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QUANTITATIVE DETERMINATION OF PHOSPHOSERINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AS THE PHENYLTHIOCARBAMYL-S-ETHYLCYSTEINE

APPLICATION TO PICOMOLAR AMOUNTS OF PEPTIDES AND PROTEINS

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SUMMARY

A method is described that permits the phosphoserine content of proteins and peptides to be determined in picomolar amounts. A micro-batch reaction first converts phosphoserine into S-ethylcysteine. Hydrolysis with 6 *M* hydrochloric acid then yields the free amino acid, which is coupled with phenyl isothiocyanate to give the corresponding phenylthiocarbamylamino acid. This derivative is determined quantitatively in the range 10–20 pmol by reversed-phase high-performance liquid chromatography. The method works well with either small peptides or proteins in the low picomole range.

INTRODUCTION

The determination of inorganic phosphate after ashing of a phosphoprotein or a phosphopeptide permits the quantitation of the overall phosphoamino acid content but does not differentiate between the three known phosphoamino acids, phosphoserine, phosphothreonine and phosphotyrosine. Moreover, at least 1 nmol is required for this analysis[1].

A more suitable method was described by Downs and Pigman[2], involving beta-elimination of the phosphate group and catalytic hydrogenation of the resulting dehydroamino acids. Phosphoserine is converted into alanine, which can then be determined by standard amino acid analysis. For small peptides of low alanine content this method works well, but with larger proteins of low phosphoserine and high alanine content it is inadequate. As an alternative, Clark[3] introduced the addition of methylmercaptan following beta-elimination to yield S-methylcysteine. This method has been applied successfully to the sequence determination of phosvitin, an exceptionally phosphoserine-rich protein.

Especially for proteins of low phosphate content, an improved method is required that allows easy handling of the sample and the specific determination of one type of phosphoamino acid in picomol amounts.

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In this paper, we report an improved method that fulfills these requirements for the determination of phosphoserine. It features high sensitivity, allowing microanalysis by transformation of phosphoserine into the stable S-ethylcysteine derivative, and convenience, being based on a one-step micro-batch reaction.

EXPERIMENTAL

Materials

Kemptide (NH_2-Leu-Arg-Arg-Ala-Ser-Leu-Gly-COOH) was obtained from Serva (Heidelberg, F.R.G.), acetonitrile (HPLC grade) and sodium hydroxide (p.a. grade) from Roth (Karlsruhe, F.R.G.), ethanol (HPLC grade) from Baker (Gross-Gerau, F.R.G.), ethanethiol (sequanal grade) from Pierce (Rodgau, F.R.G.), S-ethylcysteine from Sigma (Deisenhofen, F.R.G.) and phenyl isothiocyanate (PITC) from Beckman (Munich, F.R.G.). Other chemicals and solvents used were of the highest purity available.

Methods

Phosphorylation of kemptide was carried out as previously reported[4,5]. A 0.2-mg amount of kemptide was incubated with 1 m*M* ATP, 5 m*M* magnesium acetate, 40 m*M* potassium phosphate, 7.5% glycerol, 0.1 m*M* dithiothreitol and 100 μg of protein kinase (catalytic subunit) at pH 7.3 and 26°C for 30 min in a total volume of 4.1 ml and then purified by reversed-phase high-performance liquid chromatography (HPLC). A second phosphopeptide [NH_2-Arg-Arg-Leu-Ser(P)-Ser-Leu-Arg-Ala-COOH] was graciously supplied by Prof. Kruppa (University of Hamburg, F.R.G). Bovine cardiac troponin with a high phosphate content was isolated according to the method of Tsukui and Ebashi[6] as modified by Beier[7]. The troponin I subunit was separated from the holo-troponin complex by the method of Crabb and Heilmeyer[8] as modified by Swiderek[9], using a preparative reversed-phase column (250 × 35 mm I.D.), packed with HD-gel RP-7s-300 from Orpegen (Heidelberg, F.R.G.).

Derivatization of phosphoserine to S-ethylcysteine

To dried samples (500 pmol or less) in a small reaction vial (50 × 5 mm I.D. Pyrex tube) were added 50 μl of a reaction mixture prepared by mixing of 80 μl of ethanol, 65 μl of 5 *M* sodium hydroxide, 60 μl of ethanethiol and 400 μl of water. The mixture was incubated for 1 h at 50°C under nitrogen, acidified with 10 μl acetic acid and then vacuum-dried at room temperature.

PTC-amino acid analysis

Hydrolysis was carried out in a 40-ml screw-capped vial with 300 μl of 6 *M* hydrochloric acid for up to 10 samples at 150°C for 1 h. Amino acid analysis was performed after derivatization with PITC[10,11]. The PTC-amino acids were separated by reversed-phase HPLC. For the separation in Fig. 1a a Beckman Ultrasphere ODS 5 μm column (250 × 4.6 mm I.D.) was used, applying a gradient of 160 m*M* potassium acetate (pH 5.2) against 84% acetonitrile at a flow-rate of 1 ml/min at 40°C. For all other separations the buffer system C of Heindrikson and Meredith[12] was applied, consisting of a gradient of 50 m*M* ammonium acetate (pH 6.8) against 100

m*M* ammonium acetate containing 44% acetonitrile and 10% methanol (pH 6.8), using a 3-μm Spherisorb ODS-2 column (125 × 4.6 mm I.D.) at 42°C and a flow-rate of 1 ml/min. In both instances PTC-amino acids were detected at 260 nm with a Kratos photometer (Kratos, Karlsruhe, F.R.G.). The standard deviation did not exceed 10% for any amino acid.

RESULTS

For standardization of the method, kemptide was phosphorylated with the catalytic subunit of the cAMP-dependent protein kinase[5]. The product was characterized by measuring phosphate incorporation and found to contain one phosphoserine per mole of peptide[4].

Fig. 1a shows the chromatogram of the PTC-amino acids obtained by hydrolysis of phosphorylated keptide (P-kemptide). PTC-serine is formed from phosphoserine. This amino acid derivative is almost completely absent from the derivatized sample (Fig. 1b). Instead, the new derivative S-ethylcysteine (S-Et-Cys) is obtained. Quantitative determination of serine and S-ethylcysteine in comparison with the unmodified amino acids demonstrates that phosphoserine has been almost quantitatively transformed into S-ethylcysteine in the one-step micro-batch reaction (Table I). The conversion of phosphoserine into S-ethylcysteine was carried out on a 1-nmol sample. Both the original P-kemptide and the derivatized sample were hydrolysed and the PTC-amino acids were determined by HPLC.

Analysis of a peptide that contains both phosphoserine and serine demonstrates that only phosphoserine but not serine is converted into S-ethylcysteine. The

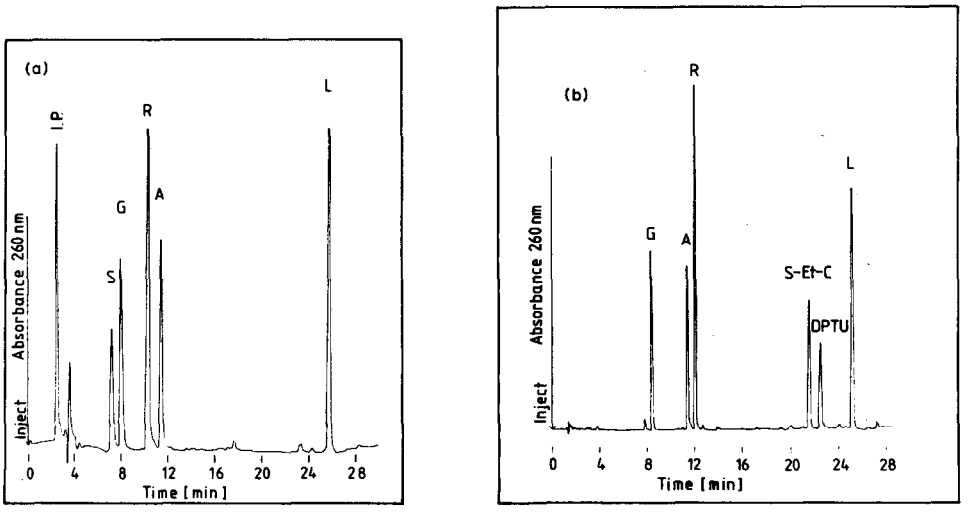


Fig. 1. PTC-amino acid analysis of phospho-kemptide. A 1-nmol amount of (a) unmodified and (b) modified phospho-kemptide was hydrolysed and coupled with PITC; (a) 500 pmol of phospho-kemptide were analysed using a Beckman Ultrasphere ODS column and (b) 350 pmol of S-ethylcysteine-kemptide were analysed on Spherisorb ODS-II. I.P. = injection peak; S = serine; G = glycine; R = arginine; L = leucine; S-Et-C = S-ethylcysteine; DPTU = diphenylthiourea (a reaction product of phenyl isothiocyanate).

TABLE I

AMINO ACID COMPOSITION OF PHOSPHO-KEMPTIDE

Duplicate samples were analysed for amino acid composition. Values given represent the mean for each amino acid of either peptide. Values for serine are not corrected for destruction during hydrolysis.

Amino acid	*Amino acid, mol per mole*	
	Phospho-kemptide	*S-Ethylcysteine-kemptide*
Leu	2.0	2.0
Arg	2.0	2.0
Ala	1.0	1.0
Gly	1.0	1.0
Ser	0.95	0.07
S-Ethyl-Cys	0.0	0.93

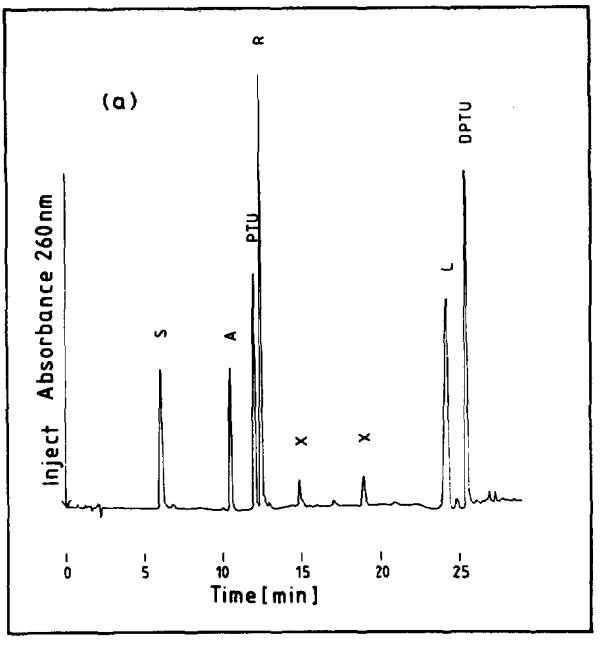
first serine of the peptide was identified as phosphoserine by sequence analysis of the modified peptide (data not shown).

Fig. 2 shows the chromatograms of the PTC-amino acids obtained from the phosphopeptide in (a) the unmodified and (b) the modified form. Only after modification S-ethylcysteine is detected. Quantitative evaluation of the amino acid composition (Table II) reveals a decrease in serine and a concomitant formation of S-ethylcysteine after the modification. Hence it can be concluded that only one of the serines was phosphorylated.

Bovine cardiac troponin I was isolated with a phosphate content of 1.2–1.9 mol per mole of protein[7,13–15] according to phosphate analysis by ashing[1,7,9]. Again, its amino acid composition was determined before and after derivatization. The chromatograms (Fig. 3) of the PTC-amino acids obtained are very similar and demonstrate an almost identical amino acid composition of the modified (b) and unmodified (a) troponin I, except for serine and threonine. The serine content is lowered after transformation of phosphoserine into S-ethylcysteine. Following modification, an additional peak emerges with the same retention time as PTC-S-ethylcysteine. The amount of 1.8 mol of S-ethylcysteine per mole of troponin I (Table III) agrees very well with the phosphate determination after ashing. It also confirms that all of the phosphate bound in troponin I is present as phosphoserine, as has been shown before by ^{31}P NMR spectroscopy[7]. The lowering of the threonine value is presumably due to some additional destruction during modification.

DISCUSSION

The transformation of phosphoserine into S-ethylcysteine, as demonstrated here, has several advantages over previously published methods. Annan *et al.*[16] suggested converting phosphoserine into β-methylaminoalanine. However, the PTC derivative of this amino acid emerges from the HPLC column just before lysine, where impurities of hydrolysed samples are often located, precluding a quantitative determination. Another drawback is that commercially available methylamine is impure,



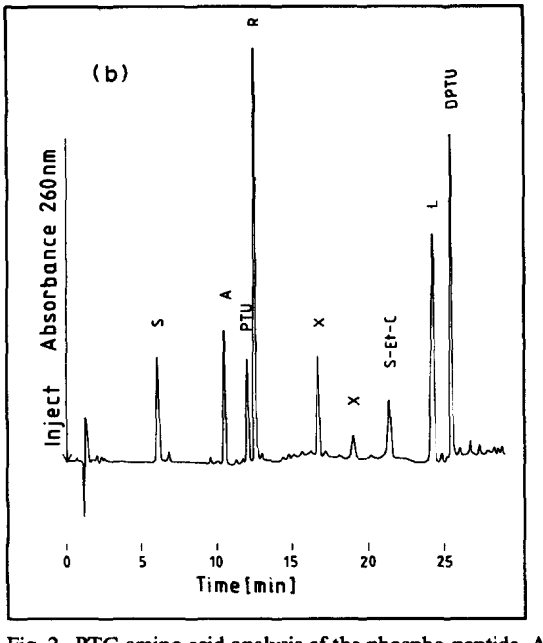


Fig. 2. PTC-amino acid analysis of the phospho-peptide. A 500-pmol amount of the phosphopeptide was hydrolysed (a) unmodified and (b) after derivatization and then coupled with PITC; 100 pmol of each sample were analysed for amino acid composition on Spherisorb ODS-II. PTU = phenylthiourea; X = unknown compounds; other symbols as in Fig. 1.

TABLE II

AMINO ACID COMPOSITION OF PHOSPHOPEPTIDE

Numbers given represent the mean values for each amino acid in duplicate determinations of either peptide. Values for serine are not corrected for destruction under hydrolysis conditions.

Amino acid	*Amino acid, mol per mole*	
	Phospho-peptide	*S-Ethylcysteine-peptide*
Ser	1.5	1.1
Ala	1.0	1.0
Arg	2.8	3.2
Leu	2.3	2.3
S-Ethyl-Cys	0.0	0.9

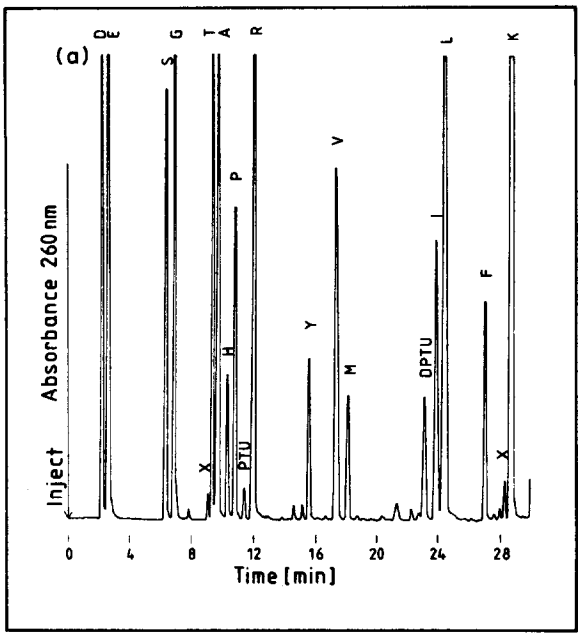
requiring distillation as an additional step. Determination of inorganic phosphate[1] requires a *ca.* 100 times larger sample than the method described here and does not differentiate between the phosphorus in phosphoserine and other phosphoamino acids. The conversion of phosphoserine into alanine according to Downs and Pigman[2] yields an amino acid that is an ordinary constituent of proteins. Hence the

TABLE III

AMINO ACID COMPOSITION OF TROPONIN I

Values represent the mean of duplicate determinations of the unmodified or modified troponin I subunit, assuming a relative molecular mass of 24 000 daltons. Cys and Trp were not determined. Values were not corrected for destruction or slow liberation of special amino acids.

Amino acid	*Amino acid, mol per mole*	
	Unmodified troponin I	*Modified S-ethylcysteine-troponin I*
Asx	10.7	11.2
Glx	23.0	24.2
Ser	9.0	7.2
Gly	13.3	12.5
His	3.3	3.2
Arg	23.6	23.5
Thr	9.0	7.2
Ala	24.9	24.8
Pro	7.2	7.4
Tyr	2.9	3.1
Val	8.6	8.2
Met	3.2	3.8
Ile	6.9	6.7
Leu	22.2	21.9
Phe	4.0	4.9
Lys	21.0	21.1
S-Ethyl-Cys	0.0	1.8



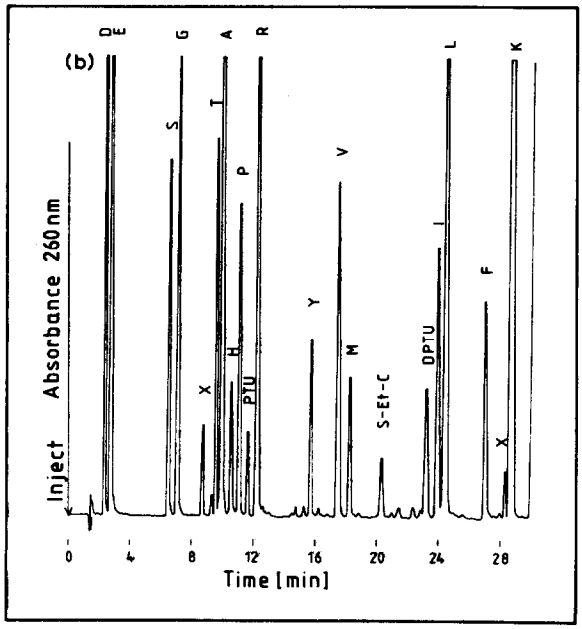


Fig. 3. PTC-amino acid analysis of troponin I. A 500-pmol amount of (a) the unmodified and (b) modified troponin I was hydrolysed as described under *Methods*; 200 pmol of each sample were analysed for amino acid composition on Spherisob ODS-II. D = asparagine or aspartic acid; E = glutamine or glutamic acid; T = threonine; H = histidine; P = proline; Y = tyrosine; V = valine; M = methionine; I = isoleucine; F = phenylalanine; K = lysine; other symbols as in Figs. 1 and 2.

quantitative determination of the phosphoserine content must be calculated from the difference in the alanine content, which is very difficult to achieve with large proteins of low phosphoserine content. Another possibility, suggested by Clark and Dijkstra[17], consists in the conversion of phosphoserine into S-methylcysteine, which would, like S-ethylcysteine, be identifiable. However, the handling of methylmercaptan, which is gaseous at normal pressure and room temperature, requires an elaborate apparatus. Addition of ethanethiol together with all other components of the reaction mixture is much more convenient.

The limitations of the method presented here are that serine residues carrying carbohydrates or other substituents will result in the same modification product as obtained from phosphoserine. However, carbohydrates can be determined by other methods beforehand. If phosphoserine posseses a free NH_2 or COOH terminus, no transformation into S-ethylcysteine will occur. Instead, pyruvate or ethylamine, respectively, is formed.

The formation of lysinoalanine, which was described by Friedman *et al.*[18] through the reaction of lysine with the intermediate dehydroalanine, is very unlikely with the present method. First, the peptide or protein concentrations and concomitant the lysine concentrations are very low (nanomolar) but the ethanethiol concentration is very high (10%) in the reaction mixture. Second, the mercapto group exhibits more nucleophilic power than the ε-amino group of lysine and will therefore react preferentially.

The S-ethylcysteine conversion described here was employed to determine phosphoserine quantitatively. However, with less than 100 pmol of substance this method also allows the localization of phosphoserines in the primary sequence unambiguously, because free serine will not be modified during the reaction, as shown recently by Meyer *et al.*[4,19].

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