Peptide sequencing by fast atom bombardment mass spectrometry: acid hydrolysis or tandem mass spectrometry?

Fast atom bombardment mass spectrometry (f.a.b.-m.s.) represents an important advance in structural studies on peptides [1]. Molecular masses can be readily determined, but fragment ion abundances are not always sufficient to obtain exhaustive sequence information. To overcome this limit, enzymic methods [2] have been proposed to degrade the peptides to small fragments prior to the instrumental measurements. More recently we have shown that additional structural information can be easily obtained by simply taking spectra of the peptide acid hydrolysates at different hydrolysis times [3]. This approach differs fundamentally from the procedure proposed by Kidwell *et al.* [4], which involves complex manipulations and, as already pointed out [5], has only been applied to a small apolar peptide and does not appear transferable to real problems.

Biemann *et al.* [5] now report the use of tandem mass spectrometry as a very efficient and, they say, unambiguous tool for peptide sequencing. The close comparison made in Biemann's paper between our hydrolysis method [3] and the m.s./m.s. procedure, as revealed by analysis of the same peptide (substance P), prompted us to expose our opinion on this argument, and to discuss both methods in the light of supplementary data in our hands.

The main advantage of the tandem mass spectrometry appears to be the possibility of studying mixtures. Actually, the m.s./m.s. technique performed by a four-sector instrument allows to select the appropriate $(M+H)^+$ ion with the first two sectors, to fragment it by collision-induced dissociation (c.i.d.) and to analyse the produced fragment ions through the second two sectors. In addition, other advantages derive from the possibility of studying in detail the fragmentation process. For example, the differentiation of leucine from the isomeric isoleucine becomes possible, on considering the abundant loss of 42 mass units from the (primary) fragment containing the former amino acid.

However, this technique also suffers from some limitations. Apart from the fact that some sequence ions are not of sufficient abundance to rely upon them with confidence, the most important ambiguity, not mentioned by Biemann *et al.* [5], is that the method does not appear to be able to differentiate the *N*-terminal from its adjacent amino acid. Counting from the *N*-terminus, amino acid 1 cannot be directly revealed, since in the c.i.d. spectrum there are several peaks at low masses, all due to immonium ions, related to single amino acids present in any position (also the *N*-terminal position) of the peptide. Thus, both the first two residues in substance P, arginine and proline, give peaks at m/z 129 and 70 respectively, regardless of which of the two positions they occupy in the peptide. The only information that is apparent in the spectrum, revealed by the appropriate peak, is the presence of two consecutive amino acids at the *N*-terminal portion of the peptide, but not their relative positions.

The isobaric amino acids glutamine and lysine are impossible to distinguish in a simple mass spectrum, and a difficulty also appears in the case of the tandem mass spectrum of substance P. In-chain glutamine residues

give fragments at m/z 707.5–57 and m/z 579.4–57 mass units, but the same happens for lysine (i.e. a fragment at m/z 354.2–57 mass units). Although these fragments are of different abundance, it would be difficult to interpret with confidence the mass spectrum of a peptide of unknown structure.

With our hydrolysis procedure [3], glutamine and lysine can be easily differentiated under favourable circumstances (substance P). In all cases, the spectra of the hydrolysis mixtures at short hydrolysis times show doublets separated by 1 mass unit for glutamine-containing fragments. The presence of glutamic acid does not represent a problem, since it is sufficient to examine also fragments free of such amino acid. It is the analysis of all fragments that allows the reassembly of the original sequence of the peptide.

It appears to us that partial acid hydrolysis combined with mass spectrometry represents a much more flexible method. When the only ambiguity in a peptide sequence is the presence of glutamine or lysine, a very mild acid hydrolysis can be carried out (1 h treatment with 5% HCl at 60 °C). Following this procedure, two varied globins, having the same nominal mass, D-Punjab β_{121} (Glu $\rightarrow$ Gln substitution) and O-Arab β_{121} (Glu $\rightarrow$ Lys substitution) can be differentiated [6].

Generally, cyclic peptides give little and unreliable fragmentation, and this could also be the case in the tandem mass spectrometric analysis of such compounds. Moreover this technique may not give any information on the cyclic nature of the peptide under analysis. In our experience (F. De Angelis, unpublished work) on gramicidin S, the rapid appearance in the hydrolysis mixture of a mass increment of 18 units with respect to $(M+H)^+$ (related to a mixture of isobaric ions, evidenced by the complexity of the spectrum) is readily attributable to a cyclic structure.

Different solvents and/or reagents (e.g. trifluoroacetic acid instead of aqueous HCl) could also be used to perform the partial peptide hydrolysis; for example, methanol could be used instead of water as the reaction medium. We have carried out such experiments using the protected peptide Z-Ala-Phe-Gly-OCH₃ as an example (where Z stands for the benzoyloxycarbonyl group). The sample (2 nmol) was dissolved in aqueous 5% HCl/methanol/deuteromethanol (5:1:1, by vol.) and heated in a closed vial at 90 °C for 1 h. The mixture was then analysed by f.a.b.-m.s. using a Kratos MS 80 instrument, a xenon beam being used to ionize the sample. The mass spectrum presents intense peaks, as equal intensity doublets separated by three mass units, at m/z 445/442, 388/385, 311/308, 254/251 and 240/237. These peaks, which are easily recognizable in the spectrum, are due to the ¹H and ²H methyl esters of the fragment peptides, and are related to the following oligomers (Me stands for ethyl and trideuteromethyl): Z-Ala-Phe-Gly-OMe, Z-Ala-Phe-OMe, H-Ala-Phe-Gly-OMe, H-Ala-Phe-OMe and H-Phe-Gly-OMe. Following this procedure, the identification in the spectrum of fragments becomes much easier and could be applied with confidence also to unknown peptides with higher molecular mass.

Other arguments presented by Biemann *et al.* [5] merit comments. The total amount of substance required following the partial acid hydrolysis, as compared with the amount required for a c.i.d. m.s./m.s. spectrum, is not 40 times as indicated by these authors. This value derives from a direct comparison between the two

experiments as actually presented in the papers, but as to our approach [3], besides the f.a.b. spectrum of the intact molecule which requires no more than 2 nmol of sample (with our instrument), only the 1 h hydrolysis experiment could be sufficient. In fact the 1 h mass spectrum of substance P reveals the formation of 34 products of the 39 present in all spectra (to be noted is the presence of peaks due to peptides where the *N*-terminal amino acid has been hydrolysed, thus revealing its identity as arginine). A great deal of information is also present in the 1 h hydrolysis mass spectrum of [2-D-Ala,5-Leu]enkephalinamide. The kinetic f.a.b. analysis of the hydrolytic process (useful in the presence of large amount of material) as presented in our paper [3] only aids the identification of fragment peptides, but the total amount of information is also present in the single 1 h hydrolysis spectrum.

The suggestion by Biemann *et al.* [5] to use a two-sector instrument in the 'linked scan' mode is appropriate, provided that in the instrument a collision chamber is fitted in the first field-free region (with reference to the MS 80 or other instruments with 'normal' geometry), otherwise signals will probably be too low. However, as already pointed out by Biemann *et al.* [5], machines such as a four-sector tandem instrument and even a triple quadrupole or a simple two-sector mass spectrometer could be too expensive. Most importantly, our hydrolysis procedure can be performed also on a single quadrupole instrument.

In conclusion, we wish to point out that, very often in biochemical problems, there is not a single preferred method, and this appears to be the case here. In some cases it will be faster and simpler to use the m.s./m.s. approach, provided that the instrumentation is available. In other cases, the hydrolytic approach will be more useful especially because, being a chemical approach, it is much more flexible than the other one, which is purely instrumental. Probably a combination of both methods, in connection with others such as enzymic cleavage [2], must be used in order to solve complex problems.

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Received 25 March 1987