JOURNAL OF INTEGRATED OMICS
A METHODOLOGICAL JOURNAL
HTTP://WWW.JIOMICS.COM

ORIGINAL ARTICLE

A novel phosphate-affinity bead with immobilized Phos-tag for separation and enrichment of phosphopeptides and phosphoproteins

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Received: 29 October 2010 **Accepted:** 1 December 2010 **Available Online**: 2 December 2010

ABSTRACT

A simple and efficient method was developed for separating and enriching phosphoproteins from crude biological samples containing solubilized cellular proteins by immobilized zinc(II) affinity chromatography. The phosphate-binding site of the affinity gel is an alkoxide-bridged dinuclear zinc(II) complex, Phos-tag, which is linked to a hydrophilic vinylic polymer bead. A novel phosphate-affinity bead (Phos-tag Toyopearl) was prepared by reaction of N-hydroxysuccinimide-activated Toyopearl AF-Carboxy-650M gel with a 2-aminoethylcarbamoyl derivative of Phos-tag. Phosphopeptides were retrieved quantitatively and selectively on a μL-scale column at room temperature. The column was stable for long-term storage and could be reused many times. The technique was used to separate and enrich phosphoproteins from an epidermal growth factor-stimulated human epidermoid carcinoma A431 cell lysate. The operations necessary for 1-mL-scale open-column chromatography were conducted at a physiological pH during 1 h. The strong enrichment of the phosphoproteins into the eluted fraction was evaluated by gel electrophoresis, followed by Western blotting with Phos-tag Biotin and several antibodies, Pro-Q Diamond phosphoprotein gel staining, and mass spectrometry.

Keywords: Affinity chromatography; Phosphopeptide; Phosphoprotein; Phosphoproteomics; Phosphorylation; Phos-tag.

Abbreviations

2-DE, two-dimensional gel electrophoresis; **CBB**, Coomassie Brilliant Blue; **ECL**, enhanced chemiluminescence; **EGF**, epidermal growth factor; **HPLC**, high-performance liquid chromatography; **HRP**, horseradish peroxidase; **IEF**, isoelectric focusing; **IgG**, immunoglobulin G; **IMAC**, immobilized metal ion affinity chromatography; **MALDI-TOF**, matrix-assisted laser desorption/ionization-time of flight; **MES**, 2-morpholin-4-ylethanesulfonic acid; **MOAC**, metal oxide affinity chromatography; **MS**, mass spectrometry; **NHS**, N-hydroxysuccinimide; **PAGE**, polyacrylamide gel electrophoresis; **p*I***, isoelectric point; **PVDF**, poly(vinylidene difluoride); **RIPA**, radio-immunoprecipitation assay; **SDS**, sodium dodecyl sulfate; **TFA**, trifluoroacetic acid.

1. Introduction

Phosphorylation of proteins dramatically increases the diversity of genetically encoded proteins and plays a key role in regulating the function, localization, binding specificity, and stability of target proteins [1]. To evaluate the role of phosphorylation, it is necessary to identify phosphorylated amino acid residues, to analyze activities of kinases and phosphatases, and to elucidate interactions among proteins. Therefore, techniques for the specific separation and enrichment of native phosphoproteins have attracted considerable interest in relation to studies on the phosphoproteome in the fields of biology and medicine.

A number of studies have been reported on the enrichment of phosphoproteins from biological samples such as cell lysates. The method that is most frequently used is affinity chromatography using immobilized antibodies to phosphorylated amino acids [2–4]. However, the antibody-based procedure has a drawback in that it is incapable of comprehensive enrichment of phosphoproteins. Other widely accepted

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methods are immobilized metal ion affinity chromatography (IMAC) [5] and metal oxide affinity chromatography (MOAC) [6], which provide more-comprehensive enrichment. However, these techniques have a problem in that the solubility of proteins is reduced under the acidic conditions that are required in the workup for chromatography. These procedures are, however, generally useful for enriching phosphopeptides produced by digestion of phosphoproteins. Recently, improvements in the specificity of IMAC/MOAC have been accomplished in a number of ways for mass spectrometry (MS)-based studies on the phosphoproteome [7–13].

In 2005, we reported a novel type of immobilized zinc(II) affinity chromatography, known as Zn(II)-IMAC, in which a phosphate-affinity gel, Phos-tag Agarose, is used for the separation and enrichment of phosphoproteins and phosphopeptides [14]. The gel consists of biopolymer beads (cross-linked agarose) containing an immobilized alkoxide-bridged dinuclear zinc(II) complex {Phos-tag: 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex}. The Phos-tag molecule, which was synthesized to mimic the active center of an alkaline phosphatase, acts as a phosphate-binding tag molecule in aqueous solutions at neutral pH {e.g., K_d = 25 nM for the phenyl phosphate dianion [PhOP(=O)(O^-)$_2$]} [15]. The anion selectivity indexes of the phenyl phosphate dianion against SO_4^{2-}, CH_3COO^- (AcO^-), Cl^-, and the diphenyl phosphate monoanion [$(PhO)_2P(=O)O^-$] at 25 °C are 5.2×10^3, 1.6×10^4, 8.0×10^5, and $>2 \times 10^6$, respectively. The characteristic of Phos-tag molecule permits a comprehensive enrichment of various biological phosphorylated compounds, providing a major advantage in obtaining information on intact native phosphoproteins present in complex samples of cellular lysates [16,17]. However, the biopolymer beads are physically and chemically unstable, and they are therefore unsuitable for multiple use or long-term storage.

To increase the opportunities for analyses of phosphoproteins in a range of applications (from, for example, processing of microvolume samples by column chromatography to high-speed processing of high-volume samples in fast-flow rate liquid chromatography) [18,19], we have developed a more-stable phosphate-affinity bead by using an amino-pendant Phos-tag molecule and a hydrophilic vinylic polymer gel (Toyopearl; Tosoh, Tokyo, Japan) [20–22]. We have also demonstrated the efficiency and advantages of our new method by using the phosphate-affinity beads (Phos-tag Toyopearl) to analyze phosphopeptides and phosphoproteins in model experiments.

2. Material and methods

2.1 *Reagents*

Phos-tag Agarose [14,16,17] and Phos-tag Biotin [23–25] are commercially available from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Toyopearl AF-Carboxy-650M, a proprietary vinylic resin functionalized with pendant carboxyl groups, was purchased from Tosoh (Tokyo, Japan). Bovine β-casein, chicken egg white ovalbumin, epidermal growth factor (EGF), proteomics-grade trypsin, and PHOS-select Iron Affinity Gel were purchased from Sigma-Aldrich (St. Louis, MO, USA). Src peptide 521–533 and phosphorylated Src peptide 521–533 were purchased from CalBiochem (La Jolla, CA, USA). Phosphorylated PKC substrate, 4A/4B peptide, insulin receptor 1142–1153, kinase domain of insulin receptor-2, and kinase domain of insulin receptor-5 were purchased from AnaSpec (San Jose, CA, USA). PKC substrate peptide was purchased from Stressgen (San Diego, CA, USA). [Ala-97]-RII 81–99 (PKA substrate), RII phosphopeptide (calcineurin substrate), MAP 177–189 pT/pY peptide, and EGFR 988–998 (PTP1B substrate) were purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). The ECL Advance Western Blotting Detection Kit, horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) antibody, HRP-conjugated anti-rabbit IgG antibody, and HRP-conjugated streptavidin were purchased from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). Pro-Q Diamond phosphoprotein gel stain, SYPRO Ruby protein gel stain, and anti-pSer antibody were purchased from Invitrogen (Carlsbad, CA, USA). Anti-pMAPK1/2 antibody (clone 12D4; against phosphorylated Thr202/Tyr204), anti-MAPK1/2 antibody, anti-pShc antibody (against phosphorylated Tyr317), and anti-pErbB-2/HER-2 antibody (against phosphorylated Tyr1248) were purchased from Millipore (Billerica, MA, USA). Anti-pMAPK substrates (PXTP) antibody (clone 46G11) was purchased from Cell Signaling Technology (Danvers, MA, USA). A PhosphoProtein Purification Kit was purchased from Qiagen (Hilden, Germany). Titansphere TiO was purchased from GL Sciences (Tokyo, Japan). A protein assay kit and the ReadyPrep 2-D Clean Up Kit were purchased from Bio-Rad Laboratories (Hercules, CA, USA). An EzApply 2D Kit and the first dimensional precast agarGEL (A-M310, pH 3–10) were purchased from Atto (Tokyo, Japan).

2.2 *Preparation of the immobilized Zn(II) Phos-tag Toyopearl affinity beads*

Phos-tag Toyopearl without any bound zinc(II) was prepared by a condensation reaction between an amino-pendant Phos-tag ligand (75 μmol, Fig. 1) and Toyopearl AF-Carboxy-650M (5.0 mL, ~100 μmol carboxyl group per mL of gel) by a similar method to that reported previously [14]. In the presence of 0.50 mmol N-hydroxysuccinimide (NHS; 1-hydroxypyrrolidine-2,5-dione) and 0.50 mmol N-[3-(dimethylamino)propyl]-N'-ethylcarbodiimide hydrochloride, the amide formation proceeded quantitatively, as evaluated from an analysis of the amount of unbound Phos-tag molecule by high-performance liquid chromatography (HPLC) [eluent: 90% (v/v) aqueous $HClO_4$ (pH 2) and 10% (v/v) CH_3CN] at 254 nm. The unreacted NHS ester groups on the beads were hydrolyzed overnight in 0.10 M $NaHCO_3$–Na_2CO_3 (pH 10.5, 10 mL) to reform carboxylate groups. The beads were then washed five times with aqueous 50% (v/v) EtOH (10 mL) and five times with 10 mM 2-morpholin-4-

ylethanesulfonic acid (MES)–NaOH (pH 6.0, 10 mL). Finally, the beads were incubated in a buffer solution of 10 mM MES–NaOH (pH 6.0, 10 mL) containing 0.10 M $Zn(OAc)_2$ for 1 h at room temperature to form the zinc(II)-bound beads. The resulting zinc(II)-bound beads were washed five times with 0.10 M Tris–AcOH (pH 7.4, 10 mL) containing 20% (v/v) propan-2-ol, and the washed Phos-tag Toyopearl beads (15 μmol/mL-gel of Phos-tag) were stored as a 50% (v/v) slurry in the same buffer at 4 °C (see Figure 1).

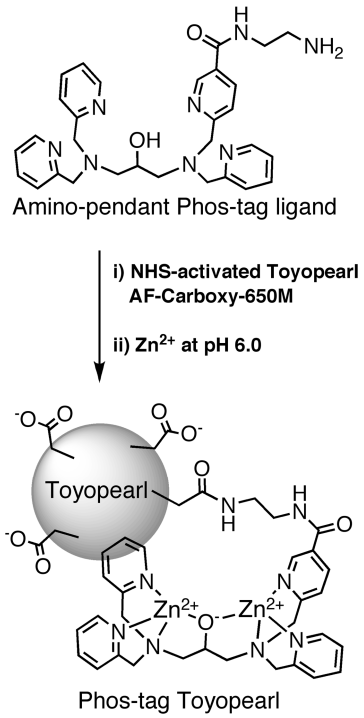

Figure 1. Preparation of Phos-tag Toyopearl, the novel Zn(II)-IMAC bead, by using a Phos-tag derivative having a 2-aminoethylcarbamoyl group (amino-pendant Phos-tag) and NHS-activated Toyopearl AF-Carboxy-650M gel.

2.3 *Preparation of the lysed proteins from the cultured cells*

A431 human epidermoid carcinoma cell line was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer at Tohoku University (Japan). The cells were grown in an RPMI1640 medium containing 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The cells (7×10^6) were placed in the same medium on a 100-mm culture plate. When the cells had adhered to the plate, the medium was removed and replaced with serum-free medium. After incubation for 16 h, the cells were stimulated with 250 ng/mL of EGF for 5 min. To terminate the stimulation, the medium was removed and the remaining cells were rinsed with Tris-buffered saline at room temperature. The saline was then removed and the culture plate was placed on ice. The cells were exposed to 0.30 mL of a cold radioimmunoprecipitation assay (RIPA) buffer consisting of 50 mM Tris–HCl (pH 7.4), 0.15 M NaCl, 0.25% (w/v) sodium deoxycholate, 1.0% (v/v) Nonidet P-40, 1.0 mM EDTA, 1.0 mM phenylmethanesulfonyl fluoride, 1.0 μg/mL aprotinin, 1.0 μg/mL leupeptin, 1.0 μg/mL pepstatin, 1.0 mM Na_3VO_4, and 1.0 mM NaF. The plate was gently rocked for 15 min on ice, and the adherent cells were then removed from the plate with a cell scraper. The resulting suspension was transferred to a microcentrifuge tube. The plate was washed with 0.20 mL of an RIPA buffer, and the washing solution was combined with the first suspension in the microcentrifuge tube. The mixed sample was incubated for 60 min on ice and then centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatant fluid was used as the cell lysate. The concentration of solubilized proteins was adjusted to 2.0 mg/mL by dilution with an appropriate amount of the RIPA buffer. Quantification of proteins was performed by using a Bio-Rad protein assay kit and a Biotrak II microplate reader (GE Healthcare Bio-Sciences). The lysed protein samples were subjected to phosphate-affinity chromatography with a spin column and with an open column as described below in Sections 2.4 and 2.12.

2.4 *Phosphate-affinity chromatography with a spin column*

Phos-tag Toyopearl beads (a 50-μL bed of compressed gel) were placed in a sample reservoir (an Ultrafree-MC centrifugal filter unit; Millipore). The filter unit was centrifuged at 2000 *g* for 20 s to remove the storage buffer [0.10 M Tris–acetic acid (AcOH) (pH 7.5) containing 20% (v/v) propan-2-ol], and the supernatant was discarded. To form the zinc(II)-bound Phos-tag Toyopearl beads, a balancing buffer (0.10 mL) was placed in the sample reservoir. For separation of protein samples, the balancing buffer consisted of 0.10 M Tris–AcOH (pH 7.5), 0.50 M NaOAc, and 10 μM $Zn(OAc)_2$, whereas for the separation of peptide samples, it consisted of 10 mM MES–NaOH (pH 6.0), 0.10 M NaCl, 1.0 mM disodium oxalate, and 10 μM $Zn(OAc)_2$. The filter unit was centrifuged at 2000 *g* for 20 s, and the supernatant buffer was discarded. A binding/washing buffer (0.10 mL) was placed in the sample reservoir. For the separation of protein samples, the binding/washing buffer consisted of 0.10 M Tris–AcOH (pH 7.5) and 0.50 M NaOAc, whereas for the separation of peptide samples it consisted of 10 mM MES–NaOH (pH 6.0), 0.10 M NaCl, and 1.0 mM disodium oxalate. The unit was then centrifuged at 2000 *g* for 20 s and the supernatant buffer was discarded; this washing operation was repeated twice.

For the preparation of the sample of proteins, the EGF-stimulated A431 cell lysate (20 μg proteins in 10 μL of an RIPA buffer) was diluted with 40 μL of the binding/washing buffer for separation of phosphoproteins. For the preparation of the sample of peptides, commercially available peptides (5.0 nmol phosphopeptides and 5.0 nmol nonphosphopeptides) were dissolved in the binding/washing buffer for separation of phosphopeptides (0.10 mL). Each sample solution was added to the sample reservoir and allowed to incubate for 5 min. Next, the filter unit was centrifuged at 2000 *g* for 20 s, and the filtrate was collected as a flow-through fraction. Binding/washing buffer (0.10 mL) was then added to the sample reservoir to wash the Phos-tag Toyopearl beads. The

filter unit was again centrifuged at 2000 *g* for 20 s, and the filtrate was collected as a washing fraction containing unbound proteins/peptides. For the separation of the protein sample, this washing operation was repeated twice, and the filtrate was collected as washing fractions, whereas for separation of the peptide sample, the washing operation was repeated three times, and the filtrate was collected as washing fractions. The flow-through and washing fractions were combined and used for subsequent analysis.

To elute the gel-bound proteins or peptides, an elution buffer (0.10 mL) was added to the sample reservoir. For proteins, the elution buffer consisted of 0.10 M Tris–AcOH (pH 7.5), 1.0 M NaCl, and 10 mM NaH_2PO_4–NaOH (pH 7.5), whereas for peptides it consisted of 0.10 M NaH_2PO_4–NaOH (pH 7.5). The filter unit was centrifuged at 2000 *g* for 20 s, and the filtrate was collected as an elution fraction. For the separation of the protein sample, the eluting operation was repeated twice, and the filtrate (0.20 mL) was collected as two elution fractions, whereas for the separation of the peptide sample, the eluting operation was repeated four times. In the case of separation of the protein sample, to analyze the proteins remaining in the column, a column-washing buffer (0.10 mL) consisting of 0.10 M Tris–AcOH (pH 7.5), 1.0% (w/v) sodium dodecyl sulfate (SDS), and 10 mM EDTA was added to the sample reservoir. The unit was heated for 5 min at 95 °C and then centrifuged at 2000 *g* for 20 s. The filtrate was collected as a column-washing fraction that contained the residual proteins from the column. Each fraction was desalted and condensed by using a Microcon YM-10 centrifugal filter unit (Millipore) and then dissolved in 20 μL of distilled water. The resulting solution was divided into two fractions of 10 μL each. One of these fractions was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) with SYPRO Ruby gel staining and the other was analyzed by SDS-PAGE with Western blotting. In the case of the separation of the peptide sample, all the fractions were analyzed by reversed-phase HPLC to estimate the relative recovery of the peptides. All the spin column chromatography operations were performed at room temperature.

2.5 *Reversed-phase HPLC*

HPLC for analysis of peptides was performed by using a Tosoh LC-8020 Model II multi-station with a CO-8020 column oven (at 40 °C), a UV-8020 UV detector (at 215 nm), two DP-8020 pumps (operating at a flow speed of 1.0 mL/min), an AS-8021 auto sampler, and a Shiseido reversed-phase column (CAPCELL PAK C18 UG80 S-5, 4.6 mm I.D. × 150 mm) (Tokyo, Japan). The HPLC analysis was conducted by gradient elution with an aqueous solution [from 95% to 60% (v/v)] of 0.10% (v/v) trifluoroacetic acid (TFA) and a CH_3CN solution [from 5% to 40% (v/v)] of 0.10% (v/v) TFA for 30 min. The HPLC retention times of each peptide are as follows: phosphorylated PKC substrate, 6.7 min; kinase domain of insulin receptor-5, 9.4 min; phosphorylated Src peptide 521–533, 13.4 min; kinase domain of insulin receptor-2, 14.5 min; PKC substrate peptide, 15.6 min; insulin receptor 1142–1153, 17.1 min; Src peptide 521–533, 17.5 min; EGFR 988–998, 18.1 min; MAP 177–189 pT/pY peptide, 21.7 min; RII phosphopeptide, 26.7 min; 4A/4B peptide, 27.1 min; and [Ala-97]-RII 81–99, 27.7 min.

2.6 *SDS-PAGE*

SDS-PAGE, conducted according to the Laemmli's method [26], was usually performed at 35 mA/gel and room temperature in a 1-mm-thick, 9-cm-wide, and 9-cm-long gel on a PAGE apparatus (AE6500; Atto). The gel consisted of a stacking gel [4.0% (w/v) polyacrylamide, 125 mM Tris–HCl (pH 6.8), and 0.10% (w/v) SDS] and a separating gel [7.5–12.5% (w/v) polyacrylamide, 375 mM Tris–HCl (pH 8.8), and 0.10% (w/v) SDS]. The acrylamide stock solution was prepared as a 29:1 (w/w) mixture of acrylamide and *N*,*N*'-methylenebisacrylamide. The electrophoresis running buffer (pH 8.4) consisted of 25 mM Tris and 192 mM glycine containing 0.10% (w/v) SDS. Each sample was prepared by mixing the separated fraction with half its volume of an SDS-PAGE loading buffer consisting of 195 mM Tris–HCl (pH 6.8), 9.0% (w/v) SDS, 15% (v/v) 2-mercaptoethanol, 30% (v/v) glycerol, and 0.10% (w/v) bromophenol blue [4,4'-(1,1-dioxido-3*H*-2,1-benzoxathiole-3,3-diyl)bis(2,6-dibromophenol)]. All the samples were heated for 5 min at 95 °C before they were loaded onto the gel.

2.7 *Western blotting*

The SDS-PAGE gel was electrotransferred to a poly(vinylidene difluoride) (PVDF) membrane (Fluorotrans W; Nihon Pall, Tokyo, Japan) for 1 h at 100 V by using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories) as a blotting system. The gel was soaked in a solution containing 25 mM Tris, 192 mM glycine, and 10% (v/v) MeOH for 10 min. The protein-blotted PVDF membrane was steeped in a solution containing 10 mM Tris–HCl (pH 7.5), 0.10 M NaCl, and 0.10% (v/v) Tween 20 (TBS-T solution) for 1 h, and then blocked with a solution of 1.0% (w/v) bovine serum albumin in TBS-T for 1 h. The membrane was probed separately with anti-MAPK1/2 antibody, anti-pMAPK1/2 antibody, anti-pSer antibody, anti-pMAPK substrates (PXTP) antibody, anti-pShc antibody, and anti-pErbB-2/HER-2 antibody. The membrane was then probed with HRP-conjugated anti-mouse IgG antibody for detection of the anti-pMAPK antibody, and with HRP-conjugated anti-rabbit IgG antibody for detection of the other primary antibodies. The enhanced chemiluminescence (ECL) signals were detected by means of an ECL Advance Western Blotting Detection Kit and were observed by using an LAS 3000 image analyzer (Fujifilm, Tokyo, Japan). For comprehensive detection of phosphoprotein, we prepared a complex of Phos-tag Biotin and HRP-conjugated streptavidin as described previously [24]. The membrane was probed with the complex for 30 min without blocking, and then washed twice with TBS-T at room temperature for 5 min each time. The ECL signal was detected as described above.

2.8 *Tryptic digestion of β-casein*

Bovine β-casein was subjected to tryptic digestion in a mixed solution of 1.0 mg β-casein in distilled water (0.10 mL) and 0.10 M NH_4HCO_3 (pH 8.1, 0.40 mL) containing 20 μg of proteomics-grade trypsin for 12 h at 37 °C. The trypsin and other large molecules (>10,000 Da) were removed by ultrafiltration through a Microcon YM-10 ultrafilter unit at 14,000 *g* for 40 min. An aqueous solution (0.20 mL) of 0.10% (v/v) TFA was then added to the ultrafiltration unit, and ultrafiltration was performed again as described above. The resulting filtrates were combined (~1.0 mL) and a 10-μL aliquot of the filtrate containing ~10 μg β-casein digest was freeze-dried and stored below 0 °C. The tryptic digestion samples were subjected to phosphate-affinity chromatography on a pipette microtip column as described below (Section 2.10).

2.9 *In-gel tryptic digestion of ovalbumin*

The SDS-PAGE gel bands for ovalbumin (total ~10 μg) were excised with a razor blade and cut into approximately 1–2 mm^3 pieces. The pieces were placed in a microcentrifuge and destained by washing with 0.10 M NH_4HCO_3 (pH 8.1) containing 50% (v/v) CH_3CN (0.50 mL) for 30 min. The waste was discarded and the washing step was repeated three times. The sample proteins were reduced with 0.10 M NH_4HCO_3 (pH 8.1) containing 10 mM EDTA and 10 mM dithiothreitol (DTT; 1,4-dimercaptobutane-2,3-diol) (0.50 mL) for 15 min, and the waste was discarded. The samples were also alkylated with 0.10 M NH_4HCO_3 (pH 8.1) containing 10 mM EDTA and 0.10 M acrylamide (0.50 mL) for 15 min, and the waste was discarded. The gel pieces were washed with 10% (v/v) AcOH containing 50% (v/v) CH_3OH (0.50 mL) for 40 min with intense mixing, and the waste was discarded. The washing step was repeated four times. The gel pieces were then incubated with 0.10 M NH_4HCO_3 (pH 8.1, 0.50 mL) for 10 min, and the waste was discarded. The gel pieces were shrunk with CH_3CN (0.50 mL) for 10 min, and the waste was discarded. The shrunken gel pieces were completely freeze-dried for 15 min, and the dried gel pieces were swollen in 50 μL of trypsin solution [0.10 M NH_4HCO_3 (pH 8.1) containing 2.5 μg proteomics-grade trypsin] then incubated for 15 min at 37 °C. The incubated gel pieces were washed with 0.10 M NH_4HCO_3 (pH 8.1, 0.25 mL) then incubated again in an identical trypsin solution for 12 h at 37 °C. The tryptic digest was extracted from the gel pieces by ultrasonication in 0.10% (v/v) aqueous TFA containing 50% (v/v) CH_3CN for 15 min and then in 0.10% (v/v) aqueous TFA containing 70% (v/v) CH_3CN for 15 min. The extracted fraction containing ~10 μg ovalbumin digest was freeze-dried and stored at below 0 °C. The tryptic digestion samples were subjected to phosphate-affinity chromatography with a pipette microtip column as described below (Section 2.10).

2.10 *Phosphate-affinity chromatography for phosphopeptides with a pipette microtip column*

A chromatographic microtip column for enrichment of phosphopeptides for subsequent MS analysis was prepared as follows (see Fig. 4A). The end (~25 mm) of a GELoader tip (Eppendorf Japan, Tokyo, Japan) was cut off and then the bottom of the microtip was packed with a custom-made sintered plastics filter (Nichiryo, Koshigaya, Japan). The tip was loaded with Phos-tag Toyopearl beads (~10 μL bed of compressed gel) and then another sintered plastics filter was packed on top of the beads. The resulting microtip column (the Phos-tag tip) was attached to a 20-μL micropipettor (for example, a Gilson P-20; Middleton, WI, USA). The plunger of the micropipettor was gently depressed and released during the analytical processes. First, the storage buffer was discarded. To form the zinc(II)-bound Phos-tag Toyopearl beads, a balancing buffer (20 μL) consisting of 10 mM MES–NaOH (pH 6.0), 0.10 M NaCl, 1.0 mM disodium oxalate, and 10 μM $Zn(OAc)_2$ was aspirated into the pipette microtip column and then dispensed; the waste was discarded. The column was washed with a binding/washing buffer (20 μL) consisting of 10 mM MES–NaOH (pH 6.0), 0.10 M NaCl, 1.0 mM disodium oxalate, and 20% (v/v) CH_3CN. This washing operation was repeated three times. Protein tryptic digests (β-casein or ovalbumin, each 10 μg) were dissolved in the binding/washing buffer (20 μL). The sample solution was aspirated into the column, and the plunger of the micropipettor was then depressed and gently released. This pipetting operation was repeated ten times to ensure maximal binding of the phosphopeptides, and then the waste was discarded. Next, the column was washed with the binding/washing buffer (20 μL) and the waste was discarded. This washing operation was repeated five times. To elute the gel-bound peptides, an elution solution (20 μL) consisting of 1.0% (v/v) aqueous TFA was aspirated, dispensed, released, and finally collected as an elution fraction. This eluting operation was repeated five times, and all the fractions were combined and analyzed by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) MS. All the operations involved in the pipette microtip column chromatography were performed at room temperature.

2.11 *MALDI-TOF MS analysis*

The elution fraction containing phosphopeptides, obtained by using a Phos-tag tip as described above, was desalted and concentrated by using a ZipTip-C18 (Millipore). MALDI-TOF mass spectra (positive linear mode) were obtained on a Voyager RP-3 BioSpectrometry Work Station (PerSeptive Biosystems; Framingham, MA, USA) equipped with a nitrogen laser (337 nm, 3-ns pulse). A saturated solution of (2*E*)-2-cyano-3-(4-hydroxyphenyl)acrylic acid (10 mg/mL) in an aqueous solution containing 0.10% (v/v) TFA and 33% (v/v) CH_3CN was used as the matrix. The sample solution (2.0 μL) applied to the MS plate was immediately mixed with the matrix solution (1.0 μL), then dried completely. Each mass spectrum was produced by accumulating data from 128 laser shots. Time-to-mass conversion was performed by means of external calibration using $[M + H]^+$ peaks for two peptides, angiotensin I (*m/z* 1297.51) and adrenocorticotropin hormone/corticotropin-like intermediate lobe peptide 7–38 (*m/z* 3660.19).

2.12 *Open-column phosphate-affinity chromatography for phosphoproteins*

The bottom filter of a polypropylene column (Qiagen) was washed with 20% (v/v) propan-2-ol and the column was then loaded with Phos-tag Toyopearl beads [~2.0 mL of 50% (v/v) suspension] to form a 1.0-mL bed of compressed gel. The gel was washed with 2.0 mL of a binding/washing buffer consisting of 0.10 M Tris–AcOH (pH 7.5) and 0.50 M NaOAc to remove the storage buffer. To form the zinc(II)-bound Phos-tag Toyopearl beads, 2.0 mL of a balancing buffer consisting of 0.10 M Tris–AcOH (pH 7.5), 0.50 M NaOAc, and 10 μM $Zn(OAc)_2$ was placed in the column, and the mixture was incubated for 5 min. The column was then washed with the binding/washing buffer (5.0 mL) to remove excess $Zn(OAc)_2$. The cell lysate from the EGF-stimulated cultured cells (0.50 mg proteins dissolved in 0.25 mL of an RIPA buffer) was diluted with 1.0 mL of the binding/washing buffer. The sample solution (1.25 mL) was applied to the gel and then a flow-through fraction containing unbound proteins was collected. Next, the binding/washing buffer (5.0 mL) was loaded, and a washing fraction was collected. The flow-through and washing fractions were combined and used for subsequent analyses. To elute the gel-bound proteins, an elution buffer (5.0 mL) consisting of 0.10 M Tris–AcOH (pH 7.5), 1.0 M NaCl, and 10 mM NaH_2PO_4–NaOH (pH 7.5) was placed in the column. Each fraction was desalted and condensed by using a Microcon YM-10 centrifugal filter unit and then dissolved in distilled water (0.20 mL). The recovery of proteins in each concentrated fraction was estimated by using a Bio-Rad protein assay kit as described above. All the open-column chromatography operations were performed at room temperature.

2.13 *Two-dimensional gel electrophoresis (2-DE)*

The protein samples were purified by using a ReadyPrep 2-D Clean Up Kit. The purified samples were resolved in the loading buffer supplied with an EzApply 2D Kit and then treated with the alkylation solution with the same kit for 10 min. The sample solution (0.10 mg protein, 20 μL) was applied onto an agarGEL (pH 3–10, 25-mm diameter and 75-mm long), and layered 10 μL of 2.0 M urea. The anode buffer was 10 mM H_3PO_4, and the cathode buffer was 0.20 M NaOH. Isoelectric focusing (IEF) was performed using an Atto AE-6540 at 300 V for 210 min. After electrophoresis, the agarGEL was steeped in 2.5% (w/v) trichloroacetic acid for 3 min twice, next in distilled water for 3 min twice, and then in distilled water for more than 2 h. Finally, the agarGEL was steeped in 0.25 M Tris–HCl (pH 6.8) buffer containing 2.5% (w/v) SDS and 0.10% (w/v) bromophenol blue, then immobilized on the top of the SDS-PAGE gel with the same buffer containing 1.0% (w/v) agarose. SDS-PAGE was performed as described above.

2.14 *Staining of the gel with Pro-Q Diamond, SYPRO Ruby, and Coomassie Brilliant Blue*

The SDS-PAGE gel was fixed in an aqueous solution containing 50% (v/v) MeOH and 10% (v/v) AcOH for 30 min twice. To stain the phosphoproteins with Pro-Q Diamond, the fixed gels were washed three times in distilled water for 30 min each time, then incubated with Pro-Q Diamond phosphoprotein gel stain for 3 h, and finally washed in 50 mM NaOAc–AcOH (pH 4.0) buffer containing 20% (v/v) CH_3CN for 3 h. For SYPRO Ruby staining, fixed gels or Pro-Q Diamond-stained gels were incubated with SYPRO Ruby protein gel stain for 2 h then washed in 10% (v/v) MeOH and 7.0% (v/v) AcOH for 2 h. For Coomassie Brilliant Blue (CBB) staining, fixed gels or SYPRO Ruby-stained gels were incubated with a CBB solution [0.10% (w/v) CBB, 10% (v/v) AcOH, and 40% (v/v) MeOH] for 1 h, and then washed in an aqueous solution containing 10% (v/v) MeOH and 7.0% (v/v) AcOH until the background was clear. Fluorescence images of the gel were acquired on an FLA 5000 laser scanner (Fujifilm). An LAS 3000 image analyzer was used for the observation of CBB-stained gel.

3. Results and Discussion

3.1 *Affinity spin column chromatography for phosphopeptides*

To determine the optimal binding/washing conditions for the separation of peptides in phosphate-affinity chromatography using Phos-tag Toyopearl, we adopted a spin column method (50-μL gel scale) using twelve commercially available neutral, acidic, and basic peptides, some of which were phosphorylated; these are listed in Table 1.

Table 2 shows the effects of the pH of the binding/washing buffer and of additional salts in the buffer on separation of phosphopeptides and nonphosphopeptides. A mixture containing 5.0 nmol each of [Ala-97]-RII 81–99 (No. 4), RII phosphopeptide (No. 5), PKC substrate peptide (No. 7), phosphorylated PKC substrate (No. 8), and 4A/4B peptide (No. 9) was applied. After spin column chromatography, the relative recovery of sample peptides in each fraction was determined by reversed-phase HPLC analysis. Some binding/washing buffer conditions were examined. We selected buffer pH values of 7.4 (0.10 M Tris–AcOH) and 6.0 (10 mM

Table 1. Amino acid sequences of the phosphopeptides and nonphosphopeptides. Phosphorylated amino acids are underlined.

	No.	Peptide Name	Amino Acid Sequence
Neutral Peptides	1	Insulin receptor 1142–1153	TRDIYETDYYRK
	2	Kinase domain of insulin receptor-2	TRDIpYETDYYRK
	3	Kinase domain of insulin receptor-5	TRDIpYETDpYpYRK
	4	[Ala-97] RII 81–99	DLDVPIPGRFDRRVSVAAE
	5	RII phosphopeptide	DLDVPIPGRFDRRVpSVAAE
	6	MAP 177–189 pT/pY peptide	DHTGFLpTEpYVATR
Basic Peptides	7	PKC substrate peptide	QKRPSQRSKYL
	8	Phosphorylated PKC substrate	KRPpSQRHGSKY-NH_2
Acidic Peptides	9	4A/4B peptide	DEMEECSQHLPNI
	10	Src peptide 521–533	TSTEPQYQPGENL
	11	Phosphorylated Src peptide 521–533	TSTEPQpYQPGENL
	12	EGFR 988–998	DADEpYQPGENL

Table 2. Effects of pH values and additional salts in the binding/washing processes on the separation of phosphopeptides and nonphosphopeptides by affinity spin column chromatography using Phos-tag Toyopearl.

(A)		Peptides[#]				
	Washing buffer (pH, additional salts)	Neutral No. 4	No. 5	Basic No. 7	No. 8	Acidic No. 9
(a)	pH 7.4 + 0.10 M NaCl	1%	82%	0%	52%	46%
(b)	pH 7.4 + 0.10 M NaOAc	1%	74%	0%	52%	21%
(c)	pH 6.0 + 0.10 M NaCl	1%	91%	1%	88%	34%
(d)	pH 6.0 + 0.10 M NaOAc	2%	85%	1%	74%	3%

(B)		Peptides[#]				
	Washing buffer	Neutral No. 4	No. 5	Basic No. 7	No. 8	Acidic No. 9
(a)	+ 0.10 M F_3CCO_2Na	3%	91%	11%	87%	57%
(b)	+ 0.10 M NaOAc	2%	92%	11%	88%	67%
(c)	+ 5.0 mM $(COONa)_2$	1%	73%	99%	100%	1%
(d)	+ 0.10 M NaCl and 1.0 mM $(COONa)_2$	1%	88%	1%	88%	4%

MES–NaOH). To prevent nonspecific binding to the phosphate-affinity beads, 0.10 M NaCl or 0.10 M NaOAc was added to the buffer solutions. The separation results in terms of the percentage recovery of each peptide in the elution fractions are shown in Table 2A. In buffer systems with a pH of 6.0 (entries c and d), the elution fraction contained a greater proportion of phosphopeptides (Nos. 5 and 8) and a smaller proportion of nonphosphopeptides (Nos. 4, 7, and 9) than did the buffer systems with a pH of 7.4 (entries a and b). In particular, the nonspecific binding of acidic peptide (No. 9) decreased to only 3% in a buffer system of pH 6.0 containing NaOAc (entry d), and the separation was markedly improved. The acidic peptides are in many cases the most critical factor in the enrichment of phosphopeptides. In this case, the presence of an excess of acetate anion and lower pH should competitively inhibit nonspecific binding between the carboxylate groups of the peptides and Phos-tag. To observe the effects of other additional salts, F_3CCO_2Na, NaOAc, and disodium oxalate were added to the 10 mM MES–NaOH buffer (pH 6.0). The separation results in terms of the percentage recovery of each peptide in the elution fractions are shown in Table 2B. In buffer systems containing 0.10 M F_3CCO_2Na (entry a) or 0.10 M NaOAc (entry b), a considerable degree of nonspecific binding of peptide No. 9 was observed in the elution fractions (57% and 67%, respectively). When the concentration of F_3CCO_2Na was increased to 0.25 M, specific binding of phosphopeptides was decreased (data not shown). In the buffer system containing disodium oxalate (entry c), an oxalate concentration of the order of only mM (5.0 mM of disodium oxalate) had a marked effect in preventing nonspecific binding of peptide No. 9, but had no effect on peptide No. 7. In the buffer system that contained both 1.0 mM disodium oxalate and 0.10 M NaCl (entry d), the separation was improved compared with that in the 10 mM MES–NaOH buffer (pH 6.0) containing 0.10 M NaOAc (see entry d in Table 2A). These findings showed that the most effective binding/washing buffer system for the separation of phosphopeptides by phosphate-affinity chromatography on Phos-tag Toyopearl in this model study consisted of 10 mM MES–NaOH (pH 6.0) containing 0.10 M NaCl and 1.0 mM disodium oxalate.

Next, we examined the optimal elution conditions for phosphate-affinity chromatography using the same spin column method and a triphosphorylated peptide, kinase domain of insulin receptor-5 (No. 3, Table 1). A higher concentration of phosphate in the elution buffer was required to elute the triphosphorylated peptide. The relative recovery of peptide No. 3 in each fraction from spin column chromatography was determined by reversed-phase HPLC analysis. Some eluent systems were found to be suitable for separation of phosphopeptides on Phos-tag Toyopearl. One was 0.10 M to 0.20 M NaH_2PO_4–NaOH (pH 7.4) as a neutral pH eluent. Others were volatile acidic solutions, such as aqueous 0.50% (v/v) TFA, or basic solutions, such as aqueous 2.0 M NH_3. Furthermore, we succeeded in separating a triphosphopeptide from a monophosphopeptide (kinase domain of insulin receptor-2; peptide No. 2, Table 1) present in 5.0 nmol amounts by stepwise elution with two kinds of phosphate buffer (Fig. 2). Elution was conducted with 1.0 mM NaH_2PO_4–NaOH (pH 7.4) (repeated six times, fractions 1–6) and with 0.10 M NaH_2PO_4–NaOH (pH 7.4) (repeated four times, fractions 7–10). Neither the flow-through fraction nor the washing fraction contained both phosphopeptides. Fractions 1–6 contained almost all the monophosphopeptide (recovery 99%, purity 96%) and fractions 7–10 contained almost the triphosphopeptide (recovery 96%, purity 99%). The total time required to complete the procedure shown in Fig. 2 was less than 30 min.

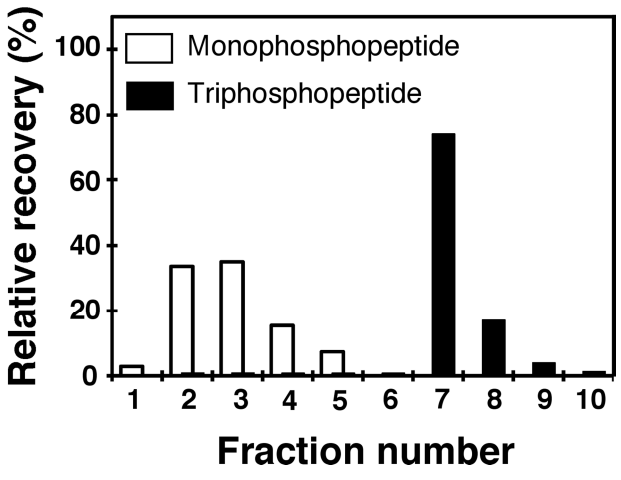

Figure 2. Relative recoveries of a monophosphopeptide (kinase domain of insulin receptor-2, see entry 2 in Table 1) and of a triphosphopeptide (kinase domain of insulin receptor-5, see entry 3 in Table 1) by affinity spin column chromatography using Phos-tag Toyopearl. Stepwise elution with NaH_2PO_4–NaOH (pH 7.4) from 1.0 mM (fractions Nos. 1–6) to 0.10 M (Nos. 7–10) was performed. Open and closed bars represent the recovery values of mono- and triphosphorylated peptide, respectively.

3.2 *Comparison of novel Zn(II)-IMAC using Phos-tag Toyopearl with other methods*

To evaluate the performance of the novel Zn(II)-IMAC using Phos-tag Toyopearl, we compared Phos-tag-based phosphate-affinity chromatography with other existing methods in the analysis of a sample mixture of twelve peptides (5.0 nmol of each peptide), as described above (Table 1). Phos-tag Agarose (Wako Pure Chemical), PHOS-select Iron Affinity Gel (Sigma-Aldrich), PhosphoProtein Purification Kit (Qiagen), and Titansphere TiO (GL Sciences) were used as objects for comparison, and we adopted the spin column procedure (50-µL gel scale). Figure 3 shows HPLC chromatograms for the mixture of peptides before separation (upper), for the flow-through/washing fractions (center), and for the eluted fractions (lower). The underlined numbers correspond to phosphopeptides. The results for the separation of phosphopeptides by the novel Zn(II)-IMAC using Phos-tag Toyopearl are shown in Fig. 3A. In the chromatographic separation, a binding/washing buffer consisting of 10 mM MES–NaOH (pH 6.0) containing 0.10 M NaH_2PO_4–NaOH (pH 7.4). In the flow- through/washing fraction, phosphopeptides No. 5 and No. 8 showed slight leakages, but all the nonphosphopeptides were washed out from the column, and only phosphopeptides were observed in the eluted fraction. The recovery of phosphopeptides was nearly 100% except in the cases of No. 5 and No. 8.

The results obtained by using Phos-tag Agarose areshown in Fig. 3B. A binding/washing buffer of 10 mM MES–NaOH (pH 6.0) containing 0.10 M NaCl and 5.0 mM disodium oxalate and an elution buffer consisting of 0.10 M NaH_2PO_4–NaOH (pH 7.4) were used. A higher concentration of oxalate in the binding/washing buffer was required to perform quality chromatography. The chromatograms are almost the same as those shown in Fig. 3A. Therefore, in the separation of phosphopeptides, Phos-tag Agarose provided a degree of selection similar to that of Phos-tag Toyopearl.

The results obtained by using the Sigma-Aldrich beads are shown in Fig. 3C. These beads consist of a matrix of agarose on which the active metal center is mono-Fe(III). A binding/washing buffer of 0.25 M AcOH (pH 2.7) containing 30% (v/v) CH_3CN, and an elution buffer of 0.20 M NaH_2PO_4–NaOH (pH 7.4) were used according to the standard protocol suggested by Sigma-Aldrich. The elution fraction contained not only phosphopeptides but also a small amount of the acidic peptide No. 9 (7%). The relative recovery of phosphopeptides (49% of No. 11, 88% of No. 2, and 70% of No. 12) was inferior to that achieved with Zn(II)-IMAC using Phos-tag Toyopearl. In addition, this method requires acidic pH conditions in the binding/washing process, and is therefore not suitable for the separation of native phosphoproteins.

The results achieved with the Qiagen beads are shown in Fig. 3D. The binding/washing buffer (pH 6.0) and the elution buffer (pH 7.4) supplied with the kit were used. In the elution fraction, phosphopeptides were observed exclusively. However, the relative recovery of phosphopeptides was inferior to that achieved with the Phos-tag Toyopearl beads.

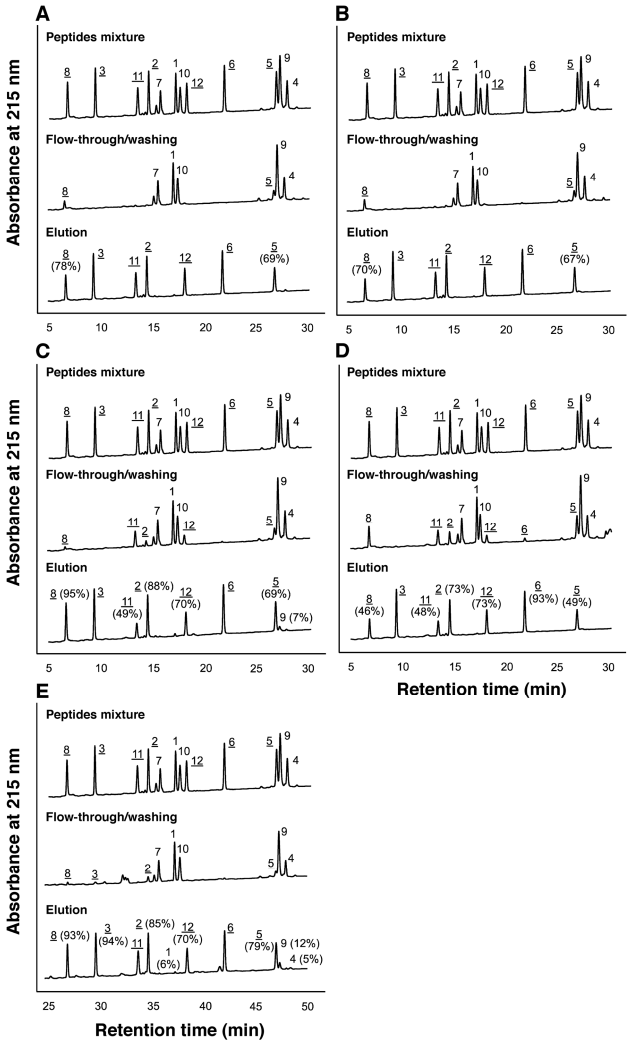

Figure 3. Comparison with other existing techniques. Phosphopeptides were separated from a mixture of twelve peptides (see Table 1) by affinity spin column chromatography using Phos-tag Toyopearl (A), Phos-tag Agarose (B), PHOS-select Iron Affinity Gel (C), PhosphoProtein Purification Kit (D), and Titansphere TiO (E). The peptides mixture prior to loading on each resin (upper chromatograms), in the flow-through/washing fraction (middle chromatograms), and in the elution fractions (lower chromatograms) were analyzed by reversed-phase HPLC. The underlined numbers correspond to phosphopeptides.

The results obtained with GL Sciences beads are shown in Fig. 3E. The beads consist of spherical particles of titanium dioxide (TiO_2). A binding/washing solution of aqueous 0.10% (v/v) TFA containing 0.30 g/mL lactic acid and 80% (v/v) CH_3CN, and both solutions of 0.20 M NaH_2PO_4–NaOH (pH 7.4) and aqueous 2.0 M NH_3 were used for elution in accord with a previous report [11]. Phosphopeptides were found in the eluent, together with small amounts of nonphosphopeptides (6% of No. 1, 12% of No. 9, and 5% of No. 4). The relative recovery of phosphopeptides was excellent. This method requires an acidic pH during the binding/washing process (pH 1.3) and, like the method using the PHOS-select Iron Affinity Gel, cannot therefore be used to separate native phosphoproteins. Furthermore, this method suffers from a

problem in the HPLC analysis: Because the flow-through/washing fraction (the high viscosity of the mixture) contained large amounts of lactic acid, the HPLC elution process of the acid with a eluent consist of aqueous 0.10% (v/v) TFA and 1.0% (v/v) CH_3CN for 20 min was necessary before the peptide analysis as described in the Methods section.

We have therefore demonstrated that our new method for analyzing phosphopeptides by using Phos-tag Toyopearl has advantages over other existing methods.

3.3 *Affinity pipette microtip column chromatography for phosphopeptides*

We developed a pipette microtip column filled with the Phos-tag Toyopearl beads (Phos-tag tip; 10-μL gel scale, see Fig. 4A), and we demonstrated its use in the separation of small amounts of phosphopeptides from a protein tryptic digest for subsequent MALDI-TOF/MS analysis. The tryptic digest of β-casein and in-gel tryptic digest of ovalbumin were used as the first model samples (each 10 μg). A binding/washing buffer of 10 mM MES–NaOH (pH 6.0) containing 0.10 M NaCl, 1.0 mM disodium oxalate, and 20% (v/v) CH_3CN, and an elution solution of 1.0% (v/v) aqueous TFA were used in the chromatographic process. The total time required to complete the microchip column chromatography and desalting procedures was less than 30 min.

Two phosphopeptides were obtained from the tryptic digest of β-casein. One was a monophosphopeptide consisting of 16 residues (Phe_{33}-Gln-*pSer*-Glu-Glu-Gln-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys_{48}, P1), and the other was a tetraphosphopeptide consisting of 25 residues (Arg_{16}-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-*pSer*-Leu-*pSer*-*pSer*-*pSer*-Glu-Glu-Ser-Ile-Thr-Arg_{40}, P2). In the mass spectrum of the tryptic digest of β-casein before the separation, no peaks for the phosphopeptides were detected (data not shown). Figure 4B shows a typical mass spectrum of the elution fraction (β-casein). We detected two peaks and we were able to identify the P1 monophosphopeptide (33–48; m/z = 2062.9) and P2 tetraphosphopeptide (16–40; m/z = 3124.8).

Similarly, we obtained two monophosphopeptides from the in-gel tryptic digest of ovalbumin separated by SDS-PAGE. One consisted of 23 residues (Leu_{62}-Pro-Gly-Phe-Gly-Asp-*pSer*-Ile-Glu-Ala-Gln-Cys-Gly-Thr-Ser-Val-Asn-Val-His-Ser-Ser-Leu-Arg_{84}) and the other consisted of 20 residues (Glu_{340}-Val-Val-Gly-*pSer*-Ala-Glu-Ala-Gly-Val-Asp-Ala-Ala-Ser-Val-Ser-Glu-Glu-Phe-Arg_{359}). The Cys_{73} residue was alkylated with acrylamide during the process of in-gel digestion. In the mass spectrum of the tryptic digest of ovalbumin, no peaks corresponding to phosphopeptides were detected (data not shown). Figure 4C shows a typical mass spectrum of the elution fraction (ovalbumin). We detected three distinct peaks, and we were able to identify a monophosphopeptide (P3, 340–359, m/z = 2089.1), a second monophosphopeptide (P4, 62–84, m/z = 2526.1), and a nondigested monophosphopeptide (P5, 59–84, m/z = 2916.9).

We succeeded in separating and identifying phosphopeptides from the peptides mixture by using the Phos-tag tip without detecting any nonphosphopeptides. The advantages of Phos-tag tip were that it requires only simple operation of pipetting without any special devises, and that it has a short operational time. In addition, the novel Phos-tag Toyopearl beads are much more stable during long-term storage than are Phos-tag Agarose biopolymer beads, and they can be reused many times for separation of low-molecular weight phosphorylated compounds containing phosphopeptides. We confirmed that previously used Phos-tag Toyopearl beads that had been stored in the binding/washing buffer for 6 months at room temperature had the same potency in separation as beads prepared shortly before use (data not shown).

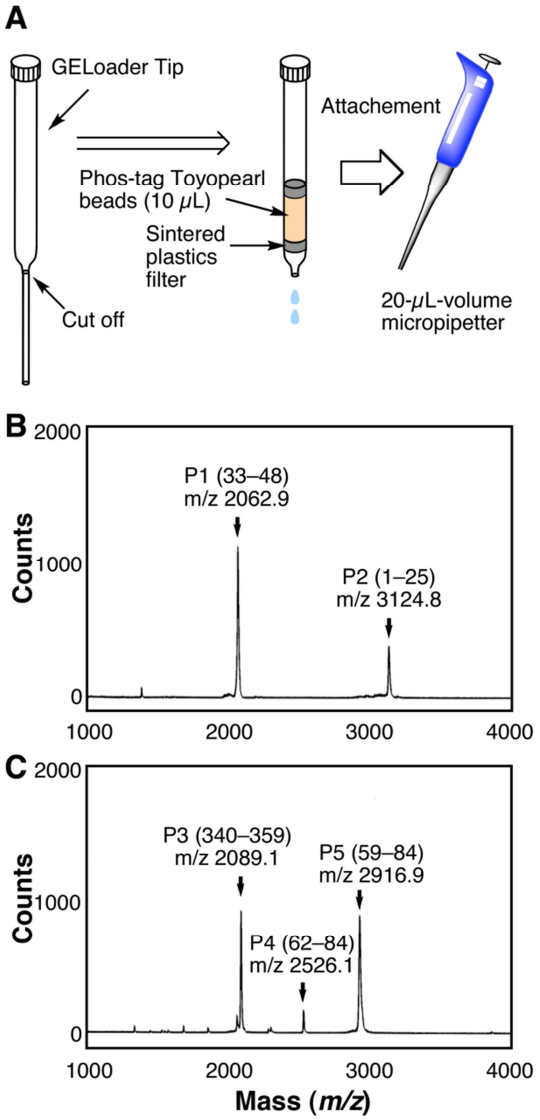

Figure 4. Affinity pipette microtip column chromatography for the separation of phosphopeptides. Scheme for the preparation of the pipette microtip column packed with Phos-tag Toyopearl (Phos-tag tip) (A). MALDI-TOF/MS analysis of each elution fraction after the affinity pipette microtip column chromatographic separation of the tryptic digest of β-casein (B) and in-gel tryptic digest of ovalbumin (C) as samples.

3.4 *Optimization of buffers for the separation of phosphoproteins*

We extended the application of the Phos-tag Toyopearl beads to the separation of phosphoproteins. In our previous study on the separation of native phosphoproteins from cell lysates by using Phos-tag Agarose, we conducted the binding/washing process with 0.10 M Tris–AcOH (pH 7.5) containing 1.0 M NaOAc, and the elution process with 0.10 M Tris–AcOH (pH 7.5) containing 10 mM NaH_2PO_4–NaOH (pH 7.5) and 1.0 M NaCl [16]. During the Zn(II)-IMAC process, no detergent or reducing agent was used. In addition, we have proposed that appropriate concentration of NaOAc (1.0 M) should be added to the binding/washing buffer to avoid nonspecific binding of proteins to the Phos-tag Agarose biopolymer matrix. We applied a similar buffer system with the Phos-tag Toyopearl beads and, in particular, we performed a detailed optimization of the concentration of NaOAc in the binding/washing buffer. We used an EGF-stimulated A431 cell lysate as a real biological sample, and we adopted the spin column method (50-μL gel scale). After elution, the residual proteins remaining in the column were analyzed by washing the column with a buffer containing 0.10 M Tris–AcOH, 1.0% (w/v) SDS, and 10 mM EDTA. Figure 5 shows typical results for SDS-PAGE with SYPRO Ruby gel staining and with Western blotting for the flow-through/washing fraction (lane 1), elution fraction (lane 2), and column-washing fraction (lane 3) for a binding/washing buffer containing 0, 0.25, 0.50, or 1.0 M NaOAc. The SYPRO Ruby images (Fig. 5A) show the distribution of proteins in each fraction. In the absence of the NaOAc, there were scarcely any proteins in the flow-through/washing fraction; in other words, almost all the proteins, including nonphosphorylated ones, were bound to the column. In addition, many proteins remained on the column after elution. As the concentration of NaOAc was increased, fewer proteins were left in the column after elution, and many proteins were distributed in the flow-through/washing and elution fractions.

The efficiency of the separation of phosphoproteins was determined by means of Western blotting using the complex of Phos-tag Biotin with HRP-conjugated streptavidin (Fig. 5B). Probing with Phos-tag Biotin permitted the comprehensive detections of phosphoproteins as ECL signals (the single positive control band corresponding to the standard phosphoprotein, ovalbumin, can be seen in the molecular-weight markers lane M in Fig. 5B). In the presence of less than 0.25 M NaOAc, ECL signals were detected from both the elution and column-washing fractions. On the other hand, in the presence of 0.50 or 1.0 M NaOAc, the signals were detected from the elution fraction only. We therefore decided to use a 0.50 M concentration of NaOAc in the binding/washing buffer for this model study. Under the optimized conditions, we also examined the separation of the phosphorylated and nonphosphorylated MAPK1/2 proteins (Figs. 5C and 5D). It is known that MAPK1/2 (Erk1/2) is phosphorylated from EGF signaling. The strong ECL signal obtained by probing with an anti-pMAPK1/2 antibody was detected in the elution fraction

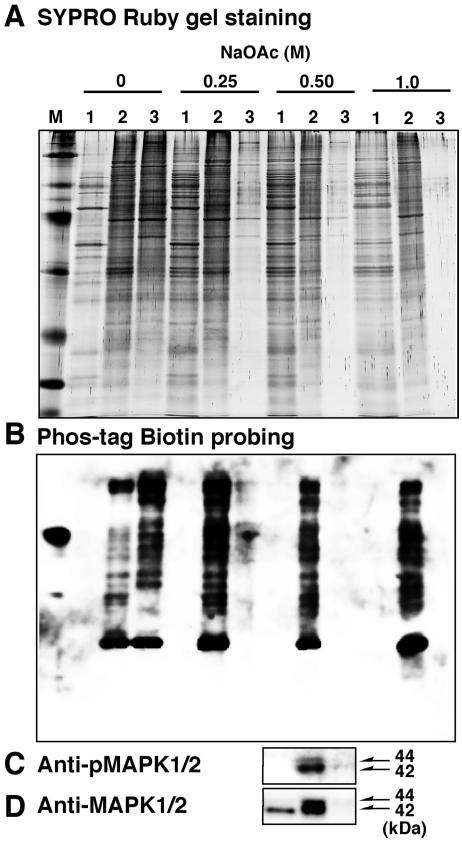

Figure 5. Optimization of the concentration of NaOAc in binding/washing buffer on the separation of phosphoproteins in an EGF-stimulated A431 cell lysate. Concentrations of 0, 0.25, 0.50, and 1.0 M NaOAc were used in the binding/washing processes. The flow-through/washing, elution, and column-washing fractions were applied in lanes 1, 2, and 3, respectively. The SDS-PAGE gels were analyzed by SYPRO Ruby gel staining (A) or by Western blotting with Phos-tag Biotin (B). Molecular-weight standards for 97, 66, 45 (standard phosphoprotein, ovalbumin), 29, 20, and 14 kDa are shown, in order from the top, in lane M. The fractions collected when 0.50 M NaOAc was used were analyzed by Western blotting with anti-pMAPK1/2 (C) and anti-MAPK1/2 (D) antibodies.

exclusively (C). In contrast, the signal obtained by probing with an anti-MAPK1/2 antibody was detected in both the flow-through/washing and elution fractions (D). These results of Western blotting indicated that phosphorylated proteins were separated from their corresponding nonphosphorylated counterparts by the Phos-tag Toyopearl beads.

3.5 *Enrichment of phosphoproteins using an open-column method*

To evaluate the enrichment of phosphoproteins in the elution fraction, we initially performed phosphate-affinity chromatography using a comparative large amount of the cell lysate and an open column with the Phos-tag Toyopearl beads (1-mL gel scale). An EGF-stimulated A431 cell lysate (0.50 mg solubilized cellular proteins in 0.25 mL of an RIPA buffer) was prepared from the cultured cells and then diluted with 1.0 mL of the binding/washing buffer. The resulting

solution (1.25 mL) was loaded onto the open column. Details of the procedures adopted for affinity column chromatography are described in the Methods section. The total time required for the phosphate-affinity chromatography procedure was less than 40 min. For SDS-PAGE followed by Western blotting (Fig. 6), fractions of the cell lysate before chromatography (lane 1) and after elution (lane 2), and the flow-through/washings (lane 3) (6.0 μg of proteins per lane) were sequentially applied. The CBB-staining image of the blotted PVDF membrane (A) showed that the amounts of proteins in each lane were almost equal. Next, we determined the distribution of the various phosphoproteins by Western blotting using Phos-tag Biotin (B), anti-pSer antibody (C), anti-pMAPK substrates (PXTP) antibody (D), anti-pMAPK1/2 antibody (E), anti-pShc antibody (F), and anti-pErbB-2/HER-2 antibody (G). Shc and ErbB-2/HER-2, as well as MAPK1/2, are candidates for *in vivo* phosphorylation in EGF signaling. In all blottings, the ECL signals corresponding to phosphoproteins were stronger in the elution fraction (lane 2) than in the lysate (lane 1). There were very small amounts of phosphoprotein in the flow-through/washing fractions (lane 3 of B and C). These results confirmed that the cellular phosphoproteins are strongly enriched in the elution fraction.

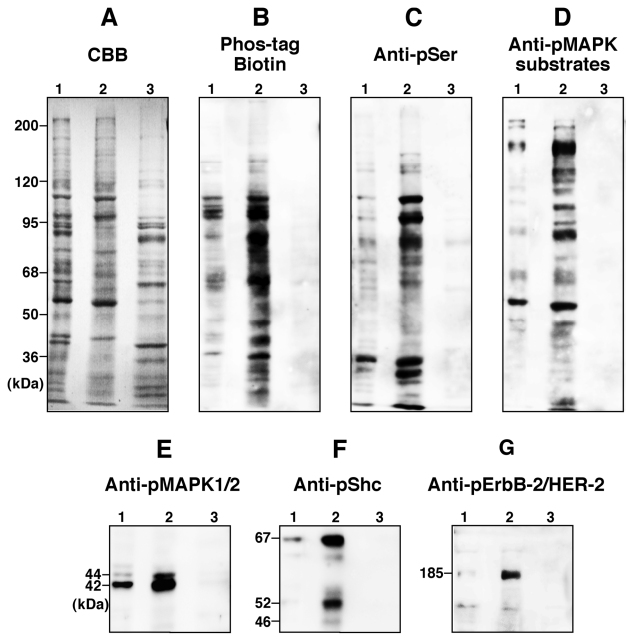

Figure 6. Comparison of the relative amounts of phosphoproteins from an EGF-stimulated A431 cell lysate before and after affinity open column chromatography. The cell lysate before loading on the column (lane 1), the elution fraction (lane 2), and flow-through/washing fraction (lane 3) were subjected to SDS-PAGE. Each lane contained 6.0 μg proteins. The proteins on the SDS-PAGE gels were electrotransferred to PVDF membranes and visualized by CBB gel staining (A) or by Western blotting using Phos-tag Biotin (B), anti-pSer antibody (C), anti-pMAPK substrates (PTXT) antibody (D), anti-pMAPK1/2 antibody (E), anti-pShc antibody (F), or anti-pErbB-2/HER-2 antibody (G). The molecular weights are shown on the left-hand side.

We quantified the proteins in each fraction by using 0.25 mL of an RIPA buffer containing 0.50 mg protein of the EGF-stimulated A431 cell lysate. The amounts of recovered protein in the flow-through/washing and elution fractions were 50% and 22%, respectively. The yield of the elution fraction is consistent with the general content of phosphoproteins in mammalian cells (10–30%) [27]. When larger amounts of the lysate proteins (e.g., 0.60 mg in 0.25 mL of an RIPA buffer) were loaded into the column, some of the phosphoproteins were eluted in the flow-through/washing fraction (data not shown). A similar leakage into the flow-through/washing fraction resulted from the use of twice the volume of an RIPA buffer (0.50 mL) containing the solubilized proteins (0.50 mg), possibly as a result of competitive binding of $HOVO_3^{2-}$ or of elimination of zinc(II) by EDTA. Thus, the appropriate capacity of the 1 mL-compressed Phos-tag Toyopearl column is ~0.50 mg of lysed proteins in 0.25 mL of RIPA buffer. These results that we obtained by using the Phos-tag Toyopearl beads were almost identical to those that we obtained by using Phos-tag Agarose, as reported previously [16].

Finally, we demonstrated the value of phosphate-affinity chromatography with Phos-tag Toyopearl as an efficient procedure for the separation and enrichment of phosphoproteins by examining the 2-DE (IEF and SDS-PAGE) of each fraction obtained from the open column with 1 mL of gel, followed by total protein gel staining with SYPRO Ruby, phosphoprotein gel staining with Pro-Q Diamond, Western blotting, and MS analysis (Fig. 7). The cell lysate before the chromatography (left panels), after elution (center panels), and after flow-through/washing (right panels) (100 μg of proteins each) were separated on a 2-DE gel that was then stained with Pro-Q Diamond (B). In subsequent SYPRO Ruby gel staining (A), many spots that were not detected by Pro-Q Diamond staining were observed in the cell lysate and in both the elution and flow-through/washing fractions. That is partially explained by the fact that the cell lysate and flow-through/washing fraction contain many nonphosphorylated proteins. However, SYPRO Ruby staining of the elution fraction permitted the detection of many protein spots, especially in alkaline isoelectric point (p*I*) region, that were not detected by Pro-Q Diamond staining. We next performed Western blotting analysis by treatment with the anti-pSer antibody (C). Some protein spots in the alkaline p*I* region that showed no fluorescent signal with Pro-Q Diamond were detected by probing with the anti-pSer antibody. We noticed that the Pro-Q Diamond stain tended to detect phosphoproteins in region of acidic p*I* much more strongly than it did in region of alkaline p*I*. Furthermore, we identified 10 proteins in the SYPRO Ruby-stained gel (Nos. 1–10, see center panel of A) by LC-MS/MS analysis performed by a commercial contract research company, Applied Cell Biotechnologies (Yokohama, Japan). The results were examined by using the Mascot search engine (Matrix Science, London, UK). All the proteins were well-known phosphoproteins, as summarized in Table 3. In addition, all the molecular weights and p*I* values were

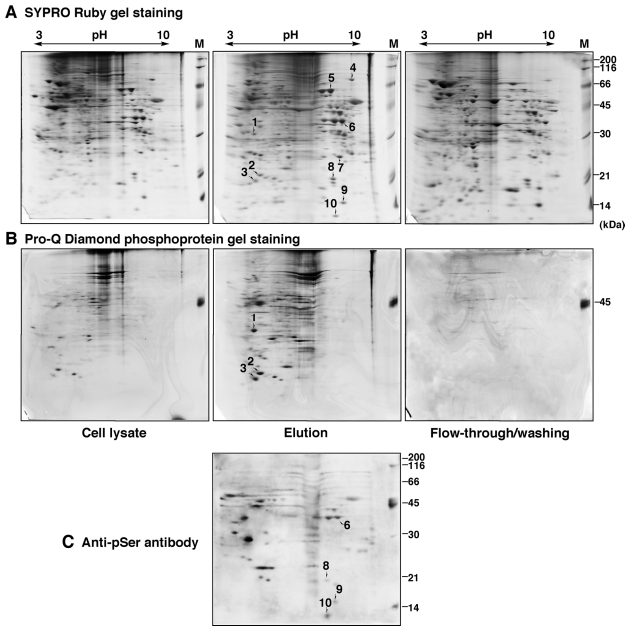

Figure 7. 2-DE (IEF and SDS-PAGE) analysis of an EGF-stimulated A431 cell lysate before and after affinity open column chromatography. The cell lysate before loading on the column (left), the elution fraction (center), and the flow-through/washing fraction (right) were subjected to 2-DE. Each 2-D gel contains 100 μg proteins. The gels were stained with SYPRO Ruby gel stain (A) and Pro-Q Diamond phosphoprotein gel stain (B). The elution fraction was probed by Western blotting analysis using anti-pSer antibody (C). The molecular-weight standards for 200, 116, 66, 45 (standard phosphoprotein, ovalbumin), 30, 21, and 14 kDa are shown, in order from the top, in lane M.

consistent with the location of the corresponding protein spot on the 2-DE gel. These results confirm that phosphoproteomics could progress markedly if our phosphate-affinity chromatography were combined with existing methods involving 2-DE and recent advances in MS analysis.

Table 3. Proteins identified in the elution fraction of affinity open column chromatography with Phos-tag Toyopearl.

	Identified proteins	MW	p*I* value
Spot 1	Glutathione S-transferase P1-1	23225	5.44
2	Nucleophosmin	32575	4.64
3	Nucleophosmin	32575	4.64
4	78 kDa gastrin-binding protein	82999	9.36
5	Pyruvate kinase isozymes M1/M2	57937	7.96
6	Glyceraldehyde-3-phosphate dehydrogenase	35922	8.58
7	Peroxiredoxin-1	22110	8.27
8	Cofilin-1	19371	8.26
9	Profilin-1	14923	8.47
10	Macropharge migration inhibitory factor	12476	7.96

MW = molecular weight

4. Concluding remarks

We have demonstrated a simple and efficient method for the separation and enrichment of phosphopeptides and phosphoproteins by means of Zn(II)–IMAC with a novel phosphate capture bead, Phos-tag Toyopearl. Phos-tag Toyopearl (15 μmol/mL-gel of Phos-tag) was synthesized by coupling a primary amine derivative of Phos-tag with NHS-activated Toyopearl AF-Carboxy-650M gel. Although there are other well-known types of IMAC that use other metal ions such as Fe(III) or Ga(III) and types of MOAC that use metal oxides such as titanium dioxide (TiO_2) or zirconium dioxide (ZrO_2), neither group of techniques functions quite satisfactorily in phosphoproteomics in terms of their selectivity and versatility. The method based on the metal oxide TiO_2 has been shown to be the best among these techniques [11,13]; however, to perform high-quality chromatography, it is necessary to use an acidic solution (pH <2) containing a high concentration of lactic acid in the binding/washing process. Our Zn(II)-IMAC provides a simple, rapid, and specific procedure for separating phosphopeptides and phosphoproteins in an aqueous solution, and physiological pH values are maintained throughout the analysis. In addition, column of Phos-tag Toyopearl are stable to long-term storage and can be reused many times. Because the procedure is nondenaturing and maintains the conformation and activity of the proteins, the separated phosphoproteins are ideal for use in many downstream applications and in top-down proteomics strategies. Quantitative phosphoproteomics could progress greatly if our new method were combined with the iTRAQ technology [28] or with Phos-tag SDS-PAGE methodology [29–37], as reported previously. The new separation and enrichment procedure is bound to lead to an increase in the sensitivity of evaluation of protein phosphorylation involving the characterization of multiple alternative forms of heterogeneous phosphoprotein, detection of activities of kinases and phosphatases, and elucidation of interactions among proteins, and it should provide an opportunity for a variety of types of experiment.

Acknowledgements

We wish to thank the Research Center for Molecular Medicine and the Analysis Center of Life Science, Hiroshima University, Japan, for the use of their facilities. This work was supported in part by Grants-in-Aid for Scientific Research (B, 22390006; C, 22590037) from the Japan Society for the Promotion of Science (JSPS), by a Grant-in Aid for Young Scientists (B, 22790037) from the Ministry of Education Culture, Sports, Science, and Technology (MEXT), by research grants for Feasibility Study from the Japan Science and Technology Agency (JST), and by a research grant from Chugoku Industrial Innovation Center. MANAC Incorporated, Fukuyama, Japan, also provided some financial support.

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