Sequence analysis of phosphoserine-containing peptides

Modification for picomolar sensitivity

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Received 29 May 1986

Sequencing of phosphoserine-containing peptides yields normally no identifiable PTH-derivatives at those positions where phosphoserine is located. Here a new method is described which allows identification of the position of phosphoserine by chemical modification just before sequence analysis. In a one-step micro-batch reaction, phosphoserine present in the intact peptide can be transformed quantitatively into stable derivatives such as β-methylaminoalanine (MAA), *S*-ethanolcysteine or *S*-ethylcysteine. These derivatives are detectable during microsequencing with less than 100 pmol peptide using an Applied Biosystems gas-phase sequencer equipped with an on-line PTH amino acid analyzer.

Phosphoserine derivatization *Sequence analysis* *Phosphopeptide* S-*Ethylcysteine* S-*Ethanolcysteine* *β-Methylaminoalanine*

1. INTRODUCTION

In eukaryotes, protein phosphorylation-dephosphorylation is one of the most important mechanisms for regulation of many intracellular functions [1]. Until now there have been many difficulties in localization of a phosphorylated residue. The strategy often employed consists of splitting such a phosphoprotein or peptide into small pieces. Then the amino acid composition rather than the amino acid sequence is used to identify the phosphorylated residue [2–4]. To overcome such difficulties Simpson et al. [5] have suggested transforming phosphoserine into cysteic acid and, alternatively, Kolesnikova et al. [6] into β-methylaminoalanine (MAA) by β-elimination and subsequent sulfite or methylamine addition, respectively. Clark and Dijkstra [7] improved the method by using dimethylamine or methylmercaptan as reagents for addition. With the latter thiol substitution the amino acid sequence of phosvitin, a protein exceptionally rich in phosphoserine, was established [8].

We have now succeeded in performing a one-step modification reaction allowing easy microsequencing of less than 100 pmol of a peptide containing phosphoserine.

2. MATERIALS AND METHODS

Kemptide (LRRASLG) was obtained from Serva. Ethanol was from Baker, dimethyl sulfoxide from Merck, methylamine from Linde and dithiothreitol (DTT) from Sigma. Acetonitrile, NaOH and mercaptoethanol were from Roth. Trifluoroacetic acid and ethanethiol were purchased from Pierce. Phenylisothiocyanate (PITC) was from Beckman. All reagents for sequence analysis were from Applied Biosystems. All other chemicals or solvents were of the highest purity available.

[γ-^{32}P]ATP was prepared according to Walseth and Johnson [9]. The catalytic subunit of the cAMP-dependent protein kinase was prepared according to Beavo et al. [10]. Phospho-Kemptide was prepared by incubation of 200 μg Kemptide

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00145793/86/$3.50

(260 nmol) in 5 mM magnesium acetate, 40 mM potassium phosphate, 7.5% glycerol, 0.1 mM dithiothreitol, 23 μg protein kinase (catalytical subunit) and 1 mM ATP containing 5×10^7 cpm [γ-^{32}P]ATP at pH 7.3 and 26°C for 30 min in a total volume of 4.1 ml [11].

Phospho-Kemptide was purified by reverse-phase HPLC using an 8 × 50 mm column filled with Vydac 218TP 5u material.

The phosphoserine residue was modified as follows: 1 nmol phospho-Kemptide was dried in a screw cap test tube (14 × 100 mm, Corning) and solubilized in 50 μl of the different modification mixtures. These consisted of 60 μl of either 10 M methylamine (freshly prepared from a gas container), or mercaptoethanol, or ethanethiol and 200 μl water, 200 μl dimethyl sulfoxide, 80 μl ethanol and 65 μl of 5 N NaOH. The addition of organic solvents protects the peptide from destruction [12]. These mixtures were incubated for 1 h at 50°C under nitrogen, the tubes then cooled and 10 μl acetic acid added. An aliquot of each mixture was directly analyzed in the gas-phase sequencer. Amino acid analysis was done by reverse-phase HPLC using precolumn modification with PITC [13,14].

Sequence analysis was performed on an Applied Biosystems gas-phase sequencer equipped with an on-line PTH amino acid analyzer, using the 03 RPTH program.

3. RESULTS AND DISCUSSION

Kemptide, one of the best small peptide substrates for cAMP-dependent protein kinase [11], was taken as a model peptide in this study. Phospho-Kemptide can easily be separated from the unphosphorylated peptide by reverse-phase HPLC as shown in fig.1. After purification both Kemptide and phospho-Kemptide were taken for subsequent experiments.

Fig.2 shows the PTH-chromatograms of all 7 degradation steps of Kemptide. It is clearly seen that the serine residue in cycle 5 yields two products, PTH-serine and the DTT adduct thereof. Under the conditions employed the peak area ratio is 25:75, as can be seen in fig.2. The same results were obtained when Kemptide was subjected to the modification conditions for phospho-Kemptide, demonstrating that the unmodified serine is not affected during the treatment with different modification mixtures (not shown). Upon Edman degradation of unmodified phospho-Kemptide, surprisingly, almost 100% of the phosphoserine in cycle 5 yields the DTT adduct of PTH-serine (fig.3). The ratio between the two possible PTH derivatives emerging from an unmodified serine during Edman degradation, as shown above, depends on the program used and other parameters of the gas-phase sequencer. An observed shift of this ratio towards the DTT adduct is therefore only unambiguously indicative of phosphoserine in our sequencer and under our conditions as given in section 2 (note given by Applied Biosystems). To establish a general procedure we have modified phospho-Kemptide with three different modification mixtures. Phosphoserine was converted into either MAA, *S*-ethanolcysteine or *S*-ethylcysteine by β-elimination and subsequent addition of either methylamine, mercaptoethanol or ethanethiol, respectively. Fig.4 shows the result

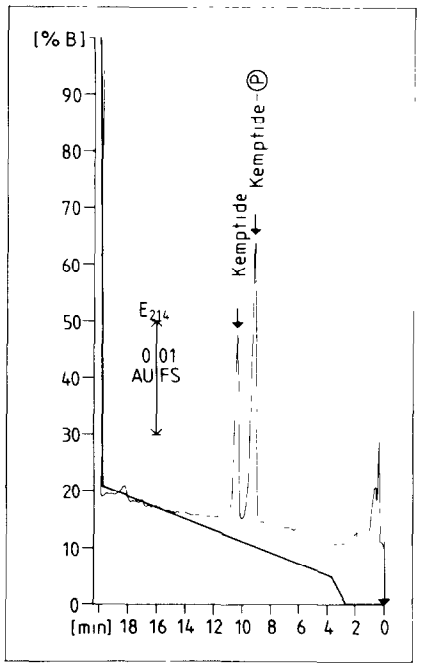

Fig.1. 5.5 nmol Kemptide and 7 nmol phospho-Kemptide were separated on a reverse-phase HPLC column and eluted with a gradient consisting of 0.1% trifluoroacetic acid in water (A) and 0.08% trifluoroacetic acid in 84% acetonitrile/16% water (B) at a flow rate of 3 ml/min.

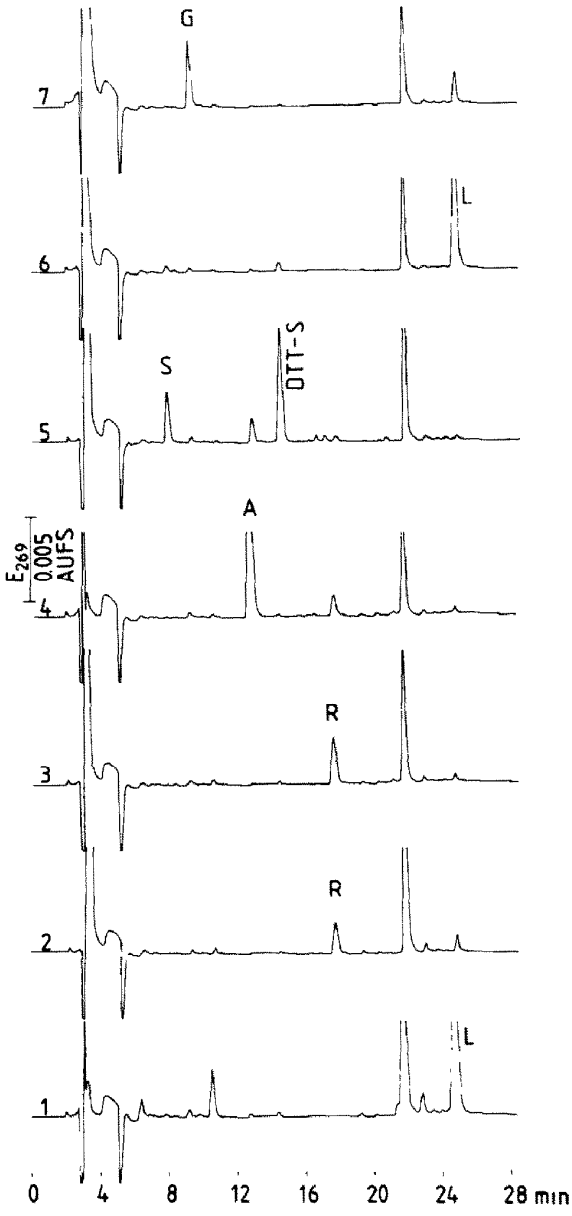

Fig.2. Sequence analysis of 120 pmol Kemptide.

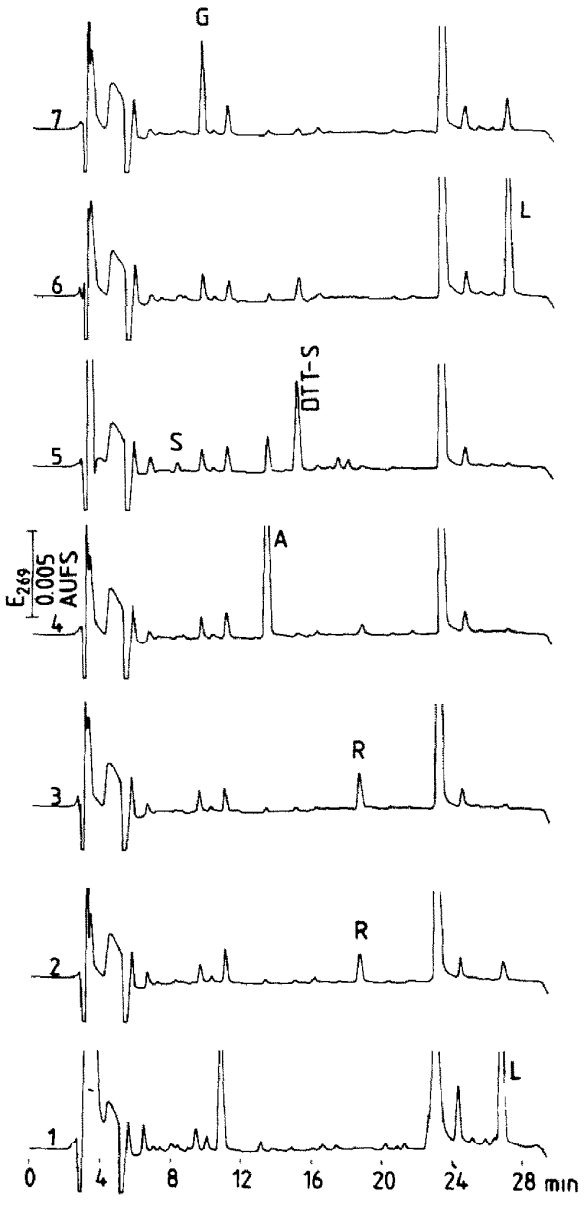

Fig.3. Sequence analysis of 100 pmol phospho-Kemptide.

of sequence analysis of the *S*-ethanolcysteine-containing Kemptide. The PTH derivative of *S*-ethanolcysteine elutes with the same retention time as the DTT adduct of PTH-serine [15].

Analogous chromatograms of the sequence analysis of MAA-Kemptide are shown in fig.5. PTH-MAA is detected in cycle 5 as a peak eluting just behind PTH-tryptophan as confirmed with synthetic PTH-MAA, which was kindly provided by Dr Annan [16]. In fig.6 the results of Edman degradation of the *S*-ethylcysteine-modified Kemptide are shown. The PTH derivative of *S*-ethylcysteine appears as a well-separated narrow peak just before the elution position of DPTU. This elution position is in agreement with the PTH-amino acid analysis of this derivative as

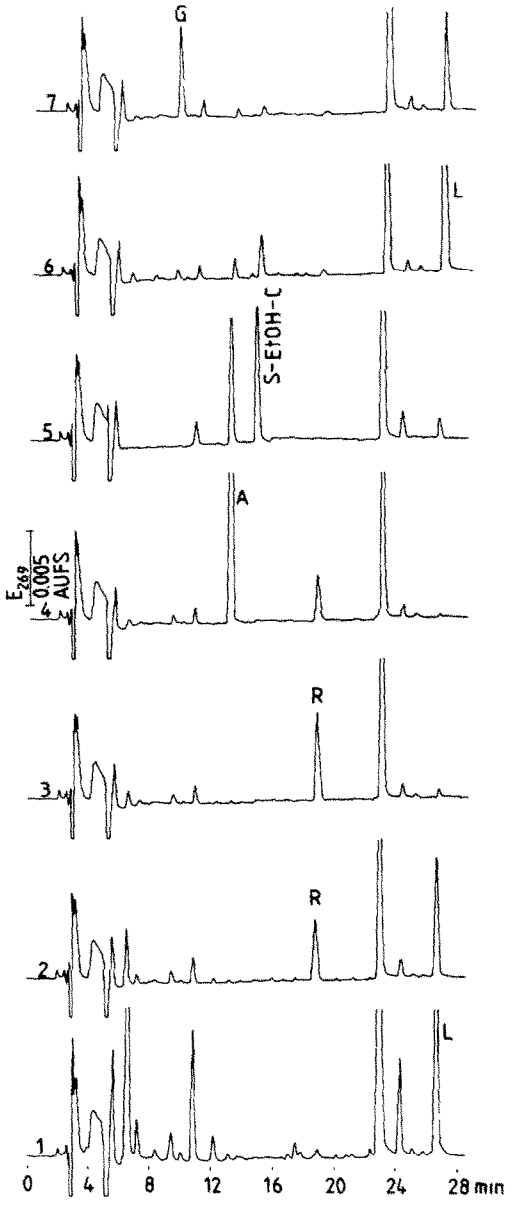

Fig.4. Sequence analysis of 130 pmol *S*-ethanolcysteine (S-EtOH-C)Kemptide.

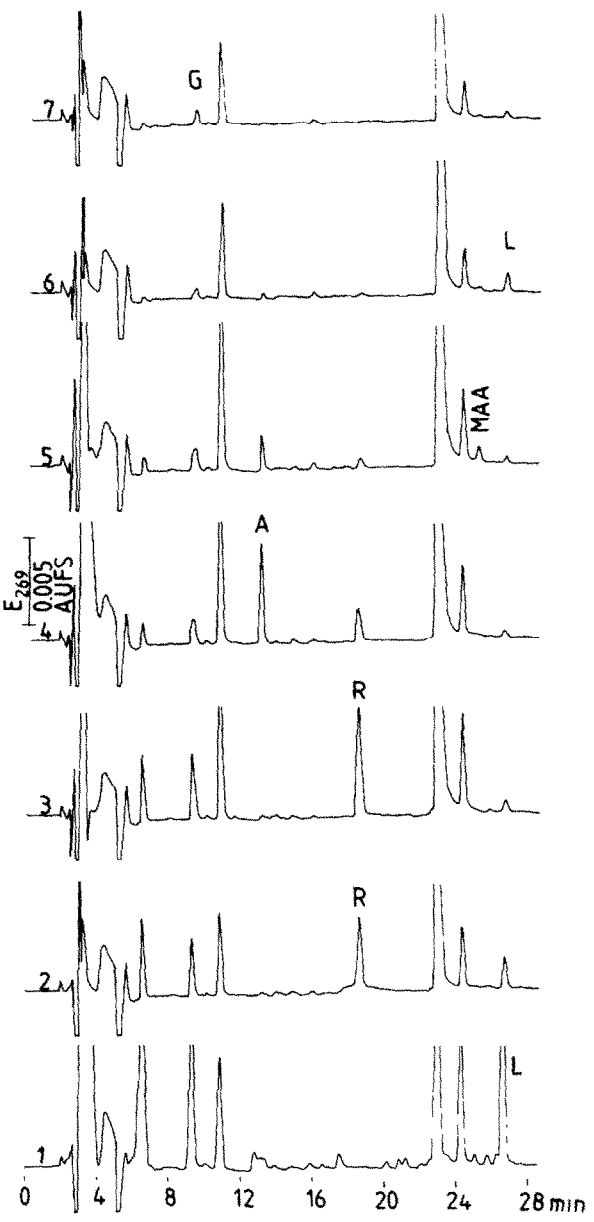

Fig.5. Sequence analysis of 150 pmol MAA-Kemptide.

reported by Pavlik and Kosta [15]. Some of the DTT adduct of PTH-serine can also be seen, resulting from partial β-elimination and dithiothreitol addition which occurs during conversion to the PTH derivative inside the conversion flask. However, this amount never exceeds 20% of the peak area of the PTH-*S*-ethylcysteine.

Of the different modification methods studied here, the transformation of phosphoserine to *S*-ethylcysteine seems to be best. All the others also work but the DTT and mercaptoethanol adducts of serine emerge at the same elution position, precluding definite identification of a phosphoserine residue. PTH-MAA yields a well-separated peak in the chromatogram. However, the introduction of MAA results in the incorpora-

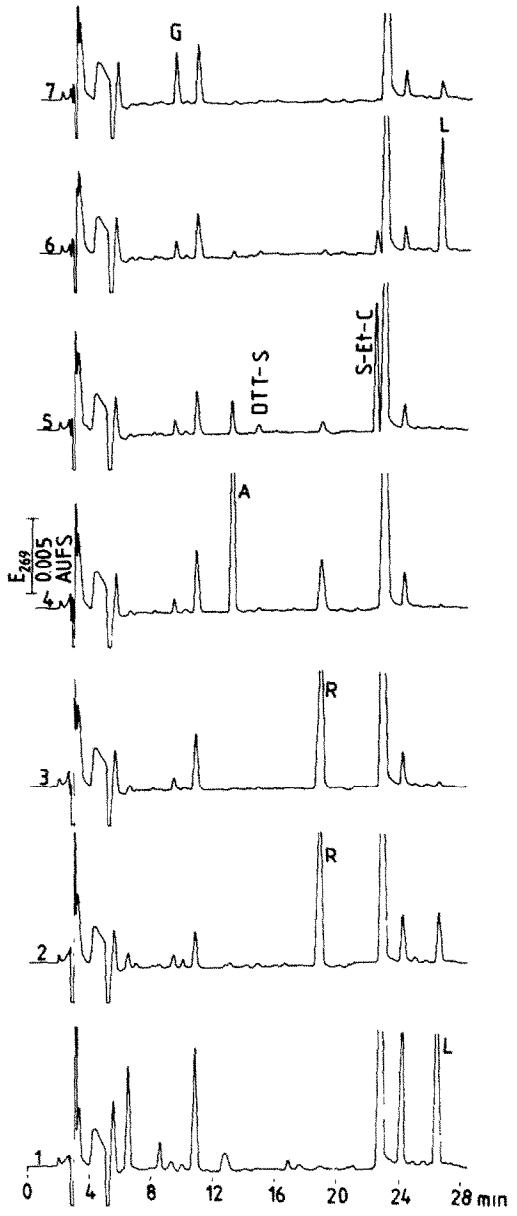

Fig.6. Sequence analysis of 120 pmol *S*-ethylcysteine (S-Et-C)Kemptide.

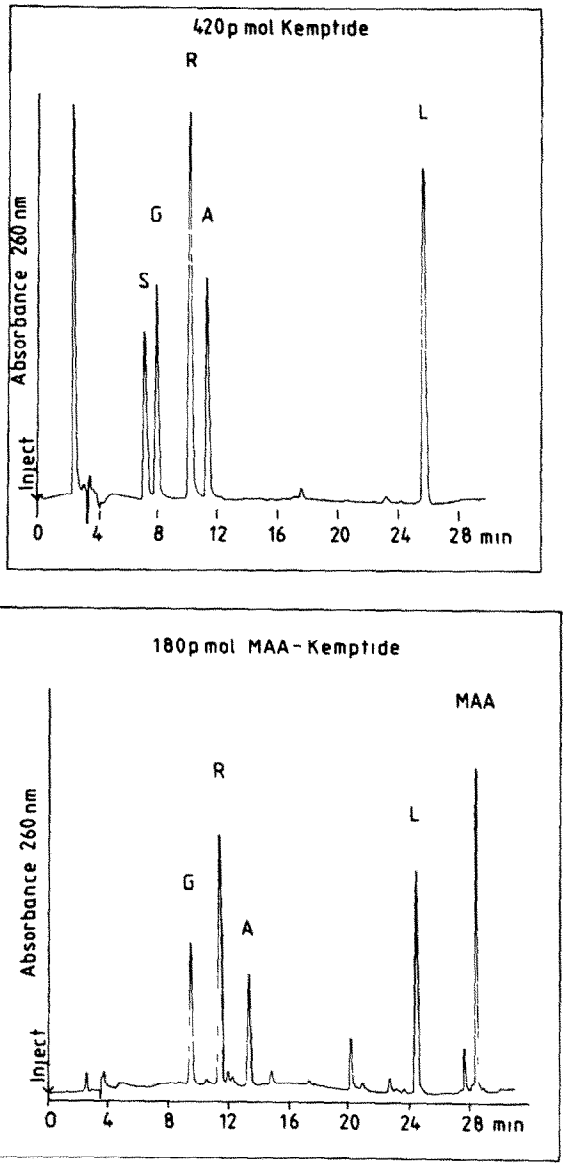

Fig.7. PTC-amino acid analysis of Kemptide and MAA-Kemptide. The PTC-amino acids were separated on a Beckman Ultrasphere ODS 5u column (4.6 × 250 mm) at 40°C and a flow rate of 1 ml/min. A gradient of 160 mM potassium acetate, pH 5.2, vs 84% acetonitrile was applied.

tion of a phenylthiocarbamyl residue into the β-amino group. Thereafter the peptide exhibits a more hydrophobic character following the first degradation step which lowers the repetitive yield due to enhanced wash out (see fig.5). Therefore the addition of ethanethiol following β-elimination of the phosphate group is the best choice out of the series shown. It also seems to be better suited than methylmercaptan, as suggested by Clark and Dijkstra [7], since liquid ethanethiol is safer to handle than gaseous methylmercaptan.

A suggested modification reaction is also

suitable for the determination of phosphoserine residues during amino acid analysis [17]. Fig.7 shows the results of the phenylthiocarbamyl-amino acid analysis of Kemptide and MAA-Kemptide. The sensitivity for the MAA derivative is similar to that of the other amino acid derivatives and determination of phosphoserine residues at the picomolar level is possible. It is a much more sensitive method than phosphate analysis [18], which requires at least 1 nmol phosphate for one determination.

A limitation to all three modification methods is a possible *O*-glycosylated serine which will result in the same derivatives as phosphoserine [12]. However, with prior enzymatic splitting off of a possible carbohydrate moiety the results will be unambiguous. No problems arise with phosphoserine-containing peptides if the phosphate is labeled with ^{32}P, the nature of the serine derivative therefore being known. Even if the peptide contains more than one serine residue there should be no problem in exact determination of the correct position of phosphoserine, since unmodified serine will be unaltered during the modification reaction.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft, SFB 168. The authors are grateful to Dr H.-H. Kiltz for valuable suggestions during this study.

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