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Identification of unusual (modified and non-encoded) amino acid residues in peptides by combinations of high-performance liquid chromatography and high-performance capillary electrophoresis with matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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Abstract

The techniques for micro-level analysis of some widespread unusual amino acids (phosphorylated and hydroxylated ones) as well as of some genetically non-encoded amino acids were developed for their subsequent identification in the peptide and protein amino acid sequence by narrow-bore column high-performance liquid chromatography (~10 pmol of the sample), high-performance capillary electrophoresis (~1–10 pmol), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (~1–10 pmol), and automatic protein gas phase sequencing (~1–50 pmol).

Keywords: Protein sequencing; Amino acids; Peptides; Amino acids, PTH derivatives; Proteins

1. Introduction

The amino acid composition and amino acid residue sequence analysis of peptides and proteins are necessary stages for investigation of structure–function relationship. The primary structure is one of the most important determinants of physiological activities and properties of peptides and proteins. Besides 20 ‘‘usual’’ amino acids, there are many other representatives of compounds differing from these ‘‘usual’’ amino acids in their side chain radical or in an attachment to the polypeptide chain, or in the polypeptide chain structure (most uncommon). Generally, these compounds represent results of post-translational modifications of amino acids encoded by the organism genome. Post-translational amino acid modifications are differentiated in various on the polypeptide skeleton or the side chain (modification at the aminoacyl-tRNA) as alkylation, arylation, desamidation, glycosylation, acylation, hydroxylation, phosphorylation, reduction, oxidation, halogenation, etc.

Post-translational modification of amino acid residues in peptides and proteins is an essential process occurring in different living organisms – from some viruses to mammals. Meanwhile, identification of the modified amino acids remains problematic upon structural investigations of peptides and proteins.

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Classical methods of amino acid analysis and protein sequence determination employ reaction conditions that may alter or destroy covalently modified residues. Furthermore, as identification usually envisages on the known chromatographic mobility of the 20 genetically coded amino acids, the modified residues may be lost or unrecognized.

There is another amino acid modification type called non-encoded amino acids, the synthetic analogs non-encountered in native peptides and proteins. Synthetic peptides containing these amino acids are of interest now, because of the necessity to obtain biologically active peptide analogs with altered properties and to investigate the mechanisms of fermentative reactions and intercellular interactions.

This study deals with the development of the technique for identification of modified and non-encoded amino acid residues in peptides by using a combination of instrumental micro-methods, including narrow-bore reversed-phase high-performance liquid chromatography (RP-HPLC), high-performance capillary electrophoresis (HPCE), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and peptide chemical sequencing.

The model synthetic peptides, containing non-encoded amino acid residues with cyclohexyl type radicals, such as 1-aminocyclohexane-1-carboxylic acid; 4-aminotetrahydropyrane-4-carboxylic acid and 1-aminocyclohexane-1,4-dicarboxylic acid, modified *O*-phosphorylated amino acid residues (*O*-phosphoserine, *O*-phosphothreonine and *O*-phosphotyrosine), and modified hydroxylated amino acid residue (δ-hydroxy-L-lysine) became the subjects of inquiry. The selection of the subjects, containing these unusual amino acids was made to develop identification techniques for medical diagnostics of several human disorders, including carcinogenic diseases as well as for intra- and extracellular process studies. The schedule of microanalysis of the unusual amino acids in peptides covers (i) micro-preparative synthesis of 3-phenyl-2-thiohydantoin (PTH) amino acid standards; (ii) isolation and purification of PTH-amino acids studied by RP-HPLC; (iii) confirmation of their homogeneity by HPCE; (iv) MALDI-TOF-MS confirmation of their nature; (v) identification of PTH-amino acids by narrow-bore RP-HPLC and micellar electrokinetic capillary chromatography (MECC) of PTH-amino acid mixtures, and (vi) sequencing of peptides containing unusual amino acid residues on gas phase sequencer with the subsequent identification of split amino acids in the form of the PTH by narrow-bore HPLC (~10 pmol), MALDI-TOF-MS (~1 pmol), and MECC (~1 pmol). Thus, the effectiveness of the hyphenated micro-analytical tools for unambiguous identification of the modified and non-encoded amino acid residues in peptides is demonstrated. In addition, a possible micro-quantitative identification of phosphorylated amino acid residues in peptides and proteins is shown without using radioactive isotope ^{32}P. Reliability, reproducibility, and high sensitivity of these methods provide a profound analysis of the above compounds at a micro-quantitative level.

2. Experimental

2.1. Chemicals and reagents

Boric acid, 2,5-dehydroxybenzoic acid, dimethylsulfoxide, β-mercaptoethanol were from Sigma (St. Louis, MO, USA). Benzene and pyridine were purchased from Merck (E. Merck, Darmstadt, Germany). Sodium dodecyl sulfate was from Bio-Rad Labs., Vienna, Austria. Ethyl acetate, 12.5% aqueous trimethylamine, 5% *n*-heptane phenylisothiocyanate, PTH-amino acids standards, *n*-heptane, *n*-butylchloride, polybrene and 25% aqueous trifluoroacetic acid (TFA) were from Perkin-Elmer (Warrington, UK). Sodium hydroxide, tetrahydrofuran, borax, hydrochloric acid, sulfuric acid, acetic acid, methanol and ethanol were from PO Azot (Novgorod, Russia). Acetonitrile was from Criochrom (Sankt-Petersburg, Russia). Purified water from a Milli-Q system (Millipore, Molsheim, France) was used throughout the experiments. All liquids used for HPLC systems were filtered through 0.22-μm (Millipore) membranes and degassed by helium for 3 min at 25°C. All solutions were mixed on the vibration mixer. Chloroform was distilled over magnesium sulfate, trifluoroacetic acid was refluxed over chromium oxide and then distilled.

2.2. Instrumentation

Peptide sequencing was performed on the gas phase protein peptide sequencer Model 477 A (Applied Biosystems, Foster City, CA, USA), equipped with a PTH Analyzer Model 120 A (ABI), dual channel recorder Kipp and Zonen (Amsterdam, The Netherlands) and a printer Hewlett-Packard (Singapore). HPCE system Model 270 A (ABI) was used. The chromatograms were recorded on Shimadzu Chromatopac C-R3A integrator (Shimadzu, Kyoto, Japan). HPLC analyses were performed on two systems. System 1 consisted of the narrow-bore high-performance liquid chromatograph Milichrom A-02 (Envirochrom A-02) (Chromatography Institute Eko-Nova, Novosibirsk, Russia) equipped with two 2.5-ml syringe pumps, thermostated column compartment, an autosampler, photometric cell with 1.2 μl volume, and UV spectrophotometer (190–360 nm). System 2 included two pumps Model 114M, spectrophotometer Model 160A, Controller Model 421, and an injector Model 210 (all from Beckman Instruments, San Ramon, CA, USA). The photometer detector output was recorded on Shimadzu Chromatopac C-R3A integrator and on a dual channel recorder Model 2210 (LKB, Sweden). For spectrophotometric data acquisition and analysis Milichrom software and ''MultiChrom for Windows'' program (Ampersend, Moscow, Russia) were employed. Mass spectra were obtained on a MALDI-TOF-MS system Vision 2000 (Thermo Bioanalysis, UK). The Alkalit indicator (pH 6.0–10.0) (E. Merck, Darmstadt, Germany), universal indicator (Brno, Czech Republic) and Digital pH meter pH 525 (Wissenschaflich-Technische Werkstatten, Germany) were used for pH measurement. The centrifuge Model CLN 12 (Nauchpribor, Lvov, Ukraine), microcentrifuge Eppendorf 1540 (Eppendorf, Germany), vibrating mixer IKA Vibro Fix (IKA, Germany), and water multi block heater (Lab-Line Instruments, IL, USA) were applied during the procedure. A rotor evaporator Rotavapor Re 111 (Buchi, Switzerland) with water bath Model 461 (Buchi), and the analytical balance Model H-12210-00 (Sartorius, Germany) were in use. The lyophilizing dryer Alpha 2-4 Loc-1 (Christ, Germany) with oil pump 2NVR-5D-M (Moscow, Russia) was employed.

2.3. Synthesis of PTH 1-aminocyclohydrohexane-1-carboxylic acid (PTH-AA1), PTH 4-aminotetrahydropyrane-4-carboxylic acid (PTH-AA2), PTH 1-aminocyclohydrohexane-1,4-dicarboxylic acid (PTH-AA3)

A 1-mg amount of amino acid was dissolved in 38 μl of pyridine–water (1:1) in the 0.4-ml polypropylene tube, then pH was increased up to ~8.6 and kept at this level by addition of 2 *M* sodium hydroxide. The temperature of the mixture increased up to 40°C and kept for 10 min; 10 μl of 10% phenylisothiocyanate in acetonitrile was pipetted into the reaction mixture, the tube was capped and mixed, pH was kept at 8.6 by addition of 2 *M* sodium hydroxide (5–10 μl). The reaction mixture was incubated at 40°C for 30 min, extracted by 0.2 ml of benzene, and 10 μl of 2 *M* hydrochloric acid was added to the remaining water solution. Another portion (2–4 μl) of hydrochloric acid was added to the reaction mixture, then cooled by ice to decrease the reaction pH to ~1. The temperature of the reaction mixture was raised up to 80°C and kept for 10 min. Phenylthiocarbamyl amino acid was precipitated from the solution as white sediment. The reaction mixture was rapidly extracted by ethyl acetate (3×0.2 ml), the ethyl acetate extract was evaporated to dryness, 0.05 ml of hot 100% acetic acid was added to the residue, and water (30–80 μl) was pipetted to the tube to precipitate PTH-amino acid. The tube content was then evaporated to dryness.

2.4. Preparation of the modification mixtures

The mixture for phosphoserine modification contains 33% (v/v) of dimethyl sulfoxide (DMSO), 13.2% (v/v) of ethanol, 10.7% (v/v) of 5 *M* sodium hydroxide, 9.2% of β-mercaptoethanol and water. The mixture for *O*-phosphothreonine modification contains 10.7% of 7 *M* potassium hydroxide instead of sodium hydroxide.

2.5. Synthesis of PTH-S-ethanolcysteine

A 1-mg amount of *O*-phosphoserine (5.41 μmol) was dissolved in 50 μl of the modification mixture in the 0.4-ml polypropylene tube and incubated at 50°C

for 60 min. The reaction mixture was cooled to room temperature and 10 μl of 100% acetic acid was added. Pyridine–water (1:1, 40 μl) was added to the tube, then pH was increased to ~8.6 and kept at this level by adding 2 *M* sodium hydroxide. The mixture temperature was increased up to 40°C and kept for 10 min; 10 μl of 10% phenylisothiocyanate in acetonitrile was pipetted into the reaction mixture, the tube was capped and mixed, pH was kept at 8.6 by addition of 10 μl of 2 *M* sodium hydroxide. The reaction mixture was thermostated at 40°C for 30 min, the white precipitate came out during thermostating, extracted by of benzene (3×0.2 ml) with the dissolving precipitate, 10 μl of 2 *M* hydrochloric acid was added to the remaining water solution. Another portion (2–4 μl) of hydrochloric acid was added to the reaction mixture cooled by ice to decrease the reaction pH to ~1. The temperature of the reaction mixture was raised up to 80°C and kept for 10 min. Phenylthiocarbamyl ethanolcysteine was precipitated from the solution as white sediment. The reaction mixture was rapidly extracted by ethyl acetate (3×0.2 ml), which was then evaporated to dryness, 0.05 ml of hot 100% acetic acid was added to the residue and water (30–80 μl) was pipetted to the tube to precipitate PTH-ethanolcysteine. The tube content was then evaporated to dryness.

2.6. Synthesis of PTH-3-methyl-S-ethanolcysteine

The procedure was the same as in Section 2.4, but the volume of 10% phenylisothiocyanate in acetonitrile was doubled and the corresponding modification mixture was in use.

2.7. Synthesis of PTH-ε-phenylthiocarbamoil-δ-hydroxylysine

The procedure was the same as in Section 2.3, but the doubled volume of 10% phenylisothiocyanate was used.

2.8. Synthesis of PTH-O-phosphotyrosine

The procedure was the same as in Section 2.3, but the stage of 10% phenylisothiocyanate was prolonged up to 20 min under a higher temperature (55°C).

2.9. Modification procedure of O-phosphoserine containing peptide

A 1-mg amount of *O*-phosphorylated serine (S*) containing peptide R-K-S*-I-R-I-G-P-G-Q-T-F-Y-A-T-G was dissolved in 400 μl of modification mixture (see Section 2.3) and incubated at 50°C for 60 min, then the reaction mixture centrifuged at 10 000 *g* for 5 min and subjected to HPLC analysis (Section 2.10).

2.10. Chromatography

2.10.1. RP-HPLC of PTH-AA1, PTH-AA2 and PTH-AA3 reaction mixtures

System 2 (see Section 2.2): Samples (10–50 μl) were injected manually into the Model 210 injector loop by the Hamilton syringe (Hamilton, Reno, NV, USA). A column (250×4.6 mm) packed with 5 μm particles of Ultrasphere ODS (Beckman Instruments) was used. The column temperature was 25°C, the flow-rate was 0.5 ml/min, detection wavelength was 280 nm, and 0.1% TFA in acetonitrile–water (50:50, v/v) was used for isocratic elution. The analysis time was 40 min.

2.10.2. RP-HPLC of the PTH-S-ethanolcysteine, PTH-3-methyl-S-ethanolcysteine and PTH-O-phosphotyrosine reaction mixtures

System 2 (see Section 2.2): The reaction mixture (10-50 μl) was injected manually into the Model 210 injector loop by the Hamilton syringe. A column (250×4.6 mm) packed with 5 μm particles of Ultrasphere ODS (Beckman Instruments) was used. The column temperature was 25°C, the flow-rate 0.4 ml/min, detector wavelength 280 nm. The elution gradient profile was as follows: profile 2.1.: 0 min – 10% B; 30 min – 90% B; 35 min – 90% B; 40 min – 10% B. The analysis time was 45 min. The elution solvent A was 0.1% TFA in water (pH 2.2), solvent B was 0.1% TFA in acetonitrile.

2.10.3. RP-HPLC of the PTH-ε-phenylthiocarbamyl-δ-hydroxylysine reaction mixture

System 2 (see Section 2.2): The reaction mixture (10–50 μl) was injected manually into the Model 210 injector loop by the Hamilton syringe. A column

(250×4.6 mm) packed with 5 μm particles of Ultrasphere ODS (Beckman Instruments) was used. The column temperature was 25°C, the flow-rate 0.4 ml/min, detector wavelength 280 nm. The elution gradient profile was as follows: profile 2.2.: 0 min – 20% B; 30 min – 90% B; 35 min – 90% B; 40 min – 20% B. The analysis time was 45 min. The elution solvent A was 0.1% TFA in water (pH 2.2), solvent B was 0.1% TFA in acetonitrile.

2.10.4. RP-HPLC of peptides containing modified or non-encoded amino acid residues

2.10.4.1. RP-HPLC of the phosphorylated and non-phosphorylated peptides

System 1 (see Section 2.2): Samples were injected using autosampler into a 75×2 mm narrow-bore column packed with 5 μm particles of Nucleosil C_{18}, pore size 120 Å (Machery–Nagel, Germany). The temperature was 35°C, the flow-rate 75 μl/min; detector dual wavelengths were 210 nm and 280 nm. The elution gradient profile was as follows: profile 1.1: 0 μl – 0% B, 2800 μl – 45% B. Elution solvent A was 0.1% TFA in water (pH 2.2), solvent B was acetonitrile.

2.10.4.2. RP-HPLC of modified S-etanolcysteine containing peptide reaction mixture

System 1 (see Section 2.2): Using an autosampler, the samples were injected into a 75×2 mm narrow-bore column packed with 5 μm particles of Nucleosil C_{18}, pore size 120 Å (Machery–Nagel). The temperature was 35°C, the flow-rate 75 μl/min; detector dual wavelengths were 210 nm and 280 nm. The primary isolation elution gradient profile was as for profile 1.1, and re-chromatography gradient profile was as follows: profile 1.2: 0 μl – 8% B, 1250 μl – 27% B, 2800 μl – 29% B. The elution solvent A was 0.1% TFA in water (pH 2.2), solvent B was acetonitrile.

2.11. Mass spectrometry

Mass spectrometric analyses of PTH-amino acid reaction mixtures, RP-HPLC refined PTH-amino acids, and of the peptides were performed on matrix-assisted laser desorption ionization time-of-flight Vision 2000 (Thermo Bioanalysis) in the reflectron mode with the 2,5-dehydrobenzoic acid matrix. The sample volume deposited on a target was 0.2–0.4 μl. The nitrogen laser (337 nm) with pulse energy 47–55 mW (~120 μJ) was employed. Laser shots varying from 10 to ~100 required to obtain good spectra. Laser shot pulse was 3 ns. Accelerating voltage was 50 002 mV, polarity positive.

2.12. High-performance capillary electrophoresis

2.12.1. HPCE of synthesized PTH-amino acids

Homogeneity of the synthesized and RP-HPLC refined PTH-amino acids was evaluated by MECC on the HPCE system Model 270 A (ABI) in fused capillary [75 cm (effective length 50 cm)×55 μm I.D.] with photometric detection at 269 nm in 200 m*M* sodium dodecyl sulfate, 5 m*M* sodium borate (pH 9.0) buffer. The sample was injected by vacuum for 1–10 s. The voltage was 30 kV, the current was ~35 μA, and analysis temperature 30°C.

MECC analyses of the PTH-amino acid mixtures containing the studied ones were performed under the same conditions.

2.12.2. HPCE of peptides

Homogeneity of RP-HPLC isolated peptides containing modified (R-K-S*-I-R-I-G-P-G-Q-T-F-Y-A-T-G) or non-encoded (I-G-AA1-F-G, F-G-AA2-Y-A) amino acid residues was tested by HPCE on the Model 270 A HPCE system (ABI) in fused capillary [75 cm (effective length 50 cm)×55 μm I.D.] with photometric detection at 210 nm, in 30 m*M* sodium phosphate (pH 9.5) buffer. The sample was injected by vacuum for 1–10 s. The applied voltage was 20 kV, the current was ~45 μA.

2.13. Peptide sequencing

The peptides studied were sequenced on a gas phase protein sequencer Model 477 A (ABI) and PTH-amino acids cleaved were identified on a PTH Analyzer Model 120 A (ABI) at the 269 nm wavelength. The elution mobile phase was: (A) standard ABI acetate buffer containing 5% tetrahydrofuran, (B) acetonitrile.

3. Results and discussion

Peptides, containing genetically non-encoded amino acids, in particular, with cyclic side-chain radicals (cyclopentyl, cyclohexyl and their substituted analogs) are more often used for research into the ligand–receptor interaction and mechanisms of fermentative reactions. Methods for identifying these amino acids in amino acid sequencing are necessary for verification of synthesized peptide structures. Their presence could be determined by mass spectrometric fragmentation [1–3,22] or direct chemical Edman degradation [4–6,23]. We prefer to choose joint application of instrumental micro-methods (HPLC, HPCE, MS and automatic sequencing) that allow us to increase the reliability of the above amino acid residue determination at a micro-level. For identifying non-encoded amino acids [1-aminocyclohexane-1-carboxylic acid (AA1), 4-aminotetrahydropyrane-4-carboxylic acid (AA2), and 1-aminocyclohexane-1,4-dicarboxylic acid (AA3)] (Figs. 1–13), PTH-amino acid derivatives were synthesized, then RP-HPLC refined, their homogeneity was tested by HPCE, and the structure was confirmed by MALDI-TOF-MS (Figs. 4, 8 and 11). RP-HPLC and MECC techniques for separation of the mixtures containing PTH derivatives of standard amino acids as well as PTHs of unusual amino acids were developed for common Edman degradation method applied for sequencing the peptides with AA1, AA2, AA3 incorporated residues (Figs. 1, 5, 9

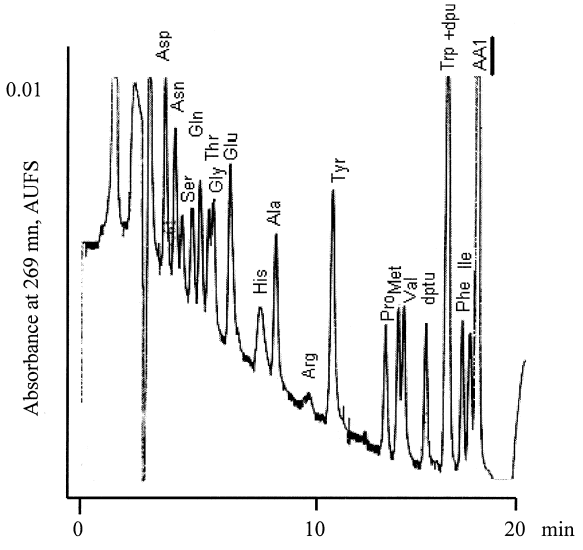

Fig. 1. RP-HPLC of the PTH-amino acid mixture (~20 pmol each of 19 usual PTH amino acids and ~35 pmol of PTH-AA1). Column was PTH C_{18} (220×2.1 mm, 5 μm, Brownlee); flow-rate 325 μl/min; detection at 269 nm; eluents: (A) 5% tetrahydrofuran in water, (B) acetonitrile; gradient profile was 0–0.04 min – 8% B, 8–44% B during 17.6 min, 44–90% B during 0.5 min, 90% B during 2.5 min, 90–8% B during 0.5 min. The total analysis time was 21.5 min. dpu=1,3-diphenylurea.

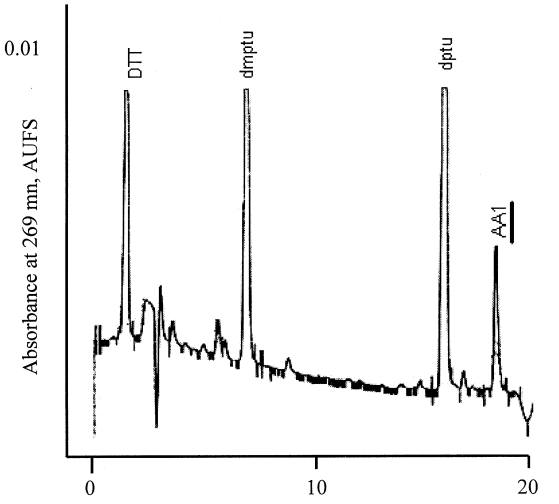

Fig. 2. RP-HPLC of the synthesized PTH-AA1 (~15 pmol) performed under the same conditions as described in Fig. 1. Time scale in min. DTT=dithiothreitol, dmptu=N,N-dimethyl-N′-phenylthiourea, dptu=N,N′-diphenylthiourea.

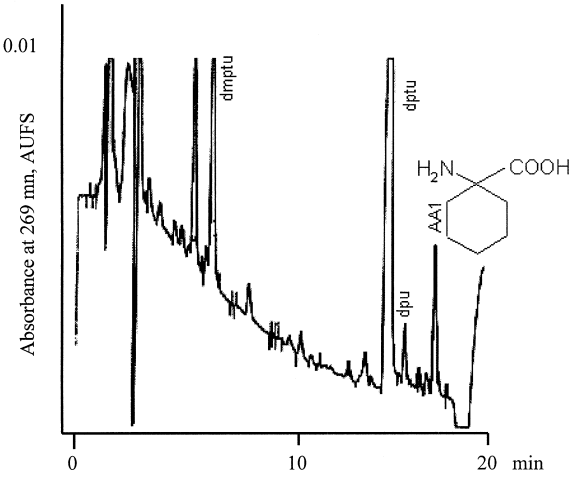

Fig. 3. RP-HPLC of the third N-terminal degradation cycle of the peptide Ile–Gly–AA1–Phe–Gly (~15 pmol). RP-HPLC performed under the same conditions as described in Fig. 1.

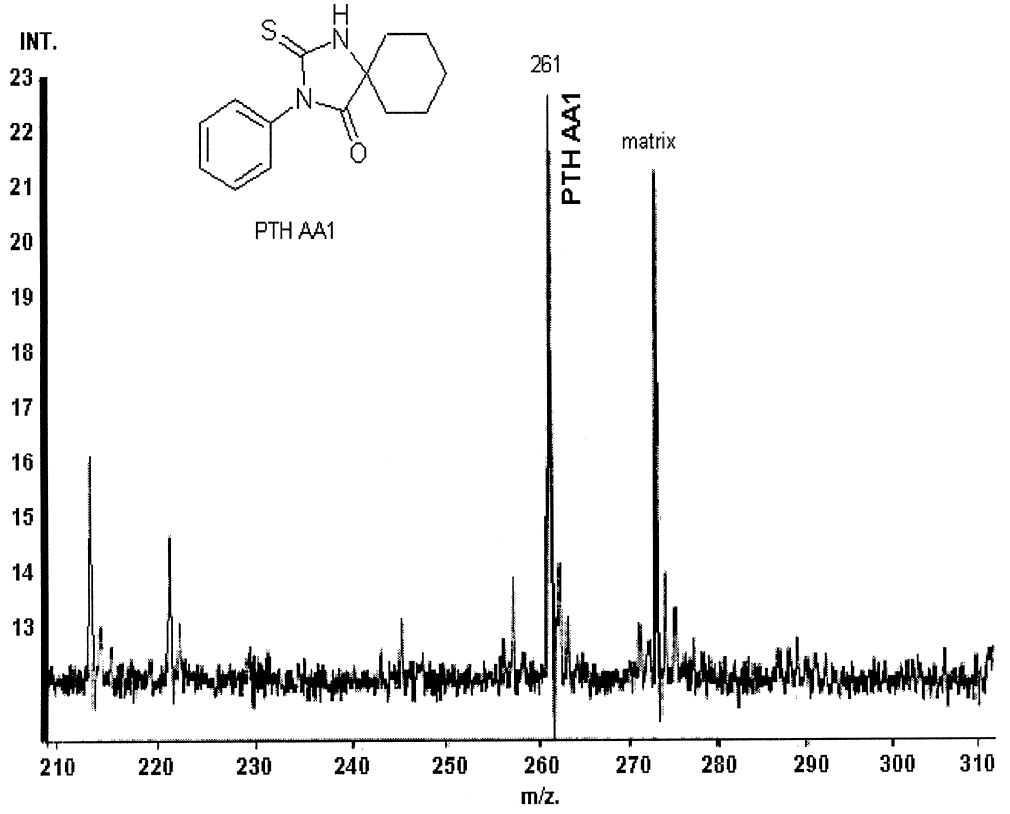

Fig. 4. Mass spectrum of PTH-AA1 (molecular formula $C_{14}H_{16}N_2OS$; M_r 260.36). *y*-Axis: intensity (%).

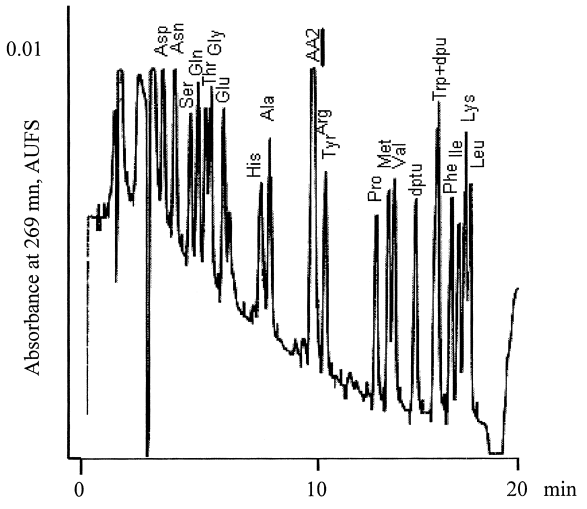

Fig. 5. RP-HPLC of the PTH-amino acid mixture (~20 pmol each of 19 usual PTH amino acids and ~35 pmol of PTH-AA2) performed under the same conditions as described in Fig. 1.

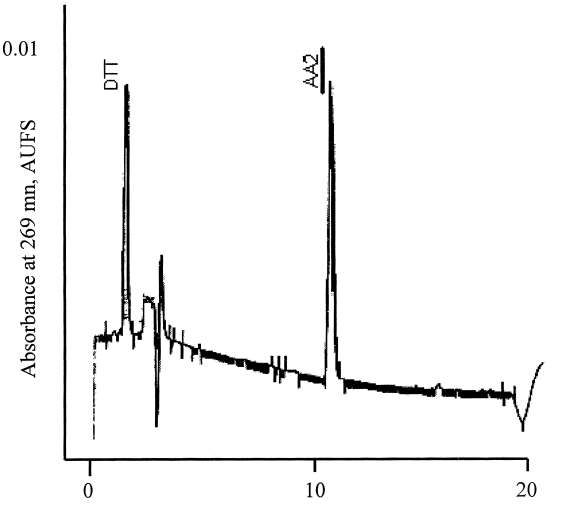

Fig. 6. RP-HPLC of the synthesized PTH-AA2 (~20 pmol) performed under the same conditions as described in Fig. 1. Time scale in min.

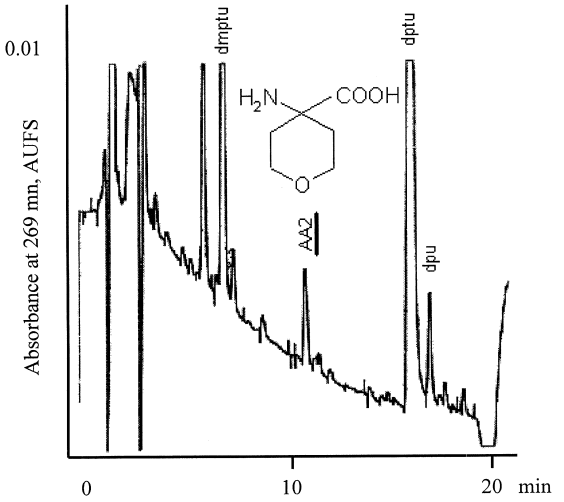

Fig. 7. RP-HPLC of the third N-terminal degradation cycle of the peptide Phe–Gly–AA2–Tyr–Ala (~15 pmol). RP-HPLC performed under the same conditions as described in Fig. 1.

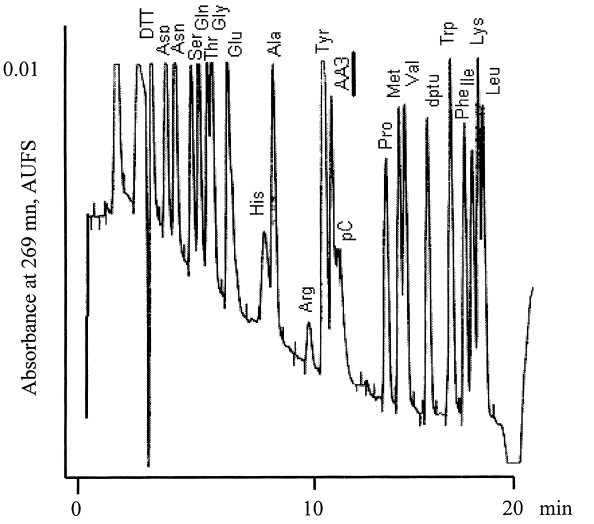

Fig. 9. RP-HPLC of the PTH-amino acid mixture (~20 pmol each of 19 usual PTH amino acids and ~15 pmol of PTH-AA3) performed under the same conditions as described in Fig. 1.

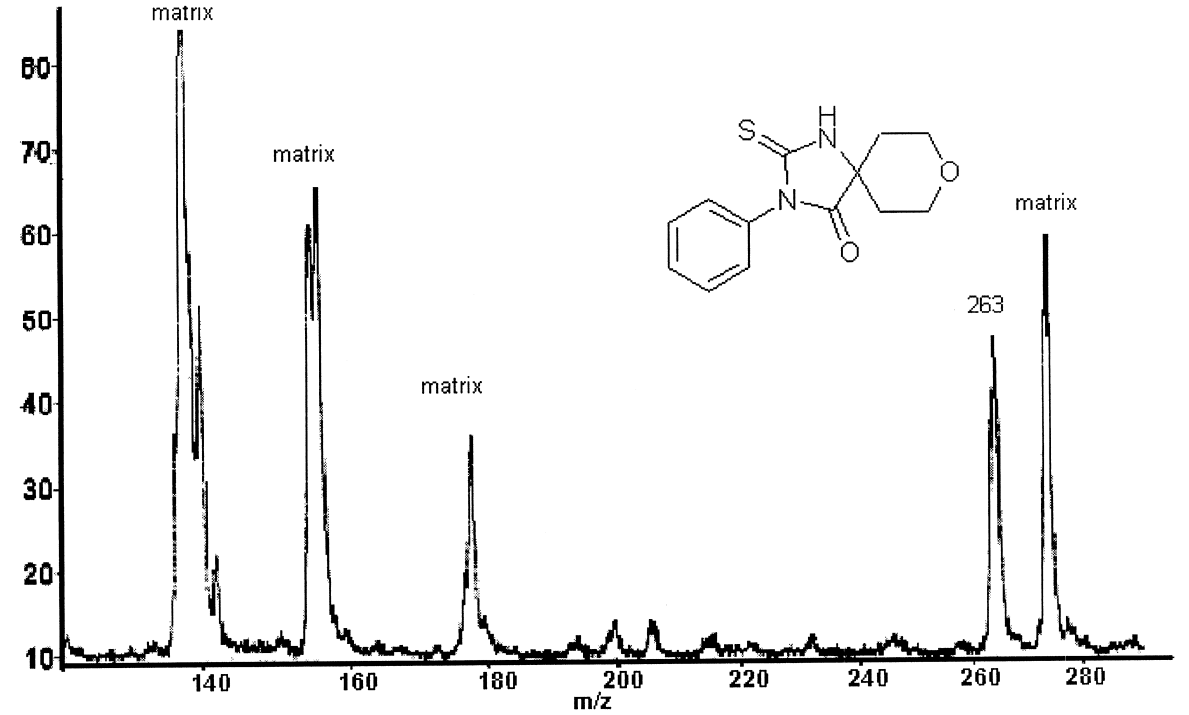

Fig. 8. Mass spectrum of PTH-AA2 (molecular formula $C_{13}H_{14}N_2O_2S$; M_r 262.33). *y*-Axis: intensity (%).

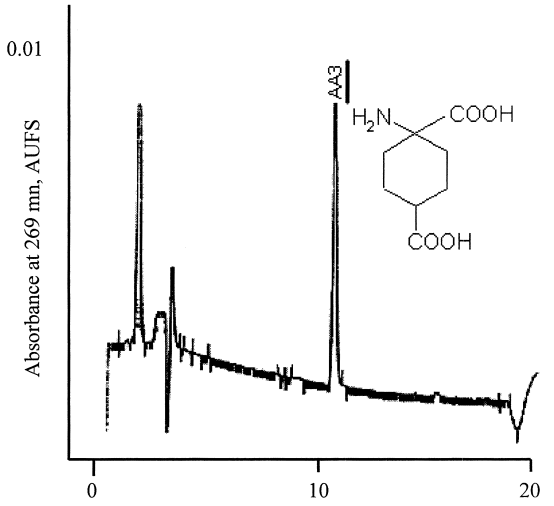

Fig. 10. RP-HPLC of the synthesized PTH-AA3 (~20 pmol) performed under the same conditions as described in Fig. 1. Time scale in min.

and 12). The difference in electrophoretic (Fig. 12) and chromatographic (Figs. 1–3, 5–10) mobility demonstrated their side-chain radical distinctions. The enumerated techniques were employed for primary structure analysis of the model peptides containing AA1 and AA2 residues (Figs. 3 and 7). For the peptide structure elucidation the PTH-δ-hydroxylysine derivative was synthesized and underwent HPLC and MS analyses (Fig. 19), which we propose to use for collagen peptides sequencing.

Protein phosphorylation is a reversible post-translational modification catalyzed by protein kinases, while dephosphorylation is catalyzed by phosphatases. Phosphorylation of specific amino acid residues alters protein activities and functions and plays an important role in cell processes, especially in signal transduction leading to normal cell proliferation and differentiation [6]. Moreover, mutations or overexpressions of cellular kinases were implicated in the progressive transformation of a normal cell to a cancer one [7]. Indeed, most onkoproteins known today are protein tyrosine kinases [8]. In eukaryotic cells, it is reported that approximately 90% of all cellular phosphorylation occurs on serine, whereas 9.9% occurs on threonine and only 0.1% on tyrosine residue [7].

Determination of protein phosphoamino acids is very important for elucidating molecular mechanisms of cellular functions controlled by protein phosphorylation. Commonly employed techniques for qualitative or semiqualitative determination of phosphorylated residues involve in vitro or in vivo labeling of the examined protein with ^{32}P followed by chromatography or electrophoresis and au-

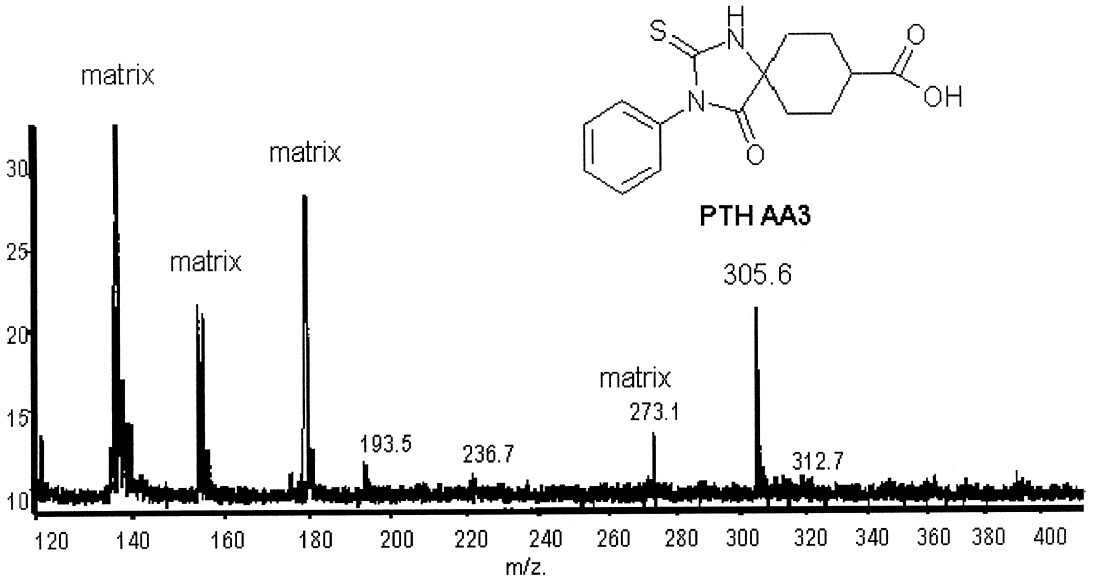

Fig. 11. Mass spectrum of PTH-AA3 (molecular formula $C_{15}H_{16}N_2O_3S$; M_r 304.37). *y*-Axis: intensity (%).

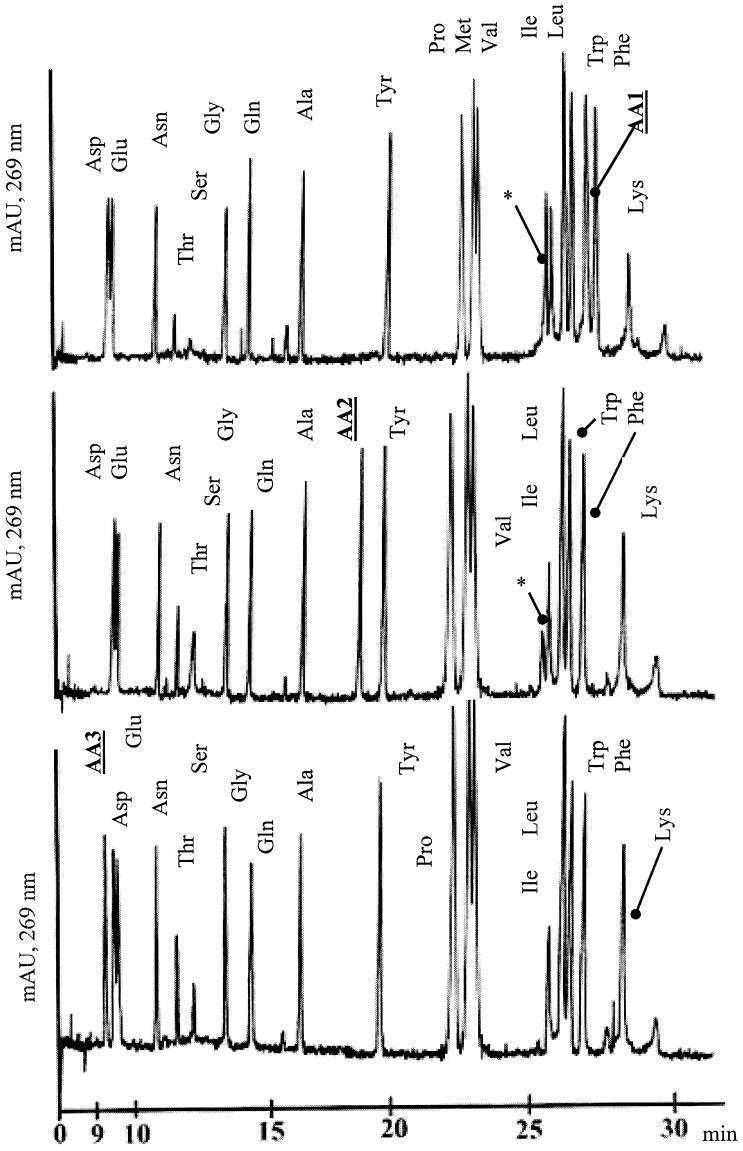

Fig. 12. MECC identification of PTH-AA1, PTH-AA2, PTH-AA3 in PTH-amino acid mixture. Peaks marked by asterisks correspond to sample impurities.

toradiography [7,9,10,24]. There is a great interest in identifying the locations of phosphorylation sites on phosphoproteins in order to explore the protein kinase specificity and to understand the phosphorylation influence on different biological functions. A problem encountered in sequencing phosphopeptides is that the standard Edman degradation procedure fails to yield stable PTH derivatives of phosphoserine and phosphothreonine. The PTH derivatives of these residues produced upon Edman degradation spontaneously break down during the acid cleavage step with a release of inorganic phosphate [4]. The common methods used to locate phosphorylation sites embraced radioactively labeled phosphopeptides with subsequent measuring ^{32}P release in the eluate of each cycle of Edman degra-

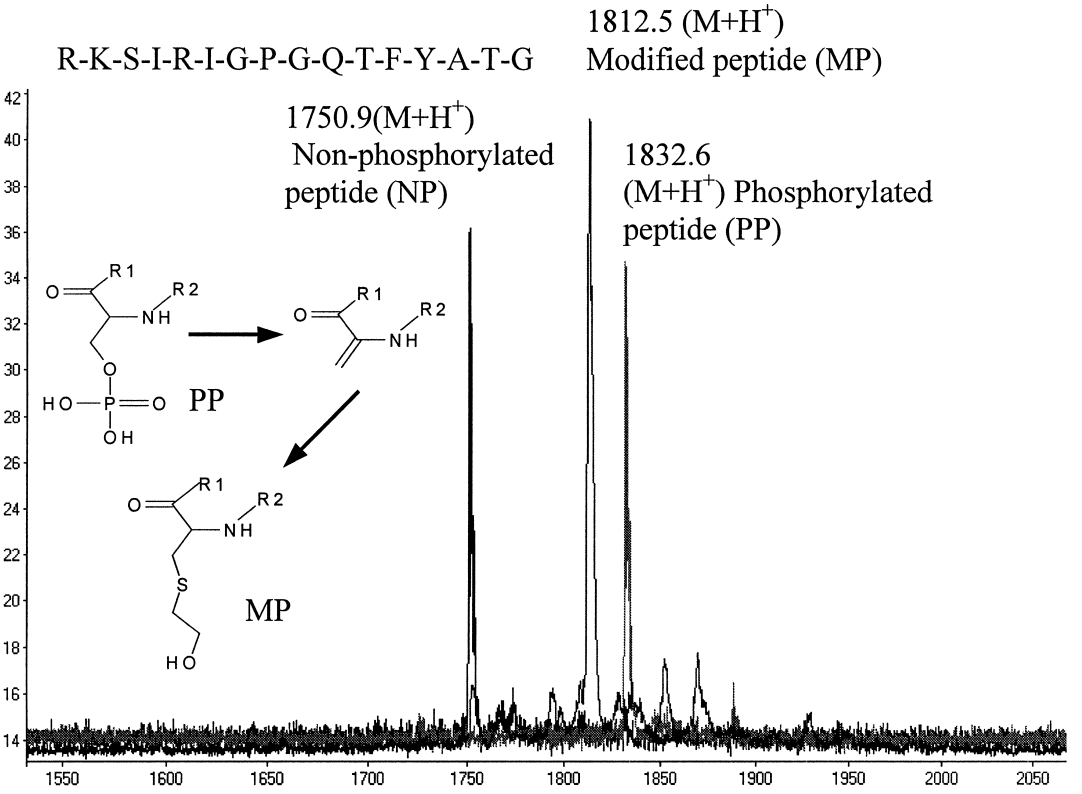

Fig. 13. Mass spectra of the peptide R-K-S-I-R-I-G-P-G-Q-T-F-Y-A-T-G forms. *x*-Axis: *m*/*z*; *y*-axis: intensity (%).

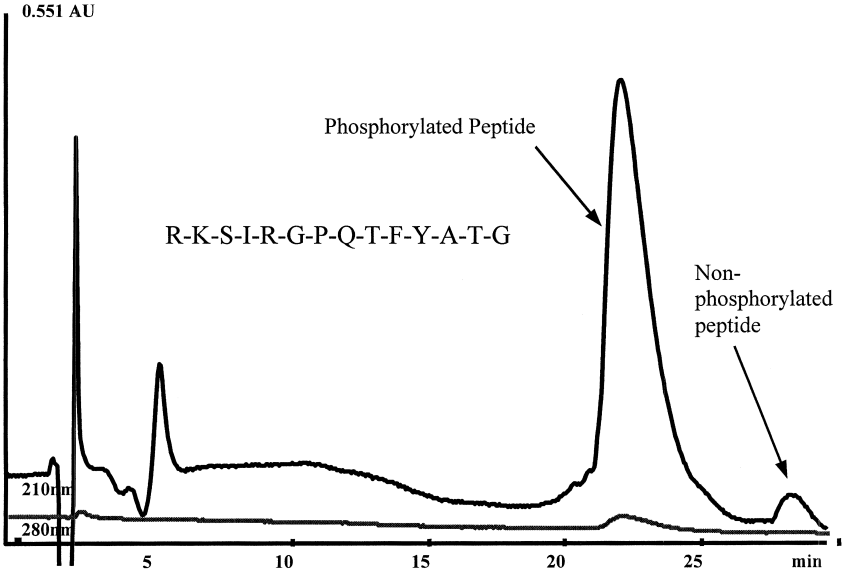

Fig. 14. Separation of phosphorylated peptide R-K-S*-I-R-I-G-P-G-Q-T-F-Y-A-T-G from its non-phosphorylated one in HPLC System 1 with the 1.1 elution gradient profile.

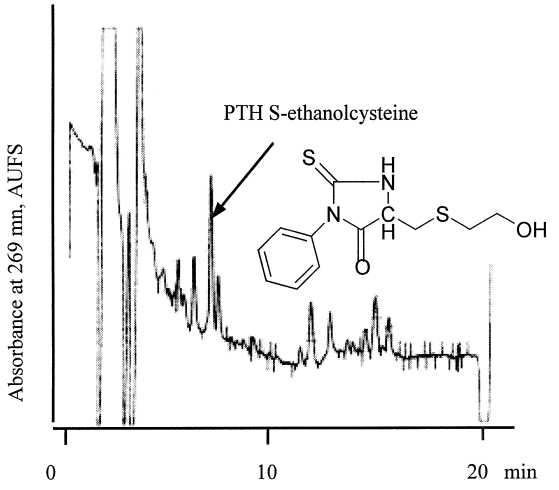

Fig. 15. RP-HPLC of the third N-terminal degradation cycle of the modified R-K-S*-I-R-I-G-P-G-Q-T-F-Y-A-T-G peptide (~10 pmol). RP-HPLC performed under the same conditions as described in Fig. 1.

dation in a solid-phase or a spinning cup sequencer [11,12]. Gas phase sequencing is less suitable for this purpose, because ^{32}P radioactivity is not detected in the eluate from the cycles corresponding to phosphorylated residues, almost all ^{32}P radioactivity remains on the reactor filter unless special extraction cycles are employed [13]. To overcome the problems in localization of phosphorylated amino acid residues, Kolesnikova et al. [14] suggested phosphoserine transformation into β-methylaminoalanine, and Simpson et al. [15] preferred the conversion into cysteic acid by β-elimination and subsequent methylamine or sulfite addition. Clark and Dijstra [16] improved the method using dimethylamine or methylmercaptan as modifying reagents. Meyer et al. [17] developed that approach using β-mercaptoethanol and ethanethiol. All these methods are based upon exposure phosphoserine containing peptide to β-elimination under alkaline conditions to form a dehydroalanyl residue, which can be reduced

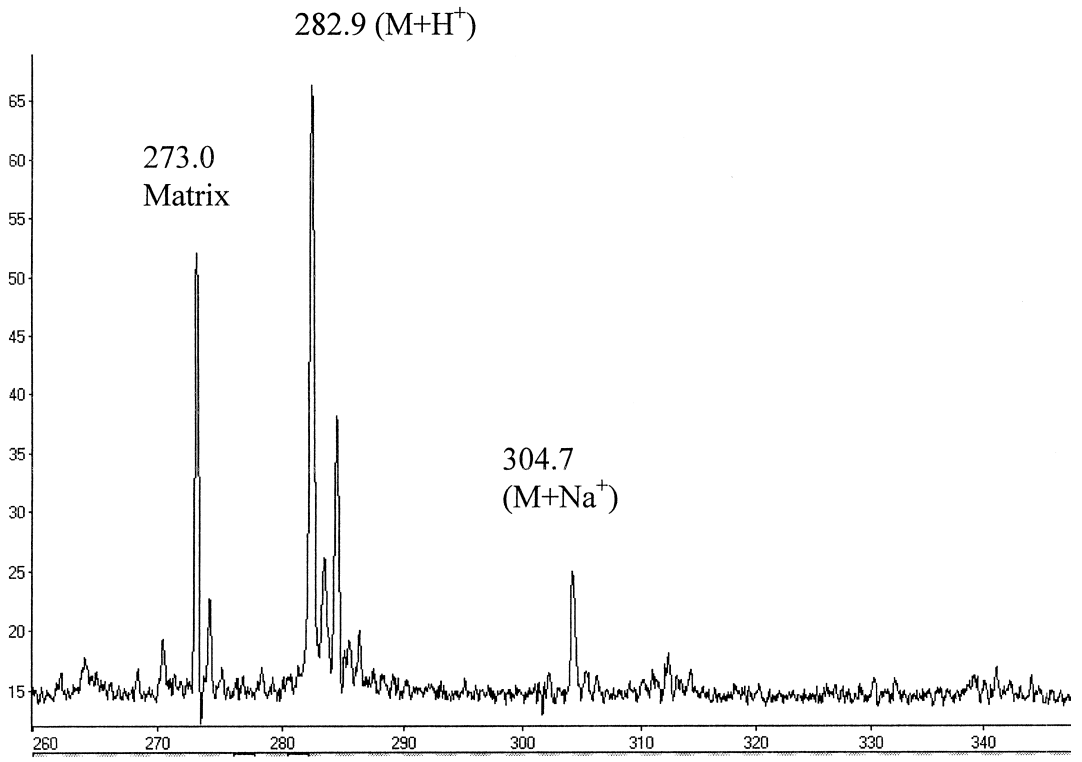

Fig. 16. Mass spectrum of PTH-*S*-ethanolcysteine. *x*-Axis: *m*/*z*; *y*-axis: intensity (%).

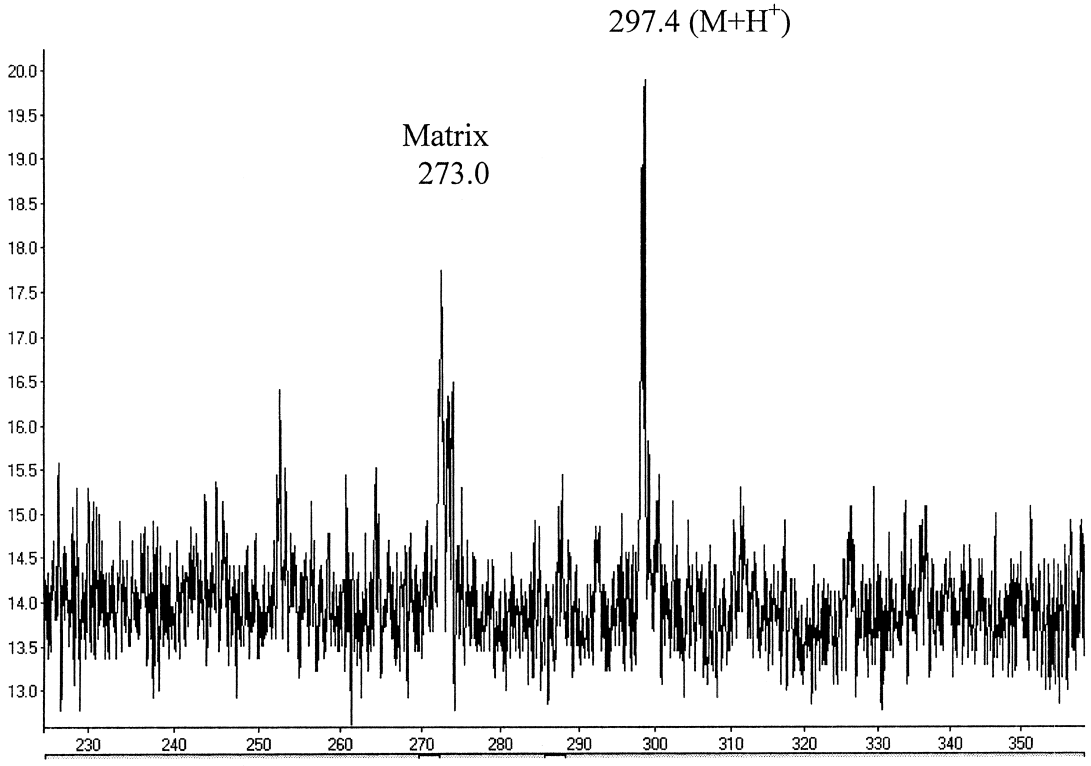

Fig. 17. Mass spectrum of PTH-3-methyl-*S*-ethanolcysteine. *x*-Axis: *m*/*z*; *y*-axis: intensity (%).

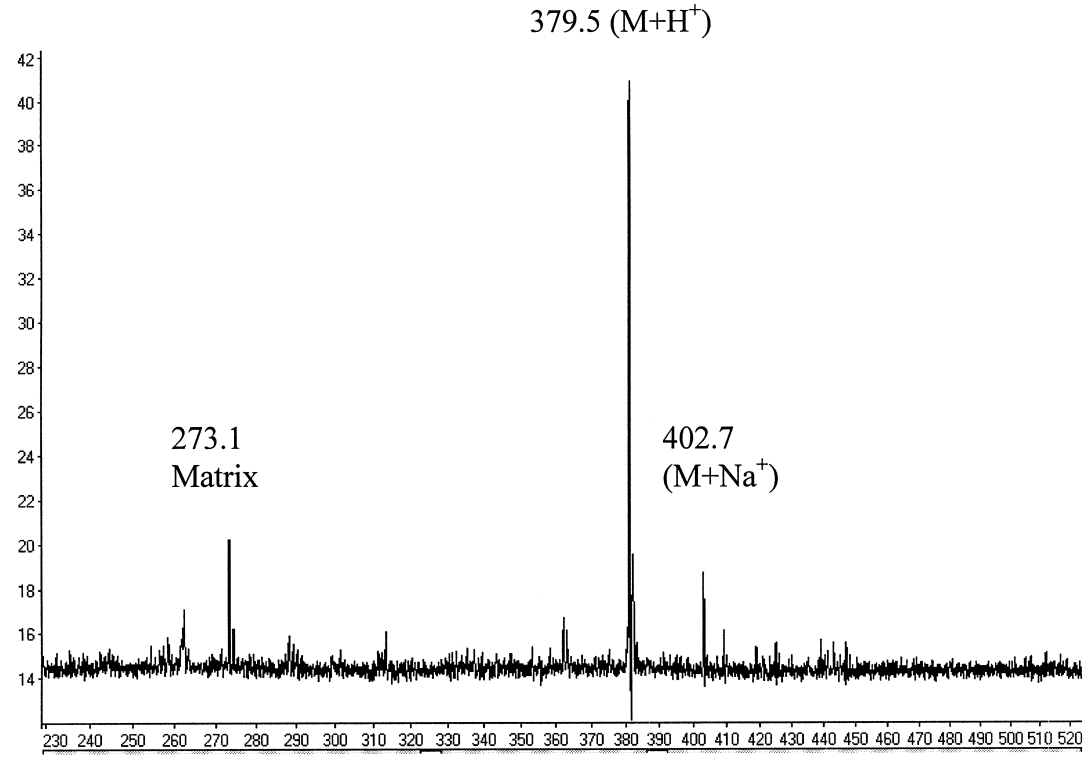

Fig. 18. Mass spectrum of PTH-*O*-phosphotyrosine. *x*-Axis: *m*/*z*; *y*-axis: intensity (%).

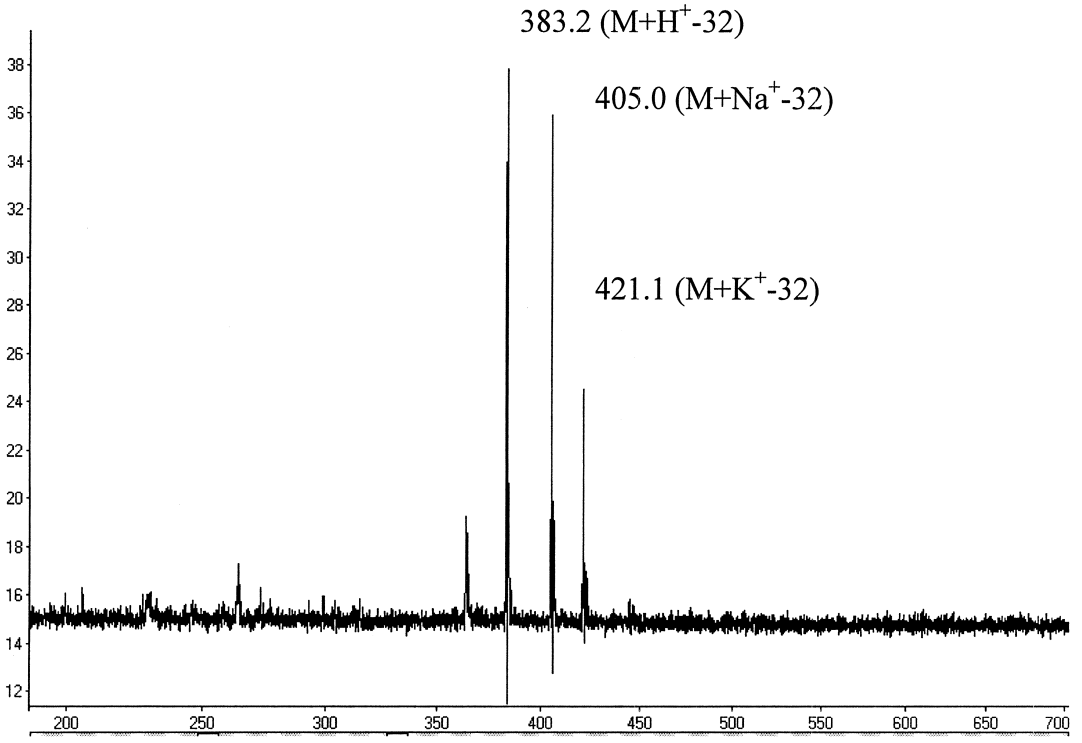

Fig. 19. Mass spectrum of PTH-ϵ-phenylthiocarbamyl-δ-hydroxylysine. *x*-Axis: *m*/*z*; *y*-axis: intensity (%).

[18] or modified. We applied modification by β-mercaptoethanol [17] under alkaline conditions for the peptide R-K-S*-I-R-I-G-P-G-Q-T-F-Y-A-T-G primary structure investigations. The corresponding PTH-amino acids were synthesized from *O*-phosphoserine, *O*-phosphothrenine and *O*-phosphotyrosine. Reaction mixtures were analyzed by MALDI-TOF-MS and target compounds were refined by RP-HPLC (not shown) with subsequent MALDI-TOF-MS analysis (Figs. 11 and 16–18). The synthesized PTH-*S*-ethanolcysteine was applied to identify *O*-phosphoserine (S*) in the peptide under study (R-K-S*-I-R-I-G-P-G-Q-T-F-Y-A-T-G). Limitation to this modification method is stipulated by its applicability to internal phosphorylated serines only. *O*-Glycosylated serine undergoes simultaneous β-elimination under the alkaline conditions and forms the same derivatives as a *O*-phosphoserine residue during the modification action. Thus, another usually mentioned limitation to the β-elimination mechanism modification method [14–18] is a possible *O*-glycosylated serine identification as the same derivatives, but our approach embracing combined microinstrumental analysis makes the method universal for *O*-phosphorylated and *O*-glycosylated serines with unambiguous identification of each post-translational modification (especially, with MALDI-TOF-MS analysis before and after the modification procedure).

It is very difficult to separate phosphorylated, non-phosphorylated, and modified peptides; there are a lot of publications on optimization of the separation [19,20]. We suggested convenient narrow-bore RP-HPLC procedure for isolating phosphorylated peptide R-K-S*-I-R-I-G-P-G-Q-T-F-Y-A-T-G from its non-phosphorylated one using common eluents, such as TFA and acetonitrile (Fig. 14). The peptide investigated was then undergone modification by β-mercaptoethanol and refined by RP-HPLC. Each peptide form was analyzed by MALDI-TOF-MS (Fig. 13). Its homogeneity was tested by HPCE. The peptide was then sequenced on a gas phase sequencer and cleaved PTH-amino acids were identified by RP-HPLC. The phosphoserine residue was

unambiguously identified on the third cycle of Edman degradation as the PTH-*S*-ethanolcysteine (Fig. 15). We did not analyze PTH-*O*-phosphotyrosine upon peptide sequencing, but there are some examples of successful direct sequencing of *O*-phosphotyrosine containing the peptides reported [21,24,25]. Thus, synthesized and RP-HPLC refined PTH-3-methyl-*S*-ethanolcysteine and PTH-*O*-phosphotyrosine were established by MALDI-TOF-MS and could be used for the identification procedure of *O*-phosphothreonine and *O*-phosphotyrosine in peptides and proteins (Figs. 16–19).

In conclusion, we have developed the combination of the instrumental analysis micro-methods for unusual amino acids of natural and synthetic origin. This combination could be employed for unambiguous determination of unusual amino acids with different side-chain radical types on ~10–100 pmol of the starting peptide material.

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References

[1] A.P. Hunter, D.E. Games, Rapid Commun. Mass Spectrom. 8 (1994) 559.
[2] V. Merand, E. Forest, J. Gagnon, C. Monnet, P. Thibault, M. Neuburger, R. Douce, Biol. Mass Spectrom. 22 (1993) 447.
[3] W.H. Fischer, C.A. Hoeger, J. Meisenhelder, T. Hunter, A.G. Craig, J. Prot. Chem. 16 (1997) 329.
[4] Y. Wang, C.J. Fiol, A.A. DePaoli-Roach, A.W. Bell, M.A. Hermodson, P.J. Roach, Anal. Biochem. 174 (1998) 537.
[5] H.E. Meyer, E. Hoffman-Posorske, H. Korte, L.M.G. Heilmeier, FEBS Lett. 204 (1986) 61.
[6] A.M. Edelman, D.K. Blumenthal, E.G. Krebs, Annu. Rev. Biochem. 56 (1987) 567.
[7] P.A. de Witte, J.F. Cuveele, W.J. Merlevede, J.R. Vandenheede, Anal. Biochem. 226 (1995) 1.
[8] L.C. Cantley, K.R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, S. Solotoff, Cell 64 (1992) 3275.
[9] E. Neufeld, H.J. Goren, D. Boland, Anal. Biochem. 177 (1989) 138.
[10] M. Manai, A.J. Cozzone, Anal. Biochem. 124 (1982) 12.
[11] A.C. Borthwick, W.H. Holms, H.G. Nimmo, FEBS Lett. 174 (1984) 112.
[12] J.C. Shtein, C.S. Rubin, J. Biol. Chem. 256 (1985) 10991.
[13] R.M. Hewick, M.V. Hunkapiller, L.E. Hood, W.J. Dreyer, J. Biol. Chem. 256 (1981) 7990.
[14] V.Y. Kolesnikova, V.A. Sklyankina, L.A. Baratova, T.I. Nazarova, S.M. Avaeva, Biokhimiya (Engl. translation) 39 (1974) 235.
[15] D.I. Simpson, J. Hranislavljevic, E.A. Davidson, Biochemistry 11 (1972) 1849.
[16] R.C. Clark, J. Dijkstra, Int. J. Biochem. 11 (1979) 577.
[17] H.E. Meyer, E. Hoffman-Posorske, H. Korte, L.M.G. Heilmeyer, FEBS Lett. 204 (1986) 61.
[18] F. Downs, C. Peterson, V.L.N. Murty, W. Pigman, Int. J. Peptide Protein Res. 10 (1977) 315.
[19] H. Matsumoto, E.S. Kahn, N. Komori, Anal. Biochem. 251 (1997) 116.
[20] H. Ohguro, K. Palcsewski, FEBS Lett. 368 (1995) 452.
[21] C.W. Turck, J. Herrmann, J.A. Escobedo, L.T. Williams, Peptide Res. 4 (1991) 36.
[22] S.A. Carr, K. Biemann, Methods Enzymol. 106 (1984) 29.
[23] P. Edman, A. Henschfn, in: S.B. Needleman (Ed.), Protein Sequence Determination, Springer, Berlin, Heidelberg, 1975, p. 232.
[24] J.A. Cooper, B.M. Sefton, T. Hunter, Methods Enzymol. 99 (1983) 387.
[25] H.E. Meyer, E. Hoffman-Posorske, A. Donella-Diana, H. Korte, Methods Enzymol. 124 (1990) 131.