Investigation of Transglutaminase-Induced Peptide Cross-Linking by Matrix-Assisted Laser Desorption / Ionization Time-of-Flight Mass Spectrometry

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used to demonstrate cross-linking of peptides induced by transglutaminase. The presence of ε-(γ-glutamyl)lysine isopeptide cross-link in the acid hydrolysate of the cross-linking reaction mixture was also demonstrated by MALDI-TOF-MS without prior separation. MALDI-TOF-MS quickly provided peptide mass maps after pronase digestion of the cross-linked peptide adduct, which enabled us to monitor the hydrolytic sequence. Pronase appears to preferentially hydrolyze peptide bonds distant from the cross-link before hydrolyzing peptide bonds around the cross-link. The results suggest that pronase digestion followed by MALDI-TOF-MS could be used for determination of amino acid sequence around a modification site.

Introduction

Once sequencing the entire human genome is completed in a few years, the structure-function relationship of the numerous gene products, namely proteins, will be investigated with renewed vigor and prospect of many benefits. Investigation of protein structure and function would greatly benefit from recent advances in bioanalytical mass spectrometry (MS) as reviewed by Biemann[1] and McLafferty.[2] Of particular interest to protein chemists are ionization techniques for peptides and proteins such as fast atom bombardment[3] (FAB), matrix-assisted laser desorption/ionization time-of-flight[4] (MALDI-TOF) and electrospray ionization[5] (ESI) mass spectrometry.

Amino acid sequencing and determination of posttranslational modifications of proteins are two examples of mass spectrometric investigations of interest in protein research. Partial sequencing of unknown proteins could provide a clue to the complete sequence if the gene corresponding to the partial amino acid sequence could be identified unequivocally by matching the partial sequence with the nucleotide sequence informations.

MALDI-TOF-MS is a powerful technique for determining molecular weight of proteins and peptides with high accuracy and sensitivity. In MALDI-TOF-MS proteins are desorbed from a crystal formed with excess matrix molecules and ionized by a laser pulse. The ions formed are separated in the flight tube and their m/z values are determined from the time of flight to the detector. It enables one to investigate proteins and peptides in a complex mixture with a picomole to attomole detection limit.

We have been using MALDI-MS for investigation of protein modifications such as glycosylation[6] and hydroxyl radical-induced cross-linking.[7] Recently, we became interested in how the protein modifications change the mode of action of proteolytic enzymes. In this paper we show how MALDI-MS can be used to demonstrate peptide cross-linking by transglutaminase and formation of the ε-(γ-glutamyl)lysine cross-link. We also show that the nonspecific hydrolysis of the peptide bonds by pronase is modified by the presence of the ε-(γ-glutamyl)lysine cross-link in such a way that the C-terminal amino acids are removed sequentially if the cross-link is between lysine and glutamine residues near the N-terminal of both peptides.

Experimental Section

Materials. Pro-Gln-Arg-Phe-NH_2 (PQRF; called peptide A, MW = 545.65 Da), Lys-Phe-Ile-Gly-Leu-Met-NH_2 (KFIGLM; called peptide B, MW = 706.96 Da), transglutaminase from guinea pig liver, pronase (protease type XIV from *Streptomyces griseus*), and α-cyano-4-hydroxy-cinnamic acid were purchased from Sigma (St. Louis, MO, USA). Other chemicals were reagent grade.

Cross-Linking Reaction. A 0.4 mL reaction mixture containing 1.0 mM each of peptides A and B, 2 μM transglutaminase, 5 mM $CaCl_2$ in 95 mM Tris · HCl buffer (pH 8) was incubated at 37 °C. The reaction was stopped after predetermined time intervals by withdrawing a 20 μL aliquot and boiling for 5 min. The mixture was diluted 5- and 50-fold with deionized water for HPLC and MALDI-MS, respectively.

Acid Hydrolysis. An aliquot of the cross-linking reaction mixture containing approximately 0.2 micromoles of both peptides A and B was freeze-dried and dissolved in 1 mL of 6 N HCl. Hydrolysis was carried out at 110 °C in a sealed glass ampule. After 24 h, HCl was removed by vacuum evaporation. The hydrolysate was dissolved in 20 μL of deionized water and analyzed by MALDI-MS.

Pronase Digestion. The peptide mixture incubated with transglutaminase for 24 h was freeze-dried and hydrolyzed by pronase at room temperature. Pronase equivalent to 0.1% w/w of total peptide was added to the reaction mixture in a 50 mM ammonium bicarbonate buffer (pH 7.8) every 20 min in order to sustain the hydrolytic activity. An aliquot of the digestion mixture was removed after 1, 2, and 4 h and analyzed by MALDI-MS.

HPLC. A Hewlett Packard 1100 Series HPLC system with hypersil BDS-C18 reversed phase column (3 μm pore size, 100 × 4 mm) and diode array detector was used. Injection volume was 20 μL. The binary gradient consisted of deionized water containing 0.1% trifluoroacetic acid and acetonitrile. The percentage of acetonitrile was constant at 7% between 0 and 8 min. Between 8 and 20 min the percentage of acetonitrile was increased linearly to 45%. Since both peptides contain phenylalanine, the chromatogram was obtained at 260 nm.

MALDI-TOF-MS. One microliter of the sample was mixed on the sample plate with another microliter of the α-cyano-4-hydroxy-cinnamic acid matrix solution (10 mg of the matrix in 1 mL of a 1 : 1 mixture of acetonitrile and water containing 0.1% trifluoroacetic acid) and let dry. Mass spectra were recorded using a Voyager Biospectrometry workstation with a linear mass analyzer (PerSeptive Biosystems, Framingham, MA, USA). The instrument was equipped with a 337 nm nitrogen laser and a 1.2 m flight tube. Positive ion spectra were obtained at a 25 kV accelerating voltage. External calibration was done with a nonapeptide (MW = 848.80 Da) or with the matrix (MW = 189.04 Da).

Results and Discussion

Peptide Cross-Linking. The top mass spectrum in Figure 1, obtained from the mixture of peptides A and B immediately after adding transglutaminase, shows peptide A at m/z of 546.5 (expected at 546.7 for $M + H^+$) and peptide B at 707.6 (expected at 708.0 for $M + H^+$). The peak at 568.5 is due to [peptide A + Na^+]. The peaks at 730.1 and 746.8 are due to [peptide B + Na^+] and [peptide B + K^+], respectively. After incubation with transglutaminase the cross-linked adduct was observed at 1236.8 (545.7 + 707.0 – NH_3 + H^+ = 1236.7 expected) in addition to peptides A and B (bottom mass spectrum in Figure 1). The peak at 379.1 is due to the matrix dimer and was used as internal reference.

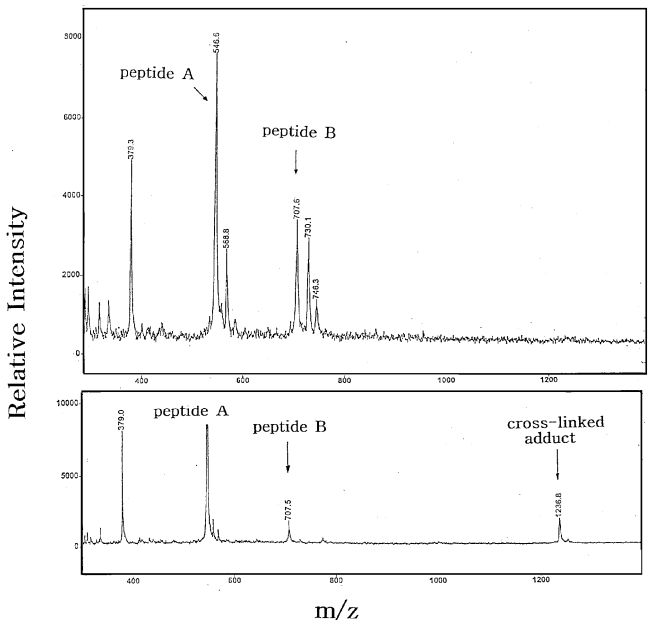

Figure 1. MALDI-TOF mass spectrum showing peptides A and B before (top) and after (bottom) cross-linking induced by transglutaminase.

Cross-linking of the peptides was also monitored by HPLC. Peptides A and B were eluted at 6.9 and 16.3 min, respectively (Figure 2, top). As the cross-linking reaction progressed, another peak at 17.2 min increased in intensity and approached saturation after 24 h. The chromatogram also showed several other peaks at 3.4, 4.3, 8.8, 14.5, and 16.7 min, which were not present before incubation and increased in intensity with increased incubation time. Analysis of the large peak at 16.7 min by MALDI-MS showed m/z value of 934.8. These peaks might be due to side reactions and need further investigation.

The eluate corresponding to the peak at 17.2 min was collected and analyzed by MALDI-MS without concentration. A predominant peak was observed at 1237.1 with a smaller peak at 1253.1 corresponding to an oxidized compound (Figure 2, bottom). Therefore, we concluded that the 17.2 min peak corresponds to the cross-linked peptide.

Observation of ε-(γ-Glutamyl)lysine. It is well known that transglutaminase forms ε-(γ-glutamyl)lysine cross-link (also known as isopeptide bond) *via* acyl transfer reaction from the glutamine side chain to primary amines. ε-amino group in the lysine side chain is the acyl acceptor in proteins.

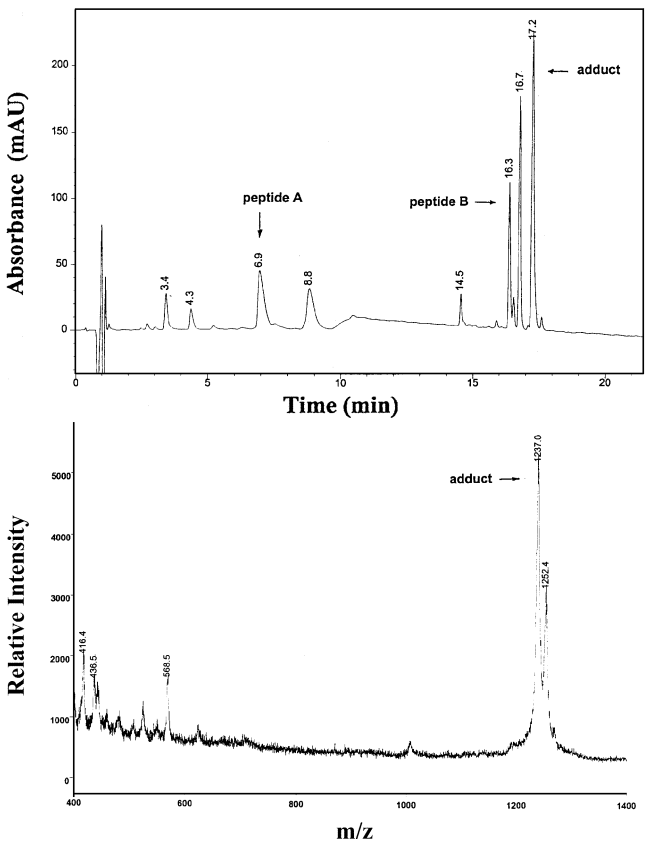

Figure 2. Reversed phase HPLC chromatogram of the reaction mixture after 8 h cross-linking by transglutaminase (top) and the MALDI-TOF mass spectrum obtained from the 17.2 min eluate (bottom).

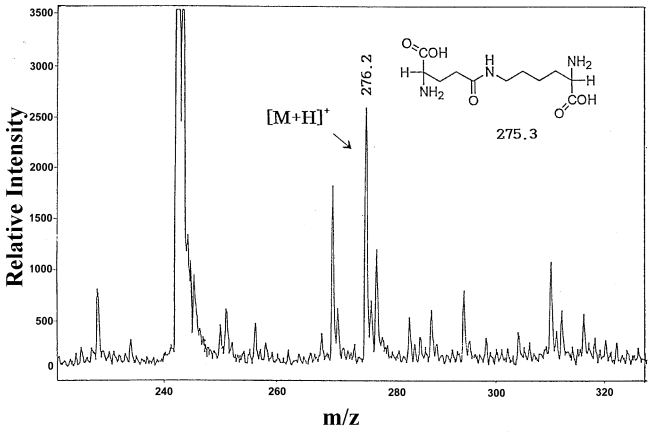

Figure 3. MALDI-TOF mass spectrum showing the ε-(γ-glutamyl) lysine dipeptide obtained by acid hydrolysis of the cross-linking reaction mixture.

The ε-(γ-glutamyl)lysine dipeptide was first discovered in 1968 by Pisano *et al.*[8] from wool hydrolysate and is believed to play a key role in maintaining a rigid structure in many biological systems.[9]

Observation of ε-(γ-glutamyl)lysine dipeptide by mass spectrometry has not been reported. Since the ε-(γ-glutamyl)lysine cross-link is not acid hydrolyzed unlike the peptide bond, we attempted to observe the dipeptide directly by MALDI-MS after acid hydrolysis without separating the peptide adduct from unreacted peptides.

The mass spectrum in Figure 3, obtained from the acid hydrolysate of the incubation mixture, shows a peak at m/z of 276.2 in good agreement with 276.3 expected for the ε-(γ-glutamyl)lysine adduct. Amino acid peaks are buried under the matrix peaks. In the past, the ε-(γ-glutamyl)lysine adduct was separated by HPLC and the retention time was compared against that of synthetic ε-(γ-glutamyl)lysine. In the present approach, the 20 min chromatographic separation is replaced by separation of the ions in the flight tube, which takes place typically within milliseconds. Clearly, MALDI-MS is a powerful technique for observing, without chromatographic separation, modified proteins or modification of a particular residue. The large peaks around 242 are due to the matrix. Other peaks between 260 and 320 were not identified. They are probably derived from the side reaction products as suggested in the HPLC chromatogram in Figure 2.

Sequential Hydrolysis by Pronase. In the past, FAB-MS was often used for investigating the site and structure of the cross-link. In 1984 Kim *et al.*[10] observed FAB-MS signals corresponding to three different phenylalanine cross-links induced by hydroxyl radicals generated by gamma irradiation. In 1988 Porta *et al.*[11] and Pucci *et al.*[12] investigated the specificity of transglutaminase reaction by FAB-MS. They showed that not all glutamine or lysine residues participate in the acyl transfer reaction. FAB-MS was quite useful for generating the peptide mass map and pointing out which peptide segments containing the glutamine or lysine residues are involved in cross-linking.

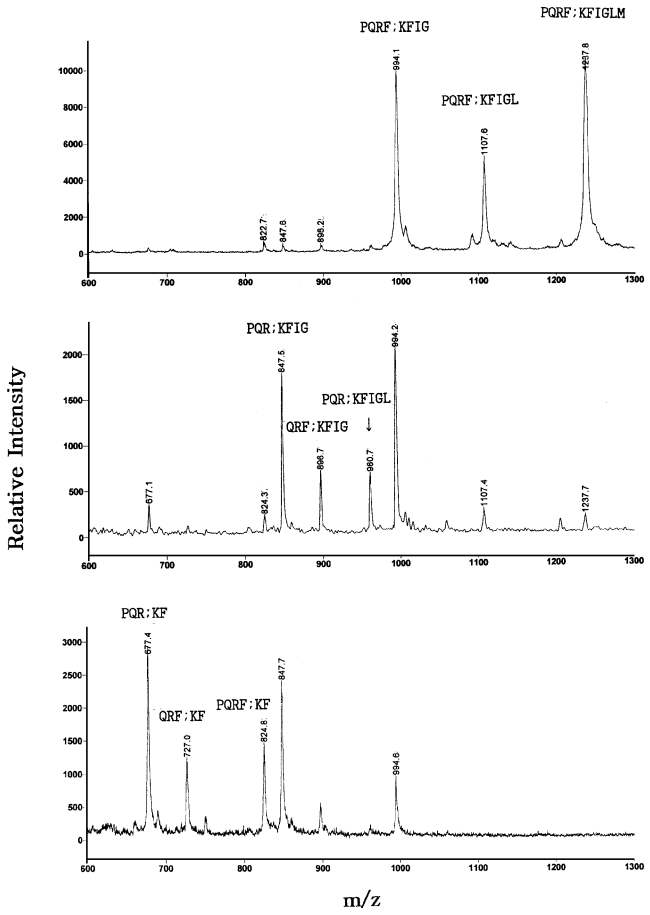

Figure 4. MALDI-TOF mass spectrum obtained from the cross-linking reaction mixture after 1, 2, and 4 h digestion by pronase.

We were interested in how MALDI-MS could be used to probe the hydrolytic behavior of pronase around the cross-link. In order to introduce the cross-link near the N-terminal and investigate how the cross-link affects hydrolysis of peptide bonds toward the C-terminal, we selected peptides with glutamine and lysine residues near the N-terminal. Glutamine is the second residue from the N-terminal of the tetrapeptide A, and lysine is the N-terminal amino acid of the hexapeptide B. Thus, the cross-link induced by transglutaminase is expected to render peptide bonds near the cross-link less accessible to proteolytic enzymes than those near the C-terminal.

Pronase is known to have very broad specificity.[13] It was of interest to find out whether this broad specificity would be modified by the presence of the ε-(γ-glutamyl)lysine cross-link. All MALDI-TOF mass spectra in Figure 4 were obtained from the cross-linking reaction mixture after 24 h incubation with transglutaminase. The reaction mixture was then digested for different time intervals with a periodic addition of pronase.

The top mass spectrum in Figure 4 corresponds to 1 h digestion (3 additions of 0.1% w/w pronase at room temperature) and shows the cross-linked adduct at 1237.8 (0.1% error from 1236.7 expected). The adduct is denoted as PQRF; KFIGLM where the semicolon is used to denote ε-(γ-

glutamyl)lysine cross-link between Q in peptide A and K in peptide B. Two major peaks were observed in addition to the unhydrolyzed adduct. The peak at 1107.6 corresponds to a decrease of 130.3 from the parent adduct, which is consistent with the expected mass change (130.2) due to the removal of methionine-NH_2. Clearly, the 1107.6 peak corresponds to PQRF; KFIGL in our notation. The difference between 1107.6 and 994.1 was recognized as due to leucine or isoleucine and we concluded that the 994.1 peak represented PQRF;KFIG.

Three small peaks were also observed at 896.2, 847.6, and 822.7. The 896.2 peak represents QRF; KFIG resulting from removal of N-terminal proline from the peptide A portion of PQRF;KFIG. The 847.6 peak (PQR;KFIG) is due to removal of phenylalanine from PQRF;KFIG. Removal of IG from PQRF;KFIG leads to the 822.7 peak (PQRF;KF). It appears that pronase does not hydrolyze the peptide bond between isoleucine and glycine and removes them as a group.

The middle mass spectrum in Figure 4 shows that, after 2 h pronase digestion, the peaks at 1237.8 (PQRF;KFIGLM) and 1107.6 (PQRF;KFIGL) are significantly decreased, whereas the peak at 994.1 (PQRF;KFIG) is increased. This observation suggests that the C-terminal methionine is removed first and leucine is subsequently removed. Pronase may hydrolyze the glycine-leucine bond before hydrolyzing the leucine-methionine bond. However, the results indicate that pronase is more likely to hydrolyze the leucine-methionine bond before hydrolyzing the glycine-leucine bond probably due to steric hindrance around the cross-link.

The 847.5 peak (PQR;KFIG), which was also observed after 1 h digestion, greatly increased after 2h at the expense of PQRF;KFIGLM and PQRF;KFIGL, whereas PQRF;KFIG appeared to maintain a steady state concentration. The 960.7 peak corresponds to PQR;KFIGL and could be an alternative precursor of PQR;KFIG. QRF;KFIG also increased after 2 h. PQRF;KF remained small and another peak corresponding to PQR;KF appeared at 677.1.

The bottom mass spectrum, corresponding to 4 h digestion, shows that the intensities of the PQRF;KF peak and the PQR;KF peak increased at the expense of the PQRF;KFIG peak. The mass spectrum also shows a strong peak at 727.0 (QRF;KF) corresponding to removal of glycine and isoleucine from the QRF;KFIG peak. Removal of glycine and isoleucine as a group is consistent with earlier result. Results of further hydrolysis was difficult to discern because of the matrix peaks.

It is clear from above results that pronase preferentially hydrolyzes peptide bonds distant from the ε-(γ-glutamyl)lysine cross-link probably due to steric hindrance of the cross-link. Once the two most distant amino acids were removed, pronase hydrolyzed peptide bonds around the cross-link. Demonstration of this effect by traditional separation and sequencing methods would have been laborious and subject to uncertainties. The power of the MALDI-TOF-MS technique is evident in this example.

We are currently extending this work to other types of protein modifications. In the present example direct sequencing around the cross-link would have been difficult due to the fact that amino acids are released from both sides of the cross-link. The interpretation would be more straightforward if the modification does not involve cross-linking between peptides, because successive removal of amino acids would take place from a single peptide chain.

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