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Antioxidative Effects of Mushroom *Flammulina velutipes* Extract on Polyunsaturated Oils in Oil-in-water Emulsion

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Abstract The antioxidative activities of the water solution of crude extract from edible mushroom *enokitake Flammulina velutipes* were compared with those of ascorbic acid and ascorbic acid 6-palmitate in oil-in-water (O/W) emulsions of cod liver oil. Oxidation of the emulsions was carried out at 40 and 50°C in the dark. The antioxidant activities were measured by *in vitro* assay against oxygen uptake, 2-thiobarbituric acid value, hydroperoxide formation of the oils. Also, residual docosahexaenoic acid content was measured as indices of lipid oxidation. The cod liver oil in O/W emulsions with added *enokitake* crude extract (ECE) was significantly more stable against lipid oxidation than the control emulsions without the extract in terms of any oxidation indices used. Moreover, ECE provided remarkable antioxidative properties to eicosapentaenoic acid ethyl ester in emulsion system. These observations demonstrate that *F. velutipes* can be used as a natural antioxidant, which effectively prevents oxidation of polyunsaturated oils in emulsion system.

Keywords: antioxidant, *Flammulina velitipes*, emulsion, hydroperoxide, mushroom

Introduction

Polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), especially abundant in fish oils, are important ingredient as edible oils and a source of energy in different formulations for animal feed (1). The high content of EPA, DHA, and other PUFA usually makes fish oils susceptible to oxidative deterioration. Lipid oxidation is an autocatalytic process where the products of the initial reaction propagate the oxidative process resulting in unpleasant rancid or off (2), which can be degraded into secondary oxidation products such as carbonyls and hydrocarbons (3). The addition of antioxidant to foods is one of the most effective methods to retard the oxidation of PUFA. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate (PG) are commonly used to prevent oxidation of fats and oils, because they are efficient and relatively cheap. However, the food hygienic safety of synthetic antioxidants has been questioned (4); BHA used in fats and oils were found to be carcinogenic by increased secretion of microsomal enzymes of liver and extra-hepatic organs such as the lungs and gastrointestinal tract mucosa (5,6). Therefore, the increase in consumer concern over such risks has stimulated the research for alternative natural antioxidants such as tocopherols and ascorbic acid (7). In recent years, potential antioxidative compounds have been found in the crude extracts of several plants, including fruits, herbs, vegetables, cereals, barks, roots as well as oilseeds (8-13). Also, the antioxidative properties of inedible mushrooms have been reported; an inedible mushroom *Cryptoporus volvatus* (polyporaceae) contained a large amount of novel bitter drimane sesquiterpenoids, cryptoporic acids A-G (14), which showed the inhibitory activities toward superoxide anion radical release. The analogues of novel neogrifolin, including 3-hydroxyneogrifolin, and 1-formyl-3-hydroxyneogrifolin, which had more potent antioxidative properties than α-tocopherol or BHA, were isolated from the inedible mushroom *Albatrellus ovinus* (15). In addition, the antioxidative activities of various species of edible mushroom have been investigated (16). For example, the methanol extracts of several edible mushrooms, including brown mushroom *Lentinula edodes*, oyster mushroom *Pleurotus cystidiosus*, *enokitake* mushroom *Flammulina velutipes* have been reported to possess antioxidative properties in bulk (17). Recently, the crude extract of *enokitake* clearly showed antioxidative properties in purified cod liver oil-in-water emulsion system compared to dechlorophyllized green tea extract and catechin during oxidation at 40°C (18). In several reviews, Porter (19-21) postulated the general rule that in food systems of low surface-to-volume ratio (e.g., bulk vegetable oils) polar antioxidants with high hydrophilic-lipophilic balance, such as propyl gallate, *tetra*-butyl hydroquinone, and Trolox C, are more effective than nonpolar lipophilic antioxidants, such as BHA, BHT, and tocopherols. However, the literature lacks information on the antioxidative properties of *enokitake* mushroom extract to polyunsaturated oils in oil-in-water (O/W) emulsions.

This paper reports a study aimed at testing lipophilic antioxidant (ascorbic acid 6-palmitate) and hydrophilic antioxidant (ascorbic acid) with purified cod liver oil in O/W emulsion system during oxidation at 50°C to comparison the antioxidative properties. Also, antioxidative properties of ECE were examined on purified EPA ethyl ester O/W emulsions system during oxidation at 40°C. Antioxidative properties were measured with the same methods: oxygen

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absorption, TBA value, and hydroperoxide formation. In order to better understand the antioxidative properties of *enokitake* crude extract (ECE), the present study were measured DHA and EPA contents in purified cod liver oil and EPA ethyl ester O/W emulsions.

Materials and Methods

Materials and chemicals Fresh *enokitake* was purchased from local retailers in Tokyo. EPA ethyl ester of over 92% purity was recovered from the gelatin capsules of Epadel® (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan). Cod liver oils were purchased from Toho Co., Ltd. (Tokyo, Japan). 2-Thiobarbituric acid (TBA) and butylated hydroxytoluene (BHT) were from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). L(+)-Ascorbic acid (AA) sodium salt was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-Ascorbic acid 6-palmitate (AP) was from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Polyoxyethylene (20) sorbitan monolaurate (Tween-20™) was from Wako Pure Chemical Industries, Ltd. 1-Palmitoyl-2-[12-(7-nitro-2-1,3-benzoxadiazole-4-yl)amino[dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-labeled PC) was from Avanti Polor Lipids, Inc. (Alabaster, AL, USA).

Preparation of *enokitake* crude extract *Enokitake* crude extract (ECE) was extracted as follows (18). Briefly, fruiting body of fresh *enokitake* (750 g) was freeze-dried and 97 g of lyophilized powder was obtained. A portion (2 g) of the lyophilized powder was homogenized in 40 mL of 70%(v/v) aqueous acetone with an Ultra Turrax T25 (Janke and Kunkel, IKA-Larbortechnik, Staufen, Germany) at 10,000 rpm for 4 min. The homogenate was centrifuged at 3,000×g for 15 min at 4°C, and the supernatant was collected. The precipitate obtained was homogenized again in 40 mL of the 70%(v/v) aqueous acetone and centrifuged. The combined supernatant was evaporated with a rotary evaporator at 40°C *in vacua* gave a gummy residue and lyophilized (0.43 g). The residue was dissolved in water to obtain the ECE.

Purification of oils According to Frankel *et al*. (22) methods, the cod liver oil was purified by column chromatography on silica gel 60 (6 cm i.d.×72 cm, Spherical, 40-50 μm, Kanto Chemical Co. Inc, Tokyo, Japan) to remove polar compounds and tocopherols. The oil (35 g) was charged on the column and eluted with *n*-hexane, followed by 7%(v/v) diethyl ether in *n*-hexane.

α-Tocopherol added in EPA ethyl ester, which was recovered from the gelatin capsules of Epadel®, was removed by column chromatography on silica gel 60 under similar conditions as mentioned above.

Preparations of oil-in-water emulsions The O/W emulsions (50 mL) were prepared by mixing purified cod liver oil (or purified EPA ethyl ester, 2.78 g) and Tween-20™ (0.28 g) as emulsifier in 0.05 M phosphate buffer (pH 7.0) using ultrasonication for 6 min on ice. Two O/W emulsions with different contents of the ECE (380 and 38 μg/mL emulsion) were prepared as the test samples. For the studies of cod liver oils, the emulsion containing ascorbic acid (500 μg/mL) or ascorbic acid 6-palmitate (500 μg/mL) was prepared for a comparison.

Oxidizing conditions of lipids in oil-in-water emulsions For the determinations of hydroperoxide contents, TBA reactive substances (TBARS) and DHA contents, 25 mL each of the control or the test emulsions was placed in an Erlenmeyer flask. To determine of oxygen absorption amount, 5 mL portion of the emulsions were placed in a glass vial (61 mL in volume), which were then sealed with an aluminum cap using a polytetraflonethylene silicone liner. The emulsion samples were stored in an oven controlled at 40 or 50°C in the dark with continuous shaking.

Oxygen absorption In the oxygen absorption assay, 0.1 mL portion of the headspace air in the vial was withdrawn with a gas tight micro syringe with a 24 G needle and immediately subjected to a Shimadzu gas chromatograph GC3BT equipped with a glass column (2.5 mm i.d.×1.7 m) packed with molecular sieve 5A (80-100 mesh, Nihon Chromato Co., Ltd., Tokyo, Japan) and a thermal conductivity detector. Helium was used as a carrier gas at an inlet pressure of 1.2 kg/cm^2.

Hydroperoxide formation Hydroperoxide contents were determined by a slightly modified flow-injection system with a diphenyl-1-pyrenylphosphine (DPPP, Dojindo Laboratories Co., Ltd., Tokyo, Japan) fluorescent post-column detection (23). Briefly, a mixture of 1-butanol: methanol (2:1, v/v) was used as a mobile phase at a flow rate of 0.5 mL/min. The DPPP solution (5 mg DPPP and 100 mg BHT in a 200 mL mixture of methanol and 1-butanol (1:2, v/v) was pumped to a reaction coil (0.25 mm i.d.×40 mm) at a flow rate of 0.3 mL/min. The hydroperoxide in the samples reacted with DPPP to form DPPP oxide in the coil and subsequently detected with a fluorescence HPLC monitor RF 535 (Shimadzu, Kyoto, Japan) at excitation wave length (Ex) 535 nm and emission wavelength (Em) 380 nm. The NBD-labeled PC used as an internal standard was detected at Em 534 nm and Ex 460 nm with a Shimadzu spectrofluorometric detector RF 10AXL which was set in the flow line behind the front fluorescent detector. A calibration curve was obtained as follows: cumene hydroperoxide (Aldrich Chemical Co., Inc.) was mixed with the NBD-labeled PC as an internal standard in methanol. The peak area ratios of cumene hydroperoxide vs. the internal standard were plotted against molar ratios of the compounds to obtain a calibration curve.

Thiobarbituric acid (TBA) value TBA value was determined by AOCS Official Method Cd 19-90 (24).

Fatty acid analysis Fatty acid methyl ester (FAME) was prepared according to AOCS Official Method Ce 1b-89 (25). Quantitative analysis of the FAME was carried out by gas liquid chromatography using a Shimadzu model GC 15APF instrument equipped with a Supelcowax-10™ fused silica open tubular capillary column (0.25 mm i.d.× 30 m, 0.25 μm in film thickness, Sigma-Aldrich, St. Louis, MO, USA) and a flame ionization detector. The column oven temperature was programmed from 150 to 240°C at a rate of 1°C/min. Helium was used as carrier gas under a column inlet pressure of 2 kg/cm^2.

Statistic analysis The data presented are mean±standard deviation (SD) of 5 replicates. Data were analyzed using the statistical analysis system software package (26). Significant differences between means were determined by Pearson's tests.

Results and Discussion

Changes in oxygen absorption Changes in oxygen absorption of the EPA ethyl ester O/W emulsions during oxidation at 40°C are shown in Fig. 1. For the control sample without added extract, the absorbed amount of oxygen decreased rapidly during 120 hr of oxidation time. At 120 hr of oxidation, 67% of the initial oxygen in the head space air of the vials decreased. In contrast, the oxygen uptake was minimal for each of the emulsions with added ECE. The initial amounts of headspace oxygen decreased on 10.9 and 14.3% in the test samples containing 380 and 38 μg/mL emulsion of ECE, respectively. These results clearly showed that addition of the *enokitake* extract to the emulsion was effective to suppress oxygen absorption due to lipid oxidation.

Changes in oxygen absorption of the cod liver oil emulsions during oxidation at 50°C are shown in Fig. 2. Remarkable oxygen absorption was observed after 48 hr of oxidation time in the control sample without any additives as well as in the reference sample with added AA. For the reference sample with AP, there was no change in oxygen absorption during oxidation for up to 96 hr, however approximately 33% of the oxygen was absorbed at 168 hr of oxidation. In contrast, the rates of oxygen absorption in the test samples with added ECE were significantly lower compared to other samples. Especially, significant prevention of oxygen absorption occurred in the test sample containing 380 μg/mL emulsion of the ECE.

Theses results clearly showed that addition of the *enokitake* extract to the emulsion was effective to suppress oxygen absorption more than well-used the chemical antioxidants, AA and AP.

Changes in TBA values The relatively high TBA values may arise owing to the presence of carbonyl groups, which react TBA reagent that may contribute to off-flavor of oxidized oil (27). Changes in TBA values during oxidation at 50°C are shown in Fig. 3. The addition of AA to the emulsions accelerated the increase in TBA values. The addition of AP was effective to suppress increase in TBA values of the cod liver oil emulsions. The addition of ECE successfully controlled the increase in TBA values of the oils in emulsions. These result coincident with those

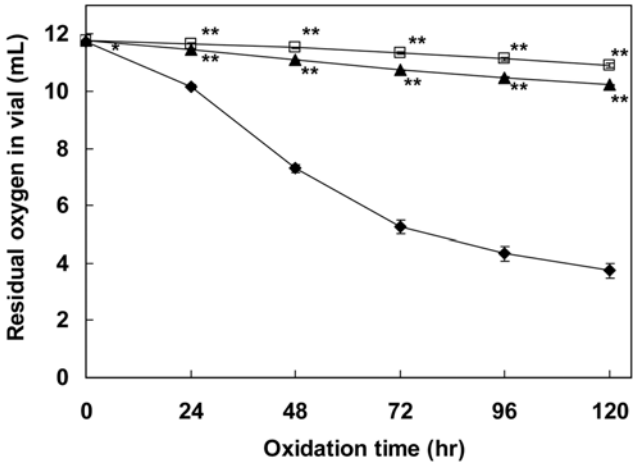

Fig. 1. Effect of added *enokitake* crude extract (ECE) on the residual amount of oxygen absorbed by eicosapentaenoic acid ethyl ester in O/W emulsion during incubation at 40°C in the dark. Data were expressed as mean value±SD (n=5). (◆) Control without ECE, (□) with 380 μg ECE/mL emulsion, and (▲) with 38 μg ECE/mL emulsion. Significant difference from the control are at p<0.01 (**) and p<0.05 (*).

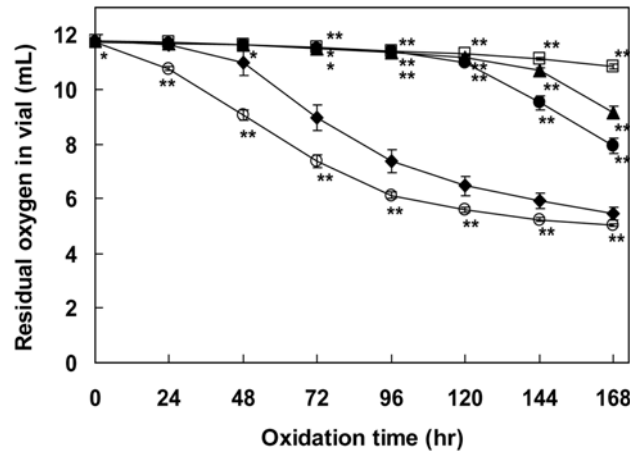

Fig. 2. Effect of added *enokitake* crude extract (ECE) on the residual amount of oxygen absorbed of cod liver oil in O/W emulsion during incubation at 50°C in the dark. Data were expressed as mean value±SD (n=5). (◆) Control without ECE, (□) with 380 μg ECE/mL emulsion, (▲) with 38 μg ECE/mL emulsion, (○) with 500 μg/mL ascorbic acid to oil, and (●) with 500 μg/mL ascorbic acid 6-palmitate to oil. Significant difference from the control are p<0.01 (**) and p<0.05 (*).

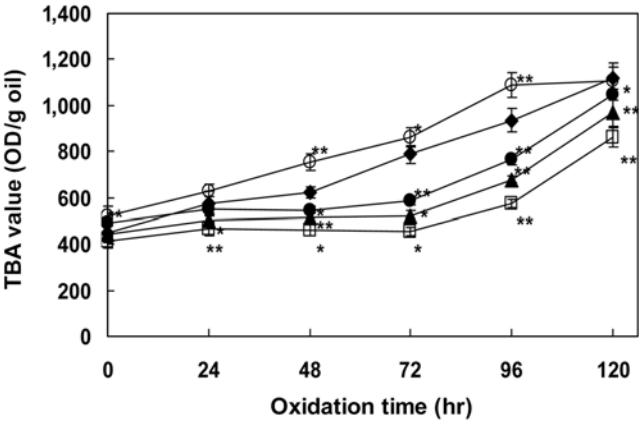

Fig. 3. Effect of added *enokitake* extract on the TBA value of cod liver oil in O/W emulsion during incubation at 50°C in the dark. Data were expressed as mean value±SD (n=5). (◆) Control without ECE, (□) with 380 μg ECE/mL emulsion, (▲) 38 μg ECE/mL emulsion, (○) with 500 μg/mL ascorbic acid to oil, and (●) with 500 μg/mL ascorbic acid 6-palmitate to oil. Significant difference from the control are at p<0.01 (**) and p<0.05 (*).

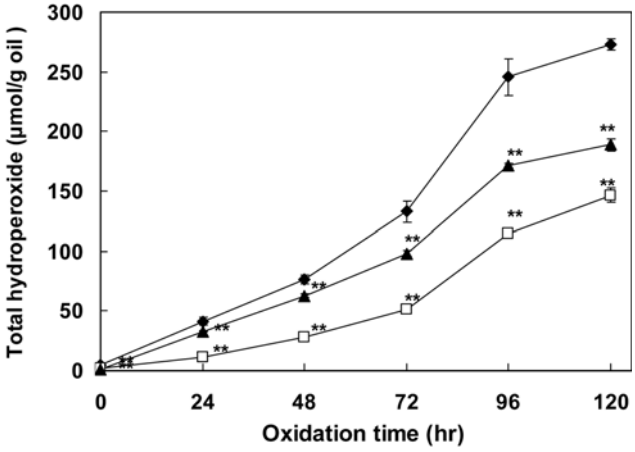

Fig. 4. Effect of added *enokitake* crude extract (ECE) on the hydroperoxide formation of eicosapentaenoic acid ethyl ester in O/W emulsion during incubation at 40°C in the dark. Data were expressed as mean value±SD (n=5). (◆) Control without ECE, (□) with 380 μg ECE/mL emulsion, and (▲) with 38 μg ECE/mL emulsion. Significant difference from the control are at p<0.01 (**) and p<0.05 (*).

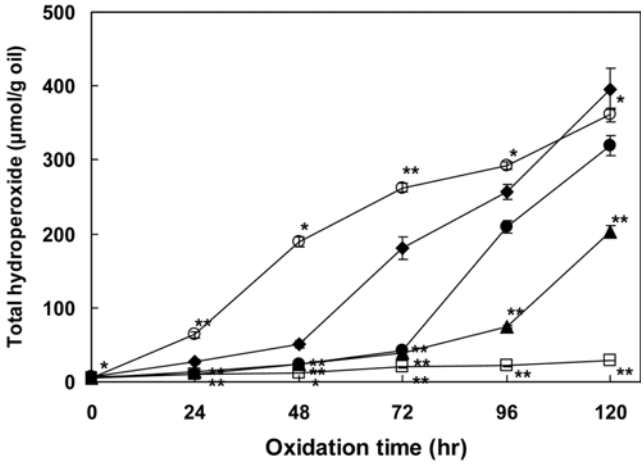

Fig. 5. Effect of added *enokitake* crude extract (ECE) on the hydroperoxide formation of cod liver oil in O/W emulsion during incubation at 50°C in the dark. Data were expressed as mean value±SD (n=5). (◆) Control without ECE, (□) with 380 μg ECE/mL emulsion, (▲) with 38 μg ECE/mL emulsion, (○) with 500 μg/mL ascorbic acid to oil, and (●) with 500 μg/mL ascorbic acid 6-palmitate to oil. Significant difference from the control are at p<0.01 (**) and p<0.05 (*).

obtained in the determination of oxygen absorption (Fig. 2). Also, similar trends were obtained in antioxidant activity for ascorbyl palmitate and in prooxidant activity for ascorbic acid on the hexanal formation in corn oil-in-water emulsion (28). Frankel *et al.* (29) reported that the mechanism of this process may be related to the affinities of the antioxidants toward the air-oil interfaces in bulk oil and the water-oil interfaces in emulsions. Thus, in the oil-in-water emulsion system, lipophilic antioxidants are sufficiently surface active to be oriented in the oil-water interface to better protect oil against oxidation. The hydrophilic antioxidants become diluted and cannot adequately protect the oil-in-water interface.

Changes in the levels of hydroperoxide The primary products of lipid peroxidation are hydroperoxides. Therefore, the results from determining of hydroperoxide formation are a clear index of lipid oxidation (30). Changes in the total hydroperoxide levels of EPA ethyl ester in O/W emulsions during oxidation at 40°C are shown in Fig. 4. The levels of hydroperoxide in the emulsions were lower in the samples with added ECE. The levels of hydroperoxide were lower in the test samples with higher concentration of the extract. The ECE exhibits remarkable antioxidative properties to eicosapentaenoic acid ethyl ester in emulsion system. These results clearly showed that the addition of the extract was effective at prevention, hydroperoxide formation in the emulsion compared to the control.

Changes in hydroperoxide levels of the emulsions during oxidation at 50°C are shown in Fig. 5. The levels of hydroperoxides in the test sample with added AA remarkably increased from an early stage of oxidation. The control sample had an induction period of 46 hr and subsequently showed a drastic increase in hydroperoxide levels. The hydrophobic AP showed antioxidative properties in O/W emulsion system compared to hydrophilic AA after 72 hr of oxidation in terms of hydroperoxide formation. The ECE was significantly more effective than AP in emulsion system. Similar results were obtained in a report of Frankel *et al.* (29) which hydrophobic antioxidant was more effective than hydrophilic antioxidant in O/W emulsions of corn oil. This effect may be allowing the some compounds to be more soluble at the oil in water (or water in oil) interphase in the oil and some more soluble in the water (30).

Thus, the addition of the ECE resulted in prolongation of an induction period: approximate 94 hr for the test sample with a lower amount (38 μg/mL emulsion) of the ECE. At the level of 380 μg/mL emulsion of ECE in the emulsions, the levels of hydroperoxides remained almost unchanged during oxidation time for up to 120 hr. Moreover, we reported that the ECE showed more effective antioxidative properties than those of dechlorophyllized green tea extract and catechin under the present experimental conditions (18). The cod liver oil and EPA ethyl ester in O/W emulsions with added *enokitake* crude extract was significantly more stable against lipid oxidation than the control emulsions without the extract in terms of any of above oxidation indices. These observations clearly show that *enokitake* extract contains certain hydrophilic compound which effectively prevents oxidation of polyunsaturated oils in emulsion system.

Changes in polyunsaturated fatty acid content Changes in EPA content of the O/W emulsion are shown in Table 1. For the control, the residual amount of EPA accounted for 92% at 144 hr of oxidation time. In contrast, 94% of the original EPA remained in the samples with the 380 and 38 μg/mL emulsion level of the ECE after 144 hr of oxidation. These observations strongly suggest that the *enokitake* extract provided antioxidative effects to EPA ethyl ester in emulsion system.

Changes in DHA content of the O/W emulsion are shown in Table 2. In the control sample the DHA contents decreased gradually to 27% of the initial content after 144

Table 1. Changes in the content of eicosapentaenoic acid ethyl ester O/W emulsions containing *enokitake* crude extract (ECE) during storage at 40°C in the dark (mg/g oil)

20:5*n*-3[1)]	Storage time (hr)		
	0	72	144
Control	668±2.29	632±1.82	611±1.66
Sample 1	668±2.21	657±1.52**	631±1.52**
Sample 2	668±2.23	632±1.65**	630±1.71*

[1)]Control, without ECE; sample 1, with 380 μg ECE/mL emulsion; sample 2, with 38 μg ECE/mL emulsion. Mean±SD (n=5); significant difference from the control are at **p<0.01 and *p<0.05.

Table 2. Changes in the content of docosahexaenoic acid of cod liver oil O/W emulsions containing *enokitake* crude extract (ECE) during storage at 50°C in the dark (mg/g oil)

22:6*n*-3[1)]	Storage time (hr)		
	0	72	144
Control	1.13±0.09	0.90±0.09	0.83±0.08
Sample 1	1.13±0.01	1.09±0.01**	1.15±0.01**
Sample 2	1.13±0.07	1.16±0.07**	1.03±0.06**
AP	1.13±0.04	1.16±0.05**	0.92±0.04*
AA	1.13±0.01	0.99±0.01**	0.73±0.01*

[1)]Control, without ECE; sample 1, with 380 μg ECE/mL emulsion; sample 2, with 38 μg ECE/mL emulsion; AA, with 500 μg/mL ascorbic acid to oil; AP, with 500 μg/mL ascorbic acid 6-palmitate to oil. Mean±SD (n=5); significant difference from the control are at **p<0.01 and *p<0.05.

hr of oxidation. In the reference sample with added AA, approximately 36% of DHA were oxidized after 144 hr of oxidation. Addition of AP was effective to prevent decrease of DHA for up to 72 hr of oxidation time, however approximately 19% of DHA decreased after 144 hr of oxidation time. In contrast, addition of the ECE was significantly effective to prevent decrease in DHA content: no remarkable decrease in the amount of DHA was observed in the test sample with higher amount of the extract after 144 hr of oxidation. Those results claimed that the *enokitake* extracts showed high antioxidative properties on the lipid oxidation.

Addition of the *enokitake* extract was effective to prevent decrease of DHA content on the O/W emulsion. DHA render more sensitive to oxidative deterioration, synthetic antioxidants such as BHA, BHT, and PG have been widely used as antioxidants in foods containing fish oils to prevent loss of DHA. However, use of synthetic antioxidants in food products is under strict regulation due to the potential health hazards caused by such compounds (2). For this reason native material extracts from leaves, fruits, and vegetables have been used in foods as replacement of synthetic antioxidants. Indeed, the crude green tea catechins reduced the formation of peroxides more effectively than α-tocopherol or BHA (31). Fresh strawberry extracts has 15 times higher total antioxidant capacity than Trolox in an artificial peroxyl radical model system (9). Fruits and vegetables contain different antioxidant compounds such as vitamin C and E, carotenoids, and phenolic compounds, especially flavonoids (12). Since research has tended to focus on the dietary value of edible mushrooms, there is relatively little in formation pertaining to their antioxidant properties and their possible use to treat oxidative stress. Jose and Janardhanan (32) have reported that the ethyl acetate and methanol extracts of *Pleurotus florida* exhibited potent hydroxyl radical scavenging and lipid peroxidation inhibition activities. *Hypsizigus marmoreus* mushroom was found to be medically active in several therapeutic effects such as antitumor (33), antifungal and antiproliferatives (34). In another study, Fu *et al.* (35) examined the efficiency of the ethanol extracts of certain edible mushrooms including *enokitake* and found that radical scavenging activity of the *enokitake* toward corn oil in O/W emulsion was weak. However, no results on the antioxidative potentials of the extracts on polyunsaturated oils were obtained in their work

The antioxidant activity of ECE was evaluated by oxygen absorption, TBA value, hydroperoxide formation, and polyunsaturated fatty acid content. According to the results of this study, it is clearly showed that the crude extract of *enokitake* contained certain compounds having potential antioxidative property. Although the antioxidative compound in *enokitake* has not been identified, the results of ^{13}C-NMR and ^{1}H-NMR suggested that the inhibitory compound is with pentose with alcohol group configuration. Utilization of the *enokitake* crude extract will be promised as a natural antioxidant for the prevention of unsaturated lipid oxidation.

Acknowledgments

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