Electrospray Mass and Tandem Mass Spectrometry Identification of Ozone Oxidation Products of Amino Acids and Small Peptides

Tapio Kotiaho
VTT Chemical Technology, VTT, Finland

Marcos N. Eberlin
State University of Campinas—UNICAMP, Institute of Chemistry CP6154, Campinas, SP Brazil

Pirjo Vainiotalo
Department of Chemistry, University of Joensuu, Joensuu, Finland

Risto Kostiainen
Department of Pharmacy, Division of Pharmaceutical Chemistry, University of Helsinki, Helsinki, Finland

Aqueous ozonation of the 22 most common amino acids and some small peptides were studied by electrospray mass (ESI-MS) and tandem mass spectrometry. After 5 min of ozonation only His, Met, Trp, and Tyr form oxidation products clearly detectable by ESI-MS. For His, the main oxidation product is formed by the addition of three oxygen atoms, His + 3O; for Met and Tyr by the addition of one oxygen atom, Met + O and Tyr + O, and for Trp by the addition of two oxygen atoms, Trp + 2O. Ozone oxidation occurs rapidly, products are already detected after 30 s of ozonation, and the reactivity order is Met > Trp > Tyr > His. The structures of the oxygen addition products were investigated by electrospray product ion mass spectra, and by comparing these spectra to those of protonated intact amino acids, and when available, to those of model compounds. His + 3O was assigned as 2-amino-4-oxo-4-(3-formylureido)butanoic acid (**1**) formed by oxidation of the His imidazole ring, Met + O as methionine sulfoxide (**2**), Trp + 2O as *N*-formylkynurenine (**4**), and Tyr + O as a mixture of dihydroxyphenylalanines (**7** and **8**). Ozonation of peptides show that the same number of oxygen atoms are added as expected from the ozonation of the free amino acids. The product ion mass spectra of both the protonated intact peptides, MH^+, and the main ozonation products $(M + nO)H^+$ (n = 1–3) revealed b and y type ions as the main fragments, which allow one to assign the type and location of modified amino acid in the model peptides. (J Am Soc Mass Spectrom 2000, 11, 526–535) © 2000 American Society for Mass Spectrometry

Ozone, owing to its unique dual nature, is an interesting and vital compound in the environment. Ozone is harmful and toxic [1], yet it is a vital ultraviolet light screen in the upper atmosphere [2]. The chemical and physical properties of ozone and its practical applications have been studied vastly; its gas phase chemistry is well characterized [3, 4] and ozone is a major agent of water purification and disinfection [5].

Its toxicity, strong reactivity, and especially strong oxidizing ability makes ozone potentially harmful to biomolecules. Peptides and proteins can be oxidized under physiological conditions, storage or various analytical and synthetic procedures [6–14]. Accumulation of oxidized proteins have been related to diseases (e.g., cataract and rheumatoid arthritis) [6, 10, 14], whereas oxidation of proteins can decrease nutritional value of foodstuffs [7, 9, 14]. It is therefore important to identify the products of aqueous ozonation of peptides and proteins.

Typically, the products of aqueous ozonation of free amino acids [15–20], peptides, and proteins [15–17, 21–29] have been identified using chromatographic and spectrophotometric methods. Recently, electrospray ionization (ESI) [30] and matrix-assisted laser desorp-

Address reprint requests to Dr. Risto Kostiainen, Department of Pharmacy, Division of Pharmaceutical Chemistry, P.O. Box 56, FIN-00014 University of Helsinki, Helsinki, Finland. E-mail: risto.kostiainen@helsinki.fi or Dr. Tapio Kotiaho, VTT Chemical Technology, P.O. Box 1401, FIN-02044 VTT, Finland. E-mail: tapio.kotiaho@vtt.fi

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Table 1. Compounds used in the study. Amino acids are presented by the standard three-letter codes. All the amino acids were L isomers

Amino acids:
Ala, Arg, Asn, Asp, Cys, L-Cystine, Glu, Gln, Gly, His, Hyp, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val
Peptides:
Lys-Phe, His-Leu
Gly-His-Lys, Lys-Trp-Lys, Lys-Tyr-Lys
Gly-Gly-Phe-Met
Tyr-Gly-Gly-Phe-Met
Standard compounds:
L-methionine sulfoxide
L-methionine sulfone
L-3,4-dihydroxyphenylalanine

tion/ionization (MALDI) [31] have been used successfully to identify oxidized peptides and proteins [13, 32–41]. Fast-atom bombardment (FAB) has also been applied to characterize structures of oxidized peptides [8, 14, 42–45]. For instance, FAB tandem mass spectrometry has been used to determine the position of the oxidized methionine [45] and tryptophan [14] in peptide chains.

In this work ESI-MS was used to investigate which of the 22 most common amino acids (Table 1) are rapidly oxidized by aqueous ozonation. We also tested whether the same amino acids would be oxidized when they are a part of a peptide chain. ESI product ion mass spectra were used to assign the structures of the ozonation products of the amino acids and the peptides.

Methods

Table 1 lists the amino acids and peptides studied. Aqueous solutions of each of the samples were prepared using deionized water (pH 5.8) (Milli-Q Plus, Millipore, Beford, MA), typical concentration being 0.3 mg/mL. Ozonation of the aqueous samples was performed by bubbling an ozone/oxygen gas mixture at a flow rate of about 60 mL/min through the samples (sample volume typically 3 mL). The ozonation times varied from 30 s to 20 min. Ozone enriched oxygen gas stream was made using a commercial ozone generator (Fischer 500, Bonn, Germany) and high purity oxygen (O_2 > 99.999%, AGA Gas, Hamburg, Germany). The ozone concentration in the ozone/oxygen gas stream was 2.6% (about 70 μmol/min) as determined by the KI–$Na_2S_2O_3$ titration method. For the mass spectrometric analysis, the ozonated solutions were diluted using a 1:1 mixture of methanol and water with 0.1% of acetic acid. Samples were stored at +5 °C before mass spectrometric analysis.

ESI-MS and ESI-MS/MS spectra were measured using a Sciex API 300 triple quadrupole mass spectrometer (Sciex, Toronto, Canada). A micro syringe pump (Harvard Apparatus, Edenbridge, Great Britain) was used for liquid delivery and the samples were introduced with a Rheodyne injector (Cotati, CA). The injection volume was typically 5 μL and flow rate 5 μL/min. The eluent composition was the same as in the corresponding samples. Synthetic air (99.998%) was used as a nebulizing gas and nitrogen generated with a Whatman 75-720 nitrogen generator (Whatman, Haverhill, MA) was used as a curtain gas and as the collision gas in MS/MS experiments. The voltage of the stainless steel capillary was 4.5 kV. The scan range was 100–800 (3 s/scan) for the ESI-MS spectra and 20 to 30 *m/z* units above the *m/z* value of the precursor ion (3 s/scan) for the ESI-MS/MS spectra.

Accurate mass measurements on collision induced dissociation (CID) fragments were made using a Bruker (Bruker Daltonics, Billerica) BioAPEX 47e Fourier transform ion cyclotron resonance mass spectrometer equipped with a 4.7 tesla, shielded 160 mm bore superconducting magnet (Magnex Scientific, Abingdon, UK), Infinity cell and electrospray source (Analytica of Branford, Branford, CT). Sample solutions were continuously introduced to the interface sprayer through a glass microliter syringe at a flow rate of 40 μL/h under atmospheric pressure. A 50:50:1 mixture of methanol: water:acetic acid was used as a solvent, sample concentration being 10 pmol/μL. The CID data was measured using internal calibration by activating the parent ion without isolation using argon as a collision gas [46–48]. As internal calibrants, Leu, His, and Tetragly were used for Trp and Leu, Met and Trp for His. All data was acquired with 128k data points and zero filled to 512k by averaging 32 scans. The masses of the fragment ions were identified with an average error of 2.8 ppm.

Amino acids, peptides, and standard compounds were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany (Table 1).

Results and Discussion

Ozonation of Amino Acids

ESI-MS

From the 22 amino acids studied, after 5 min of ozonation of the aqueous samples, only His, Met, Trp, and Tyr form oxidation products clearly observable in the measured ESI-MS mass spectra. To show, that the oxidation products were really formed by aqueous ozonation, blank amino acid and peptide samples (no ozonation) were also analyzed. This was done especially because it has been reported that oxidation of peptides, e.g., methionine and phenylalanine residues, may occur during capillary electrophoresis-nanoelectrospray mass spectrometry analysis [37] and that oxidized peptides may even be formed under infusion electrospray experiments [38]. In this study, oxidation was not observed to occur during ESI-MS analysis.

ESI-MS mass spectra of the individual ozonized amino acid samples show that His forms mainly one oxidation product corresponding to addition of three oxygen atoms, $(His + 3O)H^+$ of *m/z* 204; Met forms

Table 2. Abundances (arbitrary units) of the protonated molecules and the main oxygen addition products as a function of time for His, Met, Trp, and Tyr

	Amino acid							
	His		Met		Trp		Tyr	
Reaction time	MH^+ *m/z* 156	$(M + 3O)H^+$ *m/z* 204	MH^+ *m/z* 150	$(M + O)H^+$ *m/z* 166	MH^+ *m/z* 205	$(M + 2O)H^+$ *m/z* 237	MH^+ *m/z* 182	$(M + O)H^+$ *m/z* 198
0	4110	–	1750	11	2150	–	1940	60
30 s	4200	24	820	550	1550	616	3170	220
1 min	3920	54	470	880	970	743	3070	370
2 min	4110	90	180	1210	210	943	2970	550
5 min	3830	211	21	1570	–	892	2660	670
10 min	3270	422	21	1570	–	616	4600	470
20 min	2050	714	21	1430	–	255	1940	1030

mainly $(Met + O)H^+$ of *m/z* 166; Trp forms mainly $(Trp + 2O)H^+$ of *m/z* 237; and Tyr forms mainly $(Tyr + O)H^+$ of *m/z* 198. Formation of these four main oxygen addition products and consumption of the amino acid (MH^+) were followed as a function of ozonation time (Table 2). Some other minor oxygen addition products were also observed. For His, Met, and Tyr, $(M + 2O)H^+$ product ions were observed at *m/z* 188, 182, and 214, respectively. The results obtained for Met show that when the intensity of $(Met + O)H^+$ starts to decrease $(Met + 2O)H^+$ still keeps increasing (Figure 1), possibly indicating that Met + O is the precursor of Met + 2O. Traces of $(Trp + O)H^+$ at *m/z* 221, $(Trp + 3O)H^+$ at *m/z* 253, and $(Tyr + 3O)H^+$ at *m/z* 230 were also detected.

As Table 2 shows, Met and Trp are the amino acids most easily oxidized by ozone as measured by the $(M + nO)H^+/MH^+$ abundance ratios and all products are formed rapidly under the conditions used; after just 30 s of ozonation products are already abundant. Using the $(M + nO)H^+/MH^+$ abundance ratio to estimate the reactivity differences, the reactivity order is Met > Trp > Tyr > His. This order agrees well with the reactivity order reported by Mudd et al. [15]: Cys > Met > Trp > Tyr > His > Cystine > Phe. By performing detailed studies of kinetics of aqueous ozonation of amino acids, Pryor et al. [20] derived an almost similar reactivity order at pH 7.0: Cys > Trp > Met > Tyr > His. The authors also observed a pH dependency for the reactivity order, that is, at lower pH's: Trp > Met > Cys > Tyr. The main difference between this study and the results reported [15, 20] is that under the present ozonation conditions cystine does not form oxygen addition products and Cys (product Cys + 3O) and Phe (products Phe + O and Phe + 2O) form only traces of oxygen addition products. The poor O_3-oxidation reactivity of Cys under the present ozonation conditions is surprising since cysteic acid (Cys + 3O) is known to be formed easily by several other oxidizing agents, e.g., performic acid [35, 44], acid hydrolysis in the presence of sodium azide [12], and peroxide [36]. An explanation to this experimental result could be that cysteic acid is labile and therefore difficult to detect. However, other research groups have observed that peptides containing cysteic acid modification are stable under both FAB ionization [44] and electrospray ionization [36]; even tandem mass spectrometric experiments of these modified peptides have been successfully performed [36]. The only reasonable explanation is the difference in reaction conditions, i.e., in the present study, ozonation has been performed in deionized water (pH 5.8), whereas Mudd et al. [15] (pH's 4.6, 7.2, 8.7) and Pryer et al. [20] (pH range 1.7–3.6) used various buffer solutions.

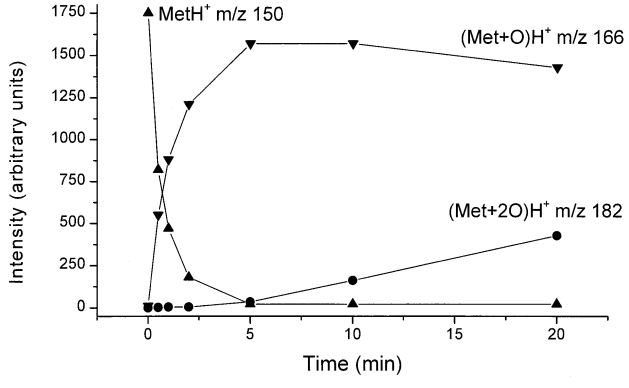
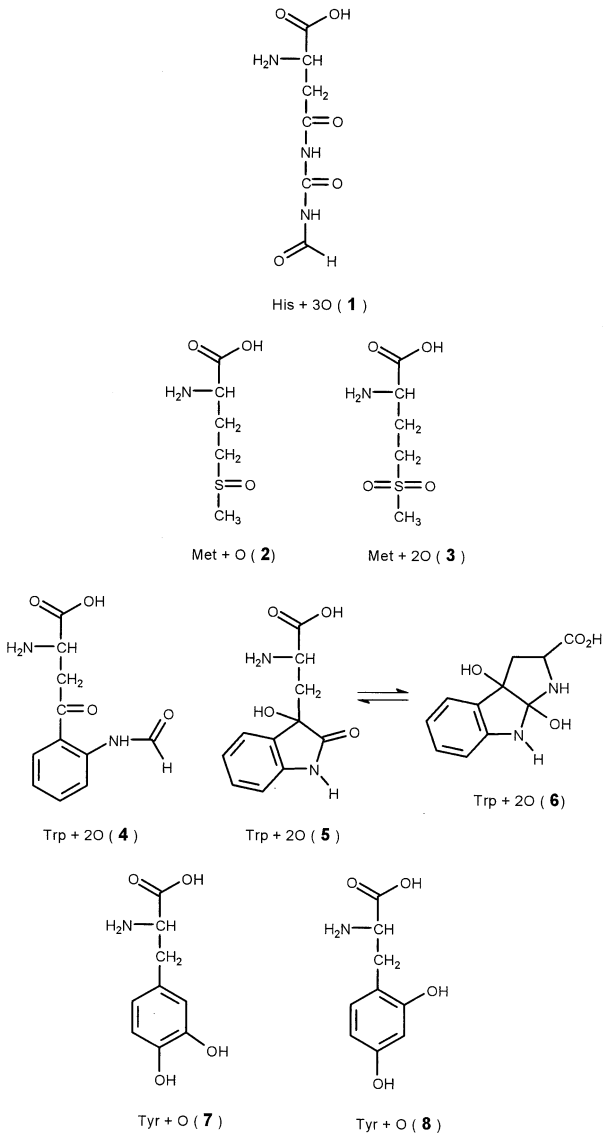


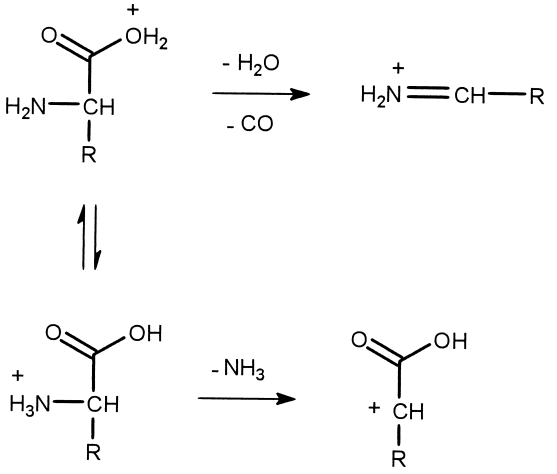
Figure 1. Intensity change of $MetH^+$ of *m/z* 156, $(Met + O)H^+$ of *m/z* 166 and $(Met + 2O)H^+$ of *m/z* 182, as a function of ozonation time.

ESI-MS/MS

The structures of the $M + nO$ (n = 1–3) products were studied by measuring their product ion mass spectra and by comparing them to those of protonated intact amino acids and, when available, to those of protonated model compounds. The product ion mass spectra of the protonated intact amino acids (MH^+) show loss of ammonia (17 u) and loss of 46 u as the main dissociation routes (Table 3, Scheme **2**), as expected from previous studies [14, 45, 49, 50]. The loss of 46 u involves mainly a rapid sequential loss of H_2O and CO to form a stable immonium ion (H_2N^+=CHR, R = a side chain), but minor loss of formic acid (HCOOH) has also been suggested [50].



Scheme 1



Scheme 2

Histidine

Figure 2 presents the product ion mass spectrum of $(His + 3O)H^+$ of *m/z* 204, which mainly dissociates by loss of 45 u to form *m/z* 159. The elemental composition of *m/z* 159 was determined to be $C_5H_7N_2O_4$ (calculated exact mass 159.0400 and measured exact mass 159.0400). Hence, $(His + 3O)H^+$ most likely loses either a neutral formamide molecule (Scheme **3**) or CO plus NH_3. Neutral losses of H_2O + CO (*m/z* 158) and NH_3 (*m/z* 187) also occur but to moderate extents (Scheme **2**). Another relatively intensive product ion is that of *m/z* 116, likely formed by NH=C=O (43 u) loss from *m/z* 159 (Scheme **3**).

Mudd et al. [15] tentatively identified the main ozone oxidation product of His as the amino acid proline, but Stadtman et al. [28] reported that His is oxidized during ozonolysis to aspartyl residue via a primary His + O oxidation product, 2-oxohistidine. Support for the interpretation of Stadtman et al. comes from the identification of aspartic acid as one of the secondary oxidation products of His under various other oxidation conditions [51]. In addition, an analog of 2-oxohistidine, *N*-benzoyl-*β*-(2-oxoimidazolonyl)alanine, is the main primary oxidation product formed by Cu^{2+}/ascorbate oxidation of the model compound *N*-benzoylhistidine [51]. The present results show, however, that under the conditions used, His forms mainly His + 3O product and none of the His + O product. From the fragmentation pattern observed for $(His + 3O)H^+$ (Scheme **3**), it is proposed that His + 3O is 2-amino-4-oxo-4-(3-formylureido)butanoic acid (**1**) (Scheme **1**), formed by oxidation of the imidazole ring. An analog of **1** has also been

Table 3. Product ion mass spectra of the protonated intact amino acids[a]

Amino acid, MH^+ ion *m/z* ratio	Neutral loss, *m/z* ratio and (relative abundance, RA)		
	$-NH_3$	$-(H_2O + CO)$	Others
His, *m/z* 156	—	110 (100)	95(5)
Met, *m/z* 150	133 (52)	104 (100)	102 ($-CH_3SH$, 24), 87 (2), 74 (4), 61 (3), 56 (104 − CH_3SH, 13)
Trp, *m/z* 205	188 (100)	159 (7)	146 (−59, 19), 144 (5), 132 (2)
Tyr, *m/z* 182	165 (62)	136 (100)	147 ($-NH_3 - H_2O$, 15), 123 (−59, 23) 119 [$-(H_2O + CO) - NH_3$, 14]), 91 (2)

[a]Peaks with relative abundance of more than 2% from the base peak are shown.

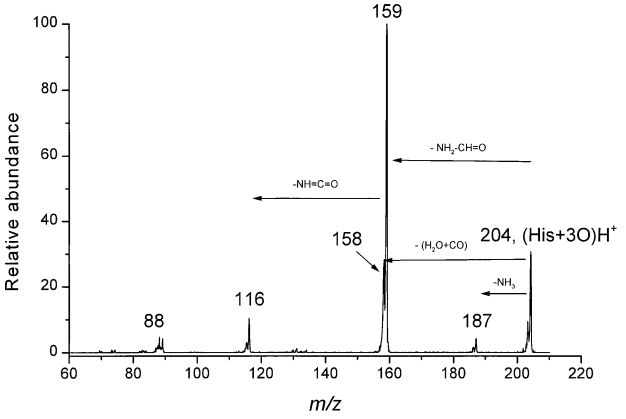


Figure 2. Product ion mass spectrum of (His + 3O)H^+ of *m/z* 204.

proposed as an intermediate between the primary oxidation product, *N*-benzoyl-*β*-(2-oxoimidazolonyl)alanine, and the secondary oxidation products during oxidation of *N*-benzoylhistidine [51]. However, its formation could not be experimentally verified.

Methionine

Figure 3 presents the product ion mass spectra for (Met + O)H^+ of *m/z* 166 and (Met + 2O)H^+ of *m/z* 182. The corresponding spectra of the protonated model compounds, L-methionine sulfoxide of *m/z* 166 and L-methionine sulfone of *m/z* 182, agree very well with the results presented in Figure 3 and with those published by Lagerwerf et al. [45], although here less fragmentation is observed most probably owing to different experimental methods, i.e., ESI and low energy CID vs. FAB and high energy CID.

The most abundant product ion for protonated methionine sulfoxide and for (Met + O)H^+ (Figure 3a) is that of *m/z* 74, probably formed by a single loss of C_3H_8SO or a sequential loss of CH_3SOH and $CH_2{=}CH_2$ (Scheme **4**). Support for a sequential loss comes from the ion of *m/z* 102, formed by CH_3SOH loss. Direct cleavage of the side chain to form the fragment of *m/z* 75 (Scheme **4**), likely ionized glycine [52], is also an important fragmentation route [45]. Additionally, loss of NH_3

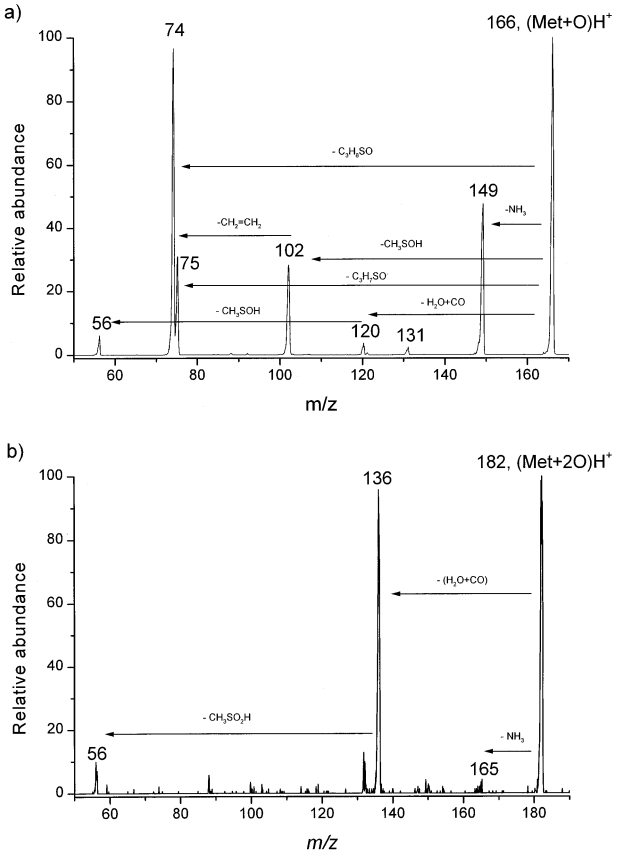
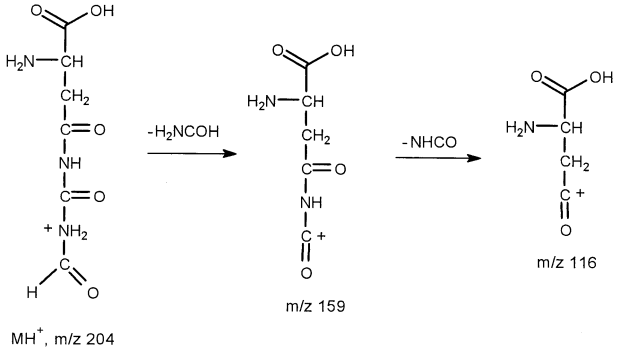
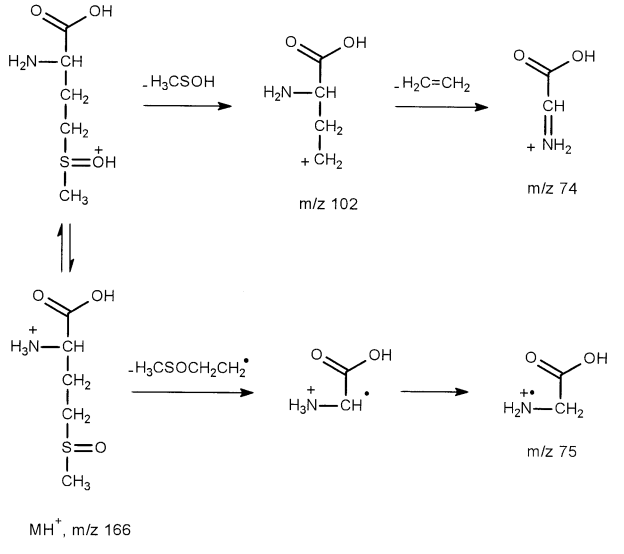


Figure 3. Product ion mass spectrum of (**a**) (Met + O)H^+ of *m/z* 166 and (**b**) (Met + 2O)H^+ of *m/z* 182.

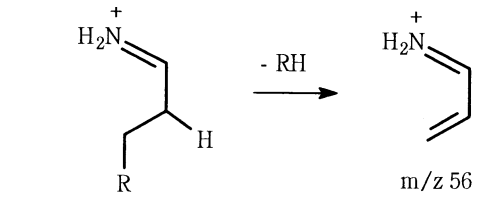
(*m/z* 149) and H_2O + CO (*m/z* 120) also occur (Scheme **2**). The product ions of *m/z* 149, 102, 75, and 74 indicate that methionine sulfoxide is protonated considerably both at the amino and sulfoxide groups. Protonated methionine sulfone and (Met + 2O)H^+ (Figure 3b) dissociate to form mainly the product ions of *m/z* 136



Scheme 3



Scheme 4



$R = SCH_3, SOCH_3, SO_2CH_3$

Scheme 5

(H_2O + CO loss, Scheme **2**), *m/z* 56, and a minor fragment ion of *m/z* 165 by NH_3 loss. Interestingly, the *m/z* 56 fragment is common for both mass spectra displayed in Figure 3. Its structure is proposed to be H_2N^+=CH–CH=CH_2, which suggests that the oxygen addition site of methionine is the sulfur atom [45]. The *m/z* 56 fragment is most probably formed from further dissociation of the corresponding immonium ions; *m/z* 104 for $MetH^+$, *m/z* 120 for $(Met + O)H^+$, and *m/z* 136 for $(Met + 2O)H^+$, by neutral loss of CH_3SH, CH_3SOH, and CH_3SO_2H, respectively (Scheme **5**). These findings indicate that aqueous ozonation of Met produces mainly L-methionine sulfoxide (**2**) and L-methionine sulfone (**3**) as the primary oxidation products. Formation of methionine sulfoxide as a primary ozone oxidation product of Met was also observed by Mudd et al. [15], but L-methionine sulfone was not observed.

Tryptophan

Figure 4 presents the product ion mass spectrum of $(Trp + 2O)H^+$ of *m/z* 237, which dissociates mainly by NH_3 loss (calculated exact mass 220.0604 and measured exact mass 220.0606) and NH_3 + H_2O loss (calculated exact mass 202.0499 and measured exact mass 202.0497) (Scheme **6**). The abundant fragment of *m/z* 192 can be formed from $(Trp + 2O)H^+$ by NH_2COH loss (calculated exact mass 192.0655 and measured exact mass 192.0658), but because the product ion of *m/z* 209 is formed by CO loss (calculated exact mass 209.0921 and measured exact mass 209.0917), sequential loss of CO + NH_3 may also occur (Scheme **6**). Product ions formed by H_2O loss (*m/z* 219) and by H_2O + CO loss (*m/z* 191) are also formed (Scheme **2**), but to minor extents.

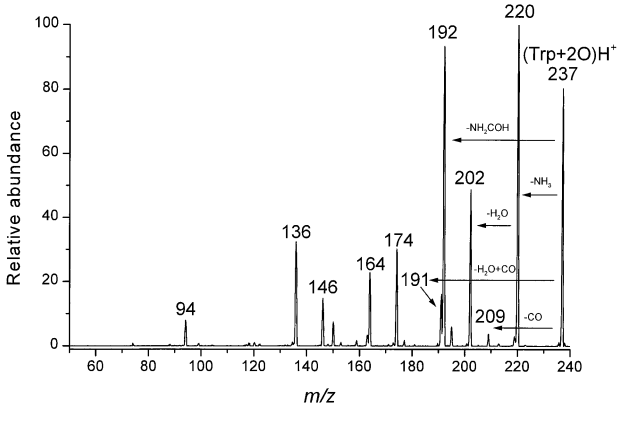


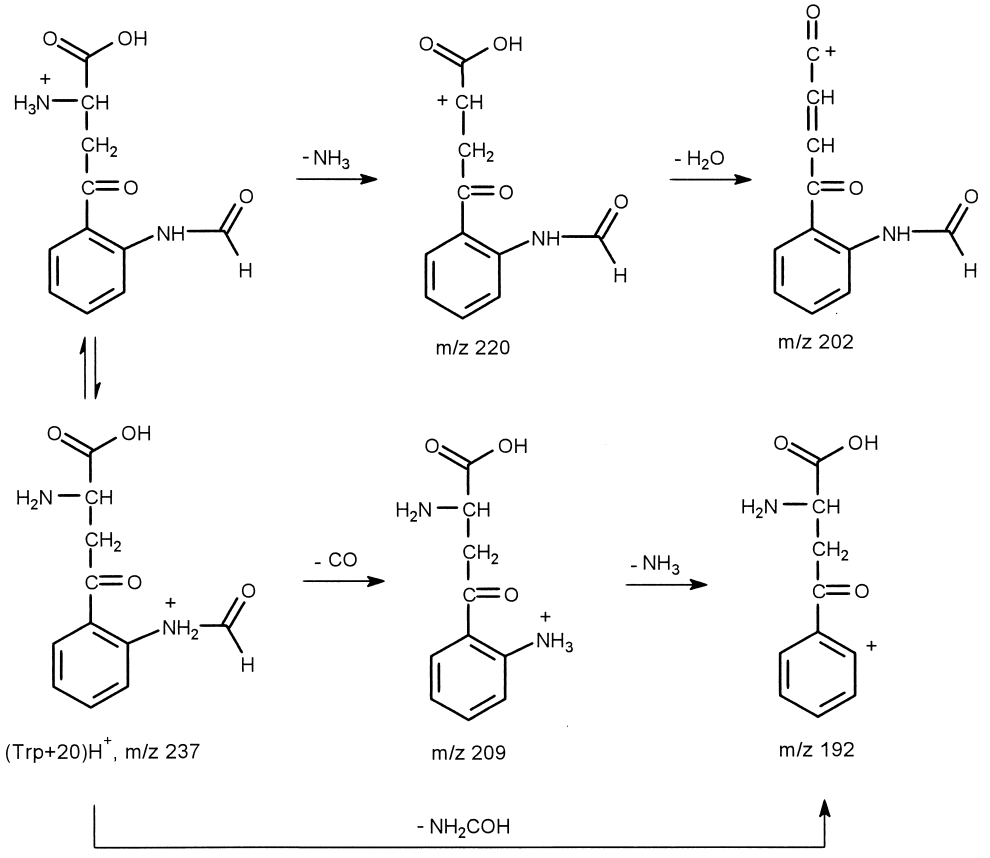
Figure 4. Product ion mass spectrum of $(Trp + 2O)H^+$ of *m/z* 237.

The oxidation products of Trp under various oxidizing conditions (chemical or electrochemical) have been well characterized [15, 16, 53–58] and Trp oxidation products formed under dimethyl sulfoxide/hydrochloric acid/acetic acid oxidizing conditions have been studied by FAB and high energy CID [14]. Two structures have been proposed for the Trp + 2O product: *N*-formylkynurenine (**4**) and dioxindolylalanine (**5**), which can exist in aqueous solution also as 2-carboxy-3a,8a-dihydroxy-1,2,3,3a,8,8a-hexahydropyrrolo-(2,3-b)-indole (**6**) (Scheme **1**). van de Weert et al. reported also a product ion spectrum for $(Trp + 2O)H^+$ product measured by FAB and high energy CID [14]. However, this spectrum differs considerably to the present spectrum. The $(Trp + 2O)H^+$ ion formed by van de Weert et al. dissociates mainly by H_2O and H_2O + H_2O loss to form the fragments of *m/z* 219 and 201, most probably indicating a structure with multiple OH groups. The authors also observed a minor fragment ion formed by NH_3 loss, which could indicate that their main product does not include primary amine to a considerably extent. These findings indicate that, under dimethyl sulfoxide/hydrochloric acid/acetic acid oxidizing conditions, Trp forms dioxindolylalanine (**5**) especially its three ring isomer (**6**). However, the $(Trp + 2O)H^+$ ion formed under the present ozonation conditions show abundant product ions formed by NH_3 loss (*m/z* 220) and by NH_3 + H_2O loss (*m/z* 202), indicating presence of both a primary amino and a hydroxyl group. CO loss (*m/z* 209), but particularly the possible formamide loss (*m/z* 192), from $(Trp + 2O)H^+$ accounts for the presence of a *N*-formyl group. We therefore suggest that the structure of the Trp + 2O product formed under the present ozonation conditions is *N*-formylkynurenine (**4**) resulting from oxidation of the pyrrole-ring double-bond, in agreement with the results of Mudd et al. [15].

Tyrosine

Figure 5 presents the product ion mass spectra for $(Tyr + O)H^+$ of *m/z* 198 and for the protonated model compound L-3,4-dihydroxyphenylalanine (L-DOPA) of *m/z* 198 (**7**). When these two spectra are compared, similar features are observed. Both spectra show abundant product ions of *m/z* 181 and *m/z* 152 formed, respectively, by NH_3 and H_2O + CO loss (Scheme **2**). Sequential loss of NH_3 + H_2O from both of the precursor ions forms *m/z* 163; loss of NH_3 + H_2O + CO forms *m/z* 135. Formation routes of the product ions *m/z* 166 and 139 are difficult to explain, but they could involve loss of CH_3OH and loss of NH_3 + CH_2CO, respectively. In contrast to the protonated L-DOPA (Figure 5b), product ions formed by H_2O loss (*m/z* 180) and H_2O + H_2O + CO loss (*m/z* 134) are detected in the product ion spectrum of $(Tyr + O)H^+$ (Figure 5a).

Mudd et al. [17] and Verweij [18] reported that the Tyr + O product, formed by ozonolysis, is 3,4-dihy-



Scheme 6

droxyphenylalanine (**7**). However, based on the present results, the Tyr + O product has a structure close, but not identical, to that of **7**. Possibly, a mixture of isomers, including **7** as a major component, is formed. 2,4-dihydroxyphenylalanine (**8**) should lose water more favorably than 3,4-dihydroxyphenylalanine; hence **8** is proposed as the minor component of the mixture. Protonation of neighboring hydroxyl substituents of the aromatic ring of **7** can cause intramolecular hydrogen bonding stabilizing the protonated molecule and therefore hindering H_2O loss. For the 2,4-dihydroxy isomer (**8**), however, hydrogen bonding is not possible, thus H_2O loss is favored.

Ozonation of Peptides

ESI-MS

For the model peptides studied (Table 1), ozone oxidation products were detected in the ESI-MS mass spectra of the peptides containing His (His-Leu, Gly-His-Lys), Met (Gly-Gly-Phe-Met, Tyr-Gly-Gly-Phe-Met), and Trp (Lys-Trp-Lys). As expected from the O_3 reactivity observed for the free amino acids, the His-containing peptides formed mainly M + 3O, the Met-containing peptides formed mainly M + O, and Trp-containing peptides formed mainly M + 2O products. The Tyr-containing peptide Lys-Tyr-Lys was also oxidized by O_3, as expected, but only traces of M + O and M + 2O products were observed, the intensity of the M + O being a little larger. Only Lys-Phe forms no oxygen addition products, again as expected from the poor O_3 reactivity of both Phe and Lys.

Formation of the M + nO (n = 1–3) products of peptides was also followed as a function of ozonation time. As for the free amino acids, oxidation products for the peptides were formed rapidly, and for the Met, His, or Trp containing peptides the M + nO products were present with high abundances after 30 s of ozonation. The Lys-Tyr-Lys peptide was, however, an exception, it forms only traces of the M + O product. Using the $(M + nO)H^+/MH^+$ abundance ratio as an indication of reactivity, the reactivity order of amino acids in the model peptides was Met > His > Trp > Tyr, whereas that for the free amino acids was Met > Trp > Tyr > His. The O_3-oxidation reactivity order of amino acids in peptide chains has been shown to differ from that of free amino acids [21, 26, 28], a likely result of different environmental and steric factors surrounding the reactive amino acid [21, 26, 29].

ESI-MS/MS

The product ion spectra for the protonated intact peptides, MH^+, and for their main $(M + nO)H^+$ products were recorded and compared as to search for structur-

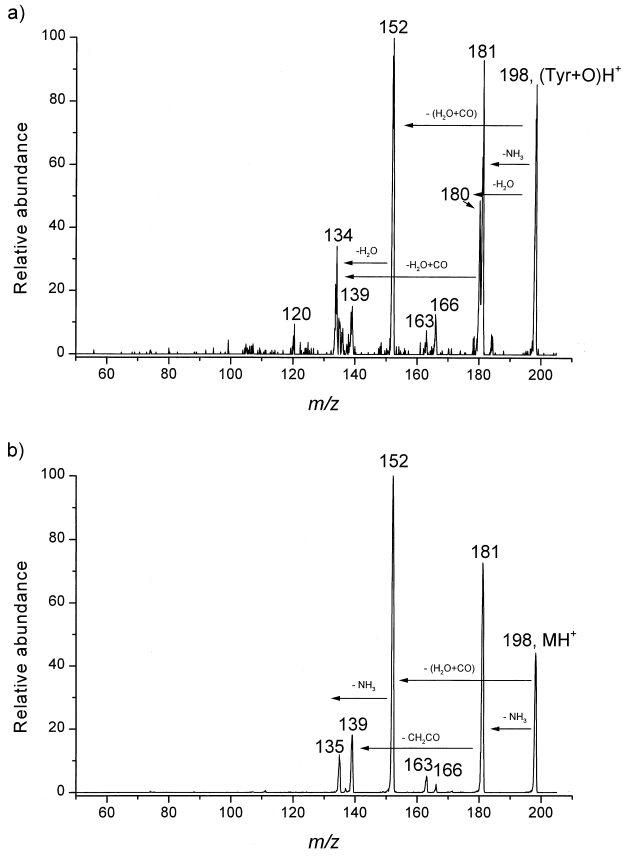


Figure 5. Product ion mass spectrum of (**a**) $(Tyr + O)H^+$ of *m/z* 198 and (**b**) protonated L-3,4-dihydroxyphenylalanine of *m/z* 198.

ally diagnostic fragment ions indicative of the type and the position of the oxidized amino acids in the peptide chains (Table 4). Oxidation of peptides have already been used to locate selected amino acids. For example, performic acid oxidation and FAB-MS have been used to locate disulfide bonds [43] and dimethylsulfoxide/hydrochloric acid/acetic acid oxidation have been used to detect peptides containing Met or Trp residues [42]. FAB-MS/MS has been used to selectively identify Met residues by hydrogen peroxide oxidation [45] and Trp residues by dimethylsulfoxide/hydrochloric acid/acetic acid oxidation [14].

Table 4 shows, as expected [61], that the b- and y-type fragment ions are dominant under CID for both MH^+ and $(M + nO)H^+$. Ozone oxidation also favors the y series of fragment ions, especially well seen here for the Met-containing peptides, and as also observed by Gaskell et al. for Cys-containing peptides [44]. The fragmentation of $(M + 3O)H^+$ of His-containing peptides differs slightly from that of other oxidized peptides (Table 4), because in addition to the b and y ions, very intense product ions are formed by the loss of the oxidized side chain. The $(M + 3O)H^+$ products of His, His-Leu, and Gly-His-Lys dissociate commonly by the loss of a neutral formamide (45 u) and formamide plus NH=C=O (88 u), to form for His the ions of *m/z* 159 and 116, for His-Leu those of *m/z* 272 and 229, and for Gly-His-Lys those of *m/z* 344 and 301. Hence, these similar fragment ions are characteristic for peptides ozonated at the His residue. The fragment $(a_1 + 3O)^+$ of *m/z* 158 also shows that His is oxidized by addition of three oxygen atoms in the His-Leu dipeptide. For the tripeptide Gly-His-Leu, this ion is not observed, but the product ions $(a_2 + 3O - 17)^+$ or $(b_2 + 3O - 45)^+$ of *m/z* 198, $(b_2 + 3O - 17)^+$ of *m/z* 226 and $(y_2 + 3O - 45)^+$ of *m/z* 287 indicate oxygen addition to the His residue. This conclusion is based on the ease by which the oxygen addition products of His, His-Leu, and Gly-His-Lys dissociate by NH_3 or formamide loss.

Lys-Trp-Lys and Lys-Tyr-Lys both form intense b- and y-type fragment ions under the low energy CID conditions used (Table 4), and the oxidized amino acid site is easily assigned. Lys-Trp-Lys forms M + 2O and the oxidized amino acid is assigned to be Trp as indicated both by the product ions $(b_2 + 2O)^+$ of *m/z* 347 and $(y_2 + 2O)^+$ of *m/z* 365. The present results differ from those presented earlier [14, 19] in which one oxygen atom addition product was used to identify Trp and from those in which ozonation of lysozyme and 10 other proteins in reverse micelles was reported to form Criegee ozonide or its tautomer as the primary oxidation product of Trp [57]. For Lys-Tyr-Lys a M + O product is formed and the product ions $(b_2 + O)^+$ of *m/z* 308 and $(y_2 + O)^+$ of *m/z* 326 indicate oxidation of the Tyr residue.

For the Met-containing peptides, Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Met, the type and the position of the oxidized amino acid residue is revealed by a series of y-type fragment ions. No oxygen addition b-type ions are formed, because the Met residue oxidized is the C-terminal amino acid. Further evidence for oxidation of the Met residue is provided by the fragment $(MH - 64)^+$ of *m/z* 363 for Gly-Gly-Phe-Met, and that of *m/z* 526 for Tyr-Gly-Gly-Phe-Met. CH_3SOH (64 u) loss, characteristic of oxidation of the Met residue [33, 44], also occurs for some of the y-type fragment ions (Table 4) providing further evidence for the presence and location of the Met residue. The greater reactivity of the Met residue also prevents oxidation of the Tyr residue in the Tyr-Gly-Gly-Phe-Met peptide. This is evidenced by the product ion spectrum of $(M + O)H^+$, which shows no $(a + O)^+$ or $(b + O)^+$ type ions.

Conclusion

ESI-MS and ESI-MS/MS have proven to be very good methods for the identification of aqueous ozone oxidation products of amino acids and small peptides. Compared to some other analytical techniques (e.g., chromatographic methods) the main strength of mass spectrometry, especially of the ESI-MS/MS technique, is its identification capability using only a very small amount of sample (10 fmol–10 pmol), although in many cases unambiguous identification of unknowns requires the use of model compounds.

More specifically, ESI-MS experiments show that

Table 4. Product ion spectra of the protonated peptides and the main protonated oxygen addition products. Only the product ions which can be named by the Biemann modification [59] of the Roepstorff classification [60] and product ions significant for the assignment of the oxygen addition site are presented

Peptide	Products of the MH^+ ion and the main oxygen addition products, type of ion, *m/z* ratio and (relative abundance)	
His-Leu	His-Leu, MH^+, *m/z* 269 (100) a_1 *m/z* 110 (5), b_1 *m/z* 138 (3)	His-Leu, $(M + 3O)H^+$, *m/z* 317 (95) $(M + 3O)H^+ - 17$ *m/z* 300 (100), $(M + 3O)H^+ - 45$ *m/z* 272 (76), $(M + 3O)H^+ - 88$ *m/z* 229 (38), $a_1 + 3O$ *m/z* 158 (16)
Gly-His-Lys	Gly-His-Lys, MH^+, *m/z* 341 (12) a_2 *m/z* 167 (26), b_2 *m/z* 195 (100), y_1 *m/z* 147 (7), y_2 *m/z* 284 (6)	Gly-His-Lys, $(M + 3O)H^+$, *m/z* 389 (13) $(M + 3O)H^+ - 45$ *m/z* 344 (100), $(M + 3O)H^+ - 88$ *m/z* 301 (41), $a_2 + 3O - 17$ or $b_2 + 3O - 45$ *m/z* 198 (2), $b_2 + 3O - 17$ *m/z* 226 (17), $y_2 + 3O - 45$ *m/z* 287 (27)
Lys-Trp-Lys	Lys-Trp-Lys, MH^+, *m/z* 461 (100) b_1 *m/z* 129 (35), b_2 *m/z* 315 (56), y_1 *m/z* 147 (7), y_2 *m/z* 333 (76)	Lys-Trp-Lys, $(M + 2O)H^+$, *m/z* 493 (81) b_1 *m/z* 129 (100), $b_2 + 2O$ *m/z* 347 (91), $y_2 + 2O$ *m/z* 365 (80)
Lys-Tyr-Lys	Lys-Tyr-Lys, MH^+, *m/z* 438 (17) b_1 *m/z* 129 (100), b_2 *m/z* 292 (29), y_1 *m/z* 147 (25), y_2 *m/z* 310 (60)	Lys-Tyr-Lys, $(M + O)H^+$, *m/z* 454 (31) b_1 *m/z* 129 (100), $b_2 + O$ *m/z* 308 (58), y_1 *m/z* 147 (44), $y_2 + O$ *m/z* 326 (47)
Gly-Gly-Phe-Met	Gly-Gly-Phe-Met, MH^+, *m/z* 411 (12) a_2 *m/z* 87 (<1), a_3 *m/z* 234 (4), b_3 *m/z* 262 (21), y_1 *m/z* 150 (18), y_2 *m/z* 297 (26)	Gly-Gly-Phe-Met, $(M + O)H^+$, *m/z* 427 (15) $(M + O)H^+ - 64$ *m/z* 363 (6), b_3 *m/z* 262 (13), $y_1 + O$ *m/z* 166 (100), $y_2 + O$ *m/z* 313 (46), $y_3 + O$ *m/z* 370 (6), $y_2 + O - 64$ *m/z* 249 (11)
Tyr-Gly-Gly-Phe-Met	Tyr-Gly-Gly-Phe-Met, MH^+, *m/z* 574 (26) a_1 *m/z* 136 (7), a_4 *m/z* 397 (100), b_2 *m/z* 221 (13), b_3 *m/z* 278 (58), b_4 *m/z* 425 (59) y_1 *m/z* 150 (20), y_2 *m/z* 297 (47), y_3 *m/z* 354 (21)	Tyr-Gly-Gly-Phe-Met, $(M + O)H^+$, *m/z* 590 (100) $(M + O)H^+ - 64$ *m/z* 526 (4) a_1 *m/z* 136 (3), a_4 *m/z* 397 (49), b_2 *m/z* 221 (5), b_3 *m/z* 278 (25), b_4 *m/z* 425 (45), $y_1 + O$ *m/z* 166 (61), $y_2 + O$ *m/z* 313 (53), $y_3 + O$ *m/z* 370 (40) $y_2 + O - 64$ *m/z* 249 (2), $y_3 + O - 64$ *m/z* 306 (2)

from the 22 most common amino acids studied, the ones most susceptible to oxidation under aqueous ozonation conditions are, in order of reactivity: Met > Trp > Tyr > His. Met and Tyr formed mainly Met + O and Tyr + O products, Trp mainly a Trp + 2O product and His mainly a His + 3O product. The ESI-MS/MS experiments allowed identification of the Met + O product as methionine sulfoxide (**2**) and the Trp + 2O product as *N*-formylkynurenine (**4**). The product ion mass spectra measured show that the most probable structure of Tyr + O product is a mixture of dihydroxyphenylalanines (**7** and **8**) and that of His + 3O product is 2-amino-4-oxo-4-(3-formylureido)butanoic acid (**1**).

The ESI-MS and ESI-MS/MS experiments reveal also that in the model peptides used the most reactive amino acids are the same as the most reactive free amino acids and that the oxygen addition products of the amino acids in the peptides have the same structures as the free oxidized amino acids. However, the reactivity order of the amino acids in the model peptides, Met > His > Trp > Tyr, differs from that of free amino acids, most probably owing to environmental and steric effects.

The product ion mass spectra of the oxidized peptides allow one to assign the type and location of the modified amino acids in these model peptides. The main fragment ions of both the intact and oxidized peptides were b- and y-type ions, except that the fragment ions, containing the oxidized residue, of the modified peptides typically occurred at $n \times 16$ (n = 1–3) mass units higher mass numbers depending on how many oxygen atoms were added to the peptide. Additionally, His and Met containing peptides showed some product ions formed by characteristic side chain losses, His containing peptides NH_2–CH=O loss and NH_2–CH=O + NH=C=O loss and Met containing peptides a CH_3SOH loss. These findings show that ozone oxidation together with ESI-MS/MS can be used as one of the tools, in determination of structures of unknown peptides.

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