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Identification of Oxidized Methionine in Peptides

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The positive- and negative-ion collision-induced dissociation spectra of peptides containing methionine, methionine sulphoxide and methionine sulphone have been studied. Characteristic fragmentations were identified and evaluated as possible indicators for the presence of oxidized methionine residues in peptides. It was found that the elimination of CH_3SOH (−64 u) from $[M+H]^+$ is unique for peptides that contain methionine sulphoxide. Sequence ions containing the oxidized methionine undergo the same elimination, allowing unambiguous sequence determination. Methionine sulphone exhibits an analogous elimination of CH_3SO_2H (−80 u) from the protonated molecule, but not from sequence ions.

The oxidation of methionine is a commonly occurring phenomenon that alters the physicochemical and functional properties of proteins and peptides.[1] During storage and sample handling, the presence of even low concentrations of oxidizing agents causes conversion of methionine to methionine sulphoxide,[2,3] often resulting in problems during subsequent analysis. The oxidized protein or peptide behaves differently on, e.g. reversed-phase high-performance liquid chromatography (HPLC), from which it elutes earlier than the non-oxidized compound.[4–6] However, the reaction is reversible and when a suitable reducing agent is added to the oxidized protein the original primary structure is restored.[1] Conversion of methionine to methionine sulphoxide may also occur *in vivo*.[1,7,8] In the biological environment, oxidizing agents formed during aerobic metabolism can give rise to this conversion. Enzymatic mechanisms are often present in organisms to reduce the oxidized products *in vivo*. Accumulation of oxidized proteins and inactivation of enzymes by oxidation have been related to diseases such as cataract formation, emphysema and rheumatoid arthritis, whereas the reversible oxidation process is also thought to play a role in regulating the activity of proteins.[1]

When methionine or methionine sulphoxide is treated with a strong oxidizing agent such as peroxyformic acid, irreversible oxidation to methionine sulphone occurs. Since strong oxidizers are not usually formed in biological systems, proteins containing methionine sulphone are rarely found. Recently, however, an enzyme from a peroxide resistant mutant of *Proteus mirabilis* was isolated in which one of the methionines was oxidized to the sulphone form.[9] Conversion to sulphone *in vitro* is more likely, e.g. as a side reaction in oxidation procedures used for fission of disulphide bridges.[10,11]

Although oxidized forms of methionine are relatively frequently encountered, a direct method for identifying their presence in peptides and for determining their position in the peptide chain is not available. In Edman sequencing, the products of methionine oxidation are not detected or coelute with other amino acids, resulting in misidentification. Additionally, routine sequence analyses are performed in the presence of a reducing agent to prevent oxidation from taking place during the procedure, but also preventing the detection of methionine sulphoxide. Mass spectrometric identification of the oxidized forms is generally only based on a mass increase of 16 or 32 u. Localization of the oxidized methionine is then performed by hydrolysis of the protein and mass determination of the resulting peptides.[2,3] This method, however, requires knowledge of the sequence and mass of the non-oxidized peptide and is thus not applicable to unknowns. An alternative method for determining the location of oxidized methionine in a peptide chain is using tandem mass spectrometric analysis (MS/MS). Sequence determination based on MS/MS, however, is limited, since it is generally not possible to differentiate between isobaric residues; methionine sulphoxide and methionine sulphone are isobaric with the common amino acids phenylalanine and tyrosine, respectively.

This paper describes characteristic fragments of methionine sulphoxide and methionine sulphone residues in peptides under high-energy collision-induced dissociation (CID) MS/MS conditions, that allow unequivocal determination of their presence and position in the peptide.

EXPERIMENTAL

The peptides studied are listed in Table 1. Conversion of methionine-containing peptides to the corresponding methionine sulphoxide forms was performed by adding about 200 μL of an aqueous 30% hydrogen peroxide solution to about 200 μg of the dry compounds. After addition of 1 μL trifluoroacetic acid and mixing, the solution was kept at room temperature for 5–20 h. Oxidation to methionine sulphone was performed similarly, but a

Table 1. Compounds used in this study. Amino acids are represented by the standard one-letter code; Mo represents methionine sulphoxide, Mo_2 represents methionine sulphone and mF represents methylphenylalanine

M[a]	MAS[a]
Mo[b]	MGMM[d]
Mo_2[b]	YGGFM[e]
AM[a]	MEHFRW[e]
MG[c]	MoEHFKF[e]
ME[c]	Mo_2EHFKF[e]
MD[c]	RWmFWLMo-amide[a]
GM[c]	MNYLAFPRM-amide[e]
LM-amide[a]	RPKPQQFFGLM-amide[e]
VM[d]	RPKPQQFFWLM-amide[e]
MV[d]	GMDSLAFSGGL-amide[e]
MM[d]	

Compounds obtained from: [a] unknown, [b] ICN Biochemicals Inc. (Costa Mesa, CA, USA), [c] Serva (Heidelberg, Germany), [d] Sigma (Bornem, Belgium) and [e] Saxon Biochemicals (Hannover, Germany).

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premixed reagent (30% aq. hydrogen peroxide+formic acid, 1:10) was used. The reaction products were concentrated under vacuum whenever necessary. Samples were either analysed immediately or stored at −20 °C.

Positive- and negative-ion fast-atom bombardment (FAB) mass spectra were recorded on a Jeol JMS-SX/SX102A four-sector instrument of B_1E_1–B_2E_2 geometry operating at an accelerating voltage of 10 kV. Xenon was used as the FAB gas; the FAB gun was operated at 6 kV and a 5 mA emission current. The scan rate was 30 s for the *m/z* range 0–2400 and the resolution was 1000. About 2 μL of the sample solution, containing approx. 10 nmol of the compound, were loaded into approx. 1 μL of glycerol or thioglycerol as matrix. Precursor-ion spectra were obtained from ions decomposing in the first field free region (FFR) using linked scanning at constant B^2/E with MS-1. CID spectra were acquired by selecting the desired precursor ion with MS-1 and colliding the ion in the collision cell located in the third FFR. The nitrogen collision gas was introduced so that the intensity of the main beam from MS-1 was reduced to about 50%. The resulting fragment ions were monitored by scanning MS-2. Identification of sulphur-containing fragment ions in the spectra of the amino acids was achieved by comparing the CID spectra of the pseudomolecular ion ($[M-H]^-$ or $[M+H]^+$) and spectra of the [pseudomolecular ion+2]. The latter mainly represents the species containing ^{34}S. Fragment ions containing the sulphur atom are detected in the [pseudomolecular ion+2] spectrum at an *m/z* value 2 units higher than in the pseudomolecular ion spectrum, whereas fragment ions that do not contain sulphur are observed at identical *m/z* values in both spectra.

RESULTS AND DISCUSSION

The negative-ion CID spectra of deprotonated methionine, methionine sulphoxide and methionine sulphone are given in Fig. 1(a)–(c). All spectra contain an abundant ion originating from the side chain, representing $[CH_3S]^-$ (*m/z* 47), $[CH_3SO]^-$ (*m/z* 63) and $[CH_3SO_2]^-$ (*m/z* 79), respectively. An ion at *m/z* 100 is present in the spectra of all three compounds and its formation from the $[M-H]^-$ ion involves the elimination of a sulphur-containing molecule from the side chain. In the case of methionine, methanethiol (CH_3SH) has been reported to be lost,[12,13] while methionine sulphoxide expels a corresponding molecule (methanesulphenic acid, CH_3SOH), most likely with a structure identical to the species obtained upon pryolysis of methyl *tert*-butyl sulphoxide.[14] The elimination of methanesulphinic acid (CH_3SO_2H) from the $[M-H]^-$ ion of methionine sulphone results in the formation of a less abundant *m/z* 100 (Fig. 1(c)). In spectra of small peptides (<5 amino acid residues), these characteristic side chain ions were easily detected and elimination of the neutral was also observed (data not shown). The characteristic ions described can be used to detect both forms of oxidized methionine in peptides, but the applicability is limited to small peptides. Ion yields for peptides in the negative ion mode are usually lower than in the positive ion mode and the usefulness of negative ion spectra of larger peptides is limited.

The fragmentation behaviour of methionine under positive-ion CID conditions is more complex than under negative conditions. The base peak in the spectrum of protonated methionine is observed at *m/z* 133 and represents the loss of NH_3. Elimination of CO_2H_2 results in formation of the immonium ion which is detected at *m/z* 104. Additional, less intense, ions observed in the positive-ion CID spectrum of methionine are *m/z* 56, 61, 75 and 102 (Fig. 1(d) and Table 2).[15,16] The ion at *m/z* 61, $[CH_3SCH_2]^+$, represents part of the side chain and the majority of the *m/z* 75 ion abundance corresponds to the backbone of the amino acid after loss of the complete side chain as a radical. The *m/z* 102 ion is of low abundance and is formed by the elimination of CH_3SH from the protonated molecule. Precursor ion scans of *m/z* 56 revealed that this ion results from a similar elimination process from the immonium ion and from loss of 48 from $[M+H]^+$ followed by elimination of CO_2H_2.

When methionine sulphoxide and methionine sulphone are subjected to positive ion CID analysis the spectra appear to be very different from the spectrum obtained from methionine, but these apparent differences mainly arise from variations in relative ion intensities (Fig. 1(d)–(f)). Most fragment ions observed in the spectrum of methionine were also observed in the spectra of the two oxidized compounds, except for the side-chain ion (*m/z* 61 in the methionine spectrum), where the corresponding ions in the spectra of methionine sulphoxide and methionine sulphone (*m/z* 77 and 93, respectively) were not detected. The relative abundance of the ion at *m/z* 102 in the methionine sulphoxide spectrum, which is formed by the loss of CH_3SOH from $[M+H]^+$, is greater than that of the corresponding ion in the methionine spectrum. Additionally, the *m/z* 102 ion in the methionine sulphoxide spectrum is accompanied by a less abundant ion at *m/z* 103, indicating that not only can CH_3SOH be eliminated, but also the $CH_3SO^{\bullet}$ radical (−63 u). The same ions were observed in the methionine sulphone spectrum, but here the signals representing the loss of CH_3SO_2H (−80 u) and $CH_3SO_2^{\bullet}$ (−79 u) are of equal intensity. Both oxidized species also undergo immonium ion formation, although this ion is of low abundance in the methionine sulphoxide spectrum. Since the latter spectrum does contain an abundant *m/z* 56 signal, it is likely that the methionine sulphoxide immonium ion is unstable and easily eliminates CH_3SOH. Direct loss of the complete side chain from the protonated molecule of methionine sulphoxide, $[M+H-91]^+$, seems to result in a relatively stable ion, as can be concluded from the abundant *m/z* 75 ion in the spectrum (Fig. 1(e)). The base peak in this spectrum at *m/z* 74 results from hydrogen transfer to, and subsequent loss of, the complete side chain.

Most of the characteristic fragment ions that are observed in the spectra of the free oxidized forms of methionine are also observed in CID spectra of peptides containing the oxidized amino acids. However, not all these fragment ions were found to be suitable for recognizing the presence of oxidized methionine in an unknown peptide. The ions in the low mass region (*m/z* 56 and immonium ions) are usually of low abundance. Apart from the poor intensity, which is especially apparent in the spectra of larger peptides, most fragment ions listed in Table 2 are not specific for these compounds. The immonium ions of methonine sulphoxide and methionine sulphone appear at the same *m/z* as the immonium ions of phenylalanine and tyrosine, respectively, and an *m/z* 56 ion also occurs in the spectra of threonine, histidine, lysine and glutamine.[15] The ions resulting from the loss of the complete side chain radical can also be observed in spectra of peptides containing the isobaric amino acids, which leaves only the losses of 64 u and 80 (and/or 79) u as being unique for methionine sulphoxide and methionine sulphone, respectively.

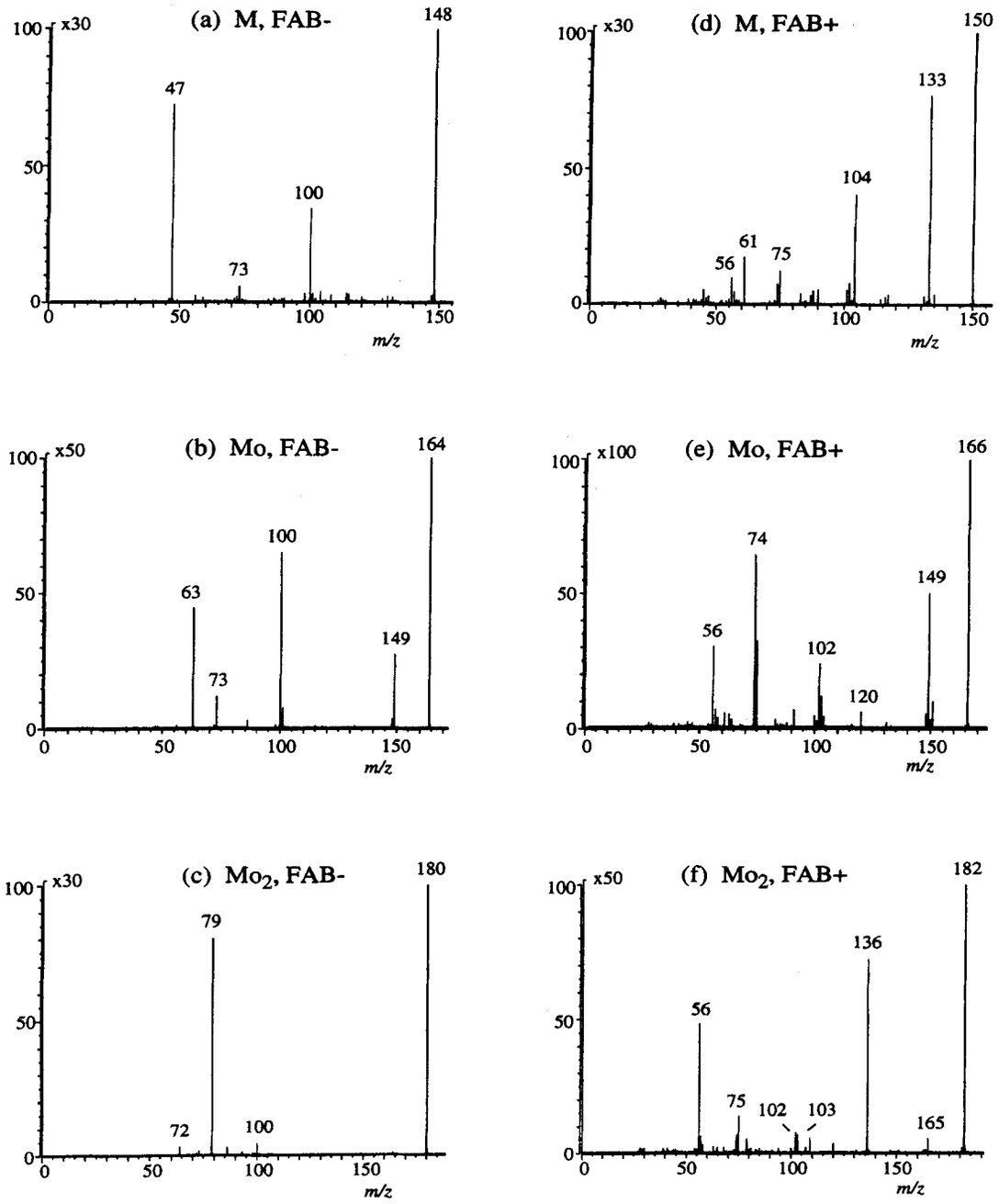


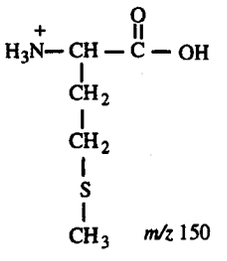
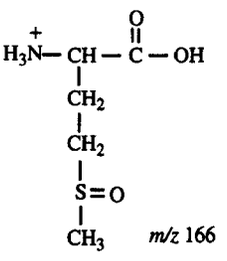
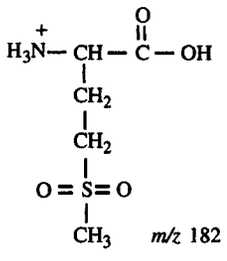
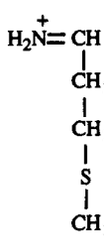
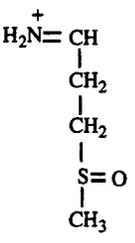
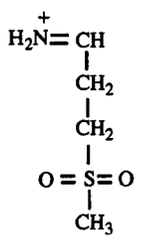
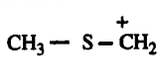
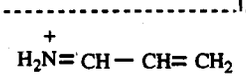
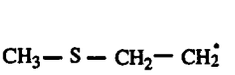
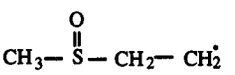
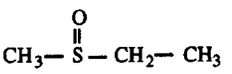
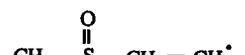
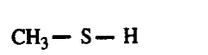
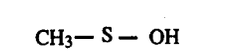
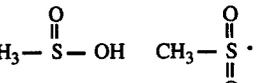
Figure 1. Negative-ion FAB-CID-MS/MS spectra of deprotonated methionine (a, M), methionine sulphoxide (b, Mo), and methionine sulphone (c, Mo_2), and positive ion FAB-CID-MS/MS spectra of the protonated compounds ((d), (e) and (f), respectively).

The loss of CH_3SO_2H (-80 u), and to a lesser extent $CH_3SO_2^{\bullet}$, from the pseudomolecular ion is observed for all methionine sulphone-containing peptides, but is often of low abundance. Moreover, the $[M+H-80]^+$ ion has been described as indicative of the presence of sulphated tyrosine,[17] where it represents the elimination of SO_3. Distinction between both modified amino acids is possible from their negative ion CID spectra, where methionine sulphone produces an m/z 79 ion, $[CH_3SO_2]^-$.

In the spectra of all peptides compiled in Table 1, that were modified to contain a methionine sulphoxide residue (see experimental), the $[M+H-64]^+$ peak was observed. Even multiple losses of CH_3SOH were detected when more than one methionine sulphoxide residue was present. Although the loss of CH_3SO_2H was only found to occur from the molecular ion, elimination of CH_3SOH was also observed from fragment ions (C-terminal and N-terminal ions). These additional sequence ions allow determination of the position of the methionine sulphoxide in the peptide chain, even if isobaric phenylalanine is also present. In Fig. 2(a) the positive-ion CID spectrum of oxidized buccalin (GMDSLAFSGGL-amide) is given. This peptide contains a phenylalanine residue at position 7, whereas the methionine at position 2 was converted to methionine sulphoxide. A series of B-ions and $[B-64]$-ions was detected, confirming that the oxidized methionine was located near the N-terminus. The $[B_2-64]$-ion corroborates the presence of the methionine sulphoxide at position 1 or 2. The exact position in the peptide chain, however, can not be determined from these data, since B_1 or $[B_1-64]$-ions cannot be observed due to their instability.[18–21]

Although all the spectra of the sulphoxide-containing peptides did contain the additional sequence ions, these were not always B-type ions. Loss of 64 u was also

Table 2. Fragmentation of protonated methionine, methionine sulphoxide, and methionine sulphone in positive-ion FAB-CID-MS/MS

	 *m/z* 150 Methionine	 *m/z* 166 Methionine sulphoxide	 *m/z* 182 Methionine sulphone
Characteristic ions: immonium ion:	 *m/z* 104	 *m/z* 120	 *m/z* 136
side chain ion:	 *m/z* 61		
	 *m/z* 56		
Characteristic eliminations: loss of complete side chain:	 -75	 -91 or  -92	 -107
loss of part of the side chain:	 -48	 -64	 or -80 -79

observed from A, Y″, V and W-type ions. The CID spectrum of the sulphone derivative of buccalin (Fig. 2(b)) just contains ions resulting from the specific losses of 79 and 80 u from $[M+H]^+$ and no additional sequence ions. This is typical for all sulphone derivatives of the peptides in Table 1.

The CH_3SOH elimination process seems not to be limited to high-energy CID conditions. Morris *et al.*[22] published a low-energy CID spectrum of a peptide containing either phenylalanine or methionine sulphoxide. They identified the residue as methionine sulphoxide using an enzymatic procedure. However, the spectrum already provides the answer, since at least 4 fragment ions were present, each accompanied by signals with m/z values 64 u lower.

In summary, the presence of methionine sulphoxide in a peptide can easily be recognized from the loss of CH_3SOH (-64 u) from $[M+H]^+$. The elimination also occurs from sequence ions, which allows determination of the position of the methionine sulphoxide residue(s) in the peptide chain when isobaric phenylalanine is present. Peptides containing

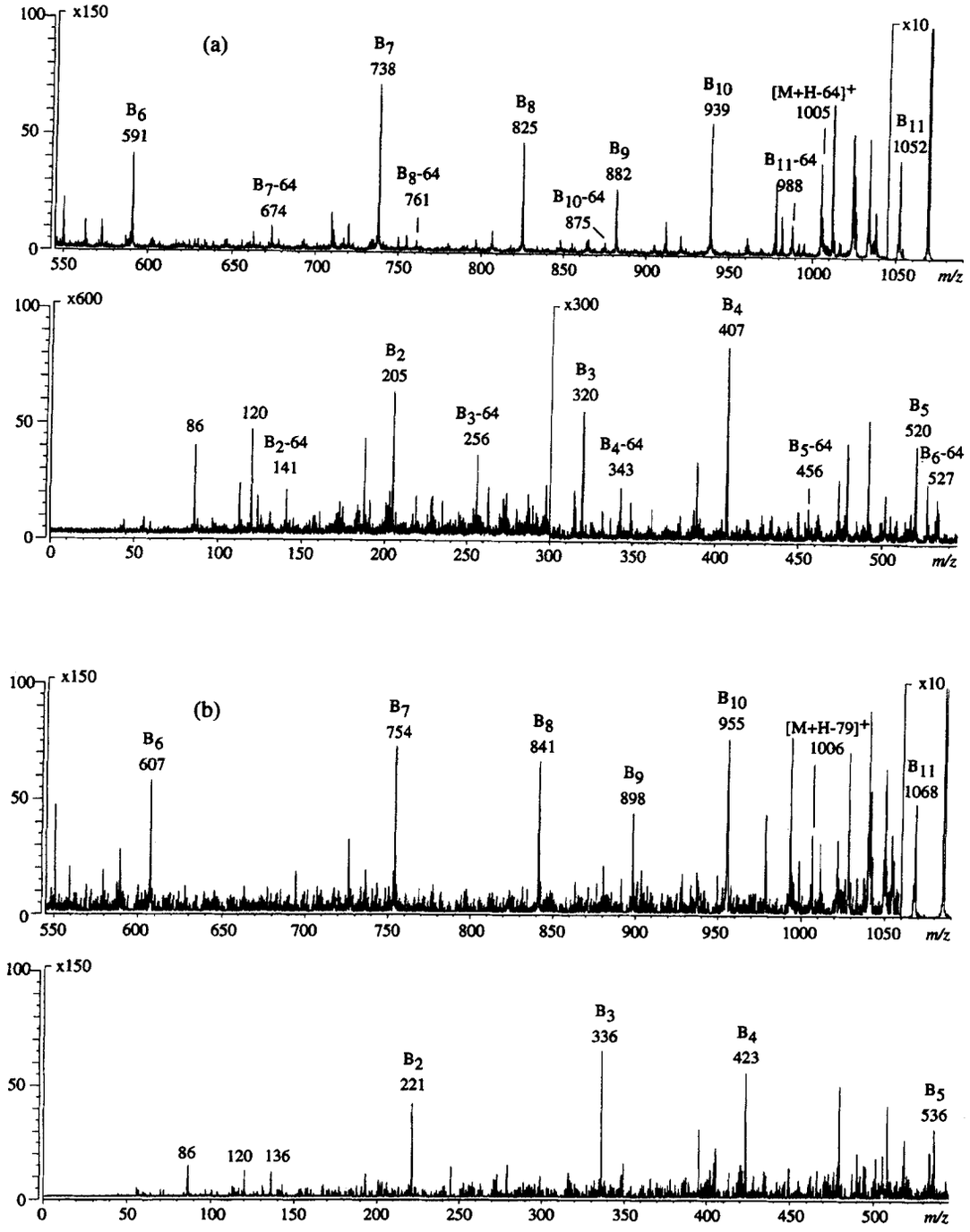


Figure 2. Positive-ion FAB-CID-MS/MS spectra of the $[M+H]^+$ ion of buccalin containing (a) methionine sulphoxide (*m/z* 1069) and (b) methionine sulphone (*m/z* 1085).

methionine sulphone undergo elimination of CH_3SO_2H (-80 u) and $CH_3SO_2^{\bullet}$ (-79 u) from $[M+H]^+$. Ions arising from this process are usually of low abundance and not observed from sequence ions.

REFERENCES

1. N. Brot and H. Weissbach, *BioFactors* **3**, 91 (1991).
2. B. Bouchon, M. Jaquinod, K. Klarskov, F. Trottein, M. Klein, A. van Dorsselaer, R. Bischoff and C. Roitsch, *J. Chromatogr. B* **662**, 279 (1994).
3. L. Dillen, X. Y. Zhang, M. Claeys, F. Liang, W. P. De Potter, W. van Dongen and E. L. Esmans, *J. Mass Spectrom.* **30**, 1599 (1995).
4. A. von Eckardstein, M. Walter, H. Holz, A. Benninghoven and G. Assmann, *J. Lipid Res.* **32**, 1465 (1991).
5. G. Teshima and E. Canova-Davis, *J. Chromatogr.* **625**, 207 (1992).
6. J. L. E. Reubsaet, J. H. Beijnen, A. Bult, E. Hop, R. Vermaas, Y. Kellekule, J. J. Kettenes-van den Bosch and W. J. M. Underberg, *Anal. Chem.* **67**, 4431 (1995).
7. P. W. D. Scilowski, I. Harris, K. Pickard, D. S. Brown and V. Buchan, *J. Chromatogr.* **619**, 299 (1993).
8. Q.-F. Gan, G. L. Witkop, D. L. Sloane, K. M. Straub and E. Sigal, *Biochemistry* **34**, 7079 (1995).
9. A. Buzy, V. Bracchi, R. Sterjiades, J. Chroboczek, P. Thibault, J. Gagnon, H. M. Jouve and G. Hudry-Clergeon, *J. Prot. Chem.* **14**, 59 (1995).
10. Y. Sun and D. L. Smith, *Anal. Biochem.* **172**, 130 (1988).
11. S. K. Chowdhury, J. Eshraghi, H. Wolfe, D. Forde, A. G. Hlavac and D. Johnston, *Anal. Chem.* **67**, 390 (1995).
12. R. J. Waugh, J. H. Bowie and M. L. Gross, *Rapid Commun. Mass Spectrom.* **7**, 623 (1993).
13. A. M. Bradford, R. J. Waugh and J. H. Bowie, *Rapid Commun. Mass Spectrom.* **9**, 677 (1995).
14. F. Turecek, D. E. Drinkwater and F. W. McLafferty, *J. Am. Chem. Soc.* **111**, 7696 (1989).
15. W. Kulik and W. Heerma, *Biomed. Environ. Mass Spectrom.* **15**, 419

(1988).
16. N. N. Dookeran, T. Yalcin and A. G. Harrison, *J. Mass Spectrom.* **31**, 500 (1996).
17. T. Yagami, K. Kitagawa and S. Futaki, *Anal. Sciences* **11**, 1025 (1995).
18. M. M. Cordero, J. J. Houser and C. Wesdemiotis, *Anal. Chem.* **65**, 1594 (1993).
19. S. Beranová, J. Cai and C. Wesdemiotis, *J. Am. Chem. Soc.* **117**, 9492 (1995).
20. T. Yalcin, C. Khouw, I. G. Csizmadia, M. R. Peterson and A. G. Harrison, *J. Am. Soc. Mass Spectrom.* **6**, 1165 (1995).
21. T. Yalcin, I. G. Csizmadia, M. R. Peterson and A. G. Harrison, *J. Am. Soc. Mass Spectrom.* **7**, 233 (1996).
22. H. R. Morris, T. Paxton, A. Dell, J. Langhorne, M. Berg, R. S. Bordoli, J. Hoyes and R. H. Bateman, *Rapid Commun. Mass Spectrom.* **10**, 889 (1996).