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Nanoscale separations combined with tandem mass spectrometry

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ABSTRACT

High-efficiency separations of peptide mixtures, tryptic digests and other biological compounds have been achieved using nanoscale packed capillaries and capillary zone electrophoresis (CZE). The coaxial continuous-flow fast atom bombardment design is an excellent interface for coupling these separation techniques with mass spectrometry (MS). In addition, this interface is very useful for the acquisition of MS–MS data from compounds separated by nanoscale packed capillary liquid chromatography and CZE. Structurally informative daughter-ion spectra can be obtained at the low picomole to femtomole level.

INTRODUCTION

Fast atom bombardment mass spectrometry (FAB-MS), as first developed by Barber *et al.* [1], has become a widely used desorption technique for the analysis of polar, non-volatile, and/or thermally labile compounds such as biomolecules. Because both liquid chromatography (LC) and FAB-MS are suitable for the analysis of biomolecules, the coupling of LC with FAB-MS is currently an area of great interest. One of the most commonly used interfaces between flowing liquid streams and mass spectrometry for the analysis of polar molecules is continuous-flow fast atom bombardment (CF-FAB) [2,3]. Generally, LC–CF-FAB interfaces employ a single fused-silica capillary to deliver the column analytes to the probe tip in the mass spectrometer. These systems are designed to operate with conventional and microbore LC columns (typically 4.6–0.22 mm I.D.). Addition of the FAB matrix is accomplished

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by either adding the matrix to the mobile phase solvents or by postcolumn addition. The presence of the matrix in the mobile phase, however, can compromise the chromatography due to changes in the polarity and viscosity of the mobile phase. On the other hand, post-column addition of the matrix sometimes leads to chromatographic peak broadening, particularly with microbore LC columns.

Miniaturization of LC columns has also drawn a considerable amount of interest. Two approaches to this miniaturization that we have been developing are nanoscale packed capillary liquid chromatography (nCLC) and capillary zone electrophoresis (CZE). These techniques hold great promise in the analysis of biological mixtures, due to their high separation efficiencies (greater than $1 \cdot 10^6$ theoretical plates [4]). In addition, the low flow-rates associated with these methodologies (less than 100 nl/min, with injection and detection volumes of less than 20 nl/min) facilitates the coupling of these separation techniques with the high vacuums required by MS.

To avoid the problems associated with the addition of the FAB matrix in the mobile phase (which are especially severe with the nanoscale techniques), we have recently developed a coaxial CF-FAB interface [5–7] in which the analytes and matrix are delivered separately to the FAB probe tip. Briefly, this coaxial CF-FAB interface consists of a fused-silica capillary column or a nanoscale packed capillary column which is surrounded by a second fused-silica capillary column in which the matrix is introduced. In this manner, there is no mixing of the matrix with the LC analytes until both have reached the probe tip. This coaxial design is advantageous since (1) the chromatography is not affected by the matrix, and (2) both the LC flow-rate and composition and the FAB matrix flow-rate and composition can be independently optimized. A comparison of precolumn versus coaxial matrix delivery showed the coaxial CF-FAB interface offered higher separation efficiencies (by up to a factor of four) and lower detection limits than the precolumn addition of matrix [8]. Using the coaxial CF-FAB interface, full-scan mass spectra were acquired from 54 fmol of a tripeptide, and a detection limit of 1.8 fmol was achieved with a narrow scan range [6].

In addition, the coaxial CF-FAB design has been successfully applied as an interface between CZE and MS. While maintaining separation efficiencies of hundreds of thousands of theoretical plates, peptide mixtures can be separated and analyzed by MS. CZE–MS detection limits have been observed for peptides of less than 10 fmol [9–12].

We have also demonstrated the ability to obtain MS–MS spectra of analytes using the coaxial CF-FAB interface [7,9–13]. Structurally informative MS–MS spectra of a tripeptide have been acquired on-the-fly from 540 fmol. A 54-fmol injection resulted in an MS–MS spectrum of sufficient quality to confirm the identity of a compound. In addition, the MS–MS spectra of peptides can be obtained as they are electrophoretically separated by CZE. Because the peak widths of CZE peaks are so narrow (typically 1–5 s wide at half-height), the acquisition of on-the-fly MS–MS data from a CZE separation can be somewhat difficult. A compromise between the separation efficiencies and the CZE peak widths, however, can allow one to obtain good MS–MS spectra. As an extension of this work, we report here the MS–MS data acquired from the separation of tryptic digests and peptide mixtures acquired using nanoscale packed capillary columns and capillary zone electrophoresis.

EXPERIMENTAL

Nanoscale capillary LC system

The nanoscale packed capillary LC columns used in this work were fabricated by using a modification of the method of Kennedy and Jorgenson [14]. For the analysis of the tryptic digest of bovine growth hormone releasing factor (1–29) a 50 μm I.D. × 150 μm O.D. column packed with 10-μm Hypersil C_{18} particles (Shandon) was used. For the horse heart cytochrome *c* tryptic digest a 75 μm I.D. × 150 μm O.D. column packed with 10 μm AQ-C_{18} particles (YMC) was used. In both analyses, the column was fully packed for 2 m in length. For the analysis of adrenocorticotropic hormone (ACTH) (1–24) a 75-μm I.D. column with a 30-cm packing bed of 10-μm AQ-C_{18} particles was used. A stirred slurry of 30:1 (ml solvent:g particles) was used to pack the columns. Hexane was used as the slurry solvent and 2-propanol was used as the packing solvent. A syringe pump (μLC-500, Isco, Lincoln, NE, USA) was used to pressurize the packing slurry to 275 bar.

LC mobile phase gradients were generated using two reciprocating piston pumps (Waters Model 6000A, Waters Assoc., Milford, MA, USA) and a gradient-control system (Waters solvent programmer Model 660, Water Assoc.). HPLC-grade acetonitrile and methanol and 18 MΩ water (Milli-Q water system, Millipore, Bedford, MA, USA) were used for preparation of samples and mobile phases. All mobile phases and sample solutions contained 0.1% trifluoroacetic acid (TFA) and were filtered (0.45 μm) and degassed prior to use. Solvent delivery was accomplished by using a capillary liquid chromatography system that was modified for use with conventional LC gradient pumps [15,16]. Sample injections were made using a stainless-steel pressure vessel which contains a microvial of sample solution. The sample was injected onto the LC column by pressurizing the vessel to 1500 p.s.i. using helium gas. After the desired volume was injected, the vessel was depressurized and the LC capillary column was removed from the injection vessel and remounted in the capillary LC system.

The electrophoretic separations were performed using a CZE system which has been previously described [9]. The CZE columns were 12–15 μm I.D. × 150 μm O.D. fused-silica capillaries which terminate at the FAB probe tip. The CZE buffer was 0.005 *M* ammonium acetate. A 60-kV reversible power supply (Glassman High Voltage) was used. A safety interlock system incorporating a high-voltage relay (Kilovac High Voltage) was used for operator safety.

Mass spectrometry

The coaxial CF-FAB interface has been previously described [5]. A CZE or nanoscale packed capillary column is inserted into a sheath column (typically 160 μm I.D. × 350 μm O.D.). The separations take place in the inner column while the matrix simultaneously flows through the outer column. The mass spectrometer used to acquire the data for this work was a VG ZAB-4F of B_1E_1–E_2B_2 geometry [17]. The instrument is operated at 8 kV and is equipped with an Ion Tech atom gun and a standard VG CF-FAB source which was heated at 40–60°C. The samples were bombarded with 8-keV xenon atoms. The MS spectra were acquired by scanning MS-I (B_1E_1) with detection in the third field-free region. The MS–MS experiments were performed by focussing the parent through MS-I into the collision cell in the third

field-free region. Daughter-ion spectra were obtained by collisions at 8 keV with helium gas (50% beam reduction). The collisional activation decomposition (CAD) spectra were obtained by a linear E_2B_2 linked scan of MS-II. The data acquisition system used was a VG Analytical 11-250 data system.

Chemicals

The peptides were purchased from Sigma (St. Louis, USA). The L-(tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK) trypsin was purchased from Worthington Biochemical (Freehold, NJ, USA). The tryptic digests were carried out on bovine growth hormone releasing factor (1–29), horse heart cytochrome *c*, and adrenocorticotropic hormone (1–24) with a 1:100 ratio of enzyme to peptide in 0.2 *M* NH_4HCO_3 buffer. Digests were allowed to proceed for 1 h at 37°C. The enzymatic digestion was stopped by either freeze-drying or by the addition of TFA. All solvents were acquired from J. T. Baker, (Pittsburgh, NJ, USA).

RESULTS AND DISCUSSION

nCLC analysis

ACTH (1–24) was analyzed using a 75-μm I.D. nanoscale packed capillary column with a 30-cm packing bed. An injection corresponding to approximately 82 pmol of undigested protein was injected using the pressure injection vessel. All of the expected tryptic peptide fragments, fragment 16–17, 18–21, 17–21, 22–24, 9–15 and 1–8, were observed and separated in less than 60 min (Fig. 1). A gradient of 0%

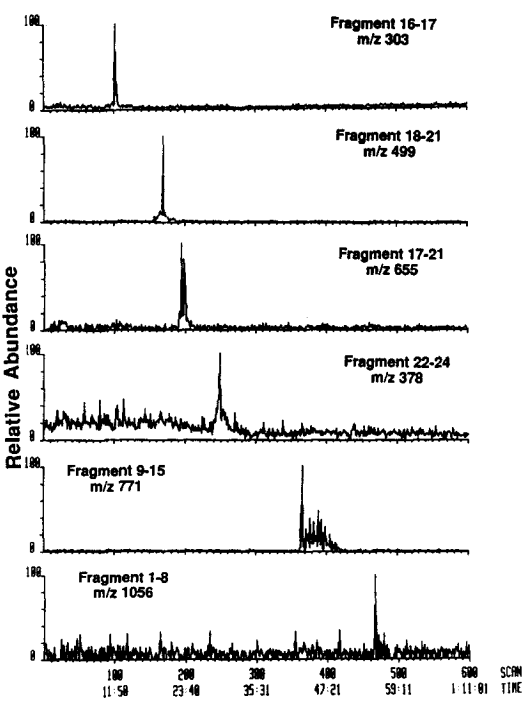

Fig. 1. Single-ion chromatograms of the tryptic fragments of ACTH (1–24) obtained from full-scan (2000–150 a.m.u.) data. Approximately 82 pmol of the protein was injected. Time in h:min:s.

acetonitrile for 5 min, then stepped to 10% and linearly programmed to 30% acetonitrile in water (0.1% TFA) was used to achieve this separation. The analysis was then repeated in order to obtain the corresponding MS–MS spectra of the tryptic fragments. An injection of approximately 250 pmol was used in order to obtain good daughter-ion information (Fig. 2). Fragments 16–17 and 18–21 (Fig. 2A and B, respectively) produce abundant side-chain cleavages as well as fragment ions corresponding to backbone cleavages of the tryptic peptide. Upon collisional activation fragment 17–21, 22–24 and 9–15 (Fig. 2C, D and E, respectively) decompose into structurally informative daughter ions including an *a*, *b* and/or *y* fragmentation at each peptide bond. Thus, information as to the identity of each amino acid in sequence is observed. The concentration of tryptic fragment 1–8 was insufficient to obtain a good MS–MS spectrum. Fragment ions corresponding to the backbone cleavages b_6 and a_7 were, however, observed for this tryptic peptide. Nomenclature for the peptide cleavages is that of Roepstorff and Fohlman [18] as modified by Biemann [19].

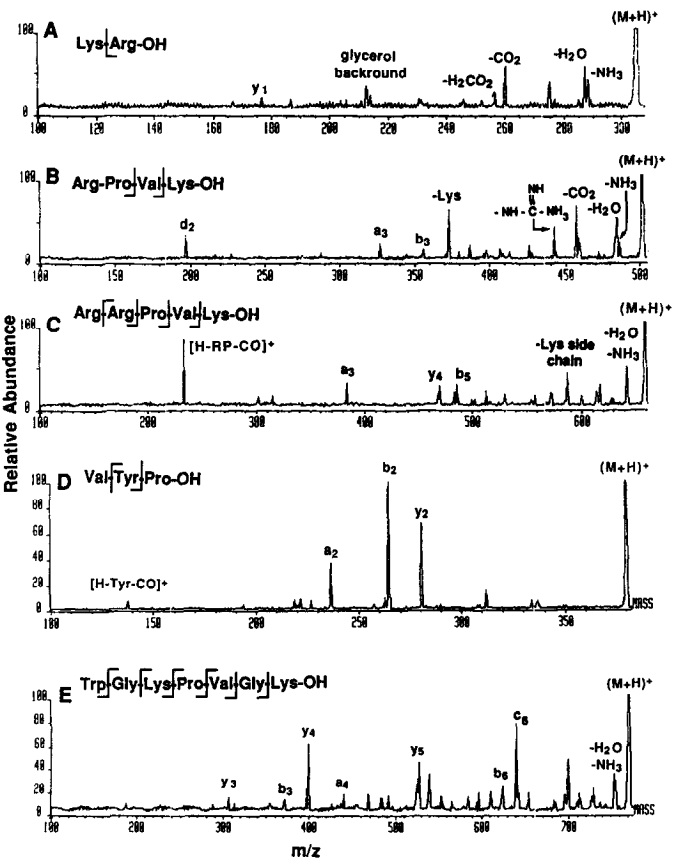

Fig. 2. MS–MS spectra of the $(M + H)^+$ ion of (A) fragment 16–17 (m/z 303); (B) fragment 18–21 (m/z 499); (C) fragment 17–21 (m/z 655); (D) fragment 22–24 (m/z 378); and (E) fragment 9–15 (m/z 771) of ACTH (1–24) acquired on-line from a nanoscale packed capillary column. Approximately 250 pmol of protein was injected.

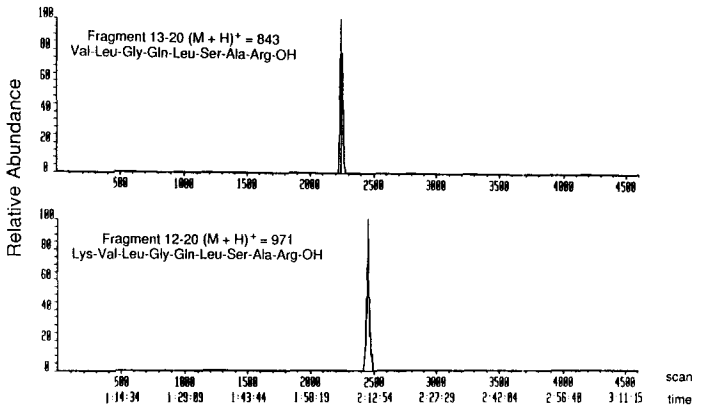

Fig. 3. Single-ion chromatograms of two tryptic fragments of bovine growth hormone releasing factor (1–29) analyzed by nanoscale LC–MS while scanning from 1160 to 800 a.m.u. The total amount of protein injected was approximately 0.8 pmol.

The tryptic digest of bovine growth hormone releasing factor was analyzed using a nanoscale packed capillary column. The total amount of protein injected was approximately 0.8 pmol in 2.5 nl of solution. At this low analyte level, a separation of four of the tryptic peptide fragments was obtained from a linear gradient of 5–70% methanol in water (0.1% TFA) over 180 min. These four peptide fragments correspond to fragments 13–20, 12–20, 22–29 and 21–29. The reconstructed-ion chromatograms of two of these tryptic fragments, fragments 13–20 Val–Leu–Gly–Gln–Leu–Ser–Ala–Arg and 12–20 Lys–Val–Leu–Gly–Gln–Leu–Ser–Ala–Arg, are shown, respectively, in Fig. 3. The MS–MS spectra of these two tryptic fragments were acquired and are shown in Fig. 4. Structurally informative daughter ions, including cleavages at five of the seven peptide bonds in fragment 13–20 (Fig. 4A) and five of the eight peptide bonds in fragment 12–20 (Fig. 4B), resulted from the collisional activation of the parent ions. Side-chain cleavages and *w* ions are the most abundant fragment ions observed. The other two tryptic fragments that were observed had a signal-to-noise ratio of approximately 3:1. At this level, the parent ions were too weak to obtain reasonable MS–MS spectra. Therefore, their reconstructed-ion chromatograms are not shown here.

The analysis of the tryptic digest of horse heart cytochrome *c* was acquired using an injection corresponding to 96 pmol of undigested protein. A large injection volume was used to insure detection of as many tryptic fragments as possible. Over 40 chromatographic peaks were found using a gradient of 0% acetonitrile for 10 min, then stepped up to 15% and linearly programmed to 35% acetonitrile in water (0.1% TFA) over 120 min. Analysis of the digest by electrospray ionization MS revealed that the horse heart cytochrome *c* had only partially digested (approximately 75% undigested protein remained); therefore, the MS–MS spectra of only three of the most abundant tryptic peptides were obtained. The reconstructed-ion chromatograms of these tryptic fragments, fragments 9–13, 28–38 and 80–86, are shown in Fig. 5, and their corresponding MS–MS spectra are shown in Fig. 6. The CAD spectrum of fragment 9–13 (Fig. 6A) reveals peptide backbone cleavages at all peptide bonds

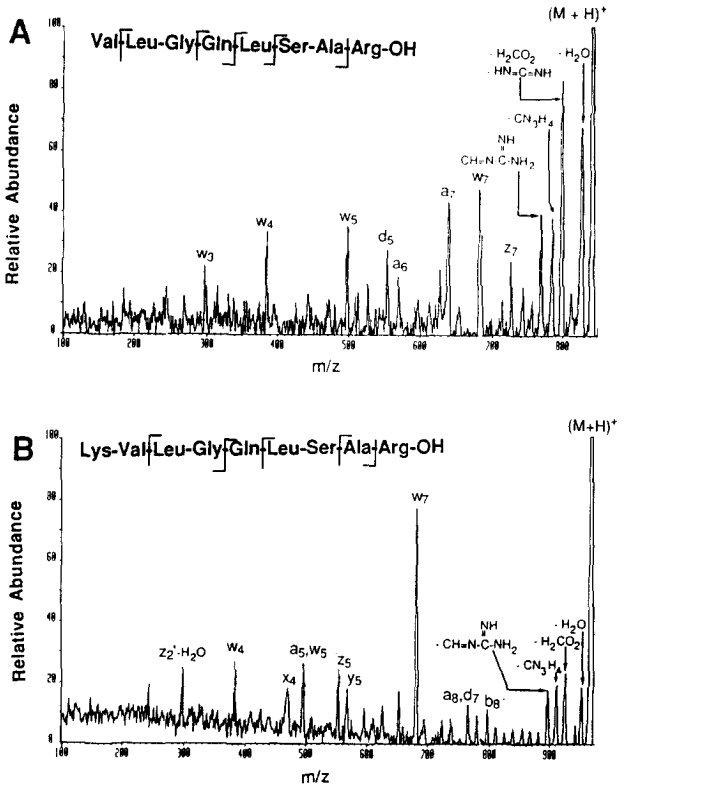

Fig. 4. MS–MS spectra of the $(M + H)^+$ ions of (A) tryptic peptide fragment 13–20 (m/z 843), and (B) tryptic peptide fragment 12–20 (m/z 971) of bovine growth hormone releasing factor (1–29).

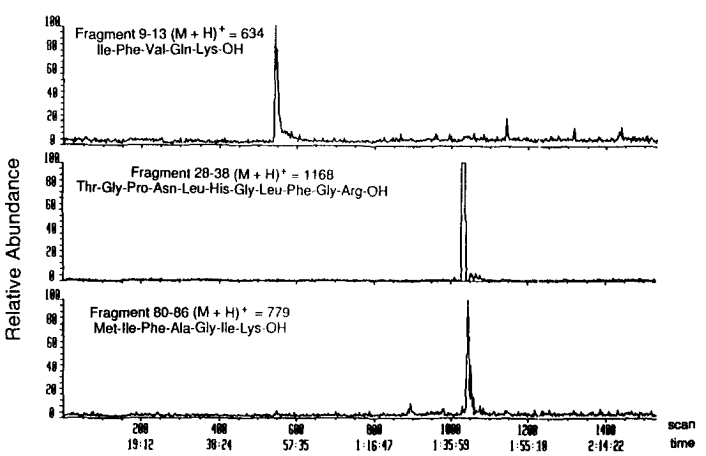

Fig. 5. Single-ion chromatograms of three tryptic fragments of horse heart cytochrome c analyzed by nanoscale LC—MS in the full-scan mode (1520– 200 a.m.u.). The total amount of protein injected was approximately 96 pmol.

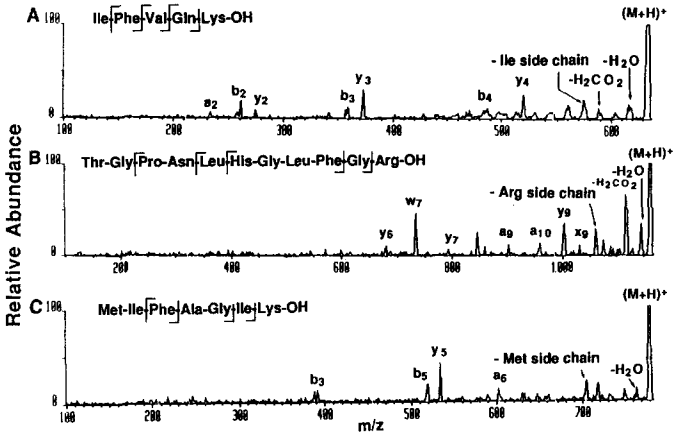

Fig. 6. MS–MS spectra of the $(M + H)^+$ ions of (A) tryptic peptide fragment 9–13 (m/z 634), (B) tryptic peptide fragment 28–38 (m/z 1168) and (C) tryptic peptide fragment 80–86 (m/z 779) of horse heart cytochrome *c*.

which can be used to determine the amino acid sequence of the peptide. The CAD spectra of the tryptic fragments 28–38 and 80–86 (Fig. 6B and C, respectively), while insufficient for complete sequence determination, provide a significant amount of structural information.

CZE analyses

The coaxial CF-FAB interface has proven to be useful for the analysis of peptide mixtures. The single-ion electropherograms of a separation of a mixture of met-enkephalinamide and met-enkephalin are shown in Fig. 7. The amount injected of each analyte is approximately 100 fmol. The CZE analysis was then repeated twice in order to acquire the MS–MS spectra of the two analytes as they migrated through the CZE column. Fig. 8 shows the MS–MS spectra of the $(M + H)^+$ ions of met-enkepha-

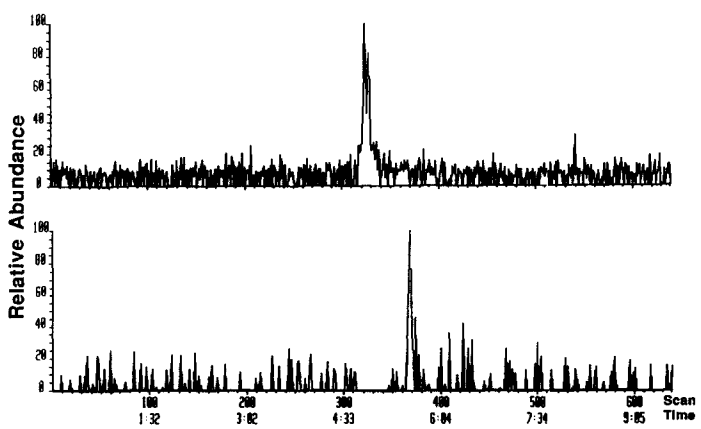

Fig. 7. Single-ion electropherograms of the $(M + H)^+$ ions of met-enkephalin-amide (m/z 573, top) and met-enkephalin (m/z 574, bottom) determined by CZE–MS (scanning from 590 to 545 a.m.u.).

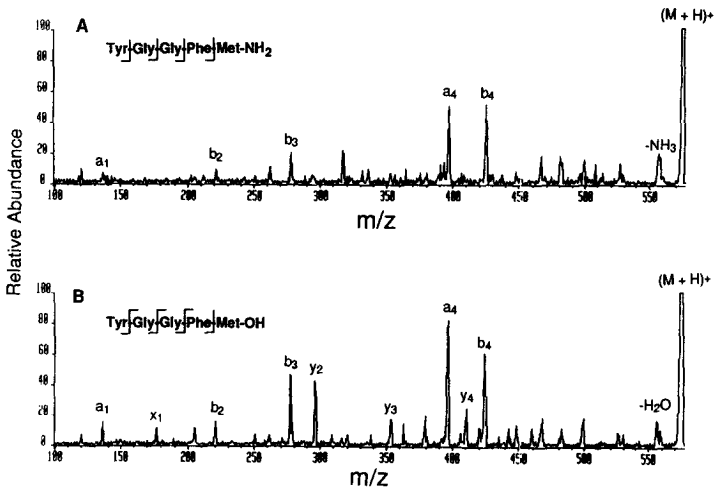

Fig. 8. MS–MS spectra of the $(M + H)^+$ ions of (A) met-enkephalinamide (m/z 573) and (B) met-enkephalin (m/z 574) electrophoretically separated by CZE.

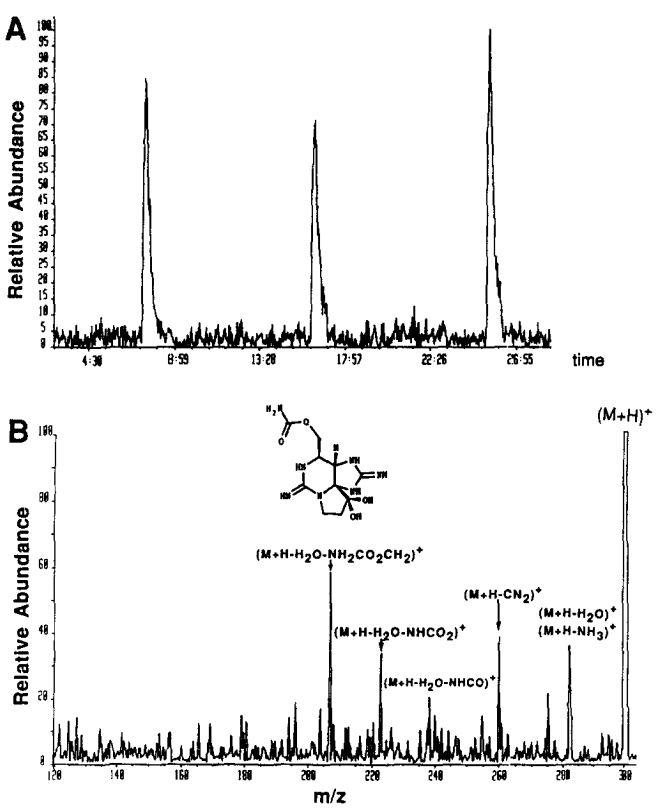

Fig. 9. (A) Single-ion chromatogram of the $(M + H)^+$ ion of saxitoxin acquired from triplicate injections of 260 fmol each while scanning from 305 to 285 a.m.u. (B) MS–MS spectrum of the $(M + H)^+$ ion of saxitoxin acquired by CZE in conjunction with coaxial CF-FAB.

linamide and met-enkephalin (approximately 400–800 fmol injected) acquired as the analytes migrated through the CZE column. Fragmentations at each peptide linkage as well as side-chain cleavages are observed in the CAD spectra. Sufficient fragmentation is observed to determine the amino acid sequence in the two peptides. In addition, the loss of NH_3 *vs.* the loss of H_2O clearly distinguishes the met-enkephalinamide from the met-enkephalin.

In addition to peptides, CZE–MS–MS is useful for the determination of other types of biological compounds. For example, saxitoxin, a paralytic shellfish toxin from the red-tide denoflagellate, can be analyzed by CZE in conjunction with coaxial CF-FAB. Excellent sensitivities can be obtained by this technique as demonstrated in Fig. 9A which shows triplicate injections of the marine toxin at the 260 fmol level while scanning the mass spectrometer over 20 a.m.u. Good reproducibility is observed at this low fmol level. The MS–MS spectrum of the $(M+H)^+$ ion of saxitoxin reveals structurally informative daughter ions (Fig. 9B). These ions compare with those which were obtained by FAB mass-analyzed ion kinetic energy spectroscopy [20] and ion spray [21].

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REFERENCES

1 M. Barber, R. S. Bordoli, R. D. Sedwick and A. M. Tyler *J. Chem. Soc. Commun.*, (1981) 325.
2 Y. Ito, T. Takeuchi, D. Ishii and M. Goto, *J. Chromatogr.*, 346 (1985) 161.
3 R. M. Caprioli, T. Fan and J. S. Cottrell, *Anal. Chem.*, 58 (1986) 2949.
4 J. W. Jorgenson and E. J. Guthrie, *J. Chromatogr.*, 255 (1983) 335.
5 J. S. M. de Wit, L. J Deterding, M. A. Moseley, K. B. Tomer and J. W. Jorgenson, *Rapid Commun. Mass Spectrom.*, 2 (1988) 100.
6 M. A. Moseley, L. J. Deterding, J. S. M. de Wit, K. B. Tomer, R. T. Kennedy, N. Bragg and J. W. Jorgenson, *Anal. Chem.*, 61 (1989) 1577.
7 L. J. Deterding, M. A. Moseley, K. B. Tomer and J. W. Jorgenson, *Anal. Chem.*, 61 (1989) 2504.
8 S. Pleasance, P. Thibault, M. A. Moseley, L. J. Deterding, K. B. Tomer and J. W. Jorgenson, *J. Am. Soc. Mass Spectrom.*, 1 (1990) 312.
9 M. A. Moseley, L. J. Deterding, K. B. Tomer and J. W. Jorgenson, *Rapid Commun. Mass Spectrom.*, 3 (1989) 87.
10 M. A. Moseley, L. J. Deterding, K. B. Tomer and J. W. Jorgenson, *J. Chromatogr.*, 480 (1989) 247.
11 M. A. Moseley, L. J. Deterding, K. B. Tomer and J. W. Jorgenson, *J. Chromatogr.*, 516 (1990) 167.
12 M. A. Moseley, L. J. Deterding, K. B. Tomer and J. W. Jorgenson, *Anal. Chem.*, 63 (1991) 109–114.
13 L. J. Deterding and M. A. Moseley in R. M. Capridi (Editor), *Continuous Flow Fast Atom Bombardment Mass Spectrometry*, Wiley, New York, 1990, p. 181–185.
14 R. T. Kennedy and J. W. Jorgenson, *Anal. Chem.*, 61 (1989) 1128.
15 J. W. Jorgenson and E. J. Guthrie, *J. Chromatogr.*, 255 (1983) 335.
16 M. D. Oates and J. W. Jorgenson, *Anal. Chem.*, 61 (1989) 432.
17 J. R. Hass, B. N. Green, R. H. Bateman and P. A. Bott, *Proceedings of the 32nd Annual Conference on Mass Spectrometry and Allied Topics, San Antonio, TX, May 1984*, p. 380.
18 P. Roepstorff and J. Fohlman, *Biomed. Environ. Mass Spectrom.*, 11 (1984) 601.
19 K. Biemann, *Biomed. Environ. Mass Spectrom.*, 16 (1988) 99.
20 K. D. White, J. A. Sphon and S. Hall, *Anal. Chem.*, 58 (1986) 562.
21 M. A. Quilliam, B. A. Thomson, G. J. Scott and K. W. M. Siu, *Rapid Commun. Mass Spectrom.*, 3 (1989) 145.