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Analysis of tyrosine- and methionine-containing neuropeptides by fast atom bombardment mass spectrometry

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ABSTRACT

A simple and unambiguous method for the detection of the amino acids tyrosine and methionine in peptide structures has been developed. The procedure, which was applied in studies of opioid peptides, is based on continuous-flow fast atom bombardment mass spectrometry (CF-FAB-MS) following chemical modification of the residue to be analyzed. Thus, for the detection of tyrosine, modification reactions such as acetylation or non-radioactive iodination were performed prior to analysis by CF-FAB-MS. O-Acetylation of the tyrosine residue with N-acetylimidazole was accompanied by a shift of 42 Da in the molecular mass of the peptide under investigation. This modification was reversed by treatment with hydroxylamine hydrochloride. Incorporation of iodine resulted in a molecular weight shift of 126 Da per iodine atom. Methionine residues were detected in methionine-enkephalin-containing peptides following S-oxidation with hydrogen peroxide. The procedures described may have a wide application in peptide chemistry, particularly for the identification of peptide fragments containing the above residues, *e.g.* in studies of processing or degradation of the enkephalins or other neuropeptides (*e.g.* endorphins and tachykinins).

INTRODUCTION

Chemical modification of various amino acids has been tested extensively during several decades, and a number of methods have been developed and applied successfully in many studies focused on peptide and protein structures. These methods were used for *e.g.* investigations of the active sites of proteinases [1,2] as an improvement in the signal-to-noise ratio or sequence information in fast atom bombardment mass spectrometry (FAB-MS) [3,4]. Covalent derivatization of neuropeptides in conjunction with multispectral analysis [5] may be useful for the determination of the cleavage site(s) after limited proteolysis by neuropeptide peptidases [6,7]. Such an approach was described recently for the identification of arginine residues [8]. This amino acid plays a crucial role in peptide precursors as a primary target of proteolytic attack.

Other amino acids of interest in neuropeptide studies are *e.g.* tyrosine and methionine. The tyrosine residue at the N-terminus of the enkephalins is necessary for their opioid activity, and an important step in their inactivation seems to involve the removal of this residue by an aminopeptidase cleavage. We have described previously a spectrophotometrical procedure for the identification of

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tyrosine-containing neuropeptides using high-performance liquid chromatography (HPLC) combined with diode-array detection [5]. That technique allowed us to avoid acidic hydrolysis, and the intact peptide was recovered without any decomposition. However, tyrosine gives a characteristic but weak UV signal as compared to tryptophan. Therefore, alternative procedures for the unambiguous identification of the tyrosine residue within peptide structures are of interest.

The other amino acid focused on here is methionine. This residue occurs in many peptides of biological importance including the opioid peptides and tachykinins. The carboxyl group of methionine is susceptible to the action of chymotrypsin-like proteinases, which should be considered when studying peptide conversion.

In the present work, we have applied a simple, one-step chemical derivatization of tyrosine- and methionine-containing neuropeptides in order to improve the amino acid sequence information obtained from FAB-MS.

EXPERIMENTAL

The peptides used in this study were purchased from Bachem (Bubendorf, Switzerland) and were used without further purification. They were, however, tested by the FAB-MS method in order to detect impurities (*e.g.* oxidized form of methionine) that could interfere with the described procedures. N-Acetylimidazole and hydroxylamine hydrochloride were supplied by Sigma (St.Louis, MO, U.S.A.). All other chemicals were of analytical grade and were purchased from various commercial sources.

Non-radioactive iodination was performed with the aid of Iodogen (Pierce, Oud Beijerland, The Netherlands), according to the protocol supplied by the producer. Tyrosine derivatization of the phenol hydroxy group with N-acetylimidazole was performed basically as described by Lundblad and Noyes [9] with some modifications. Peptide (10 nmol in 30 μl) was incubated with an equimolar amount of N-acetylimidazole (15 μl) in 10 mM sodium phosphate buffer pH 7.4 for 4 h at 37°C. Samples were then evaporated in a Speed-Vac vacuum centrifuge (Savant Vac, Hicksville, NY, U.S.A.) and stored for further studies. Tyrosine deacetylation was performed with hydroxylamine hydrochloride in a ten-fold molar excess over N-acetylimidazole, and the samples were incubated overnight at ambient temperature.

The thioether group in methionine-containing peptides was oxidized with 1% hydrogen peroxide in a total volume of 40 μl, and following evaporation, aliquots were analyzed directly in a mass spectrometer. The instrument used was a Finnigan MAT 90 (Finnigan, Bremen, F.R.G.) double-focusing mass spectrometer with reverse geometry (B,E where B = magnetic field, and E = electric field). Xenon was used as a primary atom beam in a saddle-field FAB gun (Ion Tech, Teddington, U.K.) operating at 6 kV. The source current was adjusted to 1 mA. Perfluorokerosene (PFK) and Ultramark 2500 served as mass calibration com-

pounds. Magnetic scans in the positive-ion mode were acquired at a full accelerating voltage at 5 kV, and results were processed further by a PDP-11 data system.

Samples were analyzed either on a direct stainless-steel probe (1 nmol) or by the continuous-flow (CF) FAB method (0.07 nmol) with the interface supplied by Finnigan MAT, as described by Caprioli *et al.* [10]. Peptides were redissolved in the appropriate volume of 50% acetonitrile, supplemented with 0.1% trifluoroacetic acid (TFA) and mixed either with an equal amount of glycerol (for direct probe only) or injected directly (2 μl) into the system (CF-FAB) and eluted at a flow-rate of 5 μl/min through 0.075-mm fused-silica capillary. The mobile phase consisted of 20% glycerol supplied with 0.1% TFA and was delivered with the aid of an infusion pump. The pH of the matrix was maintained between 1.9 and 2.0.

RESULTS AND DISCUSSION

Fig. 1A–E present positive-ion mass spectra of five tyrosine-containing peptides (see Table I for peptide sequences) derivatized with N-acetylimidazole. In all cases, the most abundant ion represents the molecular ion of the modified molecule, and the presence of a constant shift of 42 Da (see Fig. 2) appears as a reliable indication of the Tyr residue in the peptide. A minor sodiation (attraction of sodium ion) was also noted in several cases, which might be due to the relatively high buffer concentration (10 m*M*). This effect, however, did not significantly affect the presented spectra.

The presence of tyrosine was also verified by non-radioactive iodination as shown in Fig. 3. ^{127}I was incorporated into the tyrosine phenol ring in Dyn A(1–6), what resulted in the formation of the monoiodotyrosine peptide at *m/z* 838.6. In addition, a diiodotyrosine derivative was also observed at *m/z* 964.5,

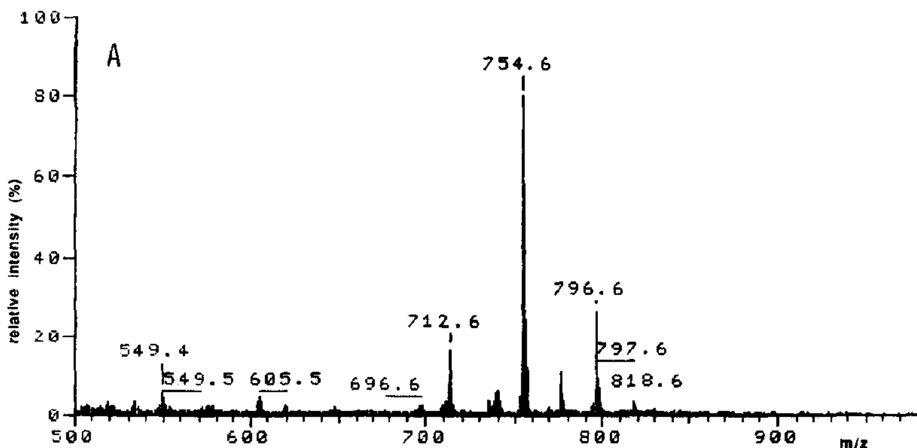

Fig. 1.

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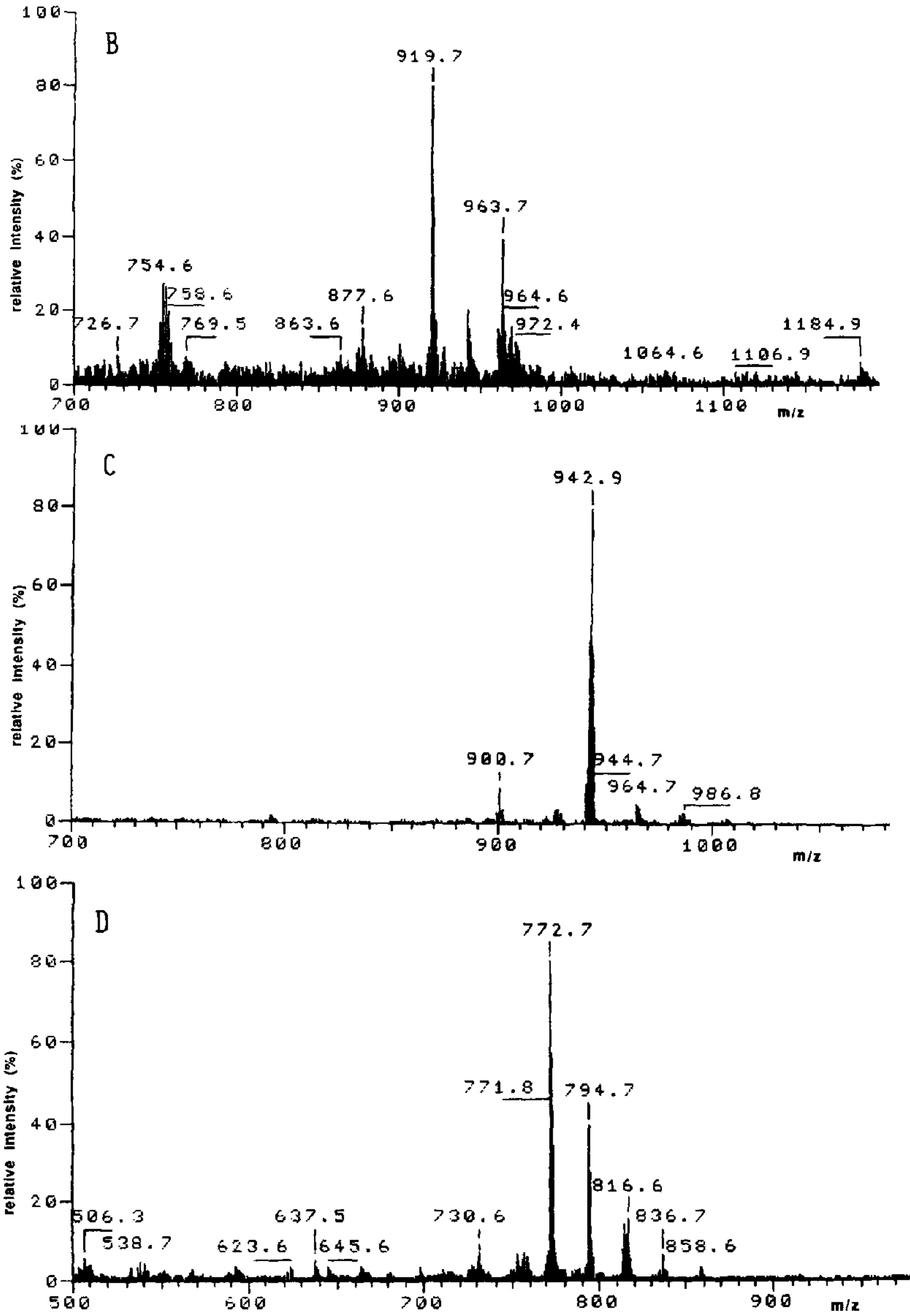


Fig. 1.

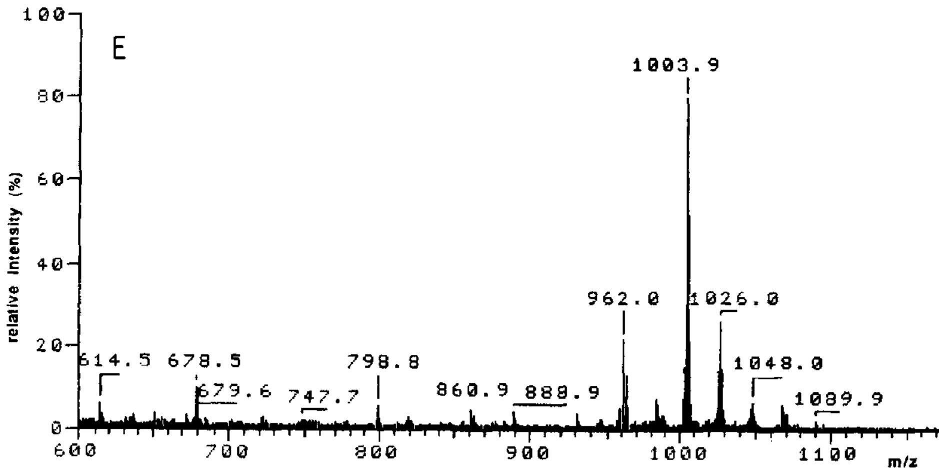


Fig. 1. Positive FAB spectra of tyrosine-containing peptides after treatment with N-actylimidazole. (A) Dyn A(1–6). The peak at *m/z* 754.6 belongs to the major derivative. The less abundant ion at *m/z* 796.6 may be due to non-specific reaction of the modifier with other groups; (B) Met-enk-Arg-Phe; (C) Met-enk-Arg-Gly-Leu. In this case, no other peaks were abundant, except that belonging to the derivatized molecule; (D) Met-enk-Arg. Minor sodiation was noted at *m/z* 794.6. Non-specific reaction with N-acetylimidazole similar to (A) causes formation of peak at *m/z* 814.6; (E) β-Casomorphin(1–8).

suggesting that even mild chemical incorporation of iodine into the protein molecule produces a mixture of tyrosine derivatives that might affect the biological or immunological activity of the substances tested. On the other hand, such a double signal may lead to an overestimation of the number of tyrosine residues present in the molecule.

Because acetylation with N-acetylimidazole under certain conditions also may result in side-effects, we applied hydroxylamine hydrochloride in a ten-fold molar excess [9]. This reagent reverses acetylation, but only when the phenol hydroxy group of tyrosine is modified. The results of deacetylation are shown in Fig. 4. The molecular ion characteristic of the intact peptide appeared in all cases. This

TABLE I

AMINO ACID SEQUENCE OF THE INVESTIGATED PEPTIDES

Name	Sequence	MW
Dyn A(1–6)	YGGFLR	711.8
Met-enk-Arg-Phe	YGGFMRF	877.0
Met-enk-Arg-Gly-Leu	YGGFMRGL	900.1
Met-enk-Arg	YGGFMR	729.6
β-Casomorphin(1–8)	YPFVEPIP	961.1

observation suggests clearly that tyrosine in fact was present in the peptide structure.

Another set of experiments was performed with methionine-containing peptides and involved a short oxidation of the thioether group in methionine-enkephalin (Met-enk) with hydrogen peroxide. The results are shown in Fig. 5. According to the reaction, presented in Fig. 2, oxidation of methionine-enkephalin and related peptides causes formation of a new signal in the mass spectrum, which was 16 Da higher than in the intact molecule. The thioether can be regenerated by reducing agents such as 2-mercaptoethanol or dithiothreitol (Fig. 5 insert).

In our previous work we used HPLC combined with photodiode-array detection for the analysis of peptides and peptide fragments containing aromatic ami-

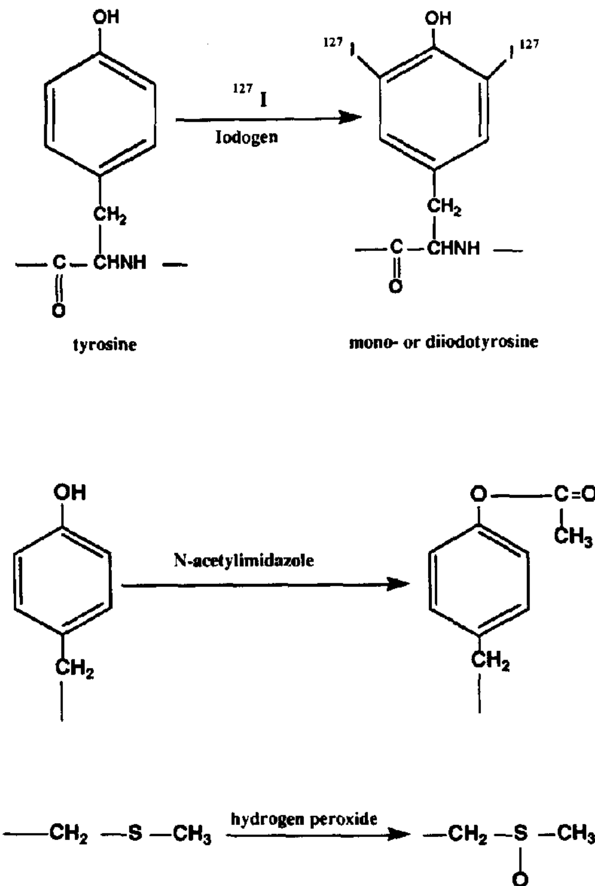


Fig. 2. Modification of tyrosine and methionine by iodination, acetylation or oxidation, respectively.

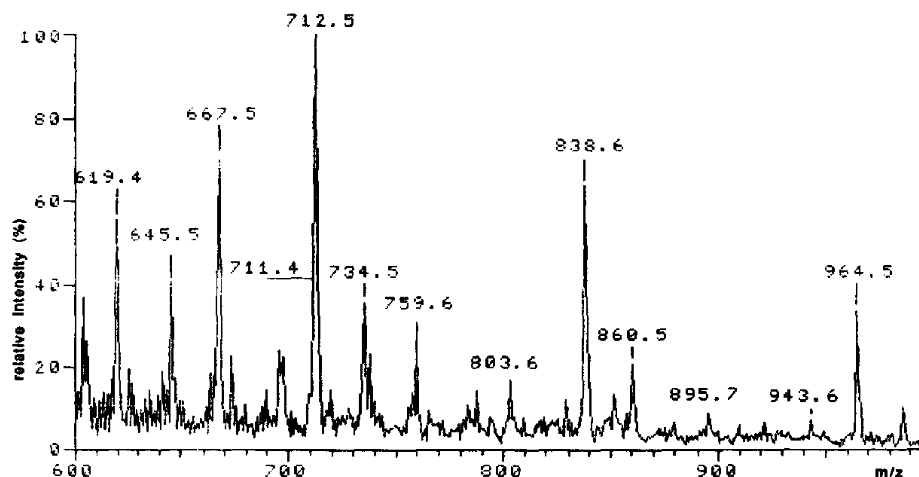
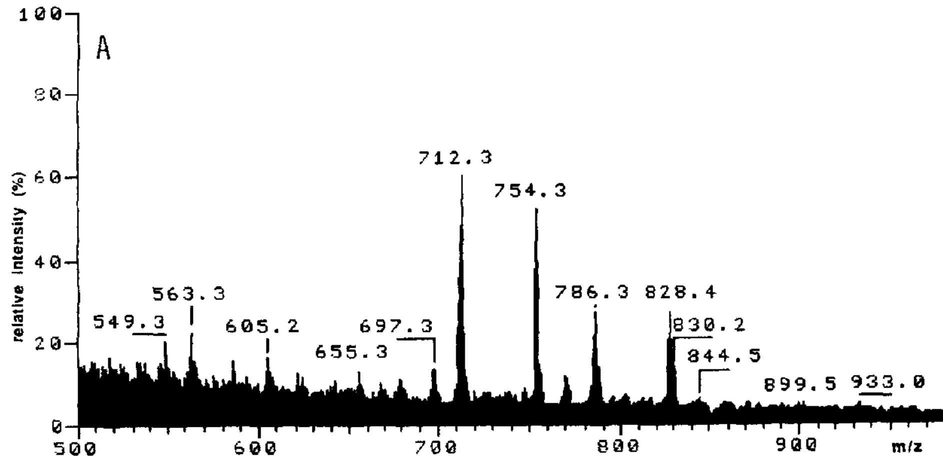


Fig. 3. Positive FAB spectrum of iodinated Dyn A(1–6). Peaks at *m/z* 838.6 and 964.5 belong to mono- and diiodotyrosine peptide, respectively.

no acid residues in studies of proteolytic enzymes [5]. The similar strategy was applied for separation and analysis of peptides and proteins in minute amounts of human cerebrospinal fluid [11]. The described technique was proved to be useful for discrimination between dipeptides differing in aromatic residues and for detection of impurities or coeluting substances. The presented method [5,11] was, however, limited to aromatic amino acids (*i.e.* Tyr, Phe and Trp) or other chromogenic substances that produce reliable UV spectra.

Application of chemical modifications of neuropeptides to determine structural information at the picomole level may be investigated with the aid of MS. This strategy, which utilized one-step derivatization without any sample transfer, may be helpful during the analysis of unknown peptides and their fragments at the concentrations, when the proper sequence information is difficult to obtain otherwise. Similar experiments for the determination of the number of thiol groups present in protein molecules were described by Morris *et al.* [12]. Samples can be processed directly, as they are collected *e.g.* during HPLC separations. It should also be mentioned that chemical derivatization may serve, in conjunction with the photodiode-array technique [5], for localization of particular amino acids during peptide mapping or for an investigation of the catalytic center in proteolytic enzymes.

In conclusion, we describe here a study of chemical modifications of the amino acids tyrosine and methionine combined with MS to identify these residues within the sequence of various neuropeptides. The procedure proved to be very effective and may apply well for similar investigations focused on other amino acid residues where the selective modifications are available.



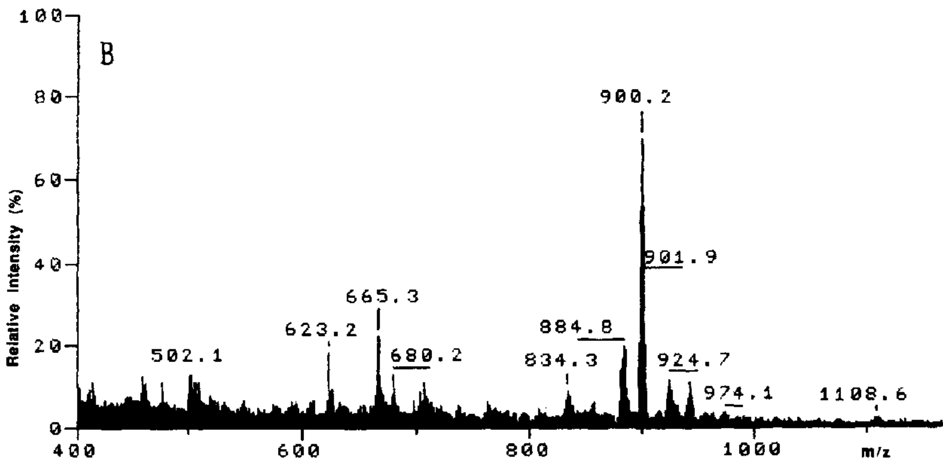


Fig. 4. Acetylated peptides after reaction with hydroxylamine hydrochloride. (A) Dyn A(1–6); (B) Met-enk-Arg-Gly-Leu.

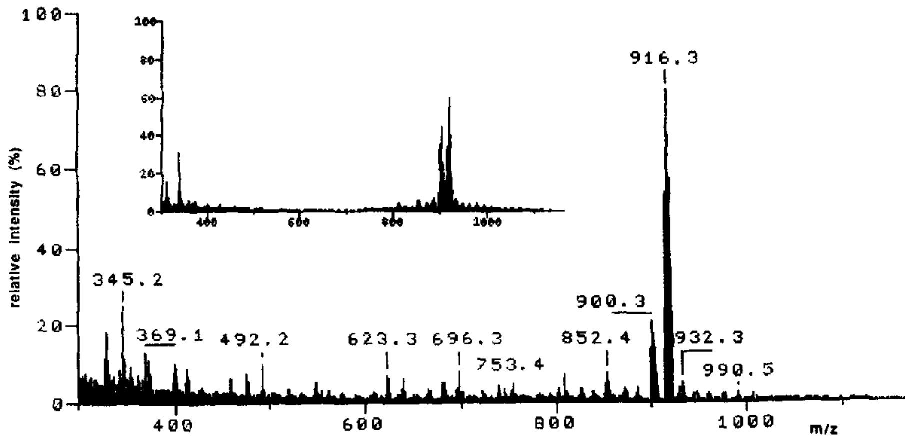


Fig. 5. Oxidation of the thioether in Met-enk-Arg-Gly-Leu with 1% hydrogen peroxide, and further reduction with 2 mM 2-mercaptoethanol (insert).

ACKNOWLEDGEMENTS

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