/min for 10 min to rapidly lower the pH to ensure the complete binding of peptides to the ODS column. After washing the column with Sol.B1 at 1 ml/min for 1 h, the phosphopeptides were eluted with a 90 min linear gradient to 45% Sol.B2 at 1 ml/min, with monitoring as to the absorbance at 210 nm, and collected in 1 ml fractions for matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) analysis. When the bound fraction from the titania column was analysed by nano-flow liquid chromatography-ion trap-tandem mass spectrometry (LC-MS/MS), the ODS column was eluted with stepwise elution to 80% Sol.B2 at 1 ml/min for 15 min and then the eluted phosphopeptides were concentrated by solvent evaporation.

In the case of reversed-phase HPLC with an ODS column by-passing the Oligo R3 and titania columns (Fig. 4A), the sample loop was directly connected to the ODS column, and then a sample was loaded using pump A with Sol.A1 at a flow rate of 1.0 ml/min

for 10 min. After reconnection of the ODS column to valve 2, linear gradient elution of the ODS column was performed using pump B. The gradient comprised Sol.B1 to Sol.B2, 0–45 % in 90 min at 1 ml/min.

When analysis of the flow-through fraction from the titania column was necessary (Fig. 4B), the ODS column was directly connected to the titania column, and the flow-through peptides were bound to the ODS column. After reconnection of the ODS column to valve 2, linear gradient elution of the ODS column was performed using pump B. The gradient comprised Sol.B1 to Sol.B2, 0–45% in 90 min at 1 ml/min.

Dephosphorylation of phosphopeptides with HF

Dephosphorylation of phosphopeptides by HF-treatment was carried out by the method of Kuyama *et al.* (*13*), as shown in the following section.

MALDI-TOF-MS

Two 500 µl aliquots of each fraction (1 ml) were placed in microtubes and the solvent was evaporated off, and then 40 µl of 46% HF was added to one tube, followed by reaction at room temperature for 90 min for dephosphorylation, and then the HF solution was evaporated off. The thus obtained HF-treated and non-treated samples were each dissolved in 1 µl of 0.1% TFA in 5% acetonitrile and then spotted onto the MALDI target, followed by spotting of 1 µl of a matrix solution (10 mg/ml 2,5-dihydroxybenzoic acid in 0.1% TFA in 50% acetonitrile). MS was performed with an Autoflex III (Bruker Daltonics). All spectra were obtained from m/z 680 to m/z 4,500 with 2,000 laser shots in the positive reflector mode. The mass axis was calibrated using the following authentic peptides as external standards: bradykinin (*1–7*) with a monoisotopic mass of 757.3992; angiotensin II, 1,046.5418; angiotensin I, 1,296.6848; substance P, 1,347.7354; bombesin, 1,619.8223; renin substrate, 1,758.9326; ACTH (*1–17*), 2,093.0862; ACTH (18–39), 2,465.1983; and somatostatin 28, 3,147.4710. The mass spectra were processed with flexAnalysis. A mass error tolerance of 0.3 Da was set for the window of error for matching the peptide mass values. The MALDI-TOF mass spectrum of a peptide showing reduction of the original molecular masses by 80, 160, 240 or 320 Da on HF-treatment was considered to be that of a phosphopeptide (*13*, Supplementary Fig. S1). In the preliminary experiments, a *Kratos-AXIMA-CFR* (Shimadzu, Kyoto, Japan) was also used for MALDI-TOF-MS.

Results

Comparison of titania and IMAC columns as to phosphopeptide recovery

High recoveries of phosphopeptides from complex peptide mixtures are a prerequisite for the purification of phosphopeptides for large-scale phosphoproteome analysis. Therefore, at first, we confirmed the superiority of a titania column over an IMAC one for this purpose. A complex peptide mixture, *i.e.* a tryptic digest of *Xenopus* egg whole soluble proteins, was used as a test sample because the affinity of each phosphopeptide should be different. As can be seen in Fig. 2, 91% and 88% of the phosphopeptides applied to the titania column were bound and eluted, respectively. However, when an IMAC column was used, the column could not retain about 40% of the phosphopepeptides, and as a consequence only 60% of the applied phosphopeptides were eluted from the column. We tried to retain phosphopeptides in the flow-through fraction from the IMAC column by re-application of the fraction to the column, and found that most peptides were again eluted in the flow-through fraction (data not shown). In the case of a titania column, when the sample was applied to a 5 cm long column (5-times longer than the standard

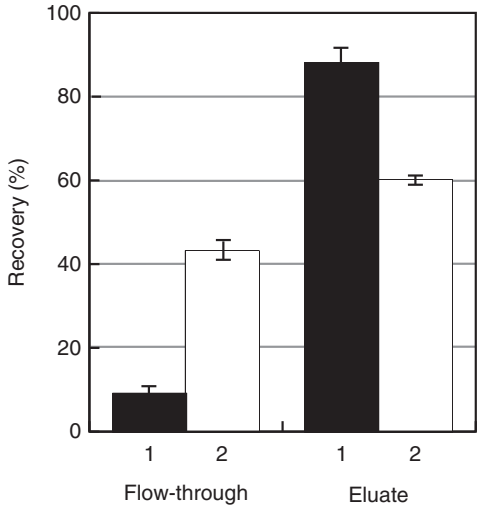

Fig. 2 Comparison of titania and IMAC columns as to phosphopeptide recovery. [^{32}P]-Labelled tryptic peptides derived from *Xenopus* egg soluble fractions were desalted with an Oligo R3 column offline. Then, the peptide mixture derived from 4 mg of protein was applied to an IMAC or titania column at 0.1 ml/min. After washing with Sol.B1, bound phosphopeptides were eluted with Sol.B3 [0.5 M phosphate buffer (pH 8.0)]. The wash fraction was combined with the corresponding flow-through fraction and designated as 'Flow-through'. The thus obtained fractions were counted and the phosphopeptide recoveries were determined. 1 (filled), titania column; 2 (empty), IMAC column. The vertical lines in the figure show the standard error ($n = 3$).

column), 99% of the phosphopeptides were bound to the column (data not shown). Therefore, we expected that all phosphopeptides contained in the sample were recovered, the yield being 88%. These results showed that the titania column bound most phosphopeptides, whereas the IMAC column could not bind some phosphopeptides in the complex peptide mixture derived on digestion of a naturally occurring sample. Thus, we chose a titania column for the purification of phosphopeptides with our system.

Optimization of the flow rate of a titania column for a high recovery

Preliminary experiments showed that a low flow rate for sample application to a titania column is essential for reproducible high recoveries of the phosphopeptides when a complex peptide mixture is applied to the column. Therefore, we examined the effect of the flow rate for sample application to the titania column. As can be seen in Fig. 3, at a flow rate of $\leqslant$0.1 ml/min, $>$84% of the applied phosphopeptides were recovered. After that, we therefore adopted a flow rate of 0.1 ml/min for the titania column. This flow rate corresponds to contact of peptides with the titania for 1.3 min.

Construction of automated phosphopeptide purification systems

In this study, we constructed three automated phosphopeptide purification systems. We constructed a prototype system based on a titania column and an ODS column to purify phosphopeptides automatically. However, as for other systems reported, samples had to be pre-cleaned prior to application to the system

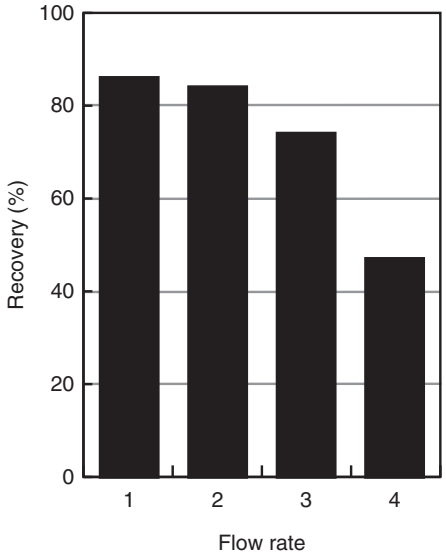

Fig. 3 Effect of the flow rate of sample application on the recovery of phosphopeptides from a complex peptide mixture. A tryptic digest (4 mg) of *Xenopus* egg cytosol proteins labelled with [^{32}P]-phosphate was desalted with a Oligo R3 column offline and then applied to a titania column. After washing with Sol.B1, bound phosphopeptides were eluted with Sol.B3 [0.5 M phosphate buffer (pH 8.0)]. The recoveries of phosphopeptides in the eluted fractions were determined by liquid scintillation counting. The flow-rate for sample application to the titania column was varied: 1, 0.025; 2, 0.1; 3, 0.2; and 4, 1.0 ml/min, respectively.

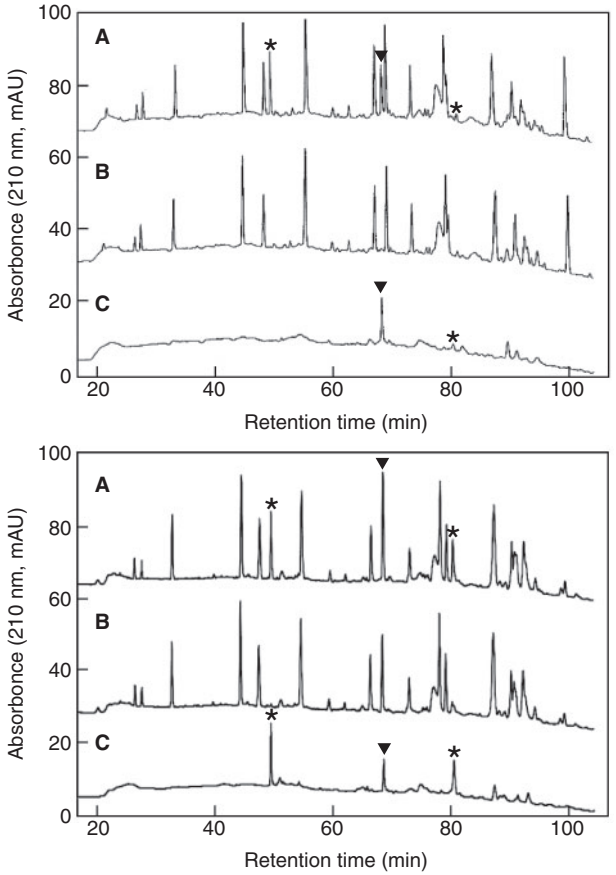

Fig. 4 Analysis of phosphopeptides in a tryptic digest of β-casein. Upper: (A) a tryptic digest of β-casein (17 μg) was directly analysed using the 2-pump system on an ODS column with a 90 min gradient of 0–45% Sol.B2 in Sol.B1, as shown under 'Experimental procedures' section. (B) A tryptic digest (17 μg) of β-casein was applied to a titania column and the flow-through fraction was analysed as in (A). (C) The bound fraction from the titania column was eluted with Sol.B3 [0.5 M phosphate buffer (pH 8.0)] and the eluate was applied to an ODS column online. Then the peptides were analysed as in (A). Lower: same as in the upper panel except that a short 3% formic acid washing step was added between the elution with Sol.B3 [0.5 M phosphate buffer (pH 8.0)] and the washing with Sol.B1 to rapidly lower the pH of the solvent flowing into the ODS column. Asterisks in the figure indicate phosphopeptide peaks, FQsEEQQQTEDELQDK (about 49 min) and ELEELNVPGEVEsLsssEESITR (about 80 min), respectively. Arrowheads each indicate an uncharacterized peptide. The differences in the elution patterns between upper A and lower A are due to the different samples.

with a desalting column offline. Treatment of a sample with a desalting column is a time-consuming step and sometimes leads to contamination by other compounds containing phosphate groups. Then, we constructed a second system, a 3-pump system. This system included a desalting column for pre-cleaning of samples to reduce non-specific binding of peptides to the titania column. Finally, we constructed a simplified system, a 2-pump system, as shown in Fig. 1. This system comprised a pre-cleaning column, a titania column and an ODS column. Phosphopeptides can be highly purified with this system including pre-cleaning of the sample automatically. With the 2-pump system, samples were desalted and pre-cleaned by the first Oligo R3 reversed-phase column. Then, all peptides bound to that column were transferred to a titania column. This column was exhaustively washed with many solvent combinations to remove non-phosphorylated peptides. Then, phosphopeptides were eluted with a phosphate buffer and bound to a second reversed-phase ODS column online. After washing this column to remove salts, phosphopeptides were separated on the column. To optimize the performance of the automated system, many conditions for the system had to be examined. The major conditions optimized are shown below.

Retention of a highly hydrophilic phosphopeptide from a titania column eluate on an ODS column

In a preliminary experiment, when phosphopeptides in a tryptic digest of β-casein were analysed with the 2-pump system, a hydrophilic phosphopeptide could not be detected in the elution profile of phosphopeptides from the ODS column (data not shown). Therefore, we examined whether such a phosphopeptide is eluted in the flow-through fraction from the titania column or not, as follows. A tryptic peptide mixture derived from β-casein was applied to a titania column, and the flow-through fraction was applied to an ODS column online and then eluted with a linear gradient (Fig. 4, upper B). On the other hand, phosphopeptides bound to the titania column were eluted with Sol.B3 [0.5 M phosphate buffer (pH 8.0)]. Then, the eluate was applied to the ODS column on-line as described above (Fig. 4, upper C). In this experiment, we could not detect a hydrophilic phosphopeptide, FQpSEEQQQTEDELQDK (Fig. 4, upper A; peak denoted by an asterisk and eluted at 49 min), in

either the flow-through (Fig. 4, upper B) or the eluted fraction from the titania column (Fig. 4, upper C). Then, we examined where the peptide was eluted, finding that it was bound to the titania column, transferred to the ODS column, and then retained on the ODS column. However, it was eluted from the column at the border of Sol.B3 (phosphate buffer) and the following washing solvent, Sol.B1 (0.1% TFA). Therefore, we added 3% formic acid elution after the Sol.B3-elution to rapidly lower the pH of the eluent (Fig. 4, lower). The peptide was now retained on the ODS column during washing with 0.1% TFA with this method and was eluted at 49 min during the gradient elution (Fig. 4, lower C). Therefore, we used the short 3% formic acid washing (10 min) for the automated system.

Washing conditions for a titania column to reduce non-phosphorylated peptides included in the phosphopeptide fraction

Then, we applied the 2-pump system to the purification of phosphopeptides from a complex peptide mixture. A tryptic digest of an M-phase cytosol of *Xenopus* eggs was applied to the system and the eluted phosphopeptide fraction was analysed by MALDI-TOF-MS. However, we could not detect phosphopeptides because the peptide fraction still contained many non-phosphorylated peptides and the ionization of phosphopeptides was suppressed. Non-phosphorylated peptides could be reduced by washing of the titania column with Sol.A3, *i.e.* 1 M NaCl in 0.1% TFA. The purity of the phosphopeptide fraction was 31% judging from the numbers of phosphorylated and non-phosphorylated peptide peaks in MALDI-TOF-MS spectra. Then, we further examined washing solvents for the titania column using a tryptic peptide mixture of purified proteins as a model sample prepared as shown under 'Experimental procedures' section with an prototype system, a 3-pump system, because washing solvents can be examined more freely with such a system.

Many solvents combinations were examined, the following results being obtained. (i) Washing with 750 mM TFA in 80% acetonitrile, which had been shown to be effective for titania column washing (*14*), was confirmed to be effective for reducing non-phosphorylated peptide peaks with our system; (ii) although washing with newly tried ethylsellosolve was effective for reducing non-phosphorylated peptides, some phosphopeptides were lost; (iii) newly-tried washing with 2-propanol was very effective; and (iv) two-cycle washing was also effective for reducing non-phosphorylated peptides (data not shown).

Analytical conditions for the 2-pump system

Examination of combinations of washing solvents for the titania column was necessary to reduce the number of solvents for use of a simplified system, *i.e.* the 2-pump system. As can be seen in Fig. 5, five washing combinations (Fig. 5B–F) were examined and the elution patterns of phosphopeptides were compared with the control one (Fig. 5A). The combination in Fig. 5C gave low non-phosphorylated peptide peaks compared

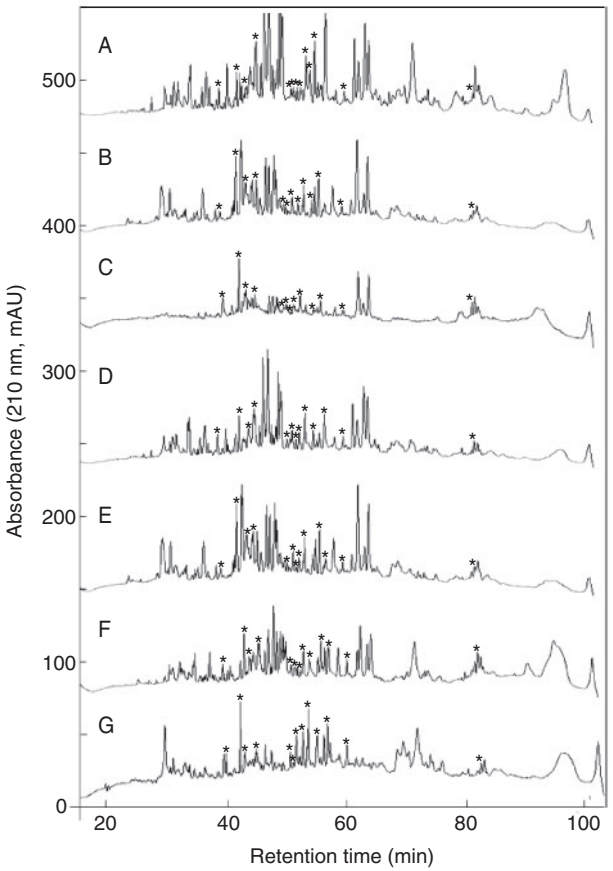

Fig. 5 Washing conditions for a titania column with the 2-pump system. A mixture of tryptic digests of phosphoproteins, *i.e.* α-, β-casein and ovalbumin, 20 μg each, and of non-phosphorylated proteins, *i.e.* bovine serum albumin, β-lactoglobulin and carbonic anhydrase, 1 mg each, were applied to the 2-pump system and the titania column was washed with the solvents indicated below. The bound peptides were eluted with Sol.B3 [0.5 M phosphate buffer (pH 8.0)] online and then analysed on an ODS column. Washing solvents: A (control), Sol.A3 (1 M NaCl in 0.1% TFA) and Sol.A1 (0.1% TFA); B, Sol.A3 and Sol.A4 (2-propanol); C, 750 mM TFA in 1 M NaCl and Sol.A4; D, Sol.A3 and Sol.A2 (750 mM TFA in 80% acetonitrile); E, Sol.A3 and 750 mM TFA in 70% 2-propanol; F, 750 mM TFA and Sol.A4; and G, Sol.A2, Sol.A3 and Sol.A4. Under washing conditions A to F, the washing solvents were put into the A3 and A4 bottles, whereas under condition G, the solvents were put into the A2, A3 and A4 bottles, respectively. These washings were each carried out for two cycles. Asterisks indicate phosphopeptides.

to the others (Fig. 5B and D–F). However, this washing solvent combination could not be used because it corroded stainless steel tubing in the apparatus. The other conditions gave relatively high non-phosphorylated peptide peaks (peaks around 50 and 65 min in Figs 5B and D–F). These results suggested that none of the three washing solvents, *i.e.* 750 mM TFA, 1 M NaCl or 2-propanol, used in the 3-pump system could be omitted. Then, we tried another combination, as shown in Fig. 5G. With this combination, to reduce the number of solvents, 750 mM TFA in 80% acetonitrile, which was the same as the washing solvent, was used instead of 0.1% TFA in 80% acetonitrile to elute peptides from the R3 column. As a result, the use of three kinds of washing solvents with

the pumps became possible. As can be seen in Fig. 5G, the peaks of non-phosphorylated peptides were greatly reduced (Fig. 5G, around 45–50 and 60–65 min) with these three kinds of washing solvents.

We confirmed that phosphopeptides were not lost from the titania column under the washing conditions with two phosphopeptides, as follows. A monophosphopeptide, FQpSEEQQQTEDELQDK (2,061.829 Da), and a diphosphopeptide, DIGSEpSp TEDQAoxMEDIK (1,942.679 Da), were applied to the titania column using the 2-pump system. After washing the column under the same conditions as in Fig. 5G, the phosphopeptides were eluted. The average recovery of each peptide was calculated from the peak areas in three independent experiments. A total of 99.7 and 96.7% of the peptides were recovered, respectively. These results suggested that washing as in Fig. 5G does not lead to loss of phosphopeptides.

Application of the 2-pump system to the purification of phosphopeptides from a complex peptide mixture

To confirm the efficiency of the 2-pump system for the purification of phosphopeptides from a complex peptide mixture, a tryptic digest of an M-phase cytosol of *Xenopus* eggs was applied to the system and phosphopeptides were eluted after washing as in Fig. 5G. The elution profile of the purified phosphopeptide fraction from the ODS column is shown in Fig. 6A, upper. As can be seen in the Supplementary Fig. S2, most peptides detected in the fraction were determined to be phosphopeptides with the HF-treatment method. In this case, the total number of 1,382 peptides determined to be phosphopeptides with the HF-treatment method were detected on MALDI-TOF-MS and the phosphopeptide content was 92%. These results clearly showed that the automated phosphopeptide purification system can be used to highly purify phosphopeptides from complex peptide mixtures. To determine whether the phosphopeptides determined with the HF-treatment method were really phosphorylated or not, the amino acid sequences of little parts of peptides were analysed by means of MALDI-TOF-TOF-MS and a Mascot search. The thus obtained results are summarized in the supplementary data (Supplementary Table SI). All peptides of which the amino acid sequences could be determined contained a phosphate group(s) on a Ser, Thr or Tyr residue (Supplementary Table SI).

To examine the reproducibility of this method, the same tryptic digest of a M-phase cytosol of *Xenopus* eggs was analysed again under the same conditions (Fig. 6A, lower). A very similar elution profile (compare Fig. 6A, upper and lower) and MS spectrum (compare Fig. 7A and B) were obtained. In the second analysis, 1,369 peptides determined to be phosphopeptides with the HF-treatment method were detected. The masses of the phosphopeptides detected in the two experiments were compared with each other, it being found that 1,252 phosphopeptides (91%) were the same (Fig. 6B). These results show that the 2-pump system gives highly reproducible results on analysis of complex peptide mixtures.

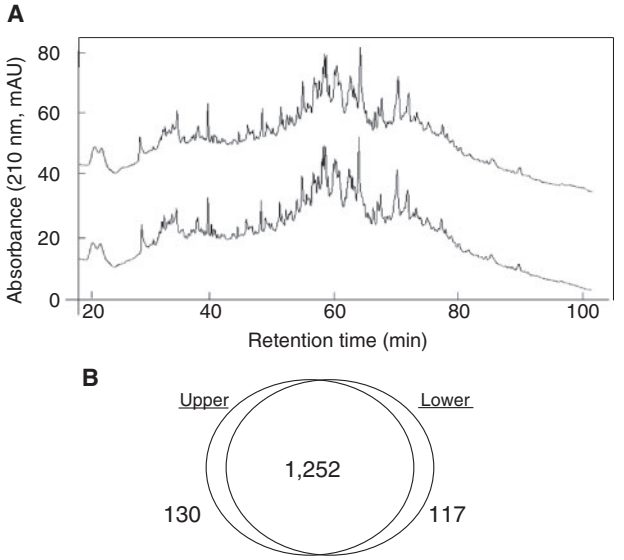

Fig. 6 Purification of phosphopeptides from a tryptic peptide mixture of a *Xenopus* egg cytosol fraction with the 2-pump system. A tryptic peptide mixture of an M-phase *Xenopus* egg cytosol fraction (4 mg) was applied to the 2-pump system under the standard conditions, and phosphopeptides bound to the titania column were eluted and applied to the ODS column online. Phosphopeptides on the column were eluted with an acetonitrile gradient and collected every 1 min (1 ml), 80 fractions in all. Each fraction was divided into equal two aliquots, one being dephosphorylated before analysis. Each fraction was analysed by MALDI-TOF-MS (Autoflex III). (A, upper) Elution profile of the purified phosphopeptide fraction from the ODS column. (A, lower) Results of a similar experiment to in A upper. B, the numbers of phosphopeptides detected in experiments A upper and A lower are shown schematically.

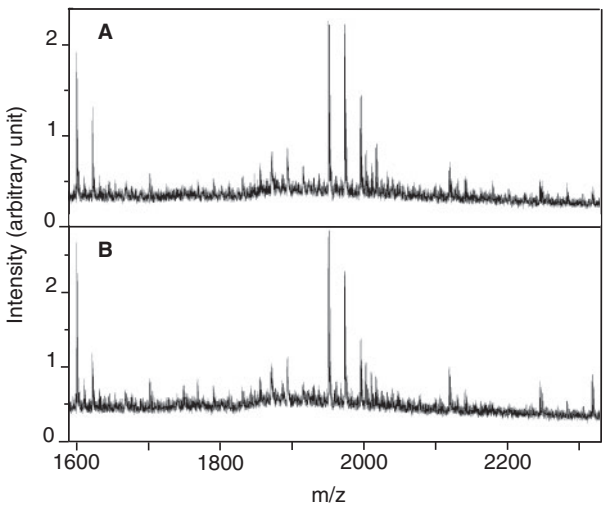

Fig. 7 Reproducible MS spectra of purified phosphopeptide fractions. Phosphopeptides in a tryptic digest of a *Xenopus* egg cytosol fraction were purified and analysed as in Fig. 6. As examples, MS spectra of fractions corresponding to 44–45 min in Fig. 6A upper and lower are shown, (A) and (B), respectively.

Discussion

Construction and optimization of an automated phosphopeptide purification system

We observed that the binding of phosphopeptides to a titania column is not as rapid as that to reversed-phase columns in the case of a complex peptide mixture.

Therefore, we examined the recovery of phosphopeptides with various flow rates for sample application to a titania column and found that $\leqslant$0.1 ml/min is necessary to optimize the binding of phosphopeptides in a crude peptide mixture to the column (Fig. 3). At the optimum flow rate, 88% or more phosphopeptides in the complex peptide mixture derived from a *Xenopus* egg extract were bound to the column and eluted in the phosphopeptide fraction (Fig. 3). The recovery was superior to that with an IMAC column (Fig. 2). Therefore, we chose a titania column for the system.

To minimize contamination of the phosphopeptide fraction by non-phosphorylated peptides, according to the method involving Sachtopore-NP TiO_2 beads used by Imanishi *et al.* (*14*), we tried 750 mM TFA for the washing of a Titansphere TiO (GL Science) column and confirmed that this solvent is effective for reducing the contamination by non-phosphorylated peptides in our system too. However, washing with DHB (*8*) and lactic acid (*9*) solutions, reported to be effective for other titania tip systems, was non-effective in our system (data not shown). We needed to reduce the contamination by non-phosphorylated peptides more. Therefore, we tried new solvents such as 20 mM glutamic acid, 20 mM malonic acid, 20 mM oxalic acid, 1 M NaCl, methanol and 2-propanol, and found that washing of the titania column with 1 M NaCl and 2-propanol was very effective. Then, combinations of these washing solvents were examined for application to the 2-pump system (Fig. 5G). On the other hand, to prevent loss of very hydrophilic phosphopeptides during transfer from a titania column to an ODS one, a short flow of 3% formic acid following 0.5 M phosphate buffer (pH 8.5) elution from the titania column was added to rapidly lower the pH of the eluent (Fig. 4). With the combination of these washing solvents and optimization of other conditions, we could obtain a $>$90% pure phosphopeptide fraction from a very complex peptide mixture. This high purity of phosphopeptides allowed us to detect 1,499 phosphopeptides on MALDI-TOF-MS without suppression by non-phosphorylated peptides from a digest of a *Xenopus* egg extract (Fig. 6). Thus, we constructed a phosphopeptide purification system applicable to large-scale phosphoproteome analysis.

Characteristics of the automated phosphopeptide purification system

The merits of an automated system for the analysis of phosphopeptides are reduction of labour, increased reproducibility of the results, and automated treatment of a lot of samples. Pinkse *et al.* (*15*) and Imami *et al.* (*16*) reported automated systems involving a titania column for the purification of phosphopeptides, however, their systems did not include a pre-cleaning column. We included in our system a polymer-based reversed-phase column, *i.e.* Oligo R3, prior to a titania column for pre-cleaning of samples on-line. A polymer-based pre-cleaning column, but not a silica-based pre-cleaning one (*17*, *18*), can be regenerated easily by removing strongly bound materials by injection of 0.7 ml of a NaOH solution after every analysis of a complex peptide mixture because the column is very stable under alkaline conditions. So, desalting and pre-cleaning of samples can be performed automatically online and the column can be used repeatedly more than 50 times. Thus, we could reduce handling of the samples and increase reproducibility. Actually, $>$90% of the phosphopeptides from a complex peptide mixture were detected reproducibly in the phosphopeptide fractions independently purified with the system (Fig. 6). Many samples can be analysed automatically by connecting an auto-sampler to the system.

Expandability of the system

In this study, 1,499 peptides determined to be phosphopeptides with the HF-treatment method were detected on MALDI-TOF-MS. This system can also be used to obtain phosphopeptide fractions for analysis by LC-MS/MS. For this purpose, phosphopeptide fractions can be obtained by stepwise elution with 0.1% TFA in 80% acetonitrile from an ODS column. In our preliminary experiments, when the obtained phosphopeptide fraction of a digest of *Xenopus* egg cytosol was subjected to nanoLC-MS/MS, many phosphoproteins were observed (data not shown). The major differences of our system from the phosphopeptide purification system connected on-line to a nanoLC-MS/MS reported by Pinkse *et al.* (*17*, *18*) are that it is offline as to MS and that it is suitable for large-scale purification of phosphopeptides. That is, a phosphopeptide fraction purified with our system can be divided and analysed by multiple MS, *i.e.* MALDI-TOF-MS and nanoLC-MS/MS, multiple dissociation methods, *i.e.* electron-capture and collision-induced dissociation, and comparison of peptides with and without dephosphorylation to obtain more complete data for large-scale phosphoproteome analysis. For the determination of further phosphoproteins, the addition of a strong cation exchanger column should be effective (*19*), and a system can be constructed easily by the addition an extra pump. For this, the 2-pump system is a flexible and expandable system for purifying phosphopeptides for large-scale phosphoprotrome analysis.

Supplementary Data

Supplementary data are available at *JB* online.

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Conflict of interest

None declared.

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