IDENTIFICATION OF ASPARAGINE, GLUTAMINE AND THE CARBOXYL-TERMINAL AMINO ACIDS IN POLYPEPTIDES VIA SEQUENCE ANALYSIS BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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1. Introduction

Gas chromatography–mass spectrometry (GC-MS) has proved in recent years to be a valuable alternative and extension to the sequential degradation method of Edman for the determination of the amino acid sequence of polypeptides; for recent reviews see [1,2]. A particular polypeptide is first degraded, either by limited acid or by enzymatic hydrolysis, to a complex mixture of oligopeptides which is then suitably converted to a corresponding mixture of volatile derivatives. Analysis is performed with the aid of a GC-MS-Computer system. From the identified oligopeptides the sequence of the original polypeptide is finally deduced.

Such approach has been successfully applied to the sequence determination of polypeptides between 20 and 39 amino acid residues long [2–6]. The peptide derivatives used are obtained by $LiAlD_4$-reduction and *O*-trimethylsilylation of *N*-perfluoroacyl oligopeptide methyl esters [7] and allowed the identification of all amino acid residues including the very polar ones (arginine, histidine, tryptophan). We found, however, that oligopeptides containing asparagine and glutamine residues are derivatised with rather low yields and also do not chromatograph as well as other derivatives. Even more important, the asparagine and glutamine residues are completely hydrolysed during acid hydrolysis to aspartic and glutamic acid residues. Thus, the positions of the asparagine and glutamine residues in the polypeptides analysed so far by GC-MS had to be determined by conventional methods [4–6]. Since the assignment of these residues is also often difficult by the Edman degradation methods, an unambiguous method for their differentiation by GC-MS is of great potential significance.

We wish to report here a new application of the diborane-reduction method of Atassi and Rosenthal [8] for the selective modification of carboxyl groups. Diborane was recently also used by Airoldi and Doonan during the sequence analysis by the dansyl-Edman procedure [9]. In the approach described in this paper the diborane-reduced polypeptide is degraded by limited hydrolysis and the resulting oligopeptides are further reduced by $LiAlD_4$ to obtain derivatives of maximum volatility. The differentiation between asparagine and glutamine and their acid analogs can unambiguously be made via measurement of the deuterium contents of the molecules by GC-MS. The carboxyl terminal amino acid can also be determined in the same experiment.

2. Materials and methods

Glucagon and the eledoisin-related peptide were obtained from Sigma Chem. Co.; the 1 M diborane solution in tetrahydrofuran was purchased from Aldrich. Reduction of peptides (0.4–0.8 μmol) with diborane was carried out at 0°C for 2 h essentially as described [8]. The reaction mixture was evaporated in vacuo and treated 3 times which methanol acidified by trifluoroacetic acid.

The remaining dry residue was transferred with 0.5 ml 6 N HCl (made from 'Ultrex' Grade, Baker Chem. Co.) to a hydrolysis tube, which was then evacuated and heated at 110°C for 30 min (eledoisin-

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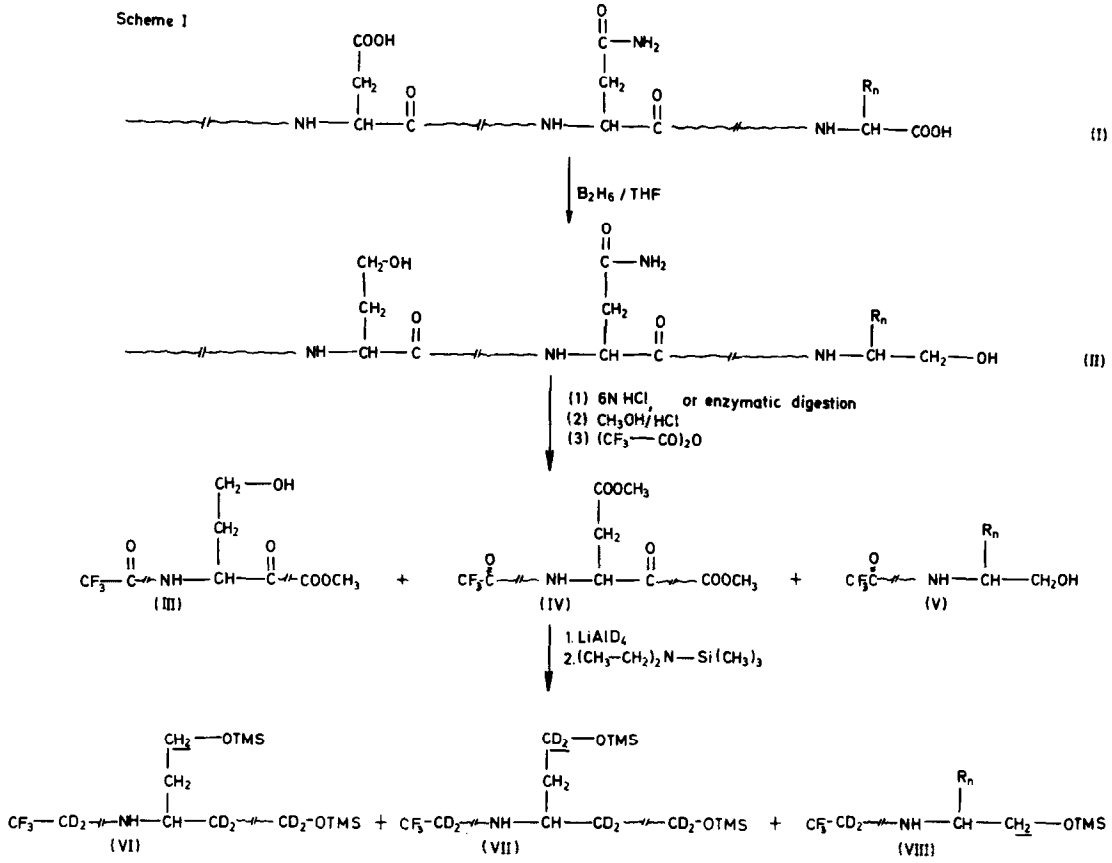
related peptide) or 35–45 min (glucagon); the optimum hydrolysis time was determined before the hydrolysis by gas chromatography with electron capture detection as described [10]. The hydrolysis mixture was evaporated in vacuo and derivatised by methyl esterification, trifluoroacetylation, $LiAlD_4$-reduction and *O*-trimethylsilylation [7].

Aliquots of 2–10% of the final mixture of the volatile derivatives were injected 'on column' into a Perkin-Elmer F-22 gas chromatograph; a 1 m glass column (1/4 inch o.d.) filled with 2% Dexsil 300 GC on Chromosorb W-AW DMCS was used and the temperature was raised from 60°C to 340°C at a rate of 12.5°C/min. The column was coupled via a 1/4 inch Swagelock union and a membrane separator to a MS-30 mass spectrometer (AEI, Manchester, England). A DS-50 data system (AEI) was used for the continuous acquisition of low resolution mass spectra (every 7 s). Selected ion records (mass chromatograms), i.e. plots of the intensity of ions vs. time, were then generated from the mass spectral data [12] to measure the deuterium content of the peptide derivatives.

3. General approach

The novel method described here is outlined in Scheme 1 (asparagine, aspartic acid and the C-terminal carboxyl group in (I) represent the potential reaction sites for diborane). The polypeptide (I) is selectively reduced by diborane – all carboxyl groups present are reduced to - CH_2-OH groups; the primary amide groups of asparagine and glutamine are not affected. The resulting peptide (II) is then partially hydrolysed and the oligopeptides obtained are methyl



Scheme 1.

esterified and trifluoroacetylated. In the resulting mixture (III–V) the carboxyl groups of the aspartic and glutamic acid residues as well as the carboxyl terminal amino acid have been reduced (III,V), while the carboxamide groups of the side chains of asparagine and glutamine residues are present as methoxycarbonyl groups (IV). The volatility of the peptide derivatives is further increased by an exhaustive $LiAlD_4$-reduction, by which the remaining $-COOCH_3$ groups as well as all peptide bonds -CONH- are reduced to $-CD_2$-OH and $-CD_2$-NH- groups. Thus, in the final *O*-trimethylsilylated mixture the aspartic and glutamic acid residues as well as the carboxyl terminal residue are represented by side chains containing $-CH_2$-OTMS groups (VI, VIII), while the asparagine and glutamine residues are represented by side chains containing $-CD_2$-OTMS groups (VII).

4. Results and discussion

The results obtained are exemplified by the GC-MS data shown in fig.1 for glucagon. This polypeptide contains three glutamine, three aspartic acid and one asparagine residues - all of which could be unambiguously identified by their mass spectra; the peptides containing these residues were assigned to the corresponding gas chromatographic peaks in fig.1a (for the sake of clarity, all other peptides identified were omitted from fig.1a, but are shown elsewhere [13]; however, in that earlier experiment [13] no distinction of asparagine and glutamine from their corresponding acid analogs could be made). As an example, the selected ion records of m/e 266 and 268 are shown in fig.1b. These ions indicate dipeptides containing carboxyl terminal aspartic acid (266) and asparagine (268) residues and have the following structures:

$$CD_2{=}NH\text{-}\underset{\oplus}{C}H\text{-}\underset{|}{C}H_2\text{-}CH_2\text{-}OTMS$$

m/e 266

$$CD_2{=}NH\text{-}\underset{\oplus}{C}H\text{-}\underset{|}{C}D_2\text{-}CH_2\text{-}OTMS$$

m/e 268

Even though the two peptide derivatives Gln–Asp[21] and Met–Asn[28] were not completely separated by gas chromatography, their presence is clearly indicated by the selected ion records. The complete structure of the peptides present is elucidated by inspection of the mass spectra of the corresponding scan numbers (e.g. scan 129 for Gln–Asp and scan 131 for Met–Asn) and by selected ion records of other sequence ions: e.g., ions m/e 349 and 351 are plotted in fig.1c, indicating peptides with the terminal sequence Met–Asp– and Met–Asn–, respectively. Indeed, one maximum

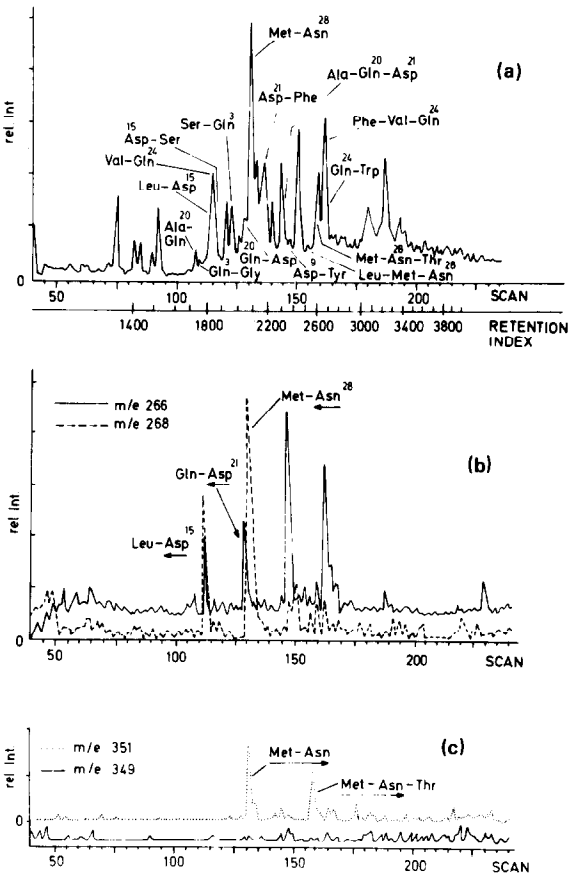
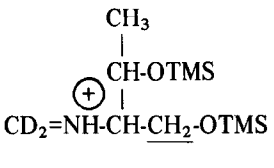


Fig.1. GC-MS analysis of a mixture of oligopeptide derivatives obtained by selective reduction of glucagon by diborane, partial acid hydrolysis, methyl esterification, trifluoroacetylation, $LiAlD_4$ reduction and *O*-trimethylsilylation. A sample of 10% of the derivatised mixture was injected into the GC-MS-computer system (see Materials and methods for experimental details). (a) Reconstructed gas chromatogram (total ion plot). (b,c) Selected ion records (mass chromatograms).

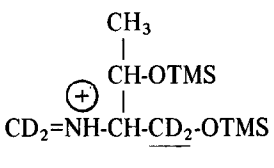
in the plot of ion 351 (but *not* 349) was found at scan 131 (for Met–Asn) and another one at scan 158, which, from additional data, was shown to be Met–Asn–Thr. Similarly, all other peptides shown in fig.1a were identified by computer plots of the selected ion records of the sequence ions.

Thus, Gln^3 of glucagon is represented by Ser–Gln and Gln–Gly; Asp^9 by Asp–Tyr; Asp^{15} by Leu–Asp and Asp–Ser; Gln^{20} and Asp^{21} by Ala–Gln, Ala–Gln–Asp, Gln–Asp and Asp–Phe; Gln^{24} by Phe–Val–Gln, Val–Gln and Gln–Trp; Asn^{28} by Leu–Met–Asn and Met–Asn–Thr.

Since the carboxyl-terminal amino acid is also modified by diborane, that residue can be identified by the mass spectral data as well. As one example, the threonine residue of Met–Asn–Thr eluting at scan 158 (see above) must be derived from the carboxyl end of the polypeptide since the carboxyl group had been reduced by diborane, thus giving rise to a m/e 264 ion (not shown) and *not* to a m/e 266 ion:



m/e 264



m/e 266

Ion m/e 266 would have been produced if that threonine residue had been derived from any other than the carboxyl terminal position. Also, if internal peptide bonds were partially reduced by diborane (see below), the affected bonds could neither be cleaved by acid nor by enzymes and thus would never appear at a carboxyl terminus of a fragment.

In another experiment, the eledoisin-related peptide was treated with diborane prior to hydrolysis and derivatisation. In this case, the terminal residue was not affected by diborane: the carboxyl terminal group must therefore have been blocked in the original oligopeptide.

The carboxyl groups were usually reduced by diborane between 40 and 80%, which leads to characteristic doublets in the mass spectra and selected ion records (fig.1b). Diborane reduction of the side chains of glutamine and asparagine was negligible (fig.1c). Diborane reduction of the peptide bonds of the polypeptides investigated was usually below 1% with the exception of the carboxyl terminal peptide bond, which was reduced to a considerable extent: e.g., the carboxyl terminal peptide bond (Leu–Met–NH_2) in the eledoisin-related peptide as judged from the relative areas of the selected ion records of ion m/e 287 and 289 (sequence ion of Leu–Met–) was calculated to be 36–38%. Partial reduction of peptide bonds by diborane does not significantly decrease the yields obtained (which was the case when the diborane-reduction was applied during the dansyl-Edman procedure [9]), since all peptide bonds are reduced subsequently by $LiAlD_4$; by interpreting the mass spectral data of the resulting derivatives, one can easily distinguish between the diborane-reduction of the carboxyl groups of the side chains and of the peptide bonds.

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