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Separation of phospho- and non-phosphopeptides using reverse phase column chromatography

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Abstract Peptides containing phosphoserine, phosphothreonine or phosphotyrosine and their parent non-phosphorylated forms were chromatographed using standard C18 reverse phase chromatography in the presence of a water/acetonitrile gradient supplemented with different counter ions. We obtained the best separation of phosphorylated from non-phosphorylated peptides in the presence of heptafluorobutyric acid, with differences in retention times as large as ~20 min. The chromatographic method was reliable in separation of the same peptides phosphorylated at different positions, acidic or basic phospho-Ser/Thr-peptides or phospho-Tyr-containing peptides. The described separation conditions are useful in studying the kinetics of phosphorylation/dephosphorylation and in analysis of phosphorylation sites in vivo.

Key words: Ser/Thr-phosphopeptide; Tyr-phosphopeptide; HPLC separation; Heptafluoroacetic acid; Rhodopsin

1. Introduction

Posttranslational phosphorylation/dephosphorylation of proteins is the key regulatory mechanism of many cellular processes. Identification of kinases and phosphatases involved in these processes and sites of phosphorylation of a protein substrate are important components in understanding the functional consequences of this reversible protein modification at the molecular level. Several methods have been developed to isolate phosphopeptides. For example, radioactively labeled phosphopeptides have been separated from non-phosphorylated peptides using conventional HPLC column chromatographies [1–2], they were enriched using Chelex-Fe^{3+} chromatography before mass spectrometric analysis [3–5] or they were separated from acidic peptides and ATP using ion exchange paper [6–7]. These methods have significant limitations, including a poor separation of phospho- from parent non-phosphopeptides, non-specific binding of acidic peptides to Chelex-Fe^{3+}, and use of radioactive ATP that is precluded in many in vivo studies. In this paper, we present a separation method of phospho- from parent non-phosphopeptide using a conventional C18 HPLC column with heptafluorobutyric acid (HFB) as a counter ion. We found that the separation is applicable to both phospho-Ser/Thr and phospho-Tyr containing peptides. The simplicity and reproducibility of the method suggest broad applications.

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Abbreviations: HFB, heptafluorobutyric acid; HPLC, high-performance liquid chromatography; PFP, pentafluoropropionic acid; TFA, trifluoroacetic acid.

2. Materials and methods

2.1. Materials

Mouse rhodopsin peptides, [330]DDDASATASKTETSQVAPA, [330]DDDA(P)SATASKTETSQVAPA and [330]DDDASA(P)TASKTETSQVAPA, were purchased from Quality Controlled Biochemicals (MA). The sequences were verified by amino acid composition and mass spectrometric analysis. Kemptide (LRRASLG), tyrosine kinase substrate (RRLIEDNEYTARG) and protein kinase A catalytic subunit were obtained from Sigma (St. Louis, MO). Casein kinase II and its substrate (RRREEEEESEEE) were purchased from Boehringer Mannheim (Indianapolis, IN). Tyrosine kinase, $p60^{c\text{-}src}$ was obtained from Oncogene Science (San Diego, CA). Counter ions HFB, PFP and H_3PO_4 and TFA were from Pierce (Rockford, IL), Sigma and Fisher Scientific (Pittsburgh, PA), respectively. All other reagents used were HPLC grade purity.

2.2. Reverse phase HPLC

The HPLC system consisted of a quaternary pump solvent delivery system, a UV absorbance variable-wavelength detector (Hewlett Packard 1050 series) and an HP 3395 integrator. The peptides were separated using a microbore C18 column (1.0 × 250 mm, Vydac 218TP51) and an acetonitrile/water gradient from 0 to 5% for 5 min, from 5 to 24% for the next 95 min. The flow rate was 0.12 ml/min and the peptides were detected at 215 nm.

2.3. Enzymatic preparations of phosphokemptide

Kemptide was phosphorylated according to Kemp et al. [8]. Briefly, kemptide (20 μg) was incubated with protein kinase A catalytic subunit (4 μg) in 25 μl of 50 mM bis-tris-propane (BTP) buffer, pH 7.5, containing 1 mM [γ-^{32}P]ATP (160 cpm/pmol), 5 mM $MgCl_2$ and 1 mM DTT for 2 h at room temperature. The phosphokemptide and kemptide were separated as described above.

2.4. Enzymatic preparation of phosphorylated casein kinase II peptide

Recombinant casein kinase II (0.05 mU) was incubated with RRREEEEESEEE (20 μg) in 50 μl of 20 mM MES buffer, pH 6.9, containing 1 mM [γ-^{32}P]ATP (160 cpm/pmol), 130 mM KCl, 10 mM $MgCl_2$, 5 mM DTT for 30 min at room temperature. The phosphopeptide was separated as described above.

2.5. Enzymatic preparation of phosphotyrosine peptide

$p60^{c\text{-}src}$ kinase (20 U) was incubated with a tyrosine kinase substrate peptide, RRLIEDNEYTARG (20 μg), in 50 μl of 50 mM BTP buffer, pH 7.5, containing, 1 mM [γ-^{32}P]ATP (160 cpm/pmol), 5 mM $MgCl_2$, 1 mM DTT for 1 h at room temperature. The phosphopeptide was separated as described above.

2.6. Preparation of phosphorylated C-terminal peptides of bovine rhodopsin

Rhodopsin phosphorylation was performed using fresh bovine rod outer segment membranes containing endogenous rhodopsin kinase as described previously [9]. The phosphorylated C-terminal peptides of

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rhodopsin were obtained from rhodopsin digested with endoproteinase Asp-N and purified using a narrow-bore C18 column [10].

3. Results and discussion

Two mono-phosphopeptides (phosphorylated at Ser-334 or Thr-336) and non-phosphorylated parent peptide encompassing the C-terminus of mouse rhodopsin ([330]DDDASATASKTETSQVAPA) were subjected to chromatographic separation, individually and mixed, using a C18 micro-bore column in the presence of one of four counter ions (Fig. 1). In the presence of H_3PO_4, the non-phosphorylated peptide co-eluted with the peptide mono-phosphorylated at residue [334]Ser, while the mono-phospho-[336]Thr peptide was eluted earlier (Fig. 1). Comparable results were obtained when H_3PO_4 was replaced by TFA; however, a complete separation of these three peptides was obtained when the length of the counter ion was extended to pentafluoropropionic acid (PFP) or heptafluorobutyric acid (HFB) (Fig. 1). In the presence of HFB, the elution time was 39 min for the phospho-[336]Thr peptide, 43 min for the phospho-

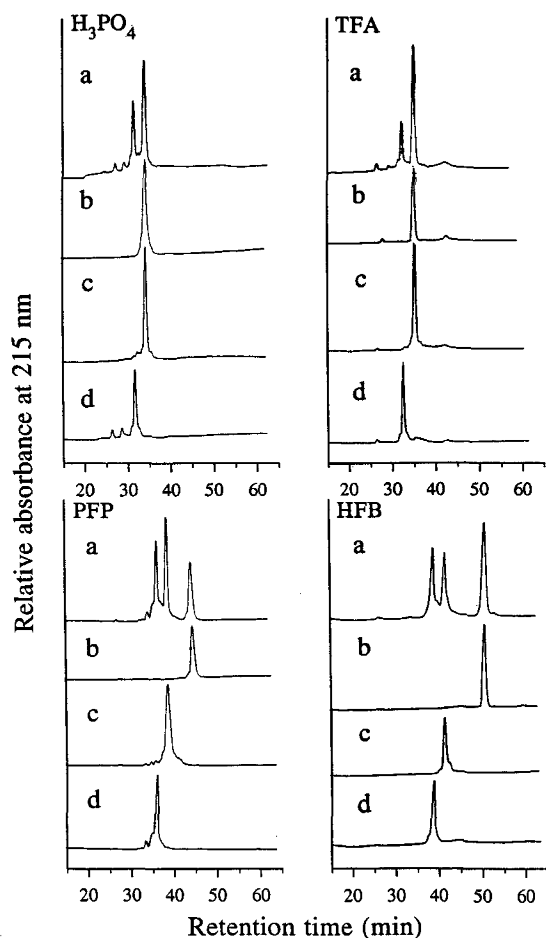

Fig. 1. Elution profiles of phospho- and non-phosphopeptides from a reverse phase HPLC column (1.0 × 250 mm, Vydac 218TP51) using different counter ions. (a) Mixture of mono-phosphorylated peptides and non-phosphorylated peptide (4–10 μg each): [330]DDDASATASKTETSQVAPA, [330]DDDA(P)SATASKTETSQVAPA and [330]DDDASA(P)TASKTETSQVAPA; (b) [330]DDDASATASKTETSQVAPA; (c) [330]DDDA(P)SATASKTETSQVAPA; (d) [330]DDDASA(P)TASKTETSQVAPA. The peptides were eluted with an acetonitrile/water gradient (0–24%) during 100 min at a flow rate of 0.12 ml/min in the presence of either H_3PO_4 (0.1%, pH 2.0), TFA (0.03%, pH 1.9), PFP (0.03%, pH 2.1) or HFB (0.03–0.05%, pH 2.4) and detected at 215 nm.

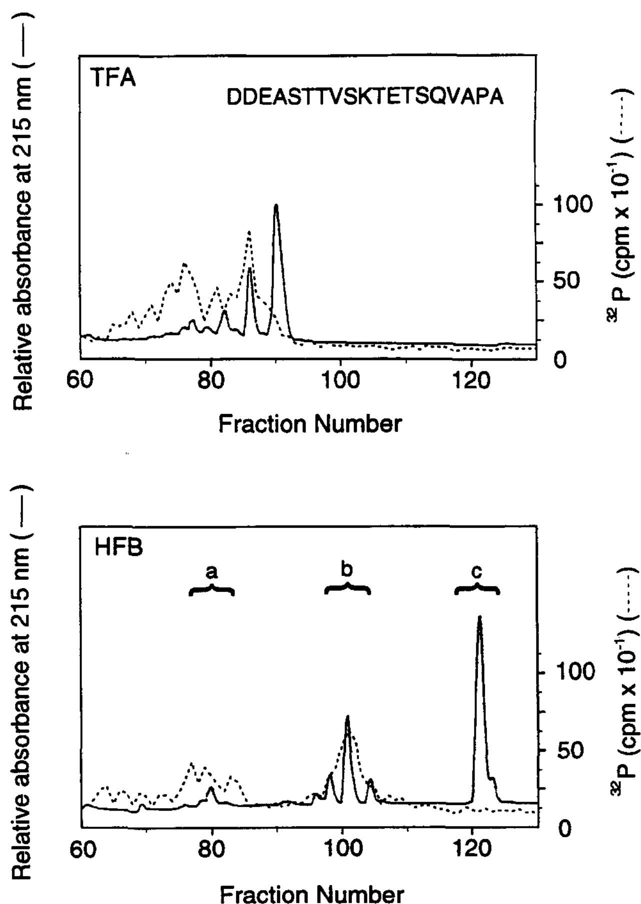

Fig. 2. Elution profiles of phosphorylated and non-phosphorylated C-terminal peptides of bovine rhodopsin in the presence of TFA or HFB. Bovine rod outer segments were incubated with [γ-^{32}P]ATP (50 cpm/pmol) under intense illumination according to Ohguro et al. [9]. The mixture of phosphorylated and non-phosphorylated peptides derived from the C-terminus of rhodopsin, [330]DDEASTTVSKTETSQVAPA, was obtained by the digestion of rhodopsin with endoproteinase Asp-N [10]. The soluble C-terminal peptides were purified on a reverse phase C18 HPLC column chromatography in the presence of 0.05% TFA (upper panel) or 0.04% HFB (lower panel) as described in Section 2. Peptides were monitored by UV absorption at 215 nm and the radioactivity was measured by Cherenkov's counting in each fraction (0.06 ml). Note that in the presence of HFB, multi- (a), mono- (b) and non-phosphorylated peptides (c) were completely separated.

Table 1
Effect of pH on the separation of phospho- and non-phosphopeptides derived from the C-terminus of mouse rhodopsin[a] using a C18 column in the presence of HFB

pH	Retention time (min)		
	Phospho-[334]Ser peptide	Phospho-[336]Thr peptide	Non-phospho-peptide
1.5	39	37	43
2.4	43	39	51
3.5	38	35	47

The conditions of separation were as described in Section 2.
[a] The peptide was [330]DDDASATASKTETSQVAPA.

Table 2
Separation of acidic, basic and Tyr-containing peptides and their phosphorylated derivatives using a C18 column in the presence of HFB or TFA

Peptides	Retention time (min)			
	HFB		TFA	
Phosphopeptide	Phosphopeptide	Non-phosphopeptide	Phosphopeptide	Non-phosphopeptide
Kemptide[b]	55	72	28	30
Casein kinase II peptide[c]	44	65	22	22
Tyr-containing peptide[d]	80	88	38.5	43.5

The conditions of separation were as described in Section 2.
[a] The peptide was LRRAS*LG. S* is phosphorylated by catalytic subunit of protein kinase A.
[b] The peptide was RREEEEES*EEE. S* is phosphorylated by casein kinase II.
[c] The peptide was RRLIEDNEY*TARG. Y* is phosphorylated by $p60^{c\text{-}src}$.

^{334}Ser peptide and 51 min for the non-phosphorylated peptide. PFP was slightly less effective than HFB.

We also studied the separation of these three peptides at different pHs in the presence of HFB (Table 1), and we obtained good resolution at either pH 2.4 or 3.5 and poor separation at pH 1.5. These results suggest that the hydrophobicity and charge of the counter ion affected the separation of the model peptides.

We also tested phosphopeptides that were basic, acidic or contained a phospho-Tyr residue. Phosphopeptides were separated from their non-phosphorylated parent peptides using a C18 column in the presence of HFB as described above. Basic kemptide (LRRASLG) was eluted at 72 min, while its phosphorylated form was eluted at 55 min (Table 2). An acidic substrate of casein kinase II (RRREEEEESEEE) was eluted at 44 min after phosphorylation, whereas the corresponding non-phosphorylated peptide was eluted at 65 min. However, TFA did not allow separation of these peptides (Table 2). The data suggest that this method could be applicable to many peptides that contain phosphorylated Ser or Thr residues. Furthermore, peptides phosphorylated at different positions can be separated using this method. The separation of phospho- from non-phosphorylated peptides was also applicable to a peptide containing phosphorylated Tyr residue, RRLIEDNE(P)YTARG (Table 2).

In vitro, rhodopsin kinase phosphorylates photolyzed rhodopsin heterogeneously at the C-terminal region [9], with a stoichiometry as high as 7–9 phosphates per rhodopsin [11–12]. When the mixture of differently phosphorylated peptides was applied to a C18 column in the presence of TFA, poor resolution of phosphopeptides from the dominant non-phosphorylated peptide was apparent (Fig. 2). In contrast, in the presence of HFB, the mixture of peptides was separated as individual mono-phosphorylated peptides. As the ratio of phosphate groups per peptide increases, the resolution decreased.

The described separation of phospho- from parent non-phosphorylated peptides may have broad applications in studies related to protein phosphorylation. Overall, the initial separation of peptides can be achieved using TFA and a step gradient of acetonitrile in water [13]. Then the peptides can be separated into individual components (for example the same peptide phosphorylate at different positions) using a shallow gradient of organic solvent in the presence of HFB as exemplified in the report. This technique of phosphopeptide separation and mass spectrometric analysis allowed us to identify phosphorylation sites on rhodopsin in vivo without a ^{32}P-radioactive tracer [13].

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References

[1] Perich, J.W., Nguyen, D.L. and Reynolds, E.C. (1991) Tetrahedron Lett. 32, 4033–4034.
[2] Turner, R.S., Kemp, B.E., Su, H. and Kuo, J.F. (1985) J. Biol. Chem. 260, 11503–11507.
[3] Andersson, L. and Porath, J. (1996) Anal. Biochem. 154, 250–254.
[4] Arendt, A., Palczewski, K., Moore, W.T., Caprioli, R.M., McDowell, J.H. and Hargrave, P.A. (1989) Int. J. Peptide Protein Res 33, 468–476.
[5] Michel, H., Hunt, D.F., Shabanowitz, J. and Bennet, J. (1988) J. Biol. Chem. 263, 1123–1130.
[6] Witt, J.J. and Roskoski, Jr., R. (1975) Anal. Biochem. 66, 253–258.
[7] Glass, D.B., Masaracchia, R.A., Feramisco, J.R. and Kemp, B.E. (1978) Anal. Biochem. 87, 566–575.
[8] Kemp, B.E., Graves, D.J., Benjamin, E. and Krebs, E.G. (1977) J. Biol. Chem. 252, 4888–4894.
[9] Ohguro, H., Johnson, R.S., Ericsson, L.H., Walsh, K.A., and Palczewski, K. (1994) Biochemistry 33, 1023–1028.
[10] Palczewski, K., Buczyłko, J., Kaplan, M.W., Polans, A.S. and Crabb, J.W. (1991) J. Biol. Chem. 266, 12949–12955.
[11] Aton, B.R., Litman, J.J. and Jackson, M.L. (1984) Biochemistry 23, 1737–1741.
[12] Wilden, U. and Kühn, H. (1992) Biochemistry 21, 3014–3022.
[13] Ohguro, H., Van Hoosier, J.P., Milam, A.H. and Palczewski, K. (1995) J. Biol. Chem. (in press).