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DETECTION OF CROSS-LINKED PEPTIDES BY FAST ATOM BOMBARDMENT MASS SPECTROMETRY

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Abstract

The possibility of chemical modification of peptides and proteins under the condition of proteolytic digestions and FABMS analysis was investigated. The results indicate that among the amino acid constituents of peptides and proteins: serine/cysteine, and cystine are the most sensitive residues which undergo chemical modifications under the experimental conditions. The chemical modification of these amino acids which is governed by the intrinsic properties of the peptides will result in the formation of a molecular ion mass which is 16 mu lower than the molecular ion of the parent peptide, MH^+. Exact mass measurements of $(MH-16)^+$ molecular ions indicate that these ions may correspond to$(MH-H_2O+H_2)^+$ but not$(MH-CH_4)^+$ molecules. In addition, the results indicate the presence of no inter-and /or intrachain disulfide bond rearrangements under the experimental conditions of degradations and FABMS analyses of Lysozyme and Ribonuclease A.

Introduction

Chemical modification of Amino Acid residues of proteins and peptides under the acidic and /or basic condition of processing or degradation have been reported by many groups [1-12]. The chemical nature of the modified residues along with their probable impact on the overall biological function of these proteins and peptides have been of growing concern to many pharmaceutical and food industries [13-15]. It is known that alkali treatment of proteins for ten minutes or longer may cause racemization of some amino acid residues [16], disulfide bond exchanges [8,9], and chemical modification of some of the less-reactive chemical groups of amino acid side chains into more reactive and unusual structures [1,3,5,7,11]. In the latter case, the newly formed reactive species may bring about further chemical reactions, mainly cross-linking processes. For example, dehydroalanine containing proteins may form from the loss of H_2O and /orH_2S molecules from serine and /or cysteine, respectively. This intermediate may cross-link with a nearby nucleophilic group such as $-NH_2$ group of a Lysine residue with the formation of Lysinoalanine containing peptides or proteins as shown in Fig. 1.

As shown in Figure 1, dehydroalanine residue in proteins may be formed from many different based catalysed elimination reactions of proteins. As illustrated, cystine, cysteine, and serine can serve as primary precursors for dehydroprotein under alkali conditions. The newly formed intermediate may serve as the imidate precursor of Lysinoalanine containing proteins or lanthionines among other probable derivatives. Due to the unusual chemical nature of these

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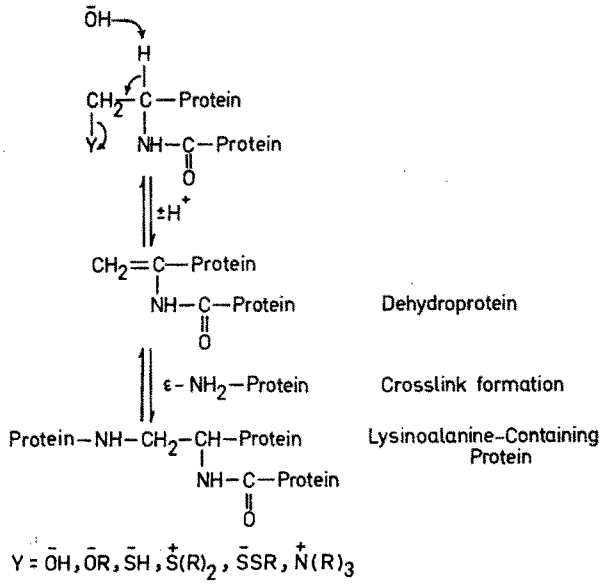


Figure1: Possible mechanism for alkali-induced Lysinoalanine formation [1,3,5,7,11]

derivatives compared to natural and unmodified proteins, there is the possibility of altered biological function for the modified proteins. Consequently, the knowledge of their mechanism of formation will be of great benefit to both pharmaceutical and nutritional industries. This information may probably assist the establishment of a correlation between the extent of modification and functional alterations.

The presence of unusual amino acids in proteins is usually achieved by amino acid analyzer using the authentic sample of the modified amino acid residues. This approach will demand a large quantity of the sample for complete identification of the modified residues. In addition, it is not always possible to investigate the possibility of disulfide bond exchanges by amino acid analyzer. These limitations will call for a more sensitive and reliable technique which will determine the chemical nature of the modified residues using the least amount of sample. In this investigation we attempt to use Fast Atom Bombardment Mass spectrometry to investigate the chemical nature of all possible intermediates formed through the proteolytic degradation of lysozyme and ribonuclease A under alkali environment. It is expected that this approach due to its inherent sensitivity and capability in structure elucidation will provide an ideal method for quick analysis of pharmaceutical and nutritional proteins and peptides which have been under acidic or basic conditions.

Materials and Methods

Materials: Bovine pancreatic ribonuclease A type IIIA and bovine pancreatic trypsin were purchased from the Sigma Chemical Company. Salt-free hen egg-white lysozyme was obtained from Cooper Biomedical. The following synthetic peptides were obtained from Peninsula Laboratories, Inc.: Somatostatin-14, oxytocin, pressinoic acid, Urotensin II, Conotoxin GI, Peptide A, kemptide and kemptamide, N-ethylmorpholine was obtained from the Aldrich Chemical Company. Phenylisothiocyonate was obtained from the Pierce Chemical Company. Organic reagents were reagent grade and have been used without additional purification. Glass distilled water has been used for all sample preparations. Protein and peptides have been used without further purifications.

Methods: Chemical and enzymic cleavage of proteins and peptides CNBr treatment of lysozyme and ribonuclease A and enzymic cleavages of all peptides with trypsin were achieved as described previously [17,18] and reduction of disulfide bonds with a mixture of DTT/DTE and desalting of peptides prior to FABMS analysis using RP-8 sep-pak cartridges were done according to reference 17 and 18.

FABMS Analysis

The dried salt-free digested samples were dissolved in 50% formic acid and /or 0.03 N HCL solution at a final concentration of 2mg/ml. An aliquot of this solution was mixed with a 3 to 1 mixture of DTT and DTE, respectively. One u l of this solution was immediately loaded on a stainless steel probe tip for FABMS analysis.

Mass Spectra

Positive ion fast atom bombardment mass spectra were recorded using a kratos MS-50 double focusing mass spectrometer (Kratos Scientific Instrument Manchester, UK). The samples and stainless steel probe tip were introduced into the FAB source and bombarded by a 7 kev xenon atom beam. Although the flux of the atomic beam at the sample was unknown, the beam

source (Ion tech) was operated in a normal mode at a power level of approximately 8 watts. Each spectrum was recorded on an oscillographic recorder and calibrated manually against a spectrum of cesium iodide. All spectra have been recorded using an 8 KV accelerating voltage.

Results and Discussion

a. Disulfide bond exchanges: Our analysis of several interchain disulfide-containing peptides indicate that disulfide bond rearrangements do not occur appreciably under the conditions used throughout our investigations. This conclusion is based on the absence of peaks in the FAB mass spectra of trypsinized somatostatin-14, urotensin II, and conotoxin GI which correspond to hypothetical disulfide containing peptides as shown in Table 1 . Our observation is in complete contrast to the findings of Buko and his co-workers who have observed isotopic envelopes corresponding to dimers or cluster ions of oxytocin, an intrachain disulfide containing peptide, during FABMS analysis [19]. However, the same group has noticed the lack of dimers in the FAB mass spectra of Tocinoic acid (a shorter analogue of oxytocin) under all identical experimental conditions. This discrepancy in behavior of disulfide-containing peptides during FABMS analysis may indicate the influence of intrinsic properties of individual peptides on dimer or cluster ion formation in addition to differences related to sample preparation.

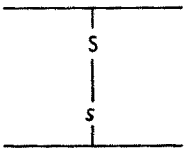
To investigate further the possibility of disulfide bond rearrangements in the crude sample of tryptic digest of CNBr- treated hen egg-white lysozyme and ribonuclease A, the masses of all peptides which could result from specific cleavages by CNBr and Trypsin were calculated for all different arrangements of eight cysteine residues in each protein, and looked for in the corresponding FAB mass spectra. Although not all of the peaks observed in the FAB mass spectra of the crude sample could be assigned to specific portions of the original intact protein, nevertheless none of the mass spectra for either hen egg-white lysozyme or ribonuclease A contained MH^+, MNa^+, or MH_2^{2+} ions related to the hypothetical peptides. As in the case of the small model peptides discussed above, these results show that disulfide rearrangements did not occur to a significant degree for the experimental conditions used in this study.

Table1- Normal and hypothetical disulfide-containing peptides of Trypsinized somatostatin-14, urotensin II, and conotoxin GI. (+) Signs indicate the observation of the peak in FAB mass Spectra and the (-) signs indicate the lack of observation of the related peak.

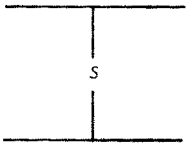
Compound	Residues	MH^+	Observation
Trypsinized somatostatin 14	1-4/10-14	933.4	+
	1-4/1-4	753.4	-
	10-14/10-14	1113.4	-
Trypsinized urotensin II	1-9/10-12	1361.6	+
	1-9/1-9	1993.8	-
	10-12/10-12	765.4	-
Trypsinized conotoxin GI	1-9/10-13	1456.5	+
	1-9/1-9	1901.6	-
	10-13/10-13	1011.4	-

b. Lanthionine and sulfoxide derivatives: In addition to disulfide bond rearrangements, we also searched the FAB mass spectra for the presence of peaks corresponding to lanthionine and its sulfoxide derivative in the tryptic digest of CNBr- treated hen egg-white

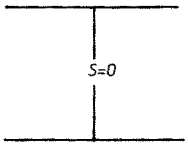
lysozyme and bovine ribonuclease A. Our approach was based on the assumption that proteolytic digestion would not be influenced by this transformation which might occur before or after enzymatic digestion. Based on this



A Disulfide-containing peptide molecular ion is MH^+



A lanthionine-containing peptide molelura ion with respect to A is $(MH-32)^+$



A sulfoxide-containing peptide molecular ion with respect to A is $[MH-16]^+$

Figure2: Shift in the molecular ion mass of a disulfide containing peptide from MH^+ (A), to $[MH-32]^+$, or $[MH-16]^+$ as a result of transformation to a lanthionine-containing peptide (B) or a lanthionine sulfoxide-containing peptide (C), respectively.

assumption, the masses of all lanthionines and their corresponding sulfoxide derivatives, which could result from the combination of two cysteine-containing peptides, were calculated. This was achieved simply by correcting the related masses of disulfide-containing peptides for the appropriate modifications as shown in Figure 2. For example, transformation of a disulfide-containing peptide to a sulfoxide-containing peptide or a lanthionine will shift the MH^+ ion to 16 and 32 mu lower than the corresponding MH^+ ions, repectively. Complete analysis of the FAB mass spectra of the tryptic digests before and after chemical reduction of disulfide bonds with DTT/DTE mixture did not support the formation of lanthionine containing peptides.

However, one of the common features of FAB mass spectra of both the tryptic digest of CNBr-treated hen egg-white lysozyme and ribonuclease A was the presence of a peak, of relatively low intensity, 16 mu lower than the molecular ions of some of the peptides. For example, the MH^+ ion at m/z 1974.8 corresponding to 62-66/67-79 tryptic peptide of ribonuclease A showed a peak at m/z 1963 which is 16 mu lower than the molecular ion at 1979.8. These peaks were not present in the FAB mass spectrum of the chemically reduced sample. The absence of the peak at m/z 1963 in the mass spectrum of the chemically reduced sample might imply that the peak at m/z 1963 is a gas phase fragment of the ion at m/z 1979.8. In this case, this peak, $(MH-16)^+$ ion, should not be present in the FAB mass spectrum of the chemically reduced sample. On the other hand, the alternative is that the peak at m/z 1963 could represent a sulfoxide-containing peptide. In this case the peak at m/z 1963 is expected to be present in the FAB mass spectrum of the chemically reduced sample. FABMS analysis of the chemically reduced sample did not contain any new peaks except the two peaks related to the constituent peptides of 62-66/67-79 tryptic fragment. In addition, both peaks at m/z 1963 and 1979 were absent from the FAB mass spectrum of the chemically reduced sample. This result might suggest the lack of formation of lanthionine sulfoxide which has a molecular weight 16 mu lower than the corresponding disulfoxide-containing peptide.

To explore further the structural identity of peaks 16 mu lower than their corresponding MH^+ ions, a set of small synthetic peptides, with and without -S-S- and -SH functional groups were subjected to FABMS analysis. The data revealed that peptides with serine residues are usually accompanied by a low-intensity peak 16 mu lower than the related MH^+ ions. This feature is shown in the positive FAB mass spectrum of a synthetic peptide with one serine residue (Figure 3). Since peptide A does not contain any cystine or cysteine residues, the presence of the peak 16 mu lower than the related MH^+ ion could not correspond to a lanthionine sulfoxide, but it might represent the formation of $[MH-H_2O+H_2]^+$ or $[MH-NH_2]^+$ ions. In addition, the peak at $(MH-16)^+$ might also represent the loss of a

CH_4 molecule from the peptide. To differentiate between these alternatives, the exact mass was determined for the m/z $(MH-16)^+$ peak of kemptide. Our data showed that the difference between the experimental and the calculated value of $(MH-16)^+$ ion (related to $MH^+-H_2O+H_2$) was less than the difference corresponding to a CH_4 or a NH_2 loss. The same observations were made using kemptamide. These results showed that the peak at $(MH-16)^+$ mu might correspond to a $(MH-H_2O+H_2)^+$ ion.

Lys-Arg-Arg-Ala-Ser-Lys-Gly

Kemptide

Lys-Arg-Arg-Ala-Ser-Lys-Gly-NH_2

Kemptamide

Besides serine-containing peptides, it was speculated that the presence of a cysteine in a peptide could also be accompanied by a characteristic peak 32 or 34 mu lower than the corresponding MH^+ ion, which might relate to $(MH-H_2S+H_2)^+$ or $(MH-H_2S)^+$ ions, respectively. Despite this speculation, these changes were not consistently observed using different peptide samples. For example, the FAB mass spectrum of pressenoic acid contained two peaks of low intensities 32 and 34 mu lower than MH^+ ion. On the other hand, the FAB mass spectra of oxytocin and urotensin II did not contain the corresponding peaks. The failure to observe these peaks in the FAB mass spectra of some of the intrachain disulfide-containing peptides might be taken to indicate that modifications which might occur during chemical or enzymic digestions, and /or during FABMS analysis are governed by the intrinsic properties of the peptides.

It is concluded from our data that dehydroalanine containing peptides may be formed depending upon their primary structure throughout the process of investigations. However, our experimental conditions did not favor the formation of cross-link products such as lanthionine, or lanthionine-sulfoxide, and lysinoalanyl containing compounds to a significant level in spite of the high sensitivity of FAB mass spectrometry technique.

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