Inhibition of Lipid Peroxidation and Nonlipid Oxidative Damage by Carnosine

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ABSTRACT: The antioxidant effects of carnosine on lipids and nonlipids, including liposomes, carbohydrates, and proteins, were investigated. Carnosine exhibited a remarkable antioxidant effect in liposome and deoxyribose model systems. Carnosine at high amounts (50 mg/mL) was effective in protecting protein against oxidation. The correlation coefficients between reducing ability and the inhibition of liposome, albumin, and deoxyribose oxidation were $r = 0.92$, 0.83, and 0.41, respectively. Carnosine exhibited metal-binding ability and scavenging ability for hydroxyl radicals generated by photolysis of H_2O_2 with UV light. The correlation coefficients between hydroxyl radical scavenging and the inhibitory effect on deoxyribose, liposome, and albumin oxidative damage were $r = 0.97$, 0.60, and 0.29, respectively. These properties may explain how carnosine protects lipids and nonlipids against oxidative damage.

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KEY WORDS: Albumin, antioxidant effect, carnosine, deoxyribose, DPPH radical, hydroxyl radical, liposome, nonlipids, oxidative damage, reducing effect.

Lipid peroxidation not only lowers the nutritive value of food and adversely affects the flavor and taste but also is associated with aging, heart disease, stroke, emphysema, mutagenesis, and carcinogenesis in humans (1). Lipid oxidation during food processing can be inhibited by the addition of antioxidants. However, efficient synthetic antioxidants such as BHT and BHA exhibit toxic properties, and they cannot be recommended for general use (2).

Some antioxidants are present naturally in raw food materials; for example, carnosine, a β-alanylhistidine dipeptide found in skeletal muscle, may contribute to the inherent antioxidant potential of muscle and may act as a natural antioxidant in processed meat products (3). Decker and Faraji (4) noted that carnosine heated at 100°C for 15 min had no antioxidant effect. Carnosine (0.5–1.5%) inhibited lipid oxidation in pork (5,6), turkey (7), and beef (8). Kansci *et al.* (2) reported that carnosine inhibited liposome oxidation. In addition, the scavenging effects of carnosine on free radicals and on the copper ion have been demonstrated (3,9–11). Although carnosine acts as an antioxidant toward lipids, its effect on the oxidation of nonlipids, including protein and deoxyribose, remains unclear. Thus, the objectives of this work were to investigate the effects of carnosine on the peroxidation of lipids and on the oxidative damage of nonlipids.

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MATERIALS AND METHODS

Materials. Upon receipt, carnosine (99.7% purity; Tokoyo Chemical Industry Co., Ltd.) was analyzed by HPLC to ensure there was no hydrazine contamination. The liquid chromatography system (Hitachi, Ltd., Tokyo, Japan) consisted of a model L-7100 pump and a model 7420 UV-Vis detector set an 220 nm. The peak was compared with that of an authentic sample (data not shown). Lecithin, H_2O_2, TCA, ascorbic acid, salicylic acid, EDTA, and $K_3Fe(CN)_6$ were purchased from E. Merck (Darmstadt, Germany). The tocopherol used was α-tocopherol (Toc), which was synthesized by and purchased from E. Merck. $FeCl_3$ and 1,1-diphenyl-2-picrylhydrazyl were purchased from Fluka Chemie AG (Buchs, Switzerland). Guanidine-HCl was obtained from Acros (Fair Lawn, NJ). BSA, deoxyribose, and dinitrophenyl hydrazine (DNPH) were purchased from Sigma Chemical Co. (Steinheim, Germany).

Determination of antioxidant effect on liposome oxidation. Lecithin (300 mg) was sonicated in an ultrasonic cleaner (Branson 8210; Branson Ultrasonic Corporation, Danbury, CT) in 30 mL of 10 mM phosphate buffer (pH 7.4) for 2 h. To the sonicated solution (0.5 mL, 10 mg lecithin/mL), were added $FeCl_3$ (0.5 mL, 400 mM), ascorbic acid (0.5 mL, 400 mM), and carnosine (0.1–60 mg). The mixture was incubated for 1 h at 37°C, and oxidation was measured by the thiobarbituric acid (TBA) method (12). The absorbance of the sample was read at 532 nm against a blank, which contained all reagents except lecithin.

Determination of antioxidant effect on protein oxidation. The effect of carnosine on protein oxidation was studied by using the method of Yang *et al.* (13) with some modification, with tocopherol as a positive control. The reaction mixture (1.2 mL), containing carnosine (0.01–100 mg), phosphate buffer (20 mM, pH 7.4), BSA (20 mg/mL), $FeCl_3$ (100 μM), H_2O_2 (2.0 mM), and ascorbic acid (200 μM), was incubated for 1 h at 37°C, then 1 mL 20 mM DNPH in 2 M HCl was added to the reaction mixture. One milliliter of cold TCA

(20%, wt/vol) was added to the mixture, followed by centrifugation at 3000 × *g* for 10 min. The protein was washed three times with 2 mL ethanol/ethyl acetate (1:1) and then dissolved in 2 mL 6 M guanidine-HCl (pH 6.5).

Determination of the effects of oxidation on deoxyribose. The determination was carried out as described by Halliwell *et al.* (14). The reaction mixture (3.5 mL), which contained carnosine (0.1–60 mg), deoxyribose (6 mM), H_2O_2 (3 mM), KH_2PO_4-K_2HPO_4 buffer (20 mM, pH 7.4), $FeCl_3$ (400 μM), EDTA (400 μM), and ascorbic acid (400 μM), was incubated at 37°C for 1 h. The extent of deoxyribose degradation was tested by the TBA method. One milliliter 1% TBA and 1 mL 2.8% TCA were added to the mixture, which was then heated in a water bath at 90°C for 20 min. The absorbance of the mixture was read spectrophotometrically at 532 nm.

Determination of radical-scavenging activity. The effects of carnosine on the DPPH radical were estimated according to the method of Hatano *et al.* (15). Carnosine (0.01–60 mg) was added to a water solution (1 mL) of DPPH radical (final concentration of DPPH was 0.2 mM). The mixture was shaken vigorously and allowed to stand for 30 min; the absorbance of the resultant solution was measured at 517 nm with a spectrophotometer (model U-2000; Hitachi). The radical-scavenging activity was expressed as: % inhibition = 100 − [(absorbance increase of sample/absorbance increase of control) × 100].

Determination of reducing ability. The determination of reducing ability was performed by the method of Oyaizu (16). Carnosine (0.01–60 mg) was mixed with phosphate buffer (2.5 mL, 2.0 M, pH 6.6) and 1% potassium ferricyanide (2.5 mL), and the mixture was incubated at 50°C for 20 min. An aliquot (2.5 mL) of TCA (10%) was added to the mixture, which was then centrifuged at 650 × *g* for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated reducing ability.

Generation and scavenging of hydroxyl radical. The method for generating hydroxyl radical from hydrogen peroxide (H_2O_2) and scavenging with salicylic acid was that of Prasad *et al.* (17). The reaction solution contained 0.5 mL of 1 mM salicylic acid in phosphate buffer (0.01 M, pH 7.4) in a 10-mL beaker, to which was added 0.05 mL of H_2O_2 (0.02 M) in deionized distilled water. The final volume of the radical-generating solution was made up to 0.9 mL by the addition of phosphate buffer. This reaction solution was then irradiated with a mineral lamp (UVP, Upland, CA) by placing the lamp directly on top of the beaker for 30 min. After irradiation, 0.1 mL of 3 mM 2,4-dihydroxybenzoic acid (2,4-DHBA) was added to the reaction solution as an internal standard for a final volume of 1.0 mL. The reaction was also carried out in the presence of carnosine (0.01–60 mg).

Chromatography. The reaction solution described above was filtered through a 0.45-μm filter before use. The 20 μL of filtrate was injected into the HPLC system (Shimadzu LC-10AD, Kyoto, Japan). The Lichrosphere RP-18 column (250 × 4 mm, 5 μm; E. Merck) and UV detector (measured at 315 nm) were used to separate the hydroxylation products of salicylic acid. The mobile phase was prepared with 80% deionized water that was 0.03 M in citric acid and 0.03 M in acetic acid buffer (pH 3.6) and 20% methanol with an isocratic system. Flow rate was 1.0 mL/min. The internal standard was 2,4-DHBA.

Measurement of chelating activity of carnosine with metal ions. The chelating activity of carnosine with Fe^{2+} was measured according to the method of Carter (18). Briefly, carnosine (0.01–60 mg) was incubated with 0.05 mL $FeCl_2 \cdot 4H_2O$ (2.0 mM). The reaction was initiated by the addition of 0.2 mL ferrozine (5.0 mM), and the mixture was diluted to 0.8 mL with methanol. After the mixture had reached equilibrium (10 min), the absorbance at 562 nm was read. EDTA served as the positive control, and an untreated sample served as the negative control.

Statistical analysis. Statistical analysis involved use of the Statistical Analysis System (19). Analysis of variance was performed by ANOVA procedures. Significant differences ($P < 0.05$) between means were determined by Duncan's multiple range tests.

RESULTS AND DISCUSSION

The antioxidant activity of carnosine in a liposome system, as induced by $FeCl_3$ and ascorbic acid and measured by the TBA method, is plotted in Figure 1. Carnosine in the range of 0–15 mg/mL showed 0–45.3% inhibition of TBARS formation compared to the control. Although the inhibitory effect of carnosine on TBARS formation was inferior to that of Toc (0–15 mg/mL), carnosine showed amount-dependent antioxidant activity on liposome oxidation.

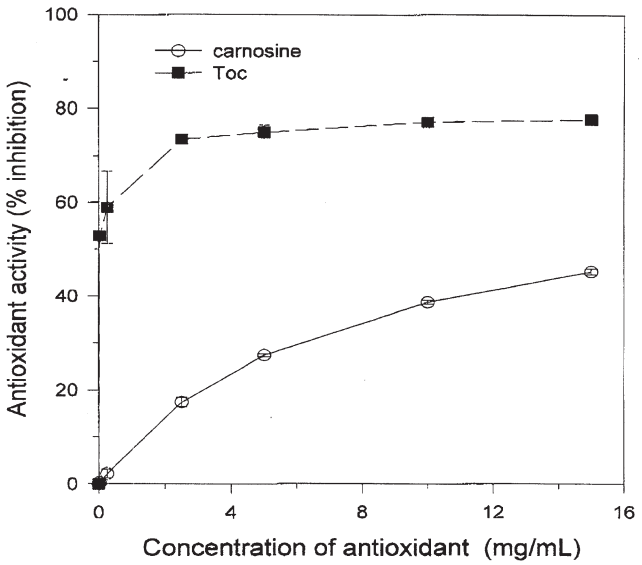

FIG. 1. Antioxidative action of different amounts of carnosine and α-tocopherol (Toc) in a liposome model system. Values represent mean ± SD ($n = 3$).

Yi *et al.* (20) reported that carnosine exhibited antioxidant activity in a linoleic acid model system. However, linoleic acid is not considered to reflect lipid peroxidation completely because of its unique physical properties in aqueous micelles (20). Phospholipids are generally thought to be the major fraction responsible for the oxidative deterioration of foods, because of their greater degree of unsaturation (21). Furthermore, phospholipids, such as PC (lecithin), are believed to be present in high amounts in cell membranes. Thus, phospholipids can be selected as a representative substrate for evaluating antioxidant activity in lipid food systems. In addition, malondialdehyde (MDA) is a TBARS, i.e., oxidative product of phospholipid peroxidation (22). MDA takes part in cross-linking reactions with DNA and proteins. Moreover, MDA is very reactive and can act as a catalyst in the formation of N-nitrosamines in foods containing secondary amines and nitrite (23). As shown in Figure 1, carnosine significally lowers TBARS when compared with the control. This result implies that carnosine may inhibit the oxidation of lipids in a food system as well as protect cell membranes against oxidative damage.

Stadtman (24) reported that some amino acid residues are oxidized to carbonyl derivatives; consequently, the carbonyl content of proteins can be used as a measure of protein damage. Figure 2 shows the effect of carnosine on protein carbonyl formation in albumin in a reaction mixture containing $FeCl_3$, H_2O_2, and ascorbic acid. In the range of 16.67–50 mg/mL of carnosine used, the protective effect against protein oxidation increased with increasing amounts of carnosine. However, in the presence of 0–16.67 mg/mL carnosine, no suppression of protein oxidation occurred. In contrast, the inhibition of protein oxidation increased with the increasing amounts of Toc up to 20 mg, and then decreased with increasing amounts. Thus,

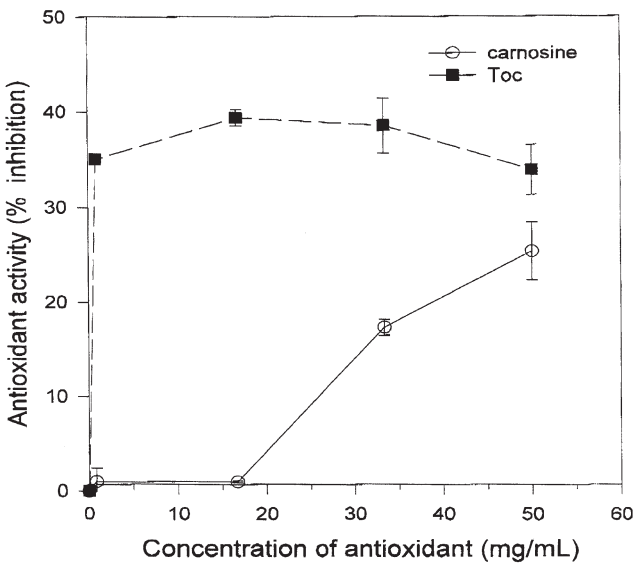

FIG. 2. Protective effect of different amounts of carnosine and α-tocopherol (Toc) on albumin oxidative damage. Values represent mean ± SD (n = 3).

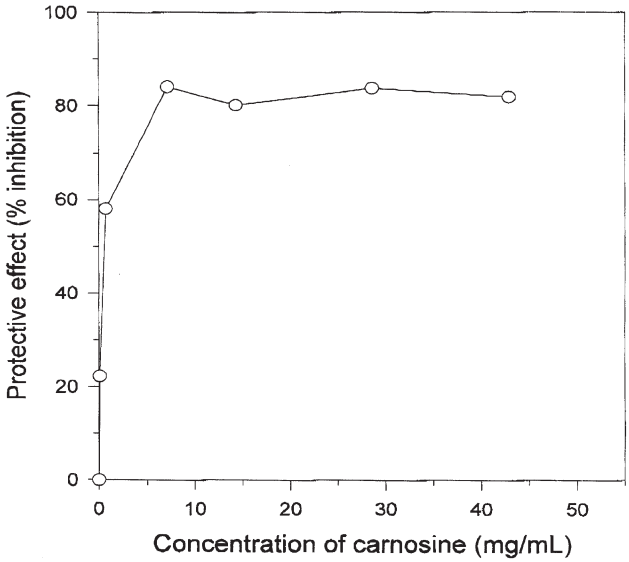

FIG. 3. Protective effect of different amounts of carnosine on deoxyribose oxidative damage. Values represent means; the SD are encompassed by the data points.

in higher amounts, carnosine has an inhibitory effect on oxidative damage and stabilizes protein against oxidation.

The effect of carnosine on deoxyribose oxidation, as induced by Fe^{3+}/H_2O_2 and measured by the TBA method, is shown in Figure 3. The inhibitory effect of carnosine on deoxyribose damage increased with increasing amount up to 10 mg. No significant differences ($P > 0.05$) were found in inhibitory activity with further increases. At 10 mg, carnosine showed 84.0% inhibition of deoxyribose damage as compared with the control.

Antioxidants are classified into four types, including chain breaker, peroxide decomposer, metal chelator, and reactive oxygen scavenger (25), based on the mechanism of action. Hence, to explain the antioxidative action of carnosine on lipids and nonlipids, one must elucidate the mechanism of its antioxidative activity.

Table 1 shows the antioxidative properties of carnosine as reflected by its reducing ability and inhibition of free radical and hydroxyl radical formation. The reducing ability of carnosine increased with an increased amount of carnosine. [Reducing ability is related to a reducing agent that contributes to the breaking of a radical chain by donation of a hydrogen atom (26).] The correlation coefficients between the reducing ability and the inhibition of liposome, albumin, and deoxyribose oxidation were r = 0.92, 0.83, and 0.41, respectively. The high positive correlation between reducing ability and the inhibitory effect on liposome and albumin oxidation indicates that the reducing ability of carnosine has an antioxidant effect on liposome and albumin.

As shown in Table 1, the scavenging effect of carnosine toward DPPH radical increased with increasing amounts of carnosine. However, carnosine in the range of 0–12 mg/L showed only 0–19.0% scavenging activity. Obviously,

TABLE 1
Antioxidative Properties of Carnosine

Amount	Reducing ability[a,b]	Inhibition (%)[b]	
(mg)	Absorbance at 700 nm	DPPH radical[c]	Hydroxyl radical[d]
0	0.05 ± 0.01^{E}	0C	0^{F}
0.1	0.04 ± 0^{E}	8.60 ± 1.27^{B}	39.7 ± 2.35^{E}
1	0.05 ± 0^{E}	8.30 ± 0.57^{B}	65.6 ± 1.57^{D}
10	0.14 ± 0^{D}	10.2 ± 5.23^{AB}	74.3 ± 1.78^{C}
20	0.27 ± 0.01^{C}	13.3 ± 1.77^{A}	81.7 ± 2.51^{B}
40	0.50 ± 0^{B}	16.7 ± 1.06^{A}	84.0 ± 0.26^{B}
60	0.70 ± 0.02^{A}	19.0 ± 0.57^{A}	87.8 ± 1.21^{A}

[a]The reducing power of carnosine was determined by absorbance at 700 nm.
[b]Values in the same column with different superscript roman letters are significantly different ($P < 0.05$).
[c]Inhibition (%) = 100 − [absorbance increase of sample/absorbance increase of control) × 100].
[d]Inhibition (%) = 100 − [amounts of salicylic acid of sample/amounts of salicylic acid of control) × 100].

carnosine at the quantity used was insufficient to scavenge DPPH radical.

The reaction of the hydroxyl radical with carnosine was investigated by generating the hydroxyl radical by photolysis of H_2O_2 with UV light. The radical was trapped by salicylic acid, which is hydroxylated in order to produce hydroxyl radical adducts 2,3- and 2,5-DHBA. Thus, the decrease in salicylic acid concentration can be used to as a measure of the ability of antioxidants to act as hydroxyl radical scavengers (17). As expected, the scavenging effect of carnosine on the hydroxyl radical increased with increasing amounts of carnosine (Table 1). Carnosine in the range of 0.083–50 mg/mL inhibited salicylic acid adduction by 39.7–87.8%, respectively, indicating that carnosine is a significant hydroxyl radical scavenger. The correlation coefficients between the scavenging hydroxyl radical and the inhibitory activity on liposome, albumin, and deoxyribose oxidation were 0.60, 0.29, and 0.97, respectively. The positive correlations indicate that the activity of the scavenging hydroxyl radical are responsible for the inhibitory effect on deoxyribose, followed by liposome, and albumin.

Halliwell and Aruoma (27) noted that when the mixture of $FeCl_3$, EDTA, and H_2O_2 is incubated with deoxyribose in phosphate buffer (pH 7.4), the hydroxyl radical generated attacks the deoxyribose and results in a series of reactions that lead to the formation of MDA. In addition, hydroxyl radical scavengers added to the reaction mixture compete with deoxyribose for ·OH to an extent depending on the rate constant of carnosine for the reaction with ·OH and its concentration relative to deoxyribose (28). Obviously, the rate constant of carnosine for reaction with the hydroxyl radical was greater than that of deoxyribose. This property may explain why carnosine can protect deoxyribose from oxidative damage.

Figure 4 shows that the chelating effect of carnosine on Fe^{2+} ion was concentration dependent. Carnosine at 0.42 mg/mL had 80.1% chelating activity. Although this effect was relatively small when compared to that of EDTA, the metal-binding capacity of carnosine is remarkable. Gordon (29) noted that chelating agents that form σ bonds with a metal are

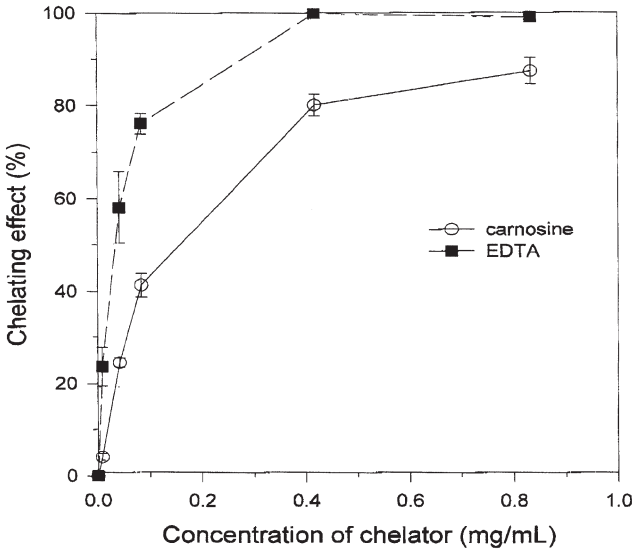

FIG. 4. Chelating effects of carnosine on iron ion. Values represent mean ± SD ($n = 3$).

effective as secondary antioxidants because they reduce the redox potential and thereby stabilize the oxidized form of the metal ion.

Smith *et al.* (30) noted that the chelating effect of metal ions plays a significant role in protecting deoxyribose against degradation and renders metal ions inactive or poorly active in Fenton reactions. In addition, both liposome peroxidation and protein damage were induced by a Fenton reaction. These results indicate that carnosine displays a capacity for iron binding and suggests that its action as a liposome, deoxyribose, or protein protector may partially relate to its interaction with iron.

Carnosine is effective in protecting lipids and nonlipids in various oxidative model systems. The mechanism by which carnosine protects against oxidation may involve reducing ability, reactive hydroxyl radical scavenging, and metal binding.

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