# Mass Spectrometric Elucidation of Phenolic Oxidation Processes with $Cu^{2+}$-Adduct

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Formation of a complex with 2-hydroxy-6-[8′(*Z*),11′(*Z*),14′-pentadecatrienyl]benzoic acid (anacardic acid) and copper ion in the respective ratios of 2 : 1 and 1 : 1 at room temperature was observed by electrospray ionization mass spectrometry. The oxidation of 3-(3,4-dihydroxyphenyl)alanine (DOPA) in the presence of anacardic acid showed competitive inhibition with temporary reduction from dopaquinone to DOPA. In the oxidation of neurotensin, the oxidized products, dopaquinone-derivatives, were observed. Mass spectrometry revealed that the enzymatic oxidation of tyrosine residue is inhibited by anacardic acid.

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## 1. Introduction

Polyphenols play an important role in preventing oxidation of not only foods but also biomolecules in human body.[1)] They have been reported to act as antioxidants, free-radical scavengers, and metal chelators. Antioxidative processes occur in radical and redox reactions. In general, the antioxidative system comprises the reactive oxygen species (ROS), substrates such as proteins, metal-related biomolecules as catalysts, and antioxidants as inhibitors. In particular, the two variants in the last group are notable for elucidating the mechanism of the antioxidation.

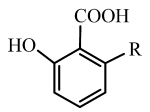
In common use, antioxidant activities against the reactive oxygen species (ROS) have been measured by various kinds of quantitation, for instance, using the absorption spectra of 2,2-di(4-*t*-octylphenyl)-1-picrylhydrazyl (DPPH), thiobaribituric acid (TBA), and the high-performance liquid chromatography of oxidized lipids.[2)] However, molecular information of the structures could hardly be obtained by these methods.

In contrast, several mass spectrometric techniques have been developed to elucidate the chemical structures of biologically occurring products. These products are mostly soluble in water, although organic compounds like terpenoids are hydrophobic. As an ionization method for water-soluble compounds, electrospray ionization (ESI) has been preferentially used. It is distinct that it is applicable to monitoring the reaction in an aqueous solution. In particular, ESI is suitable for studying the interaction of antioxidants and catalysts in a solution.[3)]

The antioxidant activity of polyphenols related to the functional structures involved in the enzymatic reaction of the biologically occurring compounds with the ROS should be rechecked. Inhibition of oxidation by the antioxidants intrinsically implies interaction between the antioxidants and metal proteins.[4)] This interaction is classified into two parts; ionic and hydrophobic interactions.

Recently the cashew, *Anacardium occidentale* L. (Anacardiaceae) apple has increased in health-care value in the equatorial area where it is grown.[5)] Chemically, the plant contains native compounds, anacardic acids, 2-hydroxy-6-[8′(*Z*),11′(*Z*),14′-pentadecatrienyl] benzoic acid (**1**), and 2-hydroxy-6-[8′(*Z*)-pentadecenyl] benzoic acid (**3**), in major composition (Fig. 1).[6)] All of the anacardic acids **1**–**4** have not only exhibited intense antitumor and antioxidation activities, but also active properties in suppressing the generation of superoxide radicals by inhibiting xanthine oxidase without radical scavenging activity. That is, the inhibition kinetics of anacardic acids do not always indicate single exponential dependence of enzyme inhibition on inhibitor concentrations (the Michaelis-Menten equation), but instead follow the Hill equation.[7)] As a result, the initial interaction of anacardic acid and the metal in the enzyme precedes the hydrophobic interaction, *i.e.*, chelation is predominant by divalent metal ions such as $Cu^{2+}$ or $Fe^{2+}$.

However, complexation in a solution is hardly detected at molecular level without using ESI-mass spectrometry (MS). In particular, changes in chemical species depending on time and intermediates are capable of




1 R1 = 8'(*Z*),11'(*Z*),14-pentadecatrienyl
2 R2 = 8'(*Z*),11'(*Z*)-pentadecadienyl
3 R3 = 8'(*Z*)-pentadecacenyl
4 R4 = pentadecyl

Fig. 1. Structures of the anacardic acids.

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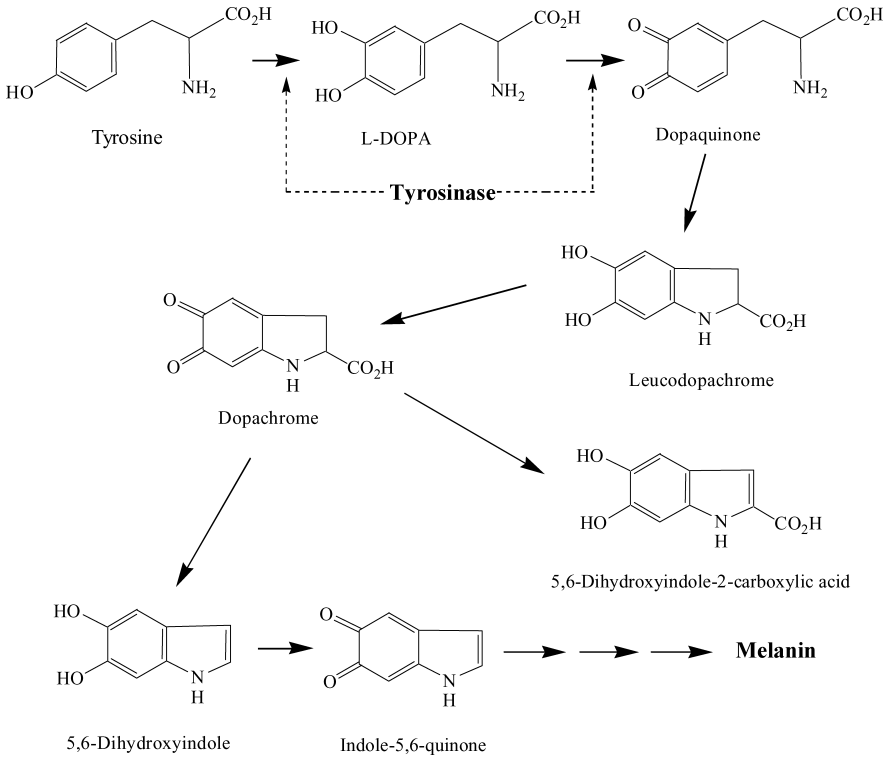


Scheme 1. Raper–Manson scheme of melanogenesis.

elucidating details of the molecular structures. Therefore, mass spectrometry of the complexation of anacardic acids and cupric ion indicates a possible alternative model for the inhibitory activity of tyrosinase.

On the other hand, the inhibitory effects of anacardic acids on melanin synthesis have been investigated by Kubo *et al.*[8] The inhibition has two aspects of competition; competitive and noncompetitive reactions. Initially, tyrosine is subject to oxidation in the presence of tyrosinase. Successive reactions give DOPA, dopachrome, eumelanin, and the final product, melanin, as shown in Scheme 1. The control of melanin biosynthesis is capable of preventing undesired pigmentation. In this report, we focus on the initial reaction of DOPA in the presence of $Cu^{2+}$ ion and tyrosinase. The purposes of this study are detection of the metal complex with the antioxidant, and observation of time-dependent change in the concentration of DOPA.

## 2. Experimental

### 2.1 Materials

The solvents and reagents were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Mushroom tyrosinase, angiotensin, and neurotensin were purchased from Sigma Co., Ltd. (St. Louis, MO, USA). Ultrapure water was obtained from the distillation of deionized water.

*Anacardium occidentale* L. was offered by Kashu Co., Ltd. The hexane extraction of anacardic acids from cashew nut shells was performed as follows. The extract was prepared by immersing the roughly crushed shells in hexane for a day at room temperature in 82 wt% of whole shells.[9] Simple column filtration on silica gel gave the mixture of anacardic acids **1** and **3** (hereafter, anacardic acid) whose composition was quantified by ESI-MS and $^1$H nuclear magnetic resonance (NMR) spectroscopy to be **1** : **3** = 2 : 1. Negative ion ESI-MS showed $m/z$ 341.3 (rel. int. 100) and 345.3 (42). $^1$H-NMR ($CDCl_3$) showed $\delta$: 5.05, 5.2–5.5, and 5.80 in the olefinic region.

### 2.2 ESI mass spectrometry

ESI-MS was performed with a ThermoElectron LCQ-Deca XP (ThermoElectron Corp., Waltham, MA, USA) mass spectrometer. The mass spectrometer was equipped with an ESI needle and the ion spray voltage was set at 3,000 V with nitrogen as the sheath gas. An MS scan ($m/z$ 50–2,000) was performed in positive and negative ion mode to detect intact molecular ions. The samples were ionized by ESI and continuously infused into the ESI chamber at a flow rate of 3 $\mu$L/min of a described solvent. The identification of complex was further confirmed by collision-induced dissociation using the MS/MS scan.

For ESI measurement of the complex-ion, a non-aqueous solution was prepared: 2-propanol : hexane : ethyl acetate = 2 : 1 : 1 with 0.05% acetic acid. A hexane solution of anacardic acid and an ethyl acetate solution of cupric acetate in the ratio of 1 : 2, respectively, were added to the solution.

### 2.3 Matrix-assisted laser desorption/ionization (MALDI) TOF MS of peptide

One microliter of the sample solution was mixed on the sample plate with one microliter of the 2,5-dihydroxybenzoic acid and matrix solution (10 mg of the matrix in 1 mL of a 1 : 1 mixture of acetonitrile and ultrapure water containing 0.1% trifluoroacetic acid) and left to dry. The mass spectra were recorded by an ABI Voyager DE PRO MALDI TOF mass spectrometer with a linear and reflectron mass analyzer. Positive ion spectra were obtained at an accelerating voltage of 30

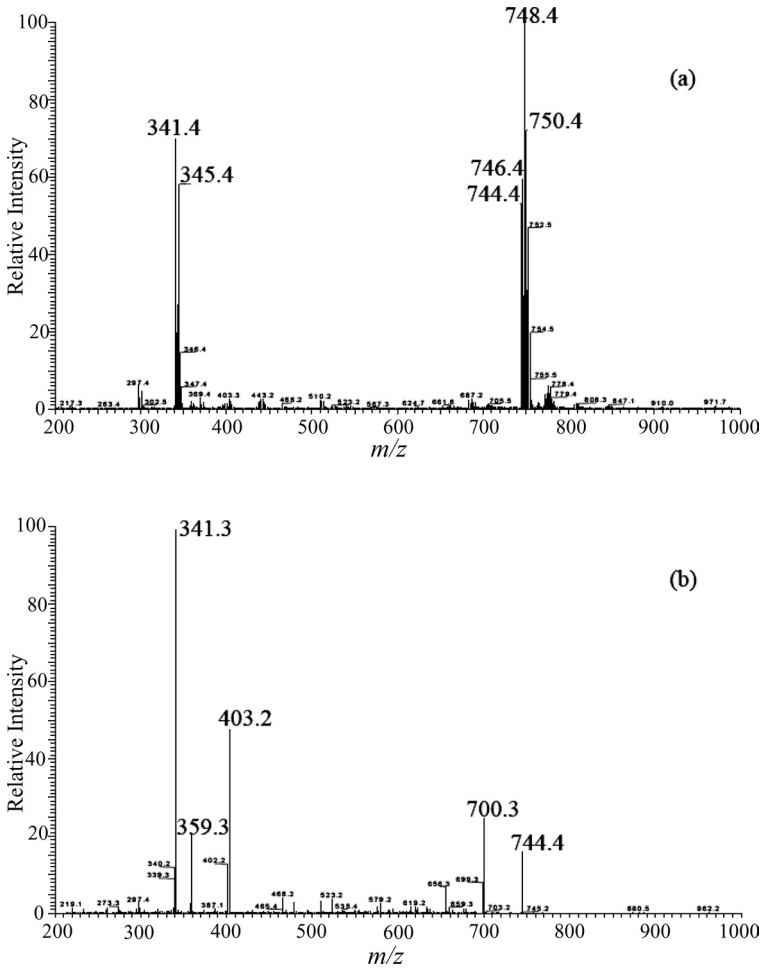


Fig. 2. (a) Negative ESI mass spectrum of anacardic acids and cupric ion. (b) Product ion mass spectrum of a precursor ion at $m/z$ 744.4.

kV. An external calibration was performed using angiotensin I human (1,295.6 Da).

### 2.4 Mass spectrometric detection of anacardic acid with cupric ion

A methanolic and aqueous solution containing the anacardic acid (1 nmol/$\mu$L), DOPA (1 nmol/$\mu$L), and cupric chloride (1 nmol/$\mu$L) was electro-sprayed and analyzed with the ion trap mass spectrometer. The solutions of DOPA and $CuCl_2$ at a concentration of 10 pmol/$\mu$L were prepared in a 1 : 1 mixture of methanol and ultrapure water. The solution of anacardic acid that was diluted in 1 : 1 mixture of methanol and ultrapure water to 4,000 times was added to the solutions. The relative concentrations of DOPA *versus* anacardic acid were measured with the peak ratios of $I_{m/z=198.2}/I_{m/z=194.9}$ ([DOPA]/[anacardic acid]).

### 2.5 Tyrosinase-induced oxidation of peptide (neurotensin)

A 2.0 mM solution of neurotensin was incubated with tyrosinase (2% w/w) in 0.1 mL of ultrapure water at 37℃ for 1 h. The reaction mixture was centrifuged for 5 min and the supernatant was analyzed by MALDI-TOF MS and ESI-MS.

## 3. Results and Discussion

### 3.1 Complexation of anacardic acid with enzyme-modeled metal

Antioxidation for tyrosinase-catalyzed oxidation of DOPA has been established as competitive inhibition of oxidation.[10] However, the intermediate phase of the complexation still remains unclear.[11] In particular, the complex of antioxidant and metal ion has been difficult to detect directly in a solution phase. Mass spectrometry enables us to detect the complex-ion in the solution with the ESI technique. Concretely, the negative ESI mass spectra were recorded for anacardic acid in the presence of copper ions ($Cu^{2+}$) in a solution of 2-propanol : hexane : ethyl acetate = 2 : 1 : 1 with 0.05% acetic acid, whereas the positive ESI was ineffective for the detection of the complex-ion in an aqueous solution. The negative spectrum showed the peaks related to a 2 : 1 complex of the anacardic acid (abbreviated as "A") and cupric ion together with the peaks composed of 1 : 1 complex as shown in Fig. 2a. Since these peaks contain a mixture of anacardic acid **1** and **3** and copper isotopes, the MS/MS spectrum was recorded as a precursor ion $[Cu^{2+}+2A-3H]^-$ at $m/z$ 744.4. The product ions appeared at $m/z$ 700.3, 403.2, 359.3, and 341.3, for which ion compositions were described as $[Cu^{2+}+2A-3H-CO_2]^-$, $[Cu^{+}+A-2H]^-$, $[Cu^{+}+A-2H-CO_2]^-$, and

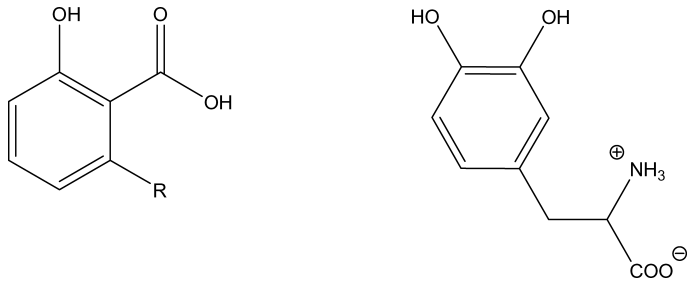


Fig. 3. Structures of anacardic acid and DOPA.

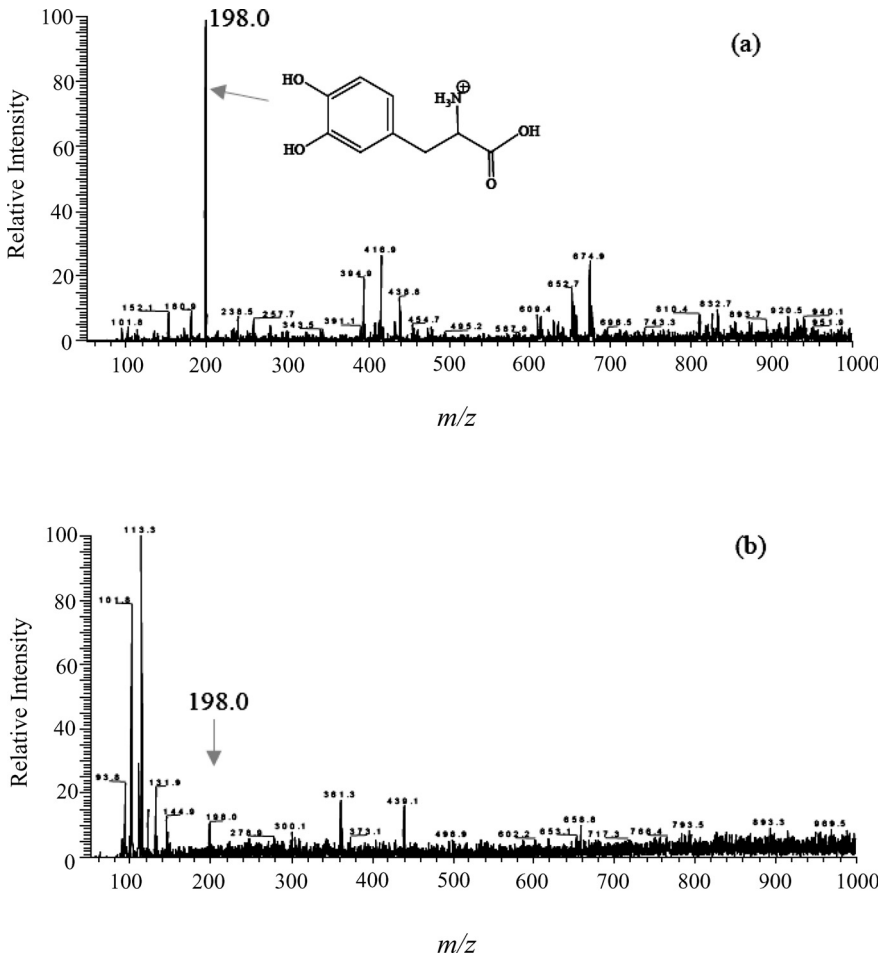


Fig. 4. (a) Positive ion ESI mass spectrum of DOPA. (b) Positive ion ESI mass spectrum of a 1 : 1 mixture of DOPA and $CuCl_2$.

$[A-H]^-$, respectively. The formation of Cu-complex ion representing the 1 : 1-composition, $[Cu^{2+}+2A-3H]^-$, was assumed to be formed in the gas phase under ESI conditions.[12), 13)]

### 3.2 Comparison of DOPA with methyl caffeate

The antioxidative activity of anacardic acids has been also discussed in the competitive reactivity of DOPA with the metal in tyrosinase.[5), 14)] The coordination of anacardic acid or DOPA to the metal is involved in a model for the competition. As shown in Fig. 3, there are two types of models in the chemical classification of the coordination; 2-hydroxybenzoic acid- and catechol-types.

The oxidation of catechol in the presence of oxidase is related to the complexation of catechol and metal ion in the coordination step. In the mass spectrometry, the complexation seems to be directly observed as peaks generated by positive ionization. However, the experimental detection of the positive ions resulted in weak intensities and showed unidentified or degraded fragments. In contrast, the negative ions of the complex consisting of DOPA and cupric ion were effectively detected as a doubly charged peak based on the dianion, as shown in Figs. 4 and 5. Furthermore, the complexation of methyl caffeate with copper ion simply gave an anion, indicating the peak derived from the formation of the mono anion, whose spectra are not shown. This implies that the addition of cupric ion to DOPA induces formation of the anions resulting from metal-complexation.

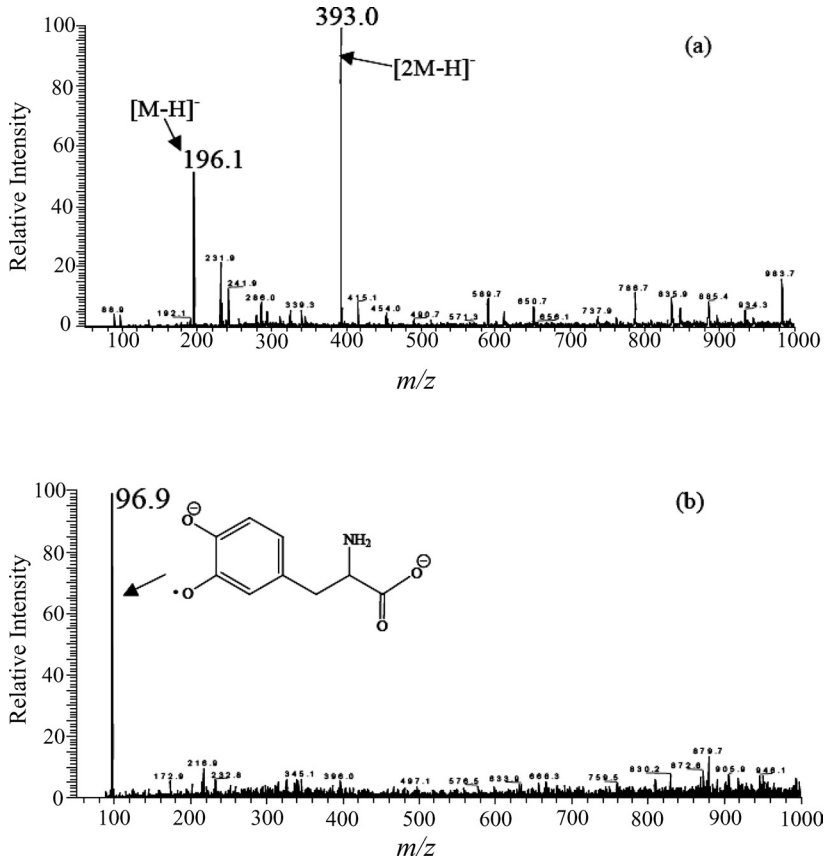


Fig. 5. (a) Negative ion ESI mass spectrum of DOPA. (b) Negative ion ESI mass spectrum of DOPA and $CuCl_2$.

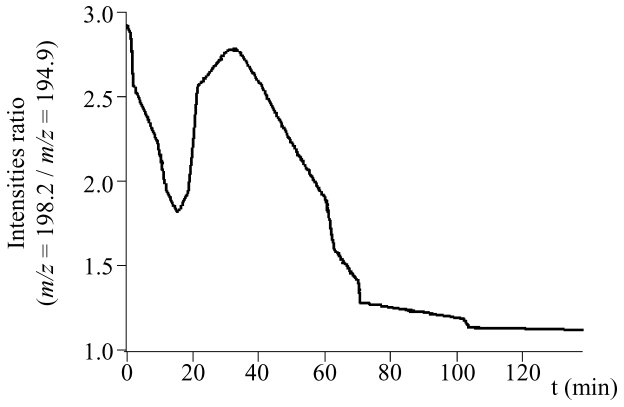


Fig. 6. Inhibition of DOPA-oxidation by anacardic acid.

### 3.3 Inhibition profile for tyrosinase-induced oxidation of DOPA

The overall competitive reactions consist of oxidations of DOPA and anacardic acid in the presence of tyrosinase. In addition, in the successive steps, dopaquinone, which is an oxidized DOPA, is reduced to DOPA with anacardic acid. Experimentally, the disappearance of DOPA was observed in the presence of tyrosinase and the antioxidant. Monitoring the relative concentrations of DOPA *versus* the antioxidant by mass spectrometry gives a qualitative profile of the redox reactions.

The antioxidation models have been proposed to explain the tyrosinase-complexation of DOPA competing with the antioxidant.[15] The competitive complexation of DOPA with tyrosinase is monotonously retarded by the presence of the antioxidant in equilibrium at the first step. The product of the gradual oxidation of DOPA is dopaquinone. However, in the reaction system consisting of DOPA, tyrosinase, and anacardic acid, the dopaquinone is capable of being reduced to DOPA by anacardic acid in the following step, as shown in Fig. 6. The intermediate increase of DOPA implies the reduction of dopaquinone to DOPA.[16]

### 3.4 Tyrosinase-induced oxidation of neurotensin

The inhibition reaction of neurotensin oxidation by anacardic acid was checked by ESI mass spectrometry. The MALDI mass spectrum was obtained after incubation of neurotensin with tyrosinase for 1 h, as shown in Fig. 7. The peaks of the ions derived from neurotensin and its oxidation product were observed at $m/z$ 1,672.2 and 1,686.2 together with 1,700.2, respectively. Weak signals of side-reaction products were obtained at around $m/z$ 1,716.2 and 1,642.2. In contrast, the MALDI mass spectrum in Fig. 8 was obtained after incubation of neurotensin with tyrosinase containing anacardic acid for 1 h. The peaks in the inhibition of the protonated molecules related to neurotensin, sodiated neurotensin, and potassiated neurotensin were observed at $m/z$ 1,672.3 together with 1,694.2 and 1,710.2, respectively.[17]

Compared with the spectra in Figs. 7 and 8, there are notable differences in the oxidation depending on the conditions that are concomitant with anacardic acid or anacardic acid-free. Fig. 7 shows that the intense peak at $m/z$ 1,686.2 could be observed as a peak derived from dopaquinone. On the other hand, Fig. 8 shows the intense peak of neurotensin together with the peaks

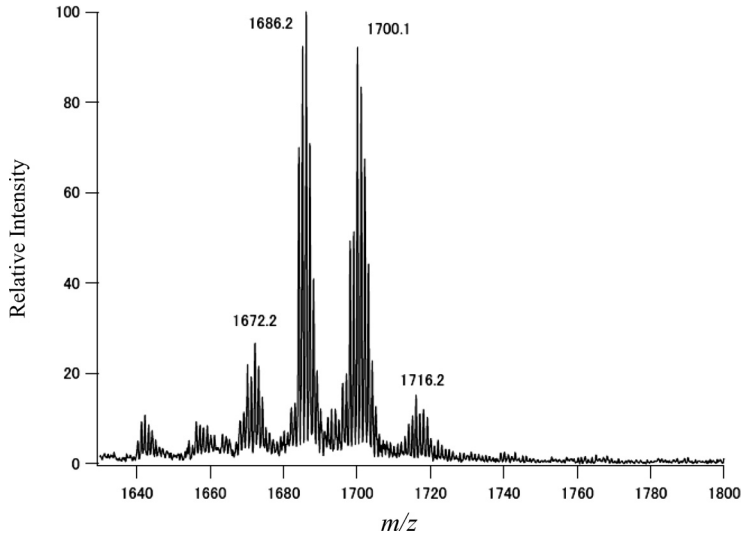


Fig. 7. MALDI-TOF mass spectrum of tyrosinase-catalyzed oxidation product.

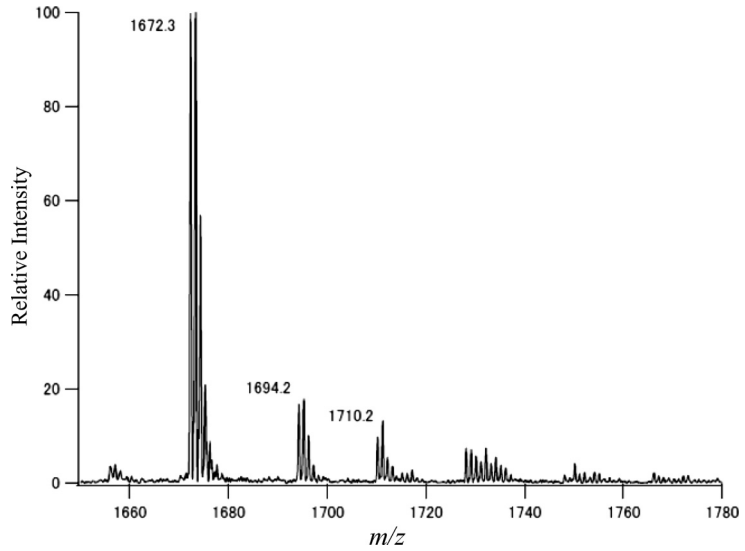


Fig. 8. MALDI-TOF mass spectrum of neurotensin in tyrosinase-oxidation inhibited by anacardic acid.

based on neurotensin consisting of sodium or potassium.

These results are summarized as two facts: First, the oxidation of neurotensin with tyrosinase gave dopaquinone-type peptide as in the oxidation of DOPA. Second, on the contrary, the oxidation was inhibited by anacardic acid. The results are consistent with the oxidation of DOPA, *i.e.*, tyrosine residue was oxidized in the presence of tyrosinase, whereas the further oxidation of DOPA was inhibited by anacardic acid.

## 4. Conclusion

Anacardic acid–metal complex in a ratio of 2 : 1 has been characterized. In addition, anacardic acid–metal complex in a ratio of 1 : 1 was slightly detected at $m/z$ 403.3. The oxidation of DOPA in the presence of anacardic acid did not show monotonous competition-reaction but indicates temporary reduction of dopaquinone to DOPA. As for neurotensin, the enzymatic oxidation was inhibited with anacardic acid.

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