A Sensitive Method for the Determination of the Primary Amide Function ($RCONH_2$) in Peptides by Mass Spectrometry

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Several model peptides have been trimethylsilylated and their FIB mass spectra obtained; reaction was detected at hydroxy groups and primary amides only, amines and carboxy groups were observed in underivatised form, and thus, in conjunction with standard procedures for the determination of hydroxy groups, this methodology represents a sensitive and extremely rapid means for the detection of primary amides in such molecules.

Trimethylsilylation is a standard method for increasing the volatility of polar compounds,[1] including amino acids and peptides, to facilitate mass spectral and GC-MS analysis by electron impact (EI) ionisation. The functional groups derivatised depend on the silylating reagent and reaction conditions employed, but in general hydroxy groups prove the most reactive followed by carboxylic acids, amines and finally amides. Here we present the results of mass spectrometric studies on the trimethylsilyl derivatives of peptides using ionisation by fast ion bombardment (FIB) (or liquid secondary ion mass spectrometry).

Small peptide samples (0.1—10 nmol) were treated with up to 40 μl of Trisil Reagent [Pierce, a mixture of hexamethyldisilazane (HMDS) and trimethylsilyl chloride in pyridine] in plastic vials. An aliquot of the reaction mixture was immediately transferred to the probe tip of the FIB source. After evaporation of excess reagents, 1 μl of matrix (3-nitrobenzyl alcohol or a 1:1 mixture of glycerol and thioglycerol) was added and mixed thoroughly with the sample. The positive ion FIB spectrum was then recorded using a Kratos MS-50 equipped with a Cs^+ gun. For all the peptides studied the signal of highest mass was found to correspond to the trimethylsilylation (addition of 72 mass units for each site of reaction) of all hydroxy groups and primary amides present in the molecule (Table 1, Figure 1). The examples demonstrate successful application of the methodology to peptides containing all amino acids except cysteine. Asparagine, glutamine, and C-terminal amides are all readily derivatised. Peaks of low intensity could also be detected arising from partial desilylation of the sample molecules and these increased with exposure to the atmosphere. The best results were obtained if the mass spectrum was recorded in the shortest possible time after addition of the silylating reagent, but for all samples the signal corresponding to complete hydroxy and amide derivatisation was still readily observable after standing the reagent mixture in air for 1 h. For the peptides studied, no sign of silylation of amino or carboxy groups was detected by FIB MS and the results were thus readily interpreted and reproduced (Figure 1). Similar spectra were obtained using bistrisilylacetamide (BSA) under the same conditions.

Thus, in the structural investigation of an unknown the number of hydroxy groups can be obtained by a standard

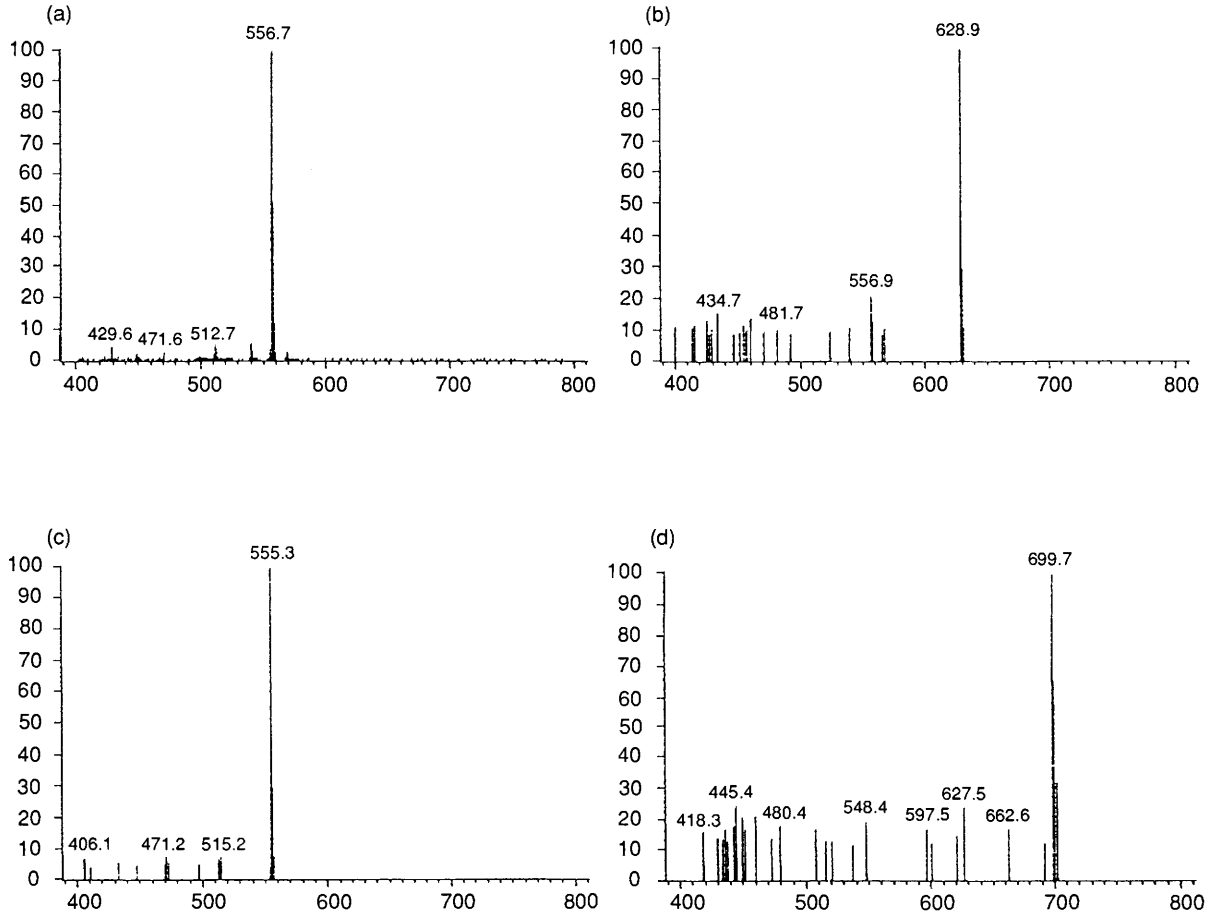

Figure 1. Positive ion FIB mass spectra of leucine enkephalin and leucine enkephalinamide and their trimethylsilyl derivatives. (a) Leucine enkephalin, (b) trimethylsilyl leucine enkephalin, (c) leucine enkephalinamide, (d) trimethylsilyl leucine enkephalinamide. The spectra were recorded on a Kratos MS-50 mass spectrometer equipped with a Cs^+ gun for ionisation. The matrices used were a 1 : 1 mixture of glycerol and thioglycerol for the parent peptides and 3-nitrobenzyl alcohol for the silylated peptides.

Table 1. Protonated molecular ions (MH^+) observed by FIB MS for model peptides and their trimethylsilyl (TMS) derivatives, the number and nature of the sites of reaction deduced.

Peptide	MH^+	MH^+ of maximum TMS derivative	No. of TMS groups added	Residues derivatised
YGGFL (Leucine enkephalin)	556	628	1	1Y
YGGFL-NH_2 (Leucine enkephalinamide)	555	699	2	C-terminal amide, 1Y
DRVYIHPFHL (Angiotensin I)	1296	1368	1	1Y
Human haemoglobin tryptic peptides				
VVAGVANALAHK (Tβ14)	1149	1221	1	1N
LLVVYPWTQR (Tβ4)	1274	1490	3	1Q, 1T, 1Y
VNVDEVGGEALGR (Tβ3)	1314	1386	1	1N
EFTPPVQAATQK (Tβ13)	1378	1666	4	2Q, 1T, 1Y
VLGAFSDGLAHLDNLK (Tβ9)	1669	1813	2	1N, 1S
FFESFGDLSTPDAVMGNPK (Tβ5)	2058	2346	4	1N, 2S, 1T
N,O-acetyl Tβ13	1546	1690	2	2Q
N,O-acetyl Tβ9	1795	1867	1	1N

procedure such as acetylation with acetic anhydride and pyridine. The silylation reaction can then be applied directly to the acetylated material or to a new sample of the compound. Application of the methodology to *N,O*-acetylated peptides is illustrated by two examples in Table 1. In either case the number of primary amides present is readily deduced; in the absence of primary amides trimethylsilylation represents a simple method for the direct detection of hydroxy groups.

The conventional reaction employed for the determination

of primary amides by mass spectrometry involves Hofmann degradation of the amide to the amine by use of *I,I*-bis(trifluoroacetoxy)iodobenzene[2] which results in the loss of 28 mass units from the molecular weight for each amide present. Trimethylsilylation has several advantages over this procedure; it is very much faster and applicable to smaller samples (for many of the peptides studied less than 1 nmol of material was required). In addition, it is specific for the desired reaction, and complicating side reactions due to the oxidation of sensitive bonds are avoided.

3-Nitrobenzyl alcohol was found to be a superior matrix for analysing the silyl peptides to glycerol/thioglycerol, since use of the latter gave unstable ion currents; this results from the generation of ammonium chloride as a byproduct of the silylation reaction. For all the peptides studied satisfactory mass spectra of the trimethylsilyl derivatives were obtained with 3-nitrobenzyl alcohol even when, as in the case of angiotensin I and leucine enkephalin for example, the parent peptide ran poorly in this matrix. In the case of very polar peptides, such as Tβ13 (MH^+ 1378) which acquire many silyl groups on derivatisation, a notable increase in signal intensity was observed, presumably due to the significantly greater hydrophobicity of the silylated compounds.

The results obtained do not follow the pattern of reactivity expected for the functional groups present in the peptides, the silyl derivatives of primary amides being anticipated to be the least thermodynamically stable. In view of the similar results obtained with both HMDS and BSA,[3] we conclude that all functional groups are initially silylated, the reaction being forced to completion by the considerable excess of reagent used. Rapid hydrolysis of the amine and carboxy derivatives must then occur, whereas the silyl amides and alcohols appear to be kinetically more stable under the matrix conditions employed and thus are still readily detectable after a few minutes on the probe tip.

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