Antioxidant and Emulsifying Activity of *N*-(Long-chain-acyl)histidine and *N*-(Long-chain-acyl)carnosine

Hironobu Murase,*,† Akihiko Nagao,‡ and Junji Terao‡

CCI Corporation, 12 Shinhazama, Seki, Gifu 501-32, Japan, and National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki 305, Japan

The antioxidant activity of *N*-(long-chain-acyl)histidine-containing compounds was investigated. In a homogeneous solution of methyl linoleate with a radical initiator, these compounds suppressed the production of methyl linoleate hydroperoxides. When the oxidation of phosphatidylcoline liposomes was induced by ferrous ion and ascorbic acid, *N*-(long-chain-acyl)histidine and *N*-(long-chain-acyl)-carnosine could suppress the oxidation more efficiently than histidine and carnosine. The emulsifying activities of these compounds were found to be higher than those of conventional surfactants, that is, casein, Tween 80, and Triton X-100.

INTRODUCTION

Carnosine (β-alanyl-L-histidine) is present at millimolar concentrations in several mammalian tissues, including skeletal muscle and brain (Crush, 1970). Although it is accepted that carnosine should play some physiological role in muscle and brain (Duane and Peters, 1988), its function has not been clarified as yet. Antioxidant activity of carnosine has recently become a subject of considerable interest. Kohen et al. (1988) reported that carnosine could scavenge peroxyl radicals. Evidence that carnosine inhibits copper-catalyzed oxidative reactions has been reported by Decker et al. (1992). Dahl et al. (1988) have found that carnosine is an efficient singlet oxygen quencher.

Amphipathic derivative of superoxide dismutase (SOD) was synthesized by linking hydrophobic fatty acids covalently to the lysyl residues of the enzyme (Ando et al., 1988). This acylated SOD derivative can dismutate extracellular superoxide radicals generating in the vicinity of membranes more efficiently than original SOD. We have an idea that the increase of solubility of carnosine (structure 1 in Figure 1) and histidine in lipids enhances antioxidant activity on lipid peroxidation. Consequently, we synthesized some *N*-long-chain-acyl derivatives of histidine and carnosine. Because of their amphipathic nature, *N*-long-chain-acyl derivatives were dissolved in apolar solvent and were efficiently incorporated into phosphatidylcholine liposomes. This study demonstrates that *N*-(long-chain-acyl)carnosine (structure 2 in Figure 1) and *N*-(long-chain-acyl)histidine possess antioxidant activity toward lipid peroxidation. Moreover, it is strongly suggested that they can suppress the peroxidation of membrane lipids when incorporated into the membranes. Excellent emulsifying activity of these compounds is described also.

MATERIALS AND METHODS

Materials. Egg yolk phosphatidylcholine (PC) and methyl linoleate were purchased from Sigma Chemical Co. (St. Louis, MO). PC was purified to remove contaminant hydroperoxides by reversed-phase liquid chromatography (Terao et al., 1987). PC hydroperoxides were prepared by photooxidation and purified by reversed-phase liquid chromatography (Terao et al., 1985).

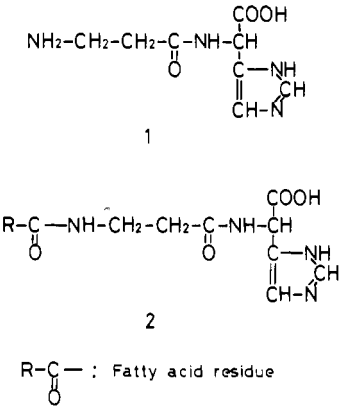

Figure 1. Structure of carnosine (1) and *N*-acylcarnosine (2).

Methyl linoleate was purified by column chromatography with Florisil (Terao and Matsushita, 1986). Carnosine was obtained from Peptide Institute, Inc. (Osaka, Japan). 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN), L-histidine (His), and glycine (Gly) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). *N*-Acyl amino acids and *N*-acyl peptides were synthesized by allowing *N*-hydroxysuccinimide esters of fatty acids to react with free amino acids or with peptides in aqueous solution (Lapidot et al., 1967). Other reagents and solvents were of analytical grade and used without purification.

Procedures. *Antioxidant Activity of N-Acyl Compounds in Solution.* The reaction mixture contained 110 μmol of methyl linoleate, an appropriate concentration of *N*-acyl compound, and 11 μmol of AMVN in 1.1 mL of hexane/2-propanol (1:1 v/v). The oxidation was initiated by adding a hexane/2-propanol (1:1 v/v) solution of AMVN, and the mixture was incubated at 37 °C. At regular intervals, aliquots of the reaction mixture were withdrawn and injected into the HPLC column. Methyl linoleate hydroperoxides were determined under the same conditions described previously (Terao and Matsushita, 1986).

Antioxidant Activity of N-Acyl Compounds in a Suspension of Multilamellar Liposomes. Multilamellar liposomes of egg yolk PC were prepared as follows. PC and PC hydroperoxides in chloroform/methanol (2:1 v/v) were put into a test tube, and the solvent was evaporated using nitrogen gas. The film prepared was dispersed in 1 mL of 10 mM Tris-HCl buffer (pH 7.4) with a Vortex mixer for 30 s followed by sonication with a Branson Sonifier 450 for 1 min at ambient temperature, and then 0.5 mL of 10 mM Tris-HCl buffer (pH 7.4) was added. The liposomes containing *N*-acyl compound were prepared by adding an *N*-acyl compound to the solution of PC and PC hydroperoxide followed by evaporation. Carnosine was dissolved in 10 mM Tris-HCl buffer (pH 7.4), and then the film was dispersed to prepare carnosine-containing liposomes. Alternatively, carnosine was

* Author to whom correspondence should be addressed.
† CCI Co.
‡ National Food Research Institute.

0021-8561/93/1441-1601$04.00/0

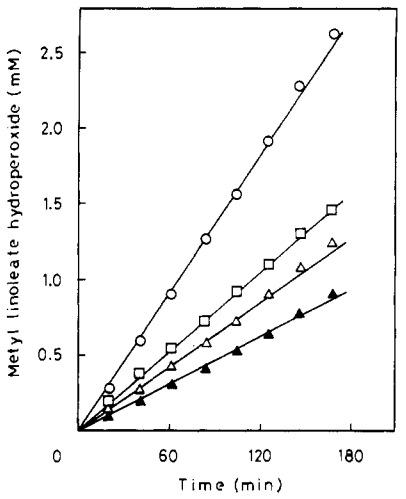

Figure 2. Effect of *N*-acylcarnosine on the oxidation of methyl linoleate in solution. Reaction system consisted of methyl linoleate (100 mM), *N*-acyl compounds, and AMVN (10 mM) in a mixture of hexane/2-propanol (1:1 v/v). (△) 0.2 mM *N*-$C_{18:1}$-carnosine; (▲) 2 mM *N*-$C_{18:1}$-carnosine; (□) 0.2 mM *N*-C_{10}-carnosine; (○) no addition.

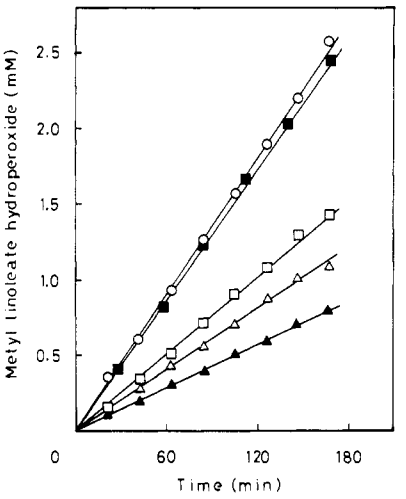

Figure 3. Effect of *N*-acylhistidine and *N*-acylglycine on the oxidation of methyl linoleate in solution. Reaction system consisted of methyl linoleate (100 mM), *N*-acyl compounds, and AMVN (10 mM) in a mixture of hexane/2-propanol (1:1 v/v). (△) 0.2 mM *N*-$C_{18:1}$-His; (▲) 2 mM *N*-$C_{18:1}$-His; (□) 0.2 mM *N*-C_{10}-His; (■) 0.2 mM *N*-$C_{18:1}$-Gly; (○) no addition.

mixed with 10 mM Tris-HCl buffer (pH 7.4) after liposomes were prepared to disperse this compound in the water phase of the liposomal suspension. The prepared liposomal suspension contained 6 μmol of PC, 0.06 μmol of PC hydroperoxide, and 0.015 μmol of antioxidant in 1.4 mL of 10 mM Tris-HCl buffer (pH 7.4). The oxidation was initiated by the addition of 0.1 mL of 10 mM Tris-HCl buffer (pH 7.4) containing 1.5 mM $FeSO_4$ and 15 mM ascorbic acid and then incubated at 37 °C with continuous shaking. At regular intervals, aliquots of the reaction mixture were withdrawn and peroxidation was monitored by measuring TBA reacting substances (TBARS) (Uchiyama and Mihara, 1978).

Assay of Emulsifying Activity. The emulsifying activity was measured in triplicate according to the turbimetric method reported by Pearce and Kinsella (1978). The solution containing *N*-acyl compounds or conventional surfactants and casein at 0.2% (w/v) in 50 mM potassium phosphate buffer (pH 7.0) was added to soybean oil in a small test tube and preincubated at 30 °C. The mixture of oil to water (ratio of 0.5) was mixed well with a Vortex mixer and sonicated with a Branson Sonifier 450. After 5 min of incubation at 30 °C, 20 μL of the resulting emulsion was diluted with 0.1% sodium dodecyl sulfate, and then absorbance at 500 nm was measured to evaluate the stability of emulsion. The ratio of the absorbance obtained by each surfactant to that obtained by *N*-oleoylcarnosine was expressed as relative emulsifying activity.

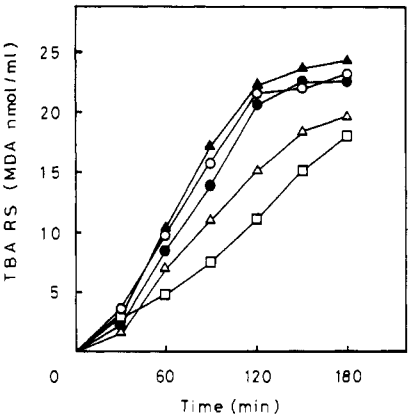

Figure 4. Effect of *N*-acylcarnosine on the oxidation of liposomal membranes induced by ferrous ion–ascorbate. Reaction system consisted of PC (4 mM), PC hydroperoxide (0.04 mM), antioxidants (10 μM), $FeSO_4$ (0.1 mM), and ascorbic acid (1 mM) in 10 mM Tris-HCl buffer, pH 7.4. (□) *N*-$C_{18:1}$-carnosine; (△) *N*-C_{10}-carnosine; (●) carnosine into the liposomal membranes; (▲) carnosine in the water phase; (○) no addition.

RESULTS

Antioxidant activity on the *N*-acyl compounds was investigated by measuring the hydroperoxidation of methyl linoleate via radical chain reaction. Figure 2 shows the effect of *n*-(long-chain-acyl)carnosine on the rate of formation of methyl linoleate hydroperoxides in solution. In the absence of *N*-acyl compound, the rate of formation of methyl linoleate hydroperoxide was 15 μM min^{-1}. *N*-Caprinoyl (C_{10})carnosine and *N*-oleoyl ($C_{18:1}$)carnosine suppressed the rate of oxidation of methyl linoleate; their rates were 8.6 μM min^{-1} (0.2 mM *N*-C_{10}-carnosine), 7.0 μM min^{-1} (0.2 mM *N*-$C_{18:1}$-carnosine), and 5.0 μM min^{-1} (2 mM *N*-$C_{18:1}$-carnosine), respectively. Figure 3 shows the effect of *N*-(long-chain-acyl)His and *N*-oleoyl-Gly on the rate of formation of methyl linoleate hydroperoxides in solution. The oxidation rate of control was 14.8 μM min^{-1}. These compounds also suppressed the rate of oxidation of methyl linoleate; their rates were 8.3 μM min^{-1} (0.2 mM *N*-C_{10}-His), 6.7 μM min^{-1} (0.2 mM *N*-$C_{18:1}$-His), and 4.7 μM min^{-1} (2 mM *N*-$C_{18:1}$-His), respectively. On the other hand, *N*-oleoyl-Gly did not exert an antioxidant effect on the formation of methyl linoleate hydroperoxides.

The effect of *N*-acyl compounds on the oxidation of liposomal membranes induced by ferrous ion–ascorbate was investigated. Figure 4 shows the effect of *N*-(long-chain-acyl)carnosine and carnosine incorporated into liposomal membranes. *N*-(Long-chain-acyl)carnosine suppressed the formation of TBARS, although carnosine did not exert any effects on this reaction. When carnosine was localized in the water phase of liposomal suspension, decreases of TBARS were not observed. Figure 5 shows the effect of *N*-(long-chain-acyl)His and *N*-oleoyl-Gly incorporated into the liposomal membranes. *N*-(Long-chain-acyl)His also suppressed the formation of TBARS. On the other hand, no effect was observed in the case of *N*-oleoyl-Gly.

N-Oleoyl-His and *N*-oleoylcarnosine formed O/W emulsion at oil to water ratios (v/v) of 0.5. Their emulsifying activities were much higher than those of conventional

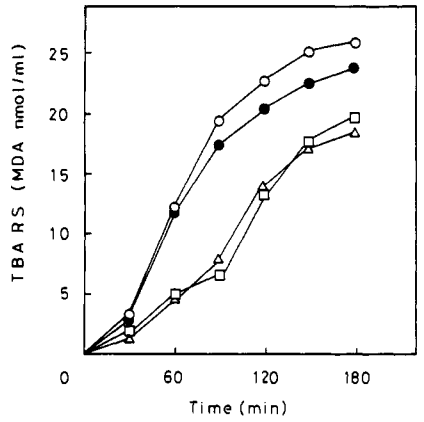

Figure 5. Effect of *N*-acylhistidine and *N*-acylglycine on the oxidation of liposomal membranes induced by ferrous ion–ascorbate. Reaction system consisted of PC (4 mM), PC hydroperoxide (0.04 mM), antioxidants (10 μM), $FeSO_4$ (0.1 mM), and ascorbic acid (1 mM) in 10 mM Tris-HCl buffer, pH 7.4. (□) *N*-$C_{18:1}$-His; (△) *N*-C_{10}-His; (●) *N*-$C_{18:1}$-Gly; (○) no addition.

Table I. Emulsifying Activity of *N*-Oleoyl Compounds, Conventional Surfactants, and Casein[a]

compound	rel act., %	compound	rel act., %
N-oleoylcarnosine	100	casein	24
N-oleoylhistidine	82	Tween 80	46
		Triton X-100	10

[a] The activity is indicated as percentage of the absorbance (500 nm) for *N*-oleoylcarnosine. The ratio (v/v) of oil to water was 0.5.

surfactants, for example, casein, Tween 80, and Triton X-100 (Table I).

DISCUSSION

Recently, considerable studies have indicated that carnosine and histidine act as antioxidants. In practice, Decker and Crum (1991) reported that carnosine could be used as an antioxidant in muscle foods. The antioxidant activity of these compounds seems to be attributed to hydrogen-donating ability (Kohen et al., 1988) and chelating ability (Lambeth et al., 1982; Uchida and Kawakishi, 1989; Decker et al., 1992) of the imidazole ring. However, carnosine and histidine are water-soluble substances, so the reactivity of these compounds toward lipid radicals seems to be low. Because *N*-(long-chain-acyl)carnosine and *N*-(long-chain-acyl)His retain the imidazole ring structure, these compounds are expected to possess antioxidant ability similar to that of carnosine and histidine. Nevertheless, the physical properties of *N*-(long-chain-acyl)carnosine and *N*-(long-chain-acyl)His are different from those of carnosine and His. These derivatives have good solubility in apolar solvent because of the presence of the hydrophobic fatty acid moiety.

To evaluate the antioxidant activity of these *N*-long-chain-acyl compounds, we used a radical initiator-induced peroxidation of methyl linoleate in an apolar solvent. *N*-(Long-chain-acyl)carnosine and *N*-(long-chain-acyl)His suppressed the formation of methyl linoleate hydroperoxides (Figures 2 and 3). These results verify that these acyl compounds possess antioxidant activity. The activity seems to be attributed to the lipid peroxyl radical-trapping ability of His moiety (Kohen et al., 1988). The antioxidant activities of *N*-$C_{18:1}$ derivatives of His and carnosine were higher than those of their *N*-C_{10} derivatives. Thus, oleoyl derivatives have higher peroxyl radical-trapping ability than caprinoyl derivatives in solution. This result may be explained by the idea that the accessibility of *N*-acyl compound toward peroxyl radical is enhanced by the increase of hydrophobicity of the fatty acid residue. On the other hand, *N*-oleoyl-Gly did not suppress that oxidation of methyl linoleate (Figure 3). This result suggests that the hydrogen next to the nitrogen of the amide bond is not responsible for the activity, as compared with the imidazole ring.

In ferrous ion–ascorbate-induced peroxidation of liposomes, *N*-(long-chain-acyl)carnosine and *N*-(long-chain-acyl)His suppressed the formation of TBARS when incorporated into the liposomal membranes (Figures 4 and 5). On the other hand, carnosine itself did not suppress the oxidation efficiently at micromolar concentrations when incorporated into the liposomal membranes or when located in the aqueous region (Figure 4). We suppose that the amino acid residue of *N*-(long-chain-acyl)carnosine and *N*-(long-chain-acyl)His is located on the surface of liposomal membranes so that its antioxidant effect is enhanced. *N*-Oleoyl-Gly is not an effective antioxidant in the ferrous ion–ascorbate system. Therefore, chelation of iron by the amide bond is not concerned in this reaction. The ability of *N*-(long-chain-acyl)carnosine and *N*-(long-chain-acyl)His to inhibit ferrous ion–ascorbate-induced peroxidation of liposomes seems to be due to the combination of the chelation of the iron by the histidine moeity (Lambeth et al., 1982) and the lipid peroxyl radical-trapping ability of histidine moiety. It has been suggested that iron is one of the major prooxidant factors in lipid peroxidation of foods (Kanner et al., 1988). Therefore, *N*-(long-chain-acyl)His and -carnosine are likely to be useful to prevent lipid peroxidation in foods.

In addition, *N*-oleoylcarnosine and *N*-oleoyl-His were found to form O/W emulsions and possess higher emulsifying activity than the tested surfactants and casein.

In conclusion, *N*-(long-chain-acyl)carnosine and *N*-(long-chain-acyl)His can serve as both effective antioxidants and emulsifying agents.

LITERATURE CITED

Ando, Y.; Inoue, M.; Utsumi, T.; Morino, Y.; Araki, S. Synthesis of acylated SOD derivatives which bind to the biomembrane lipid surface and dismutate extracellular superoxide. *FEBS Lett.* **1988**, *240*, 216–220.

Crush, K. G. Carnosine and related substances in animal tissues. *Comp. Biochem. Physiol.* **1970**, *34*, 3–30.

Dahl, T. A.; Midden, W. R.; Hartman, P. E. Some prevalent biomolecules as defenses against singlet oxygen damage. *Photochem. Photobiol.* **1988**, *47*, 357–362.

Decker, E. A.; Crum, A. D. Inhibition of Oxidation Rancidity in Salted Ground Pork by Carnosine. *J. Food Sci.* **1991**, *56*, 1179–1181.

Decker, E. A.; Crum, A. D.; Calvert, J. T. Differences in the Antioxidant Mechanism of Carnosine in the Presence of Copper and Iron. *J. Agric. Food Chem.* **1992**, *40*, 756–759.

Duane, P.; Peters, T. J. Serum carnosine activities in patients with alcoholic chronic skeletal muscle myopathy. *Clin. Sci.* **1988**, *75*, 185–190.

Kanner, J.; Sofer, F.; Harel, S.; Doll, L. Antioxidant Activity Ceruloplasmin in Muscle Membrane and in Situ Lipid Peroxidation. *J. Agric. Food Chem.* **1988**, *36*, 415–417.

Kohen, R.; Yamamoto, Y.; Cundy, K. C.; Ames, B. N. Antioxidant activity of carnosine, homocarnosine and anserine present in muscle and brain. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 3175–3179.

Lambeth, D. O.; Ericson, G. R.; Yorek, M. A.; Ray, P. D. Implications for in vitro studies of the autoxidation of ferrous ion and the iron-catalyzed autoxidation of dithiothreitol. *Biochim. Biophys. Acta* **1982**, *719*, 501–508.

Lapidot, Y.; Rappoport, S.; Wolman, Y. Use of esters of *N*-hydroxysuccinimide in the synthesis of *N*-acylamino acids. *J. Lipid Res.* **1967**, *8*, 142–145.

Pearce, K. N.; Kinsella, J. E. Emulsifying Properties of Proteins: Evaluation of a Turbidimetric Technique. *J. Agric. Food Chem.* **1978**, *26*, 716–723.

Terao, J.; Matsushita, S. The Peroxidizing Effect of α-Tocopherol on Autoxidation of Methyl Linoleate in Bulk Phase. *Lipids* **1986**, *21*, 255–260.

Terao, J.; Asano, I.; Matsushita, S. Preparation of Hydroperoxy and Hydroxy Derivatives of Rat Liver Phosphatidylcholine and Phosphatidylethanolamine. *Lipids* **1985**, *20*, 312–317.

Terao, J.; Boey, P. L.; Murakami, H.; Matsushita, S. Quinone formation from carcinogenic benzo[a]pyrene mediated by lipid peroxidation in phosphatidylcholine liposomes. *Arch. Biochem. Biophys.* **1987**, *254*, 472–481.

Uchida, K.; Kawakishi, S. Ascorbate-Mediated Specific Modification of Histidine-Containing Peptides. *J. Agric. Food Chem.* **1989**, *37*, 897–901.

Uchiyama, M.; Mihara, M. Determination of Malonaldehyde Precursor in Tissues by Thiobarbituric Acid Test. *Anal. Biochem.* **1978**, *86*, 271–278.

Received for review November 2, 1992. Revised manuscript received April 1, 1993. Accepted June 28, 1993.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts*, September 1, 1993.