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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ACID-STABLE AND ACID-LABILE PHOSPHOAMINO ACIDS

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SUMMARY

A high-performance liquid chromatographic system has been developed which permits the separation of both acid-stable and acid-labile phosphoamino acids. An anion-exchange resin and two buffers of different ionic strength and near neutral pH are used. A low-ionic-strength buffer is used for the separation of N-ω-phospho-arginine and N-ε-phospholysine, while the higher-ionic-strength buffer permits the clear separation of τ-phosphohistidine, o-phosphoserine and o-phosphothreonine. An in-stream fluorometric detection system using o-phthalaldehyde permits the rapid analysis of samples containing as little as 25 pmoles of phosphoamino acid. This method has been applied to the detection of τ-phosphohistidine from alkaline digests of chemically phosphorylated calf thymus histone 4 and bovine myelin basic protein.

INTRODUCTION

Phosphorylation of proteins on specific amino acid residues has been found to be an important modification that often results in altered catalytic activity or other biological property[1], and some phosphorylated proteins are formed as catalytic intermediates during enzymatic reactions[2,3]. Demonstration of the existence of phosphoproteins has relied on isolation of the phosphorylated form, hydrolysis of the protein by chemical or enzymatic means, and identification of the specific phosphoamino acid. Methods of identification most often used have included ion-exchange chromatography[4], paper and thin-layer chromatography[5], and high-voltage electrophoresis[6].

Many phosphorylated proteins have been found to contain o-phosphoserine and/or o-phosphothreonine which are relatively acid-stable and usually base-labile phosphomonoesters. In addition to these, phosphorylation has been shown to occur on the basic amino acids, histidine[2], and lysine[4,7], and on the sulfur of cysteine[8]. Unlike o-phosphoserine and o-phosphothreonine the phosphorylated basic amino acids are extremely acid-labile and base-stable[9]. During the isolation and characterization of proteins containing phosphoramidate (P–N) bonds, careful effort must be made to maintain neutral or slightly basic conditions in order to avoid hydrolysis.

Conventional techniques for amino acid analysis using both low[10] and high-pressure systems[11] use acidic buffers which would be unsuitable for the detection of P–N-containing phosphoamino acids. A new high-performance liquid chromatography (HPLC) method was developed which permits the separation of both types of phosphoamino acids under conditions which maintain their stability. A polystyrene-type anion-exchange resin is used along with two buffers of near neutral pH and different ionic strength.

METHODS

Chemicals

o-Phthalaldehyde (OPT) was purchased from Durrum (Palo Alto, CA, U.S.A.) or from Sigma (St. Louis, MO, U.S.A.). A protein hydrolysate standard was obtained from Pierce (Rockford, IL, U.S.A.). Phosphoserine and phosphothreonine were obtained from Sigma. N-ω-Phosphoarginine (Sigma) was found to be about 80% pure by HPLC analysis. It was purified by Dowex 1 chromatography using a 0 to 0.25 M KHCO$_3$ linear gradient. Purification yielded a single product with an identical elution volume to the enzymatic product formed by arginine kinase[12] with ATP and arginine. τ-Phosphohistidine* was prepared from phosphoramidate and histidine by described methods[6] and purified by Dowex 1 chromatography. Phospholysine was prepared by the method of Zetterqvist[4], and yielded both N-α-phospholysine and N-ε-phospholysine and a number of other products. Dowex 1 chromatography did not adequately separate the two isomers. An improved synthetic method was later developed which gave N-ε-phospholysine as the sole product and will be published elsewhere[13].

Buffers and OPT reagent

The low-ionic-strength buffer, A (15 mM KH$_2$PO$_4$), was prepared by dissolving reagent grade potassium dihydrogen phosphate in deionized water and titrating to pH 7.5 with concentrated potassium hydroxide. The high-ionic-strength buffer, B (250 mM KH$_2$PO$_4$), was prepared in a similar fashion but was brought to a final pH of 6.3. Phenol was added to a final concentration of 1 g/l. Solutions were routinely filtered through 0.2-μm filters (Millipore) before use. The OPT solution was prepared as described[11], but the final concentration of OPT was reduced to 500 mg/l.

HPLC

The arrangement of HPLC equipment is similar to that described by Voelter and Zech[14]. The column effluent enters a tee connector and mixes with a stream of OPT reagent driven by a separate pump. The mixture then travels through a piece of PTFE tubing which serves as a reaction coil before entering the flow cell of a fluorometer. An Altex Model 100 solvent metering pump was used in combination with a 250 mm × 4.6 mm I.D. column packed with Chromex DA-X12-11 anion-exchange resin (polystyrene quarternary amine-type, 12% cross-linking, Durrum). The column was held at 50°C in a water bath with a flow-rate of 0.5 ml/min. The

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* The nomenclature τ-phosphohistidine (3-phosphohistidine) and π-phosphohistidine (1-phosphohistidine) following IUPAC–IUB recommendations (*cf. Arch. Biochem. Biophys.* 160 (1972) 1–8).

column eluate passes through a short piece of PTFE tubing (200 × 0.33 mm) before entering the PTFE mixing tee (0.8 mm I.D., Altex). The OPT solution was pumped into the other inlet on the tee by a Beckman Accuflo pump at a rate of 0.5 ml/min. The combined eluates pass through a reaction coil of PTFE tubing (720 cm × 0.3 mm I.D.) held at ambient temperature prior to entering a 15-μl flow cell of the fluorometer (Spectra Glo, Gilson). The manufacturer's OPT excitation and emission filters were used. The column was equilibrated with buffer A or B prior to use and according to the separation contemplated.

Chemical phosphorylation of histone 4 and bovine myelin basic protein

Phosphoramidate, prepared by the method of Stokes[15], was used to phosphorylate calf thymus histone 4 [prepared from whole histone, Type II-A, (Sigma) according to the procedure of Böhm et al.[16]] or bovine myelin basic protein (gift of Dr. Fred Westall) under the conditions modified from Rathlev and Rosenberg[17] used to phosphorylate insulin. In some instances, [32P]phosphoramidate, prepared by the method of Sheridan et al.[18], was used. A 5-mg amount of protein was incubated with either unlabeled (20 mg) or [32P]phosphoramidate (30,000 cpm/μmole) for 4 days at room temperature in 200 mM Tris, pH 7.4, containing 6 M urea. Unreacted phosphoramidate was removed by dialysis or by passage over Sephadex G-25. Radioactivity incorporated into the protein was shown by running a portion of the sample on an 18% SDS-polyacrylamide gel prepared as described by Laemmli[19]. Neutral staining (0.25% Coomassie blue in 25% isopropanol) and destaining (10% isopropanol) procedures were used followed by autoradiography.

Alkaline hydrolysis of phosphorylated proteins

A sample of phosphorylated protein (ca. 1 mg) was dialyzed against 100 mM NaHCO$_3$,pH 8.2, and lyophilized overnight. The residue was dissolved in 3 M KOH, sealed in a glass tube, and held at >115°C for 3 h. The hydrolysate was very carefully neutralized to pH 7.5 with 2 N HClO$_4$ and the insoluble salts removed by centrifugation. The supernatant was concentrated by lyophilization and resuspended in water. Samples were filtered through 0.2-μm filters before use.

RESULTS AND DISCUSSION

The elution profile of a standard protein hydrolysate mixture (100 pmoles of each amino acid) and 100 pmoles each of o-phosphoserine, o-phosphothreonine and τ-phosphohistidine is shown in Fig. 1. At pH 6.3, a high concentration of salt (buffer B, 250 mM KH$_2$PO$_4$) was necessary for the elution of the phosphoamino acids within a reasonable period of time. However, this buffer was unsuitable for the separation of N-ω-phosphoarginine and N-ε-phospholysine since, at this concentration, they tended to coelute with the other amino acids, which is consistent with their lower overall negative charge. As shown ahead, a lower-ionic-strength buffer was used to separate N-ε-phospholysine and N-ω-phosphoarginine from one another and from the other amino acids. τ-Phosphohistidine elutes more rapidly than o-phosphoserine which most likely reflects the lower overall charge on the phosphorylated histidine due to the partial protonation of the imidazole nitrogen [pK_a 6.4 (ref. 6)]. Hydrophobic interaction between the imidazole ring and backbone of the resin may contrib-
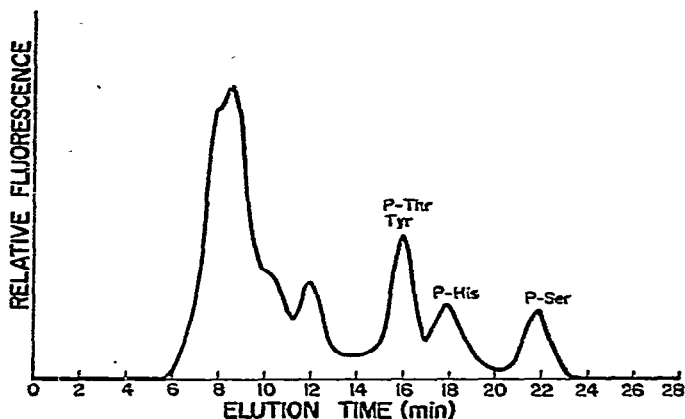
Fig. 1. HPLC separation of τ-phosphohistidine, *o*-phosphoserine and *o*-phosphothreonine. A 25-μl volume of a mixture of protein hydrolysate standard (100 pmoles each amino acid) containing 100 pmoles of τ-phosphohistidine, *o*-phosphoserine and *o*-phosphothreonine was injected. Fluorometer sensitivity R = 10. Elution buffer is 250 m*M* KH$_2$PO$_4$ pH 6.3 (buffer B), 50°C.

ute to its greater retention time relative to *o*-phosphothreonine. Using buffer B, τ-phosphohistidine and π-phosphohistidine coelute. A higher pH buffer, 250 m*M* KH$_2$PO$_4$, pH 7.2, resolved the two, giving elution times of 25 and 18 min, respectively.

Using buffer B, *o*-phosphothreonine and tyrosine coelute. The long retention time of tyrosine is presumably due to its hydrophobic interaction with the resin. Therefore, conditions were sought which would give a clear separation between tyrosine and *o*-phosphothreonine as well as between *o*-phosphoserine and τ-phosphohistidine. Slight variations in pH, temperature and salt concentration failed to give a clear separation of all four of these amino acids. However, employing a higher salt concentration (300 m*M* KH$_2$PO$_4$, pH 6.5, buffer C), *o*-phosphothreonine and tyrosine are separated, but τ-phosphohistidine now coelutes with tyrosine. Fig. 2 shows the elution profile using buffer C. Clearly, if ^{32}P-labeled phosphoamino acids are analyzed, the coelution of *o*-phosphothreonine and tyrosine (using buffer B) or τ-phosphohistidine and tyrosine (using buffer C) is not critical, as the identity of the [32P]phosphoamino acid can be determined by measuring the radioactivity of collected fractions.

Separation of N-ω-phosphoarginine and N-ε-phospholysine was achieved using the lower-ionic-strength buffer, A (Fig. 3). 15 m*M* KH$_2$PO$_4$ was found to be the optimal salt concentration for the temperatures and pH values tested. At higher salt concentrations, N-ω-phosphoarginine and N-ε-phospholysine coelute and migrate into the region of the other amino acids. Using buffer A, N-ε-phospholysine elutes at about 20 min, N-ω-phosphoarginine elutes at 22.5 min, while N-α-phospholysine elutes at a much later time. In preliminary experiments using 75 m*M* KH$_2$PO$_4$ buffer, pH 7.5 (buffer D), a mixture of the two isomers of phospholysine separated well, showing a peak at 11.0 min (N-ε-phospholysine) and 17.0 min (N-α-phospholysine). Buffer D was most useful for the rapid evaluation of the reaction products obtained during the synthesis of N-ε-phospholysine. Phospholysine, prepared by the method of Zetterqvist[4], yielded several products. However, the product formed using a new synthetic
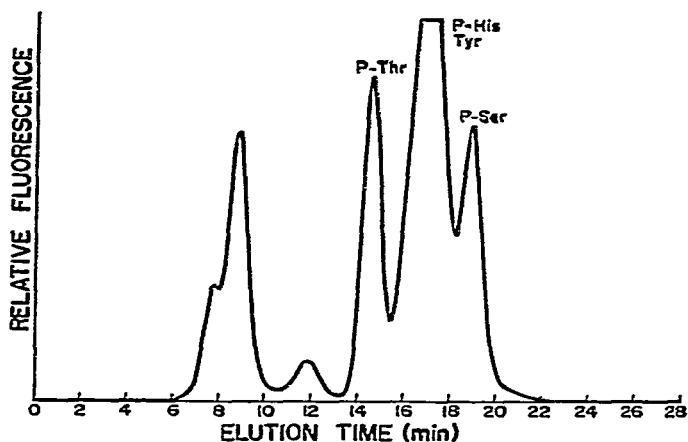
Fig. 2. HPLC separation of τ-phosphohistidine, o-phosphoserine and o-phosphothreonine. A 25-μl volume of a mixture of τ-phosphohistidine (400 pmoles), o-phosphoserine (450 pmoles), o-phosphothreonine (380 pmoles) and tyrosine (890 pmoles) was injected. Fluorometer sensitivity R = 10. Peaks eluting at 7–10 min are minor impurities in the standards. Elution buffer is 300 mM KH₂PO₄ pH 6.5 (buffer C), 50°C.
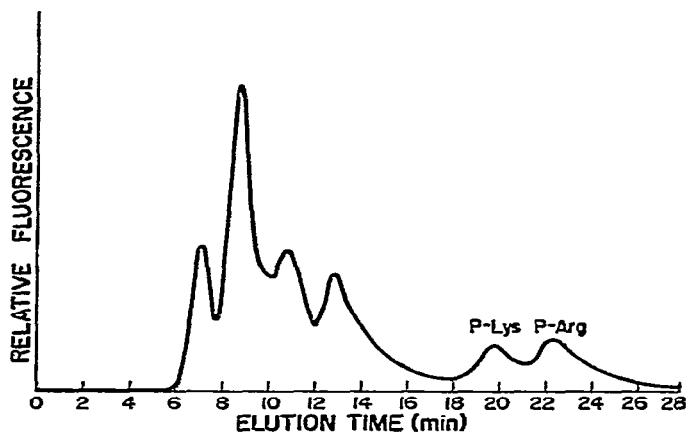

Fig. 3. HPLC separation of N-ε-pholsholysine and N-ω-phosphoarginine. A 25-μl volume of a mixture of protein hydrolysate standard (100 pmoles each amino acid) containing 100 pmoles of N-ε-phospholysine and N-ω-phosphoarginine was injected. Fluorometer sensitivity R = 10. Elution buffer is 15 mM KH₂PO₄ pH 7.5 (buffer A), 50°C.

method for N-ε-phospholysine, starting from the copper chelate of lysine, gave only the 11.0 min peak using buffer D.

Table I shows the fluorescence yield of the phosphoamino acid standards relative to tyrosine. These determinations were performed in the absence of the detergent Brij-35 which was found to have little or no effect on the fluorescence of the phosphorylated amino acids, including N-ε-phospholysine, although it is known to dramatically increase the fluorescence of lysine[11].

TABLE I

RELATIVE FLUORESCENCE OF PHOSPHOAMINO ACIDS

Fluorescence is expressed relative to tyrosine. A plot of relative fluorescence *vs.* amount of phosphoamino acid was determined for each, and the slope divided by the slope obtained for tyrosine.

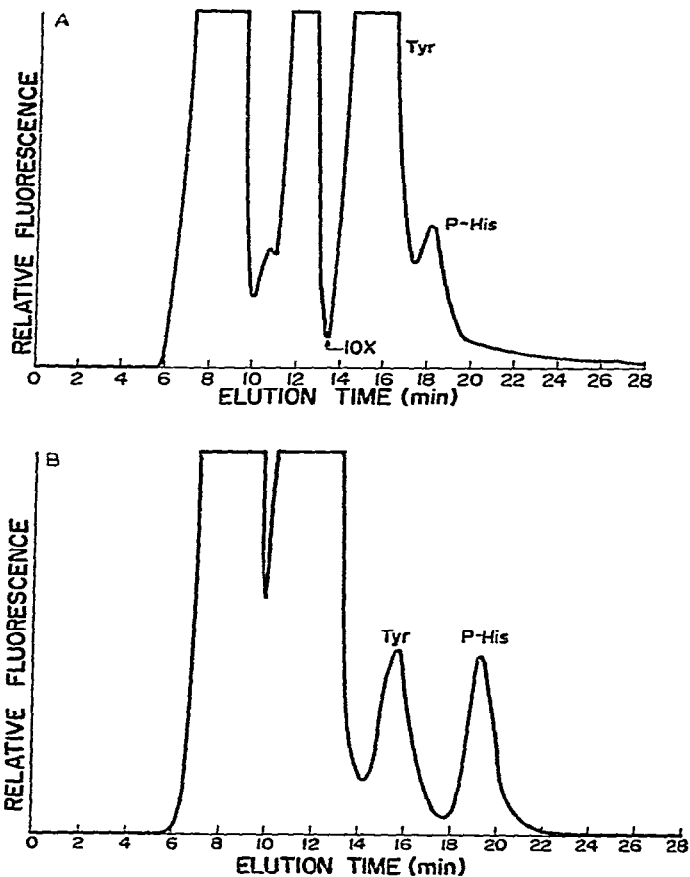|	
Phosphoserine	1.34
Phosphothreonine	1.37
N-ω-Phosphoarginine	2.61
N-ε-Phospholysine	1.00
τ-Phosphohistidine	1.63

Fig. 4.A, Identification of τ-phosphohistidine from potassium hydroxide digest of chemically phosphorylated histone 4. A 50-μl volume representing 50 μg of phosphohistone 4 was injected. Initial fluorometer sensitivity was R = 1, at 13.5 min the sensitivity was increased ten fold (R = 10). Elution buffer is 250 mM KH$_2$PO$_4$ pH 6.3 (buffer B), 50°C. B, Identification of τ-phosphohistidine from potassium hydroxide digest of chemically phosphorylated bovine myelin basic protein. 10 μl representing 50 μg of phosphorylated protein were injected. Fluorometer sensitivity R = 5 (1/2 less than R = 10). Elution buffer is 250 mM KH$_2$PO$_4$ pH 6.3 (buffer B), 50°C.

It has been reported that histone 4 and myelin basic protein are enzymatically phosphorylated on histidine residues[5,7] as well as serine residues[20]. Chemical phosphorylation of these proteins on histidine residues was performed using phosphoramidate as a phosphoryl donor. Fig. 4A and B shows an HPLC analysis of a KOH digest of phosphoramidate-treated histone 4 and myelin basic protein. Acid treatment (0.5 N HCl, 1 min at 100°C, followed by reneutralization) of the KOH digests caused the disappearance of the OPT-reactive peak at 19 min.

Due to the extreme acid lability of P–N compounds, neutral or basic conditions must be maintained during chemical analysis. Present HPLC methods for amino acid analysis[11,14] are unsuitable for P–N compounds due to the common use of acidic buffers. This places a large constraint on the parameters which may be varied for effective resolution of both acid-stable and acid-labile modified amino acids. Use of high pH buffers (pH >8) for phosphoamino acid analysis was unsuitable, as an extremely high salt concentration was required for elution. Resolution was also quite poor under these conditions. The only conditions that varied in this work were slight modifications in pH around neutrality and rather large variations in ionic strength and temperature. The use of a gradient of ionic strength and/or pH may permit the resolution of all phosphoamino acids during a single analysis.

ACKNOWLEDGMENTS

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