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MASS SPECTROMETRIC SEQUENCING OF PEPTIDES AND PROTEINS

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Abstract - Mass spectrometry had been demonstrated to be a useful technique for the determination of the amino acid sequence in oligopeptides. The extension to the sequencing of polypeptides and proteins is a complex problem involving chemical degradation and conversion techniques, separations, collection of the mass spectral information and the interpretation thereof. Partial acid hydrolyzates of primary degradation peptides (consisting of about 50 amino acids) of proteins are converted to O-TMS-polyamino alcohols which are analyzed by gas chromatographic mass spectrometry to identify all the peptides formed in the hydrolysis. From this information the original structure is then reassembled. The method is illustrated with a cyclic peptide, with the N-terminal sequence of λ repressor and the structure of the sweet protein monellin.

INTRODUCTION

Since the early beginning of the use of mass spectrometry for the determination of the structure of complex natural products almost twenty years ago, the determination of the structure and amino acid sequence of peptides has attracted the attention of those active in this field. The linear and repetitive nature of the structural elements of peptides made them attractive for mass spectrometry while the extremely low volatility of these compounds was a serious obstacle, at least until very recently. For these reasons numerous attempts to develop suitable derivatization techniques have been reported and many of them have been successfully demonstrated (Ref. 1).

The objectives of peptide sequencing fall in two general categories: relatively small oligopeptides (of less than 20 amino acids) on the one hand and larger polypeptides and proteins of any size ranging from enzymes to larger structural macromolecules on the other. To the former group belong, for example, physiologically active peptides produced by man, animals or plants, such as the thyrotropin-releasing hormone, enkephalin, α and γ endorphine, etc., and microbial peptides, many of which are useful antibiotics. Small peptides of another type are active sites of enzymes and may represent the most important segment of a large macromolecule. All these can be isolated with more or less effort in, at least mass spectrometrically, reasonable quantities. Mass spectrometric techniques to determine the structure of such molecules from the mass spectrum of the compound itself or more likely from that of a suitable derivative have been developed producing interpretable mass spectra of peptides consisting of 10 or 15 amino acids. Needless to say the structure of the individual amino acids involved plays an important role in the degree of success or the effort necessary, the more polar ones representing the more difficult problems.

The decrease in volatility and increase in thermal sensitivity and total mass puts an upper limit on the size of molecule one can hope to investigate in this way. Although the mass range and the size of the molecule that can be handled is becoming larger and larger with the advent of new ionization techniques or even new mass resolving systems one has to keep in mind that clear and unambiguous fragmentation is required if one wishes to deduce the sequence of a peptide. Unfortunately most of the recent developments which extend the mass range and have a much lower volatility requirement are characterized by the lack of uniform and reproducible fragmentation of the molecule, if any.

Work on larger polypeptides or proteins thus requires degradation to smaller parts that can be handled by mass spectrometry. The well developed chemical and enzymatic hydrolysis techniques provide the means to generate these small molecules whose structure, once it is determined, permits reassembly of the original sequence. That, of course, is the basis of conventional protein sequencing. The important contribution which mass spectrometry can make to this approach is not merely the faster and unambiguous determination of the sequence of a small peptide from its mass spectrum but rather its potential to do so without the very tedious time and material consuming step of complete separation of these very

complex hydrolysis mixtures into the individual components which then have to be purified and subjected to amino acid and end group analysis if one wishes to apply the conventional techniques. Therefore, it was the objective of this work to develop a technique that can handle a complex mixture of relatively simple, i.e. small peptides, rather than a somewhat simpler mixture of considerably larger peptides. It is for this reason that the oligopeptides of less than 15 amino acids that can be isolated in pure form are dealt with quite differently from the complex degradation mixtures obtained from proteins. For the former, it is worth the effort to modify an existing derivatization technique to make it particularly applicable to the problem at hand, even if it takes some time, while for protein degradation mixtures one has to use a generally applicable technique that can handle all small peptides expected of the structural type existing in proteins.

Mass spectrometry has been used mainly for deducing the amino acid sequence of a small peptide from a spectrum that contains clearly discernible so-called sequence peaks. These are due to characteristic ions produced by cleavage of the peptide backbone; from them the mass of the side chain or side chains of the amino acids retained in each particular fragment can be deduced. It should be noted, however, that mass spectrometry can also provide a supporting role in the Edman degradation of proteins, where it can be used to identify unambiguously the phenylthiohydantoins formed in each step. Even the quantitation of the product of each cycle, one of the crucial aspects in the Edman method, can be aided by the addition of isotopically labelled standards (Ref. 2).

Finally, there is one natural aspect of mass spectrometry that plays an important role in certain peptide structure problems, particularly of the non-protein type. The presence of unusual or rare amino acids is easily recognized and their structure can often be directly deduced from the spectrum of the peptide or of a total hydolyzate. In fact, the classical example of fortuitine is a case in point. It contained a number of N-methyl amino acids which had been previously overlooked because of their low response to ninhydrin, but were quite obvious in the mass spectrum of this acylnonapeptide (Ref. 3).

Of the derivatization techniques that have been developed over the years a few have withstood the test of time and are now most widely used (Ref. 1). For electron ionization of oligopeptides, permethylation is the method of choice, as outlined by Dr. D.H. Williams in one of the preceding papers, closely followed by the formation of N-trifluoroacetyl esters. Both derivatives are suitable for direct introduction into the ion source and produce mass spectra exhibiting significant sequence ions. When using electron ionization in conjunction with gas chromatography for the separation of complex mixtures the lithium aluminum deuteride reduction products of peptides, i.e. polyamino alcohols (which are then selectively O-trimethylsilylated) appear to be the method of choice, although at least for di- and tripeptides the N-trifluoroacetyl esters or pertrimethylsilyl derivatives are also applicable. Newer ionization techniques, such as field desorption (Ref. 4) and plasma desorption (Ref. 5) can produce mass spectra even of underivatized peptides, although they are conspicuously lacking in the sequence-revealing fragmentation characteristics which are so important for the reliable assignment of the sequence. Somewhat more informative field desorption spectra are obtained with permethylated derivatives or with polyamino alcohols.

For protein sequencing by mass spectrometry it is unavoidable to have to deal with very complex mixtures of oligopeptides and these can presently be handled best by gas chromatographic mass spectrometry (GCMS) using electron ionization. The gas chromatograph separates the mixture into the individual components or at least very simplified mixtures of two to four components eluting under one peak envelope. The mass spectra of the O-TMS-polyamino alcohols are so simple and sequence specific that even only partially resolved peptide derivatives up to hexapeptides can be reliably identified (Ref. 6-11). Peptide mixtures derived from partial degradation of polypeptides containing up to 50 amino acids have been successfully analyzed and this is just the size which one generally obtains upon tryptic or cyanogen bromide cleavage of proteins. They are also large enough to be separated from each other by conventional techniques, such as column chromatography or electrophoresis.

In this discussion on mass spectrometric techniques for protein sequencing one should, of course, not lose sight of the fact that the Edman degradation, particularly in its elegant automated form is presently the most widely used and highly successful approach (Ref. 12). As a chemical stepwise degradation from one end of the molecule it is in every respect entirely different from the mass spectrometric techniques discussed above. While the emphasis here is on the latter it should be clearly recognized that the dissimilarity of the two approaches which on the one hand leads to a different kind of information and on the other imparts on each one different strengths and weaknesses, makes the two not competitors but in fact very complementary approaches. To use both simultaneously is the most efficient and reliable way of determining the structure of a protein as the examples discussed in the following should demonstrate.

METHODOLOGY

The early concept of producing a peptide derivative that retains all the structural elements and is suitable both for gas chromatographic separation and clear mass spectrometric identification by reduction of the carbonyl group of the peptide bond with lithium aluminum deuteride (Ref. 13) has been improved over the years with respect to the chemical reactions involved and the instrumental techniques used (Ref. 6-11). In its present form it consists of converting a mixture of peptides first to methyl esters which are then N-trifluoroacetylated. The resulting mixture is then reduced by lithium aluminum deuteride in glyme, the reduction products being extracted and selectively O-trimethylsilylated with trimethylsilyldiethylamine (see Fig. 1). These derivatives are well suited to gas chromatographic separation and produce mass spectra which are dominated by peaks due to cleavage of the carbon-carbon bond of the ethylenediamine units and retention of the positive charge on either the N-terminal (A-series) or C-terminal (Z-series) fragment. Selective O-silylation greatly improves the gas chromatographic properties of these derivatives, particularly for hydroxy amino or dicarboxylic acid residues. But more importantly it increases the abundance of the C-terminal fragments,which makes it easier to read the sequence also from the C-terminal ends. The use of N-trifluoroacetyl derivatives, which are reduced to N-trifluoro-dideutero-ethyl groups enhances the N-terminal fragments further down the chain because of the destabilizing effect of the trifluoromethyl group on a positive charge at nearby nitrogen. Thus, in tri- and higher peptides the fragment containing the first three or four amino acids are most pronounced which aids the structural assignments of larger peptide derivatives without hampering the interpretation of the spectra of the derivatives of smaller peptides (Ref. 9).

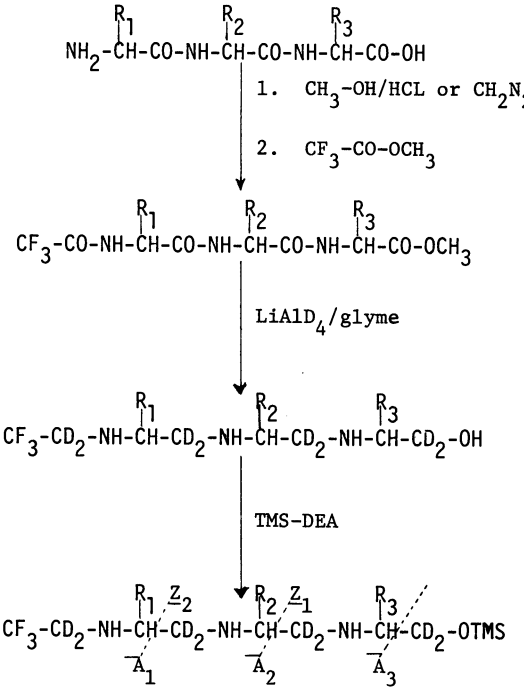


Fig. 1. Derivatization of peptides and mass spectral fragmentation of the resulting derivative.

One of the most helpful aspects in the interpretation of the mass spectra of even minor components of these complex mixtures is the fact that the gas chromatographic retention characteristics for any such polyamino alcohol trimethylsilylether can be predicted fairly accurately and the experimentally observed retention index of a particular fraction can be used to aid in the interpretation of the mass spectrum (Ref. 7, 9). Conversely, one can predict where in the gas chromatogram a particular peptide derivative should appear and this makes it easy to search for certain peptides that need to be identified to complete the structure or to differentiate one sequence from another. Finally, it should be pointed out that the simple rules that relate the mass of the mass spectral peaks to the sequence of the individual oligopeptide as well as the predictable retention time lend themselves well to an "automatic" interpretation of all the mass spectra recorded during such an experiment (Ref. 14). Furthermore, generating all these partial sequences through computer interpretation of the spectra provides the input to a program that then reassembles these data to one or more solutions for the original peptide sequence; this is finally reduced to one

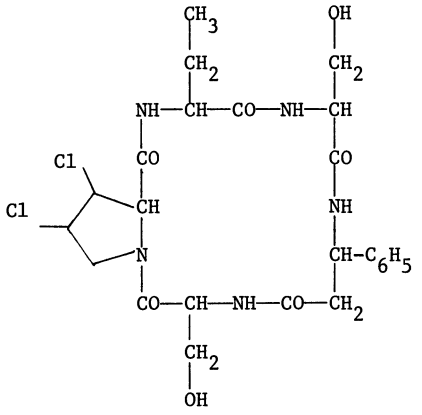
solution by accumulating more and more mass spectrometric information (Ref. 15). The following examples briefly outline some of these principles.

APPLICATIONS

Cyclic Peptides

Although not directly related to the protein problem, the application of the partial hydrolysis/GCMS technique to the determination of the structure of small cyclic peptides shall be discussed. They represent a special problem both mass spectrometrically and in conventional sequencing. It is clear that this is a class of peptides which, not containing a free N-terminus, cannot be subjected to the Edman degradation unless the molecule contains a particular amino acid which permits the specific opening of the ring to a linear molecule, and this is rarely the case. On the other hand, such cyclic peptides are often small enough and, at least after derivatization, sufficiently volatile to be subjected directly to mass spectrometry. However, the interpretation of the resulting spectrum is fraught with pitfalls because of transannular fragmentation reactions that can generate fragments which represent amino acids not attached to each other in the original molecule. Partial acid hydrolysis, on the other hand, leads to mixtures which contain very redundant and complementary information because a large number of small peptides are formed which represent multiple overlap.

The determination of the structure of a cyclic pentapeptide, malformin C, is an example where this approach led to the correct structure (Ref. 16). While a high resolution mass spectrum (Ref. 17) of a related compound was ambiguous, it appeared to confirm a structure which was later shown to be incorrect (Ref. 18). Another, more recent example (Ref. 15) which, at the same time, illustrates the ability of mass spectrometry to recognize unusual amino acids is the identification of a cyclic pentapeptide produced by a *Penicillium* species. Two isomeric compounds of this type were described in the literature, cyclochlorotine (I) (Ref. 19) and islanditoxin (II) (Ref. 20).



L-Abu-L-Ser-L-β-Phe-L-Ser-L-DCP

Cyclochlorotine
(I)

L-Abu-L-Ser-L-Ser-L-DCP-D-β-Phe

Islanditoxin
(II)

Both contain the unusual amino acid dichloroproline (DCP). Partial acid hydrolysis of a sample assumed to be either I or II produced a mixture of peptides which after derivatization gave a mixture of O-TMS-polyamino alcohols, the gas chromatogram of which is shown in Fig. 2. This particular plot represents a so-called mass resolved gas chromatogram in which the resolution is effectively improved by plotting the sum of the mass spectral intensities of only those ions which maximize at that particular point in the gas chromatogram (Ref. 21). The five di- and tripeptides identified from this gas chromatogram clearly lead to one unique sequence (see insert in Fig. 2), namely that corresponding to structure I. It should be noted that the dichloroproline did not survive the lithium aluminum deuteride reduction unchanged but was converted to dideuteroproline by substitutuion and to a pyrrole ring by elimination. This could easily be deduced from the mass spectra of the peptide derivatives, examples of which are shown in Fig. 3. It should be pointed out that the position of the chlorine atoms in the proline molecule does not show in the mass spectra of these derivatives but it is known that DCP is a 3,4-dichloroproline (Ref. 22).

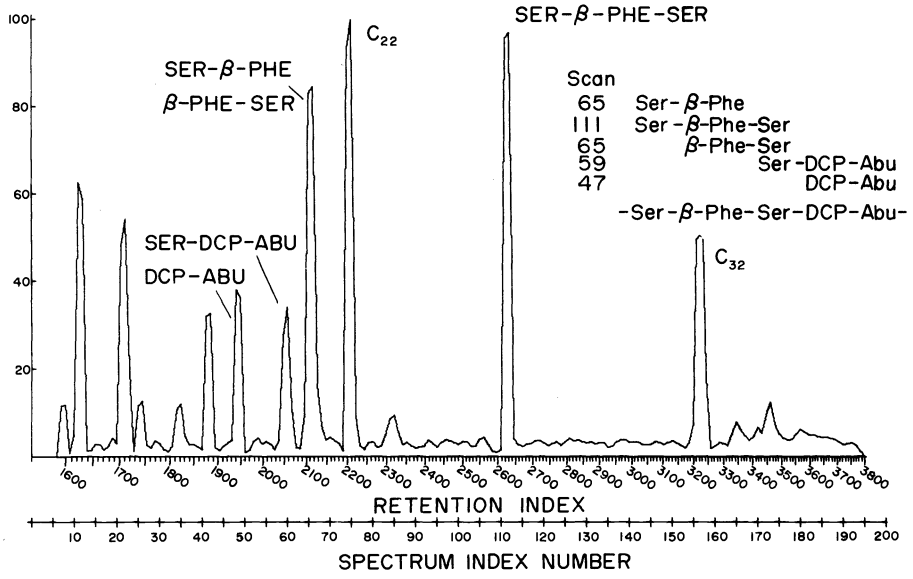


Fig. 2. Mass resolved gas chromatogram of the O-trimethylsilyl polyamino alcohols obtained by derivatization of an acid hydrolyzate of cyclochlorotine. The hydrocarbons C_{22} and C_{32} were co-injected as internal retention index standards. The insert shows the reassembly of the cyclic peptide structure.

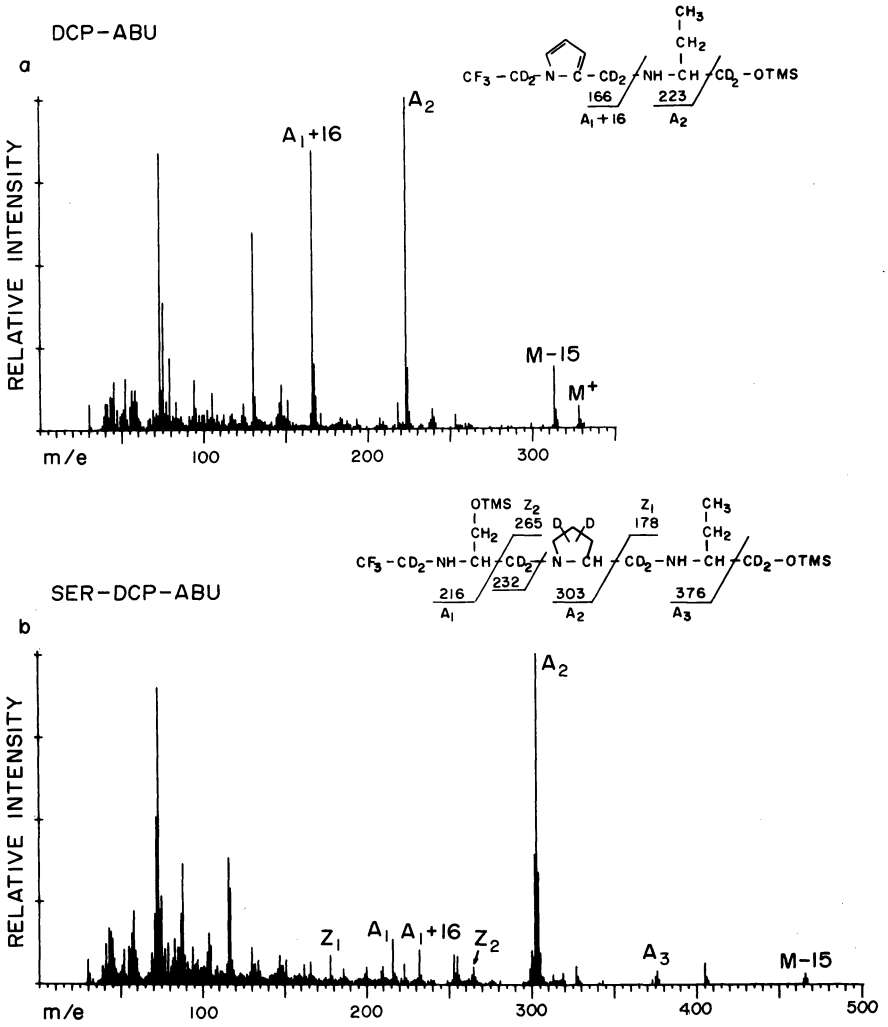


Fig. 3. Mass spectra corresponding to the derivatives of a) DCP-Abu and b) Ser-DCP-Abu.

The N-terminal Sequence of λ -Repressor Protein

A GCMS experiment on a derivatized partial hydrolyzate of even a rather large polypeptide can quickly settle ambiguities in the results of an Edman degradation. This can be illustrated with a study of tryptic peptides derived from the protein involved in the repressor-operator system of the bacteriophage lambda (Ref. 23). This protein consists of a single chain of 236 amino acids. Very recently two sequences were proposed for the N-terminal section (about 50 amino acids) which were mainly based on Edman degradation data. The first one, proposed by Beyreuther and Gronenborn (Ref. 24) differed from the second one by Ptashne et al. (Ref. 25) in position 14, 40 and 42 where the former shows Asn, Ser and Thr, respectively, while the latter group found Asp, Met and Met.

To differentiate the two proposals for residues 40 and 42 it was merely necessary to subject the tryptic peptide that starts at position 18 and is about 60 amino acids long (the lysine residues were protected during the enzymatic cleavage) to partial acid hydrolysis and derivatization and search for methionine containing peptide derivatives. This is best accomplished by the inspection of the mass chromatograms (Ref. 26) of peaks characteristic for methionine, such as its A_1 ion, Z_1 ion or m/e 61 (due to its side chain). The mass chromatogram for mass 224 (Z_1 for Met) showed three distinct peaks and the interpretation of the mass spectra at these points in the gas chromatogram revealed that they are due to Gly-Met, Ser-Met, and Lys-Met. The first and last of these correspond to position 42, 43 and 39, 40 in the sequence proposed by Ptashne, while the third one is from a region beyond position 60. Amongst the tripeptides found in this mixture was Met-Gly-Glu, corresponding to position 42,43 and 44, the glutamine having been hydrolyzed to glutamic acid during the partial acid hydrolysis. Figure 4 shows the N-terminal sequence of λ repressor through position 60 with arrows indicating the results of Edman degradations and the nucleotide sequence of the corresponding DNA segment (Ref. 25) while the horizontal lines denote the small peptides identified in the partial hydrolyzate discussed above (Ref. 15). It is clear that the area of ambiguity in the two differing published Edman sequences is well covered by mass spectrometric information and resolved in favor of Ptashne's results (Ref. 25). In this particular problem it was therefore not necessary to interpret the mass spectrometric data in more detail, or to perform additional experiments to identify the remaining parts of the sequence.

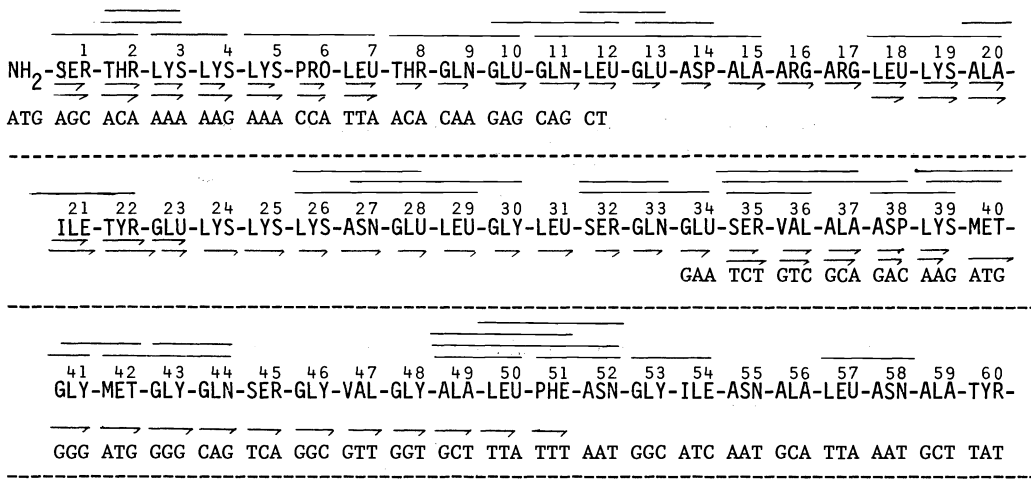


Fig. 4. Amino terminal sequence of λ repressor. Horizontal lines represent peptides identified in partial acid hydrolyzate (Ref. 15). Arrows refer to results of Edman degradations and base triplets to the DNA sequence (Ref. 24).

Monellin

A very sweet tasting material isolated from the fruits of *Dioscoreophyllum comminsii*, was found to be a small protein about 92 amino acids long (Ref. 27). It turned out to consist of two tightly associated subunits (A and C) of 44 and 50 amino acids respectively, and these were separated, purified and first subjected to partial acid hydrolysis (Ref. 28). The hydrolyzate of subunit A, upon derivatization produced a very complex gas chromatogram (Fig. 5) in which 61 peptides ranging from di- to hexapeptides could be identified. These could be assembled to large segments of the total sequence but did not lead to an unambiguous solution owing to some missing overlaps. Enzymatic digests using thermolysin, and trypsin-chymotrypsin, respectively, generated another 29 di- through hexapeptides. These finally could be assembled to a complete structure (Fig. 6) which shows the sequence except that leucine is not differentiated from isoleucine and glutamic acid and aspartic acid are not differentiated from glutamine and asparagine.

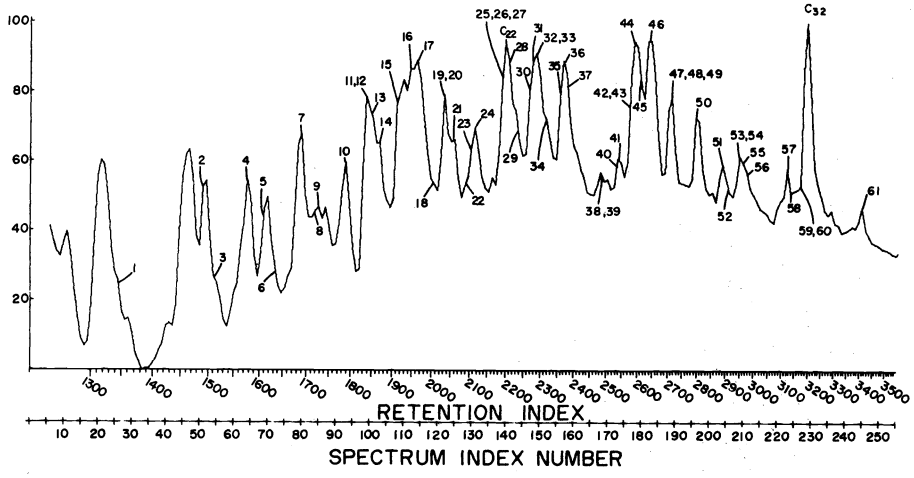


Fig. 5. Total ionization plot produced in GC-MS experiment of a partial acid hydrolysis of subunit A of Monellin. Numbers refer to peptide derivatives identified. C_{22} and C_{32} refer to retention index standards.

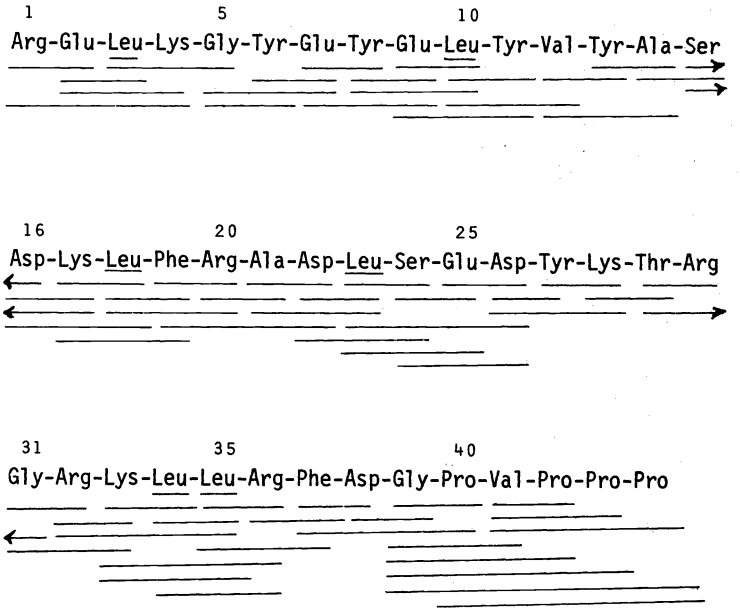


Fig. 6. Amino acid sequence of subunit A based on the peptide derivatives found (indicated by horizontal lines). Leu refers to Leu or Ile, Glu and Asp not distinguished from Gln and Asn (see text).

Experiments of the same type were performed on subunit C, the partial acid hydrolyzate of which led to the identification of 71 di- through pentapeptides. Again, enzymatic hydrolyzates provided further information and led to the assembly of the sequence shown in Fig. 7.

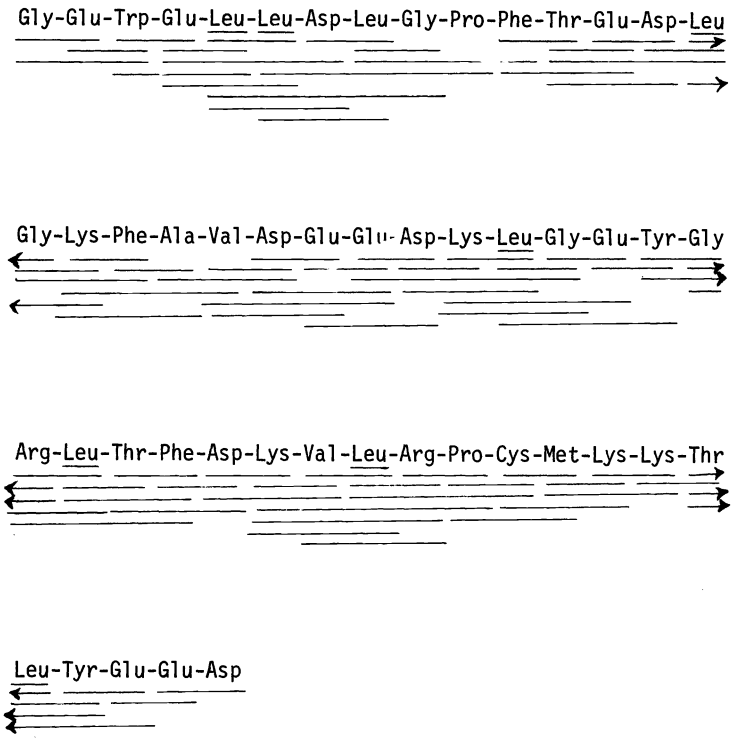


Fig. 7. The structure of subunit C based on peptides identified (underlined) in acid and enzymatic hydrolyzates. Leu refers to Leu or Ile, Glu and Asp not distinguished from Gln and Asn (see text).

The differentiation of leucine and isoleucine presents a particular problem in mass spectrometric peptide sequencing because of the isomeric nature of the two amino acids. From a large number of pairs of peptides which contain leucine or isoleucine in the same position it was found that the differences in fragmentation are small in all cases except for the derivatives of dipeptides with C-terminal leucine or isoleucine. In that case the relative intensities of the Z_1 ion at mass 206 and the rearrangement ion at m/e 150 which is formed from the former by the loss of butene are reproducibly different. The difference is sufficient to differentiate reliably the two possibilities. Using this information it could be established that position 23 in subunit A is isoleucine and position 35 is leucine. Clearly this differentiation is difficult if not impossible if a particular sequence occurs more than once in the polypeptide under investigation. For this reason it can only be stated that of residues 3 and 10 at least one must be leucine and the same holds for 18 and 34. In the case of subunit C this problem exists only for one pair: at least one of the two residues 8 and 15 must be leucine. Position 5, 6, 26, and 46 are found to be isoleucine while position 32 is occupied by leucine.

The differentiation of asparagine and aspartic acid as well as glutamine and glutamic acid is more difficult because in the partial acid hydrolysis the amide groups are always hydrolyzed and one has to rely on enzymatic hydrolyzates for these differentiations. Unfortunately, the lithium aluminum deuteride reduction products of asparagine and glutamine-containing peptides are isolated in much lower yield and thus more difficult to identify. Futhermore, when using methanolic hydrogen chloride for the esterification some methanolysis takes place thus converting the amide into the ester which is then reduced to the same product as the free acid. These assignments must therefore be based on the relative amounts of the aspartic acid and glutamic acid derivatives identified rather than on the nature of the products themselves. For subunit A, the data indicate that residues 2 and 22 are the free acid, 16 and 38 are asparagine and of the pair 7 and 9 at least one is glutamic acid while of residues 25 and 26 at least one is an amide.

While this work was in progress (Ref. 28, 29) a paper by Bohak and Li appeared (Ref. 30), presenting a complete structure of subunit C and a partial one for subunit A. The former corresponds to that shown in Fig. 7, while the structure of subunit A was incomplete because the automated Edman degradation became less certain after residue 27 and no informa-

tion could be obtained beyond position 37. Residues 30 and 32 remained unidentified while residue 33 was said to be arginine rather than lysine. From the complete structure it is quite clear why in this case the Edman degradation could not be completed: the C-terminus is loaded with hydrophobic amino acids which generally leads to rapid decrease of the yield in the Edman cycles. It is of interest to note that the C-terminal hexapeptide Gly-Pro-Val-Pro-Pro-Pro was a clearly discernible peak in the partial acid hydrolyzate (fraction 61 in Fig. 5) and gave an unambiguous mass spectrum (Fig. 8). The publication by Bohak and Li, (Ref. 29) was soon followed by that of Frank and Zuber (Ref. 31). These authors likewise used the automated Edman degradation, not only on the intact subunit but also on its selective hydrolysis products. They arrived at the same sequence for subunit A as reported here, except that they assigned residue 16 as aspartic acid rather than asparagine, and found that both residues 25 and 26 are the free acids.

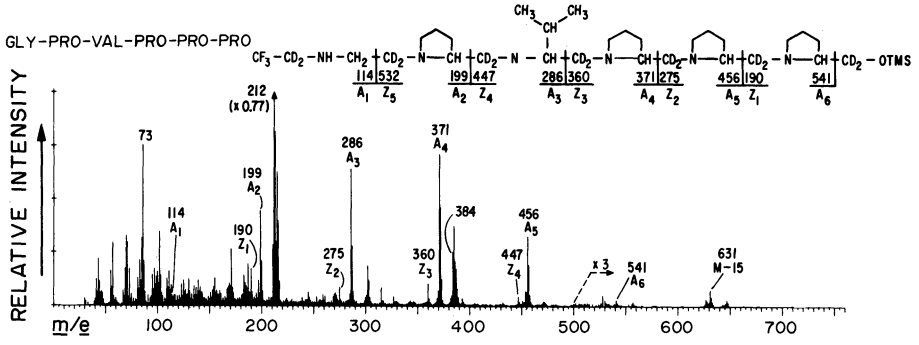


Fig. 8. Mass spectrum of Gly-Pro-Val-Pro-Pro-Pro (scan 245 in Fig. 5).

For subunit C, Frank and Zuber concurred with both Bohak and Li and this work with the exception that they proposed the C-terminal to be represented by Glu-Asn-Glu rather than Glu-Glu-Asn. The partial acid hydrolyzate of intact subunit C as well as the nonapeptide produced upon cyanogen bromide cleavage clearly showed the mass spectra of derivatives of Glu-Glu and Glu-Asp and thus would seem to settle the differences between the two Edman sequences in favor of that of Bohak and Li.

It is quite clear that the mass spectrometric technique developed in this laboratory over the years has reached the point that it can be successfully applied to the determination of the amino acid sequence of proteins. Its chief advantages are speed and clarity, as well as the abundance, redundance and permanence of the data obtained. On the other hand, there are some difficulties which center around three aspects: the differentiation of leucine and isoleucine, the differentiation between free acids and amides, and certain ambiguities which may occur when a sequence is present more than once in the polypeptide subjected to partial hydrolysis. If the latter is the case, one has to identify an intact peptide large enough to contain at least one amino acid before or beyond the repeated unit. This problem more than anything else hampers the extension of the method to much larger peptides because the probability of repeating units obviously increases with the size of the polypeptide studied. Fortunately, the cyanogen bromide cleavage and the specificity of certain enzymes, particularly trypsin in conjunction with selective masking of lysine or arginine, provides ample opportunity to generate primary degradation peptides from almost any protein one may wish to study. The two examples cited here, λ repressor protein and monellin, highlight the point that it is most efficient to use the mass spectrometric technique jointly and in parallel with sequencing by the automated Edman degradation. This generates two sets of mutually supportive data of such different nature that the likelihood of quickly arriving at the correct sequence is greatly increased.

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