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Effects of Ergothioneine from Mushrooms (*Flammulina velutipes*) on Melanosis and Lipid Oxidation of Kuruma Shrimp (*Marsupenaeus japonicus*)

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The antimelanosic and antioxidative properties of a hot water extract prepared from the fruiting body of the edible mushroom (*Flammulina velutipes*) were evaluated by dietary supplementation in Kuruma shrimp (*Marsupenaeus japonicus*) for possible aquaculture application. The extract contained ergothioneine (ERT) at a level of 2.05 mg/mL. A commercial standard of L-ergothioneine (L-ERT) and the mushroom extract showed inhibitory activity against mushroom polyphenoloxidase (PPO). Feeding of the extract had no adverse effects on the immune systems of the shrimp under the present experimental conditions. Supplementation of the extract in the diet significantly suppressed PPO activities in the hemolymphs of the shrimp. Expression of the prophenoloxidase (proPO) gene decreased in the hemocyte of the Kuruma shrimp fed with the mushroom extract. Consequently, development of melanosis was significantly suppressed in the supplement fed shrimp during ice storage. Lipid oxidation was also effectively controlled in the supplement fed group throughout the storage period. In vitro experiments showed that L-ERT effectively inhibited the activation of proPO in the hemocyte lysate supernatant (HLS). The transcript of the proPO gene in the hemocyte showed lower expression in the L-ERT-treated HLS. It was concluded that dietary supplementation of the mushroom extract in shrimp could be a promising approach to control post mortem development of melanosis and lipid oxidation in shrimp muscles.

KEYWORDS: Mushroom extract; *Flammulina velutipes*; Kuruma shrimp (*Marsupenaeus japonicus*); ergothioneine; melanosis; polyphenoloxidase; lipid oxidation

INTRODUCTION

Development of melanosis or blackspot formation during post harvest of crustaceans is a well-known post mortem phenomenon attributed to the polymerization of phenol into an insoluble black pigment, the melanin. Phenol polymerization is mainly initiated by the action of an enzymatic complex called polyphenoloxidase (also known as phenoloxidase). The term polyphenoloxidase (PPO) is generally used to refer to tyrosinase (EC 1.14.18.1) and cathecoloxidase (EC 1.10.3.1) (*1*). In live crustaceans, PPO is synthesized as prophenoloxidase (proPO), a zymogen that is activated by protease cascade triggered by microbial compounds and involves a series of other compounds. This system has been known to play an important role in the primary immune response, cuticle sclerotization, and healing of injuries in crustaceans (*1*−*3*). Severe blackspot formations can cause tremendous economic losses due to the high value commanded by these aquatic products in the market place (*4*). Its prevention has been a challenge to the industry, especially for food scientists. Many studies have focused on PPO inhibition and various techniques and mechanisms have been developed and used such as heat treatments, ionizing radiation, high pressure treatments, and application of antimelanosic compounds or inhibitors (*5*). Melanosis in crustaceans is normally controlled by means of direct application of various inhibitors such as 4-hexylresorcinol, sulphites, and phosphates (*2*). Along with the melanosis formation in crustaceans is also the oxidation of muscle lipids during post mortem storage. Lipid oxidation products significantly affect the flavor and odor of products even though present as minor components in food; thus various antioxidants are being used to control lipid oxidation in food products such as ascorbic acid, α-tocopherol, and butylated hydroxytoluene (*6*).

However, direct application of synthetic inhibitors to melanosis and antioxidants in food processing is usually restricted by considerations relevant to toxicity, wholesomeness, and effect on the taste, flavor, and texture of the products. Possible health hazards from the residue of melanosis inhibitors such as sulphites, off-color development due to excessive use of ascorbic acid, and negative effects on taste of some inhibitors such as 4-hexylresorcinol in shrimp when immersed in these compounds are some of the emerging concerns of the food industry (*4*). Moreover, increased regulatory attention and heightened consumer awareness of the

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risk associated with synthetic antimelanosic compounds and antioxidants when consumed with the food has created a need for safe and effective alternatives for food application (*7*). Thus, there have been many studies conducted on the utilization of extracts from natural foods including numerous species of mushrooms as PPO inhibitors and antioxidants.

It has been found out that the extract from the edible mushroom *Flammulina velutipes* significantly inhibited mushroom PPO activity, prevented browning in apples, and delayed melanosis in shrimp that usually develops during storage (*8*, *9*). These observations suggested that the mushroom extract contained certain compounds contributing to such actions. Recently, the antioxidative properties of a hydrophilic extract from the fruiting body of *F. velutipes* have been evaluated (*10*, *11*). The mushroom extract containing 2-thiol-L-histidine-betaine (ergothioneine, ERT) at a level of 3.03 ± 0.07 mg/mL showed remarkable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and suppressed lipid oxidation in bigeye tuna meat (*11*). The extracts also stabilized fresh color of the tuna meat during ice storage (*12*). These results strongly suggest that ERT could be one of the major compounds in the mushroom extract that effectively inhibits mushroom PPO activity, apple browning, and melanosis in shrimp in the previous studies conducted (*8*, *9*).

In vivo application of L-ERT in rats as an antioxidant (*13*) has been studied, but prior to this study there had been no attempt to determine the efficacy of extracts containing this compound by feeding in order to control melanosis and lipid oxidation in crustaceans. While immersion of post mortem shrimp in the mushroom extract-based solution was very effective to prevent melanosis during the subsequent storage of the product (*9*) and mixing the extract in beef and fish meat significantly suppressed lipid oxidation in the product (*11*, *12*), antimelanosic and antioxidative activities of the extract could be more effective when fed to the animals because of possible accumulation in the body.

This study attempted to apply a hydrophilic extract prepared from the fruiting body of *F. velutipes* to control melanosis and lipid oxidation in cultured Kuruma shrimp (*Marsupenaeus japonicus*) in vivo. Moreover, this study was conducted to evaluate the inhibitory effects of L-ERT on the activation of proPO cascade in Kuruma shrimp hemocyte in vitro.

MATERIALS AND METHODS

Materials and Chemicals. Fresh mushroom (*F. velutipes*) produced by JA (JA Zennou Nagano Co. Nagano, Japan) was purchased from a Tokyo metropolitan central wholesale market in Tokyo, Japan. L-(+)-Ergothioneine (L-ERT) was purchased from Bachem AG (Hauptstrasse, Bubendorf, Switzerland). Boc-Val-Pro-Arg-methylcoumarin amide (MCA) was purchased from Peptide Institute, INC (Osaka, Japan). 1-Myristoyl-2-(12-(7-nitro-2-1,3-benzoxadiazol-4-yl)amino)dodecanoyl-*sn*-glycero-3-phosphocoline (NBD-labeled PC) was obtained from PC Avanti Polar-Lipids Inc., (Alabaster, Alabama). Methanol, chloroform, 1-butanol, and distilled water of HPLC grades were purchased from Kokusan Chemical Co. Ltd. (Tokyo, Japan). All other chemicals of analytical grade were obtained from Sigma-Aldrich (St. Louis, MO) and Wako Pure Chemical Industries, Ltd. (Tokyo, Japan) unless otherwise indicated.

In Vivo Experiment. *Mushroom Extract*. The mushroom extract was prepared following the procedure of Jang et al. (*8*) with a slight modification. A 200 g portion of fresh mushroom fruiting body was mixed with 400 mL of distilled water and boiled gently at 90 °C for 30 min in a glass beaker. After being cooled to room temperature, 400 mL of filtrate obtained by suction filtration (6 μm, Advantec Toyo Roshi Kaisha, Ltd., Tokyo, Japan) through Hyflo supercel was evaporated at 40 °C in vacuo to obtain 200 mL of the hot water extract concentrate (1 g of wet mushroom/mL of hot water extract).

Quantification of Ergothioneine. The ERT content of the mushroom extract was quantitatively determined based on the method of Dubost et al. (*14*) with some modifications. Briefly, 1 mL of 1 mM methimazole solution was added as an internal standard (IS) to a mixture containing 0.2 mL of the mushroom extract, 4.8 mL of distilled water, and 14 mL of absolute ethanol. The ethanolic mixture was mixed and kept at 4 °C for 2 h and subsequently centrifuged at 3000*g* for 15 min at 4 °C. The supernatant was collected and evaporated at 40 °C in vacuo to remove the ethanol. The residue was dissolved in 10 mL of distilled water. Quantification analysis of the ERT was carried out using a Shimadzu model LCMS-2010EV high performance liquid chromatograph mass spectrometer 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). 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 the ERT was quantitatively determined by monitoring fragment ions for ERT and IS at m/z 230 and 115, respectively, to obtain a peak area ratio (ERT/IS). A calibration curve was obtained by different concentrations of the commercial standard. All data were expressed as milligrams of ERT per milliliter of the mushroom extract.

A 0.2 g of sample diet was used in the quantification of the ERT in the prepared diets and the analysis was carried out in triplicate using the aforementioned method previously described for the quantification of ERT in mushrooms.

For the ERT quantification in prawn muscles, 1 mL of a solution of IS was added to 5 g of the sample, and the lipid was separated following the procedure of Bligh and Dyer (*15*) with a slight modification. The homogenate was centrifuged at 3000*g* for 15 min at 4 °C. The methanol layer was taken and evaporated at 40 °C in vacuo. The methanol free residue thus obtained was dissolved in 10 mL of deionized water. The analysis was carried out in triplicate using the method previously mentioned for the quantification of ERT in mushrooms.

Assay of Mushroom PPO and Its Inhibition. The inhibitory effects of commercial L-ERT, mushroom extract, and prepared diet residues (filtrate from 1 g of diet dissolved in 10 mL of distilled water) on mushroom PPO activity were evaluated using an assay system consisting of 0.1 mL of 500 mM catechol, 0.1 mL of 500 mM L-(−)-proline, 2.9 mL of 50 mM phosphate buffer (pH 6.8), and 0.1 mL of 100 units/mL mushroom PPO (Sigma-Aldrich, St. Louis, MO). An appropriate amount of the buffer was replaced with the test solutions to determine their inhibitory activity on mushroom PPO. In the case of the prepared diet residues, 1 mL was used in the assay system. Absorbance at 530 nm (A_{530}) was monitored continuously at 25 °C for 300 s using a Shimadzu model UV-1600PC spectrophotometer. The following formula was used to calculate the inhibitory effects of the test solutions on mushroom PPO activity: % inhibition = $100 - [(A \times 100)/B]$, where $A = A_{530}$ of the test sample and $B = A_{530}$ of the control. Inhibition of PPO activity by 50% was determined using linear regression.

Determination of Radical Scavenging Activity. DPPH radical scavenging activities of the mushroom extract and prepared diet residues (filtrate from 1 g of diet dissolved in 10 mL of distilled water) were assayed following the method of Fu et al. (*16*) with some modifications. Briefly, 0.5 mL of a 0.4 mM DPPH ethanol solution was increased to a final volume of 2 mL by distilled water as a control mixture. An appropriate amount of the water was replaced with the test solutions to determine their scavenging activity. In the case of the prepared diet residues, 1 mL was used in the assay system. After thoroughly mixing, the solutions were kept at 25 °C for 30 min in the dark. The absorbance of the mixtures was measured at 517 nm using a spectrophotometer. The DPPH radical scavenging activity was calculated using the following formula: DPPH scavenging activity (%) = $(A_{control} - A_{sample}/A_{control}) \times 100$, where $A_{control}$ is the absorbance of the control mixture and A_{sample} is the absorbance of the sample mixture containing the test solution. Analyses were done in triplicate.

Experimental Animals. New stocks of live Kuruma shrimp (*M. japonicus*) weighing 10.7 ± 0.8 g per animal were purchased from Sakura Suisan, Co. Ltd. (Miyazaki, Japan). The shrimp were fed with Goldprawn (Higashimaru Co. Ltd., Kagoshima, Japan) commercial feed and kept in an aerated tank for acclimatization at a temperature of 25 °C and 28 ppt salinity until used. The nutritional composition of the commercial feed used in the feeding trial was as follows: crude protein, 53.0%; crude lipid, 9.0%; crude fiber, 3.0%; crude ash, 20.0%; calcium, 2.0%; and phosphorus, 1.0%.

Feed Preparation. The commercial feed was pulverized and kept at −20 °C prior to shrimp diet preparation. Pulverized commercial feed and hot water mushroom extract (1:1, w/v) was homogenized and pelletized manually using a 60 mL syringe. Control feed was prepared by mixing pulverized commercial feed with distilled water (1:1, w/v). Pelletized feeds were freeze-dried and kept at −20 °C until being used for feeding.

Feeding Scheme. Two groups of shrimp containing 40 animals each were then transferred to other tanks maintaining the same water temperature and salinity for further acclimatization for feeding. The shrimp were fed ad libitum with the commercial feed for a three-day acclimatization period prior to test feeding. After the acclimatization period, the shrimp were fed the prepared diet for seven days. The diet was dissolved by water (1:1, w/v, thus 25% final concentration of the extract in a wet diet) and 0.1 mL of the diet solution was administered orally by using a sterile plastic syringe for each prawn once per day for seven days. Sampling for physicochemical analyses was done after the seven-day feeding period.

Hemolymph Sampling and Total Hemocyte Count. A 500 μL hemolymph was collected from the pleaopod base of the first abdominal segment of the shrimp using a 5 mL sterile syringe preloaded with 500 μL of anticoagulant. The hemolymphs collected in eppendorf tubes were immediately stored in ice for further use. Total hemocyte counts (THC) were determined for individual shrimp (n = 5 per group) using a hemacytometer (Nitirin Co. Ltd., Tokyo, Japan). A 10 μL hemolymph was first diluted by 10 times with 50 mM 1-piperazineethanesulfonic acid (HEPES, pH 7.8) prior to loading into the hemacytometer.

Assay of the Hemolymph Polyphenoloxidase Activity. Polyphenoloxidase activity of the hemolymphs from individual shrimp (n = 4 per group) was assayed using an assay system consisting of 0.1 mL of 500 mM catechol as a substrate, 0.1 mL of 500 mM L-(−)-proline, 0.2 mL hemolymph, 2.7 mL of a 50 mM phosphate buffer (pH 6.8), and 0.1 mL of 100 units/mL mushroom PPO in a total volume of 3.2 mL. Absorbance at 530 nm was monitored continuously at 25 °C for 300 s by using a spectrophotometer.

The PPO activity of the hemolymphs from individual shrimp (n = 4 per group) was also measured spectrophotometrically using L-3,4-dihydroxyphenylalanine (L-DOPA) as its substrate following the method of Adachi et al. (*17*) with a slight modification. Briefly, a 50 μL aliquot of hemolymph was added to a mixture of 50 μL of L-DOPA (15 mM/mL) and 95 μL of 50 mM HEPES (pH 7.8) including 5 mM $MgCl_2$ and 5 mM $CaCl_2$. The mixture was incubated at 37 °C for 30 min and the absorbance at 490 nm was measured with a Beckman model DU640 spectrophotometer.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis. 500 μL of hemolymph was collected from the shrimp (n = 3 per group) using a sterile syringe preloaded with 500 μL of anticoagulant and was followed immediately by the collection of hemocytes. Total RNAs from hemocytes were isolated using an RNAiso (TaKaRa Bio Inc., Shiga, Japan) and synthesized into cDNAs using PrimeScript Reverse Transcriptase (TaKaRa Bio Inc., Shiga, Japan). The resulting cDNAs were quantified to make sure all samples were of equal concentration. To check if the proPO transcripts were effectively silenced, a RT-PCR was conducted using gene-specific primers for proPO with elongation factor 1-α gene (EF1-α) as an internal control to monitor the amount of RNA/cDNA PCR template and amplification efficacy between samples. The primer sequences were 5′-GGATCTGCCTTCTCCTTCTTCC-3′ and 5′-TAGCATCCAGGAGTCGAGATCG-3′; 5′-ATGGTTGTCAACTTTGCCC-3′ and 5′-TTGACCTCCTTGATCACACC-3′, for proPO and EF1-α, respectively. Distilled water was loaded as a template for the negative control. PCR conditions were as follows: 95 °C for 5 min, followed by 28 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. Transcripts were visualized in 1% agarose gel stained with ethidium bromide. Quantitative analyses of band intensities were obtained using ImageJ software of the National Institute of Health, Bethesda, MD (http://rsb.info.nih.gov/ij).

Evaluation of Melanosis Development in Shrimp. Shrimp covered in plastic bags were stored in ice for four days. Color images of the shrimp were acquired using a digital CCD camera (Cyber-shot 10.1 mega pixels, Sony Corp., Tokyo, Japan). Image data were taken in a dark box to prevent outer light. The carapaces (exoskeleton of the cephalothorax) of the shrimp from the acquired images in JPEG format were analyzed for mean gray value using ImageJ software. Decreasing mean gray values denoted blackening or the development of melanosis in the carapace area of shrimp. Analyses were done in triplicate.

Determination of Total Lipid Hydroperoxides. Total lipid hydroperoxide (HPO) was determined by using a flow injection analysis (FIA) system equipped with a fluorescent detection system using diphenyl-1-pyrenylphosphine as described by Sohn et al. (*18*). Briefly, 1 mL of a chloroform solution of NBD-labeled PC was added to 5 g of shrimp muscle as an internal standard, and lipid was extracted and purified according to the procedure of Bligh and Dyer (*13*). The homogenate was centrifuged at 1700*g* for 8 min at 4 °C. The lower layer was dehydrated by anhydrous sodium sulfate and filtered through a membrane filter (PTFE, 0.20 μm, Advantec Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The filtrate obtained was increased to 10 mL in total volume by chloroform. A 20 μL portion of the sample solution was injected into the FIA system for determination of total lipid HPO content.

In Vitro Experiment. *Preparation of Hemocyte Lysate Supernatant*. The hemocyte lysate supernatant (HLS) was prepared following a modified method of Adachi et al. (*19*). Briefly, a 500 μL hemolymph was collected from the pleaopod base of the first abdominal segment of the shrimp using a 5 mL sterile syringe preloaded with 500 μL anticoagulant. The hemolymph was centrifuged and pipetted out the supernatant. The pellets were washed with anticoagulant solution and sonicated at 25 μm amplitude for 5 s in 50 mM HEPES (pH 7.8) containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$. The solution was centrifuged and the resulting supernatant was collected as HLS.

Assay for Polyphenoloxidase Activity. Polyphenoloxidase activity in HLS was measured spectrophotometrically using L-DOPA as its substrate following the method of Adachi et al. (*17*) with a slight modification as previously described, except that an appropriate amount of a 50 mM HEPES buffer (pH 7.8) containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$ was replaced by a L-ERT solution and *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (*p*-APMSF). A 50 μL aliquot of HLS was used in the assay system. The final concentration of the inhibitor added to the reaction system was 1 mM.

Assay of Peptidase Activity. Assay of peptidase activity was conducted using peptidyl-MCA as the substrate according to Morita et al. (*20*) as cited by Adachi et al. (*17*) with some modifications. A 150 μL portion of the HLS was mixed with 2235 μL of 50 mM HEPES (pH 7.8) containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$, and then the mixture was preincubated at room temperature for 10 min. A 15 μL of 10 mM synthetic peptide was added to initiate the reaction and it was terminated after 30 min incubation at 37 °C by adding 600 μL of 50% acetic acid. An appropriate amount of the HEPES buffer was replaced by the solutions of L-ERT and *p*-APMSF. The final concentration of the inhibitor added to the reaction system was 1 mM. Fluorescent assays were determined at λ_{ex} = 380 nm and λ_{em} = 460 nm, using a Shimadzu model RF-1500 fluorescence spectrophotometer. The amount of the substrate hydrolyzed was calculated from the value of 7-amino-4-methylcoumarin (AMC) standard solutions. To check whether equal volumes of proteins were loaded in all samples, protein concentrations of the HLS were determined by the dye-binding method of Bradford, using a Bio-Rad protein assay kit. For a calibration curve, bovine serum albumin was used as a protein standard.

RT-PCR Analysis. Gene expressions of proPO from HLS treated with L-ERT, *p*-APMSF, and the control were quantified following the same protocol of the RT-PCR analysis as previously described. The final concentration of the inhibitor added to the reaction system was 1 mM.

Statistical Analyses. Microsoft Excel 2007 was used to calculate the means and standard deviations for all multiple measurements as well as to generate graphs. Significant differences between the mean values were determined by one-way ANOVA. The level of significance setting was $p < 0.05$.

RESULTS

In Vivo Experiment. *Ergothioneine Content of the Mushroom Extract and Shrimp Muscles*. The amount of ERT in the mushroom hot water extract used in the feeding trial was 2.05 ± 0.24 mg/mL. The ERT content of the control and extract supplement fed diets were 0.26 ± 0.21 and 2.24 ± 0.42 mg/g, respectively. Accumulation of ERT in shrimp muscle during the feeding trial was also quantified. The ERT content of the muscle of

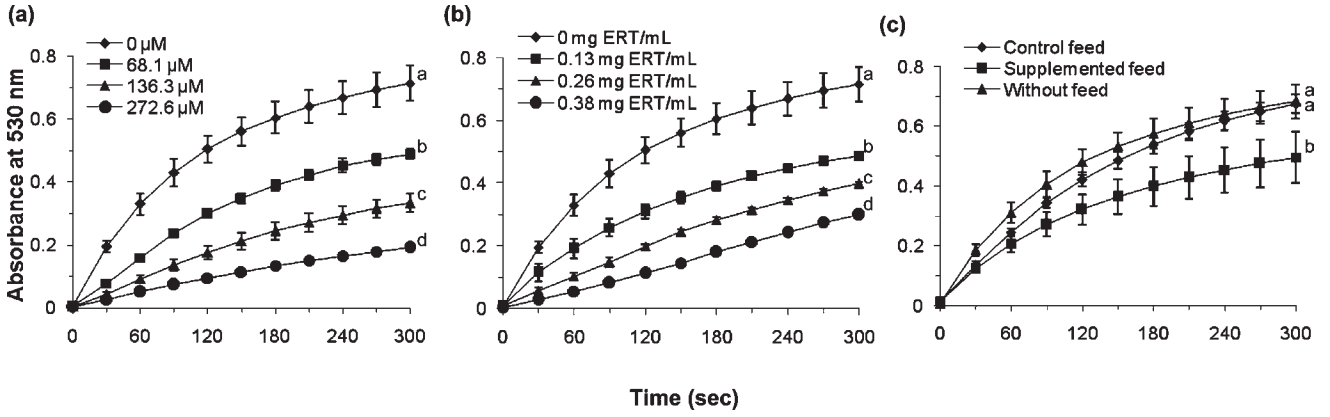

Figure 1. Inhibitory effects of commercial L-ergothioneine (a), mushroom (*Flammulina velutipes*) extract (b), and residue of diets used in the feeding trial (c) on the activity of mushroom polyphenoloxidase. Results are presented as mean ± standard deviation ($n = 3$). Values with different superscript letters represent significant difference ($p < 0.05$) at the end of the reaction period.

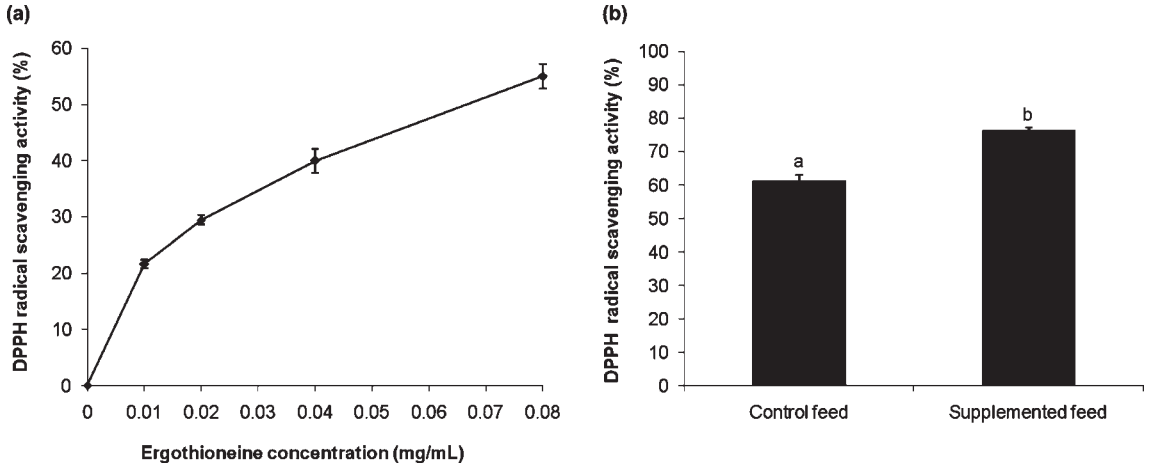

Figure 2. DPPH radical scavenging activity of the mushroom (*Flammulina velutipes*) extract (a) and residues of diets (b) used in the feeding trial to Kuruma shrimp (*Marsupenaeus japoniscus*). Results are presented as mean ± standard deviation ($n = 3$). Values with different superscript letters represent significant difference ($p < 0.05$) at the end of the reaction period.

shrimp fed the diet containing mushroom extract for seven days was significantly ($p < 0.05$) higher than the control group. The ERT content of the muscle of shrimp fed the supplemented diet was 71.2 ± 1.1 μg/g while that of the control group was 15.9 ± 0.4 μg/g.

Inhibition of Mushroom PPO Activity. For the mushroom PPO inhibition activities of the commercial L-ERT, mushroom hot water extract as well as prepared diets used for the feeding experiment are shown in **Figure 1**. With increasing concentrations of L-ERT, the mushroom PPO activity remarkably declined. At the final concentration of the commercial L-ERT in the assay system, 272.6 μM, 74% activity of the control was inhibited after 300 s. Polyphenoloxidase activity was inhibited by 50% at 122.5 μM of the L-ERT in the assay system (**Figure 1**a). In the case of the mushroom hot water extract, a final concentration of 0.38 mg ERT/mL in the assay system inhibited mushroom PPO activity at 58% of the control after 300 s (**Figure 1**b). Inhibition of PPO by the extract was also concentration dependent. Effective concentration of ERT in the extract at which the mushroom PPO activity was inhibited by 50% was 0.30 mg of ERT/mL in the assay system. These results suggest that extracts containing ERT as one of their active compounds potently inhibits mushroom PPO activity. Notably, the original PPO activity was inhibited by the control and treated diet residues by 2 and 29%, respectively (**Figure 1**c), indicating that the addition of mushroom extract to the diet significantly contributed to its inhibitory activity against mushroom PPO.

DPPH Radical Scavenging Activity. DPPH radical scavenging activity of the mushroom hot water extract and prepared diets were determined (**Figure 2**). The DPPH radical scavenging activity of the extract was dose dependent. The effective concentration of ERT in the extract at which DPPH radical was scavenged by 50% was 0.07 mg/mL in the assay system. The DPPH radical scavenging activities of the control and extract supplement fed diet residues were 61 and 76%, respectively, suggesting that the addition of the extract to the diet significantly increased its radical scavenging activity.

Mortality and Hemocyte Counts. The effect of diet supplementation with mushroom extract on the survival of Kuruma shrimp is shown in **Figure 3**. At the end of the seven-day feeding trial, no significant differences were found between the control and supplement fed groups (shrimp fed the diet containing the mushroom hot water extract) in terms of mortality and hemocyte count of the shrimp.

Hemolymph Polyphenoloxidase Activities. For the enzymatic oxidation of catechol, absorbance at 530 nm increased up to 0.14 for the control group, while it was up to 0.08 for the supplement fed group after the 300 s reaction period, suggesting that 39% of the activity of the control was inhibited. Absorbance at the end of the reaction period was significantly ($p < 0.05$) lower

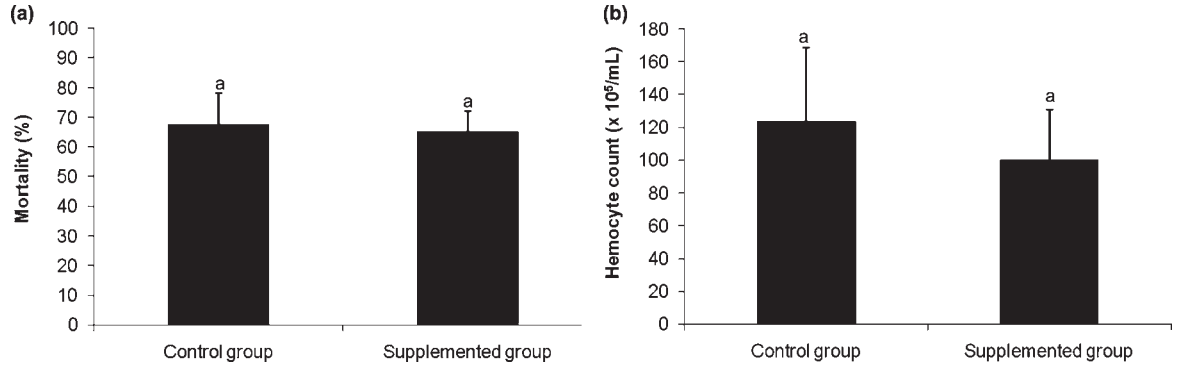

Figure 3. Cumulative mortality (a) and hemocyte counts (b) of Kuruma shrimp (*Marsupenaeus japoniscus*) after seven days of feeding. Hemocyte count is presented as the mean ± standard deviation ($n = 5$). Values with the same superscript letters represent no significant difference ($p < 0.05$).

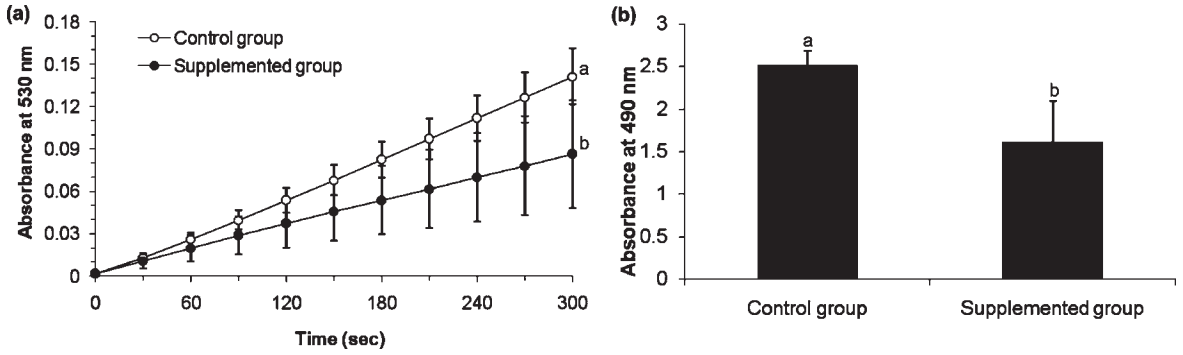

Figure 4. Enzymatic oxidation of catechol (a) and L-DOPA (b) in the hemolymph of Kuruma shrimp (*Marsupenaeus japoniscus*) after seven days of feeding. Results are presented as the mean ± standard deviation ($n = 4$). Values with different superscript letters represent a significant difference ($p < 0.05$) at the end of the reaction period for each assay.

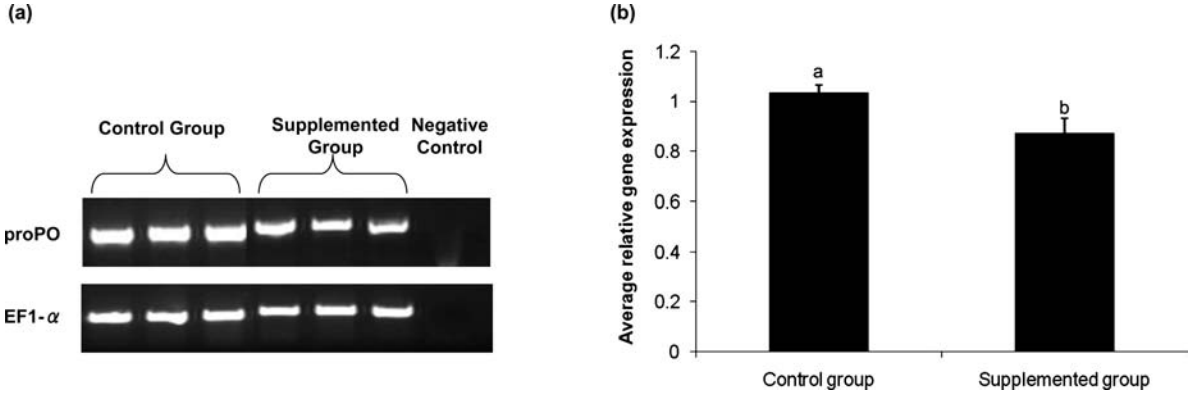

Figure 5. Gene expression analysis of prophenoloxidase (proPO) transcripts in the hemocytes of hemolymphs of Kuruma shrimp (*Marsupenaeus japoniscus*) after seven days of feeding. Representative gel (a) and quantitative analyses of band intensities obtained using ImageJ software (b). Elongation factor 1-α gene (EF1-α) was used as standard to compute relative gene expression level for each sample band. Results are presented as the mean ± standard deviation ($n = 3$). Values with different superscript letters represent significant difference ($p < 0.05$).

in the supplement fed group than in the control group. The same trend was noted when L-DOPA was used as a substrate for the PPO activity in the hemolymph of shrimp from the control and supplement fed groups. These results show that feeding the mushroom extract significantly ($p < 0.05$) reduced the total PPO activity of the control by 36% (**Figure 4**).

Gene Expression Analysis of proPO in Hemocyte. Lower expression of the proPO gene was noted in the supplement fed group. In contrast, a high-level expression of proPO genes was observed in the hemocytes of the control group. These results suggest that feeding the extract to the shrimp significantly ($p < 0.05$) suppressed the expression of proPO genes in the hemocyte (**Figure 5**).

Development of Melanosis in the Shrimp. Changes in color of the shrimp as well as the mean gray values of the carapace surfaces during ice storage are shown in **Figure 6**. By visual comparison, blackening of the carapace of the shrimp in the control group was more intense than in the supplement fed group as storage time progressed, as shown in the pictures. The mean gray values decreased abruptly in the control samples after two days of storage in ice compared to the supplement fed group. The mean gray value was determined to be significantly ($p < 0.05$) lower in the control group compared to the supplement fed group after four days of ice storage. Melanosis formation resulted in discoloration in the shrimp, particularly in the control group, so feeding the diet containing the extract

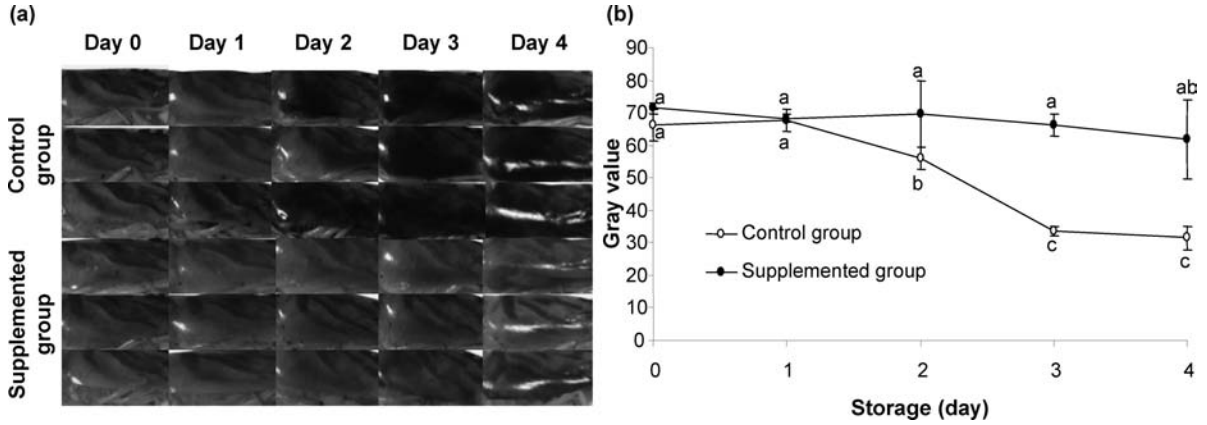

Figure 6. Development of melanosis (a) and changes in the mean gray values of carapace area (b) of Kuruma shrimp (*Marsupenaeus japoniscus*) during ice storage. Results are presented as the mean ± standard deviation ($n = 3$). Values with different superscript letters represent significant difference between groups at different storage period ($p < 0.05$).

inhibited the development of melanosis in the supplement fed shrimp.

Changes in the Lipid Hydroperoxides. Changes in the HPO content in the shrimp muscles during ice storage are shown in **Figure 7**. The HPO content of the muscle in the control group increased rapidly after two days of storage compared to the muscles of the supplement fed group. This increase in the HPO level was significant ($p < 0.05$) when the muscle from the control and supplement fed group had notable contents of HPO, amounting to 43.8 ± 4.7 and 28.9 ± 5.3 nmol/g of muscle, respectively. A slight increase in the HPO content in the muscles of the supplement fed group was observed through out the storage period.

In Vitro Experiment. *Polyphenoloxidase Activity in L-ERT- and p-APMSF-treated HLSs*. The activity of PPO in HLS was significantly lower in L-ERT- as well as *p*-APMSF-treated HLS after 30 min of incubation as shown in **Figure 8**. However, the PPO activity in L-ERT-treated HLS was significantly ($p < 0.05$) lower compared to the *p*-APMSF-treated HLS after 60 and 90 min of incubation.

Effects of L-ERT and p-APMSF on Peptidase Activity. Effects of L-ERT and *p*-APMSF on peptidase activity are shown in **Figure 8**b. The L-ERT significantly ($p < 0.05$) inhibited the activity of serine protease, but not as strongly as the *p*-APMSF. The remarkable decline in the hydrolytic rate of Boc-Val-Pro-Arg-MCA was particularly noteworthy in the *p*-APMSF treated-HLS.

Expression of proPO Gene in L-ERT- and p-APMSF-treated HLSs. Expressions of the proPO gene in the HLSs treated with L-ERT as well as *p*-APMSF were also investigated using RT-PCR. **Figure 9** shows that the transcript of proPO gene in the hemocyte showed lower relative expressions in both of HLSs treated by the L-ERT and *p*-APMSF.

DISCUSSION

In this study, the mushroom hot water extract showed a remarkable inhibitory effect on mushroom PPO. The same effect was noted for the commercial L-ERT on its activity on mushroom PPO. However, the equivalent amount of ERT in the mushroom extract necessary to inhibit the activity of the mushroom PPO by 50% (IC_{50}) was higher than the IC_{50} of the commercial L-ERT. These observations suggest that some impurities in the mushroom extract interfered with the reaction of ERT in the assay system. Bao et al. (*11*) reported that impurities in the extract also affected the activity of ERT on the DPPH radical scavenging activity and total reducing power assays. The IC_{50} of the mushroom extract in the DPPH scavenging activity assay was lower than the determined IC_{50} in the PPO activity assay. This could be

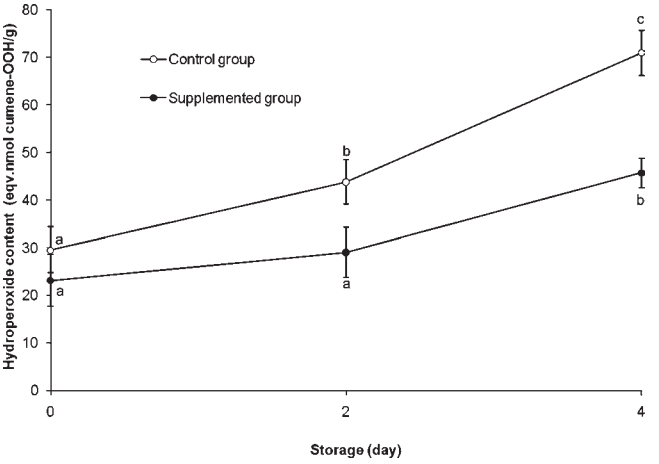

Figure 7. Changes in the total lipid hydroperoxide (HPO) content in the muscle of Kuruma shrimp (*Marsupenaeus japoniscus*) during ice storage. Results are presented as the mean ± standard deviation ($n = 3$). Values with different superscript letters represent significant difference between groups at different storage periods ($p < 0.05$).

explained by their differences in their reaction mechanism in the assay system. The DPPH scavenging activity assay was based on donating hydrogen atoms of antioxidants, whereas the mushroom PPO activity assay was based on the elimination of certain essential components from the reaction such as oxygen, enzymes, copper, or substrates (*4*). Thus, impurities have different effects on these assay systems. However, the addition of the mushroom extract in the diet still showed considerable effects on the inhibition of mushroom PPO and DPPH scavenging activities. Therefore, the mushroom hot water extract containing ERT can be used as a natural antimelanosic and antioxidative material for food application.

In the present study, dietary supplementation of the mushroom extract containing ERT effectively inhibited post mortem melanosis in Kuruma shrimp. The ERT content in the muscle of the supplement fed group of shrimp increased to seven times that of the control group. The amount of ERT in shrimp muscles has been reported to be small in quantity and below the limit of detection (*21*). Thus, results of this study strongly suggest that the ERT from the mushroom extract added in the diet accumulated in the shrimp during feeding.

The proPO activating system is an important nonself recognition system in invertebrates (*22*). This system has a key role in the

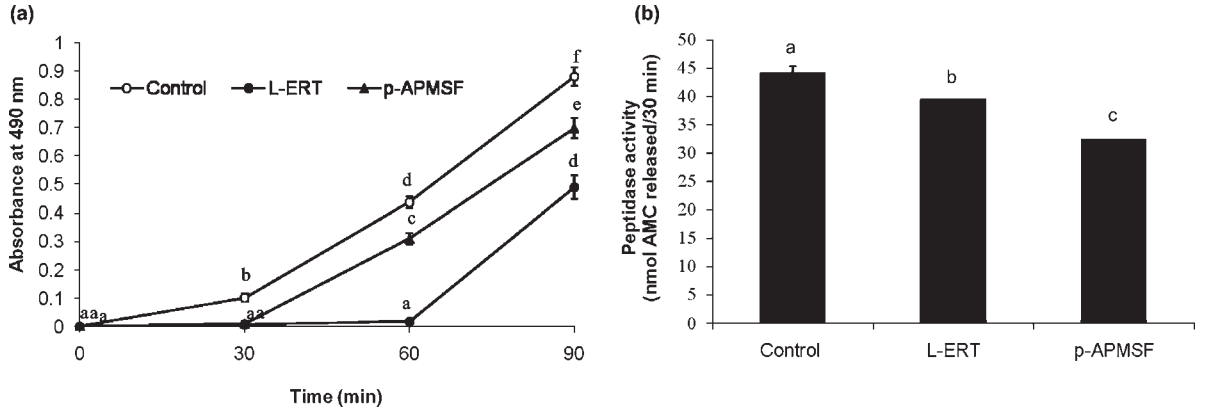

Figure 8. Inhibition of the proPO system activation in hemocyte lysate supernatant by commercial L-ergothioneine (L-ERT) and *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (*p*-APMSF). Polyphenoloxidase activity (a) and peptidase activity (b). 7-Amino-4-methylcoumarin (AMC) was used as a standard solution in the peptidase activity assay. The final concentration of each inhibitor in the reaction system was 1 mM. Results are presented as the mean ± standard deviation ($n = 3$). Values with different superscript letters represent significant difference between groups at different storage periods ($p < 0.05$).

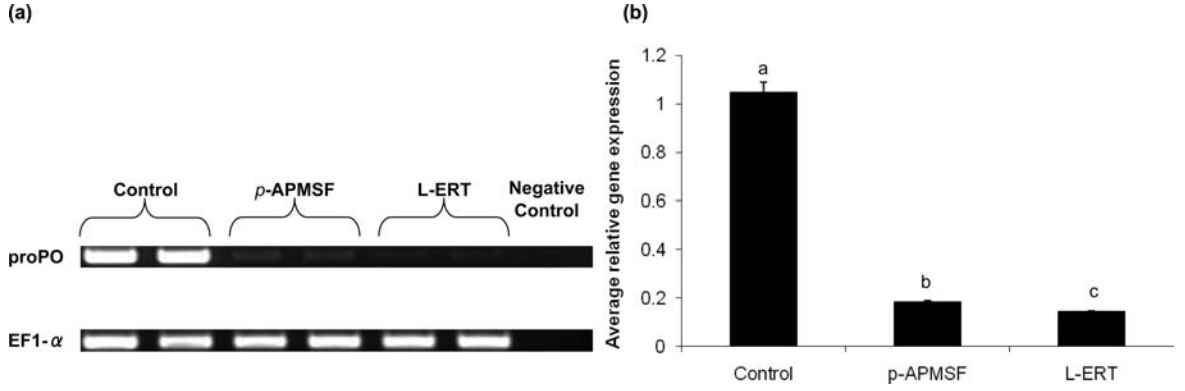

Figure 9. Gene expression analysis of proPO transcripts in the hemocyte lysate supernatant treated with commercial L-ergothioneine (L-ERT) and *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (*p*-APMSF). Representative gel (a) and quantitative analyses of band intensities obtained using ImageJ software (b). Elongation factor 1-α gene (EF1-α) was used as a standard to compute relative gene expression level for each sample band. The final concentration of the inhibitor in the reaction system was 1 mM.

primary immune response, cuticle sclerotization, and wound healing processes in crustaceans (*22*−*26*). It has been reported that two prophenoloxidases in *Penaeus monodon*, *Pm*proPO1 and *Pm*proPO2, expressed in the hemocyte, are important for the survival of *Vibrio harveyi* challenged shrimp *P. monodon* (*22*). Recognizing the significant role of the proPO system as one of the major immune responses in shrimp, inhibition of its activation could cause mortality. However, the seven-day feeding period with the mushroom extract did not significantly affect the mortality of the supplement fed shrimp when compared to the control. This was further supported by the hemocyte counts where no significant difference was found between the two groups. Thus, feeding for seven days was not critical enough to cause mortality in shrimp. Mortality in both groups could be attributed to the stress and infection due to technical injuries caused by oral feeding. Moreover, it has been known that cellular response such as encapsulation, phagocytosis, and nodule formation can also be used by invertebrates against invading pathogens (*26*). It has been reported that microbial infections in *Drosophila* did not require the activation of the proPO system for survival (*27*). This raises the question regarding the precise function of phenoloxidase activation or level of participation in the immune defense system of invertebrates since they utilize two broad but interacting categories of defense responses against pathogens, namely, the cellular and the hormonal responses.

It has been known that activations of PPO are carried out in a complex but carefully regulated series of events in the proPO activating system that consists of proteins capable of binding to polysaccharides and other compounds typically associated with microorganisms and proteases that become active in the presence of microbial products (*28*). In this study, the PPO activities of hemolymph from shrimp fed the diet containing the mushroom extract was significantly lower than that of the control group, suggesting that the mushroom extract inhibited PPO activation in the hemolymphs of the supplement fed shrimp. Interestingly, expression of the proPO genes in the hemocyte of shrimp fed with the mushroom extract was relatively lower than in the hemocyte of the control samples. Since PPO activity mainly depends on the activation of the proPO system, decreasing the expression of proPO genes in the hemocyte consequently reduces the activity of PPO. In vitro experiments conducted in this study showed that the transcript of proPO genes in the HLS showed lower expression in the L-ERT- and *p*-APMSF-treated HLSs. The PPO activity in the L-ERT-treated HLS was also remarkably low. The ERT could have been involved in the inhibition of transcriptional factors in the cascade system leading to the decrease of proPO gene expression. In human melanosis, hydroperoxy traxastane-type triterpene decreased the protein levels of PPO and its related proteins in B16 melanoma cells. It inhibited the transcriptional factor

melanocyte-type isoform of the microphthalmia-associated transcription factor, which led to the decrease of PPO and related genes (*29*). In crustaceans, it is generally believed that the enzyme responsible for the activation of proPO to PPO is a serine protease or commonly called proPO-activating enzyme (PPAE). This enzyme is believed to be the final step in the proPO cascade leading to PPO activation and in itself is tightly regulated (*3*). On the basis of the results of the peptidase activity assay, L-ERT inhibited the activity of the serine protease but not as strong as *p*-APMSF. However, the presence of L-ERT could possibly affect the overall activation of the proPO system since it showed some inhibitory activity. At least four mechanisms for PPAE regulation have been recognized: gene induction, activation by another protease, a requirement for noncatalytic serine protease homologues as cofactors and inactivation by serine protease inhibitors (*30*). Thus, other proPO activating factors such as serine protease homologues and other proteases could have been inhibited by L-ERT.

During post mortem in crustaceans, the PPO activity is usually higher in the carapace area (*1*). This study showed that the development of melanosis in the carapace area of the shrimp during ice storage was relatively inhibited in the mushroom extract-fed group. This could be the result of the inhibition of PPO activity in the hemolymphs of the supplement fed shrimp. Morover, accumulation of ERT in the shrimp muscles by feeding could directly inhibit PPO activity in the carapace during post harvest. The thiol (SH) group, containing compounds such as ERT, is a powerful nucleophile with the tendency to chelate Zn^{2+} and Cu^{2+} (*31*). Latent PPO activity of hemocyanin, a copper-binding protein in whiteleg shrimp *Penaeus vannamei*, has been shown to be involved in post mortem melanosis. Inhibitory mechanism of the ERT could be attributed also to its Cu^{2+} chelating activity; thus, the melanosis in mushroom extract-fed shrimp was inhibited.

In addition, diet supplementation of *F. velutipes* extract to shrimp also remarkably suppressed lipid oxidation. In a separate study conducted on feeding yellowtail (*Seriola quinqueradiata*) with extract prepared from *F. velutipes* cultured medium, meat discoloration due to myoglobin oxidation and lipid oxidation were effectively controlled (*32*). It has also been reported that diet supplementation of rats with L-ERT not only protected the organs against lipid peroxidation but conserved the consumption of endogenous glutathione and α-tocopherol (*13*). ERT has been known to be a powerful scavenger of hydroxyl radicals ($^{\bullet}OH$) as well as an inhibitor of metal-catalyzed generation of $^{\bullet}OH$ from hydrogen peroxide (*33*).

In conclusion, diet supplementation of ERT in *F. velutipes* in Kuruma shrimp for seven days successfully inhibited melanosis and lipid oxidation in post mortem shrimp. No adverse effects on the immune system of the shrimp were observed during the feeding period under the present experimental conditions. In vitro experiments also confirmed that ERT inhibited the activation of proPO cascade in Kuruma shrimp hemocyte. Thus, the ERT in mushrooms is a potential antimelanosic and antioxidative compound to control melanosis and lipid oxidation in aquacultured shrimp during post harvest.

ABBREVIATIONS USED

ERT, ergothioneine; L-ERT, L-ergothioneine, PPO, polyphenonol oxidase; proPO, prophenoloxidase; HLS, hemocyte lysate supernatant; MCA, methylcoumarin amide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; HEPES, 1-piperazineethanesulfonic acid; RT-PCR, reverse transcription-polymerase chain reaction; EF1-α, elongation factor 1-α; HPO, hydroperoxide; NBD-PC, 7-nitro-2-1,3-benzoxadiazol-4-yl)amino)dodecanoyl-*sn*-glycero-3-phosphocoline; FIA, flow injection analysis; *p*-APMSF, *p*-amidinophenyl methanesulfonyl fluoride hydrochloride; L-DOPA, L-3,4-dihydroxyphenylalanine; AMC, 7-amino-4-methylcoumarin.

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