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Recovery of Antioxidant Activity from Carnosol Quinone: Antioxidants Obtained from a Water-Promoted Conversion of Carnosol Quinone

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Carnosol is one of the main antioxidants in sage and rosemary. Although carnosol quinone is the antioxidation product of carnosol and has a very weak antioxidant activity, its treatment in water-containing solvent restored its strong antioxidant activity. HPLC analysis of the water-stimulated recovery reaction of the antioxidant activity revealed that the strong activity was due to the reproduced carnosol. The analysis also showed that an almost equal amount of quinone derivatives of rosmanol (rosmanol quinone) was produced in the reaction along with the carnosol. The rosmanol was formed by the addition of 1 equiv of water and the following isomerization from carnosol quinone in the water-containing solvent. The formed rosmanol was also found to be oxidized by the remaining carnosol quinone to produce rosmanol quinone. At the same time, carnosol quinone was reduced to afford carnosol. This redox phenomenon is an important part of the mechanism for the recovery of the antioxidant activity from carnosol quinone under the water-containing conditions.

KEYWORDS: Carnosol; carnosol quinone; rosmanol; epirosmanol; rosmanol quinone; epirosmanol; quinone; recovery of antioxidant activity; redox reaction

INTRODUCTION

As a general concept, the antioxidation process of a phenolic antioxidant is divided into the following two stages: (1) a radical trapping stage and (2) a radical termination stage. The first stage is a hydrogen-donating stage from the antioxidant to the hydroperoxyl radical of the biomolecule. The second stage is an irreversible radical termination stage that produces stable and nonradical products from the antioxidant radical, which is produced in the first stage (*1*). Normally, these nonradical products, which were produced by the radical termination reaction, have no antioxidant activity or a much weaker antioxidant activity than the original antioxidant. This means that the termination product is stable against attack of radical species. It should be noted that food antioxidants and their antioxidation products are always influenced by various physical and chemical conditions during food storage or processing. Recently, we found that these termination compounds recovered their strong antioxidant activity by storing in solution or heat treatment in a lipid (*2*, *3*). This recovery of the antioxidant activity depended on the production of new antioxidants from the termination product. We presume that this activity-recovery property is common and contributes to the efficient antioxidant capability of the potent antioxidant in foods.

Carnosol (CAR) is a major phenolic diterpenoid constituent having the very potent antioxidant activity of Labiatae herbs, such as sage and rosemary (*4*−*9*). Carnosol quinone (CARQ) is the *o*-quinone derivative of CAR and also the main antioxidation product of CAR (*3*). Although CARQ is a radical termination product of CAR and has almost no antioxidant activity, we recently found that its thermal treatment in a lipid restored its strong antioxidant activity (*3*). More recently, we found that the addition of water accelerates the activity recovery of the CARQ. Food is a complex system that always contains water; therefore, the effect of water could not be neglected in the clarification of the antioxidation mechanism of a food antioxidant. In this paper, we report in detail this water-stimulated recovery of the antioxidant activity from CARQ and propose a recovery mechanism from CARQ.

MATERIALS AND METHODS

Chemicals and Instruments. 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN) was purchased from Wako Pure Chemicals (Osaka, Japan). Ethyl linoleate was purchased from Kanto Chemical (Tokyo, Japan) and used after purification by silica gel (silica 60, Merck, Darmstadt, Germany) chromatography developed with 2.5% ethyl acetate in hexane. All solvents and other reagents were obtained from Nacalai Tesque (Kyoto, Japan). The NMR spectra were measured using an EX-400 spectrometer (400 MHz, JEOL, Tokyo, Japan). The mass spectra were measured using an SX-102A spectrometer (JEOL). The IR spectra were measured using an FTIR-8400 spectrometer (Shimadzu, Kyoto, Japan) according to the dry film method. The analytical HPLC was performed with a PU-980 high-pressure gradient system (JASCO, Tokyo, Japan) equipped with an SPD-M10Avp photodiode array detector (Shimadzu). Preparative HPLC was performed using the LC-6AD recycle system (Shimadzu) equipped with a UV-8011 detector (Tosoh, Tokyo, Japan). Carnosol (CAR) was prepared according to a previously described

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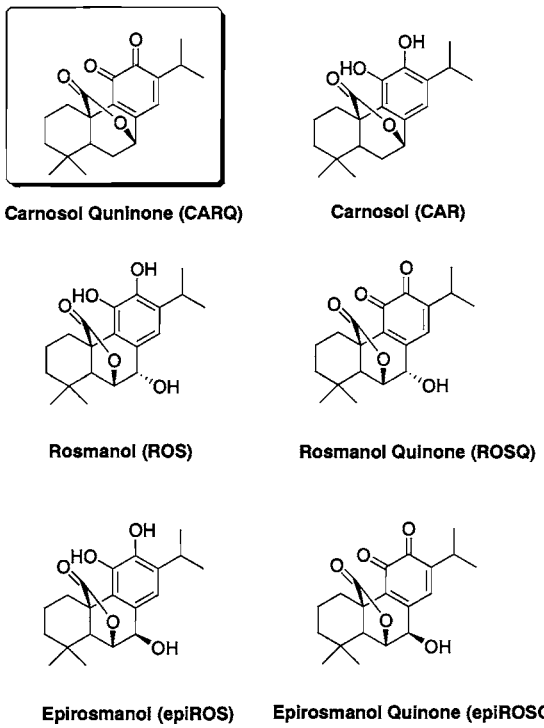

Figure 1. Chemical structures of carnosol quinone (CARQ), carnosol (CAR), rosmanol (ROS), rosmanol quinone (ROSQ), epirosmanol (epiROS), and epirosmanol quinone (epiROSQ).

method (*3*) from the methanol extract of dry sage. Carnosol quinone (CARQ) was also prepared according to a previously reported method (*3*) from CAR by $FeCl_3$ oxidation.

Quantitative Analysis of the Conversion Products from CARQ. CARQ (2 mg) was dissolved with acetonitrile (2.2 mL) in a screw-capped tube (10 mL), and then water (0.24 mL) was added. After the solution was mixed well by a vortex mixer (Scientific Industry, Bohemia, NY), the tube was incubated at 60 °C for 19 h. At 1-h intervals (from 0 to 5 h and from 17 to 19 h after the start of incubation), a 10-μL aliquot was removed from the reaction and injected into the HPLC. The conversion products were quantitatively analyzed under the following conditions: column, Daisopak ODS-AP (250 × 4.6 mm i.d.) (Daiso, Osaka, Japan); solvent, methanol/H_2O/acetic acid (75:25:1); flow rate, 0.5 mL/min; and detection, 284 nm. The concentration of each compound was calculated from the peak area under the calibration curve determined by use of pure compounds. [CAR], $Y = 1.305 \times 10^5X - 7.169 \times 10^3$ (range for X, 0.1−3.0 nmol); [CARQ], $Y = 5.737 \times 10^4X - 1.023 \times 10^4$ (range for X, 0.1−15 nmol); [rosmanol quinone] and [epirosmanol quinone] (ROSQ and epiROSQ, respectively), $Y = 2.004 \times 10^5X - 8.609 \times 10^3$ (range for X, 0.1−10 nmol)], where Y is the observed peak area of each compound and X is the amount of each sample (nmol). (Chemical structures are shown in **Figure 1**.)

Antioxidant Activity Measurement of the Mixture of Conversion Products from CARQ. An aliquot (560 μL) was removed from the above-prepared reaction mixture 19 h after the incubation and placed in a screw-capped tube (10 mL). To the solution were added acetonitrile (5.34 mL), ethyl linoleate (104 μL), and AMVN (22.4 mg). The tube was incubated at 37 °C with shaking (82 rpm) by a water bath shaker 11SD (Taitech, Koshigaya, Japan). At 1-h intervals, a 20-μL aliquot from the solution was diluted with 380 μL of methanol. Ten microliters of the solution was injected into the HPLC to analyze the ethyl linoleate hydroperoxides under the following conditions: column, YMC-ODS-A (150 × 4.6 mm i.d.) (YMC, Tokyo, Japan); solvent, CH_3CN/H_2O (9:1); flow rate, 1.0 mL/min; detection, 234 nm. The concentration of the hydroperoxide was calculated using the calibration curve: $Y = 2.29 \times 10^{-6}X - 4.38 \times 10^{-4}$ [range for X, 9.8×10^3−4.8×10^6. X is the peak area value of the 234 nm detection, and Y is the concentration of the hydroperoxide (mM)].

Isolation of Produced CAR and ROSQ from CARQ. CARQ (20 mg) was dissolved in acetonitrile (21.6 mL) and distilled water (2.4 mL). After stirring well, the mixture was incubated at 70 °C for 8.5 h. The mixture was concentrated in vacuo and purified by silica gel TLC developed with ethyl acetate/hexane (1:2) to separate the CAR (6.7 mg) and ROSQ (7.2 mg). The isolated CAR was identified by comparison with an authentic sample, which was previously prepared from sage (*3*). The isolated ROSQ (mp 167−169 °C) was also identified by an authentic sample, which was synthesized from rosmanol (ROS).

Preparation of ROS and Epirosmanol (epiROS). The preparation of ROS was carried out according to the oxidation method reported by Marrero et al. from CAR (*10*). To the acetone solution (10 mL) of CAR (104 mg) was added a 5% $NaHCO_3$ aqueous solution (12 mL). After 1.5 h of stirring at room temperature, the mixture was filtered. The filtrate was acidified with 1 N HCl and extracted three times with ethyl acetate. The ethyl acetate extract was dried over anhydrous Na_2SO_4 and concentrated. The residue was crystallized from ether to give ROS (36 mg, mp 212−213 °C). The mother liquid was purified by silica gel TLC (benzene/acetone = 9:1) to give epiROS (4 mg). The MS and ^{1}H NMR data were identical to the reported data (*10*).

Preparation of ROSQ and epiROSQ. To an acetone solution of ROS (10 mg) was added a 16% $FeCl_3$ aqueous solution (200 μL). After stirring for 20 min, the mixture was poured into CH_2Cl_2 and H_2O and then extracted three times with CH_2Cl_2. The CH_2Cl_2 extracts were combined and dried over anhydrous Na_2SO_4. After the solution was evaporated, the residue was purified on silica gel TLC developed with ethyl acetate/hexane (1:2) to give ROSQ (5.7 mg). The obtained ROSQ was identified by the reported NMR, MS, and IR data (*10*, *11*). By a similar procedure, epiROSQ (1.1 mg) was prepared from epiROS (3.7 mg). EpiROSQ: HR-EIMS, found m/z 344.1636 $[M]^+$, calcd for $C_{20}H_{24}O_5$, 344.1624; IR (film) cm^{-1}, ν_{max} 3460 (OH), 1767 (lactone CO), and 1663 (quinone CO); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.91 (3H, s, H-18 or 19), 1.01 (3H, s, H-18 or 19), 1.11 (3H, d, J = 6.0 Hz, H-16 or 17), 1.13 (3H, d, J = 6.0 Hz, H-16 or 17), 1.08−1.17 (1H, m), 1.36−1.50 (2H, m), 1.50−1.66 (2H, m), 1.95 (1H, s, H-5), 2.46 (1H, d, J = 12.0 Hz, 7-OH), 2.95 (1H, sept, J = 6.0 Hz, H-15), 3.23 (1H, brd, J = 12.0 Hz, H-1β), 4.40 (1H, dd, J = 12.0 and 4.0 Hz, H-7), 4.77 (1H, d, J = 4.0 Hz), 6.94 (1H, s, H-14).

Redox Reaction between CARQ and ROS. To a 0.5 mM acetonitrile solution (0.5 mL) of CARQ was added a 0.5 mM acetonitrile solution (0.5 mL) of ROS with stirring at room temperature. Immediately after mixing, a 10-μL aliquot was removed and analyzed by HPLC under the same conditions mentioned above. At 1-h intervals, a 10-μL aliquot was analyzed by HPLC under the same conditions for the conversion product analysis. The concentrations of the consumed CARQ and ROS and of the produced CAR and ROSQ were calculated by the following calibration curve determined using pure compounds [CARQ], $Y = 1.279 \times 10^5X - 5.582 \times 10^3$ (range for X, 0.2−30 nmol); [ROS], $Y = 2.004 \times 10^5X - 8.609 \times 10^3$ (range for X, 0.1−10 nmol); [CAR], $Y = 2.172 \times 10^5X - 1.710 \times 10^4$ (range for X, 0.25−10 nmol); [ROSQ], $Y = 1.474 \times 10^5X - 7.480 \times 10^2$ (range for X, 0.2−10 nmol)], where Y is the observed peak area detected at 284 nm for each compound and X is the amount of each compound (nmol).

RESULTS AND DISCUSSION

Analysis of the Conversion of CARQ in Water-Containing Acetonitrile. The HPLC analysis of the conversion of CARQ in a water-containing solvent was first carried out. CARQ was dissolved in acetonitrile/H_2O (9:1) at room temperature. CARQ was more stable than the carnosic acid quinone (*2*); therefore, higher temperature conditions were used. We observed that the heat treatment at 60 °C converted CARQ to other compounds. This reaction temperature was much lower than the change in CARQ in an anhydrous lipidic solvent (170 °C), which was previously reported (*3*). This indicates that the water-containing solution enhanced the conversion rate from the CARQ. **Figure 2** shows the HPLC analytical result of the conversion products from CARQ at 60 °C for 19 h. In the figure, CARQ almost disappeared, whereas two major peaks were observed at 11.9

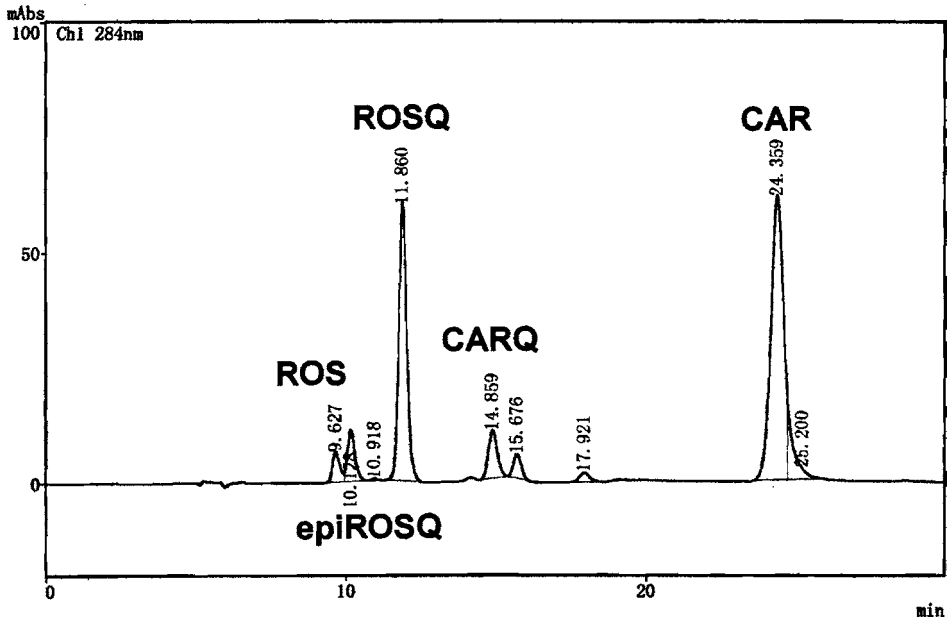

Figure 2. HPLC analytical data for 19-h solution of carnosol quinone in acetonitrile/water (9:1) at 60 °C.

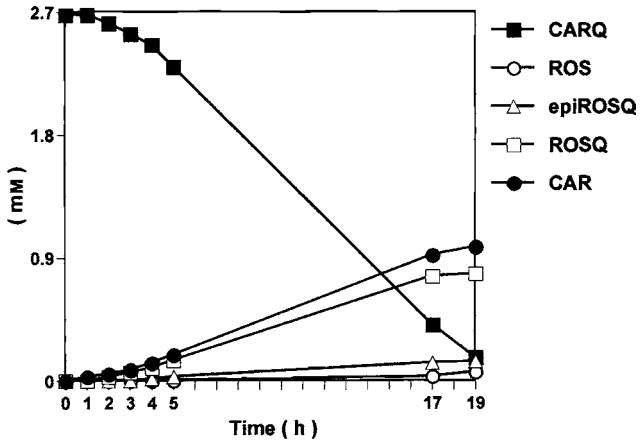

Figure 3. Time course analytical data of carnosol quinone (CARQ), carnosol (CAR), rosmanol quinone (ROSQ), epirosmanol quinone (epiROSQ), and rosmanol (ROS) observed in the HPLC analysis for the reaction of carnosol quinone in acetonitrile/water (9:1) at 60 °C.

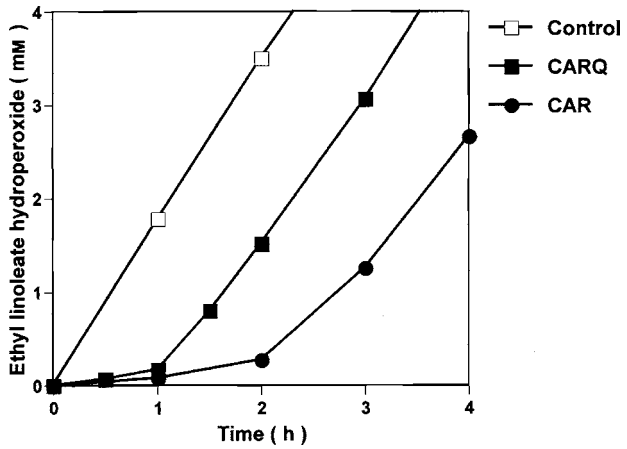

Figure 4. Antioxidant activity of the products (0.25 mM as the concentration corresponding to the starting carnosol quinone) of the 19-h reaction of carnosol quinone (CARQ) in acetonitrile/water (9:1) at 60 °C and comparison with 0.25 mM carnosol (CAR).

and 24.4 min as the retention times. The isolation and structure analysis of these two peak compounds revealed that the peak at 11.9 min was ROSQ and the peak at 24.4 min was CAR. The amounts of each produced for the 19-h reaction were similar (0.78 and 0.98 mM at 19 h, respectively). Structurally, CAR is the reduced compound of CARQ. On the other hand, ROSQ was the oxidized compound of CARQ. This structural information indicated that a self-redox reaction occurred in the CARQ itself. The other observed peak compounds in the HPLC data were screened using authentic samples by comparison of each retention time and UV spectrum. These results indicated that the peak at 9.6 min was ROS and the peak at 10.1 min was epiROSQ. The time course analysis of the production of each peak compound is shown in **Figure 3**. This figure clearly shows that CAR, ROSQ, and epiROSQ increased during the reaction along with the decrease in the starting CARQ. **Figure 4** shows the antioxidant activity of the mixture of the above 19-h reaction from CARQ against the AMVN-induced lipid oxidation. By comparison of the activity of the mixture, which was produced from the corresponding 0.25 mM CARQ with that of 0.25 mM CAR, almost half of the activity of CAR was restored from the CARQ by comparing each induction period. This activity recovery mainly depended on the activity of the reproduced CAR in the mixture, because CAR is a strong antioxidant, whereas the quinone compounds always have very weak or no activity.

Production Mechanism of CAR from CARQ in Water-Containing Solution. We found by HPLC analysis that a small amount of ROS existed in the reaction mixture used for the conversion reaction of CARQ. This ROS was presumed to be a key compound of the recovery mechanism of the antioxidant activity. ROS is a reduced compound of ROSQ and also a water-added substance of CARQ (*12*). A mixing experiment of equal amounts of ROS and CARQ was carried out to produce ROSQ and CAR as shown in **Figure 5**. This result means that ROS can very quickly reduce CARQ to CAR. This reaction was thought to be an electron-transfer reaction on a quinone−catechol system. Although redox potentials of CAR and ROS have not been reported yet, the reduction potential of CARQ should be higher than that of ROSQ. The water treatment of CARQ, which is the antioxidation product of CAR, affords ROS and epiROS (*13*) by the addition of 1 equiv of water and

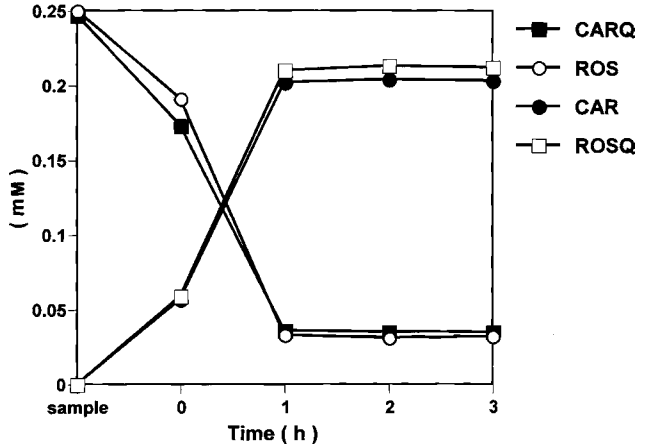

Figure 5. Time course change in the mixing reaction of carnosol quinone (CARQ) and rosmanol (ROS).

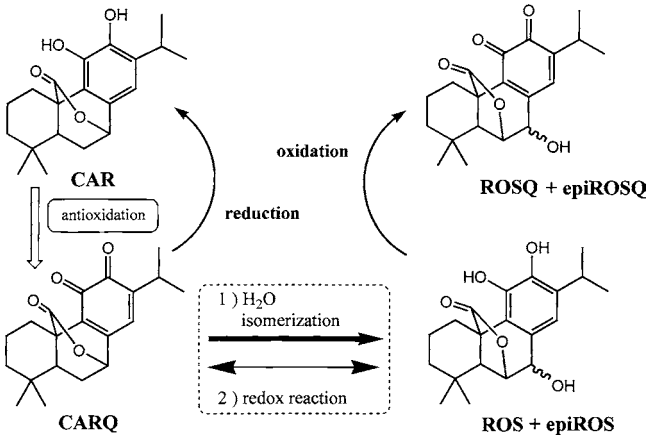

Figure 6. Proposed recovery mechanism of potent antioxidant activity from carnosol quinone (CARQ) in water-containing solvent.

following isomerization reactions as Richheimer suggested (*12*). When the rosmanols (ROS and epiROS) are produced, these rosmanols reduced the remaining CARQ to produce the potent antioxidant, CAR, as illustrated in **Figure 6**. This mechanism can explain the production of equal amounts of the rosmanol quinones (ROSQ + epiROSQ) and CAR in a water-containing solvent and the phenomenon of the half-activity recovery of CAR from CARQ.

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