# Investigation of Hydroxyl Radical-Induced Cross-Linking of Peptides and Its Inhibition by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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Reactive oxygen species collectively refer to superoxide radical ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), and hydrogen peroxide ($H_2O_2$) and are implicated in many degenerative diseases and aging as well as in carcinogenesis.[1-4] Superoxide radical is generated by electron transfer from metal ions in the low oxidation state, such as $Fe^{2+}$ and $Cu^+$, to the ground state triplet oxygen molecule. Superoxide radical reacts with hydrogen ion and produces hydrogen peroxide. Hydrogen peroxide is easily split acquiring an electron from a reduced metal ion, such as $Fe^{2+}$, and yields hydroxyl radical through the following reaction;

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH$$

Thus OH is quite abundant under physiological conditions and reacts readily with practically every type of biomolecules in living cells, such as sugars, amino acids, phospholipids, and DNA bases. Therefore, scavenging of $\cdot OH$ is considered an important reaction underlying many health benefits of food antioxidants.[5]

One of the potentially damaging hydroxyl radical-induced reactions is the protein cross-linking.[6] Therefore, the beneficial effects of food antioxidants might include inhibition of protein cross-linking. It is desirable in the search for effective radical scavengers in foods and other natural products to be able to measure the extent of cross-linking and its inhibition by radical scavengers.

Conventional techniques for observing protein cross-linking include gel electrophoresis and size exclusion chromatography. However, both methods have limited resolution and sensitivity. Direct observation of changes in molecular weight by mass spectrometric means would be more desirable. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has a great potential for biochemical and polymer applications.[7,8] However, it has not yet been used in the investigation of oxygen radical-induced protein cross-linking.

We used two tripeptides, Lys-Trp-Lys (MW=460.6 Da) and Lys-Tyr-Lys (MW=437.5 Da) to demonstrate hydroxyl radical-induced cross-linking and its inhibition using MALDI-TOF-MS. A large protein with multiple reactive sites toward $\cdot OH$ could undergo intramolecular cross-linking, in which case the changes in the molecular weight would be too small and indistinguishable from changes due to other protein modifications. Hydroxyl radical was generated from the $H_2O_2$-$Cu^{2+}$ mixture through the following reactions;[9]

$$Cu^{2+} + H_2O_2 \rightarrow Cu^+ + \cdot O_2^- + 2H^+$$
$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + \cdot OH + OH$$

Garlic extract was used as a natural source of food antioxidants.

## Experimental Section

**Cross-Linking Reaction.** The peptide-hydroxyl radical system was prepared by mixing 0.05 mL of 10 mM tripeptide (Sigma, St. Louis, MO, USA) solution in deionized water, 0.01 mL of 0.1 mM $CuSO_4$ solution, 0.01 mL of 3% $H_2O_2$, and 0.03 mL of deionized water. In the antioxidative system, 0.03 mL of garlic juice, prepared by homogenizing 0.5 g chopped garlic with 5 mL water and centrifuging the homogenate, was used instead of 0.03 mL deionized water. 0.1 mL aliquots were left at room temperature overnight in a sealed plastic vial. The aliquots were also dried in a 90 °C oven and heated further for a total of 90 min. The dried samples were dissolved in 0.1 mL water before mass spectrometric analysis.

**Acid Hydrolysis.** The dimer-size adducts derived from Lys-Tyr-Lys was separated using Bio-Gel P-2 size exclusion column and hydrolysed with 6 N HCl. The hydrolysate was neutralized with NaOH for MALDI-MS analysis.

**MALDI-TOF-MS.** The samples in the 0.1 mL volume described above were diluted 10-fold with deionized water. One microliter of the diluted sample was mixed on the sample plate with another microliter of the α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO, USA) 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 30 kV accelerating voltage. Approximately 100 scans were averaged. External calibration was done with angiotensin I (1296.5 Da).

## Results and Discussion

**Peptide Cross-Linking and Inhibition.** The top mass spectrum in Figure 1, obtained from the (Lys-Trp-Lys)-$H_2O_2$-$Cu^{2+}$ system left overnight at room temperature, shows a major peak at m/z of 461.4 corresponding to $(M+H)^+$. A small peak at 482.3, also observed from control peptide, rep-

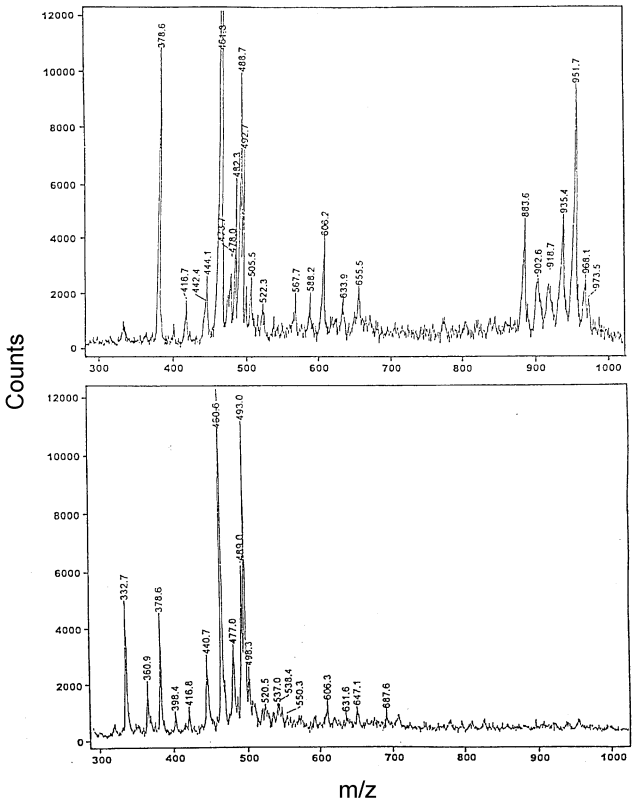


**Figure 1.** Time-of-flight mass spectrum showing hydroxyl radical-induced dimerization of Lys-Trp-Lys (top) and its inhibition by garlic extract (bottom). The reactions were carried out overnight at room temperature in solution.

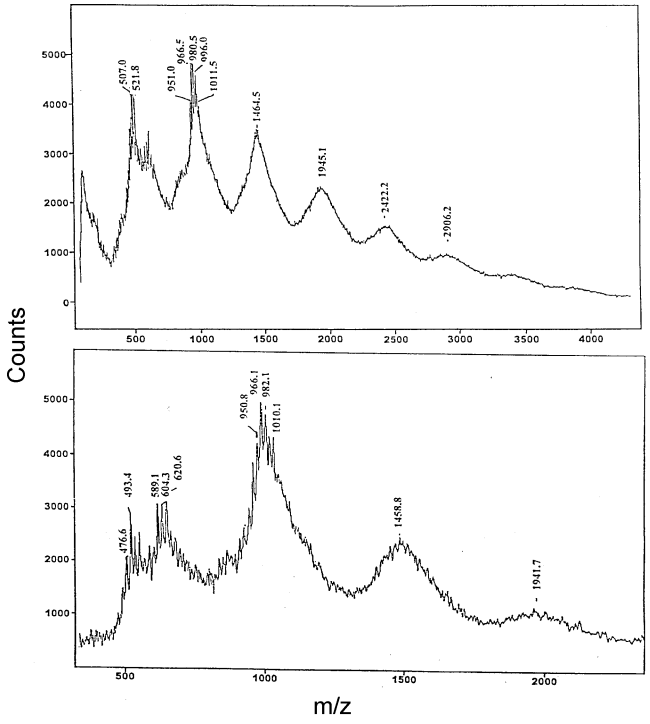


**Figure 2.** Time-of-flight mass spectrum showing hydroxyl radical-induced cross-linking of Lys-Trp-Lys up to heptamer (top) and its inhibition by garlic extract (bottom). The reactions were carried out at 90 °C for 90 min under a dehydrating condition.

resents a sodium adduct ion. The deviation of 1.3 from 483.6, the expected value for $(M+Na)^+$, represents the limit of mass accuracy (approximately 0.3%) of the instrument used. M/z of 483.3 was obtained in another run. Calibration of the instrument using a compound with molecular weight around 500 might improve the mass accuracy.

The peaks at 478.0 and 492.7 were not observed from the control peptide, but observed from the peptide exposed to air[10] and correspond to mono- and dihydroxylated peptide. Such hydroxylation reactions are known to take place on the aliphatic side chain in peptides through an initial hydrogen abstraction and a subsequent addition of the hydroxyl radical.[11] The observed m/z of 478.0 is in good agreement with the expectd value of 477.6. The peak at 492.7 corresponds to a second oxidation. We could not account for the peak at 488.7.

The presence of several peaks around 950 in the top mass spectrum in Figure 1 demonstrates formation of a peptide dimer. Both lysine and tryptophan residues are known to be reactive toward hydroxyl radical.[12,13] Cross-linking of two identical peptide radicals resulting from abstraction of the C-H hydrogen on the lysine side-chain would yield an adduct with a molecular weight of 919.2. The small peak at 918.7 is believed to correspond to the protonated adduct (920.2 expected). Peaks at 935.4 and 951.7 appear to be due to mono- and dihydroxylated adducts expected at 936.2 and 952.2, respectively. The fact that the dihydroxylated adduct peak is stronger than the monohydroxylated adduct is consistent with the dihydroxylated monomer at 492.7 being stronger than the monohydroxylated monomer at 478.0.

The bottom mass spectrum in Figure 1, obtained from the same peptide treated with ·OH in the presence of garlic juice, illustrates complete absence of the dimer-size peaks. It is clear that certain compounds in garlic inhibited dimerization of the peptide, presumably through scavenging of ·OH. Garlic contains many organosulfur compounds such as S-allyl-cysteine, alliin, and allicin, some of which might be radical scavengers.[14] The radical scavenging effects of these garlic compounds were reported recently.[5,15,16] It would be interesting to determine, using the peptide-·OH system as an assay tool, which compounds in garlic are responsible for the observed inhibition of cross-linking.

The top mass spectrum in Figure 2 shows that, under a dehydrating condition at a higher temperature, cross-linking of the peptide beyond dimerization takes place. In addition to the monomer-size peaks around 507.0 and 521.8, several groups of peaks separated by approximately 480 were observed. Monomer, dimer (around 980), and trimer (around 1465) were the major species. The weak signals around 3400 correspond to a heptamer. It is likely that formation of higher adducts from the hydroxylated peptide involves dehydration and, therefore, is facilitated by drying the sample.

The major peaks in the top mass spectrum in Figure 2 corresponding to the monomer have molecular weight (507.0 and 521.8) greater than those observed in Figure 1 (461, 489, 493). It appears that, since the peptide is exposed to air when dry, extensive oxidation as well as cross-linking takes place.

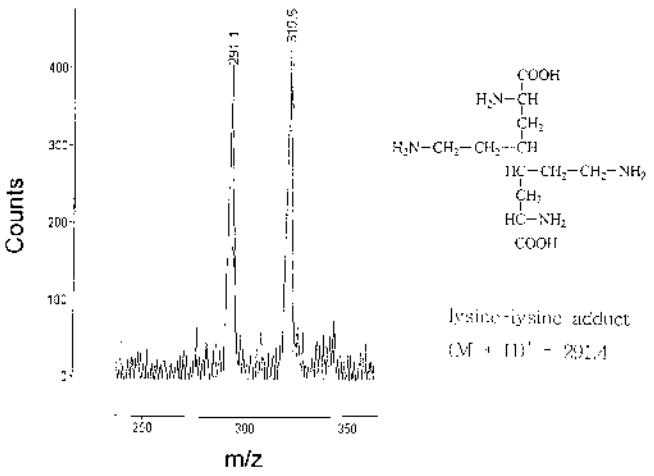


**Figure 3.** Time-of-flight mass spectrum showing lysine-lysine adducts resulting from acid hydrolysis of Lys-Tyr-Lys adducts. The structure shown represents one of many possibilities due to several hydrogen abstraction sites on the lysine molecule.

Hence, the broad peaks represent a collection of derivatives of the dimer, trimer, etc. The formation of trimer and higher adducts under the dehydrating condition was suppressed in the presence of the garlic extract, which is consistent with the results in Figure 1. Again, many oxidized species, separated by 16, were observed. Similar oxidation peaks were also observed in glucose-peptide adducts.[10]

**Cross-Linking Site.** In order to investigate the reactivity of different amino acids toward hydroxyl radical-induced cross-linking, Lys-Tyr-Lys (437.5) was subjected to a similar cross-linking condition as used for Lys-Trp-Lys. Dimer-size peaks were observed at 872.0 for $(M+H)^+$, 887.6 for $(M-O+H)^+$, and 894.0 for $(M-Na)^+$. As in Lys-Trp-Lys, direct dimerization after hydrogen abstraction will result in 874.0 (0.2% error). It appears that the same type of cross-linking takes place whether the second amino acid is tryptophan or tyrosine. Tyrosine might be as reactive as tryptophan toward cross-linking either with another aromatic amino acid or with lysine on the second peptide. Alternatively, above observation would be consistent with lysine, not tryptophan or tyrosine, being the primary cross-linking site. In this case, a lysine-lysine adduct would be a major cross-link.

In an attempt to mass spectrometrically determine the cross-linking site, the acid hydrolysate obtained from Lys-Tyr-Lys adducts was analyzed by MALDI-MS. The peaks at 291.1 and 319.6, shown in Figure 3, represent two major species in the hydrolysate. The m/z value of 291.1 corresponds to the lysine-lysin dimer derived from two identical lysine radicals resulting from abstraction of the side-chain C-H hydrogen (291.4 expected from two of 145.2 Da for hydrogen-abstracted lysine plus a proton). The m/z value of the peak at 319.6 is close to the sum of the lysine (146.2) and tyrosine (181.2) molecular weight, but it does not match any plausible lysine-tyrosine adduct. The identity of this species is under investigation. Observation of the amino acid adducts in the acid hydrolysate indicates that the cross-link is stable toward acid hydrolysis.

**Sensitivity.** Observation of the inhibitory effect or the amino acid adducts by chromatography would require a larger amount of sample and lack the resolution of mass spectrometry. In the MALDI-MS technique a picomole amount of the analyte in one microliter volume can be detected from a complex mixture without prior separation, because the separation takes place in the flight tube. Each experiment involving laser desorption, flight, and detection takes place in submillisecond. Therefore, data can be accumulated and averaged on the computer in a few minutes yielding an enhanced signal from a large number of laser shots to different regions of the sample/matrix on the sample plate.

It is anticipated that the speed and sensitivity of MALDI-MS could facilitate the search for health-beneficial compounds for food or biomedical applications as demonstrated in this Note in the case of antioxidants. MALDI-MS might be used to show radical-induced aggregation of low density lipoprotein involved in the development of artherosclerosis.[17] It would be interesting to show, using MALDI-MS, the effects of α-tocopherol and other antioxidants in the retardation of artherosclerosis[18] or cataract.[19]

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