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MICROBORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY FOR THE ANALYSIS OF PROTEOLYTIC DIGESTS BY CONTINUOUS-FLOW FAST-ATOM BOMBARDMENT MASS SPECTROMETRY

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SUMMARY

Microbore high-performance liquid chromatographic (HPLC) techniques have been combined with fast atom bombardment mass spectrometry (FAB-MS) for the mass-specific detection of mixtures of peptides produced by proteolytic hydrolysis of proteins. The continuous-flow FAB interface has been utilized for direct coupling of the microbore HPLC system and the mass spectrometer. Conditions are reported for the effective separation of 100 pmol of peptides at flow-rates of 5 μl/min with acetonitrile gradients and 1-mm I.D. C_8 columns. A comparison is also made between columns of 5 and 25 cm lengths for the separation of peptide mixtures. Data are presented for the separation of peptides on a slurry-packed C_{18} fused-silica capillary column with the continuous-flow HPLC–FAB-MS interface at flow-rates of 3 μl/min.

INTRODUCTION

Desorption ionization techniques in mass spectrometry (MS) have had a significant impact on biochemical analysis because they are capable of analyzing polar and ionic compounds without chemical derivatization. Fast atom bombardment mass spectrometry (FAB-MS) offers the additional advantage that it is performed on liquid samples[1,2]. High-energy particles, such as 6-keV xenon atoms, bombard the surface of the liquid sample and sputter the surface layers of molecules. Charged species present in the gas phase are then analyzed by the mass spectrometer. For individual samples, a highly viscous solvent (>90% glycerol, thioglycerol, ethanolamine, or similar substance) is used in order to keep the sample in the liquid state during sample insertion into the high-vacuum source of the instrument and throughout the analysis.

A recent development, termed continuous-flow or dynamic FAB, has established that aqueous sample solutions can be analyzed directly by FAB-MS[3,4]. This is accomplished by using a special continuous-flow interface which is commonly operated in two modes. (1) Aqueous samples can be analyzed by using a flow-injection technique whereby 0.5- to 1.0-μl injections of sample are made into a flowing carrier solvent. This solvent may contain as much as 95% water and only 5% glycerol. (2)

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Sample solutions may be continuously monitored by allowing them to flow directly into the source, if they contain 5% or more of glycerol or similar viscous substance. For most commercially available mass spectrometers, standard pumping systems will handle up to about 10 μl/min of direct liquid flow. The technique has been shown to be effective in following enzymic reactions and analyzing solutions containing compounds the concentrations of which change rapidly with time[5].

The continuous-flow FAB interface has also been used to couple microbore high-performance liquid chromatography (HPLC) with MS[6]. Mixtures of peptides and some oligosaccharides were analyzed by HPLC–MS, using a 25 cm × 1 mm I.D. RP-300 column at a flow-rate of 5 μl/min with a gradient up to 50% acetonitrile. This work demonstrated that the combination of low flow-rate microbore HPLC and FAB-MS is feasible and that the technique could be used to measure the specific masses of biological compounds up to *ca.* 6 kilodalton (kD). Other work involving the combination of FAB-MS and HPLC has been published and involved use of a frit interface[7] and a moving-belt interface[8].

We report here further work on the analysis of proteolytic digests by microbore HPLC–FAB-MS methods with the continuous-flow interface. The separation and mass analysis of a digest of horse heart cytochrome *c* with a 5-cm microbore column is shown. Comparisons of the performance of a 5-cm and 25-cm C_8 column for this digest as well as for chymotrypsin and subtilisin digests are made. In addition, the HPLC–FAB-MS analysis of a peptide mixture, separated on a slurry-packed C_{18} fused-silica capillary column is also described.

EXPERIMENTAL

Instrumentation

An Applied Biosystems (Foster City, CA, U.S.A.) Model 130A microbore HPLC system, fitted with a 25 cm × 1 mm I.D. Brownlee RP-300 column was used for the chromatographic separations. The unit was modified by installing a Rheodyne 7410 injector with a 0.5-μl injection loop. A splitter (1:19) was placed between the pump and the injector so that gradients could be rapidly and efficiently generated at 100 μl/min pump flow-rates while still allowing a flow-rate of 5 μl/min through the column. Gradient elutions were accomplished with a mixture of 5% glycerol in water containing 0.1% trifluoroacetic acid (TFA) as eluent A, and 5% glycerol, 60% acetonitrile, 35% water, 0.1% TFA as eluent B. The end of the column was connected directly to the continuous-flow FAB probe by 1 m of 0.075 mm (I.D.) fused-silica capillary. The end of the capillary terminated at the probe tip, where the eluent was bombarded by energetic xenon atoms inside the ionization chamber of the mass spectrometer. The instrument arrangement is shown schematically in Fig. 1.

The 5 cm × 1 mm I.D. and 25 cm × 1 mm I.D. RP-300 (C_8) microbore columns were obtained from Applied Biosystems; the 25 cm × 0.3 mm I.D. 5-μm RoSil C_{18} slurry-packed fused-silica capillary was from Alltech (Eke, Belgium); the empty 75-μm I.D. fused-silica capillary transfer line was from SGE (Austin, TX, U.S.A.), and the fittings to connect this capillary to the Rheodyne injector were from Upchurch Scientific (Oak Harbor, WA, U.S.A.). In the case of the RoSil packed capillary column, a Valco (Houston, TX, U.S.A.) submicroliter injector was used with a 60-nl loop and with connection fittings supplied by Alltech.

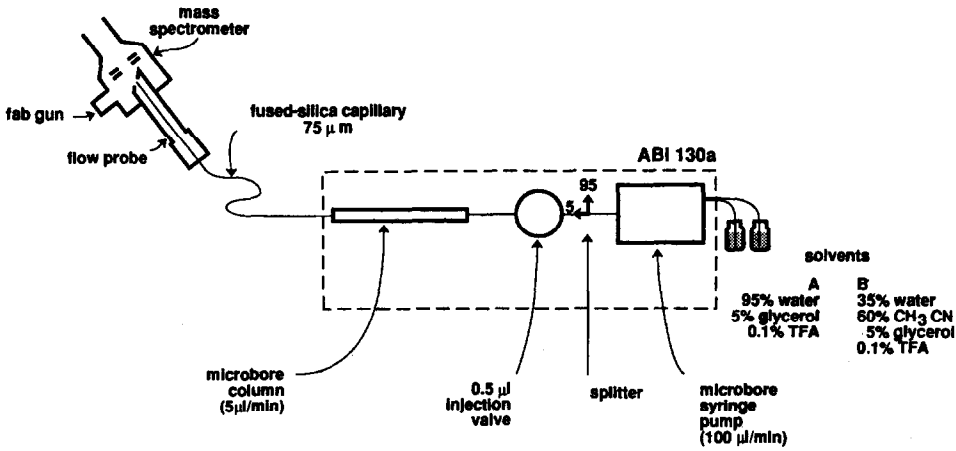

Fig. 1. Instrumental arrangement for microbore HPLC–FAB-MS with the continuous-flow interface.

A Kratos MS50RF high-resolution mass spectrometer, equipped with a DS90 (S/280) data system was used. The instrument was operated at 4 keV accelerating voltage and a resolution of about 1200. The probe tip was maintained at 40°C. The Ion Tech B11NF saddle field gun was operated at 6 keV and 40 μA current, and xenon was used. For data acquisition the raw data multi-channel-analyzer program, available with the data system, was used. Cesium iodide was the standard for mass calibration.

Enzyme digests

The protease digests were carried out on 0.2–0.4 m*M* cytochrome *c* with a 1:50 weight ratio of enzyme to substrate in 0.1 *M* Tris buffer at pH 8.6. Digests using chymotrypsin and subtilisin contained 10 m*M* calcium chloride. Digestions were allowed to proceed overnight at 37°C and digests were used directly for the HPLC–FAB-MS analysis.

Chemicals

All chemicals and reagents were obtained from Sigma (St. Louis, MO, U.S.A.) unless otherwise stated.

RESULTS AND DISCUSSION

Comparison of microbore columns

Peptide digests were separated and analyzed on both 5 cm × 1 mm I.D. and 25 cm × 1 mm I.D. RP-300 columns in order to compare elution times, resolution, and suitability for combination with a mass spectrometer detector. Fig. 2 shows the chromatograms for the analysis of a tryptic digest of approximately 50–100 pmol of horse heart cytochrome *c* on these two columns using a UV detector with a 0.5-μl cell. The same solvents were used in each case; eluent A contained 90% water, 10% glycerol, 0.1% TFA; eluent B, 40% acetonitrile, 50% water, 10% glycerol, 0.1% TFA. (Glycerol was included here because it is a necessary component of the mobile

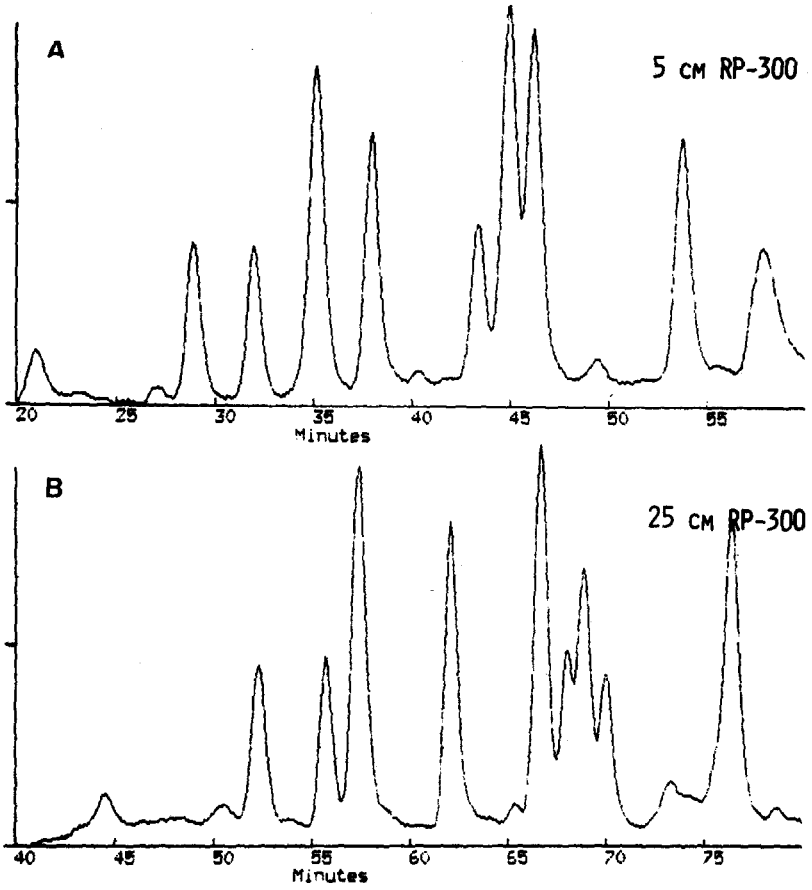

Fig. 2. Comparison of the separation of peptides in a tryptic digest of horse heart cytochrome *c* on 1-mm I.D. RP-300 (C_8) microbore columns of 5 cm (A) and 25 cm (B) lengths. Experimental conditions are given in the text.

phase for FAB-MS detection used later in the study.) The gradient program was somewhat different for each analysis in order to optimize the resolution (to a first approximation). Thus, for the 5-cm column a 0–30% acetonitrile gradient was used over the first 45 min, then it was stepped up and held at 40% acetonitrile, the gradient being started with sample injection. For the 25-cm column a 0–40% acetonitrile gradient was used for 60 min, thereafter held at 40% acetonitrile. The results showed a surprisingly similar performance in terms of resolution of the peptides at flow-rates of 5 μl/min, although it was somewhat better on the longer column. The general performance of the 5-cm column was also checked with the chymotrypsin and subtilisin digests of cytochrome *c*. In both cases, peptides were completely eluted over a time span of approximately 30–40 min, with a typical peak width of about 1 min at half-height.

HPLC–FAB-MS

The HPLC–FAB-MS analysis of 200 pmol of the tryptic digest of cytochrome *c* is shown in Fig. 3. It was obtained with a 5-cm RP-300 column and the same

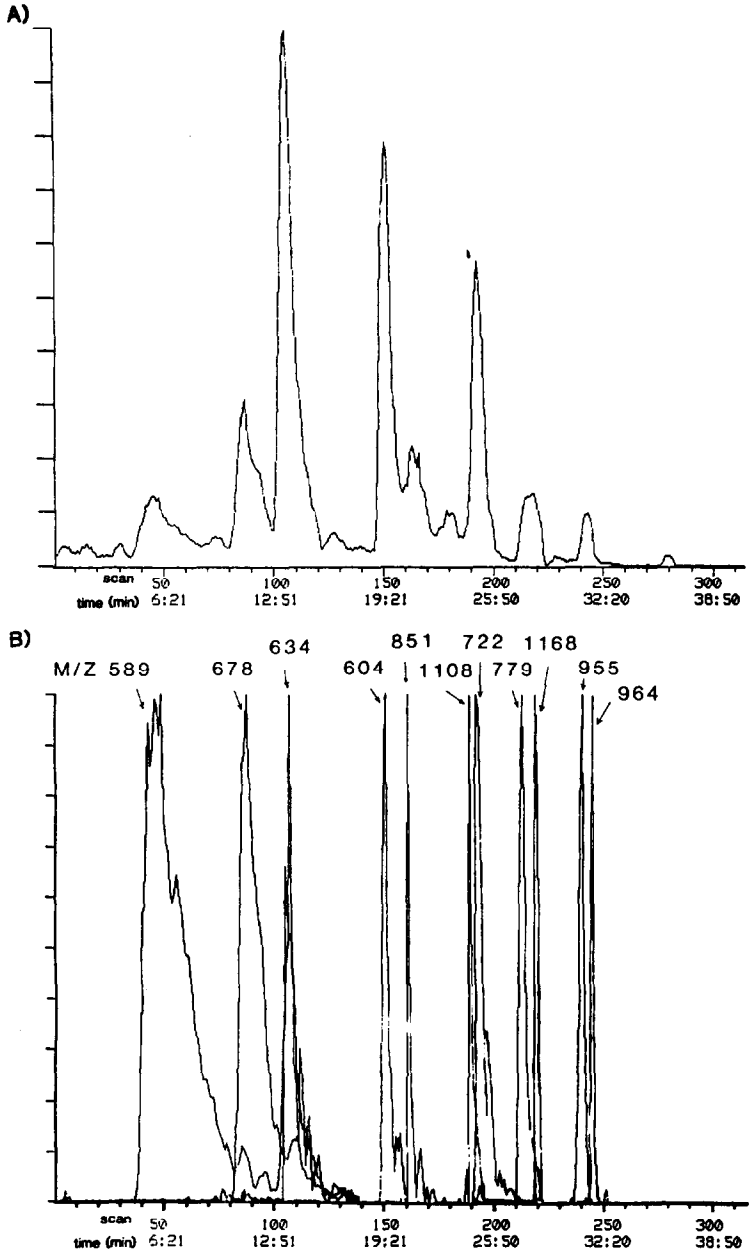

Fig. 3. HPLC–FAB-MS analysis of the tryptic digest of horse heart cytochrome *c*. (A) Total ion chromatogram, produced from scans of a mass range of 1700–570 dalton. (B) Composite peptide chromatogram, produced from independently normalized selected-ion chromatograms of the $[M + H]^+$ ions of the peptides in the digest.

gradient as that used as described above. Fig. 3A shows the total ion chromatogram and Fig. 3B the composite selected ion chromatograms for each of the major molecular species identified by the analysis. The peptide chromatogram shown in Fig. 3B was produced by overlaying individual selected ion chromatograms, which were independently normalized. Ions selected for producing these plots were obtained by performing a "mass summary" for the total chromatogram. This mass summary is simply the sum of all the mass spectra in the entire run, as shown in Fig. 4, for the analysis of the cytochrome *c* tryptic digest. Any ions that are a part of the chemical background do not produce discernable peaks in ion chromatograms and are therefore not included in the peptide chromatogram plots.

The relative response of the mass spectrometer detector and the UV detector are quite different with respect to the size of the peptides, as seen by comparison of Figs. 2A and 3A. In the case of the UV detector where the peptide bond is being monitored at 210 nm, the relative response of a typical peptide tends to increase as the number of residues increases. For the mass spectrometer detector, the number of ions recorded at the detector decreases as the mass increases. Of course, these trends are not absolute, because other factors are also involved. Nevertheless, this makes quantitative comparisons of the data obtained from these two detectors difficult, although qualitatively they are quite similar.

The mass analysis of the tryptic digest identified several small peptides which were not predicted from the known tryptic sites of cleavage and also did not show several of the larger peptides which were expected. The measured $[M + H]^+$ values

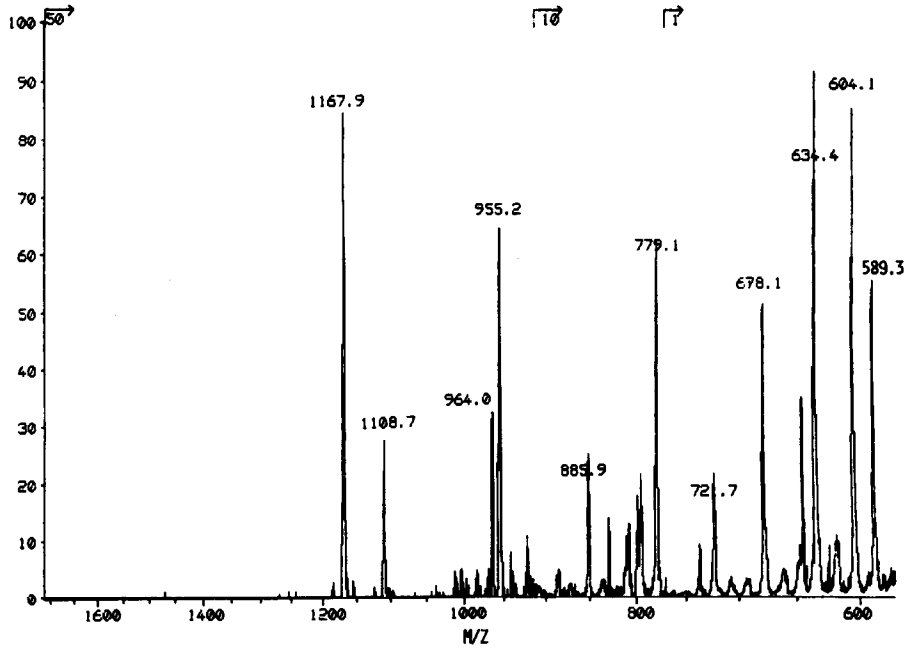

Fig. 4. "Mass summary", derived from the HPLC–FAB-MS analysis shown in Fig. 3 by summing all the mass spectra recorded during the analysis. Such a summed mass spectrum is used to identify the $[M + H]^+$ ions used to produce the "peptide chromatogram" shown in Fig. 3B.

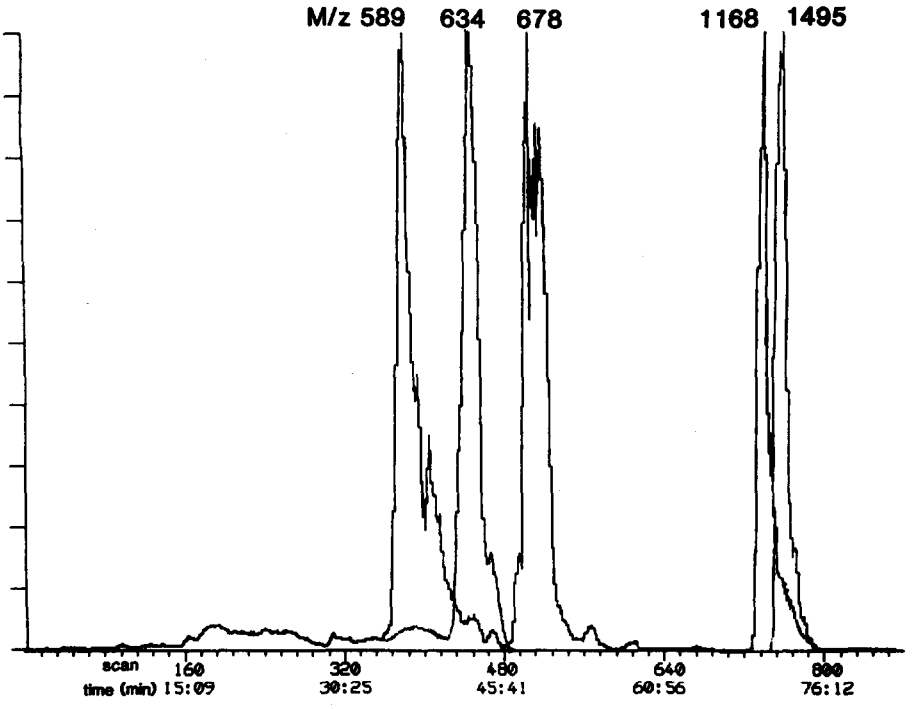

Fig. 5. Superimposed selected-ion chromatograms of some of the $[M + H]^+$ ions of peptides in the tryptic digest of cytochrome *c*, analyzed by fused-silica capillary HPLC–FAB-MS. Experimental conditions are given in the text.

of several of the masses of the unexpected peptides corresponded to those which would be produced from the action of chymotrypsin on tryptic peptides of cytochrome *c*. Evidently, even though the trypsin used had been treated to eliminate chymotrypsin activity, residual activity of this enzyme, as well as perhaps some other contaminating proteases, was sufficient to produce the smaller peptides during overnight digestion.

Slurry-packed fused-silica columns

The tryptic digest of cytochrome *c* was analyzed by HPLC–FAB-MS on the 5-μm RoSil C_{18} fused-silica capillary column (25 cm × 0.3 mm I.D.) with the continuous-flow interface. The end of the column was attached to a 25 cm × 75 μm I.D. section of unpacked fused-silica for insertion into the continuous-flow interface. An acetonitrile gradient was applied from 15 to 60% over 6 min, and held at 60% thereafter. The eluent also contained 5% glycerol and 0.1% TFA. Several of the ion chromatograms are plotted in Fig. 5 for the analysis of 120 pmol of digest. The results show good resolution of the lower-molecular-weight peptides with some separation at higher molecular weights. Undoubtedly, optimization of the gradient conditions would significantly increase the resolution of the latter. Nevertheless, this analysis demonstrates the utility of the combination of packed capillary columns and FAB-MS in the analysis of picomolar amounts of peptides by direct injection of protease digests.

CONCLUSION

The use of microbore and slurry-packed fused-silica capillary columns in combination with FAB-MS has been shown to be an effective technique for the mass-specific analysis of mixtures of peptides. Although the capillary columns are designed to operate at the 3–5 μl/min flow-rates used in this study, it is noted that the microbore columns used also performed well at these flow-rates. Use of a mass spectrometer detector makes the resolution of peptides on the column a much less critical factor in the analysis of protease digests because individual mass chromatograms can effectively distinguish between several compounds emerging at the same retention time. The low flow-rates are ideal for FAB-MS because they lead to high concentrations of compounds for maximal detection efficiency. Furthermore, the continuous-flow interface permits the use of FAB ionization for the detection of a wide range of polar and ionic biological compounds on-line with HPLC, eliminating the need for prior separation, purification and derivatization.

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