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Improved Phosphotyrosine Analysis by TLC and HPLC

Young-Me Song[1], Gyurng-Soo Yoo[1], Seung-Ki Lee[2] and Jung-Kap Choi[1]

[1]*College of Pharmacy, Chonnam National University, Kwangju 500-757 and* [2]*College of Pharmacy, Seoul National University, Seoul 151-742, Korea*

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We describe here the conditions of thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) to improve the analytical method of phosphotyrosine (P-Tyr) in biological sample. TLC was performed on silica plate with the mixture of propanol and water (2.1 : 1 v/v) as a mobile phase and R_f values were 0.42, 0.39 and 0.33 for phosphotyrosine, phosphothreonine and phosphoserine, respectively. HPLC was performed on NH_2 column with a mobile phase of potassium biphosphate solution by UV detection at 192 nm. The optimum condition of HPLC was obtained at 0.01 M, pH 4.5 with a clear separation within 12 min. These procedures have been applied to the analysis of phosphotyrosine obtained from tyrosine-phosphorylated enolase. Both TLC and HPLC methods were suitable to analyze tyrosine-phosphorylated protein without being affected by contaminants from hydrolysates.

Key words: Phosphotyrosine, Phosphoamino acids, Phosphorylated protein, TLC, HPLC

INTRODUCTION

The reversible phosphorylation of protein is the major device in signal transduction pathways which regulate several biological procedures including cell growth and differentiation. Until now, a large number of protein kinases and phosphatases of different specificities have been identified (Blithe *et al.*, 1982; Tonks *et al.*, 1988; Imes *et al.*, 1987). Although phosphorylation of protein occurs on tyrosine, threonine and serine residues, the relatively recent discovery has added further interest to the phosphorylation of tyrosine residues (Hunter and Sefton, 1980; Swarup *et al.*, 1983; Ushiro and Cohen, 1980; Sasaketal, 1985). In the course of investigation of phosphorylation/dephosphorylation reactions in protein, it is necessary to develop a suitable and convenient method for identification of phosphotyrosine residue from protein hydrolysate.

Up to date several methods for the characterization of phosphoamino acid have been presented: 1) one-dimensional or two-dimensional TLC (Munoz and Marshall, 1990; Jacobs *et al.*, 1983; Rothberg *et al.*, 1978); 2) high voltage electrophoresis alone, and in combination with TLC (Kasuga *et al.*, 1982; Richert *et al.*, 1982; Mitchell and Lunan, 1964; Kasuga *et al.*, 1983); 3) HPLC (Swarup *et al.*, 1981; Robert *et al.*, 1985) and; 4) amino acid analyzer (Martensen, 1982). One-dimensional TLC is generally insufficient for clear-out spearation. For the complete resolution of three kinds of phosphoamino acids, three sucessive cycles of TLC are necessary (Munoz and Marshall, 1990). Swarup *et al.* (1983) determined *O*-phosphoamino acids using HPLC which required 60 min for complete analysis. In 1985, Robert *et al.* were able to reduce the time required for analysis down to 20 min with a good separation. However, in all these methods, *O*-phosphoamino acids were analyzed by the use of anion exchange column. Therefore, there is no alternative for the selection of column in analyzing phosphoamino acids.

In this study we developed a TLC method capable of completely separating three phosphoamino acids with one step migration. The method is based on ascending chromatography on silica gel thin layer plates. HPLC method has been also studied using NH_2 column with a mobile phase of potassium biphosphate solution. This method has been much improved in reducing the experimental time with a good resolution. The optimum conditions and reliability of the methods are to be discussed.

MATERIALS AND METHODS

Materials

Phosphorylated amino acids, *o*-phospho-*L*-serine, *o*-phospho-*L*-threonine, *o*-phospho-*L*-tyrosine, ninhydrin, enolase and other reagents for phosphorylation reac-

Correspondene to: Jung-Kag Choi, College of Pharmacy, Chonnam National University, 300, Yongbong-dong, Buk-ku, Kwangju 500-757, Korea

tion were purchased from Sigma Chemical Co.. Calf serum was obtained from GIBCO. Methanol (MeOH), propanol (PrOH), butanol (BuOH) and glacial acetic acid (HAc) as solvents and all other chemicals were of analytical reagent grade. Distilled water was used throughout the experiment.

Apparatus

For HPLC system, pump (Waters 501, USA), injector (Waters U6K, USA), detector (Waters 484 tunable absorbance detector, USA), integrator (Waters 5052A, USA) and column (Waters, μ-Bondapak NH_2, 10 μm, 3.9×300 mm) were used. Precoated silica plates (Merck, 0.2 mm, USA) were used for TLC. Centrifugal evaporator (EYELA, CVE-100, Japan) and centrifuge (VS-15000 Vision Sci. Co., Korea) were used.

Preparation of phosphoamino acid standards for TLC

Standard solutions of phosphoamino acids were prepared in pH 1.9 buffer (HAc : formic acid : water/78 : 24 : 897 v/v) to give 0.67 μg/μl and stored at −20℃.

TLC of phospoamino acid standards

Solution of phosphoamino acid standards were spotted about 1 μl on silica TLC plates and developed with different ratio of alcohol, acetonitrile, pyridine and water. Phosphoamino acids were visualized by spraying ninhydrin in acetone, and heating.

Preparation of tyrosine-phosphorylateld enolase

To get the protein of $pp60^{c\text{-}src}$ as a kinase to phosphorylate tyrosine residue of enolase, NIH (pMc-src/focus) B cells were grown at 37℃, 10% CO_2 in Dulbeco-Vogt modified Eagle's medium (DMEM) plus 10% calf serum. Cells were lysed in 0.5 ml of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 20 mM NaH_2PO_4) supplemented with 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 50 mM NaF, 0.2 mM Na_3VO_4 and 100 KIU of aprotinin per ml. Lysates were clarified at 15,000 rpm for 30 min. The amount of total cell protein was assayed by Braford method. $pp60^{c\text{-}src}$ was immunoprecipitated with monochlonal antibody 327 for 45 min at 0℃. Immune complexes were collected on *Staphylococcus aureus* suspension that had been precoated with anti-mouse immunoglobulin G by incubation for 20 min at 0℃. Immunoprecipitates were washed with RIPA, high salt buffer [1 M NaCl, 0.5% Triton X-100, 10 mM Tris hydrochloride (pH 7.2)] and 2x phosphorylation buffer [10 mM $MnCl_2$, 40 mM Hepes (pH 7.0)]. The phosphorylation reaction was initiated by the addition of acid denatured rabbit muscle enolase to immunoprecipitated $pp60^{c\text{-}src}$ with [γ-^{32}P] ATP (1 μM, 400 Ci/mmol). After incubation at room temperature for 20 min, the reaction was terminated by the addition of sample buffer and then centrifuged at 15,000 rpm for 2 min. Tyrosine-phosphorylated enolase was analyzed by 10% SDS-PAGE and indentified by autoradiography.

Partial acid hydrolysis and TLC of tyrosine-phosphorylated enolase hydrolysates

The ^{32}P-labeled enolase to be analyzed was cut out from a polyacylamide gel. The gel pieces were reswollen in 0.05 M NH_4HCO_3, 1% SDS, 1% 2-mercaptoethanol, and pulverized and the suspension was heated at 100℃ for 5 min, then incubated overnight at 35℃. The extracts were centrifuged at 10,000 rpm for 15 min and supernatants were lyophilized in centrifugal evaporator. The ^{32}P-labeled enolase was hydrolyzed in 6 N HCl at 110℃ for 1 hr in a heating block. The hydrolyzed material was lyophilized, redissolved in 1.5 ml of water and lyophilized repeatedly to remove HCl. And then, hydrolysates were subjected to TLC in the presence of phsphoserine (P-Ser), phsphothreonine (P-Thr) and phosphotyronsine (P-Tyr) standards. The standards were detected by staining with ninhydrin and ^{32}P-labeled phosphotyrosine was identified by autoradiography.

Preparation of phosphoamino acid standards for HPLC

The concentrations of phosphoamino acid standards were 2.5 mM, 2.5 mM, and 0.12 mM for P-Thr, P-Ser and P-Tyr, respectively. Phosphoamino acids were separated on a μ-Bondapak NH_2 column (3.9×300 mm) and the column was eluted with 0.01 M KH_2PO_4, pH 4.5 at 2 ml/min of flow rate. It was detected by UV at 192 nm.

HPLC of tyrosine-phosphorylated enolase hydrolysates

Samples were obtained from partial acid hydrolysates of tyrosine-phosphroylated enolase and 1 μl of sample was injected without further purification step before HPLC. The peak identification of P-Tyr was confirmed by comparing retention time with P-Tyr standard. Potassium biphosphate aqueous solution (0.01 M, pH 4.5) was selected as a mobile phase and other HPLC conditions were identical with those of standard.

RESULTS AND DISCUSSION

TLC of phosphoamino acid standards

In the selection of the mobile phase, the nature of analytes and sorbent layer being used was consi-

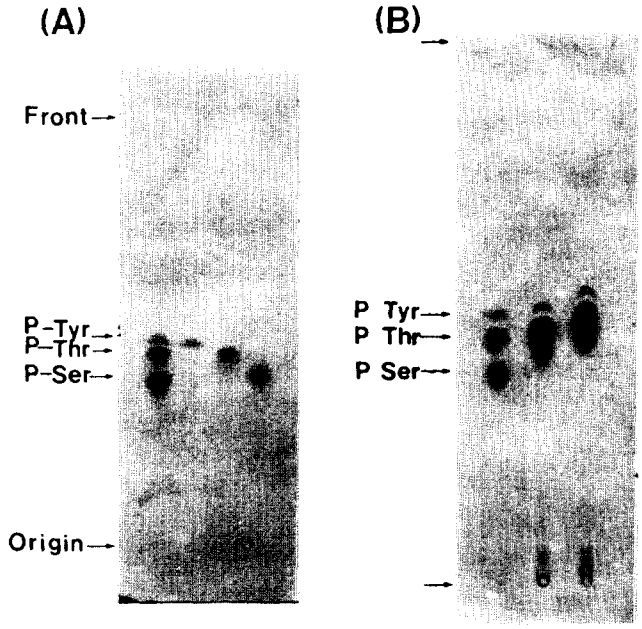

Fig. 1. TLC chromatogram of phosphoamino acids on silica plate. Ninhydrin staining was used to visualize phosphoamino acids. (A) with phosphoamino acid standards: lane 1, phosphoamino acid standards; lane 2, P-Tyr; lane 3, P-Thr; lane 4, P-Ser. (B) with tyrosine-phosphorylated enolase hydrolysates: lane 1, phosphoamino acid standards; lane 2, 3, phosphorylated enolase hydrolysates.

dered. As solvents, MeOH, PrOH, n-BuOH, water, pyridine, acetic acid and ammonium hydroxide were selected. The mixtures of two or three kinds of solvent with different ratios were tested as mobile phases.

The result of the separation of phosphoamino acids was shown in Fig. 1(A). As a mobile phase, the mixture of PrOH and water (2.1 : 1 v/v) was selected. Separation was achieved within 150 min. R_f values were 0.42, 0.39 and 0.33 for P-Tyr, P-Thr and P-Ser, respectively.

TLC of P-Tyr from phosphorylated enolase hydrolysates

The ^{32}P-labeled enolase was prepared as described under MATERIALS AND METHODS section. Under the identical condition employed in the separation of phosphoamino acid standards, the chromatography of hydrolysates was performed on silica gel.

Fig. 1(B) shows the chromatogram of tyrosinephosphorylated enolase hydrolysates. The mobility of three phosphoamino acids was not significantly altered by comigrating materials appeared between P-Thr and P-Ser. For the separation of amino acid from biological sources by TLC methods, it is sometimes necessary to remove interfering compounds prior to chromatography in order to prevent tailing and deformation of spots. However our procedure was performed without purification step.

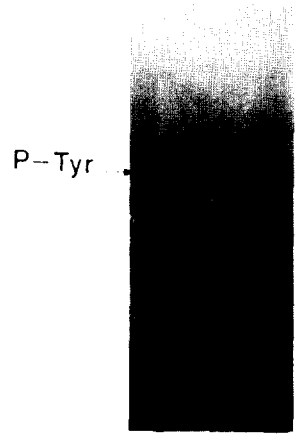

Fig. 2. Autoradiogram of ^{32}P-labeled enolase hydrolysates on silica gel plate. Phosphoamino acid standards were visualized by ninhydrin staining. Film was exposed for 7 days. lane 1, 2 were identical spots with those in lane 2, 3 in Fig. 1(B).

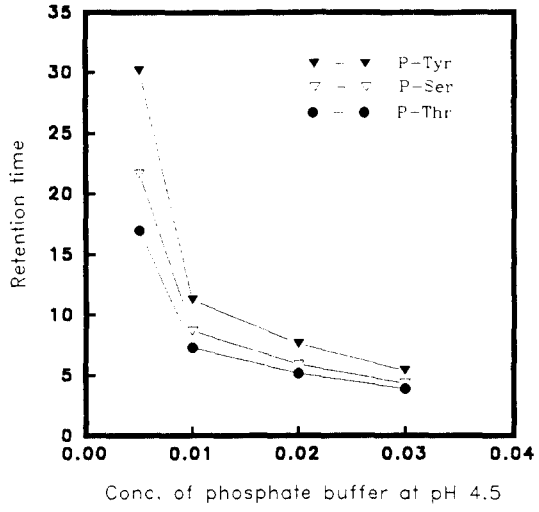

Fig. 3. Relationship of KH_2PO_4 concentration and retention time. Three phosphoamino acids were separated at different concentrations of KH_2PO_4 in mobile phase. The range of concentrations tested was 0.005 to 0.03 M KH_2PO_4 at pH 4.5

Fig. 2 shows the autoradiogram of ^{32}P-labeled enolase hydrolysates. The labeled compound was recognized as P-Tyr by the same shape and location with standard stained with ninhydrin.

HPLC of phosphoamino acid standards

μ-Bondapak NH_2 column was introduced to separate three charged phosphoamino acids based on the fact that NH_2 column acts as a weak basic ion exchanger with a suitable mobile phase.

At first we studied on the effect of salt content in mobile phase. The plot of the retention time of *o*-pho-

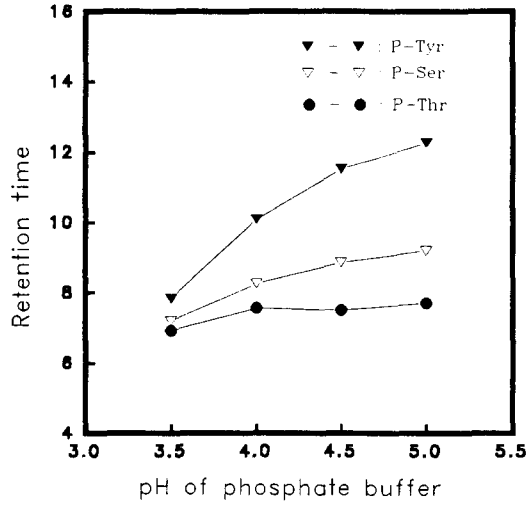

Fig. 4. Relationship of pH and retention time. Three phosphoamino acids were separated at different pH's in mobile phase. The pH range tested was 3.5 to 5.0 in 0.01 M KH_2PO_4.

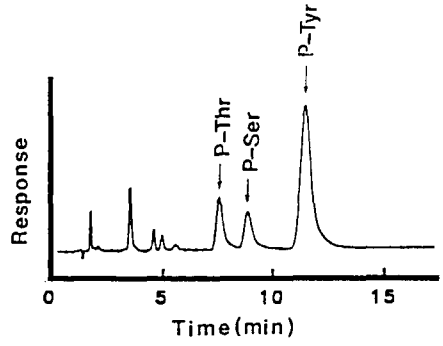

Fig. 5. HPLC chromatogram of phosphoamino acid standards. Concentrations of three phosphoamino acids were 2.5 mM, 2.5 mM and 0.12 mM for P-Thr, P-Ser and P-Tyr, respectively. Injection volume was 1 μl.

sphoamino acids versus potassium biphosphate concentrations at pH 4.5 shows that separations of P-Ser, P-Thr and P-Tyr were reasonably achieved in the whole range examined excepting at 0.03 M (Fig. 3). As the molarity of potassium biphosphate increased, the retention of o-phosphoamino acids became decreased.

The effect of the pH of potassium phosphate aqueous solution was studied at 0.01 M KH_2PO_4 (Fig. 4). It was observed that o-phosphoamino acids were separated reasonably in the range examined. The optimum condition of NH_2 column chromatography was obtained at pH 4.5, 0.01 M potassium phosphate aqueous solution as a mobile phase (Fig. 5). Each peak was confirmed by comparing the retention time of each phosphoamino acid standard. Retention times were 7.51, 8.85 and 11.53 min for P-Thr, P-Ser and P-Tyr, respectively.

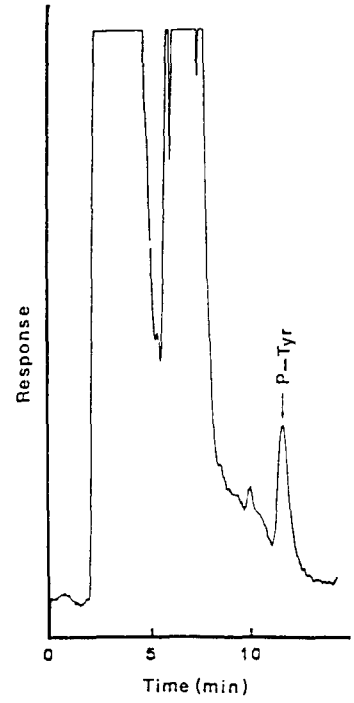

Fig. 6. HPLC chromatogram of tyrosine-phosphorylated enolase hydrolysate. Tyrosine-phosphorylated enolase was obtained by incubation with c-src protein tyrosine kinase and [γ-^{32}P] ATP. Purified tyrosine-phosphorylated enolase was hydrolyzed in 6 N HCl at 110°C for 1 hr and HCl was removed by lyophilization.

HPLC of P-Tyr from phosphorylated enolase hydrolysates

Fig. 6 shows the chromatogram of tyrosine-phosphorylated enolase hydrolysates. Enolase hydrolysates was injected without further treatment before chromatography and P-Tyr peak was obtained with a good resolution. This method has an advantage over previous HPLC methods that the separation of P-Tyr is not affected by the presence of other concomitants.

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REFERENCES CITED

Blithe, D. L., Richard, N. D., and Pastan, I. H., Purification of a tyrosine-specific protein kinase from Rous sarcoma virus-induced rat tumor. *J. Biol. Chem.*, 257, 7135-7142 (1982).

Hunter, T., and Sefton, B. M., Transforming gene pro-

duct of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. USA,* 77, 1311-1315 (1980).

Imes, S. S., Kathan, N. O., and Knowles, A. F., Plasma membrane-associated phosphatase activities hydrolyzing [^{32}P] phosphatidyl histones and [^{32}P] phosphatidylinositol phosphate. *Anal. Biochem.,* 161, 316-322 (1987).

Jacobs, S., Kull. Jr., F. C., Eaarp, H. S., Svoboda, M. E., Van Wyk, J. J., and Custrecasas, P., Somatomedin-C stimulates the phosphorylation of the β-subunit of its own receptor. *J. Biol. Chem.,* 258, 9581-9584 (1983).

Kasuga, M., Yamaguchi, Y. F., Blithe, D. L., White, M. F., and Kahn, C. R., Characterization of the insulin receptor kinase purified from human placenta membranes. *J. Biol. Chem.,* 258, 10973-10980 (1983).

Kasuga, M., Zick, Y., Blithe, D. L., Karlesson, F. A., Haring, H. U., and Kahn, C. R., Insulin stimulation of phosphorylation of the β subunit of the insulin receptor: Formation of both phosphoserine and phosphotyrosine. *J. Biol. Chem.,* 257, 9891-9894 (1982).

Martensen, T. M., Phosphotyrosine in proteins: Stability and quantification. *J. Biol. Chem.,* 257, 9648-9652 (1982).

Mitchell, H. K., and Lunan, K. D., Tyrosine-o-phosphate in Drosophila. *Arch. Biochem. Biophys.,* 106, 219-222 (1964).

Munoz, G., and Marshall, S. H., An alternative method for a fast separation of phosphotyrosine. *Anal. Biochem.,* 190, 233-237 (1990).

Richert, N. D., Blithe, D. L., and Pastan, I., Properties of the src kinase purified from Rous sarcoma virus-induced rat tumors. *J. Biol. Chem.,* 257, 7143-7150 (1982).

Robert, J. C., Soumarmon, A., Lewin, M. J. M., Determination of o-phosphothreonine, o-phosphoserine, o-phosphotyrosine and phosphate by high performance liquid chromatography. *J. Chromatogr.,* 338, 315-324 (1985).

Rothberg, P. G., Harris, T. J. R., Nomoto, A., and Wimmer, E., O^4-(5'-uridyl)tyrosine is the bond between the genome-linked protein and the RNA of poliovirus. *Proc. Natl. Acad. Sci. USA,* 75, 4868-4872 (1978).

Sasak, N., Ress-Jones, R. W., Zick, Y., Nissley, S. P., and Rechler, M. M., Characterization of insulin-like growth factor I-stimulated tyrosine kinase activity associated with the β-subunit of type I insulin-like growth factor receptors of rat liver cells. *J. Biol. Chem.,* 260, 9793-9804 (1985).

Swarup, G., Cohen, S., and Garbers, D. L., Selective dephosphorylation of protein containing phosphotyrosine by alkaline phosphatases. *J. Biol. Chem.,* 256, 8197-8201 (1981).

Swarup, G., Dasgupta, J. D., and Garbers, D., Tyrosine protein kinase activity of rat spleen and other tissuses. *J. Biol. Chem.,* 258, 10341-10347 (1983).

Tonks, N. K., Diltz, C. D., and Fisher, E. H., Purification of the major protein-tyrosine-phosphatases of human placenta. *J. Biol. Chem.,* 263, 6722-6730 (1988).

Ushiro, H., and Cohen, S., Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. *J. Biol. Chem.,* 255, 8363-8365 (1980).