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A new method for the selective isolation of phosphoserine-containing peptides

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Meyer et al. [(1986) FEBS Lett 204, 61–66] have shown that phosphoserine can be converted to *S*-ethylcysteine by β-elimination and addition of ethanethiol. I have utilised this modification to develop a rapid method for the selective purification of phosphoserine-containing peptides from complex mixtures. Changing phosphoserine to *S*-ethylcysteine increases the hydrophobicity of a peptide, altering its mobility during reverse-phase chromatography. The number of *S*-ethylcysteine residues in a peptide can be quantified at the picomolar level, following acid hydrolysis and conversion to the phenylthiocarbamyl derivative. The procedure may be particularly powerful for the analysis of peptides that are phosphorylated at multiple sites in vivo.

Protein phosphorylation; Phosphoserine; Ethanethiol; *S*-Ethylcysteine; RPLC; Amino acid analysis

1. INTRODUCTION

Reversible phosphorylation is a principal mechanism for regulating the function of proteins in response to extracellular signals, and almost all cellular processes in eukaryotes are controlled by this post-translational modification [1,2]. It is therefore important that rapid and sensitive methods are available for identifying and purifying phosphopeptides, in particular those containing phosphoserine, the most common phosphoamino acid present in proteins.

Recently, Meyer et al. [3] showed that the phosphoseryl derivative of the peptide LRRASLG could be converted to its *S*-ethylcysteinyl derivative by β-elimination of the phosphoseryl residue in NaOH and addition of ethanethiol. The phenylthiohydantoin (Pth) derivative of *S*-ethylcysteine was identified in picomolar amounts during gas-phase sequence analysis of the modified peptide [3]. The procedure was not applicable to phosphothreonine and phosphotyrosine-containing peptides.

Here, the conversion of phosphoserine to *S*-ethylcysteine has been exploited to develop a simple procedure for the selective isolation of phosphoserine-containing peptides.

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2. MATERIALS AND METHODS

2.1. *Preparation of phosphopeptides*

The peptide SPQPSRRGSESSEE, corresponding to the phosphorylation site on the glycogen-binding subunit of protein phosphatase-1 [4], was synthesized by Dr Bruce Kemp, Repatriation General Hospital, Heidelberg, Victoria, Australia. The peptide was phosphorylated by incubation with the catalytic subunit of cyclic AMP-dependent protein kinase [5] and MgATP, and separated from any residual dephosphopeptide by reverse-phase liquid chromatography on a Vydac C_{18} column (Separations Group, Hesperia, CA) as

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described in fig.1. The phosphopeptide eluted 2 min earlier than the dephosphopeptide.

Protein phosphatase inhibitor-2 was purified from rabbit skeletal muscle in the presence of NaF to prevent dephosphorylation, digested with trypsin and chromatographed on a Vydac C_{18} column [6]. The partially purified peptide EQESSGEEDSDLSPEER, containing two phosphoserine residues as judged by fast atom bombardment mass spectrometry (FABMS) [6], was collected and dried on a vacuum concentrator.

2.2. *Conversion of phosphoserine to S-ethylcysteine*

Phosphoseryl peptides (0.2–2 nmol) were dissolved in 50 μl of a reaction mixture consisting of ethanethiol (60 μl), water (200 μl), dimethyl sulphoxide (200 μl), ethanol (100 μl) and 5 N NaOH (65 μl), and incubated for 1 h at 50°C under nitrogen. These conditions are identical to those described by Meyer et al. [3], except that the volume of ethanol was increased by 20 μl. This improved the recovery of intact *S*-ethylcysteinyl peptides to at least 70%. Incubations were terminated by cooling and adding 10 μl acetic acid. After dilution to 1 ml with water, the reactions were dried, dissolved in 0.1% (v/v) trifluoroacetic acid and refractionated on the Vydac column as described below.

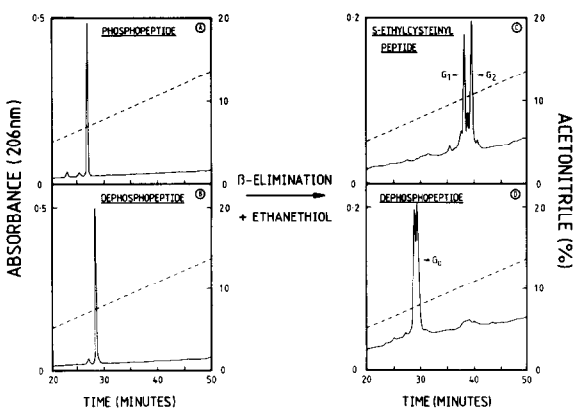

Fig.1. Fractionation of the phosphorylated and dephosphorylated forms of the peptide SPQPSRRGSESSEE on a Vydac C_{18} column, before and after modification with ethanethiol in NaOH. Reverse-phase liquid chromatography was carried out using a Gilson HPLC system equipped with a variable-wavelength UV detector. The column was developed with a linear water–acetonitrile gradient at a flow rate of 1 ml/min. (——) Absorbance at 206 nm; (– – –) acetonitrile gradient. G_1 and G_2 (C) represent the two major forms of the *S*-ethylcysteinyl derivative, discussed in the text.

Table 1

Amino acid compositions of the peptides SPQPSRRGSESSEE (G) and EQESSGEEDSDLSPEER (I) after incubation of phosphorylated and dephosphorylated forms with ethanethiol in NaOH

Amino acid	Peptide			
	G_1	G_2	G_D	I_2
Asx				2.0 (2)
Glx	4.0 (4)	3.8 (4)	4.0 (4)	6.8 (7)
Ser	4.3 (4)	4.1 (4)	5.2 (5)	2.4 (2)
Gly	1.1 (1)	1.3 (1)	1.3 (1)	2.0 (1)
Arg	2.1 (2)	1.9 (2)	2.1 (2)	1.0 (1)
Pro	2.2 (2)	2.1 (2)	2.1 (2)	1.2 (1)
S-EtCys	0.8 (1)	0.8 (1)	0	1.5 (2)
Leu				1.0 (1)

Peptides G_1 and G_2 denote the two major forms of the *S*-ethylcysteinyl derivative of peptide G (fig.1C). Peptide G_D is the dephosphopeptide doublet (fig.1D). I_2 represents the derivative of peptide I with two *S*-ethylcysteine residues (fig.3). Serine was corrected for 10% destruction during acid hydrolysis. In quantifying *S*-ethylcysteine, the extinction coefficient of its Ptc derivative was assumed to be 96% of the value for Ptc-methionine [7]

3. RESULTS AND DISCUSSION

3.1. *Purification of S-ethylcysteinyl peptides*

Following incubation with ethanethiol in the presence of NaOH, the phosphorylated and dephosphorylated forms of the peptide SPQPSRRGSESSEE were rechromatographed on a Vydac C_{18} column (fig.1). The chemically modified phosphopeptide now eluted 11–13 min later at a 4% higher acetonitrile concentration (10 vs 6%). The modified peptide was resolved into two major peaks, which after acid hydrolysis had identical amino acid compositions (table 1). Gas-phase sequencer and FABMS analysis revealed that both peaks had an identical sequence and the same molecular mass (not shown). Therefore, resolution of the modified peptide into two major

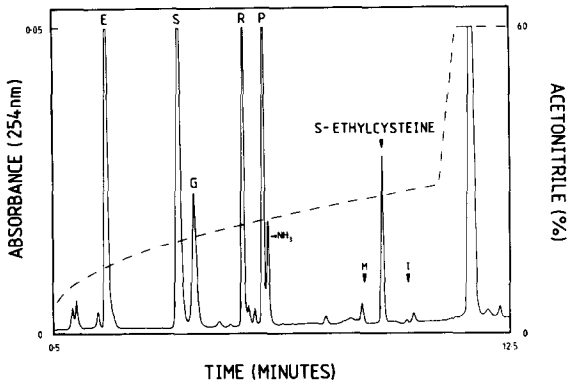

Fig.2. Amino acid analysis of the phosphopeptide SPQPSRRGS(P)ESSEE, after conversion to its *S*-ethylcysteine derivative. The peptide was hydrolysed for 16 h at 110°C in 6 N HCl-2 mM phenol in the vapour phase in vacuo. The hydrolysate was derivatised with phenylisothiocyanate as described in [8]. Ptc-amino acids were separated on a PICOTAG C_{18} column as described in the Waters Associates manual.

components is not explained by deamidation of the glutamine residue, oxidation of *S*-ethylcysteine, or peptide bond cleavage. Amino acid analysis confirmed that in both components, a single phosphoserine residue had been converted quantitatively to *S*-ethylcysteine (table 1, fig.2). The phenylthiocarbamyl (Ptc) derivative of *S*-ethylcysteine was found to elute at 9.15 min between Ptc-methionine (8.65 min) and Ptc-isoleucine (9.80 min) on the reverse-phase C_{18} column used for amino acid analysis (fig.2). These results demonstrate that the number of phosphoserine residues in a peptide can be quantified simply by chemical modification to *S*-ethylcysteine followed by amino acid analysis. Although the analysis illustrated in fig.2 was performed with 125 pmol peptide, as little as 10 pmol Ptc-*S*-ethylcysteine could be quantified by adjusting the sensitivity of the UV detector.

Incubation of the dephosphopeptide with ethanethiol in NaOH did not significantly affect its elution from the Vydac column, although it now eluted as a doublet, as observed with the modified peptide (fig.1). Gas-phase sequencer and FABMS analyses (not shown) and amino acid analysis (table 1) showed that the doublet was not explained by partial deamidation, oxidation or peptide bond cleavage. The reason why incubation with ethanethiol in NaOH generates multiple forms of the peptide is unclear. One possibility is amino acid racemisation to generate isomeric species that are partially resolvable by reverse-phase liquid chromatography.

Gas-phase sequencer analysis showed that *S*-ethylcysteine was the ninth residue in the modified peptide (not shown), the same site phosphorylated in the intact protein by cyclic AMP-dependent protein kinase. Pth-*S*-ethylcysteine was eluted just before diphenylthiourea, as reported by Meyer et al. [3]. The other four serine residues were unmodified and therefore detected as Pth-serine and its dithiothreitol adduct.

3.2. *Purification of a diphosphopeptide from inhibitor-2*

It was of interest to apply the above procedure to a peptide containing more than one site of phosphorylation. Following digestion of inhibitor-2 with trypsin and chromatography of the digest on a Vydac column, FABMS revealed the presence of a diphosphorylated form of the peptide EQESSGEEDSDLSPEER, corresponding to residues 117–133 of the protein [8]. This peptide was eluted at 41 min, 4 min earlier than the

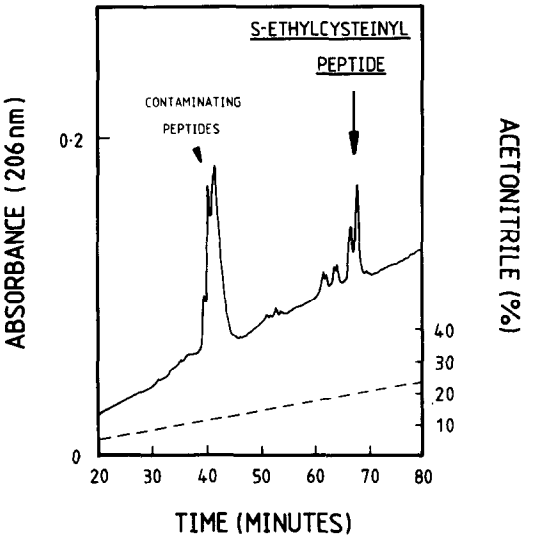

Fig.3. Separation of the diphosphopeptide EQES(P)S(P)GEEDSDLSPEER from contaminants, after conversion to its *S*-ethylcysteine derivative. Fractionation was carried out on a Vydac C_{18} column, as described in the legend to fig.1.

dephosphorylated derivative [6]. Since the diphosphopeptide was contaminated with other peptides, it was incubated with ethanethiol in NaOH and rechromatographed on the Vydac column. As shown in fig.3 the peptide now eluted approx. 25 min later at a 9% higher acetonitrile concentration (20 vs 11%). It was therefore completely resolved from all contaminating peptides whose elution positions were unchanged. Amino acid analysis of the modified peptide established that two of the four serine residues had been converted to *S*-ethylcysteine (table 1).

The presence of two *S*-ethylcysteine residues explains the much later elution of the modified peptide from the Vydac column (fig.3). A monophosphorylated form of EQESSGEEDSDLSPEER is also present in vivo, but at very low concentration [6]. The modified form of this peptide with one *S*-ethylcysteine residue is eluted from the column at 15.5% acetonitrile between the unmodified form and the peptide with two *S*-ethylcysteine residues (not shown). This demonstrates the potential of the method for the separation of multiply phosphorylated peptides.

The modified peptide containing two *S*-ethylcysteine residues was analysed on the gas-phase sequencer. *S*-Ethylcysteine was identified at residues 4 and 5, demonstrating that phosphoserine is present at these positions in vivo [6]. These sites are known to be phosphorylated in vitro by casein kinase II [9].

The procedures described in this paper provide a very sensitive method for identifying and purifying phosphoserine-containing peptides and do not require the presence of a radioactive (^{32}P) label. In conjunction with FABMS and the methodology described by Meyer et al. [3], they have recently been used to analyse the in vivo phosphorylation state of inhibitor-2 [6].

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