Inhibition of Lipid Oxidation by Carnosine[1]

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The antioxidant activity of carnosine, a β-alanine-histidine dipeptide found in skeletal muscle, was investigated. Carnosine (25 mM) inhibited the catalysis of lipid oxidation by iron, hemoglobin, lipoxidase and singlet oxygen from 35–96% suggesting that the antioxidant mechanism of carnosine is not solely due to metal chelation. Heating the carnosine at 100°C for 15 min had no effect on its ability to inhibit these lipid oxidation catalysts, and the activity of carnosine was not affected over the pH range of 5.1–7.1. Studies using tocopherol-containing liposomes suggest that carnosine and tocopherol do not act synergistically to inhibit lipid oxidation. These data indicate that carnosine has excellent potential for use as a natural antioxidant in processed foods.

KEY WORDS: **Anserine, antioxidant, carnosine, lipid oxidation.**

Lipid oxidation (peroxidation) is a major cause of chemical spoilage in foods. The major pathway of the oxidation of unsaturated fatty acid involves a self-catalytic free radical mechanism that produces lipid peroxides (1). Americans are consuming more foods high in unsaturated fatty acids because of their concern about reducing the risk of heart diseases. Foods high in unsaturated fatty acids are extremely susceptible to lipid oxidation; therefore diets high in unsaturated fatty acids could include high amounts of lipid oxidation products. Lipid oxidation products may contribute to the development or exacerbation of many of mankind's most common ills including cancer, heart attack, stroke, and emphysema (2). Therefore, it is important for food processors to control lipid oxidation in their products not only to preserve sensory quality but also to provide safe, wholesome foods.

Lipid oxidation can be catalyzed in foods by transition metals, hemoproteins, singlet oxygen and lipoxygenase (3). These four catalysts oxidize lipids by different mechanisms. Iron catalyzes lipid oxidation in the presence of reducing agents such as ascorbic acid, superoxide anion and cysteine (3) while metmyoglobin and methemoglobin initiate membranal lipid peroxidation when activated by hydrogen peroxide (4). Singlet oxygen directly reacts with double bonds of unsaturated fatty acids to produce hydroperoxides (3). Singlet oxygen is generated from triplet oxygen in presence of a sensitizer (i.e. riboflavin) and light. Lipoxidase enzymically catalyzes the formation of linoleic acid hydroperoxides (5). Lipoxidase is found in many plant foods such as legumes.

The dipeptides, carnosine (β-alanyl-methylhistidine), and anserine (β-alanyl-L-1-methylhistidine) are found in the skeletal muscle tissue of most vertebrates (6). Boldyrev and co-workers (7) reported that the concentrations of carnosine and anserine found in skeletal muscle (1–25 mM) are capable of inhibiting iron-catalyzed lipid oxidation while individually the amino acids were ineffective. These naturally occurring dipeptides have also been reported to play a role in the regeneration of α-tocopherol (7) suggesting that they could act synergistically with α-tocopherol to inhibit lipid oxidation. These data suggest that carnosine has excellent potential for use as a natural antioxidant in foods.

The purpose of this research was to characterize the antioxidant mechanism of carnosine to determine whether carnosine would be an effective antioxidant in foods. The antioxidant activity of carnosine was investigated in the presence of different lipid oxidation catalysts as well as under various conditions of pH, temperature and in the presence of α-tocopherol.

MATERIALS

Bovine hemoglobin, ascorbic acid, riboflavin, soybean phosphatidylcholine, soybean lipoxidase (Type I), linoleic acid, carnosine and α-tocopherol were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade or purer.

METHODS

Liposomes were prepared from soybean phosphatidylcholine by homogenization and sonication and quantitated by phosphate determination (8). Liposomes containing tocopherol were prepared by homogenizing phosphatidylcholine (0.26 g) and tocopherol (50 mg) in 50 mL of 0.12 M KCl, 5 mM histidine buffer (pH 7.0). Lipid oxidation studies were performed in a model system (5 mL total volume) containing 0.12 M KCl, 5 mM histidine buffer (pH 7.0 unless otherwise indicated), 0.1 mg phosphatidylcholine liposomes plus lipid oxidation catalysts and varying concentrations of carnosine (8). Heated carnosine was produced by placing a solution of carnosine (625 mM) in a boiling water bath for 15 min and then immediately cooling it on ice. All reactions were run at 37°C for 20 min except for singlet oxygen assays which were run for 3 hr. Lipid oxidation was monitored by measuring thiobarbituric acid reactive substances (TBARS) as described by McDonald and Hultin (9).

Iron-catalyzed lipid oxidation was initiated by iron redox cycling using 15 μM $FeCl_3$ and 100 μM ascorbate. Hydrogen peroxide-activated hemoglobin was prepared by incubating equal concentrations of hemoglobin and hydrogen peroxide for 3 min at room temperature. The hydrogen peroxide-activated hemoglobin was added to the model system to give a final concentration of 30 μM hemoglobin (10). Singlet oxygen was generated by adding 5.0 μM riboflavin to the model system (11) followed by illumination for 3 hr with a 200-watt incandescent light bulb. No oxidation was observed in the absence of either light or riboflavin. Lipoxidase-catalyzed lipid oxidation was initiated by the addition of 15,000 units of lipoxidase and 0.1 mM linoleic acid to the model system.

All experiments were performed on triplicate samples and were repeated a minimum of two times.

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TABLE 1

Inhibition of Different Lipid Oxidation Catalysts by Carnosine

	Percent inhibition[a]	
Catalyst	Carnosine (25 mM)	Heated carnosine (25 mM)[b,c]
Fe + ascorbate	77	76
H_2O_2 + hemoglobin	96	96
Singlet oxygen	61	65
Lipoxidase	35	39

[a] Conditions for the assays are outlined in the Methods section. Percent inhibition was calculated as {1−(TBARS produced in the presence of carnosine ÷ TBARS produced in the absence of carnosine)} × 100.

[b] Carnosine was heated for 15 min in a boiling water bath and immediately cooled on ice.

[c] Heated samples were not significantly different than unheated samples (P < 0.05).

RESULTS

The ability of heated and unheated carnosine to inhibit iron-, hemoglobin-, lipoxidase- and singlet oxygen-catalyzed lipid oxidation is shown in Table 1. Carnosine (25 mM) inhibited from 35–96% of the activity of the oxidation catalysts. Heating carnosine prior to addition to the model system did not significantly affect its activity ($p < 0.05$). Carnosine was most effective at inhibiting the production of TBARS by hydrogen peroxide-activated hemoglobin and least effective in the presence of lipoxidase.

Figure 1 shows the effect of pH on the inhibition of iron- and hemoglobin-catalyzed lipid oxidation. Over the pH range of 5.1–7.0, the production of TBARS by iron and ascorbate was inhibited from 70–79%. When hydrogen peroxide-activated hemoglobin was used as the oxidation catalyst, little to no TBARS production was observed at a pH ⩽5.78. Harel and Kanner (4) also observed the loss in activity of hydrogen peroxide-activated hemoglobin in this pH range. Over the pH range of 5.8–7.1, carnosine inhibited hydrogen peroxide-activated hemoglobin from 60–100%. The low percent inhibition of hydrogen peroxide-activated hemoglobin at pH 5.8 could be due to the low concentrations of TBARS produced in the absence of carnosine (1.5 nmole TBARS/20 min at pH 5.8 vs 25.0 nmole TBARS/20 min at pH 6.25).

The ability of carnosine to synergistically inhibit lipid oxidation in the presence of tocopherol was tested by incorporating tocopherol into the phosphatidylcholine liposomes. Figure 2 shows the percent inhibition of iron-catalyzed lipid oxidation by increasing concentrations of carnosine from 1–25 mM in the presence and absence of tocopherol. If carnosine and tocopherol were acting synergistically to inhibit lipid oxidation, then the percent inhibition by the mixture of carnosine and α-tocopherol should be greater than the sum of the inhibition by the two antioxidants separately. The sum of the percent inhibition by the two antioxidants separately was calculated by adding the percent inhibition due to the α-tocopherol alone (27%) to the percent inhibition observed by varying concentrations of carnosine (1–25 mM) in the absence of α-tocopherol (Table 2). As can be seen in Table 2, the sum of the inhibition by the two antioxidants separately (corrected % inhibition) was equal to or greater than the percent inhibition measured when the antioxidants were used in combination. Therefore, no synergistic relationships between carnosine and α-tocopherol were observed in this system.

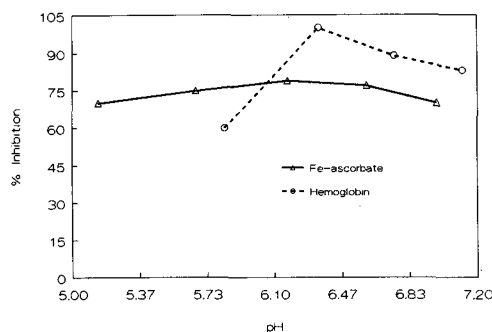

FIG. 1. The effect of pH on the ability of carnosine to inhibit iron- and hydrogen peroxide-activated hemoglobin-catalyzed lipid oxidation. All assays contained 25 mM carnosine. Experimental conditions were the same as those described in Table 1.

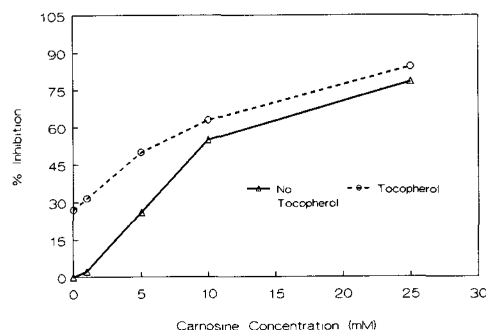

FIG. 2. Inhibition of iron-catalyzed lipid oxidation by carnosine in the presence and absence of α-tocopherol. Experimental conditions were the same as those described in Table 1. Tocopherol-containing liposomes were prepared by homogenizing 50 mg of α-tocopherol with 0.26 g phosphatidylcholine.

TABLE 2

The Effect of Tocopherol on Inhibition of Iron-Catalyzed Lipid Oxidation by Carnosine

Carnosine concentration	No tocopherol[a] (% inhibition)	Tocopherol[a] (% inhibition)	Corrected[b] (% inhibition)
0	0	27	—
1 mM	2.3	31.5	29.3
5 mM	26	50	53
10 mM	55	63	82
25 mM	79	85	106

[a] Conditions for the assays are outlined in the Methods section. Percent inhibition was calculated as {1−(TBARS produced in the presence of carnosine ÷ TBARS produced in the absence of carnosine)} × 100.

[b] Corrected percent inhibition was calculated by adding the percent inhibition due to tocopherol alone (no carnosine present) to the percent inhibition due to the carnosine alone (1–25 mM).

DISCUSSION

Antioxidants inhibit lipid oxidation by chelating metals and absorbing free radicals (3). Boldyrev and co-workers (7) reported inhibition of iron-catalyzed lipid oxidation by carnosine, but no evidence is available to determine whether this inhibition was due to chelation or free radical scavenging. In order to better understand the antioxidant mechanism of carnosine, we tested its ability to inhibit several different lipid oxidation catalysts. Kanner and Harel (12) observed that chelating agents such as ethylenediaminetetraacetic acid (EDTA) and desferrioxamine had no effect on the activity of hydrogen peroxide-activated myoglobin unless hydrogen donors or free radical scavengers were present. Singlet oxygen and lipoxidase would also not be inhibited by chelating agents since they promote the formation of lipid peroxides without the involvement of free metals (3,5). The ability of carnosine to inhibit hydrogen peroxide-activated hemoglobin, lipoxidase and singlet oxygen in addition to iron (Table 1) suggests that its antioxidant mechanism is not due solely to chelation.

The ability of carnosine to inhibit lipid oxidation catalysts besides metals could be due to its ability to scavenge free radicals or donate hydrogen ions. Uchida and Kawakishi (13,14) have found that histidine (free and in bovine serum albumin) decomposes in the presence of free radical generating systems. The observed loss of histidine was suggested to be due to oxygen radical attack of the imidazole ring. The function of β-alanine in the antioxidant activity of carnosine is not clear but it could be acting to stabilize the histidine radical. This could be due, in part, to the peptide bond since free β-alanine and histidine do not inhibit lipid oxidation (7). Boldyrev and co-workers (7) did examine the antioxidant activity of the α-alanine-histidine dipeptide and found it ineffective. This suggests a specific role of β-alanine, but no information is available on the activity of other histidine-containing dipeptides. Uchida and Kawakishi (13) also found that histidine binds Cu^{+2}; therefore the antioxidant function of carnosine could be due to a combination of chelation and free radical scavenging. More research is needed to further elucidate the properties of carnosine which make it an effective inhibitor of lipid oxidation.

Tocopherol inhibits lipid oxidation by scavenging free radicals (15). The resulting tocopherol radical is incapable of further inhibiting lipid oxidation unless the tocopherol is regenerated. Reducing substances such as ascorbate have been reported to reactivate the tocopherol radical (15). Interaction of two antioxidants such as tocopherol and ascorbate often results in a more pronounced (synergistic) inhibition of lipid oxidation (1). Boldyrev and co-workers (16) reported carnosine as capable of regenerating tocopherol but we did not observe any synergistic antioxidant interactions between the two in our model system (Table 2). This lack of synergistic interaction could be due to the artificial membrane system used. Addition of tocopherol to the liposomes by homogenization and sonication could result in a different alignment of the tocopherol compared to natural membrane systems. Therefore, interactions between carnosine and tocopherol could be sterically hindered preventing the regeneration of tocopherol. More research is needed to determine whether carnosine can be used to increase the effectiveness of tocopherol in membranes and foods.

The concentration of carnosine found in chicken, beef and pork skeletal muscle—ca. 10 mM (6)—inhibited iron-catalyzed lipid oxidation 55% (Fig. 2), and the antioxidant activity of carnosine was unaffected over the pH range found in meat—5.1–7.1 (Fig. 1). These data suggest that carnosine may act as a natural antioxidant in muscle foods. The ability of carnosine to inhibit iron-catalyzed lipid oxidation increased as the concentration was increased above *in situ* concentrations (Fig. 2) and heating carnosine had no effect on its ability to inhibit different lipid oxidation catalysts (Table 1). These properties make carnosine an excellent candidate for use as a natural antioxidant in processed foods. Research is currently underway to determine whether carnosine can inhibit lipid oxidation in meat and dairy products.

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