# Antioxidative Properties of Histidine-Containing Peptides Designed from Peptide Fragments Found in the Digests of a Soybean Protein

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The properties of 22 synthetic peptides containing histidine, which were designed on the basis of the antioxidative peptide (Leu-Leu-Pro-His-His) derived from proteolytic digests of a soybean protein, were examined with regard to their antioxidative activity against the peroxidation of linoleic acid and the scavenging effects on active oxygen and free radical species. The antioxidative activities of these peptides in an emulsion oxidation system using 2,2′-azobis(2-amidinopropane) dihydrochloride as a radical initiator correlated well within an aqueous system. Although the histidine-containing peptides had a quenching activity on singlet oxygen, they did not show antioxidative activity in an 2,2′-azobis(2,4-dimethylvaleronitrile)-induced oxidation system or scavenging effects on 1,1-diphenyl-2-picrylhydrazyl radical and superoxide. The metal-ion chelating activities and the hydrophobicities of these peptides showed no direct correlation with their antioxidative activities. Leu-Leu-Pro-His-His was modified with a hydroxyl radical in an aqueous ethanol system during the peroxidation of linoleic acid.

**Keywords:** *Antioxidative peptide; histidine; antioxidant; metal chelate; radical scavenger*

## INTRODUCTION

Proteins have been shown to have antioxidative activities against the peroxidation of lipids and/or fatty acids upon hydrolysis. Previously we had isolated six antioxidative peptides from the proteolytic digests of a soybean protein (Chen et al., 1995). Based on the smallest peptide, Leu-Leu-Pro-His-His (LLPHH), 28 synthetic peptides were constructed and their antioxidative activities against the peroxidation of linoleic acid were compared in an aqueous system (Chen et al., 1996). The segment HH in LLPHH was found to play a major role in the antioxidative activity of the peptide. The addition of Leu or Pro to the N terminus of HH increased the activity, and PHH was the most antioxidative among the tested peptides. Furthermore, the peptides showed synergistic effects with nonpeptidic antioxidants; however, the magnitude of the effects did not correlate with the antioxidative activities of the peptides.

His-containing peptides have been known to be antioxidative. For example, the antioxidative properties of carnosine, which is a $\beta$-alanyl-L-histidine dipeptide found in animal skeletal muscle, have been extensively reviewed (Chan and Decker, 1994; Quinn et al., 1992). Carnosine is antioxidative in different oxidation systems, including phosphatidylcholine liposomes, linoleic acid emulsions, skeletal muscle microsomes, and sarcoplasmic reticulum membranes. The antioxidative mechanism of carnosine is not well understood but has been suggested to be due to a combination of metal-ion chelation, free radical scavenging (Kohen et al., 1988; Decker et al., 1994), and singlet oxygen quenching (Dahl et al., 1988). The hydrophilic nature of carnosine is attributed to be of physiological significance in endogenous antioxidant systems. When the oxidation of phosphatidylcholine liposomes was induced by ferrous ion and ascorbic acid, *N*-(long-chain-acyl)-carnosine and *N*-(long-chain-acyl)-His could suppress the oxidation more efficiently than intact carnosine and His. This result was explained by the increased accessibility of carnosine and His toward peroxyl radical through the increased hydrophobicity caused by long-chain acyl groups (Murase et al., 1993).

In this study we investigated the properties of 22 synthetic His-containing peptides, which were designed on the basis of an antioxidative peptide derived from a proteolytic digest of soybean protein, to explore the antioxidative mechanism. The antioxidative activity of the peptides was examined in different oxidation systems with hydrophilic and lipophilic radical initiators. The scavenging effects of the peptides on active oxygen and free radical species were measured, as well as the metal-ion chelating activities and the hydrophobicities of the peptides.

## MATERIALS AND METHODS

**Materials.** Linoleic acid (~99%) was purchased from Sigma Chemical Co. (St. Louis, MO), and *d*-$\delta$-tocopherol (~86%) was from Eisai Co. (Tokyo, Japan). Carnosine, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), and xanthine oxidase (activity = 6.6 units/mL) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Methylene blue was obtained fron Koso Chemical Co., Ltd. (Tokyo, Japan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and other reagents were of analytical grade from Nacalai Tesque (Kyoto, Japan). L-Configuration

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amino acid derivatives, coupling reagents, and resins for peptide assembly were SynProPep reagents (Shimadzu Corp., Kyoto, Japan). Peptides were prepared according to the fluorenylmethoxycarbonyl strategy using a simultaneous multiple peptide synthesizer, Model PSSM-8 (Shimadzu), as described (Nokihara et al., 1992; Chen et al., 1996).

**Antioxidative Activity in AAPH-Induced Oxidation System.** Linoleic acid (10 mg) in 3 mL of 0.1 M sodium phosphate buffer (pH 7.0) containing Triton X-100 (1%, w/v) was emulsified by sonication for 2 min. The emulsion (3 mL) was mixed with the peptide in the test cell and stirred at 37 °C for 3 min. The cell was fitted with an oxygen electrode to monitor the dissolved oxygen content in the emulsion. As an accelerator of oxidation, 100 $\mu$L of water-soluble initiator AAPH (50 mg/mL) in distilled water was injected into the emulsion. The oxidation of linoleic acid in the emulsion was monitored by measuring the uptake of the dissolved oxygen with a YSI 5300 biological oxygen monitor (Yellow Springs Instrument Co., Inc., Yellow Springs, OH). The time required for the dissolved oxygen content to decrease by half after injection of the accelerator was defined as the induction period. The relative antioxidative activity was calculated by dividing the induction period of test samples by that of the control. All tests and analyses were run in two or three replicates and averaged.

**Antioxidative Activity in AMVN-Induced Oxidation System.** Linoleic acid (30 mg) in 0.5 mL of ethanol, peptide in 0.8 mL of 0.1 M sodium phosphate buffer (pH 7.0), and AMVN (50 mg) in 0.7 mL of ethanol were mixed in a glass test tube (5 mL volume). The tube was sealed tightly with a silicon rubber cap and incubated at 37 °C. A gas chromatographic method was employed to estimate antioxidative activity by measuring oxygen consumption in the headspace of the tube under the conditions as described (Wang et al., 1991).

**Quenching of Singlet Oxygen.** The antioxidative activity of His-containing peptides on the photosensitized oxidation of linoleic acid was measured according to the method of Yamashoji et al. (1979). Methylene blue was used as a photosensitive dye. Sample dissolved in 1.0 mL of 0.1 M sodium phosphate buffer (pH 7.0), 1.0 mL of 50 mM linoleic acid in ethanol containing 3.7 $\mu$g of methylene blue, and 0.5 mL of distilled water were mixed in a glass test tube (5 mL volume). The tubes were sealed tightly with silicon rubber caps and placed to allow equal irradiation of all samples with a fluorescent lamp (60 W) at 5 °C for 10 h. The distance between the tubes and the lamp was 40 cm. Aliquots of the reaction mixtures were withdrawn at regular intervals using a microsyringe for the measurement of antioxidative activity according to the ferric thiocyanate method (Chen et al., 1995).

**Scavenging of DPPH Radical.** Test sample was mixed with 40 $\mu$M DPPH radical in 2.5 mL of 40% aqueous ethanol. The mixture was shaken vigorously and left for 30 min at room temperature, and the absorbance of the resulting solution was measured at 517 nm.

**Scavenging of Superoxide.** Superoxide was generated in xanthine oxidase and hypoxanthine, and the superoxide scavenging effect of peptides was determined according to the method of Oyanagui (1984).

**Chelating Activity on Metal Ions.** Chelating activity of His-containing peptides on metal ions was measured on a chelating column (HiTrap Chelating Affinity Column, 1 mL, Pharmacia), on which $Cu^{2+}$ or $Zn^{2+}$ was immobilized. The column was treated with 0.5 mL of 0.1 M $CuSO_4$ (or 0.1 M $ZnSO_4$), washed with 5 mL of distilled water, and then equilibrated with at least 5 mL of solution A (50 mM phosphoric acid solution, pH 5.0, containing 0.5 M NaCl). Twenty microliters of peptide (0.2 $\mu$moL/mL) in distilled water was injected into the column, and the nonadsorbed substances were washed off with 10 mL of solution A. Adsorbed peptide was eluted at a flow rate of 1.0 mL/min at 35 °C by monitoring at 210 nm using a linear gradient of solution A to solution B (50 mM phosphoric acid solution, pH 2.0, containing 0.5 M NaCl) (0−60% in 60 min). Zinc ion chelated peptide eluted using a linear gradient of solution A (pH 7.2) to solution B (pH 3.5).

**Hydrophobicity of Peptides.** Hydrophobicity of the peptides was assessed by their retention time on a reversed-phase column. The chromatography was performed using a TSKgel ODS 120T column (5 $\mu$m, 4.6 × 250 mm) (Tosoh, Tokyo, Japan), which was eluted with a linear gradient of acetonitrile (0−20% in 45 min) in the presence of 0.1% trifluoroacetic acid. The retention time was measured by monitoring at 210 nm (UV absorbance).

**Recovery of Peptide from the Antioxidative Assay.** LLPHH (400 $\mu$M) was incubated in an aqueous ethanol solution containing linoleic acid under the conditions for the antioxidative activity measurement (Chen et al., 1995). After the absorbance attained 0.3 according to the ferric thiocyanate method, the reaction mixture was concentrated to 1 mL using a centrifugal evaporator. The peptide was recovered by reversed-phase HPLC on a TSKgel ODS 120T column (5 $\mu$m, 4.6 × 250 mm) using a linear gradient of acetonitrile (0−12% in 60 min) in the presence of 0.1% trifluoroacetic acid as the solvent system at 35 °C at a flow rate of 1.0 mL/min. The recovered peptide was subjected to electrospray ionization mass spectrometery (Chen et al., 1995).

## RESULTS AND DISCUSSION

**Antioxidative Activity of His-Containing Peptides in Radical Initiator-Induced Oxidation System.** During the process of the isolation of antioxidative peptides from the proteolytic digest of a soybean protein, the antioxidative activity was measured against the peroxidation of linoleic acid in an aqueous system (Chen et al., 1995). In our previous work, we measured the antioxidative activities of 28 synthetic peptides, which were designed on the basis of an antioxidative peptide derived from the digest, with the same assay systems (Chen et al., 1996). In this study, the antioxidative activities of the synthetic His-containing peptides were measured in AAPH- and AMVN-induced oxidation systems. AAPH and AMVN are a water-soluble and an oil-soluble radical initiator, respectively. The comparison of the antioxidative activities of the synthetic peptides in an aqueous system and in an AAPH-induced oxidation system is summarized in Table 1 (left column). The antioxidative activities of the peptides in an AAPH-induced emulsion system monitored by measuring the decrease of the dissolved oxygen content showed a correlation coefficient of 0.86 with those in an aqueous system measured according to the ferric thiocyanate method.

In contrast, PHH and HLH, which showed high antioxidative activity in an aqueous system, did not show any antioxidative activity in an AMVN-induced aqueous system monitored by measuring the oxygen absorption in the headspace using gas chromatography (data not shown).

The results indicate that the peptides can scavenge the radical induced by AAPH in an aqueous system to protect the linoleic acid against oxidation, but the oil-soluble radical attacks linoleic acid directly; hence, the peptides provide no protection. *N*-(Long-chain-acyl)His showed antioxidative activity against the oxidation of methyl linoleate in an AMVN-induced aqueous system (Murase et al., 1993). This discrepancy can be explained by the hydrophobicity of *N*-(long-chain-acyl)His, which has access to the peroxy radical generated from fatty acid. The histidine-containing peptides, containing no hydrophobic moiety, cannot interact properly with the hydrophobic peroxy radical.

**Effect on Active Oxygen and Radical Species.** The scavenger activities of peptides toward various radical species are summarized in Table 1 (right col-

**Table 1. Antioxidative Properties of His-Containing Peptides**

| | relative antioxidative activity | | radical scavenging effect | | |
|---|---|---|---|---|---|
| peptide | aqueous system[a] | AAPH-induced oxidation system[b] | singlet oxygen[c] | DPPH radical[d] | superoxide[e] |
| HPLP | 0.6 | 0.98 | ND[f] | 1.05 | 1.06 |
| HHLP | 0.9 | 0.97 | 1.29 | 1.03 | 1.03 |
| HL | 0.9 | 0.98 | ND | 1.00 | 1.06 |
| HLPH | 1.2 | ND | 1.29 | 1.05 | 1.00 |
| LLPH | 1.2 | 0.94 | 1.00 | 1.00 | 1.03 |
| PLHH | 1.6 | 1.03 | 1.18 | 1.05 | 0.95 |
| HPHL | 1.6 | 0.97 | 1.10 | 1.03 | 1.03 |
| HH | 2.3 | 1.10 | 1.25 | 1.05 | 1.03 |
| HPH | 2.4 | 1.02 | 1.15 | 1.03 | 0.97 |
| LLHH | 2.4 | 1.03 | 1.10 | 1.05 | 1.00 |
| HHPLL | 2.6 | 1.15 | 1.22 | 1.03 | 1.06 |
| HLHP | 2.7 | 1.14 | 1.25 | 1.03 | 1.00 |
| LPHH | 2.7 | 1.10 | 1.05 | 1.00 | 1.00 |
| HHPL | 2.9 | 1.07 | 1.22 | 1.03 | 1.03 |
| LHPH | 2.9 | 1.12 | 1.15 | 1.03 | 0.97 |
| HHP | 2.9 | 1.06 | 1.05 | 1.00 | 1.09 |
| LH | 3.0 | 1.08 | 1.02 | 1.02 | 1.03 |
| LLPHH | 3.0 | 1.10 | 1.13 | 1.00 | 1.06 |
| HLH | 3.2 | 1.21 | 1.36 | 1.05 | 0.97 |
| LLPHHH | 3.3 | 1.13 | 1.10 | 1.08 | 1.03 |
| LHH | 3.8 | 1.08 | 1.32 | 1.05 | 1.00 |
| PHH | 5.8 | 1.30 | 1.32 | 1.03 | 0.97 |

[a] The data are from Chen et al. (1996). The peptide concentrations were 33 $\mu$M for (*b*), 50 $\mu$M for (*c*), 33 $\mu$M for (*d*), and 40 $\mu$M for (*e*). The results are shown as relative activities by adjusting the control to be 1.00, and are the averages of three independent experiments. [f] ND, not determined.

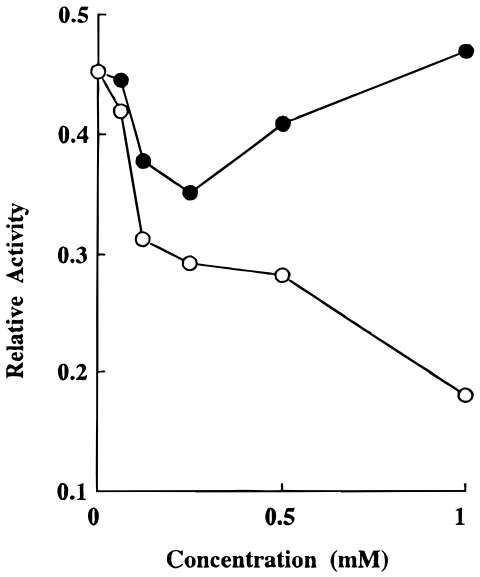


**Figure 1.** Quenching activities of PHH (●) and carnosine (○) on singlet oxygen.

umn). The His-containing peptides showed the quenching activities on singlet oxygen at 40 $\mu$M. The order of the activities of each peptides did not coincide with the order of their antioxidative activities against the linoleic acid peroxidation in an aqueous system. Figure 1 shows the quenching activities of PHH and carnosine on singlet oxygen at various concentrations. Carnosine quenched singlet oxygen in a dose-dependent manner, and 1 mM carnosine quenched 60% of singlet oxygen. However, PHH showed maximum activity (22%) at 0.25 mM, and then the activity decreased at higher concentrations. The quenching effect of carnosine on singlet oxygen was suggested to be mainly due to the imidazole group. Although reversing $\beta$-Ala and His to His-$\beta$-Ala did not affect the ability of the quenching of singlet oxygen (Dahl et al., 1988), some limited structure must be required for the quenching effect as shown in the present study.

**Table 2. Metal-Ion Chelating Activities and Hydrophobicities of His-Containing Peptides Expressed by Retention Times (Minutes)[a]**

| | metal-ion chelating activity | | |
|---|---|---|---|
| peptide | $Cu^{2+}$ | $Zn^{2+}$ | hydrophobicity |
| HPLH | 28.6 | 7.0 | 28.9 |
| HHLP | 27.2 | 13.0 | 29.2 |
| HL | 13.1 | 3.2 | 15.2 |
| HLPH | 26.9 | 11.2 | 29.4 |
| LLPH | 4.5 | 2.5 | 42.9 |
| PLHH | 24.6 | 15.0 | 28.9 |
| HPHL | 27.8 | 19.0 | 32.4 |
| HH | 27.6 | 17.0 | 8.0 |
| HPH | 28.0 | 12.0 | 15.8 |
| LLHH | 24.9 | 14.2 | 40.3 |
| HHPLL | 29.0 | 15.2 | 42.5 |
| HLHP | 30.2 | 10.8 | ND[b] |
| LPHH | 25.2 | 16.9 | 27.9 |
| HHPL | 29.3 | 16.2 | 26.6 |
| LHPH | 23.6 | 8.2 | 26.3 |
| HHP | 28.0 | 13.6 | 14.6 |
| LH | 4.9 | 3.1 | ND |
| LLPHH | 24.2 | 14.4 | 46.1 |
| HLH | 28.2 | 13.0 | 21.1 |
| LLPHHH | 29.3 | 41.5 | ND |
| LHH | 26.1 | 11.0 | 20.7 |
| PHH | 26.2 | 6.9 | 14.4 |

[a] The metal-ion chelating activities were measured on the metal-ion-loaded columns. The hydrophobicities were measured on the ODS column. Experimental conditions are described in the text. [b] ND, not determined.

Although 50 $\mu$M *d*-$\delta$-tocopherol scavenged 32% of DPPH radical, only a marginal scavenger activity was observed with the peptides at this concentration. The scavenger activity of *d*-$\delta$-tocopherol increased with the concentration, and 92% of DPPH radical was scavenged at 200 $\mu$M. However, LPHH showed no scavenger activity at the same concentration. None of the peptides scavenged the superoxide generated by xanthine oxidase and hypoxanthine at 33 $\mu$M.

**Metal-Ion Chelating Activity.** Metal-ion chelating activities of the His-containing peptides were measured by their retention times on metal-ion-loaded chelate columns. Except for LLPHHH, longer retention times were observed with the copper ion loaded column than with the zinc ion loaded column. The retention times varied according to the number and the location of His residues in the peptides with both columns (Table 2). With a few exceptions, the peptides gained in affinity for metal ions on increasing the number of His residue, and the peptides containing His residues at the N-terminal side showed higher affinity than did those with His residues at the C terminus, e.g., HL > LH, HHP > PHH, HHLP > PLHH, HHPL > LPHH, and HHPLL > LLPHH. The relationship between antioxidative activity and chelating activity of the three His-containing peptides isolated from egg white albumin hydrolysate has been presented (Tsuge et al., 1991). These authors showed that the metal-ion chelating activities of the His-containing peptides were not proportional to their antioxidative activities. Hence, the metal-ion chelating activities must affect other functions that contribute toward their antioxidative activities. It is interesting to note that the metal-ion chelates of carnosine possess superoxide scavenger activity as well as superoxide dismutase activity (Chan and Decker, 1994).

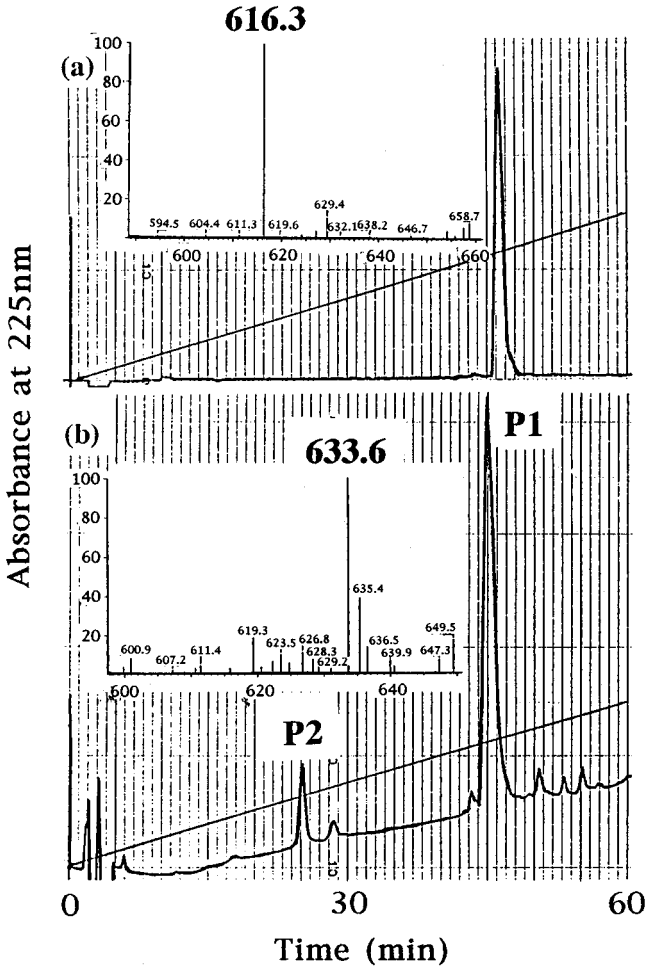


**Figure 2.** Reversed-phase HPLC of LLPHH before (a) and after incubating in an aqueous ethanol solution containing linoleic acid (b). Inset indicates mass spectrum of P1 (a and b) and P2 (b).

**Hydrophobicity of Peptides.** The relative hydrophobicities of the His-containing peptides were estimated from the retention times on an ODS reversed-phase HPLC column. The separation of the peptides depends upon the hydrophobic interactions between the hydrocarbonaceous column and the peptides: the more hydrophobic the peptide is, the stronger its retention on the column. Thus, the retention times reflect the hydrophobicity of the peptides (Meek, 1980). The retention order depended on the amino acid composition, but not on their sequence (Table 2). The addition of Leu and/or Pro residues increased the hydrophobicity of His-containing peptides. The hydrophobicity of the antioxidant is an important factor for increasing the accessibility of lipophilic fatty acids or oxidants and hence in expression of its antioxidative activity. However, such a correlation was not observed with the His-containing peptides (Table 1).

**Modification of Peptide.** LLPHH was incubated in an aqueous ethanol solution containing linoleic acid under the same conditions as those of the antioxidative activity assay. After the linoleic acid began to be autoxidized, the peptide was recovered by reversed-phase HPLC. The reaction mixture gave several peptide peaks besides the intact LLPHH (P1) (Figure 2). A minor peak (P2) was isolated and subjected to electrospray ionization mass spectrometry. The mass spectrum of P2 gave an increased mass unit of 17, suggesting the LLPHH was modified by a hydroxyl radical. The modified LLPHH was estimated to be 15% of the total from the peak area. Therefore, the peptide itself functions as a hydroxyl radical scavenger during the oxidation process of linoleic acid as reported with carnosine (Chan and Decker, 1994).

**Conclusions.** Many papers on the antioxidative mechanism of antioxidants have been published. For example, the antioxidant effect of flavonoids on lipid peroxidation is the result of scavenging of active-oxygen species and hydroxyl radicals involving termination of chain reactions during the autoxidation of polyunsaturated fatty acids (Husain et al., 1987). Butylated hydroxyanisole and butylated hydroxytoluene function as antioxidants through termination of free radical reactions, whereas citric acid functions via quenching of metal-catalyzed autoxidation (Dziezak, 1986). In conclusion, the present study demonstrated that His-containing peptides can act as a metal-ion chelator, an active-oxygen quencher, and a hydroxyradical scavenger. These properties seem to be important in explaining how the peptides possess their antioxidative activity. Although none of the properties can be correlated solely with the antioxidative activity of the peptides, the overall antioxidative action must be attributed to the cooperative effects of these properties. The different stabilities of the resulting peptide radicals, which can terminate chain reactions from autoxidation, may afford a further explanation for the discrepancies in the activities of the peptides.

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