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Effects of Protein and Peptide Addition on Lipid Oxidation in Powder Model System

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The effect of protein and peptide addition on the oxidation of eicosapentaenoic acid ethyl ester (EPE) encapsulated by maltodextrin (MD) was investigated. The encapsulated lipid (powder lipid) was prepared in two steps, i.e., mixing of EPE with MD solutions (± protein and peptides) to produce emulsions and freeze-drying of the resultant emulsions. EPE oxidation in MD powder progressed more rapidly in the humid state [relative humindity (RH) = 70%] than in the dry state (RH = 10%). The addition of soy protein, soy peptide, and gelatin peptides improved the oxidation stability of EPE encapsulated by MD, and the inhibition of lipid oxidation by the protein and the peptides was more dramatic in the humid state. Especially, the oxidation of EPE was almost perfectly suppressed when the lipid was encapsulated with MD + soy peptide during storage in the humid state for 7 days. Several physical properties such as the lipid particle size of the emulsions, the fraction of nonencapsulated lipids, scanning electron microscopy images of powder lipids, and the mobility of the MD matrix were investigated to find the modification of encapsulation behavior by the addition of the protein and peptides, but no significant change was observed. On the other hand, the protein and peptides exhibited a strong radical scavenging activity in the powder systems as well as in the solution systems. These results suggest that a chemical mechanism such as radical scavenging ability plays an important role in the suppression of EPE oxidation in MD powder by soy proteins, soy peptides, and gelatin peptides.

KEYWORDS: Autoxidation; eicosapentaenoic acid; maltodextrin; soy peptide; soy protein; gelatin peptide; antioxidant activity

INTRODUCTION

Microencapsulation of core lipid materials with wall matrices is a very useful technique to improve the storage stability and handling of the core materials (*1*). Microcapsules or powdery lipids are used widely as food, medical, and pharmaceutical stuffs (*2*). Microencapsulation consists of two steps, i.e., mixing of core materials with solutions of wall materials to prepare emulsions and drying of the resultant emulsions. The quality of powdery lipids is affected by various factors such as wall materials, drying methods and conditions, the ratio of core lipid and wall materials, the presence of other ingredients, etc. (*3*, *4*). Of these factors, the selection of wall materials seems to be crucial.

Various wall materials have been tested for the ability to encapsulate core lipids. Polysaccharides are promising for wall materials because they are easy to solubilize or disperse in aqueous medium and have good film-forming and drying properties (*5*, *6*). Proteins such as milk whey proteins (*7*−*13*), soy protein isolate (*14*), caseins (*15*), and cereal prolamins (*16*−*20*) have also been used to encapsulate core lipids. With respect to flavors, tastes, and antigenicity, polysaccharides may be better than proteins as matrix materials, although proteins can produce stable emulsions, which are prerequisite to the successful microencapsulation, more efficiently than polysaccharides. Some combination of proteins and carbohydrates including polysaccharides as wall materials has been found to be useful to improve the oxidative stability of core lipids, but the systematic formulation of wall materials has still not been established.

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 (*21*, *22*). 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

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their secondary products originating due to lipid oxidation are thought to be toxic (*23*). Preventing oxidation of the n-3 fatty acids is indispensable in allowing n-3 fatty acids to fulfill their physiological functions.

In the present study, the oxidative stability of eicosapentaenoic acid ethyl ester (EPE) encapsulated by maltodextrin (MD) was investigated. Minemoto et al. (*24*) demonstrated that the oxidation rate of lipids in MD powder was reverse by proportion to the humidity surrounding the powders; that is, the stability of lipids against oxidation was enhanced in the dry state. Therefore, in the previous study, the high humidity [relative humidity (RH) = 70] and low humidity (RH = 10) conditions were used for the storage of MD powder.

The effects of the addition of soybean and gelatin peptides to the powder system were also studied, because these peptides have been found to exhibit antioxidant activity. For comparison, the ability of soy protein isolate was tested as an antioxidant in the powder system. We also tried to understand the physical and chemical mechanism whereby the oxidation of EPE was effectively retarded by encapsulation with the mixture of MD and the peptides or soy protein.

MATERIALS AND METHODS

Materials. EPE was supplied by Nippon Suisan Co. (Tokyo, Japan). Its purity was more than 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). Soy protein (average MW = 10000) and soy peptide (average MW = 1100) were kindly given by Fuji Oil Co., Ltd. (Osaka, Japan). Gelatin peptides were supplied from Nippi Gelatin Co., Ltd. (Osaka, Japan). The molecular weights of the gelatin peptides PRA, PE-20, and PA-10 were approximately 3000, 2000, and 1000, respectively. PRA, PE-20, and PA-10 are called GP-3, GP-2, and GP-1 in the present paper. The spin probe 4-hydroxy-2,2,6,6-tetramethylpiperidine N-oxyl (Tempol) was obtained from Sigma (United States). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Wako (Japan).

Encapsulation of EPE by MD with Protein or Peptides. Before encapsulation, peroxides were removed from EPE by utilizing a Sep-Pak Vac 20 cm^3 (5 g) Florisil Cartridge twice. 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 soy peptide or soy protein 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 Sersakusho, 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. A laser diffraction particle size analyzer (model LA-500, Horiba Saisakusho, Kyoto, Japan) was used to determine the droplet diameter distribution from which the specific surface area was derived. The emulsions were dried by a freeze-drier 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 frozen emulsion was dried below 6.6×10^{-4} atm and at room temperature for 24 h.

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 RH was approximately 10% with 55% (w/w) sulfuric acid and 70% with 22% (w/w) sulfuric acid. The samples were removed at stated intervals, and the oxidation of EPE was followed by gas chromatography (GC) analysis and peroxide value (POV) measurement.

GC Measurement. Two milliliters of water was added to the powder lipid (50 mg) after the 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 3.0×10^3 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 was discarded, 3 mL of solvent (methanol/water, 1:1) was added and mixed vigorously by a homogenizer and a sonicator. After centrification, the lower layer (chloroform layer) was removed and dried by nitrogen gas, followed by being dissolved 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 (FID). 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; and air pressure, 0.5 kg/cm^2. The ratios of EPE to an internal standard SAE (internal standard) were obtained by calculation from the corresponding recorded peak areas. The oxidation degree was calculated based on the ratio of the amounts of EPE and SAE (internal standard).

Measurement of POV. The POV was measured by the ferric thiocyanate method (*16*). A 0.02 mL portion of each sample-extracted oil, which was prepared in the previous section, was diluted with 4.78 mL of chloroform−methanol (2:1, v/v), 0.1 mL of 30% ammonium thiocyanate, 0.1 mL of a mixture of equal volumes of 0.02 M ferrous sulfate and 0.02 M barium chloride. Absorbance at 500 nm was measured exactly 3 min after the start of the reaction.

Determination of Nonencapsulated Lipids. The sample powders (10 mg) were immersed in 2 mL of *n*-hexane for 30 min and were centrifuged for 5 min at 3.0×10^3 g. After the supernatant was discarded, the samples were evaporated to dryness under a stream of nitrogen gas. The residues were dissolved in 2 mL of *n*-hexane and then, the amount of lipid was analyzed by IATROSCAN TH-10, which combines the techniques of thin-layer chromatography with a FID (Iatron Laboratories, Inc., Japan).

Observation of the Powder Lipids by Scanning Electron Microscopy (SEM). The morphology of the surfaces of the powder lipids prepared by freeze-drying was evaluated by a S-4100 (Hitachi, Ltd., Tokyo, Japan) scanning electron microscope, using an acceleration voltage of 15 kV. The encapsulated samples were fixed in a stub containing a double-faced adhesive metallic tape and coated with Pt−Pd using an ion coater (IB-3, Eiko, Tokyo). The conditions used to operate the electron microscope were as follows: objective aperture, 10 μm; sample distance, 18−23 mm; accelerating voltage, 15 kV; and tilt angle, 0°. Examinations were made at 700×, 1200×, and 2200× magnifications.

Electron Spin Resonance (ESR) Measurement Analysis of Wall Material Powders. MD (± protein and peptides) was mixed with an aqueous solution of the spin probe Tempol, and the resultant solutions were freeze-dried. The ratio of the peptides (protein), Tempol, and MD in the powder was 1:0.27:40. After incubation at RH = 10% or RH = 70% for 7 days, the sample powders were introduced rapidly 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), using heating cycles over the temperature range 25−120 °C in stepwise fashion at 10 °C intervals. Samples were allowed to reach thermal equilibrium for 5 min at each temperature before recording the spectra. The microwave power was below saturation. 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; and time constant, 0.3. In another experiment, the sample powder packed in capillaries was originally kept at 90 °C without stepwise elevation of temperature, and the intensity of the peaks in the ESR spectra was recorded periodically for 40 min.

DPPH Radical Scavenging Ability. The radical scavenging ability of MD, peptides, and soy protein in the aqueous solution was tested as follows. MD, each peptide, or soy protein (50 mg) was dissolved in 2 mL of sodium phosphate buffer (10 mM, pH 7.0). To the solution, 2

Table 1. Median Diameter and Surface Area for Emulsion Stabilization by MD[a]

formulation	median diameter (μm)	surface area (cm^2/cm^3)
MD	1.588	45194
MD + soy peptide	0.954	84894
MD + soy protein	0.865	105698
MD + GP-1	10.905	7234
MD + GP-2	1.350	60248
MD + GP-3	1.423	59095

[a] Soy protein, soy peptide, and gelatin peptide were added to MD prior to emulsification.

mL of ethanol and 1 mL of DPPH solution (0.5 mM DPPH in ethanol) were added. The absorbance of the DPPH was measured at 517 nm using a Shimadzu UN-2400PD recording spectrometer (Shimadzu Co., Ltd.) 30 min after the addition of the DPPH solution.

RESULTS

Droplet Size of Emulsions. EPE was dispersed by MD prior to freeze-drying to prepare the powder lipids. **Table 1** shows the effects of the addition of soy protein, soy peptide, and gelatin peptides on the median diameter and specific surface area of the emulsions. MD could produce a fine emulsion with a median diameter of 1.588 μm and a specific surface area of 45194 cm^2/cm^3. MD is not viscous enough to stabilize the emulsion. However, the local viscosity increase or local formation of matrix caused by MD may retard the moving and/or access of oil droplets. The addition of the peptides and soy protein mostly decreased the median diameter slightly and increased the specific surface area of the MD-stabilized emulsion. Especially, soy protein is the most effective, i.e., produced the finest emulsion, with a median diameter of 0.865 μm and a surface area of 105698 cm^2/cm^3. These results can be attributed to the surface activity of the protein and peptides. However, the addition of GP-1 exceptionally destabilized the emulsion drastically, i.e., increased the median diameter to 10.905 μm and decreased the surface area to 7234 cm^2/cm^3. The molecular size of GP-1 (1000) is too small to form a thick and rigid adsorbed layer at the oil droplet surfaces of the emulsions. It is also likely that GP-1 interfered with the stabilizing effects of MD.

The prepared emulsions were freeze-dried to obtain powder lipids. During the drying process, no separation of lipids was observed for all of the samples. The obtained emulsions were stored for 7 days to be tested with respect to the stability of the lipids.

Determination of Unoxidized Lipids in Powders by GC. To evaluate the degree of lipid oxidation during the 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 were expressed as the percentage of unoxidized lipids in the total lipids (**Figures 1** and **2**). The reproducibility of the results of these figures was good.

Figure 1 shows the autoxidation processes of EPE encapsulated with MD, MD + soy peptide, and MD + soy protein at low and high humidity at 40 °C. When EPE was encapsulated with MD, the lipid was easily oxidized irrespective of humidity. Especially, the amount of EPE rapidly decreased when the powder was stored at high humidity, reaching 20 and 0% after 3 and 5 days, respectively. Also, in the case of low humidity, the lipid amount decreased to almost 0% after 5 days.

The addition of soy peptide and soy protein to MD retarded the autoxidation especially at high humidity. The inhibitory effect was more dramatic for soy peptides, namely, the oxidation

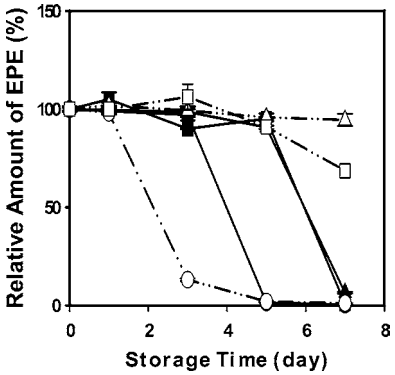

Figure 1. Effects of addition of soy peptide and soy protein on the oxidation of EPE encapsulated with MD. ■, MD + soy protein at RH = 10; □, MD + soy protein at RH = 70; ▲, MD + soy peptide at RH = 10; △, MD + soy peptide at RH = 70; ●, MD at RH = 10; ○, MD at RH = 70. Each value of the ratio is the mean of triplicate determinations.

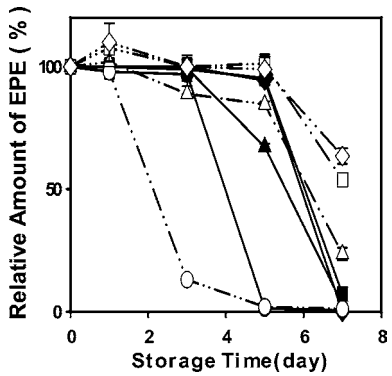

Figure 2. Effects of addition of gelatin peptide (GP-1, GP-2, and GP-3) on the oxidation of EPE encapsulated with MD. ■, MD + GP-3 at RH = 10; □, MD + Gp-3 at RH = 70; ▲, MD + GP-2 at RH = 10; △, MD + GP-2 at RH = 70; ●, MD at RH = 10; ○, MD at RH = 70; ◆, MD + GP-1 at RH = 10; and ◇, MD + GP-1 at RH = 70. Each value of the ratio is the mean of triplicate determinations.

of EPE was almost perfectly suppressed during 7 days when the lipid was encapsulated with MD + soy peptide. On the other hand, the amount of EPE decreased to approximately 70% after 7 days when the lipid was encapsulated with MD + soy protein. In the case where the powder lipids of MD + soy protein and MD + soy peptide were stored at low humidity, no change in the EPE amount was observed up to 5 days, but EPE rapidly decreased to 0% at 7 days. This means that high humidity is preferable for the suppression of lipid oxidation in the powder lipid of MD with soy protein or soy peptide, in contrast to the aforementioned results where the powder of only MD in which the lipid oxidation was more suppressed in lower humidity.

Figure 2 shows the autoxidation processes of EPE encapsulated with MD and MD + gelatin peptides (GP-1, -2, and -3). Again, the oxidation of the powder lipid including gelatin peptides was more suppressed in the case of storage at high humidity. GP-1 (MW = 1000) exhibited the best antioxidative activity, whereas the most rapid decline of the EPE amount was observed for the GP-2 (MW = 2000) case, indicating no clear relation of molecular size and antioxidative activity of the gelatin peptides. At low humidity, the amount of EPE in MD + gelatin peptides decreased to approximately 0% at 7 days irrespective of the type of gelatin peptides. Comparing the results of **Figures 1** and **2**, especially the case at the low humidity, it is obvious that soy peptide was more effective in suppressing EPE oxidation than the gelatin peptides in the MD powder system.

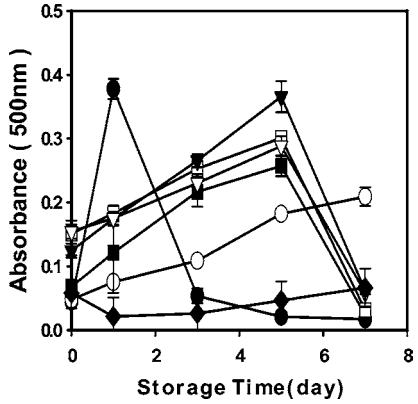

Figure 3. Time-dependent changes of peroxide value in powder lipids stored at high humidity (RH = 70). ■, MD + soy protein; □, MD + Gp-1; ●, MD; ○, MD + soy peptide; ▼, MD + GP-2; ▽, MD + GP-3; and ◆, MD + soy peptide (four times amount). Each value of the ratio is the mean of triplicate determinations.

Determination of POV. Figure 3 shows the results of the POV of EPE encapsulated with MD, MD + soy peptide, and MD + soy protein stored at high humidity and 40 °C. The POV method is believed to detect lipid oxidation more sensitively in the initial stage as compared with GC. A rapid increase in the POV was observed in MD, reaching a maximum at 1 day. The decrease in POV after reaching a maximum suggests that peroxides are converted to secondary products. When soy protein and gelatin peptides were added, the POV increased more gradually with time and reached a maximum at 5 days indicating the retardation of EPE oxidation. The reason for the high POV value in the presence of gelatin peptides at 0 days is unclear. The suppression of EPE oxidation was most effective for the MD + soy peptide system, which was in agreement with results of GC. However, soy peptides could not inhibit the POV increase completely. Even though a trend similar to the results of GC was shown, the sensitive detection by POV revealed the progress of EPE oxidation even in the presence of soy peptide. However, as shown in **Figure 3**, the increment in the soy peptide amount (four times) in MD powder enabled almost perfect inhibition of EPE. Therefore, one could reasonably conclude that soy peptide has an excellent antioxidant activity in the powder system.

Change in the Fraction of Nonencapsulated Lipids during Storage. The improved antioxidation effect of powder lipids by the addition soy protein and soy or gelatin peptides, which was shown in the previous section, could be due to the modification of encapsulating behavior in the powdery lipids. Namely, a decrease in nonencapsulated lipids or exposed lipids at the surface of the powders was expected. To confirm this point, nonencapsulated lipids were determined as hexanes−extractable oils by an IATROSCAN machine. The fraction of nonencapsulated lipids in the powder lipids did not change significantly irrespective of the presence or absence of soy protein, soy peptide, and gelatin peptides, i.e., MD:24.9%, MD + soy peptide:26%, and MD + soy protein:21%. This result means that the inhibition of lipid oxidation by the protein or peptides in the powdery system cannot be explained by a change in the encapsulating behavior of the lipids.

Observation of Powder Lipids by SEM. Figure 4 shows the surface images of powder lipids prepared by freeze-drying and stored at high humidity. The similar surface images were observed for the case of storage at low humidity (data not shown). The surface of the freeze-dried powder lipid was smooth, but there were large or small pores observed in some places, perhaps originating from the evaporation of large ice crystals formed during freezing or the bursting of air bubbles.

Comparing images at 0 days (**Figure 4a**−**c**) with those at 7 days (**Figure 4d**−**f**), slight changes were observed with black or gray rings surrounding the pores. The area of such a gray ring zone was spread more widely with more dark color. Although we have no direct evidence, this black ring may be attributable to the exposure or leakage of oil to the surface through the pores. Despite such a time-dependent change in the black ring zones, all powders presented similar external morphologies, irrespective of addition of the protein and peptides when comparing the samples at the same day of storage. This suggests that the addition of the peptides and the protein had no effect on the surface structure of the powder lipids.

Analysis of MD Matrix Mobility by ESR. Encapsulation of lipids depends on the physical properties of the wall materials. If the mobility of the wall materials matrices is increased, diffusion of oxygen as well as chemical reaction in the powder lipid is accelerated; thereby, lipid oxidation should be initiated. Therefore, by using the ESR technique, the effects of the addition of the peptides and protein on the mobility of the MD matrix were investigated. Conventional and saturation transfer ESR spectroscopy were used to study the temperature dependence of the rotational behavior of the spin probe Tempol in the powders of MD, MD + peptides, and MD + soy protein at the two humidities. **Figure 5a**,**b** shows the typical patterns of ESR spectra in the case of storage at high humidity. The signal indicating a component with slow motion was observed at 20 °C (**a**), and the signal of a component with a fast motion was shown at 90 °C (**b**). During the process of elevating the temperature from 20 to 90 °C, both of the signals appeared in the ESR spectrum. The relative ratio of the fast (F) and slow (S) components was calculated and plotted as a function of temperature.

Figure 6 shows a plot of the S/F ratio vs temperature for MD, MD + peptides, and MD + soy protein powders stored at high humidity. There was a dramatic change in the rotational mobility from 20 to 40 °C, indicating the onset of a moving MD matrix, which accelerates the lipid oxidation.

Although the S/F ratio was different among the samples at 20 °C, the decline in the S/F ratio to a similar level of approximately 2 around 40 °C happened irrespective of the presence or absence of soy protein and peptides. These results suggest that the addition of the peptides and soy protein did not affect the mobility of the MD matrix around 40 °C. This fact is important, because the powder lipids were stored for the test of lipid oxidation at 40 °C in the present study. In the case of low humidity, a dramatic decrease in the S/F ratio with a temperature increase around 40 °C could not be observed (data not shown).

Radical Scavenging Ability of MD, the Peptides, and the Protein in an Aqueous Solution. Although the oxidation of the powder lipids was suppressed by the addition of the peptides and soy protein, these ingredients were found to scarcely modify the physical properties and microstructure of the powdery lipids. This fact caused us to consider that chemical factors such as radical scavenging ability play more important roles in the suppression of lipid oxidation by the ingredients. To test the ability of MD, soy peptide, soy protein, and gelatin peptides (GP-1, GP-2, and GP-3) as radical scavengers, the disappearance of the DPPH radical (stable and water soluble radical) was followed for 30 min after the addition of MD and the other ingredients (**Figure 7**). The absorbance was expressed as the relative value (percentage) of the absorbance without the

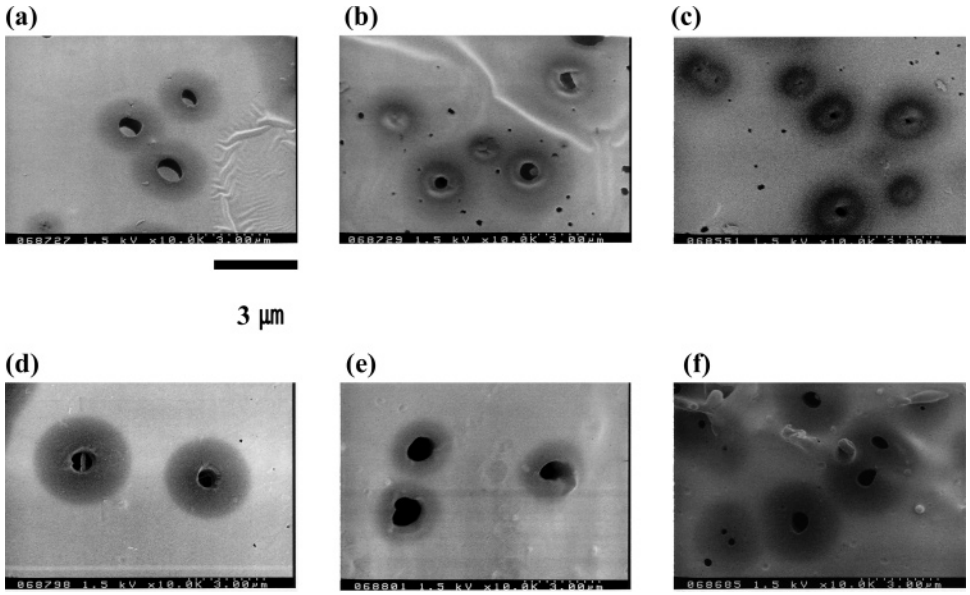

Figure 4. SEM images of powder lipids. The observation by SEM was performed before storage (0 day) and after storage for 7 days at high humidity. (**a**) MD (0 days); (**b**) MD + soy peptide (0 days); (**c**) MD + soy protein (0 days); (**d**) MD (7 days); (**e**) MD + soy peptide (7 days); and (**f**) MD + soy protein (7 days).

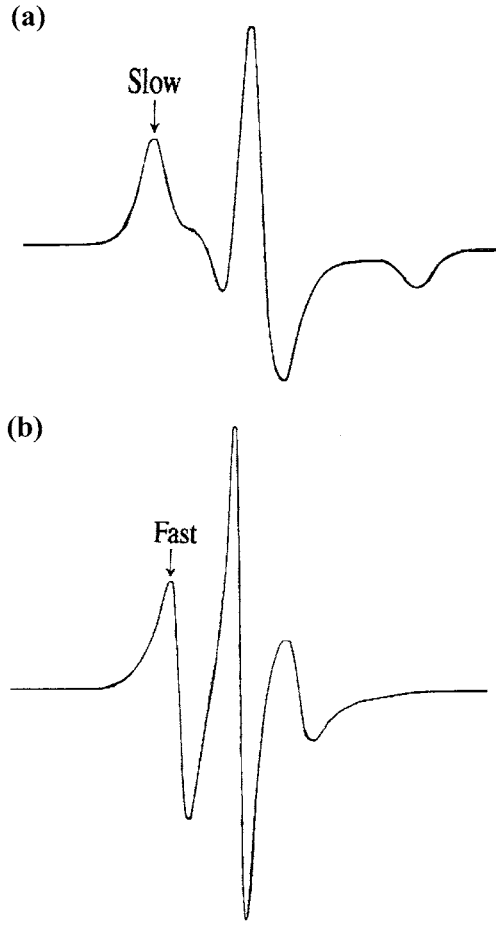

Figure 5. ESR spectra of Tempol in MD powder stored at high humidity. (**a**) Spectrum at 20 °C and (**b**) spectrum at 90 °C.

addition of MD, the peptides, and the soy protein. 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 polysaccharide. Slight decreases in the absorbance were observed with gelatin peptides (GP-2, GP-3, and GP-1). Soy protein reduced the absorbance

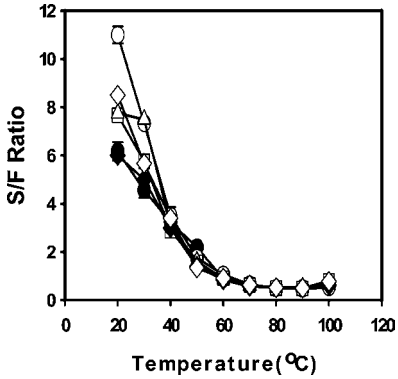

Figure 6. Ratio of slow and fast components as a function of temperature. Intensities of the signals corresponding to the component with slow motion (**Figure 5a**) and the component with fast motion (**Figure 5b**) were measured for ESR spectra during the process of temperature elevation from 20 to 90 °C. The ratio of intensity of slow and fast components was calculated and plotted against temperature. ●, MD; ○, MD + soy peptide; □, MD + soy protein; △, MD + GP-3; ◇, MD + GP-1; and ◆, MD + GP-2.

to 37%, showing an ability to scavenge radicals. However, the scavenging activity of soy peptide was the best, causing a dramatic decrease (approximately to 24%) in the absorbance of DPPH.

Radical Scavenging Ability of MD, the Peptides, and the Protein in the Powder System. Further to the radical scavenging ability in aqueous solution, the radical scavenging ability in the powder system was investigated. To test the ability, we used an ESR technique. As can be seen in **Figure 5a**, a sharp signal having the highest peak in the center position was observed for Tempol in the powder system of MD and MD + peptides or protein 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 decreased dramatically because of the high chemical reaction rate at 90 °C.

Figure 8 shows the results for the powders, which were stored at high humidity prior to the ESR measurements. In the case of MD without the other ingredients, a relatively high intensity

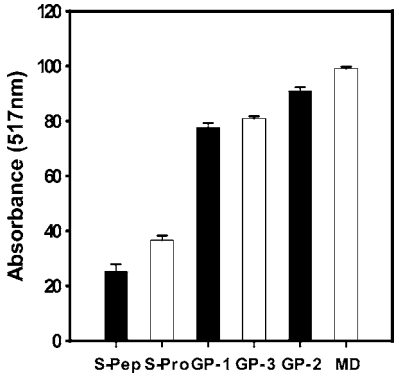

Figure 7. Radical scavenging activity of the MD, soy peptide, soy protein, and gelatin peptides (GP-1, GP-2, and GP-3). MD, soy peptide, soy protein, and gelatin peptides were mixed with DPPH, and the absorbance at 517 nm was measured after 30 min. The numbers reported are the means of four measurements.

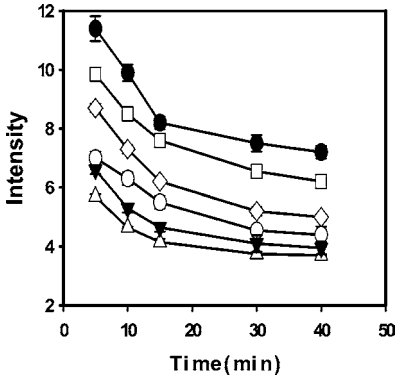

Figure 8. Time-dependent change in intensity of a center in ESR spectrum for Tempol in MD powder. Powders were kept at 90 °C, and ESR spectra were measured periodically for 40 min. ●, MD; ○, MD + soy peptide; □, MD + soy protein; △, MD + GP-3; ◇, MD + GP-1; and ▼, MD + GP-2.

(approximately 12) was observed at 2 min, followed by a gradual decrease to eight after 40 min. 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. The addition of soy and gelatin peptides enhanced the decrease in the intensity of Tempol. GP-3 and -2 were more effective than soy peptide, decreasing the intensity to nearly 4. GP-1 was less effective than soy peptide. In comparison with the peptides, soy protein exhibited a weak radical scavenging ability, i.e., the addition of soy protein to MD caused only approximately a one point decrease in intensity for the entire time. When the samples were stored at low humidity, time-dependent decreases in the Tempol signal were not observed (data not shown), indicating the importance of high humidity for the radical scavenging ability of soy peptide, gelatin peptides, and soy protein.

DISCUSSION

We have found, in the present study, that soy protein, soy peptide, and gelatin peptides have the ability to suppress lipid oxidation in the MD powder system especially in the high humidity state. The antioxidative effects of these protein ingredients were also observed at low humidity but not as strong. We assumed first that the encapsulation behavior of lipids with the MD matrix should be modified by the protein and peptides, and lipid oxidation could thereby be suppressed. Several physical properties were investigated such as the lipid particle size of the emulsions, the fraction of nonencapsulated lipids, SEM images of powder lipids, and the mobility of the MD matrix to find evidence of a change in the encapsulation behavior of the powder lipids.

The process of production of the powder lipids consists of two steps, i.e., the mixing of a lipid with solutions of wall materials to prepare the emulsions and the drying of the resultant emulsions. Minemoto et al. (*3*) investigated the effects of lipid particle size of the emulsions on the oxidation stability of the powder lipids. They demonstrated that an encapsulated lipid of small particle size oxidized more slowly than did that of large particle size. The small particle size of a lipid would seem to be undesirable for oxidation because of the large specific surface area increasing the accessibility of the lipid to external oxygen. The reason for the better stability of powder lipid with small particle size was explained by the increased degree of interaction of the lipid with the wall material protecting the lipid from the attack of external oxygen. On the basis of these findings, we also investigated the effect of the addition of soy protein, soy peptide, and gelatin peptides (GP-1, -2, and -3) on the emulsions stabilized by MD. As shown in **Table 1**, slight decreases in particle size due to soy protein, soy peptide, GP-1, and GP-2 were observed, but such small differences may not explain the large differences in the suppression of lipid oxidation by the protein and peptides of **Figures 1** and **2**. Furthermore, despite the large particle size induced by GP-2, the lipid oxidation was retarded by the addition of this peptide to MD. These results suggest no close relationship between the lipid particle size in the emulsions and the stability of the powder lipids.

Minemoto et al. (*24*) found that the ratio of the solvent extractable fraction from powder lipids was correlated to the degree of lipid oxidation. The lipid fraction exposed on the powder surface is likely to be not only more extractable than the core lipid but also more susceptible to oxidation. Therefore, in the present study, the fraction of unencapsulated lipid was determined by the hexane extraction method for the powder lipids. As described in the results, none of the soy protein, soy peptide, and gelatin peptides affected the degree of unencapsulated lipids very much, indicating that the protein and peptides have no special effects on suppressing the exposure of lipids to the MD powder surface. The results of SEM images (**Figure 4**) of the powder lipids support this speculation; namely, the surface structure of the powder could not be modified by the protein and peptides.

Finally, the ESR technique was attempted to probe the mobility of the MD matrix. The addition of soy protein, soy peptide, and gelatin peptides could not change the mobility of the MD matrix around 40 °C (**Figure 6**), which was the temperature for the test of lipid oxidation. Thus, we could not find evidence of the relationship between the mobility of the MD matrix and the suppression of lipid oxidation by the peptides and the protein.

On the basis of the experiments aforementioned, we could conclude that physical factors should not contribute to the inhibitory effects of soy protein, soy peptide, and gelatin peptides on EPE oxidation. In the next step, therefore, we focused on the chemical aspects of the protein and peptides, i.e., their radical scavenging ability. The radical scavenging abilities of soy protein, soy peptide, and gelatin peptides were demonstrated in the powder system as well as in the aqueous system. Particularly, it is worthwhile to notice that the radical scavenging ability in the aqueous phase (**Figure 7**) corresponded perfectly to the degree of suppression of lipid oxidation by the proteins and peptides especially at high humidity (**Figures 1**

and **2**). Although such agreement was not found between the results of radical scavenging ability in the powder system (**Figure 8**) and the lipid oxidation, soy peptide and gelatin peptides presented an excellent radical scavenging ability. On the basis of these results, it is likely that the suppression of lipid oxidation in the MD powder system is attributed to the radical scavenging ability of the peptides. It is known that peptides have the ability to react with free radicals (*25*−*27*). Although the radical scavenging ability of soy protein was second following that of soy peptide in the aqueous phase (**Figure 7**), its ability in the powder system was not as strong (**Figure 8**). Nevertheless, soy protein showed excellent inhibitory effects on lipid oxidation in the powder system (**Figure 1**). The molecular mechanism of the antioxidative activity of soy protein in the powder system is not clear, but some unknown factors such as the denser packing of MD and the soy protein layer surrounding EPE may contribute to protection from oxygen attack in addition to radical scavenging ability.

In addition to these factors, the moisture adsorption of each wall material is worth deliberation with respect to the inhibition of lipid oxidation. The freeze-dried powder is very hygroscopic in nature. During storage, the structure changes by the adsorbed water. The effective surface area of the powder for oxygen transferring into the powder might be changed markedly. In addition, water seems to play an important role in the chain reaction of oxidation of the lipid and forms hydrogen bonds with the hydroperoxides, which are produced on the propagation step. Furthermore, the mobility of these species should be increased with the increase of water activity; the structure changes from the glassy state to the rubbery state. We measured the amount of water adsorption after the storage for 7 days for the lipid powders. We found that MD, MD + soy peptide, MD + soy protein, and MD + gelatin peptide (GP-3) showed 5.87, 4.02, 2.47, and 3.56% of water adsorption at RH = 10 and 12.98, 10.50, 9.68, and 9.95% at RH = 70, respectively. These results indicate that the peptide and protein addition caused the decrease of water adsorption by MD powder. Such differences in water content might affect the glass transition and the physicochemical properties of the powders aforementioned, thereby modifying the lipid oxidation behavior.

The most striking feature in the results of the present study is that the oxidation suppression in MD powder by the protein and peptides was more effective in a high humidity state than in a low humidity state. The reason high humidity is desirable for the suppression of lipid oxidation by the peptides and proteins is not clear. Moisturizing the powders to some extent may be necessary to activate the radical scavenging ability of the protein and peptides. It is also likely that the elevated mobility of the wall materials by moisturizing enables the peptides and proteins to move and catch radicals more effectively. Further studies are necessary to understand the mechanism of the enhanced ability of the peptides and protein to suppress lipid oxidation at high humidity.

In conclusion, the present study revealed that the addition of small amounts of soy protein, soy peptide, and gelatin peptides to MD powder greatly improved the stability of EPE against oxidation. The storage of the powders at high humidity is more effective in suppressing the lipid oxidation as compared to storage at low humidity. However, high humidity, accelerating the growth of microorganisms and the degradation of food components by chemical and enzymatic process, is not good for food preservation. We should search for more effective peptides and better formulations to fulfill the perfect inhibition of lipid oxidation in a powder system even in the dry state. Such challenges may lead to the development of new types of powdery foods including physiologically important lipids.

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