Journal of Chromatography, 557 (1991) 359–368
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 376

Performances and limits of plasma desorption mass spectrometry in the primary structure determination of proteins

JEAN-MARIE SCHMITTER
Laboratoire de Biochimie, URA 240 CNRS, École Polytechnique, Route de Saclay, 91128 Palaiseau Cedex (France)

ABSTRACT

The resolution, sensitivity, matrix effect, cationization and spectral suppression in plasma desorption mass spectrometry (PD-MS) were investigated in the context of peptide analysis. Excessive cationization may be avoided by the addition of citric acid on the target. The importance of the relative net charge of peptides in PD-MS spectra suppression was confirmed. Esterification of peptides is shown to be an easy way to overcome spectral suppression. Provided that cationization and spectral suppression of peptides are under control, PD-MS is an excellent tool for protein sequence analysis, affording the necessary complement to automated Edman degradation.

INTRODUCTION

Primary structure determination of proteins consisting of several hundred amino acids is a delicate and lengthy task, even with the help of modern automated stepwise Edman degradation [1]. Indeed, it requires a careful piecing together of peptides generated by various cleavage techniques, and subsequently purified to homogeneity. Thus, the sequence of a large protein is preferably deduced from an alternative approach, by sequencing the gene encoding this protein. This then implies verifying the deduced sequence and identifying possible post-translational modifications and sequence heterogeneities [2,3]. Automated Edman degradation may be used for this purpose, after having generated and purified a set of peptides. However, in addition to the fact that this sequencing technique is time consuming, it cannot handle peptides having blocked N-termini and does not lead in a direct way to a complete identification of all possible post-translational structure modifications. The best choice is then to use mass spectrometry.

Recent advances in desorption–ionization techniques have opened the way to high mass measurements [4–7], and several instrumental combinations allow the analysis of peptides by mass spectrometry. Among low-resolution instruments, spectrometers based on a ^{252}Cf plasma desorption source and a time-of-flight (TOF) analyser offer several attractive features for this type of application [8–11]. In addition to a

0021-9673/91/$03.50

simple-to-operate instrumental combination, the sensitivity of this technique matches that of automated sequencing based on Edman degradation. Further, an analysis by plasma desorption mass spectrometry (PD-MS) destroys less than 1% of a sample deposited on the target; this allows the use of subsequent *in situ* chemical derivatizations and proteolytic digestions. Further structural information may then be obtained by reanalysing the same target [12,13].

The capabilities of such an instrument are presented in the context of protein structure determination, focusing on the aspects of resolution and sensitivity. As a complement to Edman degradation, it was important to evaluate the potential of PD-MS to handle peptide mixtures. Therefore, the effects of matrix, cationization and spectral suppression on peptide molecular ion signals were investigated.

EXPERIMENTAL

Mass spectrometer

The ^{252}Cf fission fragment ionization TOF mass spectrometer (Depil-X) was constructed at the Institut de Physique Nucléaire (Orsay, France) and has been described elsewhere [14–16]. An electrostatic mirror allows both an increase in resolution and access to metastable ion studies [15,16]. Spectra were acquired with an acceleration voltage of 10 kV. When the electrostatic mirror was used, a 13 kV voltage was applied to the mirror in order to reflect ions to an axial annular stop detector [14].

Mass calibration of spectra was based on the flight times of H^+ and Na^+ for positive ions and of H^- and CN^- for negative ions.

Sample probes

Aluminized Mylar was used as the primary sample support. In most instances, a thin nitrocellulose (NC) film was produced on this support by electrospraying 25 μl of a 2 mg/ml solution of NC in acetone [17].

Peptides samples were dissolved at a concentration of 0.1–1 m*M*. Various solvents were used, with the following compositions: water–methanol (1:4 or 1:1, v/v); 0.05 *M* sucrose octaacetate in methanol–water (1:1); 0.05 *M* citric acid in methanol–water (1:1); 0.05 *M* glutathione in methanol–water (1:1); and 0.05 *M* citric acid–0.05 *M* glutathione in methanol–water (1:1). A 1–3-μl volume of sample was usually applied on the NC layer and allowed to dry on the probe.

Peptides were obtained from Serva (Heidelberg, Germany), except tryptic peptides from *E. coli* thioredoxin, which were a gift from Dr. P. Decottignies (Orsay, France). The single letter code is used for amino acids.

Methanolic hydrochloric acid (3 *M*) was used for the esterification of peptides. After reaction for 1 h at room temperature, this reagent was removed under a stream of nitrogen and peptides were taken up in methanol–water (1:1).

RESULTS AND DISCUSSION

Influence of the sample matrix

Peptide samples may be directly electrosprayed onto an aluminized Mylar surface and analysed by PD-MS. Most often, this results in a difficult control of alkali

metal ion concentration on the target, and the $[M + H]^+$ ion may almost disappear to the benefit of pseudomolecular ions cationized with sodium or potassium, such as $[M + Na]^+$ and $[M + 2Na - H]^+$ (Fig. 1A). Another drawback of direct electrospraying on aluminized Mylar is that only a few solvents are suitable for this technique, those with a high water content being prohibited. For these reasons, nitrocellulose (NC), which can be electrosprayed on aluminized Mylar from a solution in acetone [17], has gained wide acceptance for peptide analysis by PD-MS. Aqueous solutions may be easily used to adsorb peptides on NC, and the amount of alkali metal ions on the target is much lower than that observed with direct electrospraying on aluminized Mylar. This is illustrated by the analysis of the tripeptide GPA on an NC backing (Fig. 1B). Various combinations of aqueous solvents, including methanol–water and acetonitrile–water, with or without the presence of 0.1% of trifluoroacetic acid, may be used in order to load peptides on the probe without altering the uniformity of the NC layer. Methanol–water (1:1) always gave good results when a 1 3-μl sample volume was allowed to dry on the target.

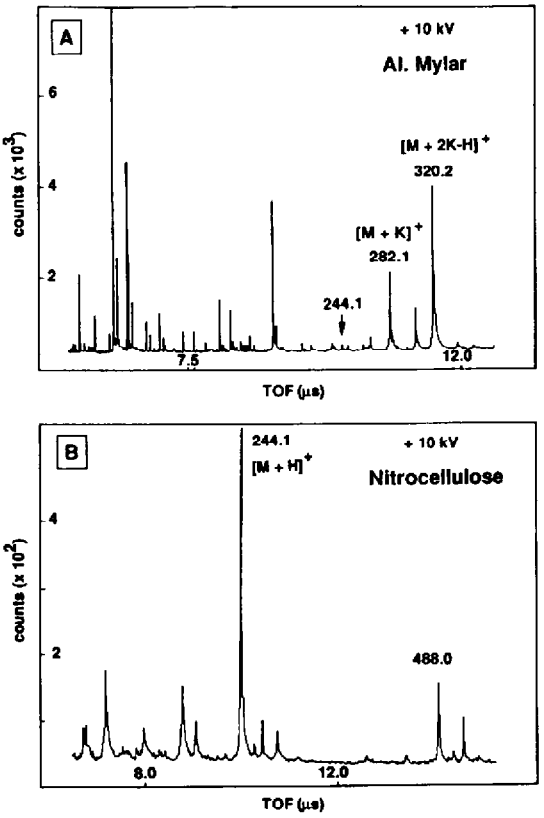

Fig. 1. (A) When the tripeptide GPA (single-letter coding for amino acids is used) is electrosprayed on aluminized Mylar from a methanol–water (4:1, v/v) solution, the $[M + H]^+$ ion (m/z 244.1) hardly appears, and major pseudomolecular ion species result from cationization with potassium. (B) By drying the tripeptide solution (same solvent) on a previously electrosprayed nitrocellulose layer, the $[M + H]^+$ ion dominates all cationized species.

Using NC as a matrix, fragmentation of peptides occurs only to a very minor extent, and only information on molecular mass is usually obtained from the spectra. This behaviour differs from that of peptides electrosprayed on Mylar, which sometimes leads to the formation of fragment ions [18].

A gain in sensitivity of 1–2 orders of magnitude is obtained with NC backings as compared with aluminized Mylar. Therefore, for all these reasons, subsequent analyses were performed with an NC matrix.

Resolution and sensitivity

A TOF mass spectrometer equipped with an electrostatic mirror allows access to the study of metastable ions, als already outlined [14–16]. Reflecting ions toward an annular stop detector with about twice the length of the initial flight path, and partly removing neutral species, results in a significant increase in resolution. This is illustrated with the analysis of an eleven-residue-long peptide (Fig. 2). With the electrostatic mirror in operation (reflex mode), the resolution reaches about 3000. This feature is of major interest when accurate mass measurements are required; such is

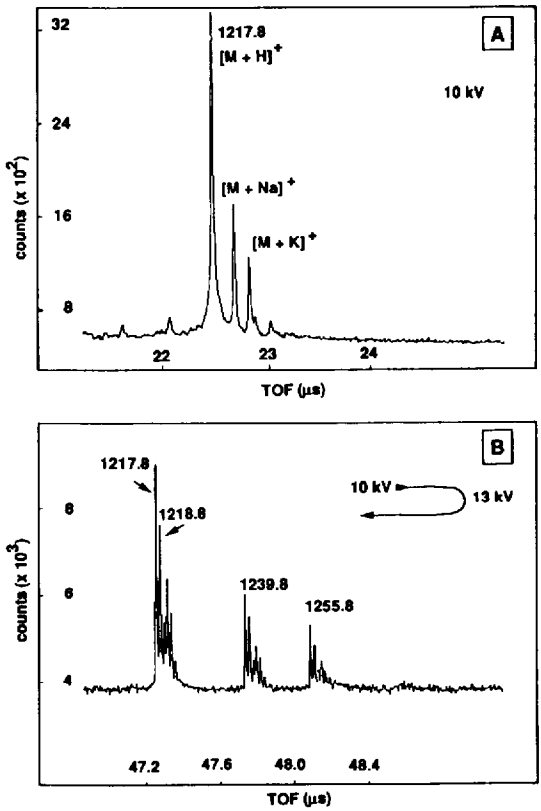

Fig. 2. Improvement in resolution achieved with the electrostatic mirror. (A) An undecapeptide (calculated relative molecular mass 1216.7, 300 pmol) loaded on an NC layer [methanol water (4:1, v/v) solution] is analysed with a 10 kV acceleration voltage. (B) By applying 13 kV to the mirror, ions are reflected and detected after about twice their initial flight time [14], and the isotopic cluster is resolved.

the case when a normal (–COOH) peptide C-terminus must be distinguished from an amidated one, with only 1 u difference.

However, the use of the electrostatic mirror is limited by the sensitivity that can be achieved. With the 80-cm long flight tube of the Depil-X instrument, a clear molecular ion signal of a peptide of relative molecular mass below 2000 can be obtained in the direct mode with less than 100 pmol of sample, and this within a few minutes of spectral accumulation time. This amount of sample has to be multiplied by a factor of about ten when relative molecular masses in the range 2000–5000 are to be measured; above 5000 successful results are strongly dependent on sample preparation and the nature of the peptide itself. In the reflex mode, the sensitivity drops by a factor of about ten. This can be partly compensated for by using longer spectral accumulation times, but the use of the electrostatic mirror in PD-MS is very limited for relative molecular masses above 3000–5000.

Control of cationization

As already evidenced with the analysis of a tripeptide presented in Fig. 1, the presence on the target of a large amount of alkali metal ions may lead to very weak signals of $[M + H]^+$ ions, and possibly result in a wrong attribution of molecular ions. Indeed, dealing with peptides of unknown sequences, strong cationization by sodium may lead to the absence of the $[M + H]^+$ ion, and to the presence of abundant $[M + Na]^+$ and $[M + 2Na - H]^+$. As these two latter ions differ by 22 u, the $[M + Na]^+$ ion may then be wrongly identified as the $[M + H]^+$ ion.

When analysing peptides of unknown sequences, cationization should therefore be kept as low as possible. After a preliminary analysis, it is safe to reanalyse the sample after having treated nitrocellulose-bound peptides in order to reduce the amount of sodium and potassium ions susceptible to form adducts with peptides.

As a first possibility, the target may be washed with deionized water or with a dilute acid solution. As already outlined by other workers [10,17], we observed that this treatment may result in important losses of peptides having relative molecular masses below 1000–1500. Another possibility consists in treatment of peptide solutions with an ion-exchange resin prior to sample adsorption on the NC layer, but losses cannot be excluded when this procedure is applied to subnanomolar amounts of peptides.

Thus, adding on the target a solution of a non-volatile carboxylic acid was found to be more generally applicable. Excellent results were obtained with solutions of citric acid (0.05–0.1 *M*), as shown in Fig. 3. This treatment was found to reduce considerably or even abolish cationization by sodium and potassium, but also to increase slightly the overall sensitivity for pseudomolecular ion species.

The addition of sucrose octaacetate to NC-bound peptides also results in a decrease in cationization, but this time to the detriment of sensitivity. As a practical rule, less than 10 nmol of this sugar derivative should be added to the target in order not to quench $[M + H]^+$ ions when subnanomolar amounts of peptides are analysed. Unfortunately, this amount is then too low to trap alkali metal ions efficiently.

It is worth mentioning that glutathione, which has been used as an additive to improve the desorption of polypeptides [19], also leads to a strong decrease in cationization of NC-bound peptides. This was experienced by adding 1–2 μl of a 0.05 *M* solution of glutathione (reduced form) to peptides adsorbed on NC. Glutathione was

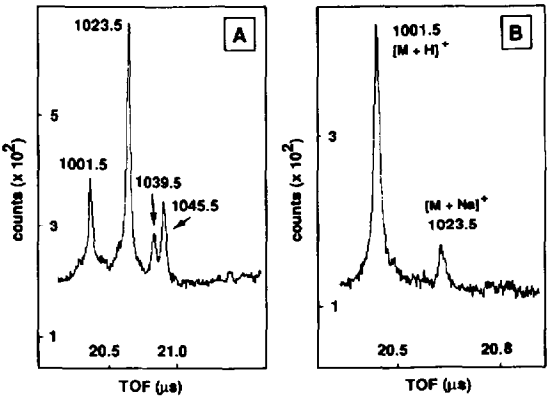

Fig. 3. Analysis in the positive-ion mode on an NC backing (10 kV acceleration voltage) of peptide GIPTLLLFK (from *E. coli* thioredoxin, calculated relative molecular mass 1000.6, 1 nmol). (A) The sample has been dissolved in methanol–water (4:1, v/v); (B) the target has been treated by drying 1 μl of a 0.1 *M* solution of citric acid on the surface and reanalysed.

found to be less efficient than citric acid for cationization removal, but for a large variety of peptides the overall sensitivity was improved after this treatment (data not shown).

As a general result for routine laboratory PD-MS analysis, peptides are now dissolved in a solution of 0.05 *M* citric acid–0.05 *M* glutathione in methanol–water (1:1). However, when subsequent enzymatic cleavages are planned to be performed on the target, peptides are dissolved in methanol–water (1:1).

Control of spectral suppression

The potential of peptide mapping by means of mass spectrometry is of particular interest for protein primary structure analysis. Indeed, in order to speed up a sequence verification, the effort on peptide purification should be kept to a minimum. However, it was experienced, with the hitherto most often used fast atom bombardment (FAB) mapping technique, that the analysis of peptide mixtures rarely led to complete peptide maps [4,20,21]. The spectral suppression phenomenon observed with FAB mapping can be related to strong differences in hydrophobicity, missing peptides being the most hydrophilic ones [20–22].

PD-MS has also been proposed as a tool for peptide mapping [12]. Spectral suppression effects were sometimes observed with this desorption–ionization mode, but were not well documented untill a recent study by Nielsen and Roepstorff [22]. According to them, spectral suppression of peptides in PD-MS is related to their relative net charge and not to their relative hydrophobicity.

An investigation of the applicability of PD-MS for both quantitative analysis and mapping of peptides was therefore undertaken, aimed at the design of optimum conditions for obtaining complete peptide maps.

Spectral suppression may not be observed at all when studying ^{252}Cf desorption of peptides. This technique has been used to characterize a carboxypeptidase Y (CPD-Y) catalysed transpeptidation of peptides [23]. Under certain conditions, this exopeptidase is able to catalyse the replacement of the carboxyl-terminal amino acid

of a peptide by an exogeneous amino acid. By analysing a transpeptidation reaction mixture, one may thus find starting material, together with hydrolysis and transpeptidation products. If the N-terminal amino acid bears an aromatic residue, such as tyrosine, a reversed-phase chromatographic system can easily lead to the quantification of the various constituents of the mixture. In the case of CPD-Y catalysed transpeptidation of peptide YPFPGPI with methionine, quantitative analysis of the reaction mixture by both high-performance liquid chromatography (HPLC) and PD-MS gave nearly identical results, *i.e.*, no spectral suppression was observed (Fig. 4).

In order to probe the relationship between the relative net charge of peptides and the spectral suppression effect in PD-MS, an equimolecular mixture of peptides YPFVEPI (net charge −1 at pH 7) and LWMRFA (net charge +1 at pH 7) was prepared. When analysed separately using an NC target in the positive-ion mode, each peptide desorbs in excellent yield. When using methanol–water (1:1) as a solvent and loading the mixture of the two peptides on NC, the spectrum shown in Fig. 5 was obtained. The signal of peptide YPFVEPI is strongly suppressed compared with LWMRFA. Also, the $[M + H]^+$ ion of YPFVEPI is very weak, this peptide being more cationized than LWMRFA by sodium. This result confirms the important role of the relative net charge of peptides in PD-MS analysis. The next step was to investigate possible ways to overcome spectral suppression effects.

By dissolving the previous equimolecular mixture of LWMRFA and YPFVEPI in methanol–water (1:1) in the presence of 0.05 *M* citric acid and 0.05 *M* glutathione, cationization disappears as expected, and spectral suppression of YPFVEPI also

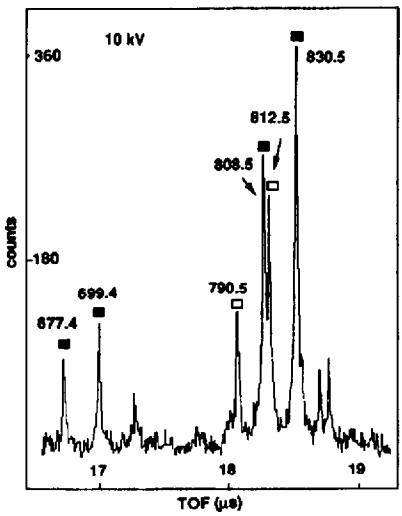

Fig. 4. Analysis of a reaction mixture resulting from carboxypeptidase Y-catalysed transpeptidation of the peptide YPFPGPI, with methionine added as exogenous nucleophile. Peak areas of the hydrolysis product YPFPGP (closed squares, $[M + H]^+$ at *m/z* 667.4), starting peptide (open squares, $[M + H]^+$ at *m/z* 790.5) and transpeptidation product YPFPGPM (shaded squares, $[M + H]^+$ at *m/z* 808.5) were found to be in good agreement with those measured by HPLC (absorbance of tyrosine at 280 nm [23]). The solvent used for loading on the NC target was methanol–water (4:1, v/v). Cationization by sodium affects the three peptides in the same proportion.

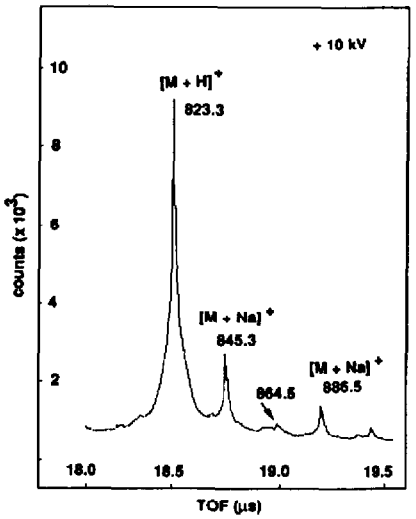

Fig. 5. Equimolecular mixture of peptides LWMRFA ($[M + H]^+$ at m/z 823.3) and YPFVEPI ($[M + H]^+$ expected at m/z 864.5) analysed with a 10 kV acceleration voltage after adsorption on an NC layer from methanol–water (1:1, v/v).

slightly diminishes in the positive-ion mode (Fig. 6A). The situation is more favourable in the negative-ion mode, but at the expense of a lower sensitivity (Fig. 6B).

Taking into account the hypothesis of the important role of the relative net charge of peptides [22], changing this net charge by derivatization of carboxylic acid groups should reduce the spectral suppression of peptides having negative net charg-

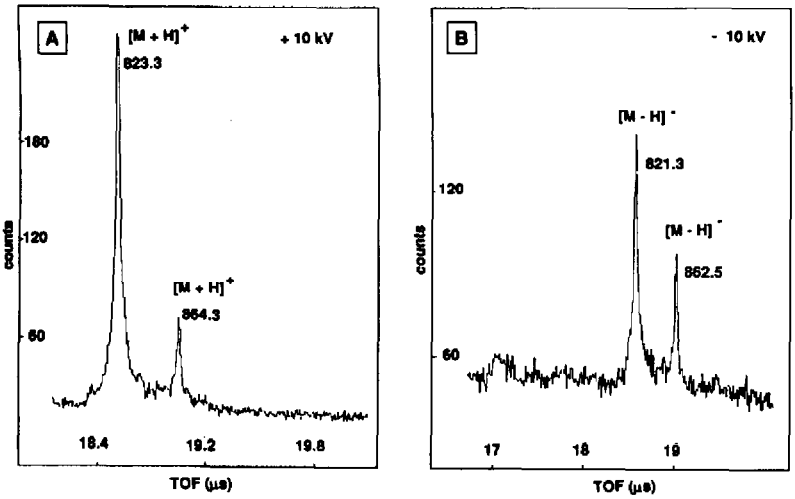

Fig. 6. Equimolecular mixture of peptides LWMRFA and YPFVEPI dissolved in 0.05 M citric acid–0.05 M glutathione in methanol–water (1:1). After loading on an NC layer, the analysis in both (A) positive-ion (+ 10 kV) and (B) negative-ion (− 10 kV) modes shows that both cationization and spectral suppression have strongly decreased in comparison with the conditions used to obtain Fig. 5.

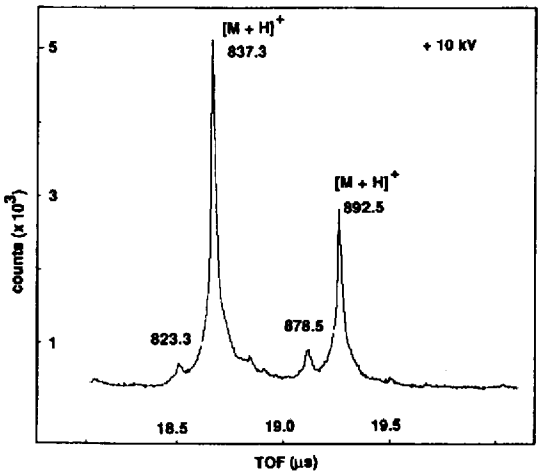

Fig. 7. Spectral suppression of peptide YPFVEPI almost disappears after esterification. An equimolecular mixture of LWMRFA and YPFVEPI, treated with methanolic hydrochloric acid, was dissolved in methanol–water (1:1) solvent mixture and loaded on an NC backing. Two methyl groups have been added for YPFVEPI ($[M + H]^+$ at m/z 892.5); this peptide is also present with a single function esterified ($[M + H]^+$ at m/z 878.5).

es. Thus, the same equimolecular mixture of YPFVEPI and LWMRFA was first esterified by means of methanolic hydrochloric acid. After evaporation to dryness, the sample was dissolved in methanol–water (1:1), adsorbed on NC and analysed in the positive-ion mode (same conditions as in Fig. 5). As shown in Fig. 7, spectral suppression almost disappeared. This single derivatization step is thus of great help in the sequence analysis of proteins, as it is an easy way to solve the problem posed by spectral suppression in PD-MS, and simultaneously provides the additional information of the number of carboxyl groups present in peptides.

As a result of this work, a general strategy for PD-MS mapping was designed. If enough peptidic material is available, the peptide mixture to be analysed is divided into two equal parts. One part is analysed in both positive- and negative-ion modes, using glutathione and citric acid as additives on the NC backing. The second part is first esterified, and subsequently analysed in the same way, in order to detect peptides suppressed under the first set of analytical conditions. In this way, PD-MS really is a valuable tool for protein sequence analysis, *i.e.*, an excellent technique to be used in combination with automated microsequencing.

ACKNOWLEDGEMENTS

The author thanks Bertrand Platel for assistance with the mass spectrometric analysis, Pierre-François Berne for providing the transpeptidation reaction mixture and Dr. Paulette Decottignies for providing tryptic peptides from *E. coli* thioredoxin. The constant interest of Professor Sylvain Blanquet in this work, and his efforts, together with those of Dr. Henri Audier, to develop this research programme are gratefully acknowledged. The Depil-X spectrometer was purchased with the financial support of INSERM and the École Polytechnique.

REFERENCES

1 R. M. Hewick, M. W. Hunkapiller, L. E. Hood and W. J. Dryer, *J. Biol. Chem.*, 256 (1981) 7990.
2 B. W. Gibson and K. Biemann, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 1956.
3 K. Biemann and H. A. Scoble, *Science, (Washington, D.C.)*, 237 (1987) 992.
4 K. Biemann and S. A. Martin, *Mass Spectrom. Rev.*, 6 (1987) 1.
5 T. R. Covey, R. F. Bonner, B. I. Shushan and J. Henion, *Rapid Commun. Mass Spectrom.*, 2 (1988) 249.
6 F. Hillenkamp, *Adv. Mass Spectrom.*, 11A (1989) 354.
7 K. D. Henry, E. R. Williams, B. H. Wang, F. W. McLafferty, J. Shabanowitz and D. F. Hunt, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 9075.
8 R. D. McFarlane and D. F. Torgerson, *Science, (Washington, D.C.)*, 191 (1976) 920.
9 R. D. McFarlane, J. C. Hill, D. L. Jacobs and P. W. Geno, *Adv. Mass Spectrom.*, 11A (1989) 3.
10 P. Roepstorff and B. Sundqvist, in S. J. Gaskell (Editor), *Mass Spectrometry in Biomedical Research*, Wiley, New York, 1986, Ch. 15, p. 269.
11 S. K. Chowdhury and B. T. Chait, *Anal. Biochem.*, 180 (1989) 387.
12 A. Tsarbopoulos, G. W. Becker, J. L. Occolowitz and I. Jardine, *Anal. Biochem.*, 171 (1988) 113.
13 K. Klarskov, K. Breddam and P. Roepstorff, *Anal. Biochem.*, 180 (1989) 28.
14 S. Della Negra and Y. Le Beyec, *Int. J. Mass Spectrom. Ion Processes*, 61 (1984) 21.
15 S. Della Negra and Y. Le Beyec, *Anal. Chem.*, 57 (1985) 2035.
16 Y. Le Beyec, *Adv. Mass Spectrom.*, 11A (1989) 126.
17 G. P. Jonsson, A. B. Hedin, P. L. Hakansson, B. U. R. Sundqvist, B. G. S. Säve, P. F. Nielsen, P. Roepstorff, K.-E. Johansson, I. Kamensky and M. S. L. Lindberg, *Anal. Chem.*, 58 (1986) 1084.
18 J. Fohlman, P. A. Petersson, P. Roepstorff, P. Højrup, I. Kamensky, B. G. S. Säve, P. L. Hakansson, B. U. R. Sundqvist, *Biomed. Mass Spectrom.*, 12 (1985) 380.
19 M. Alai, P. Demirev, C. Fenselau and R. J. Cotter, *Anal. Chem.*, 58 (1986) 1303.
20 S. Naylor, A. F. Findeis, B. W. Gibson and D. H. Williams, *J. Am. Chem. Soc.*, 108 (1986) 6359.
21 R. M. Caprioli, W. T. Moore and T. Fan, *Rapid Commun. Mass Spectrom.*, 1 (1987) 15.
22 P. F. Nielsen and P. Roepstorff, *Biomed. Environ. Mass Spectrom.*, 18 (1989) 131.
23 P. F. Berne, J. M. Schmitter and S. Blanquet, *J. Biol. Chem.*, 265 (1990) 19551.