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ANALYSIS OF SULFATE AND PHOSPHATE ESTERS OF AMINO ACIDS BY ION-EXCHANGE CHROMATOGRAPHY ON POLYMERIC DEAE

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SUMMARY

A liquid chromatographic system has been developed for the analysis of the sulfate esters of serine and tyrosine and the phosphate esters of threonine, serine and tyrosine. The system also allows the determination of tryptophan obtained by base hydrolysis of peptides and proteins. The system employs a polymer-based DEAE anion-exchange column, elution with a sodium chloride–borate gradient under basic conditions, post-column derivatization by *o*-phthalaldehyde and fluorescence detection. The method has been successfully applied to the analysis of a synthetic peptide containing tyrosine-O-sulfate.

INTRODUCTION

The determination of the complete amino acid composition of a purified peptide or protein is crucial in elucidating its primary structure. A number of different methods have been developed for amino acid analysis following acid, base or enzymatic hydrolysis. The most widely used technique for the analysis of protein hydrolyzates is the ion-exchange method developed by Moore and Stein[1]. This analysis has been traditionally performed on dedicated analyzers, but more recently[2], has been accomplished with modular high-performance liquid chromatographic (HPLC) systems. More recent methods employing pre-column derivatization have proven quite useful. Pre-column derivatization with *o*-phthalaldehyde (OPA) followed by reversed-phase separation and fluorescence detection of the derivatives is a rapid and sensitive technique. This method is useful in specific applications[3]. A more recent method with phenylisothiocyanate (PITC)[4] offers both advantages of the classical ion-exchange technique in its ability to analyze all hydrolyzate amino acids, combined with the speed and sensitivity of the pre-column OPA derivatization method.

Several ion-exchange methods have previously been used in attempts to analyze these esters. Cation-exchange chromatography of phosphoamino acids has been performed[5,6]. However, this method requires strongly acidic buffers to prevent elution of the esters together with the acidic amino acids.

Anion-exchange chromatography has also been used for the analysis of phosphate esters of amino acids. Steiner *et al.*[7] used Chromex DA-X12-11 anion-exchange resin under neutral conditions to analyze both acid-stable and acid-labile phos-

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phoamino acids. Yang *et al.*[8] used the Whatman Partisil-10-SAX anion-exchange resin to separate the phosphate esters of threonine, tyrosine and serine. Tyrosine-O-sulfate has been analyzed by both anion- and cation-exchange analysis[9].

We now report that anion-exchange chromatography on the polymer-based DEAE column developed by Toya Soda with post-column derivatization with *o*-phthalaldehyde provides a rapid and sensitive method for the analysis of the sulfate and phosphate esters of tyrosine, threonine, and serine. In addition, this system allows analysis of phenylalanine, glutamic acid, aspartic acid, tryptophan and tyrosine.

EXPERIMENTAL

Materials

o-Phthalaldehyde was purchased from Pickering Laboratories (Mountain View, CA, U.S.A.). HPLC-grade 2-propanol and acetonitrile were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Hydrolyzate amino acid standards, constant-boiling hydrochloric acid, sequanal-grade triethylamine and phenylisothiocyanate were purchased from Pierce (Rockford, IL, U.S.A.). HPLC-grade boric acid, sodium acetate and potassium hydroxide pellets were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). O-Phospho-DL-threonine, O-phospho-L-serine, O-phospho-DL-tyrosine, L-serine-O-sulfate and adenosine-5′-triphosphate were purchased from Sigma (St. Louis, MO, U.S.A.). Cholecystokinin-(26–33)-octapeptide, protease (non-specific) and aminopeptidase M were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Tyrosine-O-sulfate was synthesized by the method of Reitz *et al.*[10].

Acid hydrolysis

Samples were dried in 50 × 6 mm test tubes and hydrolyzed in a Waters Assoc. (Milford, MA, U.S.A.) protein work station at 105°C. Residual hydrochloric acid was removed under vacuum. Samples to be analyzed by ion-exchange chromatography on the DEAE-5PW column were dissolved in buffer A (see HPLC section), and those to be analyzed by reversed-phase chromatography after pre-column derivatization with PITC were prepared as described by Heinrikson and Meredith[11].

Base hydrolysis

Base hydrolysis was performed by the procedure described by Hauschka[12]. Samples were evaporated to dryness and 150 μl of 2 *M* potassium hydroxide previously sparged with nitrogen was added. Hydrolysis was performed at 105°C under nitrogen. The sample tubes were cooled and placed on ice. A volume of 30 μl of saturated potassium hydrogen carbonate solution was added to each tube and neutralized with cold 60% perchloric acid to a pH of 7. The potassium perchlorate precipitate was removed by centrifugation. The resulting supernatant was injected directly into the DEAE-5PW column after addition of 2–5 μl of 2 *M* potassium hydroxide.

Enzyme hydrolysis

The method used was a modification of that described by Glazer *et al.*[13]. Samples were dried and dissolved in 100 μl of 0.02 *M* Tris–HCl (pH 8.3), containing

0.001 *M* calcium chloride. Then, 1% (w/w) of non-specific protease was added, and the samples were incubated at 37°C for 12 h. The pH was re-adjusted to 8.3 with 0.2 *M* Tris if necessary. A second aliquot of protease was added and the incubation was continued at 37°C for an additional 12 h. Aminopeptidase (1%, w/w) was then added, and the mixture was incubated overnight at 37°C. After the addition of 2–5 μl of 2 *M* potassium hydroxide, the samples were injected into the DEAE-5PW column.

High-performance liquid chromatography

Anion-exchange chromatography was performed on a Waters liquid chromatography system consisting of a Model 680 controller, two Model 510 pumps, a WISP 710 B autosampler, a 420-AC fluorescence detector and a post-column reaction system. The chromatography system was fitted with a Waters Protein Pak DEAE-5PW column. Buffer A was 0.01 *M* boric acid titrated to pH 11.0 with sodium hydroxide and containing 5% (v/v) 2-propanol. Buffer B was 0.25 *M* boric acid and 0.05 *M* sodium chloride titrated to pH 11.0 with sodium hydroxide. The column was eluted at 45°C with a linear gradient of 0 to 60% B over 50 min at a flow-rate of 0.8 ml/min. The post-column derivatization reagent was 0.5 *M* potassium borate buffer at pH 10.5 containing 0.8 g/l *o*-phthalaldehyde and 0.1% (v/v) 2-mercaptoethanol. The reagent flow-rate was 0.4 ml/min and the resulting amino acid derivatives were monitored using a 338-nm excitation filter with 425-nm emission. Data were collated and stored on a Nelson Analytical System Model 4416 (Cupertino, CA, U.S.A.).

Reversed-phase analysis was performed on a Waters chromatography system with the M440 UV–VIS detector at 254 nm. The phenylthiocarbamyl amino acid derivatives were separated on an Altex ODS-PTH column (25 cm × 4 mm I.D.) at 50°C. Solvent A was 0.15 *M* sodium acetate containing 0.1% triethylamine, titrated to pH 6.95 with phosphoric acid. Solvent B was acetonitrile–water (60:40, v/v). A linear gradient of 5 to 55% B over 32 min after a 3-min delay was used. The flow-rate was 1.2 ml/min.

RESULTS AND DISCUSSION

The separation under optimal conditions of 1.0 nmol of standard hydrolyzate amino acids, two sulfate esters and three phosphate esters is shown in Fig. 1. Initially, a gradient to 0.25 *M* borate was used, but this resulted in delayed elution times of the esters. The addition of 0.05 *M* sodium chloride to buffer B resulted in excellent resolution of all esters in less than 60 min.

The addition of 5% 2-propanol to buffer A had little effect on the retention times of the esters but did improve the resolution of aspartic acid and glutamic acid. Increasing the 2-propanol concentration to above 5% resulted in a loss of efficiency.

Initial separations were performed at room temperature. When hydrolyzate amino acids were chromatographed with the esters it was observed that tyrosine and phosphothreonine were unresolved. Increasing the temperature to 45°C was found to give the best results.

The resolution was dependent on the steepness of the gradient. A rapid increase in % B resulted in phosphothreonine and phosphoserine emerging unresolved.

The pH of the buffers had less effect than the ionic strength. A pH of 11.0 resulted in improved resolution of lysine and arginine and had little effect on the esters.

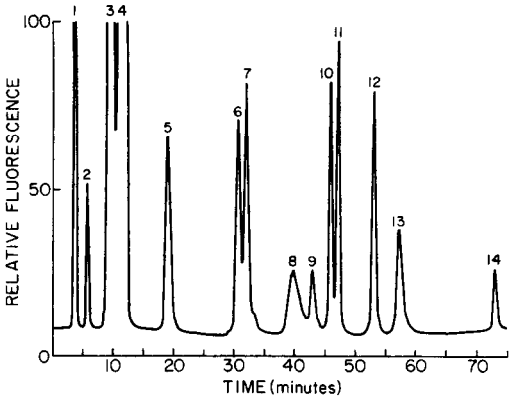


Fig. 1. Anion-exchange analysis of 1.0 nmol of standard hydrolyzate amino acids, and sulfate and phosphate esters. Chromatographic conditions are described in Experimental. Peak identification: 1 = lysine; 2 = arginine; 3 = alanine, glycine, serine and threonine; 4 = histidine, isoleucine, leucine, methionine and valine; 5 = phenylalanine; 6 = glutamic acid; 7 = aspartic acid; 8 = tryptophan; 9 = tyrosine; 10 = phosphothreonine; 11 = phosphoserine; 12 = phosphotyrosine; 13 = serine-O-sulfate; 14 = tyrosine-O-sulfate.

Methods for the acid and base hydrolysis of peptides and proteins are well documented[14]. The treatment of the standard with 6 *M* hydrochloric acid for 20 h at 105°C resulted in complete decomposition of all but one derivative. Approximately 20% of phosphothreonine was recovered. A detailed study of the decomposition of phosphoserine and phosphothreonine in hydrochloric acid has been reported[15] and limited acid hydrolysis has successfully been used for the analysis of the phosphate esters[16–18].

Table I shows the recovery of the standard sulfate and phosphate amino acid esters after incubation of 2.0 nmol of each with 2 *M* potassium hydroxide at 105°C for various times. These data, however, are valid only for the free amino acid derivatives, and cannot be extrapolated to estimate recoveries from a peptide or protein which has been phosphorylated or sulfated.

Some preliminary data on the stability of the sulfate and phosphate esters in the presence of protease and aminopeptidase M is given in Table II. Decomposition

TABLE I

STABILITY OF SULFATE AND PHOSPHATE ESTERS IN 2 *M* POTASSIUM HYDROXIDE AT 105°C

Amino acid ester	*Recovery (%)*			
	1 h	*4 h*	*6 h*	*24 h*
Phosphothreonine	100	100	96	84
Phosphoserine	98	87	65	21
Phosphotyrosine	100	97	96	96
Serine-O-sulfate	0	–	–	–
Tyrosine-O-sulfate	100	97	95	94

TABLE II

STABILITY OF SULFATE AND PHOSPHATE ESTERS IN THE PRESENCE OF PROTEASE AND AMINOPEPTIDASE M

Amino acid ester	*Recovery (%)*			
	Buffer	*Protease*	*Protease + aminopeptidase*	*Aminopeptidase*
Phosphothreonine	100	94	79	78
Phosphoserine	100	97	85	83
Phosphotyrosine	100	98	57	54
Serine-O-sulfate	100	99	99	97
Tyrosine-O-sulfate	100	100	100	98

of the phosphate esters occurred to a significant extent whereas the sulfate esters were quite stable to the enzymes. The treatment with aminopeptidase M results in the highest degree of dephosphorylation, and may be attributable to phosphatase contamination of the aminopeptidase M reagent.

The DEAE 5PW column can also be used for the analysis of inorganic phosphate. Fig. 2 shows the radioactivity profiles of the chromatograms of [γ-^{32}P]ATP before and after acid and base hydrolysis, and of a $H_3{}^{32}PO_4$ standard. The second major peak of the base hydrolyzate of [γ-^{32}P]ATP may be [^{32}P]pyrophosphate, but this has not been verified.

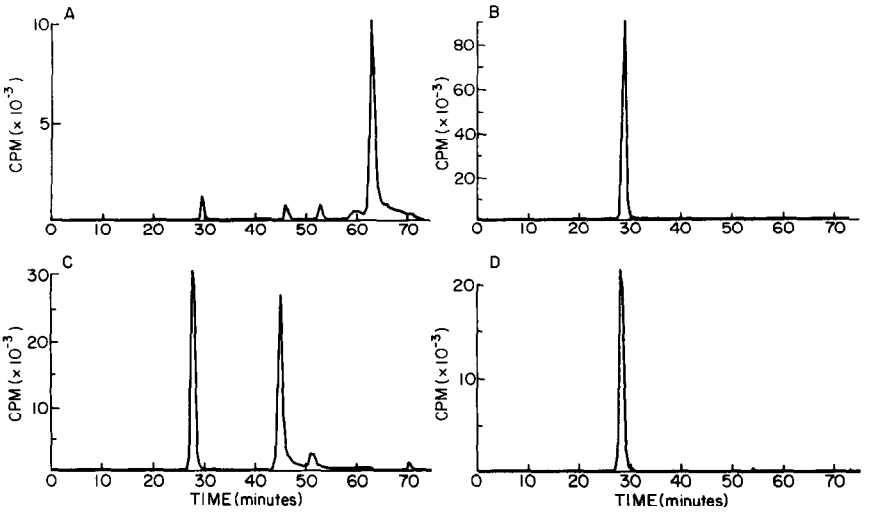
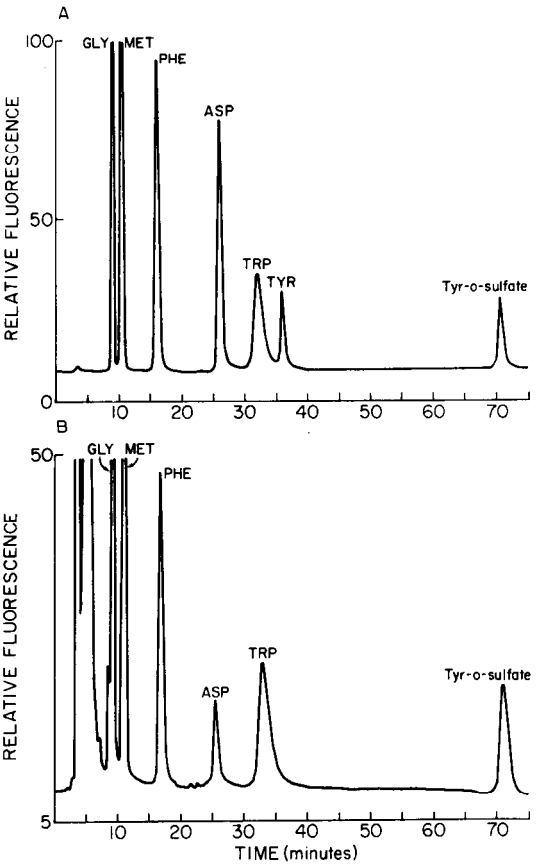


Fig. 2. Anion-exchange analysis of (A) [γ-^{32}P]adenosine-5'-triphosphate; (B) [γ-^{32}P]ATP, acid hydrolyzed for 1 h; (C) [γ-^{32}P]ATP, base hydrolyzed in 2 *M* potassium hydroxide for 1 h; (D) $H_3{}^{32}PO_4$. Chromatographic conditions are described in Experimental.

The usefulness of the DEAE-5PW analysis in conjunction with a standard reversed-phase amino acid analysis for the determination of the complete amino acid composition of peptides and proteins was demonstrated by applying the method to the cholecystokinin octapeptide (residues 26–33, Asp-Tyr-(SO_3H)-Met-Gly-Trp-



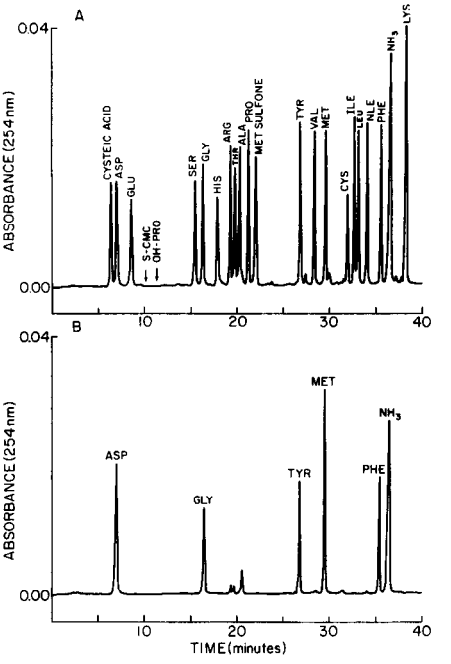


Fig. 3. Anion-exchange analysis of (A) standard amino acids of cholecystokinin-(26–33)-octapeptide plus tyrosine and (B) enzyme hydrolyzate of cholecystokinin-(26–33)-octapeptide. Chromatographic conditions are described in Experimental.

Fig. 4. Reversed-phase analysis of phenylthiocarbamyl derivatives of (A) standard hydrolyzate amino acids and (B) hydrochloric acid hydrolyzate of cholecystokinin-(26–33)-octapeptide. Chromatographic conditions are described in Experimental.

TABLE III

AMINO ACID COMPOSITION OF CHOLECYSTOKININ-(26–33)-OCTAPEPTIDE FROM HYDROCHLORIC ACID HYDROLYSIS

Amino acid	*Amount (nmol)*	
	Observed	*Theoretical*
Aspartic acid	1.94	2.00
Glycine	0.98	1.00
Tyrosine	0.95	1.00
Methionine	1.88	2.00
Phenylalanine	0.98	1.00

Met-Asp-Phe-NH_2). The cholecystokinin was enzyme-hydrolyzed as described and analysis of the hydrolyzate on the DEAE-5PW column is shown in Fig. 3. From the quantitation of Gly, Met, Phe and Trp it was determined that over 98% of the peptide had been hydrolyzed. The composition of the peptide was confirmed by acid hydrolysis, pre-column PITC derivatization, and analysis by reversed-phase chromatography, and is shown in Fig. 4. As expected, unesterified tyrosine was recovered. The compositional data is shown in Table III.

In summary, our data demonstrate that the DEAE-5PW column is useful for the analysis of sulfate and phosphate esters of tyrosine, serine, and threonine and when used in conjunction with standard reversed-phase amino acid analysis provides valuable information for determining the accurate and complete amino acid composition of peptides and proteins.

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