Journal of Chromatography, 331 (1985) 425–431
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 893

Note

Sequence determination of cyanogen bromide-peptides by combined use of high-performance liquid chromatography and fast atom bombardment mass spectrometry

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(Received May 14th, 1985)

The separation of complex mixtures of peptides generated by enzymatic and chemical cleavage of proteins has been greatly improved by the introduction of high-performance liquid chromatography (HPLC)[1–3]. In particular, reversed-phase HPLC (RP-HPLC) has been replacing more classical methods of peptide fractionation in many laboratories and represents one of the most important advances in the field of protein sequence analysis[4–7].

In recent years, a number of peptides have been sequenced by mass spectrometric (MS) methods and the advent of a new soft ionization technique, fast atom bombardment (FAB), has allowed sequence studies of peptides in the high molecular weight range[8–10].

Here we describe the complete separation of the pair homoserine–homoserine lactone containing peptides resulting from cyanogen bromide digestion of mitochondrial aspartate aminotransferase from ox heart by HPLC. We have also applied FAB MS to the identification of the peptide pairs and to the determination of their primary structure.

EXPERIMENTAL

Mitochondrial aspartate aminotransferase (mit-o-AAT) was purified from ox heart as described earlier[11].

Cleavage by cyanogen bromide (CNBr) was performed according to Gross and Witkop[12]; reduced and carboxymethylated mit-o-AAT (80 mg) was dissolved in 70% formic acid and a 100-fold molar excess of CNBr over methionine residues was added. The reaction was carried out at room temperature for 24 h, then the sample was diluted with cold water and freeze-dried.

The resulting peptide mixture was partially resolved by gel filtration chromatography on a Sephadex G-75 Superfine column; low-molecular-weight fragments were further purified by HPLC.

HPLC analyses were performed on a Beckman Model 332 liquid chromatograph with a Model 420 system controller programmer. The column was an Altex Ultrasphere ODS (5 μm, 250 × 4.6 mm I.D.). Water was doubly distilled. HPLC-

0021-9673/85/$03.30

grade trifluoroacetic acid (TFA) was purchased from Carlo Erba and HPLC-grade acetonitrile from Baker.

Amino acid analyses were performed on a Beckman Model 119 CL amino acid analyser after acidic hydrolysis. Homoserine lactone was converted into the corresponding free amino acid by boiling in 1.2 *M* pyridine–acetic acid buffer (pH 6.5) for 1 h immediately before being analysed[13].

Manual Edman degradation was accomplished using 5% phenyl isothiocyanate in pyridine as a coupling reagent. After the cleavage step the sample was dried and loaded on to the probe tip as described below.

FAB spectra were recorded on a VG ZAB HF mass spectrometer equipped with an M-Scan gun using xenon as the primary ionizing beam (current 20 μA at 8 keV).

Samples were dissolved in 5% acetic acid and loaded on to a glycerol-coated probe tip; when it was necessary, thioglycerol and/or 0.1 *M* HCl were added directly on to the probe.

Acetyl-[$^2H_3$]acetyl derivatives (1:1) were prepared by reacting the peptides with a mixture of acetic anhydride and [$^2H_6$]acetic anhydride in methanol at room temperature for 2–4 min.

Tryptic and chymotryptic digests were carried out in 0.4% ammonium hydrogen carbonate (pH 8.5) at 37°C for 15 min.

Trypsin, chymotrypsin, cyanogen bromide and thioglycerol were obtained from Sigma, acetic anhydride from BDH and [$^2H_6$]acetic anhydride from Aldrich. Sephadex G-75 Superfine was purchased from Pharmacia. All other chemicals were Baker analysed reagents.

RESULTS

The whole CNBr digest was first fractionated on a Sephadex G-75 Superfine column, equilibrated and eluted with 10% formic acid, giving rise to five major peaks.

The three lowest molecular weight fractions, 3, 4 and 5, were further purified by HPLC. Samples were dissolved in 0.1% TFA and loaded on to the column. The solvent system includes 0.1% aqueous TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B). Fig. 1 shows the HPLC profiles of Sephadex fractions 3, 4 and 5; fraction 5 was resolved into four fully separated peaks, B_5, C_5, E_5 and F_5, and fraction 4 gave two peaks, A_4 and B_4. Fraction 3 resulted in a more complicated pattern and only four homogeneous peaks were collected, A_3, B_3, C_3 and D_3.

Aliquots of these peptides were submitted to amino acid analysis and the results are reported in Table I. Each peptide seems to exist in two different forms having the same amino acid composition but different chromatographic behaviour.

The nature of the heterogeneity was hypothesized as being due to part of the sample ending in free homoserine and part in the homoserine lactone form. This hypothesis was confirmed by FAB MS analysis of the samples. Fig. 2 shows the quasi molecular ion region of the five peptide pairs.

The early eluted peptide in each pair always has a molecular weight higher than that of the later eluted peptide by 18 mass units. The former contains the free homoserine residue at the C-terminus whereas the latter ends with the lactone form.

The complete amino acid sequence of the five peptides B_3, D_3, B_4, C_5 and F_5, obtained by FAB MS, is shown in Table II.

The interpretation of the spectra in terms of fragmentation occurring at peptide bond[9] allowed the assignment of most of the peptide sequences. In some instances,

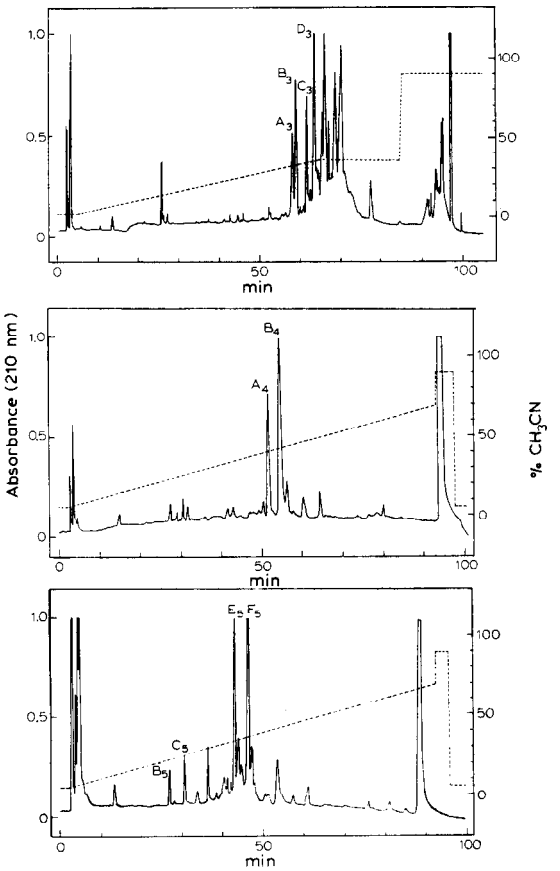

Fig. 1. HPLC traces of fractions 3 (top), 4 (middle) and 5 (bottom) from Sephadex G-75. Solvent A, 0.1% aqueous TFA; solvent B, 0.1% TFA in acetonitrile; flow-rate, 1 ml/min. The broken line indicates increasing acetonitrile concentration.

TABLE I

AMINO ACID COMPOSITION OF FIVE PEPTIDE PAIRS PURIFIED BY RP-HPLC

Amino acid	A_3	B_3	C_3	D_3	A_4	B_4	B_5	C_5	E_5	F_5
Asp	2.1	2.2	2.7	2.8	3.2	2.7	1.2	0.9	–	–
Thr	1.7	1.8	1.1	1.0	1.1	1.0	–	–	–	–
Ser	1.1	0.8	2.7	2.9	–	–	1.2	1.1	1.7	1.9
Glu	1.2	1.3	3.9	4.0	–	–	–	–	1.1	1.2
Pro	1.0	0.8	–	–	–	–	–	–	–	–
Gly	1.2	1.3	2.1	2.3	–	–	–	–	–	–
Ala	1.2	1.2	–	–	2.1	1.8	1.0	0.9	1.0	1.2
Val	0.9	1.1	1.4	1.2	2.2	1.8	–	–	1.2	1.3
Ile	1.2	1.3	2.7	2.9	–	–	1.3	1.1	–	–
Leu	1.3	1.4	1.8	2.1	1.2	1.1	–	–	–	–
Tyr	–	–	–	–	–	–	–	–	–	–
Phe	1.1	1.1	–	–	2.8	2.7	–	–	–	–
His	–	–	2.1	1.7	–	–	–	–	0.8	0.7
Lys	2.7	2.8	2.0	2.3	2.0	1.9	–	–	–	–
Arg	1.0	0.9	0.9	1.1	–	–	0.8	0.9	–	–
HomoSer	1	1	1	1	1	1	1	1	1	1

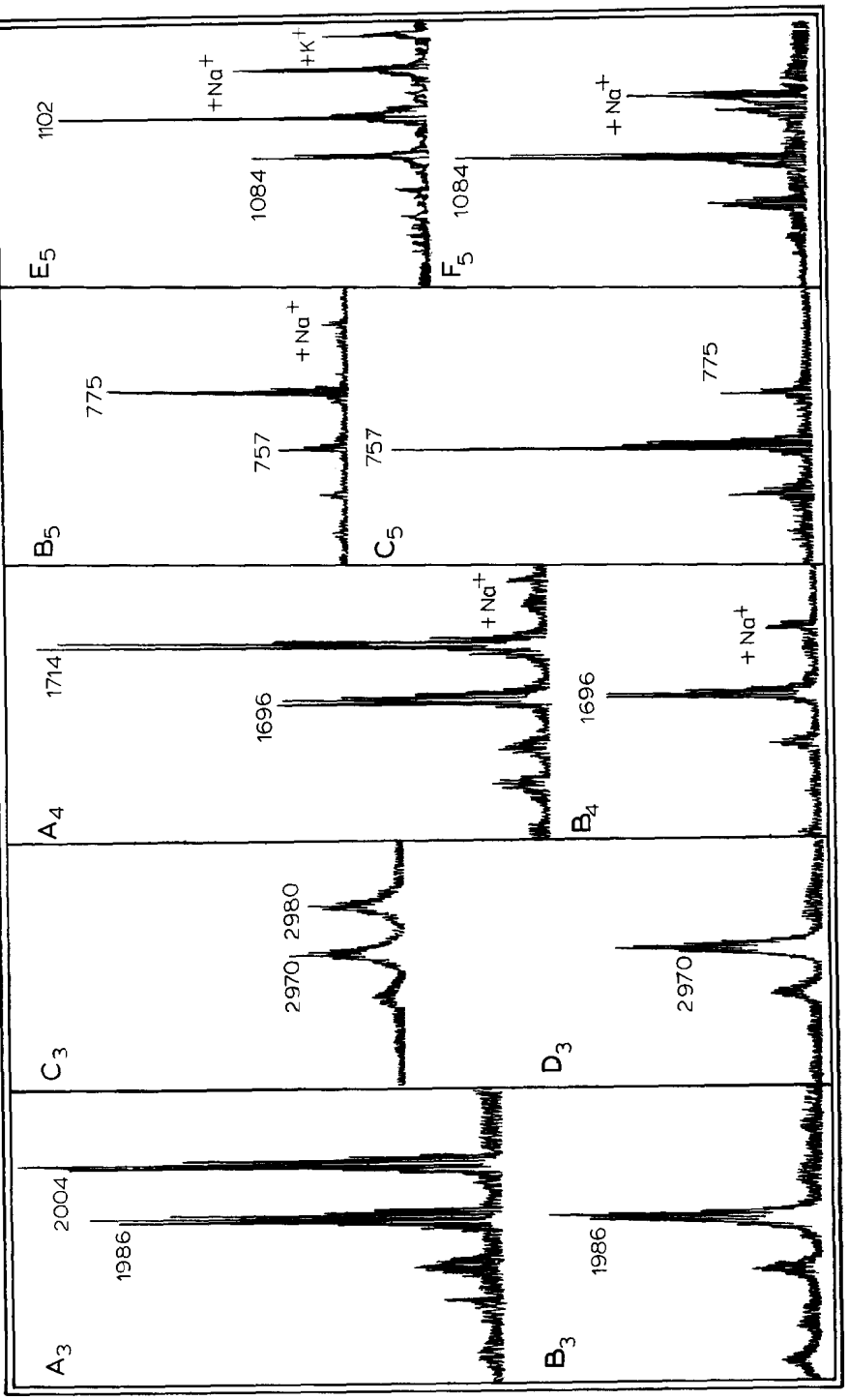

Fig. 2. Quasi-molecular ion region of FAB mass spectra of the five peptide pairs (see Fig. 1) purified by HPLC.

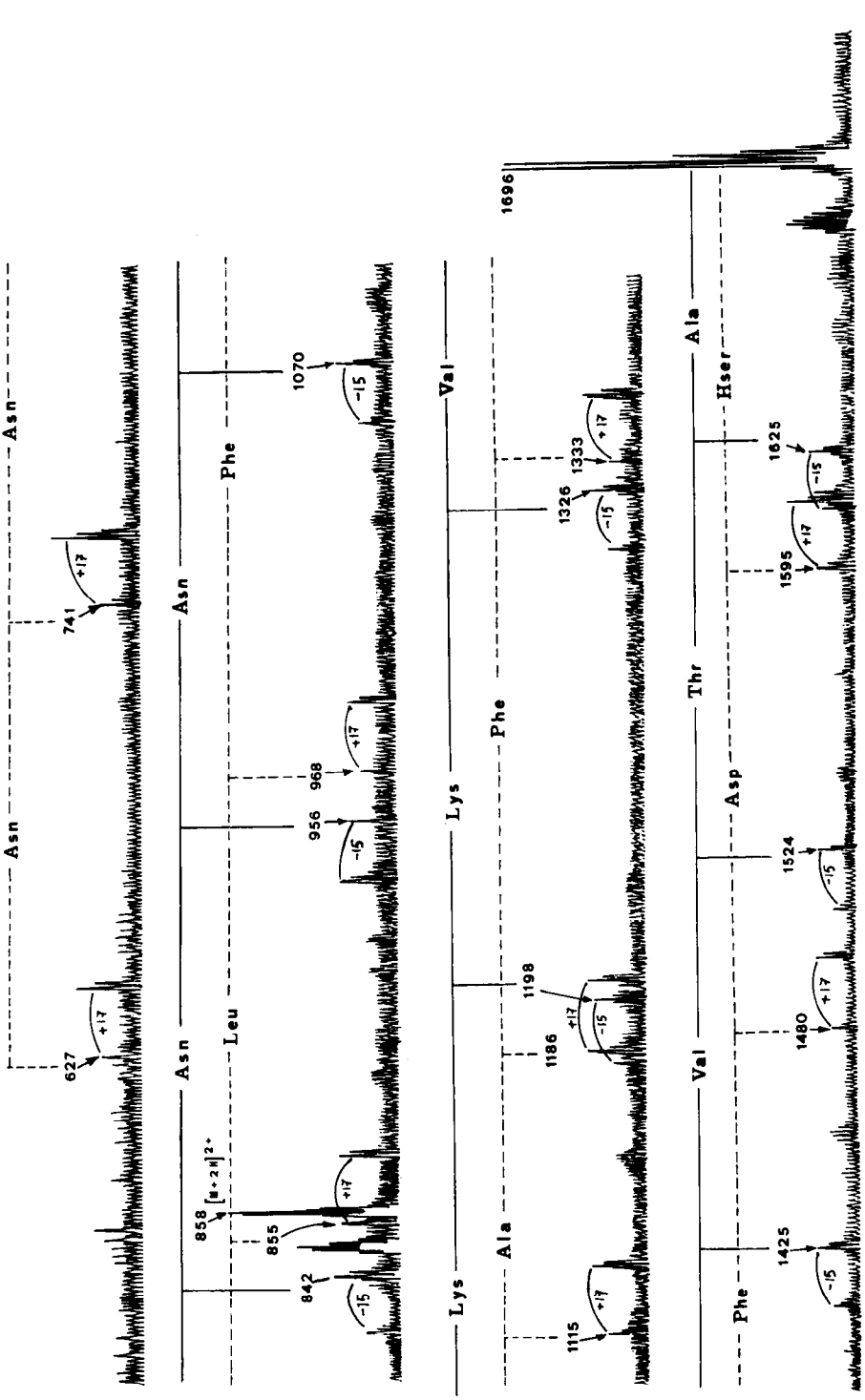

Fig. 3. FAB mass spectrum of non-derivatized peptide B_4 (see Fig. 1). Full line and broken line show N-terminal and C-terminal sequence, respectively.

TABLE II

AMINO ACID SEQUENCE OF FIVE CNBr-PEPTIDES FROM mit-o-AAT OBTAINED BY FAB MS

Peptide	*Sequence*
$A_3 = B_3$	Pro–Ile–Leu–Gly–Val–Thr–Glu–Ala–Phe–Lys–Arg–Asp–Thr–Asn–Ser–Lys–Lys–Met
$C_3 = D_3$	Arg–Thr–Gln–Leu–Val–Ser–Asn–Leu–Lys–Lys–Glu–Gly–Ser–Ser–His–Asn–Trp–Gln–His–Ile–Ile–Asn–Glu–Ile–Gly–Met
$A_4 = B_4$	Ala–Thr–Val–Val–Lys–Lys–Asn–Asn–Leu–Phe–Ala–Phe–Phe–Asp–Met
$B_5 = C_5$	Ala–Asp–Arg–Ile–Ile–Ser–Met
$E_5 = F_5$	Ser–Ser–Trp–Trp–Ala–His–Val–Glu–Met

however, samples were submitted to Edman degradation and the truncated peptides were analysed by FAB MS. The interpretation of peptide C_4 spectra was greatly facilitated by preparing the 1:1 acetyl–[$^2H_3$]acetyl derivative.

All peptide sequences were confirmed by the FAB mapping technique[10] by submitting aliquots of the peptides to limited proteolysis and directly analysing the resulting peptide mixture on the mass spectrometer.

As an example, we describe the determination of the peptide B_4 sequence. Fig. 3 shows the FAB mass spectrum of peptide B_4, having a quasi-molecular ion at m/z 1696. Fragment ions occurring at m/z 1625, 1524, 1425, 1326, 1198, 1070, 956 and 842 accounted for an N-terminal sequence Ala–Thr–Val–Val–Lys–Lys–Asn–Asn–... The C-terminal sequence was deduced to be ...–Asn–Asn–Leu–Phe–Ala–Phe–

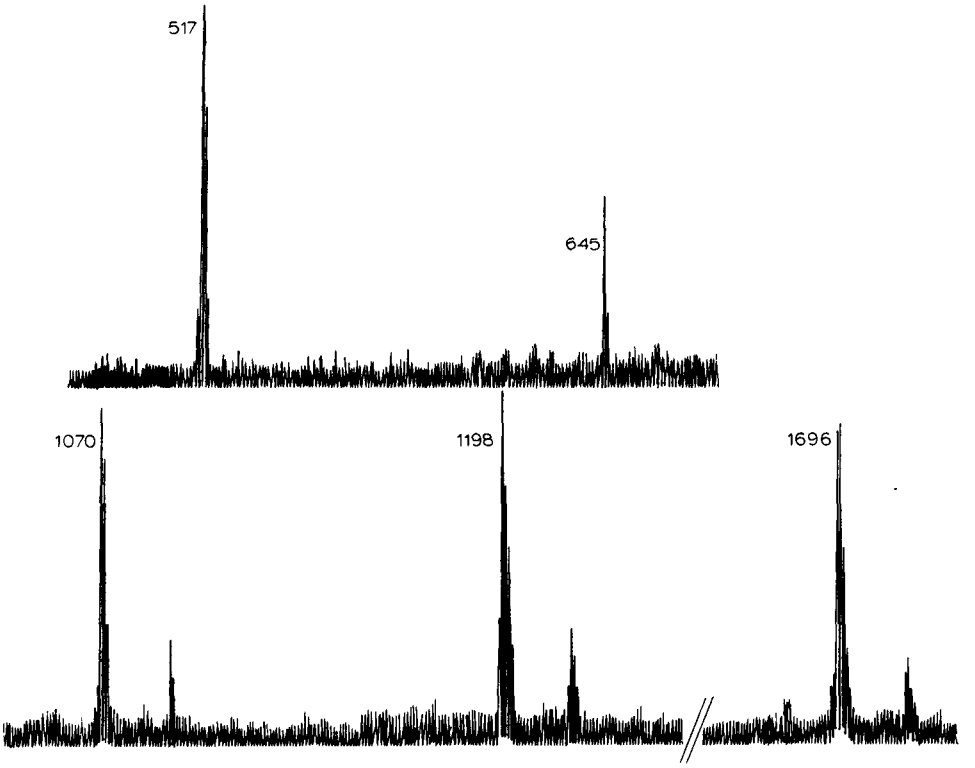

Fig. 4. FAB mass spectrum of tryptic digest of peptide B_4 (see Fig. 1).

Phe–Asp–Met from the signals at *m/z* 1595, 1480, 1333, 1186, 1115, 968, 855, 741 and 627. Because of the overlapping of the two Asn residues, the analysis of this spectrum gives the complete sequence of peptide B_4.

In order to confirm this sequence, the peptide was digested by trypsin; the FAB mass spectrum of the resulting peptide mixture is shown in Fig. 4. The quasi-molecular ion is still detectable at *m/z* 1696, while signals occurring at *m/z* 517, 645, 1070 and 1198 correspond to the expected tryptic peptides, therefore, confirming the above reported sequence.

DISCUSSION

We have described the real case of sequencing five CNBr peptides by coupling the chromatographic resolution power of HPLC and the sensitivity of FAB MS.

It should be noted that all the structural information was obtained on a portion of peptides collected from an analytical HPLC column. Further, FAB MS allowed the immediate establishment of the nature of pairs of peaks having the same amino acid composition. In fact, the mass difference (18 a.m.u.), corresponding to an H_2O molecule, clearly indicates the nature of the heterogeneity. Sometimes this microheterogeneity could represent a problem in structural studies of CNBr-peptides[14]. The FAB mapping approach was exploited when the sequence spectra did not allow confident assignment of some residues.

In conclusion, this work shows the great potential of HPLC and FAB MS when they are jointly applied to protein structural studies.

ACKNOWLEDGEMENTS

The authors thank Prof. H. R. Morris for the mass spectrometry facilities granted by the Medical Research Council at the Department of Biochemistry, Imperial College, London, U.K., where part of this work was performed during the tenure of a CNR (Rome) Fellowship (P.P.). Financial support from the Ministero Pubblica Istruzione (Rome) is acknowledged.

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