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Note

Complete analysis of protein hydrolysates containing phosphoserine and phosphothreonine using the amino acid analyzer

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In recent decades, the accumulation of knowledge about the important effects of phosphorylation on the enzymatic and other biochemical and biophysical properties of proteins has not been matched by equivalent advances in analytical methods for the quantitative estimation of the phosphorylated amino acid residues (phosphoserine and phosphothreonine) that are commonly produced by the action of protein kinases.

The following is a rapid, automated method for the complete analysis of an amino acid mixture containing phosphoserine and phosphothreonine. The method utilizes one column on a Technicon TSM amino acid analyzer to separate highly acidic amino acids (cysteic acid, phosphoserine, and phosphothreonine) while a second column is used to separate the amino acids more commonly found in protein hydrolysates. It is a modification of the method described by Niece[1], which fails to give a good resolution of these phosphoamino acids.

EXPERIMENTAL

Column modifications

The standard TSM column for basic amino acids (167-B116-01) (Technicon, Tarrytown, N.Y., U.S.A.) is replaced by the longer physiological column (167-B116-03) 4 × 328 mm. This column, packed with 280 mm of C-3 Chromobeads (T15-0360-42) and operated at 60°, allows separation of the highly acidic amino acids.

The composition of the elution buffer used to separate the highly acid amino acids (cysteic acid, phosphoserine, and phosphothreonine) is given in Table I.

The flow-rate for the 328-mm column is adjusted to 0.3 ml/min, resulting in pressures less than 500 lb./in.^{2}. This low flow-rate causes the baseline to be raised 5 absorbance units above that of the 285.8-mm column but does not obscure amino acid identification. The 0.3 ml/min flow-rate is necessary for separation of phosphoserine and phosphothreonine.

The 285.8-mm column for standard amino acid analysis uses 230.5 mm of C-3 Chromobeads and a single column buffer system (Table I), modified from Niece[1]. It is operated at 60° with a flow-rate of 0.5 ml/min.

Samples in 0.1 *M* citric acid–1% (v/v) thiodiglycol adjusted to pH 2.0 with

TABLE I

COLUMN BUFFERS

Column	Buffer	Reagents*	Final	
			pH	Volume (l)
1	1	7.5 g KCl, 970 ml 0.2 *M* HCl	1.0	2
2	2	98.49 g Trisodium citrate, 240 ml methyl cellosolve	2.98	4
2	3	78.79 g Trisodium citrate, 72 g NaCl	3.60	3
2	4	78.79 g Trisodium citrate, 72 g NaCl	4.0	2
2	5	78.79 g Trisodium citrate, 72 g NaCl, 32 g NaOH, 30 g H_3BO_3	9.5	4

* All buffers contained 1% (v/v) thiodiglycol and 1% (v/v) of 30% (w/v) Brij-35.

hydrochloric acid are loaded on cartridges which are used to elute the hydrolysate onto the 328- and 285.8-mm columns.

A 120-min analytical cycle consists of 26 min of buffer 1 in the 328-mm column, and 26 min of buffer 2, 28 min of buffer 3, 15 min of buffer 4, and 20 min of buffer 5 in the 285.8-mm column. Both columns are then regenerated with 0.2 *M* sodium hydroxide–0.1% (w/w) EDTA for 10 min, followed by equilibration with their respective starting buffers for 12 min.

Under the conditions described above, the 285.8-mm column can be run with buffer 2 during the last 14 min of the 328-mm column elution, with the 285.8-mm column eluate channeled to waste for those 14 min.

All chemicals were reagent grade or prepared for amino acid analysis by Pierce (Rockford, Ill., U.S.A.).

Analytical system

The column eluate was injected into a 2 m*M* hydrazine sulfate–0.1% (v/v) of 30% (w/v) Brij-35 solution segmented stream. Ninhydrin reagent (1% ninhydrin, 50% (v/v) methyl cellosolve, 25% (v/v) 4 *M* sodium acetate, pH 5.1) was injected into the column eluate–hydrazine mixture segmented stream at a flow-rate of 0.70 ml/min. The reaction product was monitored at 570 nm in the first flow cell and proline was monitored at 440 nm in a second flow cell.

Finally, it is important not to allow the analyzer to shut down or stand with buffer 1 in the column since that would leave the pH 1 buffer in the 328 mm column, resulting in the corrosion of its fittings. The columns should have 0.2 *M* sodium hydroxide–0.1% EDTA pumped through both columns for 10 min before the analyzer is turned off.

RESULTS AND DISCUSSION

As seen in Figs. 1 and 2, complete separation of phosphoserine and phosphothreonine is achieved, with cysteic acid eluted well before either of the phosphoamino acids. The remaining amino acids from the 328-mm column are channeled to waste by programming the TSM. The 285.8-mm column handles analysis of the standard amino acids of protein hydrolysates so there is the option of doing a complete amino acid analysis or doing phosphoserine and phosphothreonine determinations alone. The phosphoserine and phosphothreonine determinations can be accomplished within 30 min and subsequent amino acid analysis deleted if desired.

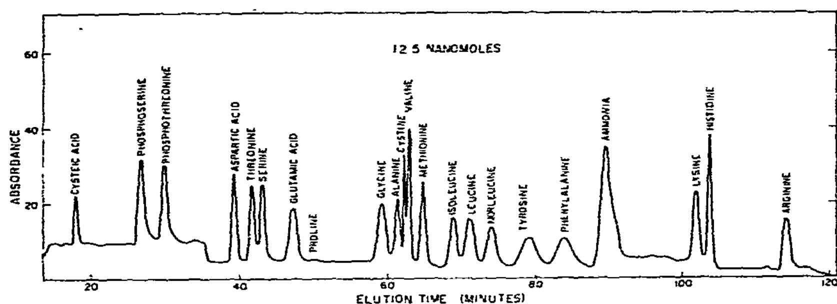

Fig. 1. Amino acid analysis of standard amino acid mixture containing 10 nmoles of each amino acid.

At present, the most widely used method for quantitative estimation of these two phosphoamino acids is high-voltage paper electrophoresis[2-6]. Although this method gives good separations of these compounds, it is not suited to automated procedures, nor does it yield information about other amino acids that may be present in the mixture (usually a protein hydrolysate). This method offers the advantage of rapid results, multiple samples, nanomolar sensitivity, and complete analysis of amino acid mixtures over alternative electrophoretic, chromatographic[7], or chemical[9] methods.

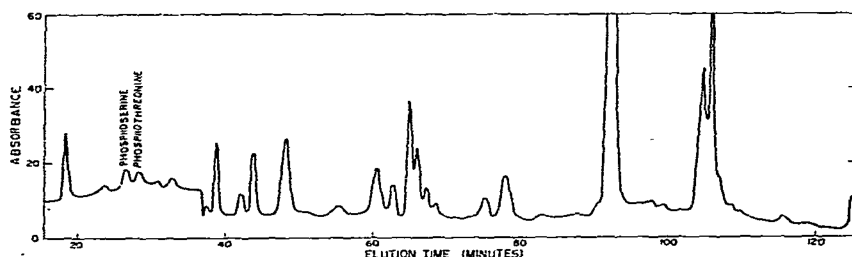

Fig. 2. Amino acid analysis of 1 mg casein hydrolyzed in 1 ml of 2 *M* hydrochloric acid for 8 h at 110°. The elution sequence is the same as in Fig. 1. The hydrolyzed sample was dried in vacuo, 60 μl of sample buffer (0.1 *M* citric acid–1 % (v/v) thiodiglycol) was added, and 25 μl was loaded onto each cartridge.

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