Eiji Kinoshita
Atsushi Yamada
Hironori Takeda
Emiko Kinoshita-Kikuta
Tohru Koike

Department of Functional Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan

Novel immobilized zinc(II) affinity chromatography for phosphopeptides and phosphorylated proteins

Immobilized metal ion affinity chromatography (IMAC) is now a widely accepted technique for the separation of natural or artificial products that is beginning to find industrial applications. Here, we introduce a novel procedure for the separation of phosphopeptides and phosphorylated proteins by immobilized zinc(II) affinity chromatography. The phosphate-binding site of the affinity gel is an alkoxide-bridged dinuclear zinc(II) complex, the 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex (*Phos-tag*), which is linked to a highly cross-linked 4% (*w/v*) agarose. The affinity gel (*Phos-tag agarose*) was prepared by the quantitative reaction of *N*-hydroxysuccinimide-activated Sepharose and a *Phos-tag* derivative having a 2-aminoethylcarbamoyl group in dry CH_3CN. Phosphopeptides were retrieved in a quantitative and highly selective manner by a spin column method using *Phos-tag agarose* at room temperature. Furthermore, in this study, we demonstrate a simple, rapid, and reusable affinity column chromatography for the separation of phosphorylated proteins such as ovalbumin, α_{s1}-casein, and β-casein at physiological pH.

Key Words: Affinity chromatography; Zinc(II) complex; Phospho-proteomics; Phosphopeptide; Phosphorylated protein

Received: April 22, 2004; revised: October 12, 2004; accepted: October 12, 2004

DOI 10.1002/jssc.200401833

1 Introduction

A wide variety of post-translational modifications of proteins, such as phosphorylation, glycosylation, methylation, and acetylation, dramatically enhance the diversity of genetically encoded proteins and play key roles in regulating the function, localization, binding specificity, and stability of target proteins [1]. Among the modifications, protein phosphorylation represents the most abundant covalent modification of proteins. Organisms utilize this reversible reaction of proteins to control many cellular activities, including signal transduction, gene expression, cell cycle progression, cytoskeletal regulation, and energy metabolism [2, 3]. Because phosphorylation, which mainly occurs on serine, threonine, and tyrosine residues in eukaryotes, is a principle for the regulation of life, much effort has been focused on the development of methods for characterizing protein phosphorylation. A widely and traditionally used method for defining a particular phosphorylation event is to label a target molecule with [^{32}P]orthophosphate followed by separation and visualization using polyacrylamide gel electrophoresis (PAGE) and autoradiography, respectively [4]. Newer, non-radioactive methods using poly- and monoclonal antibodies for the detection of phosphorylated amino acids have been reported [5–9].

While the development of more specific and efficient methods to purify phosphorylated proteins has attracted great interest toward phosphoproteome study in the post-genome era, to date there has been no satisfactory procedure for separation and purification under physiological conditions. Previously, we found that macrocyclic polyamine zinc(II) complexes are useful as a family of host molecules for phosphate monoester dianions ($K_d = 10^{-3}$ to 10^{-6} M) under physiological conditions [10–12]. Their molecular design was originally conceived from the fact that phosphates act as substrates or inhibitors by reversible coordination to zinc(II) ion in zinc-enzymes [13]. In our zinc-enzyme model studies with macrocyclic polyamine zinc(II) complexes, we reached the generalized hypothesis that the selective association of phosphate dianions is feasible with two zinc(II) ions that are within a distance of 3–4 Å [14]. Recently, we have reported that a dinuclear zinc(II) complex (i.e., the 1,3-bis[bis(pyridin-2-ylmethyl)-amino]propan-2-olato dizinc(II) complex [15], *Phos-tag*) acts as a novel phosphate capture molecule ($K_d = 25$ nM for phenyl phosphate dianion) in aqueous solution at physiological pH (see **Figure 1**) [16]. The *Phos-tag* molecule has a vacancy on the two zinc(II) ions (Zn–Zn

Correspondence: Eiji Kinoshita and Tohru Koike, Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan.
Phone: +81 82 257 5323. Fax: +81 82 257 5336.
E-mail: kinoeiji@hiroshima-u.ac.jp; tkoike@hiroshima-u.ac.jp.

Abbreviations: BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue R-250; HSA, human serum albumin; IMAC, immobilized metal affinity chromatography; NHS, *N*-hydroxysuccinimide.

Original Paper

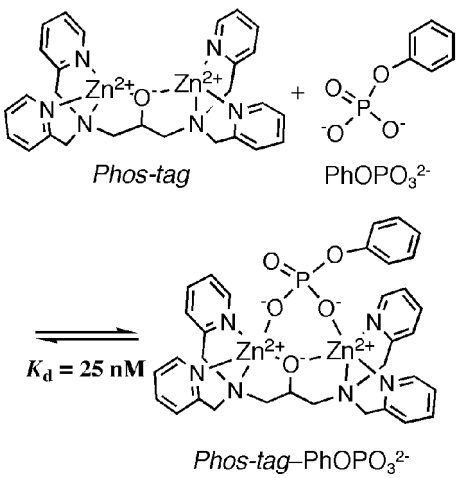

Figure 1. Complex formation equilibrium of phenyl phosphate dianion ($PhOPO_3^{2-}$) and *Phos-tag* in aqueous solution: $K_d = [\textit{Phos-tag}]\,[PhOPO_3^{2-}]/[\textit{Phos-tag}{-}PhOPO_3^{2-}]$ (M).

distance = 3.6 Å) that is suitable for the access of a phosphate monoester dianion as a bridging ligand (i.e., $Zn^{2+}{-}{}^{-}OPO^{-}{-}Zn^{2+}$). The resulting 1:1 phosphate complex ($ROPO_3^{-}{-}Zn_2L^{3+}$) has a total charge of +1. The anion selectivity indexes of the phenyl phosphate dianion against SO_4^{2-}, CH_3COO^{-}, Cl^{-}, and bisphenyl phosphate monoanion at 25°C are 5.2×10^3, 1.6×10^4, 8.0×10^5, and $>2 \times 10^6$, respectively. These findings have contributed to the development of a simple, rapid, and sensitive procedure for a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of phosphorylated compounds such as phosphopeptides and lysophosphatidic acid [17]. We here describe a novel application of the *Phos-tag* molecule, namely, a simple and rapid affinity chromatography for the separation of phosphopeptides and phosphorylated proteins. As the first practical example, we demonstrate the separation of phosphopeptides (i.e., p60c-*src* peptide, insulin receptor peptide, and β-casein digest) and phosphorylated proteins (i.e., ovalbumin, α_{s1}-casein, and β-casein) in the presence of the corresponding non-phosphopeptides and dephosphorylated proteins.

2 Experimental

2.1 Reagents

A *Phos-tag* ligand attached with a methoxycarbonyl group (*N*-(5-methoxycarbonyl-2-pyridinylmethyl)-*N,N′,N′*-tris-(pyridin-2-ylmethyl)-1,3-diaminopropan-2-ol) was provided by the NARD Institute (Amagasaki, Japan) [18]. NHS-activated Sepharose 4FF and a Tricorn 5/50 empty column were purchased from Amersham Biosciences (Piscataway, NJ, USA). An Ultrafree-MC centrifugal filter unit (UFC3 0GV) and a centrifugal filter device (Microcon YM-10) were purchased from Millipore (Bedford, MA, USA). Phosphopeptides and non-phosphopeptides (p60c-*src* 521–533 peptides and insulin receptor 1142–1153 peptides) and *Escherichia coli* β-galactosidase were supplied by Toyobo (Osaka, Japan). Chicken egg white ovalbumin, bovine α_{s1}-casein and β-casein, human serum albumin (HSA), bovine erythrocyte carbonic anhydrase, bovine intestinal mucosa alkaline phosphatase, trypsin (proteomics-grade), and a Phosphopeptide Positive Control Set (a monophosphopeptide and a tetraphosphopeptide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Takara Shuzo (Kyoto, Japan). Coomassie Brilliant Blue R-250 (CBB) for gel staining was purchased from Nacalai Tesque (Kyoto, Japan). The other reagents and solvents used were of analytical quality and used without further purification. All aqueous solutions were prepared using deionized and distilled water. Thin-layer and silica gel column chromatography were performed using a Merck Silica gel TLC plate (No. 5567, Silica Gel 60 Kieselguhr F_{254}) and Merck Silica gel 60 (No. 5009), respectively.

2.2 Apparatus

UV spectra were obtained with a Hitachi U-3500 spectrometer at 25.0 ± 0.1°C. IR spectrum was recorded on a Horiba FT-710 infrared spectrometer with a KCl pellet (Real Crystal IR Card) at 20 ± 2°C. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra at 35.0 ± 0.1°C were recorded on a JEOL LA500 spectrometer. Tetramethylsilane (in $CDCl_3$) was used as an internal reference for ^1H and ^{13}C NMR measurements. The pH measurement was conducted with a Horiba F-12 pH meter (Kyoto, Japan) and a combination pH electrode Horiba-6378, which was calibrated using pH standard buffers (pH 4.01 and 6.86) at 25°C. MALDI-TOF MS spectra (positive reflector mode) were obtained on a Voyager RP-3 BioSpectrometry Workstation (PerSeptive Biosystems) equipped with a nitrogen laser (337 nm, 3 ns pulse). A Micro Collector AC-5700 (ATTO, Tokyo, Japan) was used as a fraction collector for affinity column chromatography.

2.3 Synthesis of an amino-pendant *Phos-tag* ligand

Ethylenediamine (12.0 g, 0.20 mol) was added to *N*-(5-methoxycarbonyl-2-pyridinylmethyl)-*N,N′,N′*-tris(pyridin-2-ylmethyl)-1,3-diaminopropan-2-ol (1.00 g, 1.95 mmol) at room temperature. After the mixture was heated at 70°C for 15 h, the excess ethylenediamine was evaporated at ca. 20 mmHg and 60°C. The residue was purified by silica gel column chromatography (eluent; CH_2Cl_2/MeOH/28% aqueous NH_3 = 50:10:2) to obtain *N*-(5-(2-aminoethylcarbamoyl)pyridin-2-ylmethyl)-*N,N′,N′*-tris(pyridin-2-ylmethyl)-1,3-diaminopropan-2-ol as a brown oil (0.77 g, 73% yield): TLC (eluent; CH_2Cl_2/MeOH/28% aqueous NH_3 = 20:10:1) R_f = 0.36. IR (cm^{-1}): 3283, 3059, 2932, 2825,

1650, 1594, 1475, 1434, 1364, 1314, 1050, 755. ^{1}H NMR ($CDCl_3$): δ 2.53–2.71 (4H, m, NCCCHN), 2.96 (2H, t, CONCCH), 3.50 (2H, dt, CONCHC), 3.81–3.95 (9H, m, NCCHCN, PyCHN), 7.10–7.15 (4H, m, CONH, PyH), 7.34 (3H, d, PyH), 7.44 (1H, d, PyH), 7.58 (3H, dt, PyH), 8.01 (1H, dd, PyH), 8.46–8.51 (3H, m, PyH), 8.89 (1H, d, PyH). ^{13}C NMR ($CDCl_3$): δ 41.2, 42.3, 59.17, 59.24, 60.77, 60.80, 61.1, 67.3, 122.0, 122.1, 122.8, 123.1, 128.8, 135.5, 136.5, 147.4, 148.98, 149.05, 159.3, 159.5, 162.8, 166.0. MALDI-TOF MS: zinc(II)-free ligand (HL) in a 50% (*v/v*) CH_3CN solution containing α-cyano-4-hydroxycinnamic acid (10 mg/mL); *m/z* 540.3 for M + H^+, 1:1 phosphate-bound dizinc(II) complex (Zn_2L^{3+}–$HOPO_3^{2-}$) in a 50% (*v/v*) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (10 mg/mL), 2 mM $Zn(CH_3COO)_2$, 1 mM HL, and 12.5 mM NaH_2PO_4-NaOH (pH 6.9); *m/z* 763.1.

2.4 Affinity spin column chromatography for phosphopeptides

For the separation of phosphopeptides from a mixture containing non-phosphopeptides by affinity spin column chromatography, *Phos-tag agarose* was used. *Phos-tag agarose* (0.1 mL of swollen gel) was applied in a sample reservoir (an Ultrafree-MC centrifugal filter unit, UFC3 0GV). The filter unit was centrifuged at 2,000 × *g* for 20 s, and the filtrate was discarded. To completely form the dizinc(II) complex, a balancing buffer (0.10 M Tris-CH_3COOH, pH 7.4, 0.30 mL) containing 10 μM $Zn(CH_3COO)_2$ was applied in the sample reservoir. The unit was centrifuged at 2,000 × *g* for 20 s, and the filtered buffer was discarded. A sample solution (0.10 M Tris-CH_3COOH, pH 7.4, 0.30 mL) containing phosphopeptides and non-phosphopeptides was added into the sample reservoir and allowed to incubate for 5 min at room temperature. The filter unit was centrifuged at 2,000 × *g* for 20 s, and the filtrate was collected as a through fraction (fraction 1). To wash the *Phos-tag agarose*, a washing buffer (0.10 M Tris-CH_3COOH, pH 7.4, 0.30 mL) containing 0.50 M $NaNO_3$ was added to the sample reservoir. The unit was centrifuged at 2,000 × *g* for 20 s. The filtrate was collected as a washing fraction containing unbound peptides. The washing operation was repeated 3 times (fractions 2–4). To elute the bound phosphopeptide, an elution buffer (0.30 mL, 50 mM NaH_2PO_4–NaOH, pH 7.0) was added to the sample reservoir. The filter unit was centrifuged at 2,000 × *g* for 20 s, and the filtrate was collected as an eluting fraction. The eluting operation was repeated 4 times (fractions 5–8). From the loading to the separation of the sample, the procedure took within 15 min. As other elution buffers, 0.20 M $(NH_4)_2CO_3$–CO_2 (pH 8.0), 0.10 M Tris-CH_3COOH (pH 7.4) containing 20 mM EDTA · 2Na, 1.0 M aqueous NH_3 (pH 11.0), and CO_2 saturated water could be used. All fractions were analyzed by HPLC in order to estimate the selective recovery of the samples.

2.5 Affinity column chromatography for phosphorylated proteins

A Tricorn 5/50 empty column was utilized for the separation of phosphorylated proteins from a mixture containing unphosphorylated proteins by affinity column chromatography using *Phos-tagagarose*. *Phos-tagagarose* was introduced into the column to make 0.40 mL of a compressed gel bed and washed with a 0.10 M Tris-CH_3COOH buffer (pH 7.4) to remove the storage buffer (i.e., 20 mM Tris-CH_3COOH (pH 7.4) containing 20% (*v/v*) 2-propanol) under low pressure using a peristaltic pump. *Phos-tag agarose* in the column was equilibrated with 15 mL of a 0.10 M Tris-CH_3COOH buffer (pH 7.4) containing 10 μM $Zn(CH_3COO)_2$ and 0.50 M $NaNO_3$ to completely form the dizinc(II) complex. After equilibrating, the buffer was substituted with a binding buffer (0.40 mL) of 0.10 M Tris-CH_3COOH (pH 7.4) containing 0.50 M $NaNO_3$, and then the sample containing phosphorylated and unphosphorylated proteins (in a binding buffer, 0.33 mL) was loaded at a constant flow rate of 0.25 mL/min using a peristaltic pump. Next, a washing buffer of 0.10 M Tris-CH_3COOH (pH 7.4) containing 0.50 M $NaNO_3$ was loaded at the same rate and collected as washing fractions (0.66 mL × 6, fractions 1–6). Finally, elution of the samples was performed with increasing concentrations of NaH_2PO_4-NaOH (pH 7.0) from 1.0 mM to 50 (or 500) mM to obtain the eluting fractions (0.33 mL × 18, fractions 7–24). The total time for this affinity chromatography was within 40 min. Each fraction was desalted and condensed to ca. 10 μL using a centrifugal filter device, Microcon YM-3. In order to load onto subsequent SDS-PAGE, 10 μL of a 0.125 mM Tris-HCl buffer (pH 6.8) containing 20% (*v/v*) glycerol, 4.0% (*w/v*) SDS, 10% (*v/v*) β-mercaptoethanol, and 0.14% (*w/v*) bromophenol blue was added to the concentrated samples.

2.6 HPLC analysis

High-performance liquid chromatography (HPLC) for peptide analysis was performed using a JASCO LCSS-905 HPLC system station with a CO-2060 column oven (at 40°C), a UV-2070 UV-detector (at 215 or 266 nm), and a reversed phase column (TSKgel Octadecyl-2PW, 4.6 × 150 mm, Tosoh). A freeze-dried sample was dissolved in 0.10 M Tris-CH_3COOH (pH 7.4), and the sample solution was applied to the column and separated under the appropriate gradient conditions (described in the Results and Discussion section for each sample) for ca. 30 min at a flow speed of 1.0 mL/min.

2.7 Tryptic digestion of bovine β-casein

Tryptic digestion of bovine β-casein was performed in a mixed solution of 0.50 mg β-casein in H_2O (50 μL) and a 50 mM $(NH_4)_2CO_3$-CO_2 buffer (pH 8.0, 0.45 mL) containing 10 μg of proteomics-grade trypsin for 12 h at 37°C. The

trypsin and large molecules (i. e., >10000 Da) were removed by ultrafiltration with a Microcon YM-10 ultrafilter unit at 14,000 × *g* for 40 min. After an aqueous solution (0.20 mL) of 0.10% (*v/v*) CF_3COOH had been added to the ultrafilter unit, the same ultrafiltration procedure was conducted. The filtrates were combined (ca. 0.7 mL) and then divided into ten fractions. Each fraction containing ca. 50 μg β-casein digest was freeze-dried and stored below 0°C.

2.8 Dephosphorylation of phosphorylated proteins

Dephosphorylation of phosphorylated proteins (50 μg; ovalbumin, α_{s1}-casein, and β-casein) was performed overnight in a 50 mM Tris-HCl buffer (pH 9.0, 0.20 mL) containing 1.0 mM $MgCl_2$ and 3.3 units of bovine intestinal mucosa alkaline phosphatase at 37°C. Each reaction mixture was used as the sample of dephosphorylated protein for the following affinity column chromatography.

2.9 SDS-PAGE and gel staining

SDS-PAGE was performed at 40 mA at room temperature in a 1-mm-thick, 9-cm-wide, and 9-cm-long gel on a PAGE apparatus (model AE6500; ATTO, Japan). The gel consisted of 1.8 mL of a stacking gel (4.0% (*w/v*) polyacrylamide, 0.125 M Tris-HCl (pH 6.8), and 0.10% (*w/v*) SDS) and 6.3 mL of a separating gel (12.5% (*w/v*) polyacrylamide, 375 mM Tris-HCl (pH 8.8), and 0.10% (*w/v*) SDS). An acrylamide stock solution was prepared as a mixture of a 30:1 ratio of acrylamide to *N,N′*-methylenebisacrylamide. The electrophoresis running buffer (pH 8.4) was 25 mM Tris and 192 mM glycine containing 0.10% (*w/v*) SDS. Sample solutions were heated for 5 min at 95°C before gel loading. After electrophoresis, the gels were stained in a CBB solution (0.10% (*w/v*) CBB, 10% (*v/v*) CH_3COOH, and 40% (*v/v*) MeOH) for 1 h with gentle agitation and then washed in an aqueous solution containing 10% (*v/v*) CH_3COOH and 25% (*v/v*) MeOH until the background was clear.

3 Results and discussion

3.1 Preparation of the immobilized zinc(II) affinity gel (*Phos-tag agarose*)

A *Phos-tag* ligand, **1**, *N*-(5-methoxycarbonyl-2-pyridinylmethyl)-*N*, *N′*, *N′*-tris(pyridin-2-ylmethyl)-1,3-diaminopropan-2-ol, was treated with large excess amounts of ethylenediamine at 70°C for 15 h to obtain an amino-pendant *Phos-tag* ligand, **2**, *N*-(5-(2-aminoethylcarbamoyl)pyridin-2-ylmethyl)-*N,N′*,*N′*-tris(pyridin-2-ylmethyl)-1,3-diaminopropan-2-ol, in 73% yield (see **Figure 2**). The zinc(II)-unbound gel was prepared by condensation of **2** and a highly cross-linked agarose gel (NHS-activated Sepharose 4FF). The NHS-activated Sepharose 4FF (3.0 mL, swollen in dry CH_3CN), which has 6-aminohexanoic *N*-hydroxysuccinimide ester groups (ca. 20 μmol/mL-gel), was mixed with a dry CH_3CN solution (3.0 mL) of **2** (5.0 μmol/mL) at room temperature (see Figure 2). The amide-formation reaction proceeded quantitatively and was followed by the decrease of unbound HL (λ_{max} 260 nm in aqueous solution). The mixture was allowed to stand for 2 h at room temperature with gentle agitation using a glass rod, and then the solvent was filtered off. The obtained gel was suspended in 9.0 mL of 0.10 M $NaHCO_3$-NaOH (pH 10) for 1 h, where the unreacted *N*-hydroxysuccinimide ester groups were hydrolyzed to form carboxylate groups, and then the buffer was removed. After washing the gel 3 times with 3 mL of 20 mM Tris-CH_3COOH (pH 7.4), an aqueous solution (3.0 mL) containing 20 mM $Zn(CH_3COO)_2$ and 0.10 M 2-morpholinoethane sulfonic acid-NaOH (pH 6.0) was added. The obtained zinc(II)-bound gel (*Phos-tag agarose*) was stored in a mixed solution of 20 mM Tris-CH_3COOH (pH 7.4) and 20% (*v/v*) 2-propanol at ca. 4°C.

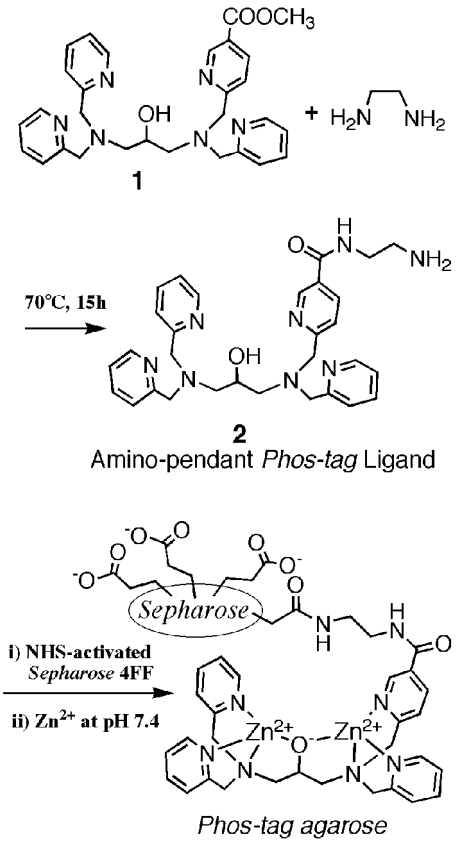

Figure 2. Preparation scheme for amino-pendant *Phos-tag* and *Phos-tag agarose*.

3.2 Affinity spin column chromatography for phosphopeptides

As the first example of the affinity spin column chromatography, p60c-*src* phosphopeptide (45 μg) was separated

and purified from a mixture of the corresponding non-phosphopeptides (45 μg). The p60c-*src* peptide consists of 13 residues (Thr_{521}-Ser-Thr-Glu-Pro-Gln-*Tyr*-Gln-Pro-Gly-Glu-Asn-Leu_{533}) of the c-*src* protein, and the phosphorylation site is the hydroxyl group of Tyr_{527} (see **Figure 3**). Figure 3.a shows the separation result for the fractions 1–8. The HPLC analysis of the peptides in each fraction was conducted by gradient elution with an aqueous solution of 0.10% (*v/v*) CF_3COOH (from 93 to 88% (*v/v*)) and a CH_3CN solution of 0.10% (*v/v*) CF_3COOH (from 7 to 12% (*v/v*)) at 266 nm. The phosphopeptide was not included in the through fraction (fraction 1) and washing fractions (fractions 2–4) and was completely recovered in eluting fractions (fractions 5–7). Thus, we could succeed in separating p60c-*src* phosphopeptide rapidly and efficiently from the mixture. In the absence of zinc(II) ions in the *Phos-tag agarose* (i.e., by zinc(II) elimination with a 0.20 M EDTA-NaOH buffer (pH 8.0) before sample loading), both peptides were washed out in the fractions 1–4 (see Figure 3.b), indicating that the selective binding of the phosphopeptide is performed via the zinc(II) ions in the *Phos-tag* molecule.

Next, we tried to perform the separation using another phosphopeptide (insulin receptor phosphopeptide, 50 μg) in the presence of the corresponding non-phosphopeptide (50 μg). The insulin receptor peptide consists of 12 residues (Thr_{1142}-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-*Tyr*-Tyr-Arg-Lys_{1153}) of an insulin receptor protein, and the phosphorylation site is the hydroxyl group of Tyr_{1150} (see **Figure 4**). Figure 4 shows the separation result for the fractions 1–8 with the chemical structures of the peptides. The HPLC analysis of the peptides in each fraction was conducted by gradient elution with an aqueous solution of 0.10% (*v/v*) CF_3COOH(from 90 to 85% (*v/v*)) and a CH_3CN solution of 0.10% (*v/v*) CF_3COOH (from 10 to 15% (*v/v*)) at 215 nm. The phosphopeptide was not included in the through fraction (fraction 1) and washing fractions (fractions 2–4) and was completely recovered in eluting fractions (fractions 5–7). In this case, 20% (*v/v*) CH_3CN (instead of 0.50 M $NaNO_3$) in the washing buffer was necessary for the selective removal of the non-phosphopeptide.

Furthermore, we investigated the separation of a phosphopeptide from the tryptic digest of β-casein (i.e., a calcium-binding phosphorylated protein). The phosphopeptide consists of 16 residues (Phe-Gln-*pSer*-Glu-Glu-Gln-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys), and the phosphorylation site is the hydroxyl group of the serine residue, whose recovery was determined by HPLC. The separation protocol is the same as that for the p60c-*src* phosphopeptide. The separation result of the phosphopeptide from the digest is presented by three chromatograms in **Figure 5**. The HPLC analyses of the peptides in the tryptic digest (Figure 5.a), the through/washing fractions (fractions 1–4, Figure 5.b), and the eluting fractions (fractions 5–8, Figure 5.c) were conducted by gradient elution with an aqueous solution of 0.10% (*v/v*) CF_3COOH (from 95 to 35% (*v/v*)) and a CH_3CN solution of 0.10% (*v/v*) CF_3COOH

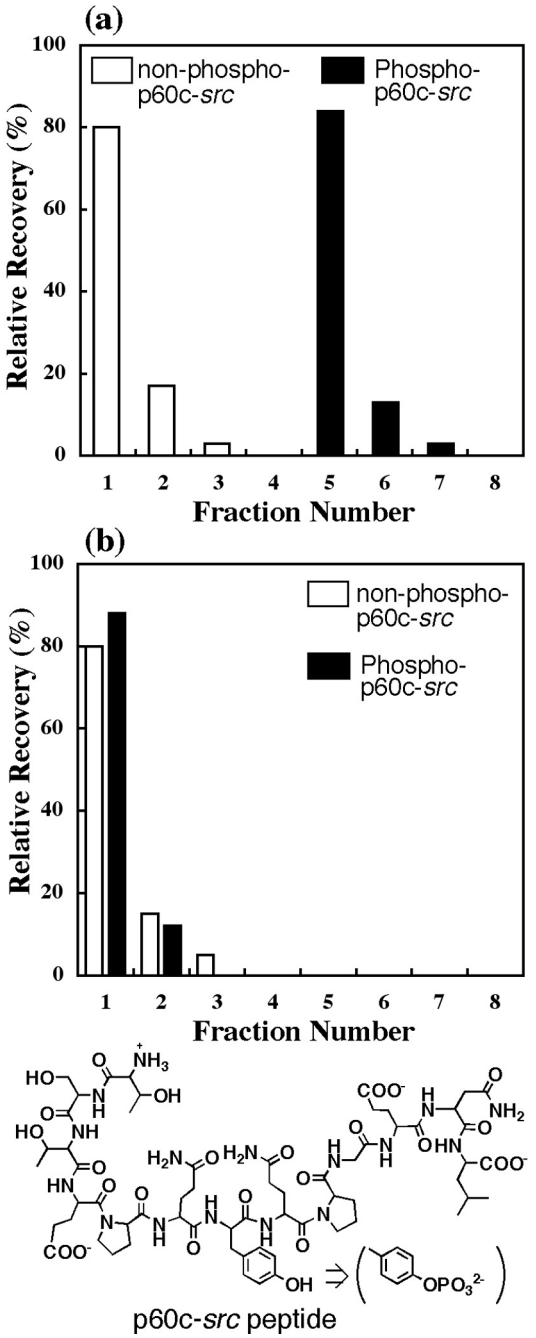

Figure 3. Relative recoveries of the p60c-*src* phosphopeptide and non-phosphopeptide by affinity spin column chromatography using *Phos-tag agarose*: (a) zinc(II)-bound *Phos-tag agarose* and (b) zinc(II)-unbound *Phos-tag agarose* (by a pretreatment with a 0.20 M EDTA-NaOH buffer (pH 8.0)). Closed and open columns show the values of phospho- and non-phosphopeptides, respectively. The chemical structure of the p60c-*src* peptide and the phosphorylation site are represented.

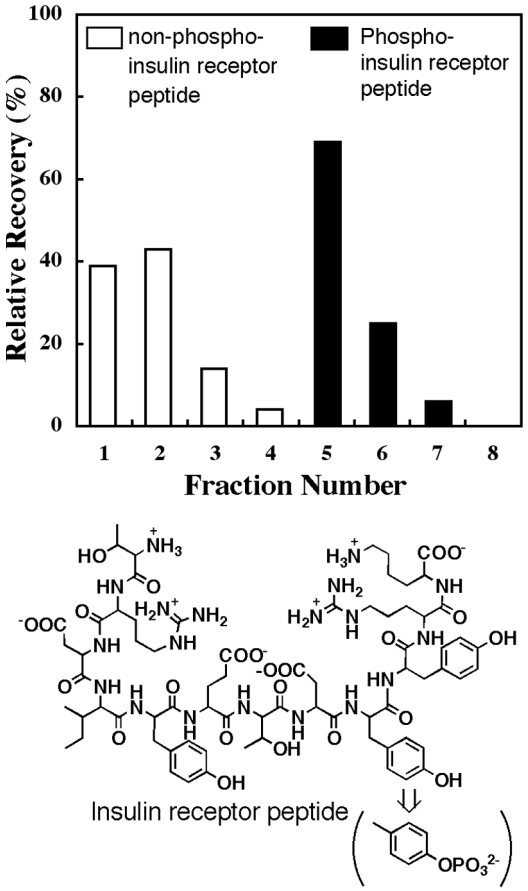

Figure 4. Relative recoveries of the insulin receptor phosphopeptide and non-phosphopeptide by affinity spin column chromatography using *Phos-tag agarose*. Closed and open columns show the values of phospho- and non-phosphopeptides, respectively. The chemical structure of the insulin receptor phosphopeptide and the phosphorylation site are represented.

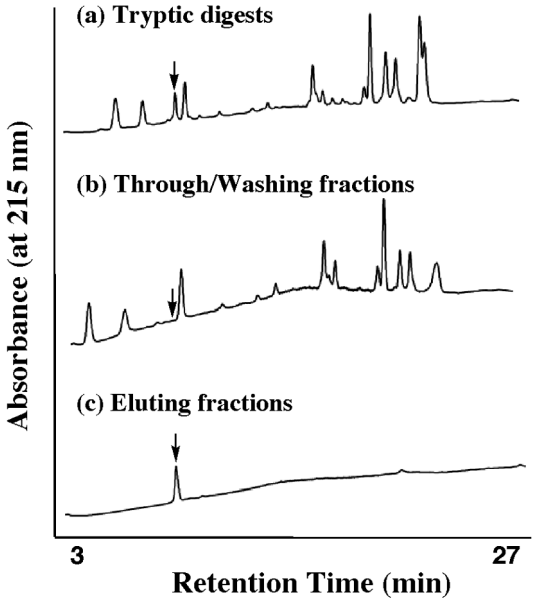

Figure 5. Reversed-phase HPLC analysis: (a) crude tryptic digest of β-casein prior to loading on *Phos-tag agarose*, (b) through and washing fractions, and (c) eluting fractions by affinity spin column chromatography using *Phos-tag agarose*. The arrows indicate the target phosphopeptide (Phe-Gln-*pSer*-Glu-Glu-Gln-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys).

(from 5 to 65% (*v/v*)) at 215 nm. According to the HPLC of the same monophosphopeptide commercially available (retention time = 8.8 min), the HPLC peak indicated by an arrow in Figure 5.a and Figure 5.c was assigned to the target phosphopeptide. During the washing with a 0.10 M Tris-CH_3COOH buffer (pH 7.4) containing 0.50 M $NaNO_3$, a phosphopeptide peak was not observed (see Figure 5.b), whereas the other peaks for the non-phosphopeptides were almost the same as those in Figure 5.a. In the eluting fractions (fractions 5–8), only one peak appeared at 8.8 min, which had the same retention time of the target phosphopeptide (see Figure 5.c). The eluting fraction was analyzed by MALDI-TOF MS analysis using a matrix solution of α-cyano-4-hydroxycinnamic acid in CH_3CN, resulting a strong signal of the monophosphopeptide ($M + H^+$) at *m/z* 2061.8. The total recovery of the phosphopeptide from the tryptic digest was >95%. The absence of another phosphopeptide (Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-*pSer*-Leu-*pSer*-*pSer*-*pSer*-Glu-Glu-Ser-Ile-Thr-Arg) in the eluting solution (see Figure 5.c) is possibly due to much stronger affinity of the four phosphate groups to *Phos-tag agarose* under the experimental conditions. We could succeed in separating and purifying the monophosphopeptide from the mixture of various peptides derived from β-casein fragmented by trypsin. Therefore, the results demonstrate that affinity spin column chromatography using *Phos-tag agarose* is a highly efficient and versatile technique to enrich phosphopeptides from proteolytic digests prior to mass analysis and other studies.

3.3 Affinity column chromatography for phosphorylated proteins

A first example of the affinity column chromatography for phosphorylated proteins was performed according to the protocol described in Experimental section. All fractions from the column were analyzed by SDS-PAGE, as shown in **Figure 6**. Ovalbumin (Figure 6.a), α_{s1}-casein (Figure 6.d), and β-casein (Figure 6.g) were not included in the washing fractions (fractions 1–6) but were instead eluted using a phosphate buffer. Ovalbumin was eluted with lower concentrations of a phosphate buffer than the other two proteins. The eluting pattern with the phosphate buffer might be dependent on the characteristics of the protein, such as the protein structure and the number of phosphorylated sites (ovalbumin, α_{s1}-casein, and β-

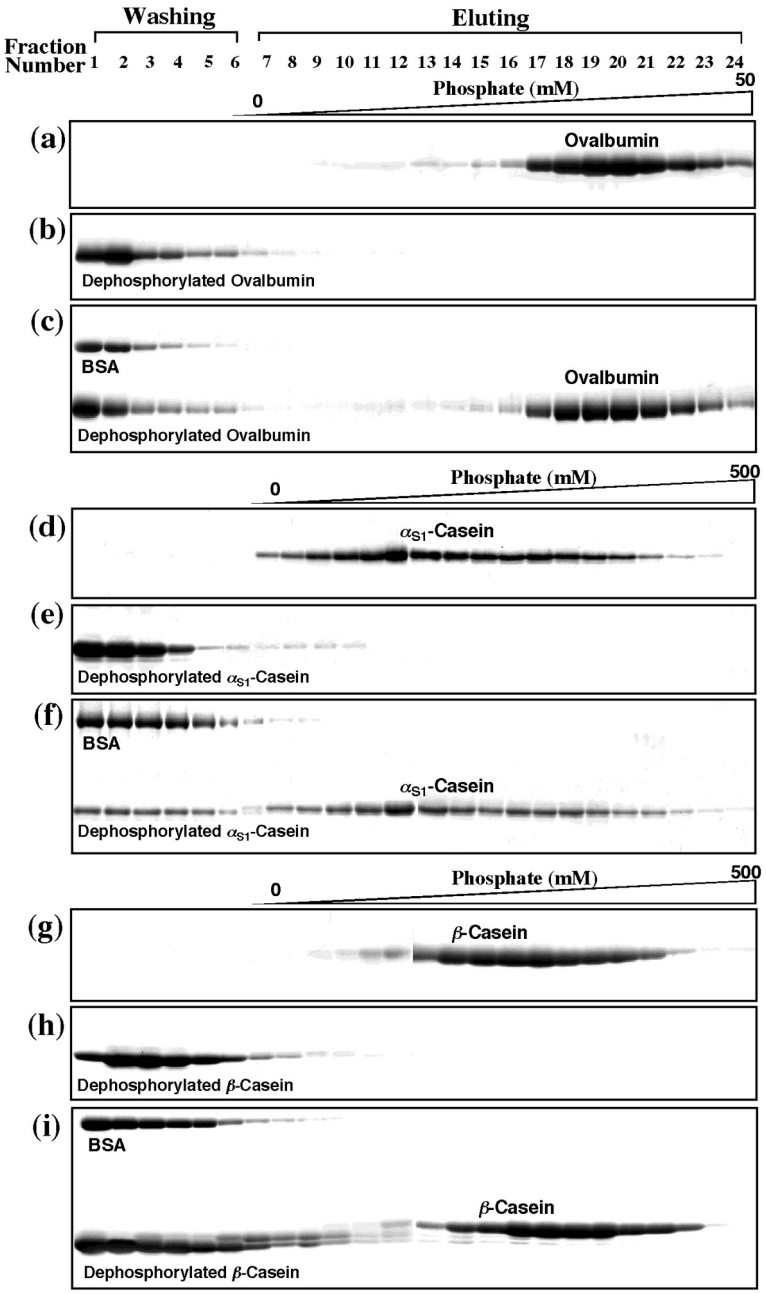

Figure 6. SDS-PAGE analyses after affinity column chromatography using *Phos-tag agarose*: (a) ovalbumin (0.3 mg), (b) dephosphorylated ovalbumin (0.3 mg), (c) BSA (30 μg) + ovalbumin (0.3 mg) + dephosphorylated ovalbumin (0.3 mg), (d) α_{s1}-casein (0.3 mg), (e) dephosphorylated α_{s1}-casein (0.3 mg), (f) BSA (30 μg) + α_{s1}-casein (0.3 mg) + dephosphorylated α_{s1}-casein (0.3 mg), (g) β-casein (0.3 mg), (h) dephosphorylated β-casein (0.3 mg), (i) BSA (30 μg) + β-casein (0.3 mg) + dephosphorylated β-casein (0.3 mg).

casein have 2, 8, and 5 phosphorylated serine residues, respectively). In contrast with the phosphorylated proteins, the corresponding dephosphorylated proteins were eluted in the washing fractions (see Figure 6.b, Figure 6.e, and Figure 6.h). From a mixture of BSA, dephosphorylated protein, and phosphorylated protein, the phosphorylated protein (i.e., ovalbumin, α_{s1}-casein, and β-casein) was separated in the eluting fractions (see Figure 6.c, Figure 6.f, and Figure 6.i). Partially dephosphorylated β-casein was eluted at lower concentrations of phosphate (see fractions 6–9 in Figure 6.i). In the absence of the zinc(II) ions of the *Phos-tag agarose* (by a pretreatment with a 0.20 M EDTA-NaOH buffer, pH 8.0), all of the phosphorylated and unphosphorylated proteins were eluted in the washing fractions (data not shown). These facts indicate that *Phos-tag agarose* selectively captures the phosphorylated proteins via the zinc(II) ions of the *Phos-tag* molecule. Other non-phosphorylated proteins, such as β-galactosidase, HSA, and carbonic anhydrase, were not trapped by *Phos-tag agarose* either and were eluted in the washing fractions just like BSA (data not shown). Total recoveries of any proteins used in this chromatography were >95%, which was confirmed by Lowry's method. When a mixture of α_{s1}-casein, β-casein, and oval-

bumin was applied on excess amounts of *Phos-tag agarose*, the phosphorylated proteins were all trapped and were eluted by an appropriate concentration of phosphate buffer. After the elution of sample proteins, the column was washed with a solution consisting of 1.0% (*v/v*) Nonident P-40 and 1.0 M NaCl and stored in 0.10 M Tris-CH_3COOH (pH 7.4). After equilibration with a 0.10 M Tris-CH_3COOH buffer (pH 7.4) containing 10.0 μM $Zn(CH_3COO)_2$ and 0.50 M $NaNO_3$, the column can be reused at least 3 times for the same affinity chromatography.

4 Concluding remarks

We have demonstrated a novel separation procedure of phosphopeptides and phosphorylated proteins by immobilized zinc(II) affinity chromatography (Zn(II)-IMAC) using a phosphate capture gel, *Phos-tag agarose*. *Phos-tag agarose* was synthesized by coupling a primary amine derivative of *Phos-tag* with NHS-activated Sepharose. Although there are other well-known types of immobilized metal ion affinity chromatography (IMAC) using other metal ions such as Fe(III) and Ga(III) in phospho-proteomics [19–22], neither functions quite satisfactorily in terms of selectivity and versatility. The Ga(III) ion has been shown to be better among them; however, to perform quality IMAC, it was necessary to adjust the acidic pH to between 2.0 and 3.5 [21]. Our Zn(II)-IMAC realizes a simple, rapid, specific, and economical procedure for separating phosphopeptides and phosphorylated proteins in aqueous solution at physiological pH throughout experiments. Since the non-denaturing procedure maintains the protein conformation and activity, the separated phosphorylated proteins are ideal for use in many downstream applications. This separation procedure is bound to lead to increased sensitivity of various kinds of phosphodetection experiments.

Acknowledgements

E.K. and T.K. thank the Ministry of Education, Science, and Culture in Japan for a Grant-in-Aid for Scientific Research (B) (No. 15390013). T.K. gratefully acknowledges helpful discussions with Mr. Yoshio Sano (MANAC Incorporated), Mr. Norio Minami (NARD Institute Ltd.), Dr. Masaaki Matsuo (NARD Institute Ltd.), and Mr. Akihiko Kawasaki (NARD Institute Ltd.).

References

[1] N.N. Nalivaeva, A.J. Turner, *Proteomics* **2001**, *1*, 735–747.
[2] T. Hunter, *Cell* **1995**, *80*, 225–236.
[3] T. Hunter, *Cell* **2000**, *100*, 113–127.
[4] T.A.J. Haystead, J.C. Garrison, in: *Protein Phosphorylation* (2nd edn.), D.G. Hardie (Ed.), Oxford University Press, New York 1999, pp. 1–31.
[5] E. Kinoshita, N. Handa, K. Hanada, G. Kajiyama, M. Sugiyama, *FEBS Lett.* **1997**, *407*, 343–346.
[6] Q.P. Weng, M. Kozlowski, C. Belham, A. Zhang, M.J. Comb, J. Avruch, *J. Biol. Chem.* **1998**, *273*, 16621–16629.
[7] Y. Kawakami, S.E. Hartman, E. Kinoshita, H. Suzuki, J. Kitaura, L. Yao, N. Inagaki, A. Franco, D. Hata, M. Maeda-Yamamoto, H. Fukamachi, H. Nagai, T. Kawakami, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 2227–2232.
[8] H. Kaufmann, J.E. Bailey, M. Fussenegger, *Proteomics* **2001**, *1*, 194–199.
[9] M.R. Larsen, G.L. Sørensen, S.J. Fey, P.M. Larsen, P. Roepstorff, *Proteomics* **2001**, *1*, 223–238.
[10] E. Kimura, S. Aoki, T. Koike, M. Shiro, *J. Am. Chem. Soc.* **1997**, *119*, 3068–3076.
[11] E. Kimura, T. Koike, *J. Chem. Soc., Chem. Commun. Feature Article* **1998**, 1495–1500.
[12] H. Fujioka, T. Koike, N. Yamada, E. Kimura, *Heterocycles* **1996**, *42*, 775–787.
[13] B.L. Vallee, in: *Zinc Enzymes*, I. Bertini, C. Luchinat, W. Maret, M. Zeppezauer (Eds.), Birkhäuser, Boston 1986, Ch. 1, pp. 1–15.
[14] T. Koike, M. Inoue, E. Kimura, M. Shiro, *J. Am. Chem. Soc.* **1996**, *118*, 3091–3099.
[15] H. Adams, D. Bradshaw, D.E. Fenton, *J. Chem. Soc. Dalton Trans.* **2002**, 925–930.
[16] E. Kinoshita, M. Takahashi, H. Takeda, M. Shiro, T. Koike, *Dalton Trans.* **2004**, 1189–1193.
[17] H. Takeda, A. Kawasaki, M. Takahashi, A. Yamada, T. Koike, *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2075–2081.
[18] Available: http://www.phos-tag.com.
[19] L. Andersson, J. Porath, *Anal. Biochem.* **1986**, *154*, 250–254.
[20] G. Muszyńska, L. Andersson, J. Porath, *Biochemistry* **1986**, *25*, 6850–6853.
[21] M.C. Posewitz, P. Tempst, *Anal. Chem.* **1999**, *71*, 2883–2892.
[22] C. Rios, V. Salvadó, M. Hidalgo, *J. Sep. Sci.* **2004**, *27*, 602–606.

