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Effects of the Addition of Amino Acids and Peptides on Lipid Oxidation in a Powdery Model System

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The effects of the addition of amino acids and peptides on the oxidation of eicosapentaenoic acid ethyl ester (EPE) encapsulated by maltodextrin (MD) were investigated. The encapsulated lipid was prepared in two steps, that is, by mixing of EPE with MD solutions (±amino acids and peptides) to produce emulsions and freeze-drying of the resultant emulsions. The addition of amino acids and peptides improved the oxidation stability of EPE encapsulated with MD, and the inhibition of lipid oxidation by the amino acids and peptides was more effective at 70% relative humidity (RH). Met, Arg, and Trp were effective amino acids for antioxidation at RH = 10 and 40%, whereas at RH = 70%, His was the most effective amino acid, preventing the oxidation of EPE almost perfectly. Carnosine also exhibited a strong antioxidant effect at RH = 70%, but the effect of anserine was inferior. The addition of Met + Trp or Met + Arg inhibited the oxidation of EPE encapsulated with MD at RH = 40%. Cys accelerated the oxidation of EPE, indicating that the thiyl radical may act as a pro-oxidant. No close relationship was observed between the radical scavenging abilities of amino acids and peptides measured in the aqueous diphenylpicrylhydrazyl solution and their antioxidative effects in the powdery system. It is possible that the radical-scavenging ability of amino acids and peptides detected by ESR in the powder system is responsible for the antioxidative activity of these compounds.

KEYWORDS: Oxidation; eicosapentaenoic acid; maltodextrin; amino acid; amino acid mixture; peptide; antioxidant activity

INTRODUCTION

Polyunsaturated fatty acids of the *n*−3 family such as docosahexaenoic acid and eicosapentaenoic acid are known to have various health benefits, such as ameliorative effects on hypertension, inflammation, immune problems, and other diseases (*1*, *2*). However, *n*−3 fatty acids are susceptible to oxidative deterioration, limiting their use in foods because of flavor degradation by oxidation. In addition, hydroperoxides and their secondary products originating by lipid oxidation are thought to be toxic (*3*). Preventing the oxidation of the *n*−3 fatty acids is indispensable in allowing *n*−3 fatty acids to fulfill their physiological functions.

Microencapsulation is a technique in which a membrane encloses small particles of solid, liquid, or gas, to offer protection to the core material from adverse environmental conditions such as the undesirable effects of light, moisture, and oxygen, thus contributing to an increase in the shelf life of the product and promoting a controlled liberation of the encapsulate. In addition to such benefits on storage stability, the handling of the core materials (*4*) can be also improved by microencapsulation. Microcapsules or powdery lipids are widely used as food, medical, and pharmaceutical stuffs (*5*).

In the previous study (*6*), the oxidative stability of eicosapentaenoic acid ethyl ester (EPE) encapsulated by maltodextrin (MD) was investigated. Also, the effects of the addition of soybean and gelatin peptides to the powder system were studied. We have found that the soy protein, the soy peptide, and the gelatin peptides have the ability to suppress lipid oxidation in the MD powder system, especially in the high-humidity state. Other papers have also pointed out that high humidity is necessary for the suppression of lipid oxidation when lipids are encapsulated by a matrix of proteins such as cereal prolamin (*7*) and egg white proteins (*8*, *9*). Therefore, it is likely that water plays an important role in the antioxidative effects of proteins and peptides in the powdery system.

In our previous study (*6*), the soy peptide exhibited the greatest antioxidative effect. Chen et al. (*10*) also reported that soy peptides have radical-scavenging activity in aqueous systems. They found the peptide with strong radical-scavenging activity, and its structure was Leu-Leu-Pro-His-His. Especially, His and Pro were shown to be essential for the antioxidant effect by the experiment using synthetic peptides in which His and Pro were displaced by other amino acids. Such results suggested that the composition and sequence seem to be of great

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importance for the antioxidative effect of peptides. A systematic approach is required with respect to the relationship of the peptide sequence to its antioxidative effect in order to design the antioxidant foods including peptides. However, it is a laborious task to prepare many kinds of peptides with varying sequences for the antioxidant experiment. Especially, larger amounts of peptides are needed in the powder experiment, compared to the radical-scavenging test in the solution system. Therefore, before performing the peptide experiment, we should collect information on the antioxidative activity of amino acids, which are constituent elements of peptides, and then design potential antioxidative peptides based on such information.

In this study, we investigated the antioxidative activity of amino acids in the powdery system consisting of MD and EPE. Seven amino acids (Ala, His, Arg, Pro, Cys, Trp, and Met) were chosen according to the previous papers (*10*−*14*) reporting the antioxidative activity of these amino acids. In addition to the case when the amino acid was used solely, mixtures of two amino acids were also investigated to test the synergistic effect. Carnosine and anserine are dipeptides that are known to have antioxidative ability (*15*−*17*), and the activities of these simple peptides were also tested for the comparison. Throughout all of the experiments, three moisture activity conditions were used, because the antioxidative effects change with water activities as previously mentioned.

MATERIALS AND METHODS

Materials. EPE was supplied by Nippon Suisan Co. (Tokyo, Japan). Its purity was >99%. It was stored at −80 °C before use. Stearic acid ethyl ester (SAE) was purchased from the Sigma Chemical Co. (St. Louis, MO). MD with a dextrose equivalent of 2−5 was purchased from Matsutani Chemical Industries (Osaka, Japan). Anserine nitrate salt was obtained from Sigma. β-Ala was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Met, His, α-Ala, cysteine hydrochloride monohydrate, Trp, Arg, Pro, and carnosine were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Encapsulation of EPE by MD with Amino Acids or Peptides. Before encapsulation, the peroxides were removed from EPE by twice utilizing a Sep-Pak Vac 20 cm^3 (5 g) Florisil cartridge. Peroxide-free EPE was dissolved in $CHCl_3$ (0.25 g/mL) and stored at −4 °C until the encapsulation. MD (30 g) was dissolved in 262.5 mL of distilled water at 40 °C. When 0.75 g of the amino acid or peptide was added to the MD solution, the total amount of distilled water decreased to 261.75 mL. The oil mixture (6 g of EPE and 1.5 g of SAE) was mixed with the wall material solution. The oil and aqueous phases were emulsified with a rotor/stator homogenizer for 3 min in a high-speed blender (Nichion Irikagakukiki Seisakusho, Tokyo, Japan) operated at 22000 rpm. The average droplet diameter was further reduced using an ultrasonic homogenizer (Nihonseiki Kaisha, Tokyo, Japan) operated at maximum power for 2 min. The emulsions were dried by a freeze-dryer (Central Scientific Co., Ltd., Osaka, Japan) to produce powder lipids according to the following process: A bottle containing an emulsion was immersed in liquid N_2 to freeze the emulsion below −150 °C for 20 min. The addition of the amino acids and peptides mostly decreased the median diameter of the MD-stabilized emulsions, that is, MD, 1.588 μm; MD + His, 1.351 μm; MD + Cys, 1.371 μm; MD + Pro, 1.353 μm; MD + Trp, 1.321 μm; MD + Met, 1.341 μm; MD + Ala, 1.327 μm; MD + Arg, 1.382 μm; MD + His + Met, 1.337 μm; MD + Met + Trp, 1.342 μm; MD + Met + Arg, 1.361 μm; MD + His + Ala, 1.373 μm; MD + His + β-Ala, 1.381 μm; MD + Car, 1.321 μm; and MD + Ans, 1.388 μm. The frozen emulsion was dried below 6.6×10^{-4} atm and at room temperature for 24 h. After freeze-drying, the dried powder was crushed before storage. No significant change in the microstructure of the powder during storage by SEM (data not shown).

Oxidation of Powder Lipids. The sample powders were subdivided into small portions (50 mg in each tube) and stored in a humidity-controlled desiccator at 40 °C. The relative humidity was adjusted to approximately 10, 40, and 70%, with 55, 38.5, and 22% (w/w) sulfuric acid, rsepectively. Samples were removed at stated intervals, and the oxidation of EPE was followed by gas chromatography (GC) analysis and peroxide measurement (POV).

GC Measurements. Two milliliters of water was added to the powder lipid (50 mg) after storage, and then this suspension was incubated at 40 °C for 30 min. This suspension was added to 6 mL of solvent (chloroform/methanol, 2:1). The mixture was dispersed by a homogenizer and a sonicator and centrifuged for 10 min at $3.0 \times 10^3 g$ to separate the upper layer (methanol + water), lower layer (chloroform), and pellets (residues). The upper layer was taken out, and 3 mL of the solvent (methanol/water, 1:1) was added to the lower layer and pellets. The mixture was dispersed and centrifuged again. After the supernatant had been discarded, 3 mL of the solvent (methanol/water, 1:1) was added and vigorously mixed by a homogenizer and a sonicator. After centrifugation, the lower layer (chloroform layer) was removed and dried by nitrogen gas, followed by dissolving in 1 mL of *n*-hexane. An aliquot (1 μL) was directly injected into the column inlet of the Shimadzu GC-14B apparatus (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a hydrogen flame ionization detector. The analytical conditions were as follows: a glass column (0.32 × 210 cm) packed with SILAR-10C (10%) on Chromosorb W (AW-DMCS, 80−100 mesh); temperature program, 140−240 °C at 4 °C/min; injection temperature, 260 °C; carrier gas (N_2) at a flow rate of 60 mL/min; N_2 pressure, 0.6 kg/cm^2; air pressure, 0.5 kg/ cm^2. The ratios of EPE to SAE (internal standard) were obtained by calculation from the corresponding recorded peak areas. The oxidation degree was calculated on the basis of the ratio of the amounts of EPE and SAE (internal standard).

Measurement of POV. The POV was measured according to the ferric thiocyanate method (*8*). A 0.02 mL portion of each sample extracted oil, which was prepared as in the previous section, was diluted with 4.78 mL of chloroform/methanol (2:1, v/v), and 0.1 mL of 30% ammonium thiocyanate and 0.1 mL of a mixture of equal volumes of 0.02 M ferrous sulfate and 0.02 M barium chloride were added. The absorbance at 500 nm was measured exactly 3 min after the start of the reaction.

Electron Spin Resonance Measurement (ESR) Analysis of Wall Material Powders. MD (±amino acid and peptide) was mixed with an aqueous solution of the spin probe, Tempol, and the resultant solutions were freeze-dried. The ratio of the amino acid (peptide), Tempol, and MD in the powder was 1:0.27:40. After incubation at a relative humidity (RH) of 10, 40, or 70% for 7 days, the sample powders were rapidly introduced into capillaries (1-mm diameter) and sealed. The length of the samples was 4 cm. ESR spectra were recorded with a controlled-temperature JEOL FR30 spectrometer (JEOL, Tokyo, Japan). The sample powder packed in capillaries was kept at 90 °C, and the intensity of the peaks in the ESR spectra was periodically recorded for 40 min. The microwave power was below saturation. The typical instrument parameters used were as follows: microwave power, 1 mW; sweep width, ±1 × 10 mT; sweep time, 2 min; modulation width, 2 × 0.1 mT; time constant, 0.3.

Diphenylpicrylhydrazyl (DPPH) Radical-Scavenging Ability. The radical-scavenging ability of MD, amino acids, and peptides in the aqueous solution was tested as follows: MD, each peptide, or amino acid (50 mg) was dissolved in 2 mL of sodium phosphate buffer (10 mM, pH 7.0). To the solution were added 2 mL of ethanol and 1 mL of DPPH solution (0.5 mM DPPH in ethanol). The absorbance of the DPPH was measured at 517 nm using a Shimadzu UN-2400PD recording spectrometer (Shimadzu Co., Ltd., Kyoto, Japan) 30 min after the addition of the DPPH solution.

RESULTS

Determination of Unoxidized Lipids in Powders Including Amino Acids by GC. To evaluate the degree of lipid oxidation during storage, the amounts of unoxidized EPE in the powdery lipids were determined by GC. The ratio of EPE and SAE (internal standard) was used to calculate the relative amount of EPE. The results are expressed as the percentage of unoxidized lipids in the total lipids.

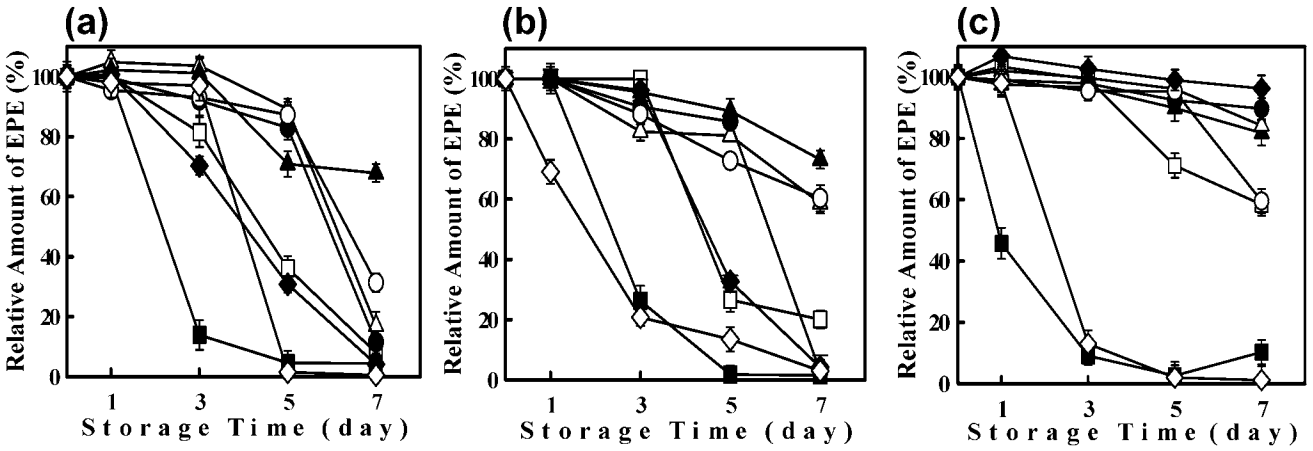

Figure 1. Effects of the addition of amino acids on the time-dependent loss of EPE encapsulated with MDL (□) MD + Pro; (■) MD + Cys; (▲) MD + Trp; (△) MD + Met; (●) MD + Ala; (○) MD + Arg; (◆) MD + His; (◇) MD. The powders were stored at relative humidities of 10% (**a**), 40% (**b**), and 70% (**c**). Each value of the ratio is the mean of triplicate determinations.

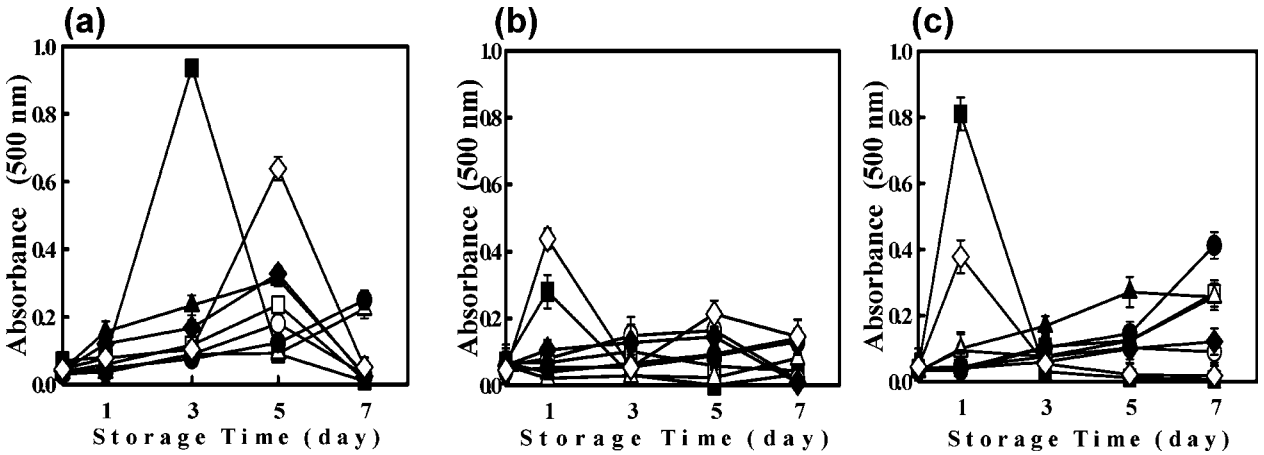

Figure 2. Effects of the addition of amino acids on the POV of EPE encapsulated with MD: (■) MD + Cys; (□) MD + Pro; (▲) MD + Trp; (△) MD + Met; (●) MD + Arg; (○) MD + Ala; (◆) MD + His; (◇) MD. The powders were stored at realtive humidities of 10% (**a**), 40% (**b**), and 70% (**c**). POV was expressed as an absorbance at 500 nm instead of using mequiv/kg. Each value of the ratio is the mean of triplicate determinations.

Figure 1 shows the autoxidation processes of EPE encapsulated with MD and MD + amino acid at RH = 10, 40, and 70% at 40 °C. The reproducibility of the results of these figures was good. When EPE was encapsulated with MD, the lipid was easily oxidized irrespective of humidity. In the case of RH = 10% (**Figure 1a**), the amount of EPE rapidly decreased to almost 0% after 5 days. When Cys was added, the oxidation process was more rapid, and the amount of lipid decreased to almost 15% after 3 days. For Pro and His, the oxidation started after 1 day and was almost completed after 7 days. The best and second best antioxidative effects were detected for Met and Arg, respectively. **Figure 1b** shows the results of the addition of amino acids at RH = 40%. The oxidation was slowed at RH = 40% compared to those at RH = 10%. The oxidation of EPE was effectively suppressed by the addition of Met, Arg, and Trp. The best antioxidative effect was detected for Met.

Figure 1c shows the results of the addition of amino acids at RH = 70%. The oxidation rates were lowest among the set relative humidities. Especially when His was added, the oxidation was suppressed almost completely for 7 days. However, Cys accelerated the oxidation under both RH conditions. These results mean that RH = 70% is preferable for the suppression of lipid oxidation in the powder lipid of MD with amino acids.

Determination of POV of Powdery Lipids Including Amino Acids. Figure 2 shows the results of the POV of the EPE encapsulated with MD and MD + amino acids stored at various humidities and 40 °C. The results of POV exhibited similar trends for the GC experiments. A rapid increase in the POV was observed in MD, reaching a maximum at 1 day irrespective of the humdity. The decrease in POV after reaching a maximum suggests that the peroxides are converted to secondary products. When amino acids were added, the POV increased more gradually with time and reached a maximum at 5 days, indicating the retardation of EPE oxidation by most of the amino acids. At RH = 10% (**Figure 2a**), the POV was depressed by the addition of Met and Arg. At RH = 40% (**Figure 2b**), the POV was depressed by the addition of Met. On the other hand, at RH = 70% (**Figure 2c**), Ala and His were shown to suppress the increases in POV very effectively.

On the basis of the results of GC and POV, we have found amino acids that have the ability to suppress the EPE oxidation in the MD powder. In the case of RH = 10 and 40%, Met and Arg were very effective, but His was the best for RH = 70%.

Effects of Mixing of Amino Acids on Lipid Oxidation. We checked whether there was any change in the antioxidative effect when two amino acids were mixed. Because Met exhibited the highest antioxidative effect and Arg showed the second best antioxidative effect at RH = 10 and 40% as shown in **Figures 1a**,**b** and **2a**,**b**, respectively, the mixtures of Met and Arg were studied. The combination of Met and Trp was also attempted, because the latter amino acid was the third most effective amino

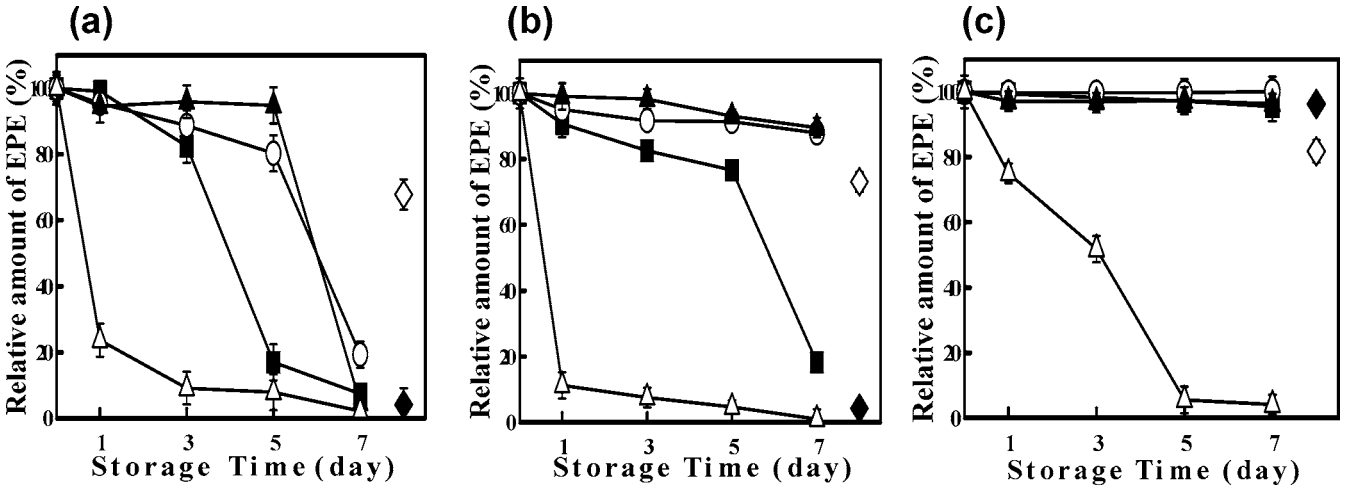

Figure 3. Effects of the addition of amino acid mixtures on the time-dependent loss of EPE encapsulated with MD: (■) MD + His + Met; (○) MD + Met + Trp; (▲) MD + Met + Arg; (△) MD + His + Ala. The powders were stored at relative humidities of 10% (**a**), 40% (**b**), and 70% (**c**). Diamond symbols indicate EPE amounts at 7 day in the case of single amino acid use: (♦) His; (◇) Met. Each value of the ratio is the mean of triplicate determinations.

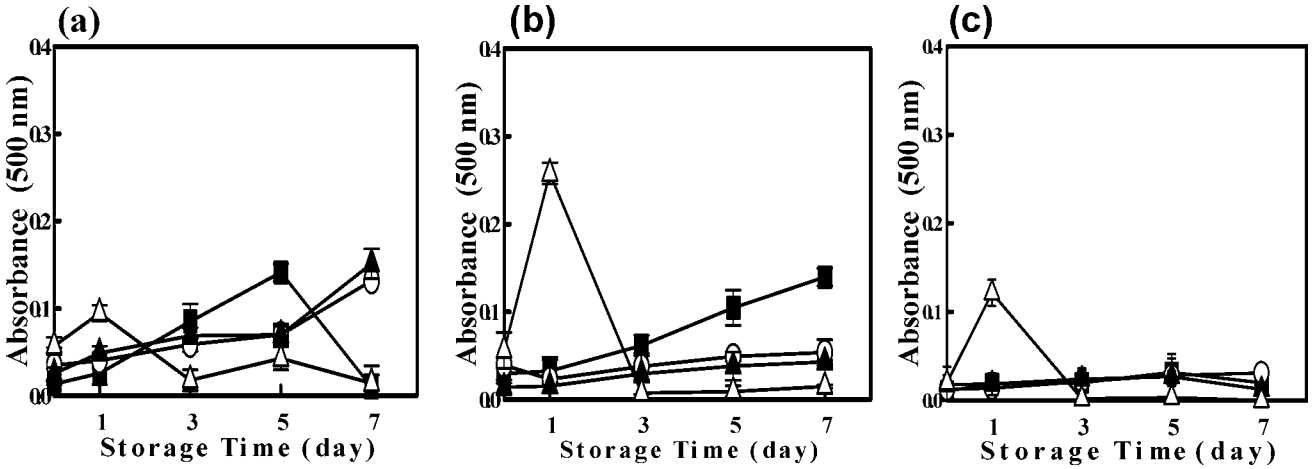

Figure 4. Effects of the addition of amino acid mixtures on the POV of EPE encapsulated with MD: (■) MD + His + Met; (○) MD + Met + Trp; (▲) MD + Met + Arg; (△) MD + His + Ala. POV was expressed as an absorbance at 500 nm instead of using mequiv/kg. The powders were stored at relative humidities of 10% (**a**), 40% (**b**), and 70% (**c**). Each value of the ratio is the mean of triplicate determinations.

acid at RH = 10 and 40%. On the other hand, at RH = 70%, the best and second best effective amino acids were His and Ala, respectively (**Figures 1c** and **2c**). On the basis of these results, His was mixed with Ala for testing antioxidative activity. As the last combination, His and Met were chosen, because His and Met were shown to be most active at RH = 70% and RH = 10 or 40%, respectively.

Figure 3 shows the results of the time-dependent loss of EPE detected by GC. EPE was almost oxidized in the case of all mixed amino acids at RH = 10% in 7 days (**Figure 3a**). From a comparison with the results for single amino acids after storage for 7 days (see diamond symbols in the figure), it is clear that the oxidation is much faster in the case of the amino acid mixtures. However, at RH = 40% (**Figure 3b**), the oxidation was almost suppressed for 7days in the case of Met + Arg and Met + Trp. Therefore, it is clear that at RH = 40%, Met and Arg or Trp showed synergistic antioxidative effects. At RH = 70% (**Figure 3c**), for the mixture of His and Ala, the oxidation started after 1 day and was almost completed after 5 days. This result indicates that the antioxidative effect was lost when His and Ala were mixed, although these amino acids showed excellent antioxidative effects when used separately. Other mixtures of amino acids suppressed the oxidation very effectively.

Figure 4 shows the results of the POV of EPE encapsulated with MD + amino acid mixture stored at various humidities and 40 °C. At RH = 10% (**Figure 4a**) when His + Met was added, the POV increased more gradually with time as compared to the control (MD only) of **Figure 2a** and reached a maximum at 5 days, indicating the retardation of the EPE oxidation. The suppression of EPE oxidation was most effective for Met + Arg and Met + Trp, which was in agreement with the GC results. However, His + Ala had no effect on the oxidation at RH = 10%. At RH = 40% (**Figure 4b**), POV was depressed by adding Met + His, Met + Trp, and Met + Arg. At RH = 70% (**Figure 4c**), His + Met, Met + Trp, and Met + Arg were shown to suppress the increase in POV very effectively. On the other hand, for the His + Ala mixture, the oxidation started after 1 day and was almost completed after 7 days. On the basis of the results of GC and POV, the synergistic effects of amino acids with respect to the inhibition of lipid oxidation were obviously demonstrated at RH = 40%, especially for Met + Arg and Met + Trp. At RH = 70%, the antioxidative activity was increased by the mixing of amino acids. However, in the

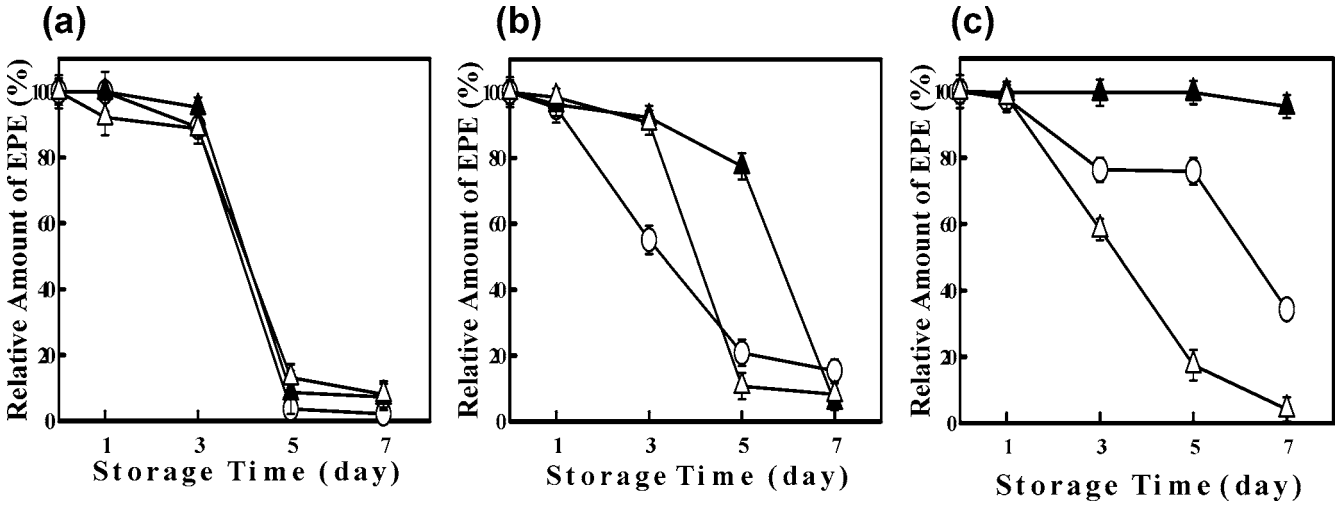

Figure 5. Effects of the addition of amino acid mixtures and peptides on the time-dependent loss of EPE encapsulated with MD: (○) MD + His + β-Ala; (▲) MD + carnosine; (△) MD + anserine. The powders were stored at relative humidities of 10% (**a**), 40% (**b**), and 70% (**c**). Each value of the ratio is the mean of triplicate determinations.

mixture of His and Ala, the oxidation process was even faster. It was quite interesting that the antioxidative effect was lost when His and Ala was mixed, although these amino acids showed excellent antioxidative effect when used separately. Therefore, the combination of His and Ala caused the negative effect.

Effects of Peptide Addition on Lipid Oxidation in Powdery Lipids. Figure 5 shows the results of the time-dependent loss of EPE detected by GC when the powdery lipid contained carnosine and anserine. For comparison, the mixture of His and β-Ala, which constitutes carnosine, was also tested. At RH = 10% (**Figure 5a**) carnosine showed high antioxidative effect until 5 days, but the amount of EPE rapidly decreased to almost 0% after 7 days. His + β-Ala and anserine also decreased to almost 0% after 7 days. At RH = 40% (**Figure 5b**), in the case of the His + β-Ala mixture, the oxidation started after 3 days, and the amount of EPE rapidly decreased after 5 days. For anserine, EPE was also oxidized after 5 days. The oxidation of EPE was almost perfectly suppressed when the lipid was encapsulated with carnosine during the 5 days of storage at RH = 40%, but EPE decreased to almost 6% after 7 days.

Figure 5c shows the results at RH = 70%. The addition of carnosine suppressed the EPE oxidation perfectly. The mixture of His and β-Ala, which constitutes carnosine, did not exhibit the strong antioxidative effects. This means that the peptide linkage is necessary for the suppression of lipid oxidation. Anserine, the derivative of carnosine, showed weak antioxidative effects. The results of the POV value for the anserine, carnosine, and His + β-Ala were in agreement with the results of GC (data not shown).

Radical-Scavenging Ability of MD, Amino Acids, and Peptides in an Aqueous Solution. To test the ability of MD, amino acids, and peptides as radical scavengers, the disappearance of the DPPH radical (a stable and water-soluble radical) was followed for 30 min after the addition of MD and the other ingredients. The results of control (MD) and amino acids are shown in **Figure 6**. The absorbance was expressed as the relative value (percentage) of the absorbance without the addition of the ingredients. The lower intensity of a peak indicates the higher radical-scavenging activity of the subject. The reproducibility of the results was good.

The absorbance value after 30 min was not affected by the MD addition, indicating no radical-scavenging ability of the

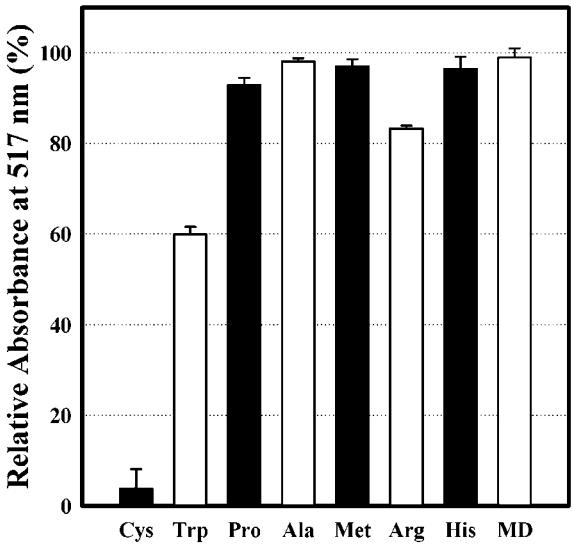

Figure 6. Radical-scavenging activity of amino acids and MD. MD and amino acids (Cys, Trp, Pro, Ala, Met, Arg, and His) were mixed with DPPH solution, and the absorbance at 517 nm was measured after 30 min. The numbers reported are the mean of four measurements.

polysaccharide. Trp reduced the radical almost to 60%. Pro, Ala, Met, Arg, and His, which exhibited excellent antioxidative effects in the results of GC and POV, did not show strong radical-scavenging activity. On the other hand, Cys, which did not suppress the oxidation as previously shown, scavenged the radical up to 4%. From these results for the amino acids, we cannot find a clear relationship between the antioxidative effect in the powdery lipid and the scavenging activity measured by DPPH.

Figure 7 shows the results of the mixture of amino acids and peptides. Met + Trp decreased the radical up to 17%, indicating the synergistic effects of both amino acids. The combination of Met + His and Met + Ala slightly decreased the radical amount as compared to the case of single use. Anserine had no radical-scavenging activity. Carnosine only decreased the radical amount up to 87%, whereas the combination of its constituent amino acids, His + β-Ala, scavenged the radical more effectively. Although coincidence between the radical-scavenging ability and antioxidative activity was found for the Met + Trp case, the other results did not support the

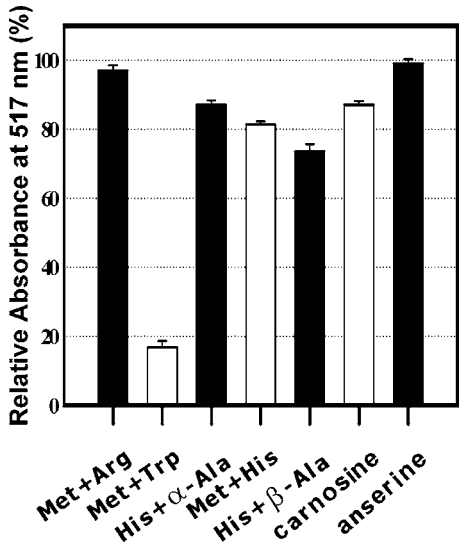

Figure 7. Radical-scavenging activity of amino acid mixtures and peptides. Amino acid mixtures (Met + Arg, Met + Trp, His + α-Ala, Met + His, and His + β-Ala) and peptides (carnosine and anserine) were mixed with DPPH solution, and the absorbance at 517 nm was measured after 30 min. The numbers reported are the mean of four measurements.

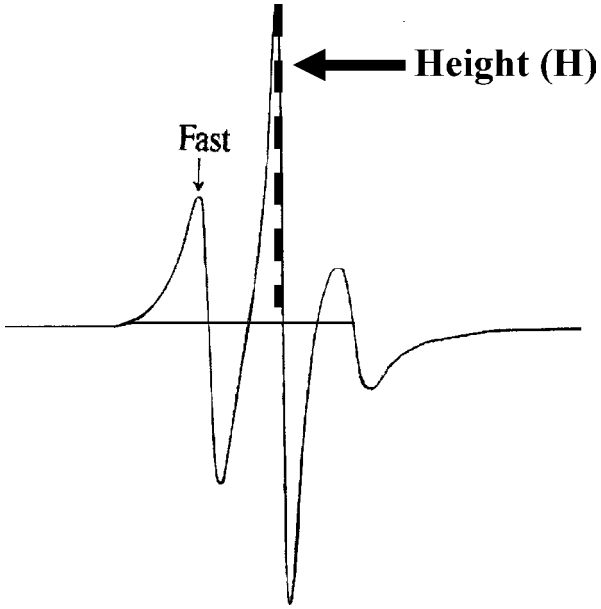

Figure 8. ESR spectra of Tempol in MD powder stored at 90 °C.

idea that the radical-scavenging ability of amino acids and peptides is responsible for their antioxidative effects in the powdery lipid systems.

Radical-Scavenging Ability of MD and Amino Acids in the Powdery System. Further to the radical-scavenging ability in aqueous solution, the radical-scavenging ability in the powder system was investigated using an ESR technique. As can be seen in **Figure 8**, a sharp signal having the highest peak in the center position was observed for Tempol in the powder system of MD and MD + amino acids at 90 °C. The decrease in the intensity of the center peak was measured for 30 min at 90 °C. If the sample powder has radical-scavenging ability, the Tempol signal could be dramatically decreased because of the high chemical reaction rate at 90 °C.

Figure 9c shows the results for the powders that were stored at RH = 70% prior to the ESR measurements. The peak intensity of Tempol declined with time even in the case of MD. In the case of MD without the other ingredients, a gradual decrease to ~67% after 40 min was observed. Such a decrease in the intensity of the Tempol signal may be due to the autodegradation of Tempol at high temperature or the weak radical-scavenging ability of MD. However, when amino acids were added, the intensity was lower and decreased more rapidly. For example, His is very effective in decreasing the intensity. This result corresponds to the strong antioxidative effect of His in the powder lipid as shown in the results of GC and POV. Therefore, the antioxidative effects of these amino acids can be partially explained by the radical-scavenging ability in the powder, although the perfect coincidence between the results of ESR and GC was not found in POV experiments. For example, Arg decreased Tempol very effectively, but exhibited a lower effect than His with respect to the inhibition of lipid oxidation (**Figures 1c** and **2c**).

DISCUSSION

We have found, in the present study, that amino acids and peptides can suppress lipid oxidation in the MD powder system. Such antioxidative effects varied with relative humidity. It is generally accepted that the mobility of the biopolymer matrix in powder lipids is elevated with an increase in water activity because of the decrease in the glass transition point, thereby accelerating the oxygen diffusion from the outside into the matrix and increasing the rate of lipid oxidation inside the powder. The present study confirmed that the lipid oxidation encapsulated by only the MD matrix was accelerated at RH = 70% compared to the cases of RH = 10 and 40% (**Figures 1** and **2**). However, in the powdery lipids including amino acid or peptide, the antioxidative activity was increased with relative humidity; that is, the most effective inhibition of lipid oxidation by amino acids was demonstrated at RH = 70%. These results indicate that water is of great importance with respect to the inhibition of lipid oxidation by amino acids or peptides in the powdery system, which is in agreement with our previous results (*6*). Water may enhance the mobility of amino acids or peptides, thereby increasing the probability of an encounter of these compounds with oxygen and/or free radicals in the matrix. This speculation was supported by ESR measurements (**Figure 9**). The decreases in the Tempol radical by amino acids were very low at RH = 10% (**Figure 9a**), whereas the decreases were dramatic at RH = 70% (**Figure 9c**), indicating that water might enhance the mobility of amino acids or peptides to react with and scavenge radicals. It is also likely that hydration at high relative humidity increases the charge density of amino acids, which may be responsible for the enhanced antioxidative activity.

Despite the high antioxidative activity at the high humidity state as previously mentioned, some amino acids were very effective in retarding the lipid oxidation even in low and medium relative humidities. For example, Met, Arg, and Trp inhibited the oxidation of EPE even at RH = 10 and 40% (**Figure 1**). These amino acids also exhibited synergistic effects at RH = 40% (**Figure 3**), protecting EPE almost perfectly during 7 days. Therefore, in the case of these amino acids, the hydration is important, but not essential, for the inhibitory effects on lipid oxidation in the powder system. Met is easily oxidized to methionine sulfoxide (*18*−*20*), and the tryptophan ring is also highly susceptible to breakage by oxidative damage (*21*, *22*). Such a well-known mechanism can probably work even at low and medium relative humidities. The antioxidant mechanism of Arg is not well-known, but the guanizyl group may act as the potential radical scavenger or trapper.

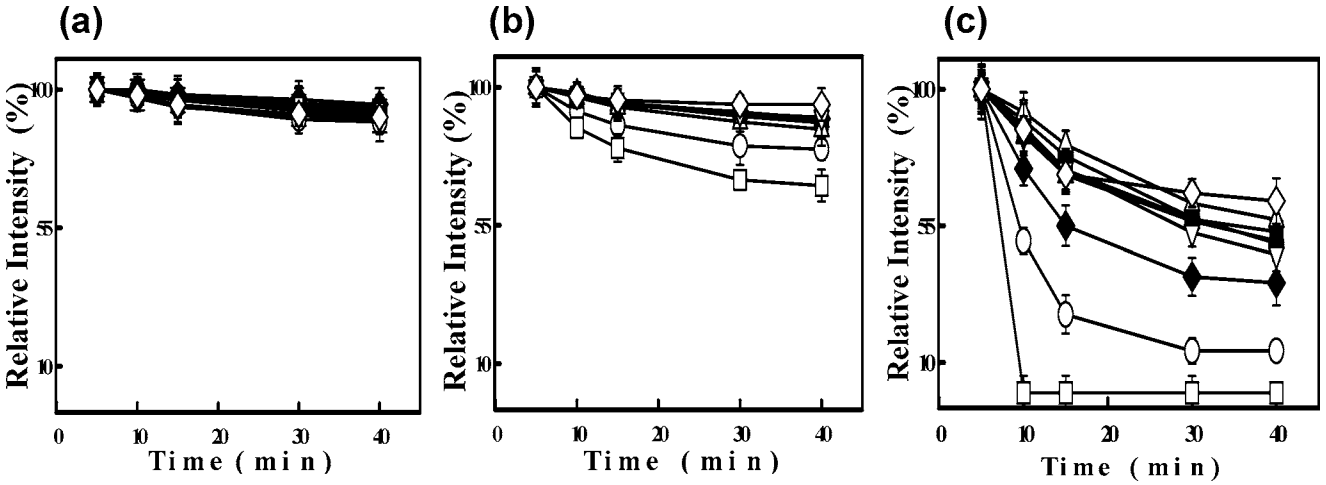

Figure 9. Time-dependent change in intensity of a center in the ESR spectrum for Tempol in MD powder: (◆) MD + His; (○) MD + Arg; (■) MD + Pro; (●) MD + Ala; (▲) MD + Trp; (△) MD + Met; (□) MD + Cys; (▽) MD + His + Met; (◇) MD. Powders were kept at 90 °C, and ESR spectra were measured periodically for 40 min. The value at the starting point (2 min) was expressed as 100%. The intensity at each time was expressed as relative value. The powders were stored for 7 days at relative humidities of 10% (**a**), 40% (**b**), and 70% (**c**).

His was found to be a unique amino acid with respect of the inhibitory effects on lipid oxidation in the powdery system. Whereas His did not demonstrate the strong antioxidative activity at RH = 10 and 40%, it inhibits the lipid oxidation almost perfectly at RH = 70%. The mechanism of the oxidative damage of His was investigated extensively (*15*, *23*). In addition to the breakage of the imidazole ring, the liberation of the α-amino group occurs by the attack of the free radicals. The latter reaction results in the formation of imidazole lactate or imidazole acetate. These reactions may need more water, although the effects of water activity on the rates of the reactions have not been sufficiently tested. That is the reason His exhibited strong antioxidative activity only at the high relative humidity.

His showed unique results with regard to the effects of mixing with other amino acids, too. Whereas Met, Arg, and Trp exhibited the synergistic effects on the protection of lipid oxidation as previously mentioned, His did not improve the effects by mixing with the other amino acids. Especially, it is worth noting that the inhibitory effect of His + Ala was inferior compared to that of the amino acids used individually (**Figure 3c**). The addition of *β*-Ala also decreased the activity of His (**Figure 5**). The mechanism of the negative effects of mixing His with other amino acids, particularly Ala, is not understood, but some competitive reaction between the two amino acids with respect to the trapping of free radicals may be responsible for the effect.

As described in the Introduction, it was already known that His plays an important role in the antioxidative effects of isolated soybean peptides (*10*). In the present study, too, carnosine including His exhibits strong antioxidative effects, whereas the effects of anserine including methyl-His were not so strong (**Figure 5**). The peptide linkage of His to other amino acids was important, because the mixture of His and *β*-Ala (the constituent amino acids of carnosine) was not so effective as previously described. These results totally suggest that His is a key amino acid for generating the antioxidative activity of peptides. Further studies are necessary to understand the role of His, but we may be able to design antioxidative foods using peptides that include His residues.

Cys showed a strong radical scavenging activity in the powdery system (**Figure 9**) as well as in the aqueous system (**Figure 6**). It is strange that Cys could not suppress or even accelerate the lipid oxidation in the results of GC and POV (**Figures 1** and **2**), despite such a strong radical-scavenging activity. It is known that Cys is turned to be a thiyl radical by the attack of free radicals. In our powdery system, too, the thiyl radical generated from Cys under oxidative stress conditions may work as a pro-oxidant to promote the lipid oxidation. It is generally accepted that SH compounds such as Cys and glutathione protect the biological system from oxidative stress. However, our results suggest that the use of SH compounds is hazardous in the powdery system.

We should emphasize the importance of the results on the amino acid mixture at RH = 40% (**Figure 3b**). Met + Trp and Met + Arg exhibited synergistic effects to protect the lipid from the oxidation almost perfectly for 7 days. It is well-known that the rate of a chemical reaction such as the Maillard reaction increases with water activity and reaches the maximum at 0.6−0.8. The growth of fungi becomes active above the water activity at 0.7. On the other hand, the lipid oxidation was accelerated at extremely low and high water activities. On the basis of such facts, storage at medium relative humidity is satisfactory for food preservation. The high antioxidative effects of Met + Trp and Met + Arg at RH = 40% suggest the possibility that we will minimize the deterioration including lipid oxidation by utilizing the mixture of appropriate amino acids in the case of food preservation at intermediate relative humidity.

In conclusion, the present study revealed that the addition of small amounts of amino acids and peptides to MD powder greatly improved the stability of EPE against oxidation. Although further research is needed, our results may supply useful information for the development of powder and/or "intermediate moisture foods" with great stability against lipid oxidation.

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