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Antioxidative Activity and Antidiscoloration Efficacy of Ergothioneine in Mushroom (*Flammulina velutipes*) Extract Added to Beef and Fish Meats

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The antioxidative property of a hydrophilic extract prepared from the fruiting body of edible mushroom (*Flammulina velutipes*) was evaluated. The mushroom extract contained ergothioneine (ERT) at a level of 3.03 ± 0.07 mg/mL, showed higher 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and suppressed lipid oxidation of bigeye tuna meat more effectively than authentic L-ERT added at the same concentration. The authentic L-ERT had stronger total reducing power than the mushroom extract and inhibited the formation of metmyoglobin (metMb) more significantly in bigeye tuna meat. Lipid oxidation in beef and fish meats to which the mushroom extract had been added was "virtually" controlled during storage on ice. Ground beef and bigeye tuna meat with the extract added kept their natural colors unchanged for longer than 12 and 7 days of ice storage, respectively. Contrary to this, browning in meat color was observed in the control samples without the extract after 6 and 2 days of storage, respectively, when stored under similar conditions. There was significant correlation between meat color and chemical parameters, including total lipid hydroperoxides, thiobarbituric acid reactive substances, and metMb. However, there was no significant correlation between pH value and meat discoloration. These results suggest that ERT in the hydrophilic extract of *F. velutipes* plays an important role as a color stabilizer of meats.

KEYWORDS: Mushroom; ergothioneine; fish meat; beef; discoloration; myoglobin; lipid; oxidation; *Flammulina velutipes*

INTRODUCTION

Myoglobin (Mb) is known as a hemoprotein imparting the color of meat products. It has been observed that rapid darkening or browning of meats containing higher concentrations of Mb such as tuna meat and beef occurs when these meats are chilled or even frozen above −40 °C (*1*−*4*). The mechanism of this phenomenon has been proposed as the generation of free radicals through a lipid oxidation process, which may initiate Mb oxidation (*5*), and also metmyoglobin (metMb) formation promotes lipid oxidation (*6*). To control discoloration of muscle foods, the addition of antioxidants has been believed traditionally to be almost the only effective way (*7*−*9*).

In recent years critical problems of food safety have begun to change the food industry as a worldwide trend. Most consumers tend to prefer foods containing natural additives such as extracts from fruits and vegetables over those with artificial food additives. Extraction of natural antioxidants from plants for food application is therefore necessary to meet the demands of these consumers. The ethanolic extracts prepared from *Flammulina velutipes* had 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (*10*). The authors (*11*) showed that an acetone extract prepared from *F. velutipes* successfully prevented oxidation of polyunsaturated fatty acids (PUFA) in cod liver oil in water emulsion systems. Later, the inhibitory effect of the water extract on the oxidation of oxymyoglobin (MbO_2) isolated from cattle meats was reported (*12*). Previous studies have shown that extracts of various mushrooms have antioxidative activities; however, the active compounds, including certain phenolic compounds, ergothioneine (ERT) and saccharides, have been found in the extracts of different species of mushroom (*13*−*15*). Especially, ERT has been known as a powerful scavenger of hydroxyl radical ($^{\bullet}OH$) and an inhibitor of $^{\bullet}OH$ generation from hydrogen peroxide, which is catalyzed by iron or copper ion (*16*). Arduini et al. (*17*) demonstrated that ferryl Mb, which was formatted when deoxy Mb and metMb were exposed to hydrogen peroxide, was reduced to metMb in the presence of ERT. Although ERT showed high potential of antioxidative activity in vitro, its application to foods and foodstuffs is still limited. Application of ERT as a common food additive is a challenge of scientists as well as an expectation of processers. To face this challenge, our approach was started by using a crude extract of the edible mushroom *F. velutipes*, which contains an appropriate amount of ERT.

This study was, therefore, conducted to evaluate the antioxidative efficacy of ERT in the hydrophilic extract of *F. velutipes*

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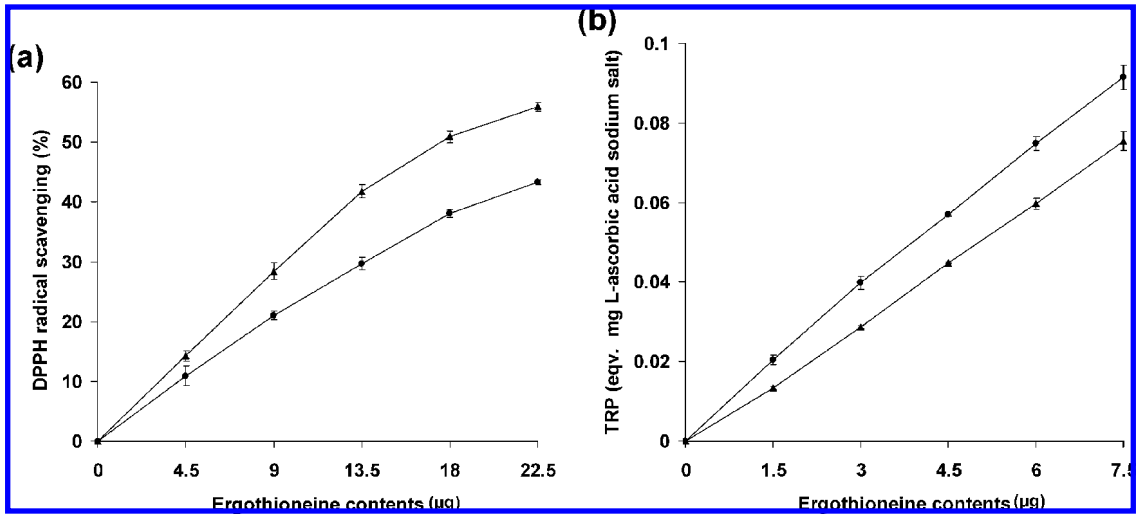

Figure 1. DPPH radical scavenging activity (**a**) and total reducing power ability (**b**) of mushroom (*Flammulina velutipes*) extract (▲) and authentic L-ergothioneine (●). The extract of 1 mL volume contained 3 mg of ergothioneine. Results are presented as mean ± SD ($n = 3$).

in comparison with an authentic ERT and its effect on preventing discoloration of raw beef and tuna meat during chilled storage.

MATERIALS AND METHODS

Materials and Chemicals. Fresh Pacific mackerel (*Scomber japonicus*) in the end of rigor stage and thawed blocks of muscle of bigeye tuna (*Thunnus obesus*) that had been frozen at − 70 °C for 3 months were purchased from the Tokyo Central Market, Japan. Fresh beef in postrigor stage, which was about 10 days after slaughtering under chilled storage, and fresh fruiting body (13−14 cm long) of mushroom (*F. velutipes*) harvested after 2 months of cultivation were purchased from local retailers in Tokyo. L-(+)-Ergothioneine was purchased from BIOMOL International, L.P. Diphenyl-1-pyrenylphosphine was purchased from Dojindo Laboratories Co. Ltd. (Kumamoto, Japan). 1-Myristoyl-2-(12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl)-*sn*-glycero-3-phosphocholine (NBD-labeled PC) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). HPLC grade methanol, chloroform, and 1-butanol were purchased from Kokusan Chemical Co. Ltd. (Tokyo, Japan). All other chemicals of analytical grade were obtained from Aldrich Chemical Co. (Milwaukee, WI) and Sigma-Aldrich (St. Louis, MO).

Mushroom Extract. Mushroom extract was prepared following a procedure of Jang et al. (*11*) with slight modifications. Briefly, 102 g of lyophilized powder of the mushroom was obtained from 1 kg of fresh fruiting body. A portion (5 g) of the mushroom lyophilized powder was homogenized in 40 mL of 70% (v/v) aqueous acetone at 10000 rpm for 4 min. The homogenate was centrifuged at 900*g* for 15 min at 4 °C, and the supernatant was collected. The precipitate thus obtained was homogenized again in 40 mL of 70% (v/v) aqueous acetone and centrifuged. The combined supernatant was evaporated at 40 °C in vacuo. The acetone free residue obtained was dissolved in 5 mL of distilled water. Consequently, the concentration of mushroom extract was 0.532 ± 0.005 g of dry materials/mL.

Preparation of Samples. Red meat of beef and ordinary muscles of bigeye tuna and Pacific mackerel were individually isolated and thoroughly minced with a National food processor model MK-K75 (Matsushita Electric Industrial Co., Ltd., Osaka, Japan). To compare the effectiveness of the mushroom extract with the equivalent content of authentic L-ERT added to fish meat, the minced tuna meat (100 g) was formulated to provide the following treatment: (i) mushroom extract (1 mL of mushroom extract contained 3 mg of ERT), (ii) L-ergothioneine (3 mg of authentic L-ERT in 1 mL of distilled water), and (iii) control (1 mL of distilled water). To evaluate the effects of mushroom extract at a higher concentration on preventing oxidation of lipid and Mb in beef and fish meats during ice storage, the samples were prepared by adding 5 mL of mushroom extract (= 15 mg of ERT) to 100 g of the minced meats and were then mixed well. The meats with 5 mL of distilled water added were used as control samples. A 100 g portion of the minced meats was packed individually in zipper-sealed polyethylene bags 140 × 100 × 0.08 mm (Seisannipponsha Ltd., Tokyo, Japan) and stored on ice.

Quantification of Ergothioneine. The content of ERT in mushroom extract was quantitatively determined according to the method of Dubost et al. (*18*) with a slight modification. Briefly, 1 mL of a solution of 1 mM methimazole was added as an internal standard (IS) to 0.2 mL of mushroom extract, and the ERT was extracted by 20 mL of 70% (final concentration) ethanol. The ethanolic solution was vortexed and left to stand at 4 °C for 2 h and subsequently centrifuged at 3000*g* for 15 min at 4 °C. Supernatant was collected and evaporated at 40 °C in vacuo to remove ethanol. The ethanol free residue thus obtained was dissolved in 10 mL of deionized water. The analysis was carried out using a model LCMS-2010EV high-performance liquid chromatograph mass spectrometer (Shimadzu) equipped with a C30 reversed-phase column (Develosil C30-UG-5, 4.6 mm i.d. × 250 mm, 5 μm particle size, Nomura Chemical Co. Ltd., Aichi, Japan). The 100% deionized water was pumped as a mobile phase at a flow rate of 0.25 mL/min. The injection volume of the sample was 20 μL, and the column temperature was kept at 25 °C. The content of ERT was quantitatively determined by monitoring a peak area ratio (ERT/IS) of fragment ions at *m*/*z* 230 and 115 for ERT and IS, respectively. A calibration curve was obtained by different concentrations of the authentic standard. All data were expressed as milligrams of ERT per milliliter of the mushroom extract. Triplicate extractions were done for ERT analysis from one crop of *F. velutipes*.

Determination of 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Activity. DPPH radical scavenging activity was determined following the method of Fu et al. (*10*) with a slight modification. Briefly, a 0.5 mL portion of 0.4 mM DPPH ethanol solution was mixed with various volumes ranging from 1.5 to 7.5 μL of the mushroom extract, and each was made up to a final volume of 2 mL by distilled water. The mixtures were mixed thoroughly and then kept at 25 °C for 30 min in the dark. The absorbance of the mixtures was measured at 517 nm against a blank without DPPH using a model UV-1700 Shimadzu spectrophotometer (Kyoto, Japan).

The DPPH radical scavenging activity was calculated using the equation

$$\text{DPPH radical scavenging activity (\%)} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

where A_{control} is the absorbance of a control mixture except the mushroom extract and A_{sample} is the absorbance of the sample mixture containing the mushroom extract.

Determination of Total Reducing Power Ability. The total reducing power ability of the mushroom extract was measured as described by Oyaizu (*19*) with a slight modification as described below.

Reaction mixtures containing 0.5 mL of 1% potassium ferricyanide and different volumes of the extract ranging from 0.5 to 2.5 μL were made up to 1.5 mL by 0.2 M sodium phosphate buffer (pH 6.6) in test tubes. The mixtures were incubated at 50 °C for 20 min, and subsequently 0.5 mL of 10% trichloroacetic acid was added, following by the addition of 2 mL of distilled water and 400 μL of 0.1% ferric chloride. The absorbance of the mixtures was measured at 700 nm against a control without mushroom extract.

pH Measurement. A portion (3 g) of minced muscle was homogenized with 10 mL of distilled water. The homogenate was centrifuged at 1700*g* for 5 min at 4 °C, and the supernatant was filtered through a no. 1 filter paper (Advantec Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The pH of the supernatant was then measured with a Horiba pH-meter model F-21 (Kyoto, Japan).

Determination of Total Lipids and Fatty Acid Composition. Total lipids in meat were extracted and determined according to the Bligh and Dyer procedure (*20*). Fatty acid methyl esters (FAME) were extracted and weighed according to the Official Method and Recommended Practices of the AOCS (*21*) using tricosanoic acid methyl ester as an internal standard. The FAME were analyzed with a Shimadzu gas chromatograph model GC-14B (Kyoto, Japan) equipped with an open tubular capillary column (0.25 mm i.d × 30 m, 0.25 μm in film thickness, Supelco, Bellefonte, PA). The initial oven temperature was kept at 140 °C for 1 min and then programmed to a final temperature of 240 °C at a rate of 1 °C/min. The temperature of both the injector and detector was kept at 250 °C. Helium was used as carrier gas at the column inlet pressure of 2 kg/cm^2.

Determination of Total Lipid Hydroperoxides (HPO). Total lipid HPO were quantitatively analyzed by a flow injection analysis (FIA) system equipped with a fluorescent detection instruments as described by Sohn et al. (*22*). Briefly, 1 mL of a solution of NBD-labeled PC (4.82 nmol/mL in methanol) was added as an internal standard to 5 g of minced meat, and lipids were extracted according to the procedure of Bligh and Dyer (*20*). The filtrate obtained was made up to 10 mL by chloroform. A 20 μL portion of the chloroform solution was subjected to analysis with the FIA system for the quantitative determination of HPO.

Determination of Thiobarbituric Acid Reactive Substances (TBARS). TBARS were determined spectrophotometrically according to a procedure of Uchiyama and Mihara (*23*). Absorbance at 535 and 520 nm of TBARS in butanol solutions was measured with the spectrophotometer. The difference between absorbances at 535 and 520 nm was used as the TBARS value.

Measurement of Metmyoglobin Formation. The percentage of metMb formed in meat was measured spectrophotometrically according to a procedure of Bito (*1*). Briefly, a 3 g portion of minced meat was thoroughly mixed with 10 mL of cooled (<4 °C) distilled water with a Teflon-coated magnetic stirring bar. The mixture was kept at 4 °C for 10 min and subsequently centrifuged at 3000*g* for 5 min. The supernatant was filtered through no. 1 filter paper. The pH of the filtrate was adjusted to 6.8−7.0 by 1 M NaOH and centrifuged again. The absorbance of the supernatant was measured at 540 nm (E_{540}) and 503 nm (E_{503}) as maximum absorbances of MbO_2 and metMb, respectively. The percentage of metMb was calculated using the absorption ratio (E_{540}/ E_{503}) according to a formula for bluefin tuna Mb (*1*).

Color Image Analysis. Color images of the surface of minced meat were acquired using a digital CCD camera (Cyber-shot 10.1 mega pixels, Sony Corp., Tokyo, Japan) equipped with a ring light (Cyber-shot; illumination intensity, approximately 100 lx; dimensions, 111 × 63 × 34 mm; Sony Corp.). Image data were acquired in a dark box to prevent outer light. The acquired images in JPEG format were analyzed for red (*R*), green (*G*), and blue (*B*) values using ImageJ software of the National Institutes of Health, Bethesda, MD (http://rsb.info.nih.gov/ij/). To remove device dependency toward the *RGB* color space and the brightness, the *RGB* values were normalized using the following expressions:

$$r = \frac{R}{R+G+B} \times 100$$

$$g = \frac{G}{R+G+B} \times 100$$

$$b = 100 - (r+g)$$

Through this normalization, the value *b* is a balance between *r* and *g*, and *b* is not taken into consideration for color evaluation.

Statistical Analyses. For all of the experiments triplicate samples ($n = 3$) were conducted from same raw materials under similar conditions. Multivariate analysis was performed using the Unscrambler version 9.7 (CAMO Process, Trondheim, Norway). The main variance in the data set was evaluated using principal component analysis. Partial least squares regression (PLSR) models were used to explore the correlation of pH, lipid oxidation indices (HPO and TBARS), metMb, and color changes. Microsoft Excel 2000 was used to calculate means and standard deviations for all multiple measurements and to generate graphs. Analysis of variance (ANOVA) was applied to the data using R software version 2.4.1 (http://cran.R-project.org). Significant differences were determined by one-way ANOVA, and Tukey's multiple comparisons of means was used to determine the statistical difference between samples.

RESULTS

Ergothioneine Content of the Mushroom Extract. The ERT content in the mushroom extract was 3.03 ± 0.07 mg/mL. Correspondingly, 1 kg of wet fruiting body of mushroom *F. velutipes* can be used to extract 300 mg of ERT according to the extraction method used in the present study.

Radical Scavenging Activity and Total Reducing Power (TRP) Ability of the Mushroom Extract. DPPH radical scavenging activities and TRP ability of the mushroom extract and authentic L-ERT with different amounts are presented in **Figure 1**. DPPH radical scavenging activity of the mushroom extract was higher ($p < 0.01$) than that of authentic L-ERT at the same concentration. The activity varied from 14.3 to 55.9%, which corresponds to 1.5−7.5 μL of the extract (= 4.5−22.5 μg of ERT). The effective volume of the extract at which DPPH radical was scavenged by 50% was 5.8 μL (= 17.6 mg of ERT).

The TRP of the mushroom extract exhibited dose-dependent activity between 0.5 and 2.5 μL (= 1.5−7.5 μg of ERT). In comparison with the authentic L-ERT, the TRP of the mushroom extract was lower ($p < 0.01$) at the same concentration.

Antioxidative Efficacy of the Mushroom Extract and Authentic L-ERT Added to Bigeye Tuna Meat. Effects of 1 mL of the mushroom extract (= 3 mg of ERT) in comparison with 3 mg of the authentic L-ERT added to 100 g of bigeye tuna meat are shown in **Figure 2**. Both samples with added mushroom extract and authentic L-ERT showed remarkable effects on controlling lipid oxidation as well as metMb formation in the tuna meat during ice storage. The samples with added mushroom extract suppressed the formation of HPO more remarkably in the tuna meat compared to those with added authentic L-ERT. The difference in HPO content of the tuna meat with added mushroom extract and the authentic L-ERT was significant after 2 days of ice storage. Contrary to the changes in HPO, the formation of metMb in tuna meat with added mushroom extract was regularly higher than that in tuna meat with added authentic L-ERT through the storage period. Although the metMb concentration was higher in the tuna meat with added mushroom extract, the bright red color of the tuna meat remained after 5 days of ice storage. Meanwhile, the control tuna meat showed significant browning after 2 days under the same storage conditions (**Figure 3**).

Changes in pH. The pH of the tuna and mackerel meats and beef without mushroom extract ranged between 5.80 and 5.93, between 5.80 and 5.89, and between 6.01 and 6.13, respectively. The pH of meats with added mushroom extract was slightly higher. However, there was no significant difference ($p > 0.05$) in the pH of individual meats during the storage period.

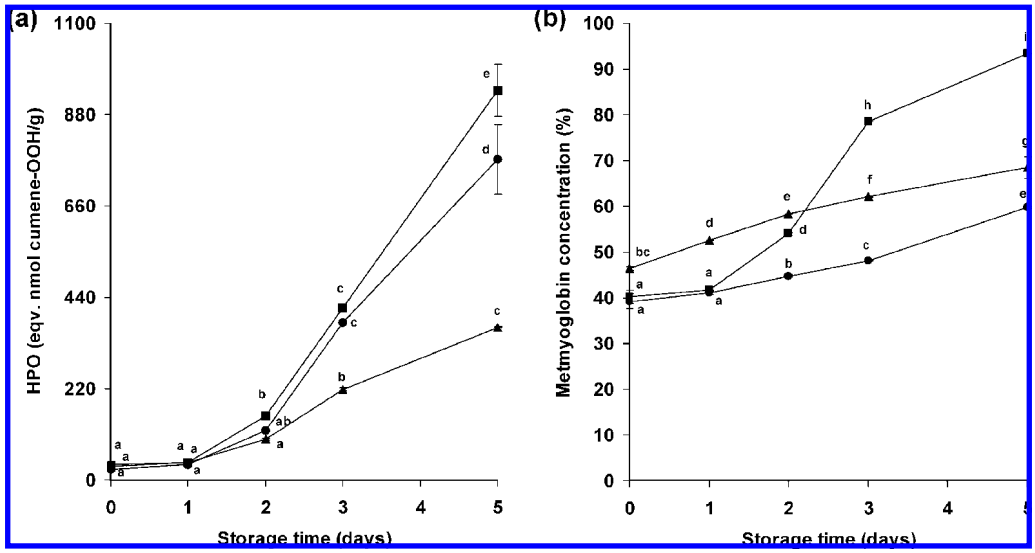

Figure 2. Changes in total lipid hydroperoxides (**a**) and metmyoglobin concentration (**b**) of minced bigeye tuna meat during ice storage: (▲) 100 g of minced bigeye tuna meat with 1 mL of mushroom extract (= 3 mg of ergothioneine) added; (●) 100 g of minced bigeye tuna meat with 1 mL of authentic L-ergothioneine solution (3 mg/mL) added; (■) control with 1 mL of distilled water added to 100 g of minced bigeye tuna meat. Data are presented as mean ± SD (n = 3). Values with different letters represent significant difference ($p < 0.05$).

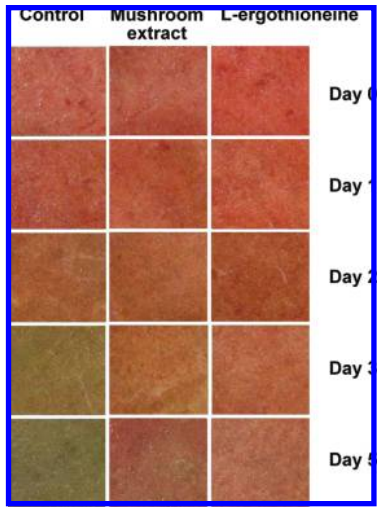

Figure 3. Changes in color tones of minced bigeye tuna meat during ice storage. Mushroom extract, 100 g of minced bigeye tuna meat with 1 mL of mushroom extract (= 3 mg of ergothioneine) added; L-ergothioneine, 100 g of minced bigeye tuna meat with 1 mL of authentic L-ergothioneine solution (3 mg/mL) added; Control, 100 g of minced bigeye tuna meat with 1 mL of distilled water added.

Changes in Total Lipids and Fatty Acid Composition. Initial total lipid contents of bigeye tuna meat with added mushroom extract and control without mushroom extract before storage were 1.30 ± 0.15 and 1.25 ± 0.21 g/100 g of meat, respectively. The lipid content in bigeye tuna meat after 7 days of ice storage was not significantly ($p > 0.05$) different from those of the control with the samples with added extract, amounting to 1.26 ± 0.10 and 1.24 ± 0.04 g/100 g of meat, respectively. Initial total lipid contents of beef and mackerel meat with added extract and control without the extract before storage were 6.80 ± 0.84, 2.20 ± 2.28, 6.70 ± 0.70, and 2.30 ± 0.42 g/100 g of meat, respectively. The total lipid contents of beef and mackerel meat were not significantly ($p > 0.05$) different from their initial contents after 12 and 7 days of ice storage, respectively.

Fatty acid contents of beef and fish meats with added mushroom extract and control before and after storage are shown in **Table 1**. 16:0 and 18:1n-9 fatty acids were dominant in both beef and fish meats. There was no significant difference between the control and the sample with added extract in terms of the total saturated fatty acids and monounsaturated fatty acids before and after storage. Significant changes were found in only certain polyunsaturated fatty acids (PUFA), including 16:4n-3, 20:4n-6, 20:4n-3, 20:5n-3, 22:5n-6, 22:5n-3, and 22:6n-3 existing in the tuna and/or mackerel meats of the control without the extract.

Changes in Total Lipid Hydroperoxides and Thiobarbituric Acid Reactive Substances. Changes in the contents of HPO and TBARS in the minced beef and fish meats during ice storage are shown in **Figure 4**. None of the samples except the mackerel meat with added mushroom extract changed significantly in the HPO content after 7 days of storage. However, the level of HPO in the mackerel meat with added mushroom extract was not higher than that of control after 1 day of storage. The HPO content of control fish meats without the extract increased rapidly after 1 day of storage. This increase in the HPO level was significant ($p < 0.05$) after 2 days of storage, when the tuna and mackerel meats had notable contents of HPO, amounting to 519.2 and 397.1 nmol/g of meat, respectively. Although lipid oxidation in the beef was not drastically altered compared to those in fish meats, a significant difference ($p < 0.05$) had been found between the beef with added mushroom extract and the control without the extract after 10 days of storage. Beef with the extract added remained at a level of HPO content below 60 nmol/g of meat for longer than 12 days of storage, whereas the control beef accumulated HPO up to 90 nmol/g of meat after 12 days of storage.

Trends of changes in TBARS were similar to those of HPO. The TBARS content all fish meats with added mushroom extract slightly increased after 7 days of storage. For the control fish meats, the TBARS content increased after 1 day of storage. The increase in TBARS was also significant ($p < 0.05$) in the control beef for up to 6 days of storage. The TBARS content in the beef with extract added remained unchanged for 12 days of storage.

Metmyoglobin and Color Changes. Changes in metMb concentration and color tones of the minced beef and bigeye tuna meats during ice storage are shown in **Figure 5**. In the

Table 1. Fatty Acid Contents (Milligrams per Gram of Oil) of Minced Bigeye Tuna and Pacific Mackerel Meats and Beef with 5 mL of Mushroom Extract (= 15 mg of Ergothioneine) Added to 100 g of Meat and Control with 5 mL of Distilled Water Added to 100 g of Meat during Ice Storage[a]

	bigeye tuna				Pacific mackerel				beef			
fatty acid	TC-0	TE-0	TC-7	TE-7	MC-0	ME-0	MC-7	ME-7	BC-0	BE-0	BC-12	BE-12
saturated fatty acids												
C14:0	19.13	18.84	14.98	18.52	16.84	16.69	16.88	16.51	16.87	12.10	17.02	16.31
C15:0	7.03	6.82	5.51	6.73	2.65	2.67	2.74	2.65	2.38	2.26	2.36	2.28
iso 16:0	7.00	6.86	4.56	6.23	2.07	2.03	1.20	1.99	5.35	5.65	5.81	6.24
C16:0	252.77	249.15	201.53	210.08	147.56	147.53	148.05	143.70	267.05	257.80	274.49	264.24
iso 17:0	6.06	5.82	4.57	5.53	3.17	2.78	2.70	2.64	2.28	2.09	2.00	2.18
C17:0	10.83	10.56	8.28	10.30	4.64	4.79	4.84	4.51	8.12	7.69	8.23	7.81
iso 18:0	1.18	1.14	0.71	1.03	0.50	0.56	0.44	0.52	1.10	1.02	1.00	1.15
C18:0	68.55	67.30	55.61	54.98	65.00	65.11	66.18	63.94	96.84	92.08	98.17	95.30
C19:0	4.26	3.87	2.90	3.81	1.71	1.62	3.17	1.68	ND	ND	ND	ND
C20:0	2.49	2.29	1.84	2.10	3.30	3.27	3.45	3.39	ND	ND	ND	ND
C22:0	1.55	1.48	1.30	1.53	1.27	1.27	1.29	1.32	2.08	1.91	1.87	1.96
C24:0	1.19	1.28	1.14	1.29	1.35	1.33	1.39	1.38	1.17	0.79	0.69	0.80
total	382.05 a	375.40 a	302.92 a	322.12 a	250.07 α	249.65 α	252.32 α	244.22 α	403.22 A	383.39 A	411.65 A	398.30 A
monounsaturated fatty acids												
C14:1n-7	2.23	2.08	1.92	1.93	1.20	1.24	1.00	1.20	4.29	4.13	4.47	4.16
C14:1n-5	0.42	0.41	0.36	0.43	0.94	0.95	1.02	0.96	ND	ND	ND	ND
C15:1n-6	0.55	0.51	0.27	0.50	0.58	0.61	0.55	0.55	0.81	0.70	0.74	0.73
C16:1n-9	0.80	0.78	1.07	0.74	2.23	2.23	2.18	2.07	2.35	2.26	2.49	2.32
C16:1n-7	44.64	43.12	33.12	42.43	17.63	17.51	17.99	17.33	30.67	29.88	31.41	30.45
C16:1n-5	1.41	1.33	1.15	1.28	1.12	1.13	1.04	1.09	1.79	1.67	1.81	1.73
C18:1n-9	209.03	204.92	154.02	170.1	180.26	180.51	187.11	176.85	570.37	558.24	582.05	566.84
C18:1n-7	10.81	7.83	13.40	17.94	1.36	1.42	1.54	1.44	5.65	3.31	4.49	5.29
C18:1n-5	1.28	0.96	1.00	1.29	1.38	1.42	1.18	1.56	2.46	1.68	1.92	2.25
C20:1n-9	17.89	16.63	12.81	15.72	40.18	40.10	42.25	39.92	5.89	3.74	4.30	3.73
C20:1n-7	1.36	1.18	0.98	1.28	2.14	2.12	2.25	2.21	ND	ND	ND	ND
C22:1n-11	1.35	1.24	0.95	1.11	22.06	22.03	22.58	22.03	ND	ND	ND	ND
C22:1n-9	2.21	2.15	1.74	2.00	4.30	4.31	4.50	4.33	ND	ND	ND	ND
total	293.98 a	283.12 a	222.79 a	256.75 a	275.37 α	275.59 α	285.19 α	271.53 α	624.27 A	605.62 A	633.68 A	617.51 A
polyunsaturated fatty acids												
C16:2n-6	1.01 a	0.94 a	0.75 a	0.90 a	1.36 α	1.32 α	1.20 α	1.32 α	5.18 A	4.73 A	5.10 A	4.89 A
C16:3n-6	8.57 a	8.12 a	6.37 a	7.82 a	3.76 α	3.72 α	2.43 β	3.68 α	ND	ND	ND	ND
C16:3n-4	7.75 a	7.33 a	5.65 a	7.06 a	2.76 α	2.73 α	2.82 α	2.71 α	8.41 A	7.96 A	8.23 A	8.00 A
C16:3n-1	2.16 a	2.04 a	1.59 a	1.92 a	1.88 α	1.96 α	1.94 α	1.90 α	2.90 A	2.98 A	3.14 A	3.26 A
C16:4n-3	0.85 a	0.75 a	0.35 a	0.64 a	1.53 α	1.57 α	1.06 β	1.56 α	ND	ND	ND	ND
C18:2n-6	10.57 a	10.43 a	11.24 a	9.41 a	10.60 α	10.60 α	6.88 α	10.49 α	41.08 A	44.30 A	43.27 A	47.63 A
C18:2n-4	3.11 a	2.72 a	2.01 a	2.48 a	1.05 α	1.07 α	0.85 α	1.07 α	1.37 A	1.30 A	1.42 A	1.54 A
C18:3n-6	3.47 a	3.02 a	2.31 a	2.78 a	2.14 α	2.12 α	2.09 α	2.10 α	1.60 A	3.31 A	1.39 A	4.06 A
C18:3n-4	1.40 a	1.17 a	0.94 a	1.24 a	1.02 α	0.98 α	1.05 α	1.03 α	ND	ND	ND	ND
C18:3n-3	3.51 a	3.58 a	4.74 a	2.97 a	4.85 α	4.75 α	1.75 α	4.74 α	ND	ND	ND	ND
C18:4n-3	1.73 a	1.49 a	1.11 a	1.39 a	2.07 α	2.10 α	1.94 α	2.05 α	ND	ND	ND	ND
C20:2n-6	2.30 a	2.03 a	1.59 a	1.89 a	1.21 α	1.21 α	1.35 α	1.41 α	0.36 A	0.38 A	0.35 A	0.38 A
C20:3n-6	1.31 a	0.70 a	0.51 a	0.63 a	1.34 α	1.41 α	1.38 α	1.56 α	2.43 A	2.47 A	2.46 A	2.65 A
C20:4n-6	28.11 a	27.06 a	20.43 a	24.11 a	16.55 α	16.52 α	13.84 β	16.59 α	7.00 A	6.87 A	6.66 A	7.41 A
C20:3n-3	1.84 a	1.74 a	1.34 a	1.69 a	0.47 α	0.47 α	0.52 α	0.48 α	ND	ND	ND	ND
C20:4n-3	3.70 a	3.44 a	2.50 a	3.11 a	2.46 α	2.36 αβ	2.20 β	2.49 α	ND	ND	ND	ND
C20:5n-3	49.12 a	48.09 a	35.49 a	42.42 a	35.04 α	34.98 α	27.61 β	34.75 α	ND	ND	ND	ND
C22:4n-6	4.29 a	3.65 a	2.75 a	3.32 a	2.38 α	2.46 α	2.11 α	2.48 α	0.92 A	1.01 A	0.95 A	1.15 A
C22:5n-6	15.93 a	15.61 a	11.94 b	13.40 ab	6.96 α	6.91 α	5.62 β	6.98 α	ND	ND	ND	ND
C22:5n-3	15.59 a	15.17 a	11.45 b	13.52 ab	13.99 α	14.02 α	12.04 β	14.00 α	ND	ND	ND	ND
C22:6n-3	256.27 a	257.80 a	191.18 b	220.68 ab	173.03 α	173.24 α	127.38 β	170.32 α	ND	ND	ND	ND
total	422.6 a	416.89 a	316.22 b	363.36 ab	286.44 α	286.49 α	218.06 β	283.72 α	71.26 A	75.31 A	72.97 A	80.98 A

[a] Data are presented as mean values of three replicates for each sample. Different letters within rows indicate significant differences between samples ($p < 0.05$). TE-0, tuna meat with added extract before ice storage; TE-7, tuna meat with added extract after ice storage; ME-0, mackerel meat with added extract before ice storage; ME-7, mackerel meat with added extract after ice storage; BE-0, beef with added extract before ice storage; BE-7, beef with added extract after ice storage; TC-0, control tuna meat before ice storage; TC-7, control tuna meat after ice storage; MC-0, control mackerel meat before ice storage; MC-7, control mackerel meat after ice storage; BC-0, control beef before ice storage; BC-7, control beef after ice storage.

case of bigeye tuna, the initial concentration of metMb in the control and the samples with added mushroom extract were 19.3 and 20.1%, respectively. It was found that the metMb concentration of samples with added extract were higher than those of controls after 1 day of storage. However, there was no significant difference ($p > 0.05$) in metMb concentration between the controls and samples with the extract added after 2 days of storage. The difference in percentage of metMb after 1 day of storage might be due to the increase in the pH of the meat with the addition of the extract to the meat. Furthermore, metMb concentration in the sample with the extract added did not increase rapidly during ice storage, whereas the metMb concentration in the control accelerated after 2 days of storage. The acceleration of metMb formation in the control sample thus resulted in the discoloration of bigeye tuna meat (see **Figure 6**).

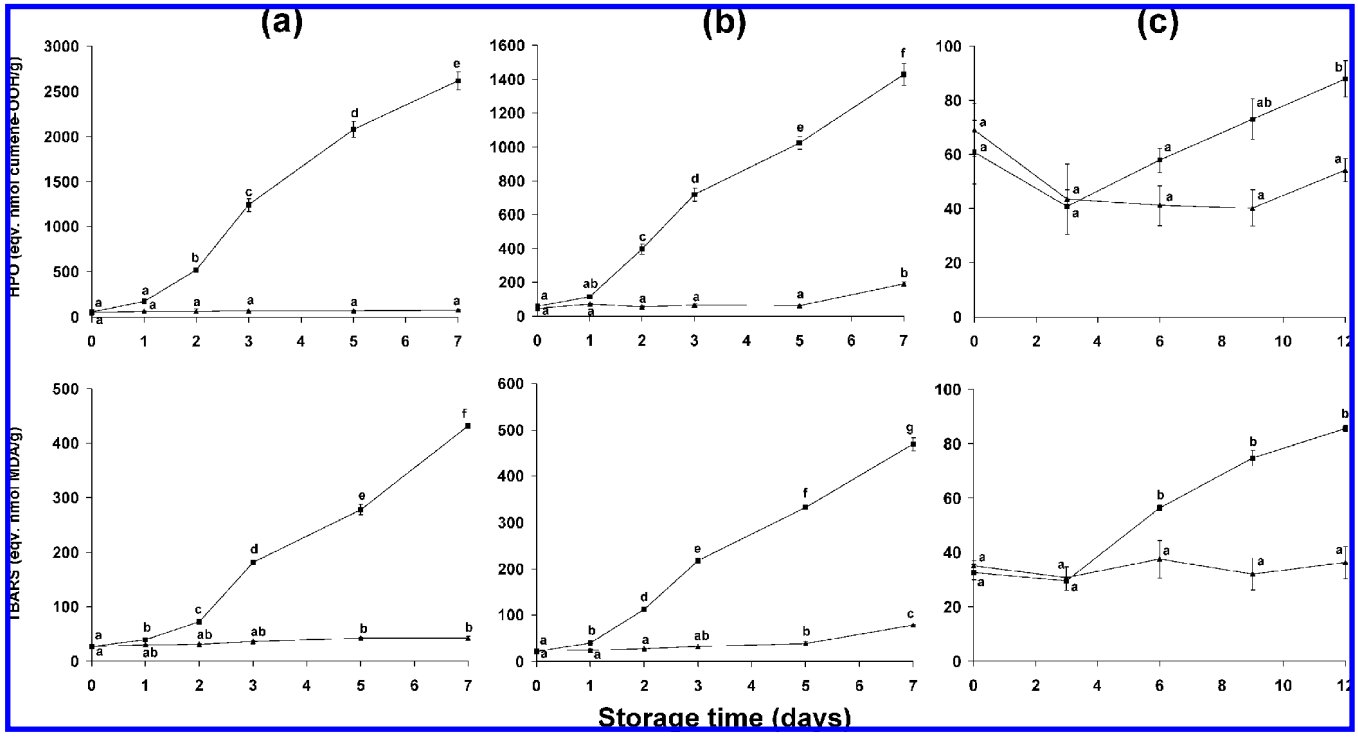

Figure 4. Changes in total lipid hydroperoxides (HPO) and thiobarbituric acid reactive substances (TBARS) of minced bigeye tuna (**a**) and Pacific mackerel (**b**) meats and of beef (**c**) with 5 mL of mushroom extract (= 15 mg of ergothioneine) added to 100 g of meat and of control with 5 mL of distilled water added to 100 g of meat during ice storage: (▲) meats with added mushroom extract; (■) control meats. Data are presented as mean ± SD ($n = 3$). Values with different letters represent significant difference ($p < 0.05$).

The changes in visual colors correlated significantly to metMb concentrations, as well as the *r* and *g* values of the bigeye tuna meat during the storage period, and are shown in **Figure 6**. By visual comparison, the fresh color of sample with the extract added remained unchanged even after 7 days of ice storage, whereas the control sample retained its fresh color for 2 days. This was further confirmed by the changes in *r* and *g* values. The decreases in *r* values and increases in *g* values of control samples were significant ($p < 0.05$) after 3 days of storage. At that time, metMb concentrations of the control sample also significantly increased.

Similar phenomena were found in the case of the beef trial, and the initial metMb concentration in beef with added mushroom extract was significantly higher than that in the control beef without extract. However, the metMb concentration in beef with added extract did not increase rapidly in the following days of storage. The metMb concentration in the control beef increased rapidly after 6 days of storage. On that day, the decrease in the *r* value and the increase in the *g* value of the sample were also significant (**Figure 5b**).

DISCUSSION

The antioxidative effects of *F. velutipes* extract on the oxidation of cod liver oil in emulsion was reported first by the authors (*11*) who demonstrated that lipid oxidation of a cod liver oil in water emulsion system was suppressed by the addition of the extract. However, any key compounds that play a role as antioxidant have not been clarified. When equivalent contents of authentic L-ERT were compared in the present study, the DPPH radical scavenging activity of the mushroom extract was significantly higher, indicating that the mushroom extract might contain other compounds acting as radical scavengers. This result explained why the addition of the mushroom extract to tuna meat could suppress the oxidation of lipid more effectively than the equivalent amount of authentic L-ERT (**Figure 2a**). The significantly higher levels in initial metMb concentration of tuna meat with added mushroom extract than in those with added authentic L-ERT as well as control suggest that some impurities in the mushroom extract interfered with the action of ERT in the mushroom extract. This interference of impurity compounds in mushroom extract was quite clear due to the fact that the mushroom extract showed a lower ability of TRP compared to the equivalent contents of the authentic L-ERT (**Figure 1b**). These results suggested that the interfering compounds might be involved in a redox circle of iron, thus resulting in the loss of the TRP ability of mushroom extract as well as the increase in initial metMb concentration of tuna meat with added mushroom extract. This could be explained by the fact that the DPPH radical scavenging test is based on donating hydrogen atom of antioxidants, whereas the total reducing power test is based on transferring electron from antioxidants to Fe^{3+}, reducing to Fe^{2+}. Therefore, the impurity compounds might have different effects on these tests. However, the mushroom extract still showed enough effects to prevent oxidations of lipid and Mb, and it is therefore compatible to be used as a natural antioxidant to prevent the oxidations of unsaturated lipid and of heme-proteins in muscle foods (*24, 25*). The mushroom extract itself has TRP ability as discussed above. This suggests that protons in the meat systems might be captured by certain compounds in the mushroom extract and result in a slight increase in the pH of the meats treated with mushroom extract.

The present study showed a significant correlation between metMb concentration and HPO content in bigeye tuna meat with a determination coefficient of 0.99 ($p < 0.05$) during the ice storage period. Our results highly confirm that lipid oxidation and Mb oxidation are coupled reactions in meats and that oxidation of PUFA catalyzes the formation of metMb and vice versa (*2*). The mushroom extract that was added to meats might

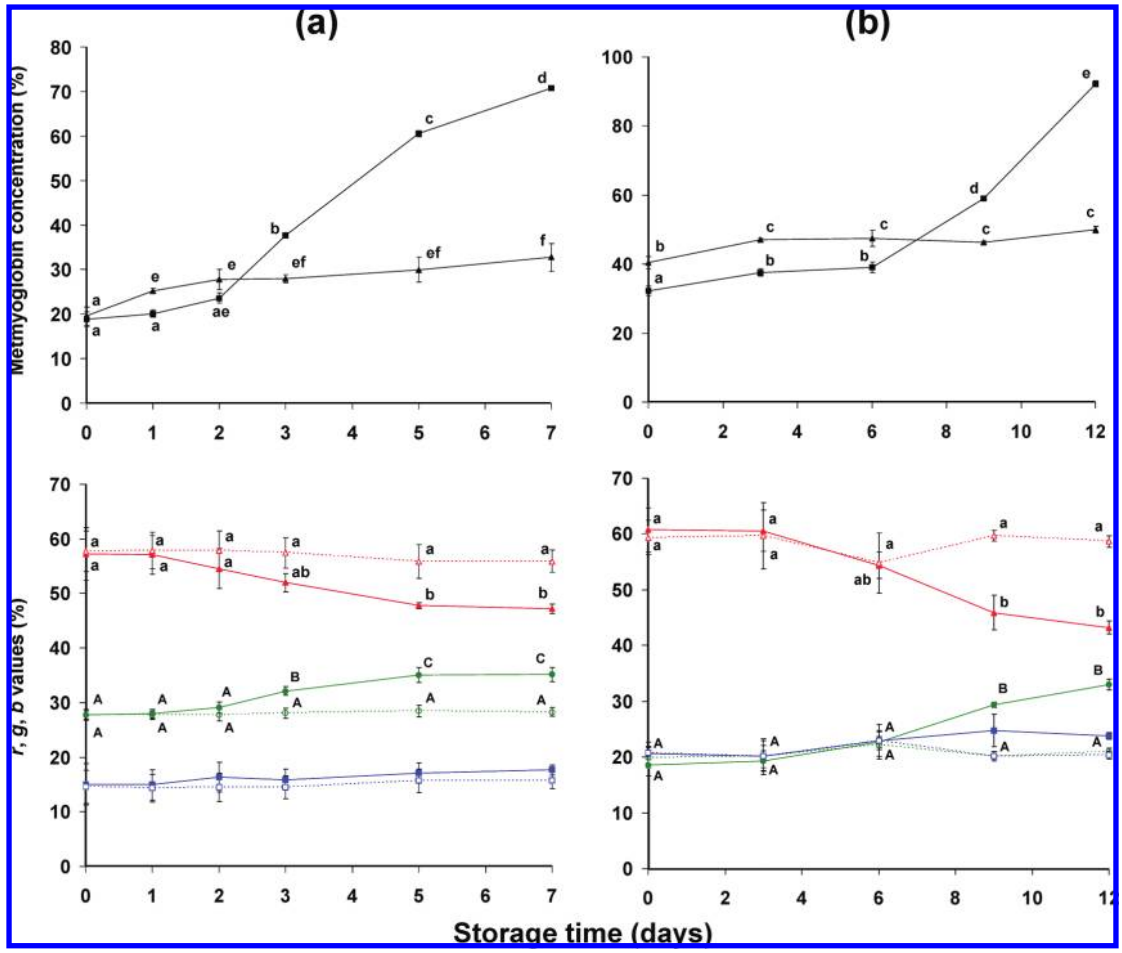

Figure 5. Changes in metmyoglobin concentration and *r*, *g*, and *b* values of minced bigeye tuna meat (**a**) and beef (**b**) with 5 mL of mushroom extract (= 15 mg of ergothioneine) added to 100 g of meat and of control with 5 mL of distilled water added to 100 g of meat during ice storage: (▲) metmyoglobin concentration of meats with added mushroom extract; (■) metmyoglobin concentration of control meats. (△) *r* value of meats with added mushroom extract, (○) *g* value of meats with added mushroom extract, (□) *b* value of meats with added mushroom extract, (▲) *r* value of control meats, (●) *g* value of control meats, (■) *b* value of control meats. Data are presented as mean ± SD ($n = 3$). Values with different letters represent significant difference ($p < 0.05$).

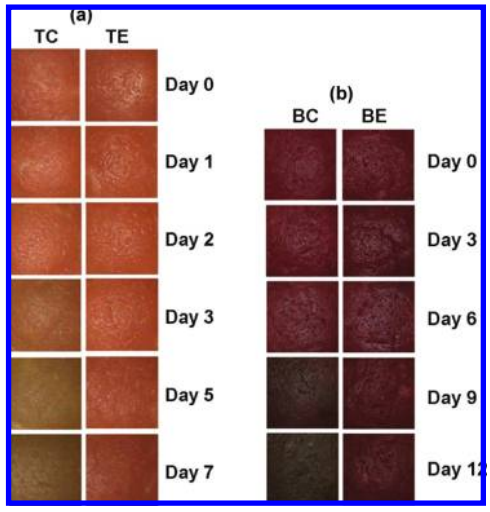

Figure 6. Changes in color tones of minced bigeye tuna meat (**a**) and beef (**b**) with 5 mL of mushroom extract (= 15 mg of ergothioneine) added to 100 g of meat and of control with 5 mL of distilled water added to 100 g of meat during ice storage. TE, tuna meat with added extract; BE, beef with added extract; TC, control tuna meat; BC, control beef.

act as a type of chain-breaking antioxidant in this particular case. This could be explained in detail on the basis of the mechanism of oxidative reactions between lipid and Mb, which was proposed by Reeder and Willson (*5*) as follows:

$$\text{deoxy}(Fe^{2+})\text{Mb} + \text{LOOH} \rightarrow \text{met}(Fe^{3+})\text{Mb} + \text{LO}^{\bullet} \quad (1)$$

$$\text{met}(Fe^{3+})\text{Mb} + \text{LOOH} \rightarrow \text{ferryl}(Fe^{4+})\text{Mb} + \text{LO}^{\bullet} \quad (2)$$

$$\text{LO}^{\bullet} \rightarrow \text{epoxyL}^{\bullet} \quad (3)$$

$$\text{epoxyL}^{\bullet} + O_2 \rightarrow \text{LOO}^{\bullet} \quad (4)$$

$$\text{epoxyL}^{\bullet} + \text{ferryl}(Fe^{4+})\text{Mb} \rightarrow \text{met}(Fe^{3+})\text{Mb} + \text{epoxyLOH} \quad (5)$$

Usually, Mb exists in three forms, which include deoxymyoglobin [deoxy(Fe^{2+})Mb], oxymyoglobin [oxy(Fe^{2+})Mb], and metmyoglobin [met(Fe^{3+})Mb] in fresh meat. The metMb is an undesirable form because of not only its brown color but also its catalytic effect on the oxidation of unsaturated lipids (*26*). The reaction between metMb and lipid hydroperoxides as described by formula 2 generated ferryl myoglobin [ferryl-(Fe^{4+})Mb], which has the ability to abstract a hydrogen atom from lipid (LH) to form an alkyl radical ($L^{\bullet}$). In the presence of oxygen, the alkyl radical then forms a peroxyl radical ($LOO^{\bullet}$), which can in turn abstract a hydrogen atom from another lipid, resulting in the production of lipid hydroperoxides. When lipid hydroperoxides form in the meats, they are continuously

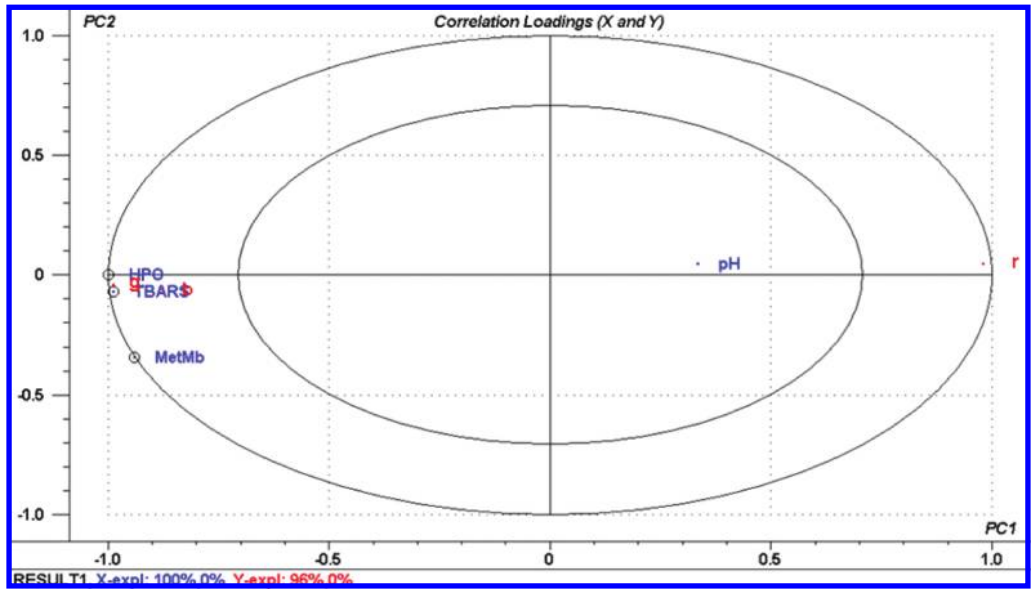

Figure 7. Partial least squares regression model correlation loadings based on all of the measured variables (*X*), pH, total lipid hydroperoxides (HPO), thiobarbituric acid reactive substances (TBARS), and metmyoglobin (metMb), to predict the changes in color (*Y*), *r*, *g*, and *b* values, of minced bigeye tuna meat with 5 mL of mushroom extract (= 15 mg of ergothioneine) added to 100 g of meat and of control with 5 mL of distilled water added to 100 g of meat during ice storage. The outer and inner ellipses indicate 100 and 50% explained variance, respectively. Significant variables ($p < 0.05$) are symbolized with small circles.

involved in the redox reactions of Mb to generate radicals as described by formulas 1 and 2. Once under way, the alkoxyl radical ($LO^{\bullet}$) formed from formulas 1 and 2 rearranges to form an epoxyalkyl radical ($epoxyL^{\bullet}$) (eq 3), which reacts with molecular oxygen to form a peroxyl radical (eq 4). The epoxyalkyl radical can also react with ferrylMb to form a metMb, which continuously plays a role as an intermediate to oxidize lipid in meats (*6, 27*). When the mushroom extract was added to meats, the $LO^{\bullet}$ radical generated in meats from formulas 1 and 2 could be scavenged by ERT existing in the mushroom extract (*16*), and ferrylMb formed from formula 2 received electrons from the thiol group of ERT returning metMb (*17*). The discoloration of meats thus can be delayed.

The previous studies had shown a relationship between lipid oxidation, metMb formation, and color changes in meats (*28, 29*), but none of them had shown a better indicator to predict the color changes in meats during storage. To distinguish this point, a PLSR was used to evaluate the correlation of the variables and their contribution to predict the changes in color (*r* and *g* values) of minced bigeye tuna meat with added mushroom extract and a control without mushroom extract during ice storage (**Figure 7**). The results of analysis showed that all of the measured variables had a high correlation to the *r* value ($r^2 = 0.96$) and the *g* value ($r^2 = 0.98$) with root-mean-square errors shown in **Table 2**. The low responses in the pH explained less variance in the data than the other variables as shown by its location in the middle of the plot in **Figure 7**. The contribution of the significant ($p < 0.05$) variables (HPO, TBARS, and metMb) to predict the color changes was studied further by exploring different PLSR models (**Table 2**). A high correlation between lipid oxidation and *r* and *g* values indicates that lipid oxidation contributed mainly to the changes in color of bigeye tuna during ice storage. However, a model based on the metMb had a lower correlation to *r* value ($r^2 = 0.88$) and *g* value ($r^2 = 0.89$), indicating that lipid oxidation gave better information about the changes in the color of bigeye tuna meat during ice storage. It is well-known that the color of tuna meat reflects the content of Mb as well as certain derivatives of Mb in the meat (*1, 3*). During ice storage, the discoloration of tuna meat was due to not only Mb oxidation but also Mb degradation. Because the percentage of metMb formation in tuna meat reflects only an aspect of Mb oxidation, it had a lower correlation with the discoloration of tuna meat during ice storage.

Table 2. Correlation and Error of Prediction (Root-Mean-Square-Error of Prediction) for Different Partial Least Squares Regression Models Based on the Measured Variables, pH, Total Lipid Hydroperoxides, Thiobarbituric Acid Reactive Substances, and Metmyoglobin, To Predict the Changes in Color, *r*, *g*, and *b* Values, of Minced Bigeye Tuna Meat with 5 mL of Mushroom Extract (= 15 mg of Ergothioneine) Added to 100 g of Meat and of Control with 5 mL of Distilled Water Added to 100 g of Meat during Ice Storage

	r value			*g* value			*b* value		
measured variable	r^{2a}	RMSE[b]	N^c	r^{2a}	RMSE[b]	N^c	r^{2a}	RMSE[b]	N^c
all variables (pH, HPO, TBARS, metMb)[d]	0.96	0.71	12	0.98	0.43	12	0.77	0.50	12
pH	0.12	3.46	12	0.10	2.59	12	0.15	0.96	12
lipid oxidation (HPO, TBARS)	0.96	0.71	12	0.98	0.43	12	0.77	0.50	12
metMb	0.88	1.28	12	0.89	0.91	12	0.72	0.56	12

[a] r^2, correlation coefficient. [b] RMSE, root-mean-square error of prediction. [c] *N*, number of elements. [d] HPO, total lipid hydroperoxides; TBARS, thiobarbituric acid reactive substances; metMb, metmyoglobin.

In conclusion, ERT in mushroom (*F. velutipes*) extract is definitely a powerful antioxidant that can be used to controlling the oxidation of not only lipid but also myoglobin in meats, thus delaying discoloration of meats during low-temperature storage.

LITERATURE CITED

(1) Bito, M. Studies on retention of meat color of frozen tuna. II. Effect of storage temperature on preventing discoloration of tuna meat during freezing storage. *Bull. Jpn. Soc. Sci. Fish.* **1965**, *31*, 534–539.

(2) Renerre, M. Review: Factors involved in the discoloration of beef meat. *Int. J. Food Sci. Technol.* **1990**, *25*, 613–630.

(3) Matthews, A. D. Muscle colour deterioration in iced and frozen stored bonito, yellowfin and skipjack tuna caught in Seychelles waters. *J. Food Technol.* **1983**, *18*, 387392.

(4) Chow, C.; Ochiai, Y.; Watabe, S. Effect of frozen temperature on autoxidation and aggregation of bluefin tuna myoglobin in solution. *J. Food Biochem.* **2004**, *28*, 123–134.
(5) Reeder, B. J.; Wilson, M. T. Mechanism of reaction of myoglobin with the lipid hydroperoxide hydroperoxyoctadecadienoic acid. *Biochem. J.* **1998**, *330*, 1317–1323.
(6) Baron, C. P.; Andersen, H. J. Myoglobin-induced lipid oxidation. A review. *J. Agric. Food Chem.* **2002**, *50*, 3887–3897.
(7) Georgantelis, D.; Blekas, G.; Katikou, P.; Ambrosiadis, I.; Fletouris, D. J. Effect of rosemary extract, chitosan and α-tocopherol on lipid oxidation and colour stability during frozen storage of beef burgers. *Meat Sci.* **2007**, *75*, 256–264.
(8) Li, S. J.; Seymour, T. A.; King, A. J.; Morrissey, M. T. Color stability and lipid oxidation of rockfish as affected by antioxidant from shell waste. *J. Food Sci.* **1998**, *63*, 438–441.
(9) Lee, B. J.; Hendricks, D. G.; Cornforth, D. P. A comparison of carnosine and ascorbic acid on color and lipid stability in a ground beef pattie model system. *Meat Sci.* **1999**, *51*, 245–253.
(10) Fu, H.; Shieh, D.; Ho, C. Antioxidant and free radical scavenging activities of edible mushrooms. *J. Food Lipids.* **2002**, *9*, 35–46.
(11) Jang, M.; Eun, J.; Ushio, H.; Ohshima, T. Antioxidative properties of mushroom (*Flammulina velutipes*) crude extract on the oxidation of cod liver oil in emulsion. *Food Sci. Biotechnol.* **2004**, *13*, 215–218.
(12) Ashida, S.; Sato, R.; Sato, M. Screening of edible plants for reducing activity by monitoring their effects on the oxidation of oxymyoglobin. *Food Sci. Technol. Res.* **2005**, *11*, 349–354.
(13) Wasser, S. P. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 258–274.
(14) Dubost, N. J.; Ou, B.; Beelman, R. B. Quantification of polyphenols and ergothioneine in cultivated mushrooms and correlation to total antioxidant capacity. *Food Chem.* **2007**, *105*, 727–735.
(15) Quang, D. N.; Hashimoto, T.; Asakawa, Y. Inedible mushroom: a good source of biologically active substances. *Chem. Record* **2006**, *6*, 79–99.
(16) Akanmu, D.; Cecchini, R.; Aruoma, O. I.; Halliwell, B. The antioxidant action of ergothioneine. *Arch. Biochem. Biophy.* **1991**, *288*, 10–16.
(17) Arduini, A.; Eddy, L.; Hochstein, P. The reduction of ferryl myoglobin by ergothioneine: a novel function for ergothioneine. *Arch. Biochem. Biophy.* **1990**, *281*, 41–43.
(18) Dubost, N. J.; Beelman, R.; Peterson, D.; Royse, D. Identification and quantification of ergothioneine in cultivated mushrooms using liquid chromatography−mass spectroscopy. *Int. J. Med. Mushr.* **2007**, *8*, 215–222.
(19) Oyaizu, M. Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn. J. Nutr.* **1986**, *44*, 307–315.
(20) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917.
(21) Fatty Acid Composition by GLC—Marine Oils. AOCS Official Method Ce 1b-89.
(22) Sohn, J. H.; Taki, Y.; Ushio, H.; Ohshima, T. Quantitative determination of total lipid hydroperoxides by a flow inspection analysis system. *Lipid* **2005**, *40*, 203–209.
(23) Uchiyama, M.; Mihara, M. Determination of malonaldehyde precursor in tissue by thiobarbituric acid test. *Anal. Biochem.* **1978**, *86*, 271–278.
(24) Porter, N. A.; Caldwell, S. E.; Mills, K. A. Mechanisms of free radical oxidation of unsaturated lipids. *Lipid.* **1995**, *30*, 277–290.
(25) Giulivi, C.; Cadenas, E. Heme protein radicals: formation, fate, and biological consequences. *Free Radical Biol. Med.* **1998**, *24*, 269–279.
(26) Grunwald, E. W.; Richards, M. P. Studies with myoglobin variants indicate that released hemin is the pramary promoter of lipid oxidation in washed fish muscle. *J. Agric. Food Chem.* **2006**, *54*, 4452–4460.
(27) Grunwald, E. W.; Richards, M. P. Mechanisms of heme protein-mediated lipid oxidation using hemoglobin and myoglobin variants in raw and heated washed muscle. *J. Agric. Food Chem.* **2006**, *54*, 8271–8280.
(28) Lee, S.; Joo, S. T.; Alderton, A. L.; Hill, D. W.; Faustman, C. Oxymyoglobin and lipid oxidation in yellowfin tuna (*Thunnus albacares*) loins. *J. Food Sci.* **2003**, *68*, 1664–1668.
(29) Kannan, G.; Kouakou, B.; Gelaye, S. Color changes reflecting myoglobin and lipid oxidation in chevon cuts during refrigerated display. *Small Ruminant Res.* **2001**, *42*, 67–74.

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