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Identification and Quantification of Adducts between Oxidized Rosmarinic Acid and Thiol Compounds by UHPLC-LTQ-Orbitrap and MALDI-TOF/TOF Tandem Mass Spectrometry

Chang-bo Tang,[†,§] Wan-gang Zhang,[†] Chen Dai,[‡] Hui-xia Li,[†] Xing-lian Xu,[†] and Guang-hong Zhou*[,†]

[†]Synergetic Innovative Center of Food Safety and Nutrition, Key Laboratory of Meat Processing and Quality Control, Ministry of Education, and Key Laboratory of Animal Products Processing, Ministry of Agriculture, College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, People's Republic of China

[‡]Laboratory Center of Life Science, College of Life Science, Nanjing Agricultural University, Nanjing 210095, China

[§]Department of Food Nutrition and Detection, College of Education and Humanity, Suzhou Vocational University, Suzhou 215104, China

ABSTRACT: LTQ Orbitrap MS/MS was used to identify the adducts between quinones derived from rosmarinic acid (RosA) and thiol compounds, including cysteine (Cys), glutathione (GSH), and peptides digested from myosin. Two adducts of quinone−RosA/Cys and quinone−RosA/2Cys, one quinone−RosA/GSH adduct, and three quinone−RosA/peptide adducts were identified by extracted ion and MS^2 fragment ion chromatograms. By using MALDI-TOF/TOF MS, the adduction reaction between RosA and myosin in myofibrillar protein isolates was determined, demonstrating that the accurate reaction site was at Cys949 of myosin. The effect of reaction conditions, including stirring time, temperature, and oxidative stress, on the formation of adducts was further investigated. The formation of quinone−RosA/Cys and quinone−RosA/GSH increased with stirring time. Both adducts increased with temperature, whereas the reactivity of the addition reaction of GSH was higher than that of Cys. With increasing oxidation stress, the formation of quinone−RosA/GSH adduct increased and that of quinone−RosA/Cys adduct decreased.

KEYWORDS: *LTQ Orbitrap, MALDI-TOF/TOF, rosmarinic acid, thiol compounds, myofibrillar protein isolate, adduct, reaction conditions*

INTRODUCTION

Many plant extracts containing polyphenol compounds have been widely used to inhibit the formation of protein oxidation and lipid products in food systems.[1−4] However, phenolic compounds containing di- or trihydroxybenzene in food products, apart from donating hydrogen to prevent SH from oxidation, can be oxidized to quinones, which react with protein thiol groups to form thiol−quinone adducts (Scheme 1, reaction I).[5] Adducts may indirectly protect against disulfide formation (Scheme 1, reaction II) and block the thiol groups in food proteins that contribute to improved meat quality (including tenderness and water-holding capacity).[6−9] Several proteins, such as myofibrillar protein isolate (MPI),[10] α-lactalbumin and lysozyme,[11] bovine serum albumin,[12] whey protein,[13] and myoglobin,[14] have been demonstrated to react with quinones oxidized from phenolic compounds to form thiol−quinone adducts. However, the structures of those adducts remain unknown, and high mass accuracy measurements on molecular ions and MS/MS fragment ions are necessary for structural characterization.

In this study, the content of thiol−quinone adducts was qualitatively and quantitatively measured by employing liquid chromatography coupled with hybrid MS consisting of a linear ion trap and Orbitrap mass analyzer. High resolution and mass accuracy data can be obtained by using the LTQ Orbitrap MS; therefore, structural elucidation of target analytes can be achieved by the multiple levels of fragmentation (MS^n) coupled to external accumulation devices (e.g., linear trap).[15,16] The LTQ Orbitrap MS has been recently applied in the analysis of small molecules, structural characterization of phenolic compounds and macromolecules, sequence of amino acids and binding site of peptides, and identification of LTA-binding proteins.[17−19] MALDI-TOF MS has been widely used to determine biological macromolecules due to high throughput, simple operation, and high precision.[20,21] MALDI-TOF/TOF MS was herein applied to determine the adduction reaction between RosA and myosin in MPI extracted from meat.

Rosmarinic acid (RosA) contains two catechol moieties and is a potential polyphenol in antioxidant, which is widely

Table 1. Final Concentrations of Reagents in the Model Solutions

thiol source	(mM)	rosmarinic acid (mM)	$FeSO_4$ (mM)	H_2O_2 (mM)
cysteine	1.0	0.5	0.1	30.0
glutathione	1.0	0.5	0.1	30.0
hexapeptide-1	0.5	0.5	0.1	30.0
heptapeptide-2	0.5	0.5	0.1	30.0
nonapeptide-3	0.5	0.5	0.1	30.0

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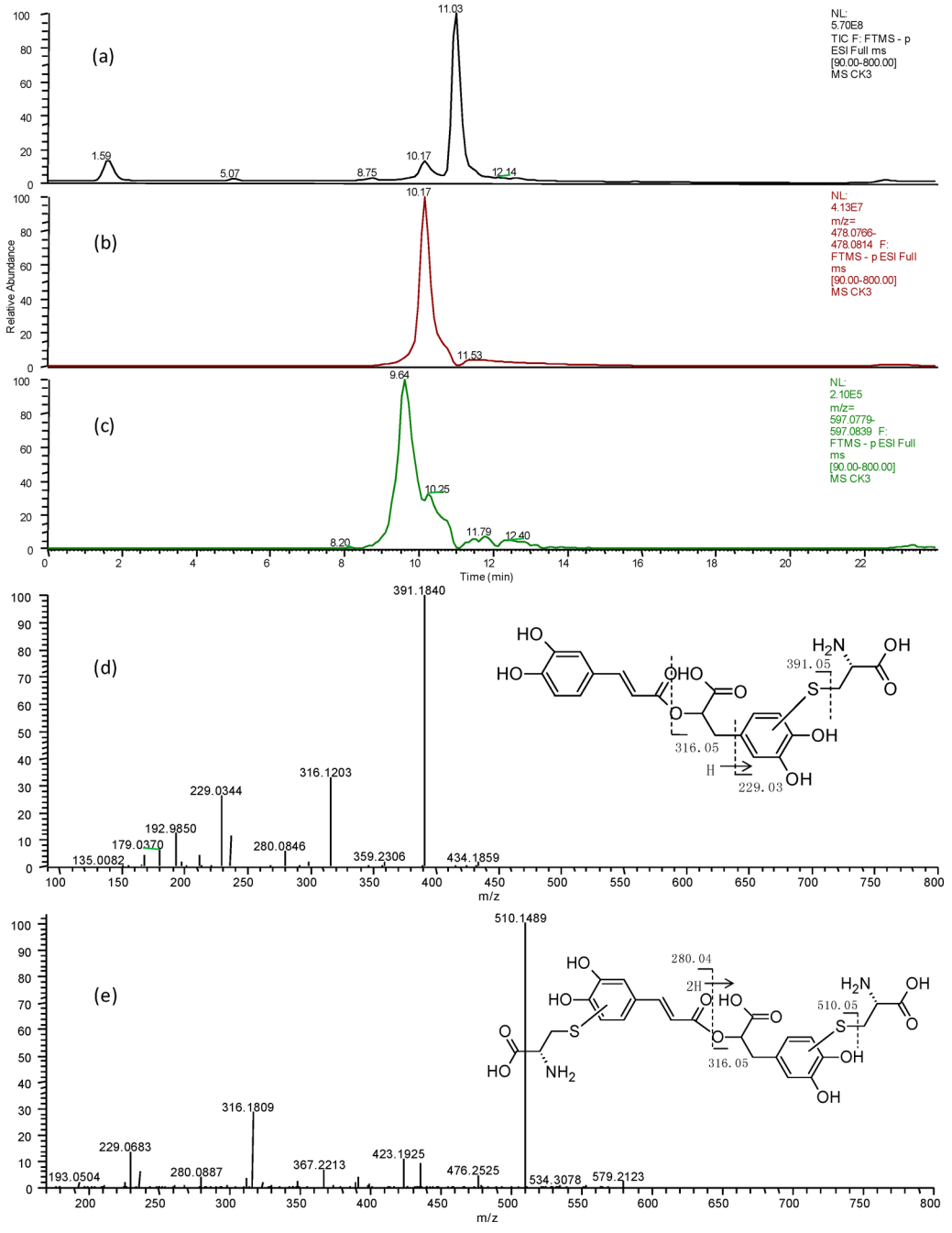

Figure 1. Cys (1.0 mM) and RosA (0.5 mM) incubated in maleic acid buffer (0.1 M) at pH 5.8, with $FeSO_4$ (0.1 mM) and H_2O_2 (30 mM) at 30 °C: (a) TIC of the incubation solution; extracted chromatograms of (b) M1 and (c) M2 from TIC; MS^2 fragmentation patterns of (d) M1 and (e) M2 show the structures of the products with fragmentation pathways noted.

distributed in Lamiaceae herbs.[22] The Lamiaceae herbs, including rosemary, sage, and melissa, are rich in RosA; these herbs are used as food additives in many food products.[23−26] Although Fujimoto[33] has demonstrated the addition reaction between RosA and 1-dodecanethiol, there are still questions about whether RosA can react with myofibrillar proteins, which is closely related to the quality of resulting meat products. As of this writing, little information is available to elucidate the effects of processing conditions, including stirring time, temperature, and oxidative stress, on the formation of adducts. Thus, the objective of this study was to identify the structures of adducts by the reaction of RosA and thiol compounds including cysteine (Cys), glutathione (GSH), and peptides digested from myosin; determine the occurrence of the adduction reaction between RosA and myosin, which is the main protein of myofibrillar proteins in meat; and investigate the influence of processing conditions on the formation of adducts.

MATERIALS AND METHODS

Materials. RosA (96%, MW 360.33), maleic acid, formic acid, Cys (96%, MW 121.15), and GSH (reduced Biotech grade, MW 307.32) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Hydrochloric acid, sodium hydroxide, acetonitrile, sodium dihydrogen phosphate, sodium chloride, magnesium chloride, ethylenebis-(oxyethylenenitrilo)tetraacetic acid, and hydrogen peroxide (30%) were obtained from Sino Pharm Chemical Reagent Co., Ltd. (Shanghai, China). Water was purified by a Milli-Q system (Millipore,

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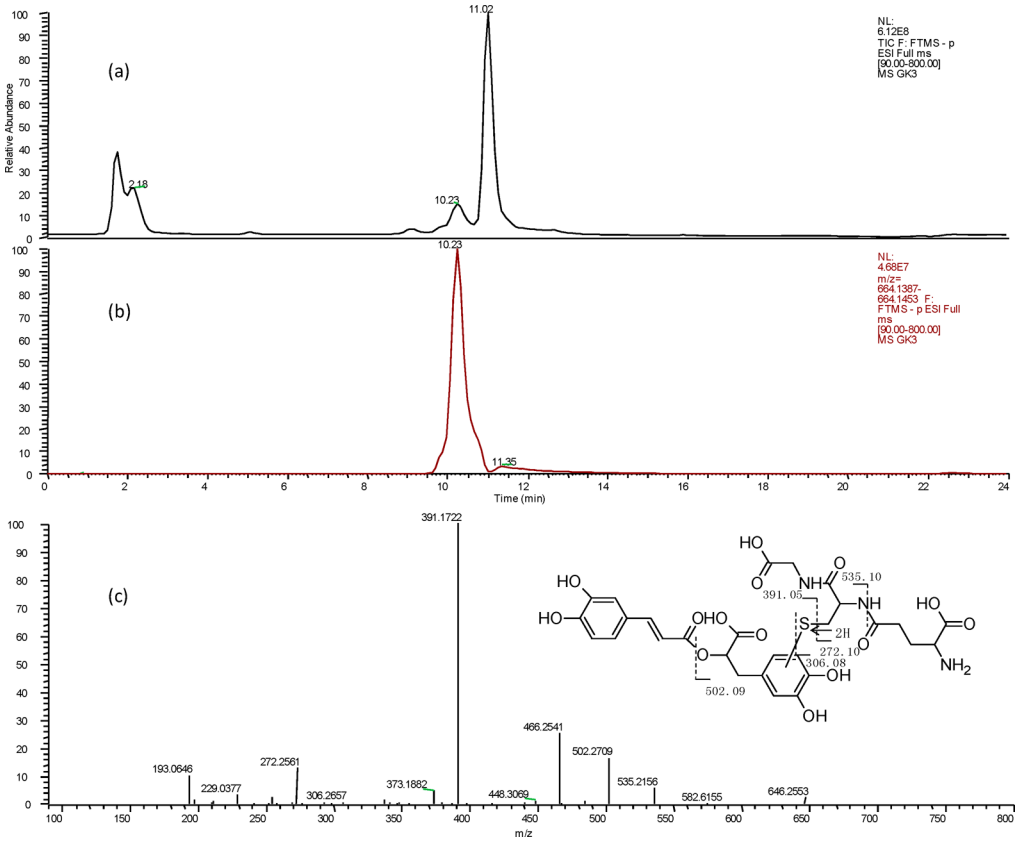
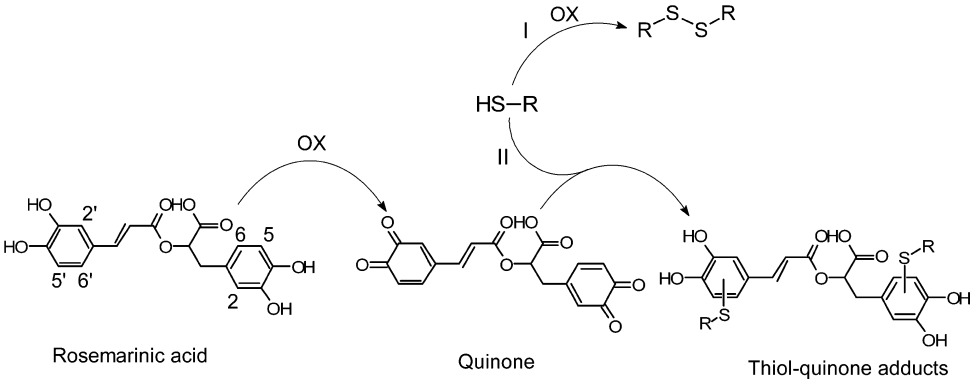


Figure 2. GSH (1.0 mM) and RosA (0.5 mM) incubated in maleic acid buffer (0.1 M) at pH 5.8, with $FeSO_4$ (0.1 mM) and H_2O_2 (30 mM) at 30 °C: (a) TIC of the incubated solution; (b) extracted chromatogram of M3 from TIC; (c) MS^2 fragmentation patterns of M3 show the structures of the products with fragmentation pathways noted.

Scheme 1. Proposed Oxidation Mechanism of Rosmarinic Acid in Meat Model Systems: (I) Oxidation of Compounds Containing Thiol Groups (R−SH) Resulting in Dipolymer by Disulfides to Form Cross-Linked Proteins; (II) Quinone of Rosmarinic Acid Reacted with Compounds Containing Thiol Groups (R−SH), Forming a Thiol−Quinone Adduct



Thermo, Bedford, MA, USA). The following three synthetic peptides (96% enrichment) were obtained from China Peptides Co., Ltd. (Shanghai, China): hexapeptide-1, Cys-Ala-Ser-Leu-Glu-Lys (MW 649.76); heptapeptide-2, His-Asp-Cys-Asp-Leu-Leu-Arg (MW 870.98); and nonapeptide-3, Leu-Glu-Asp-Glu-Cys-Ser-Glu-Leu-Lys (MW 1065.16).

Preparation of Model Solution Sample. The method used for generating adducts was based on the method formerly described;[10,27] thiol compounds including Cys, GSH, and peptides from myosin were diluted in 0.1 M maleic acid buffer adjusted to pH 5.8 with NaOH. The solutions were then separately incubated with RosA, $FeSO_4$, and H_2O_2 at 30 °C and stirred in the dark for 5 h. The concentrations of samples are presented in Table 1.

Preparation of Meat Samples. Freshly ground pork was purchased from Walmart supermarket, Nanjing, China. The meat was chopped and divided in two parts (100 g): to one part was added 50 mg of RosA, and the other part without RosA was used as the control. Aliquots of 5.0 g samples were transferred to plastic bags, which were filled with pure oxygen and sealed. The meat samples in triplicate were kept in storage in the dark at 4 °C for 6 days.

Extraction of MPI. MPI was extracted from the meat samples after 6 days of storage according to the method of Park et al.[28] with slight modifications. An aliquot of 4.0 g of meat was homogenized in 20 mL of isolation buffer (100 mM NaCl, 10 mM NaH_2PO_4, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.0) in 50 mL centrifuge tubes. The homogenate was centrifuged at 2600*g* at 4 °C for 12 min, and the pellet was

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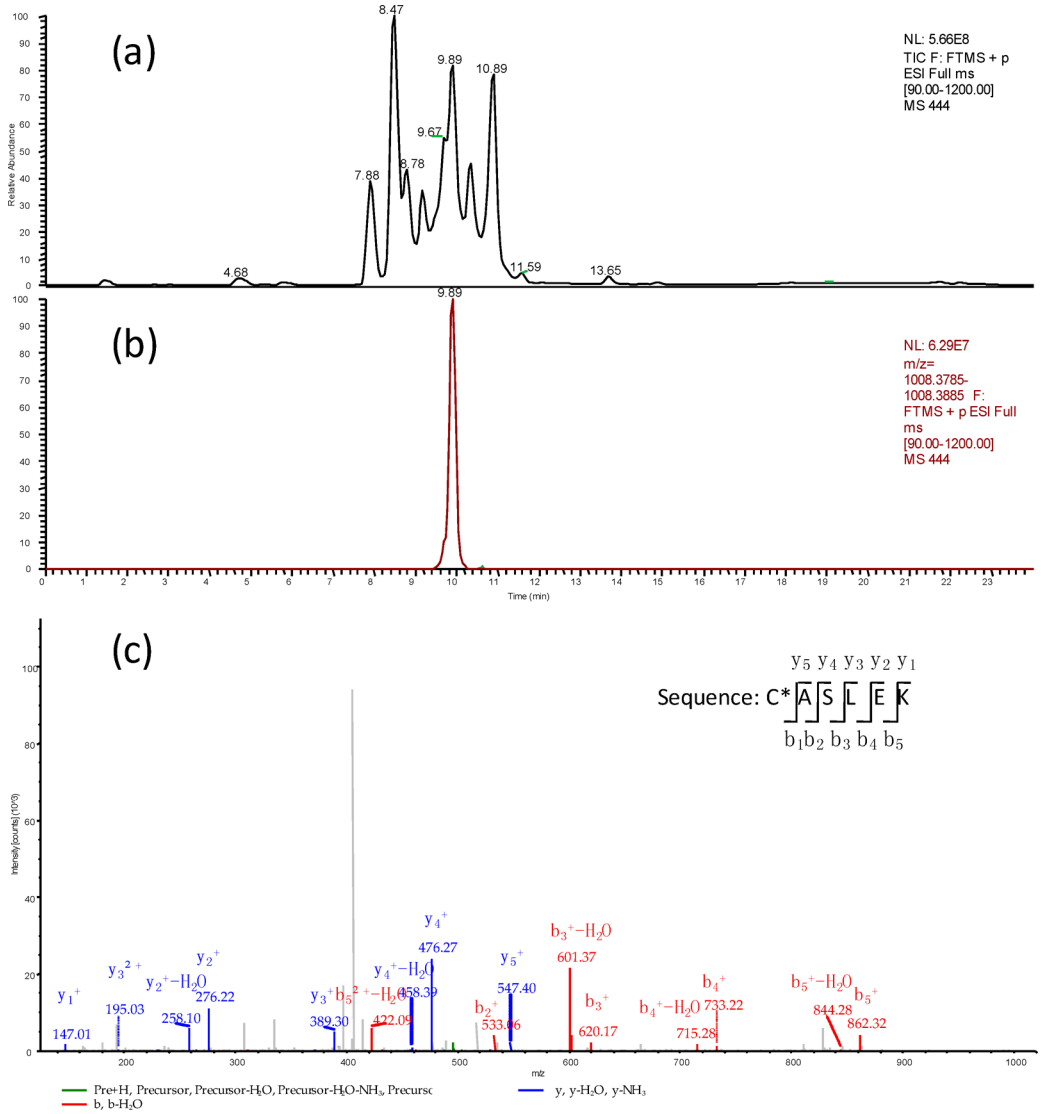

Figure 3. Hexapeptide (0.5 mM) and RosA (0.5 mM) incubated in maleic acid buffer (0.1 M) at pH 5.8, with $FeSO_4$ (0.1 mM) and H_2O_2 (30 mM) at 30 °C: (a) TIC of the incubated solution; (b) extracted chromatogram of M4 from TIC; (c) MS^2 fragmentation patterns of M4 show the structures of the products with fragmentation pathways noted.

resuspended in 10 mL of isolation buffer by homogenization and centrifugation, which was repeated three times. Before the last centrifugation, the suspension was filtered through cheesecloth, and the protein pellet was stored in ice for use.

Influence of Processing Condition on Adducts. To evaluate the effects of stirring time, temperature, and oxidizing intensity on adduct production, the above reaction mixtures were incubated at 30, 40, and 50 °C and stirred for either 1, 3, 5, or 7 h in the presence of 10, 30, 50, or 70 mM H_2O_2. Aliquots (10 mL) of each reaction were prepared in amber vials. Reactions were stopped by the addition of 50 mM potassium bisulfate solution. All reactions were performed in triplicate. The residue was filtered through a 0.45 μm membrane filter for mass spectrometer analysis.

UHPLC-LTQ-Orbitrap MS Analysis. The thiol sources, including Cys, GSH, and peptides of myosin, and their adducts with quinones oxidized from RosA were monitored by the UHPLC-LTQ-Orbitrap system, which consisted of UHPLC (Dionex, ThermoFisher Scientific, New York, USA) coupled with LTQ Orbitrap XL MS (Thermo Fisher Scientific). Instruments were charged by Tune 2.6.0 and Cheomeleon programs. The chromatographic column used was Hypersil GOLD C18 (100 mm × 2.10 mm, 3 μm particle size, Thermo Fisher Scientific). UHPLC mobile phase A was water with 0.1% formic acid, and UHPLC mobile phase D was 100% acetonitrile. Separations were performed in 20 min with a flow rate of 0.2 mL/min under the following programs: 0−4 min, 5% D; 4−12 min, 5−95% D; 12−15 min, 95% D; 15−15.5 min, 95−5% D; and 15.5−20 min, 5% D. The oven temperature of the column was set at 35 °C, and the injection volume was 5 μL.

MS was operated in negative mode to analyze adducts formed by Cys and GSH and monitor the calculated ions of $[M - H]^-$ = 478.0813 (Cys−RosA, M1), $[M - H]^-$ = 597.0854 (Cys−RosA−Cys, M2), and $[M - H]^-$ = 664.1454 (GSH−RosA, M3). The positive mode was used to analyze the adducts formed by peptides and monitor the calculated ions of $[M^+H]^+$ = 1008.3866 (hexapeptide−RosA, M4), $[M + 2H]^{2+}$ = 615.2428 (heptapeptide−RosA, M5), and $[M + 2H]^{2+}$ = 712.2767 (nonapeptide−RosA, M6).

MALDI-TOF/TOF MS Analysis. MALDI-TOF/TOF-MS equipped with a 355 nm laser was applied to examine the precise sites of adduct between RosA and myosin in MPI. The protein samples were reduced and alkylated by the routine method of Biotech Proteome kit (Shimadzu, Osaka, Japan). Finally, the sample (2 μg) was digested in 5 μL of 100 mM NH_4CO_3 (pH 8.0) containing 10 μg/mL trypsin for 10 h at 37 °C. After desalting by C18 Tip, 1 μL of sample solution was spotted on the MALDI plate (Shimadzu GLC, Osaka, Japan) with DHB as the matrix. The digests were analyzed on a MALDI-TOF/TOF

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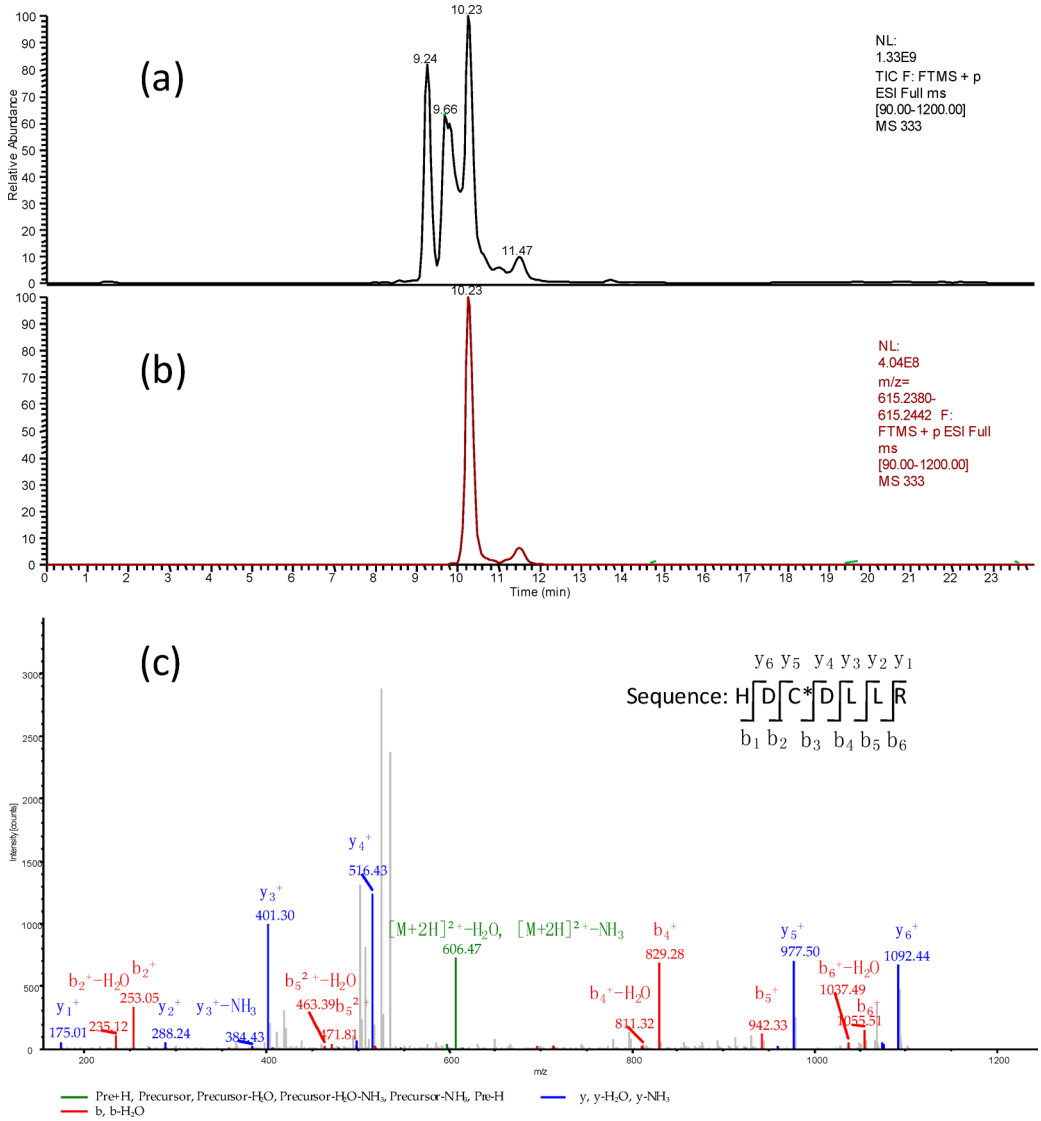

Figure 4. Heptapeptide (0.5 mM) and RosA (0.5 mM) incubated in maleic acid buffer (0.1 M) at pH 5.8, with $FeSO_4$ (0.1 mM) and H_2O_2 (30 mM) at 30 °C: (a) TIC of the incubated solution; (b) extracted chromatogram of M5 from TIC; (c) MS^2 fragmentation patterns of M5 show the structures of the products with fragmentation pathways noted.

instrument (AXIMA Performance; Shimadzu, Manchester, UK) with Kompact version 2.8.

Protein identification was operated by using the MS ion and MS/MS ion search of Mascot. The following parameters were set for searching peptides containing cysteine modified by rosmarinic acid: database, SwissProt; allowing one missed cleavage; enzyme, trypsin; peptide mass tolerance, 100 ppm; fragment mass tolerance, ±1.5 Da; fixed modification, carbamidomethyl (C); and variable modification, oxidation (M). A Mascot threshold of 63 was used to achieve a false identification rate of 5%.

RESULTS AND DISCUSSION

Identification of Adducts Formed between Cys and Quinone Derived from RosA. Two adducts formed between Cys and quinone derived from RosA were named quinone−RosA/Cys adduct (M1) and quinone−RosA/2Cys adduct (M2), respectively. A typical total ion chromatogram (TIC) for adducts in the solutions is presented in Figure 1a. Extracted chromatograms from the TIC with different accurate mass settings are shown in Figure 1b,c. M1 and M2 were identified using the exact mass of two peaks at retention times of 2.81 and 2.96 min, displaying the accurate mass of 478.0813 (Figure 1b) and *m/z* 664.1454 (Figure 1c) for each peak, respectively.

The structure of M1 was identified by the data-dependent MS/MS spectra and fragments for further examination (Figure 1d). The ratio of *m/z* 391.05 suggested the base peak of the RosA moiety with sulfur residue. Given the ester bond cleavage, *m/z* 316.05 was attributed to the 2-oxyphenylpropanoyl moiety with the conjunction Cys, which implied that the Cys residue occurred on the right benzene moiety of RosA. In M2 (Figure 1e), which consisted of RosA with two Cys residues caused by ester bond cleavage, *m/z* 316.05 was attributed to the 2-oxyphenylpropanoyl moiety with the conjunction Cys, and *m/z* 280.04 suggested the loss of the caffeoyl moiety with the conjunction Cys. The ion fragmentation patterns of the adducts were similar to those of RosA, which confirmed our results.[29−31]

According to the 1,4-Michael addition reaction, Cys could add to the 2- and 2′-positions (Scheme 1) of quinone derived from RosA. Cys reacts with 4-MC on the 2-position.[10] Caftaric acid, which is similar to the left benzene moiety of the RosA ring, also reacts with 3SH on the 2′-position.[32] However, RosA

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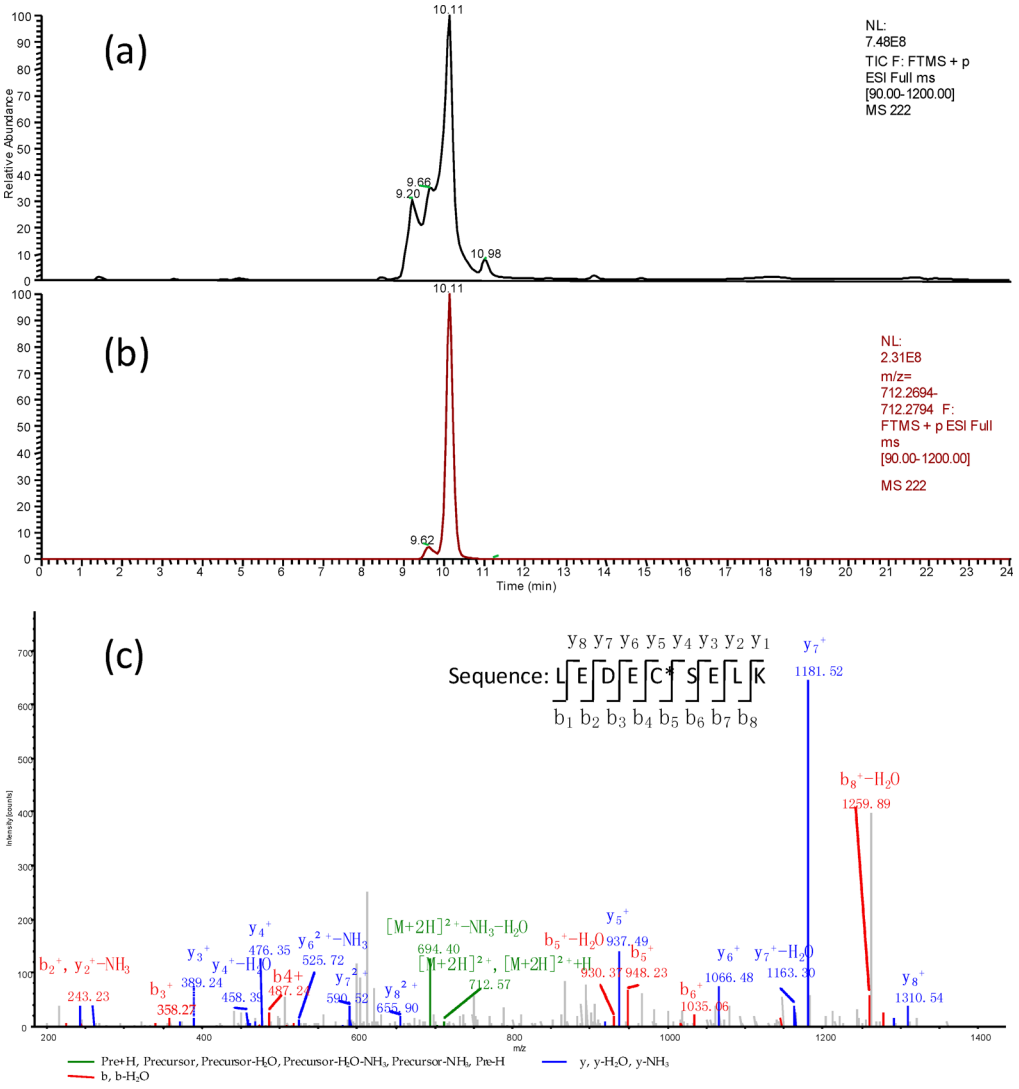

Figure 5. Nonapeptide (0.5 mM) and RosA (0.5 mM) incubated in maleic acid buffer (0.1 M) at pH 5.8, with $FeSO_4$ (0.1 mM) and H_2O_2 (30 mM) at 30 °C: (a) TIC of the incubated solution; (b) extracted chromatogram of M6 from TIC; (c) MS^2 fragmentation patterns of M6 show the structures of the products with fragmentation pathways noted.

reacted with 1-dodecanethiol to form three thiol adducts, in which the binding sites were on the 5-, 6-, and 2′-positions of the benzene rings.[33] Further research should be conducted to confirm the reaction position on the benzene moiety of the RosA ring.

Identification of Adducts Formed between GSH and Quinones Derived from RosA. M3, which was formed between GSH and quinones derived from RosA, was identified using the exact mass of the peak at a retention time of 10.23 min, and an accurate mass of 664.1413 was obtained (Figure 2b). Fragments were performed to identify the structure of M3 (Figure 2c). The ratio of m/z 502.09 suggested the 2-oxyphenylpropanoyl moiety with the conjunction of GSH. This finding indicates that the GSH residue occurred on the right benzene moiety of RosA. Given the fracture of the GSH thiol, m/z 391.05 was attributed to the base peak of RosA, and m/z 272.10 indicated the GSH residue with the removal of 2H. The ratio of m/z 353.10 suggested the loss of the glutamyl residue, and m/z 306.08 suggested the GSH residue.

Other quinones from 4-methylcatechol,[10] tocopherol,[34] and epigallocatechin gallate (EGCG)[35] were confirmed to undergo an adduct reaction with GSH. The structure of those adducts could not be understood because MS/MS ion fragmentation analysis was not accomplished in the current study. However, we can confirm that only one GSH reacted with the corresponding phenolics.

Identification of Adducts Formed between Peptides of Myosin and Quinones Derived from RosA. A series of trypsin-digested peptides of myosin with Cys residue were selected to evaluate the formation of adducts between quinones derived from RosA and myosin, and the positive mode was used for analyzing adducts. The results of peptides were CASLEK, HDCDLLR, and LEDECSELK, and RosA acted as a modification group on the Cys residues for analysis. Adducts were named M4, M5, and M6.

UHPLC-MS TICs for adducts in the solution are presented in Figures 3a, 4a, and 5a. As shown in Figures 3b, 4b, and 5b, the ions from the molecular masses of adducts (M4, M5, and M6) were determined to be $[M + H]^+$ = 1008.3835 m/z (retention time = 9.89 min), $[M + 2H]^{2+}$ = 615.2412 m/z (retention time = 10.23 min), and $[M + 2H]^{2+}$ = 712.2744 m/z (retention time = 10.11 min), respectively. The MS/MS spectra

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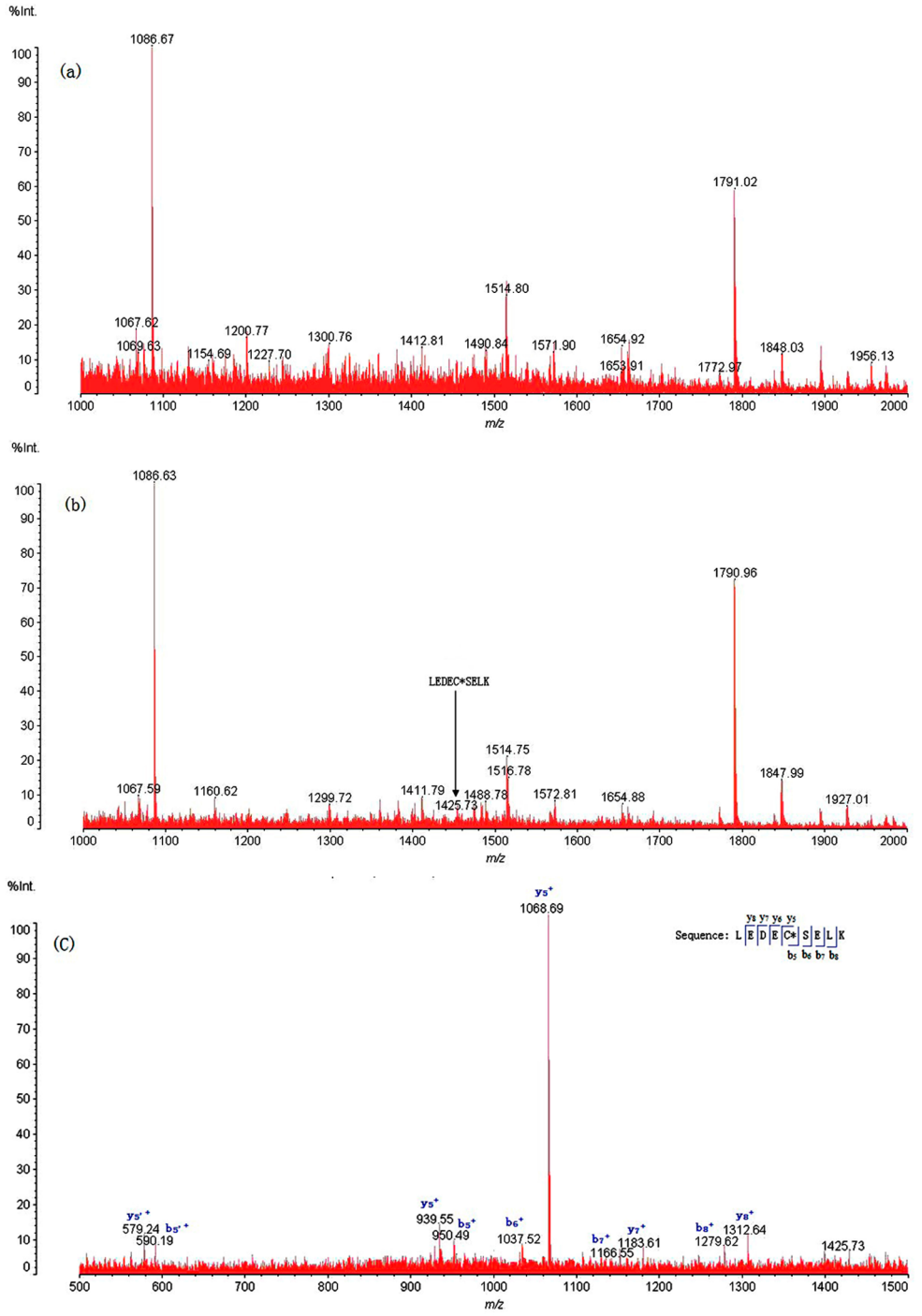

Figure 6. MALDI-TOF/MS spectrum of the tryptic digest of (a) myosin in MPI extracted from sample as control, (b) myosin in MPI extracted from sample with RosA (500 ppm), (c) MS/MS spectrum of m/z 1425.73 peptide (LEDEC*SELK) acquired with MALDI-TOF/TOF MS (C*, cysteines adducted with RosA).

and fragments were studied to identify the structures of M4, M5, and M6 (Figures 3c, 4c, and 5c), and Xcalibur 2.1.0 was used for analysis. Peptides were identified by peptide mass mapping using the Mascot database search program. We found the corresponding ions, from which we confirmed that RosA could react with peptides on the Cys residues.

The Cys−quinone adduct was determined to investigate the adduction reaction between MPI and 4-MC.[10] Rawel[38] found that whey proteins are modified by reactions with selected phenolic compounds using MALDI-MS. However, no study has identified and further analyzed the structures of adducts formed between RosA and peptides digested from myosin. To analyze the mechanism of adduction between phenolic compounds and proteins, structural identification of adducts is necessary.

However, quinones may also react with delta-amino groups located in the side chains of alkaline amino acids (e.g., lysine, proline, and arginine) to form the corresponding semialdehydes.[39,40] The outcome of this reaction is the carbonylation of that side chain, which may also influence the quality of muscle foods.[41,42]

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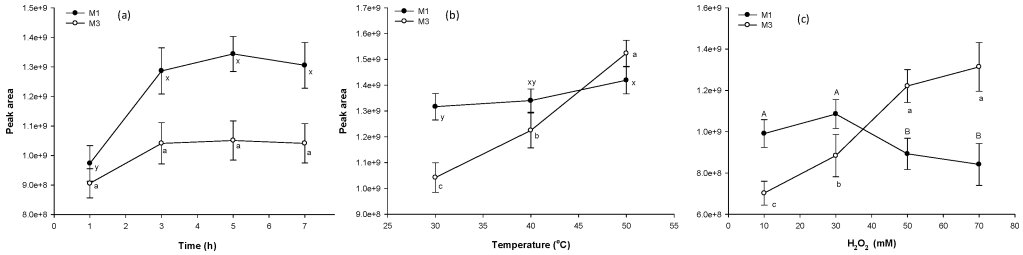

Figure 7. Stirring time effects of (a) oxidation reaction, (b) temperature, and (c) oxidation stress on the concentrations of M1 and M3. Data are expressed as the mean ± standard deviation (SD) (n = 3).

Identification of Adduction Reaction Sites between RosA and Myosin in MPI. To identify adduction sites between myosin and RosA, we performed MALDI-TOF/TOF analysis with PTM Finder. Figure 6a shows the spectrum of peptides digested from MPI sample as control. A Swissprot database search of the MS data yielded 11% sequence coverage with "MYH4-PIG". Figure 6b shows the spectrum of peptides digested from MPI sample with RosA, identifying the adduction peptide m/z 1425.73 LEDEC*SELK (C*, cysteines adducted with RosA). With the MS/MS spectrum of "LEDEC*SELK" in Figure 6c, we confirmed the peptide sequence of the observed fragment ions and demonstrated Cys949 was the adduction site. We herein, for the first time, managed to determine the accurate adduction site between RosA and myosin and to demonstrate the adduction between polyphenols and proteins during meat storage and processing.

Influence of Processing Condition on the Reactivity of RosA and Thiol Compounds. To understand the influence of processing conditions (stirring time, temperature, and oxidation stress) on the reactivity of RosA and thiol compounds, 1 mM Cys or 1 mM GSH was reacted with 1 mM RosA and incubated by chemical oxidation (30 mM H_2O_2). Adduct formation was determined by UHPLC-LTQ-Orbitrap with a proton detector for ion peak area. M2 was ignored because its contents were significantly lower than those of M1 and M3.

Different incubation times (1, 3, 5, and 7 h) were used. The effect of stirring time was examined, as shown in Figure 7a. The formation of M1 and M3 initially increased followed by a lag phase, and the maximum yield was observed at 3 h. In the study of adducts between oxidized grape phenolic compounds and odoriferous volatile thiol in grape wine, seven adducts showed similar increasing trends.[36] The chemical oxidation of RosA to yield peroxyl radicals results in the formation of quinones (Scheme 1).[37] Quinones, which are reactive electrophilic intermediates, are inclined to react easily with thiol compounds, including Cys and GSH, to form thiol−quinone adducts by 1,4-Michael addition reactions. However, the yield rate of M1 was faster than that of M3 from 1 to 3 h. Some similar findings were reported with EGCG[35] or quercetin,[43] in which Cys was more reactive than GSH. Nikolantonaki[44] explained that Cys, a primary thiol, is expected to have less sterically hindered sulfhydryl groups than GSH thiols; thus, Cys has greater reactivity with the quinones.

The effect of different temperatures on the formation of M1 and M3 was investigated. As shown in Figure 7b, two adducts presented an increasing trend as the temperature increased from 30 to 50 °C. The generation of M1 significantly increased with temperature (ion intensity of M1 increased by 46.15%), whereas that of M3 changed more gradually from 30 to 50 °C (M3 increased by only 7.58%). Besides accelerating RosA to quinone, an increase in temperature converted the autoxidation of thiol compounds to polymerization (Scheme 1, reaction I), which could hinder 1,4-Michael addition reactions to form the thiol−quinone adduct. Given its nucleophilic properties,[44] Cys autoxidation reacts faster than GSH, which hinders the formation of adduct 1. Thus, the addition reaction also increased with rising temperature. The order of the reactivity of the addition reaction was GSH > CYS.

The effect of different types of oxidation stress on the formation of M1 and M3 is investigated in Figure 7c. With increasing oxidation stress, M3 demonstrated an increasing trend. Growing oxidation stress can increase the oxidation of RosA to quinone and improve the addition reaction.[45] M1 showed a decreasing trend with higher oxidation stress (50−70 mM). Besides accelerated oxidation of RosA, high H_2O_2 concentration also induced the polymerization reaction of Cys to form the Cys−Cys dimer (Scheme 1, reaction I), which could inhibit M1 formation. Considering the less sterically hindered sulfhydryl group, M1 itself could further oxidize or polymerize, thereby causing the decreasing trend of M1. These findings indicate that oxidation potentials played an important role in adduct formation, and sulfhydryls of different molecular weights exhibited different reaction capacities to form adducts.

Overall, we successfully identified the structures of adducts between RosA and thiols under chemical oxidation conditions. The amount of adducts was regulated by chemical reaction conditions including stirring time, temperature, and oxidation stress. We further determined the accurate adduction site between RosA and myosin in meat samples. In addition, polyphenols and proteins were subjected to adduction reaction during meat storage and processing. Further studies are necessary to investigate how these adducts regulate the quality and nutritional value of food products in processing.

AUTHOR INFORMATION

Corresponding Author

*(G.Z.) Phone/fax: +86 025 84395341. E-mail: ghzhou@njau.edu.cn.

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Notes

The authors declare no competing financial interest.

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