The Separation and Sequencing of Permethylated Peptides by Mass Spectrometry Directly Coupled to Gas–Liquid Chromatography

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The use of g.l.c. coupled to mass spectrometry to separate and sequence permethylated acetyl- and trifluoroacetyl-peptides in a single operation is described. Both electron impact and chemical ionization were used to induce fragmentation, and the latter was found to be more sensitive. Chromatographic retention data are presented which suggest that peptide derivatives of molecular weight of at least 750 are accessible to the technique. The application of our methods to the determination of the primary sequence of proteins is discussed.

This paper describes, we believe for the first time, the sequencing of permethylated peptides by mass spectrometry after their separation in a gas–liquid chromatograph directly coupled to the mass spectrometer. Both electron-impact and chemical ionization have been used to obtain the fragmentation patterns from which the amino acid sequences were deduced. *NO*-Permethylation, which enhances volatility and leads to a more informative fragmentation pattern, has been used on many previous occasions in mass-spectrometric sequence studies, including some on peptides obtained in quite small quantities by the chromatographic and electrophoretic methods of separation usual in conventional sequence work (e.g. Priddle, 1974; Priddle & Offord, 1974). However, the acylated permethylated peptides have hitherto been inserted directly into the ion source of the mass spectrometer as a deposit on the end of a probe and mixtures have been separated, if at all, by differential evaporation as the probe is progressively heated (Morris *et al.*, 1971). Ambiguities can result when the signals from two or more components are detected simultaneously, and it has been suggested that high-resolution spectrometry (Wipf *et al.*, 1973) or the analysis of duplicate samples with and without isotopic labelling (Roepstorff *et al.*, 1971) should be used to overcome the problem. We believe that it is the direct coupling of gas chromatography to mass spectrometry that offers the best hope of realizing the full potential of the mass-spectrometric method in sequence studies. The method should provide reliable sequence data with a significantly decreased expenditure of time and resources in comparison with conventional methods. The capacity to use one means of fragmentation or another further augments the power of the approach.

The gas chromatography of higher peptides, as opposed to derivatives of dipeptides or free amino

acids, has received little attention since the early work on *N*-trifluoroacetyl-peptide methyl esters [reviewed by Kolb (1971)], except for the studies on *N*-acyl-polyaminoalcohols (Kelley *et al.*, 1975; Nau *et al.*, 1975). Both techniques use trimethylsilylation of side-chain polar groups. Most recent peptide work has concentrated on the separation of dipeptides with a view to using aminodipeptidase enzymes (cathepsins) for sequential degradation of longer peptides from their *N*-terminus (Ovchinnikov & Kiryushkin, 1972; Calam, 1972; Caprioli *et al.*, 1973; Lindley & Davis, 1974). Calam (1972) demonstrated that acetylation and permethylation made dipeptides (and also some tripeptides) amenable to gas chromatography. We have now been able to separate larger peptides, and, more importantly for our purpose, found it possible to use the spectrometer to deduce sequences after the injection of realistically small quantities on to the column. We have chosen the approach described here, rather than that of reduction to polyaminoalcohols, because permethylated peptides can give more readily interpretable fragmentation patterns. They approach the polyaminoalcohols in volatility, particularly when there are several side-chain functional groups.

Experimental

The peptide derivatives were prepared as described by Priddle (1974), or by trifluoroacetylation with trifluoroacetic anhydride/trifluoroacetic acid (1:4, v/v) for 1 h at room temperature (18–22°C) followed by permethylation as previously described (Priddle, 1974). They were eluted from a 0.3 m glass column, 2 mm internal diameter, packed with 3% OV-1 or 1% Dexsil 300GC on Diatomite CQ. A Pye 104 gas chromatograph (Pye–Unicam, Cambridge CB1 2PX, U.K.) was used. The columns were exhaustively

conditioned at 330°C with helium flowing. The peptides were injected as a chloroform solution (1 μ l) directly into the column packing, with the injection zone held at 305°C. He carrier-gas flow was 15 ml/min and effluent was directly coupled to the Micromass 30F mass spectrometer (VG Organic Ltd., Altrincham, Cheshire WA14 5TL, U.K.) via an all-glass tee-splitter held at 280°C, which allowed 5 ml of gas/min to enter the source. The temperature of the source was 275°C. The machine is differentially pumped, with a 1200 litres/s diffusion pump on the source housing. This allows coupling without any form of molecular separator, which might have had adsorptive effects. At 5 ml of He/min, resolution was maintained at 1000–1200 (10% valley).

Chemical ionization was carried out by using the narrower source slit of the mass spectrometer and with isobutane as the reagent gas. The flow of reagent gas was adjusted until the ratio of the intensities of the peaks at m/e 57 and m/e 43 was 3:1.

Results

Fig. 1 shows the electron-impact mass spectrum of a derivative of the pentapeptide Ser-Gly-Leu-Phe-Asp on elution from the dimethylsilicone column. There was a maximum of 25 nmol present in the source during the period of the scan. The progressive decrease in intensity of the ions with increasing mass can be seen. In contrast, the chemical-ionization spectrum of a derivative of the tetrapeptide Ser-Gly-Leu-Phe in Fig. 2 shows good intensity at high mass; the quasimolecular ion has 37% of the intensity of the first sequence ion. A maximum of 17 nmol was consumed in the source during the scan. The quantities that were injected on the packed columns were 100–150 nmol, and it will probably be necessary to use open tubular columns to realize the full potential

sensitivity of the method. Such a column gives much sharper peaks, and it has proved possible to admit the total effluent into the source (Priddle *et al.*, 1976). Fig. 2 shows that even without this improvement it would already be possible to sequence much smaller quantities of peptide than 17 nmol when using chemical ionization.

The graph of approximate retention index versus molecular weight (Fig. 3) implies that many pentapeptides would be accessible to the technique. Dexsil 300 is slightly polar and retards the peptide derivatives more than does OV-1, which is a simple dimethylsilicone. Fig. 3 shows that a significant increase in volatility results from acylation with the trifluoroacetyl group (Nau, 1974). Other workers have experienced difficulties in permethylation, particularly of *N*-trifluoroacetyl-peptides (Calam, 1972; Lindley & Davis, 1974). We are using the gas-chromatographic separation of by-products such as those formed by *C*-methylation and by anomalous reactions at the *C*-terminus to assess the overall yields of the permethylation. We have found that the short permethylation technique of Morris (1972) yields acceptable products, but that none of the permethylation procedures currently in use are ideal.

We have also found that retention data provide useful confirming evidence on length of peptides. This has been helpful in gas-chromatographic studies of mixtures containing unexpected components and in preliminary runs where insufficient material meant that molecular ions were not observed. We anticipate that knowledge of retention indices will make it possible to place limits on amino acid compositions and vice versa. The contributions from each amino acid residue would be expected to be additive (Kolb, 1971).

The techniques described in this paper could be used to separate and sequence mixtures of peptides

Fig. 1. Electron-impact mass spectrum of 25 nmol of trifluoroacetylated permethylated Ser-Gly-Leu-Phe-Asp

The peptide derivative emerged from the OV-1 column at 284°C during a temperature programme from 200°C at 4°C/min. Below m/e 530, peaks of <5% intensity have been omitted from the drawing. The usual three-letter code is used, in the Figures only, to denote the *NO*-permethylated amino acid residues.

Fig. 2. Chemical-ionization mass spectrum of 17 nmol of acetylated permethylated Ser-Gly-Leu-Phe

The derivative was eluted from the OV-1 column at 265°C under the same conditions as in Fig. 1. The high yield of the direct-effluent technique and the enhancement of sequence ions of higher mass by chemical ionization is well illustrated by the spectrum shown. Peaks of <5% intensity have been omitted from the drawing.

Fig. 3. Graph of retention index against molecular weight of various peptide derivatives sequenced by directly coupled mass spectrometry

The retention indices were determined approximately by linear interpolation of the apparent elution temperatures between those of normal alkanes with the linear temperature programme described in the legend to Fig. 1. The values are within the range where the relationship of carbon number to elution temperature was shown to be linear. ○, ●, Elution from the OV-1 column; ■, elution from the Dexsil 300 GC column. Filled symbols denote acetylated permethylated derivatives; open symbols denote trifluoroacetylated permethylated derivatives. The peptides were: (1) Gly-Phe; (2) Leu-Gly-Val; (3) Gly-Leu-Phe; (4) Leu-Ala-Gly-Val; (5) Ser-Gly-Leu-Phe; (6) Phe-Phe-Phe; (7) Ser-Gly-Leu-Phe-Asp; (8) a derivative of peptide (7) that was α -C-methylated at the aspartic acid residue but not at the glycine residue; (9) Ala-Phe-Leu; (10) Thr-Pro-Gly-Ser; (11) Ala-Phe-Tyr; (12) Val-Lys-Ala.

derived by chemical or enzymic cleavage of longer chains. More than one type of degradation method would probably be necessary to yield a complete set of overlapping fragments from which the original sequence could be reconstructed, but the ease,

sensitivity and rapidity of the method even at this stage is consistent with such a strategy. As mentioned above, very considerable increases in sensitivity can be expected from the use of small-bore columns and chemical ionization. The approach described in this paper would have the advantage over methods relying on sequential Edman degradation that it would provide more than one piece of experimental evidence for the position of each residue in the peptide being analysed.

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