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Novel Approach for Controlling Lipid Oxidation and Melanosis in Aquacultured Fish and Crustaceans: Application of Edible Mushroom (*Flammulina velutipes*) Extract *In Vivo*

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1. Introduction

The demand for fish and fishery products in the global market has been increasing with increase in the world population. This has necessitated the introduction of more aquaculture technologies in the fisheries industry in order to meet such demand. Data from the Food and Agriculture Organization (FAO, 2006) shows that the world aquaculture contribution to the global supplies of fish, crustaceans, molluscs and other aquatic animals continues to grow, increasing from 3.9, 27.1, and 32.4% of total production by weight in 1970, 2000, and 2004, respectively. It has been reported that aquaculture continues to grow more rapidly than all other animal food-producing sectors. Furthermore, the FAO (2006) reported that the aquaculture sector has grown at an average rate of 8.8% per year since 1970, compared with only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems over the same period worldwide. Notably, production from aquaculture has greatly outpaced population growth, with per capita supply from aquaculture increasing from 0.7 kg in 1970 to 7.1 kg in 2004, representing an average annual growth rate of 7.1%.

However, the generally acknowledged limitations of production from aquaculture and capture fisheries, coupled with the widening gap between the supply of and demand for fish for human consumption, reaffirms that postharvest technology is a very important component of this industry. Undoubtedly, postharvest losses are an unacceptable waste given our scarce natural resources.

Postharvest losses of fish occur in various forms during handling, processing, and preservation. Significant economic losses occur when spoilage of fish and crustaceans decrease market value or the product needs to be reprocessed, thereby increasing the cost of the finished product. Improper handling and processing methods can also reduce nutrient levels and conversion of large quantities of fish catches into fish meal for animal feeds can be considered under certain conditions as a "loss" for human food security. According to FAO (2006), fish losses caused by spoilage are estimated to be 10 to 12 million tonnes per

year, accounting for approximately 10% of the total production from capture fisheries and aquaculture. Thus, the postharvest losses in fisheries can be among the highest for all the commodities in the entire food production system. An appropriate preservation method can significantly reduce these losses, particularly those incurred during the handling, processing, distribution, and marketing of fishery products.

2. Postharvest technology for fisheries

Our fisheries resources have not only been a source of food for people but are also economically important because they are a source of livelihood for many. However, the potential for harvesting more products is now very limited because of overexploitation and the demand for food protein is increasing worldwide. Applying effective postharvest technology could reduce gap between production and demand and result in effective utilization of aquatic resources. This includes the different processes and techniques used during the postharvest handling, processing, and marketing of aquatic products.

2.1 Methods for handling and preserving fresh seafood products

Seafood products are among the world`s most perishable commodities, and their spoilage begins soon after the death of the fish. Improper handling could hasten spoilage, resulting in gradual development of undesirable qualities in seafood products. Thus, effective handling and preservation techniques need to be used to address the problems of postharvest losses.

Many developments have taken place in postharvest technology. Freezing at sea was a major development in the advance nations in the 1950`s along with the bulk chilling in chilled and refrigerated seawater systems on board fishing vessels and no new major technologies have been recently introduced but only a consolidation and anticipated development of current ones (Hermes, 1998). The industry tends to be more perceptive of the necessity of satisfying the requirements and demands of consumers, particularly with regards to the safety and quality of seafood products.

2.1.1 Chilling

Chilling is most commonly used in the industry to keep seafood fresh after harvest. Chilling is used to reduce the temperature of seafood products to some point below (-2 to -4°C for superchilling) or above (0 to 5°C) the freezing point of water (Hermes, 1998). The methods used for chilling include wet icing and use of chilled seawater, ice slurry, refrigerated air, dry ice, and gel ice mats. Icing is the most common and useful way of chilling the fish catch and is affected by direct contact between the melted ice and the fish. This requires sufficient ice and proper arrangement of ice and the product to allow rapid cooling. The use of chilled seawater or slush ice involves seawater and ice. The amount of ice used depends on the initial temperature of the water and fish, the size of the container and the quality of its insulation, and the length of the trip. Refrigerated air has been used in some big commercial boats, wherein chilled air is circulated by a finned evaporator and a fan situated at the end of the cold room. Cooling of fish through dry ice (solid carbon dioxide) is affected by evaporation of the ice, but dry ice should not be used in direct contact with the product in order to avoid cold burns (Shoemaker, 1991 as cited by Hermes, 1998). This method is normally used for air shipment. However, these chilling methods do not stop spoilage but

slow it down considerably; therefore, they are used only to delay spoilage of the fish catch but not for longer storage of the product.

2.1.2 Freezing

Freezing seafood becomes essential when other methods of preserving fish such as chilling are unsuitable for longer storage. Under proper conditions, it can keep the product frozen for several months without considerably changing its quality. Freezing is a method of stopping, either partly or entirely, the deteriorative activities of microorganisms and enzymes.

Microorganisms stop multiplying at approximately around -10°C and below, and the enzymatic activity is generally controlled when the temperature is reduced below the freezing point, to approximately -1°C (Cornell, 1995 as cited by Hermes, 1998). The water in the flesh begins to freeze at temperatures ranging from -1°C to -3 °C, and most of the water is converted into ice during freezing. At -5°C, approximately 75% of the water in fish muscles is frozen.

In the fishing industry, there are 3 basic methods available for freezing seafood products, namely, air-blast freezing, contact or plate freezing, and spray or immersion freezing. Air-blast freezing involves the continuous flow of cold air over the product. Uniform freezing is attained only if the temperature and speed of the air over the product is constant. This type of freezing system is very versatile, and is thus very useful in producing individual quick frozen (IQF) products for crustaceans, fish fillets, and value-added products. In contrast, contact or plate freezing allows the product to come into direct contact with hallow freezing metal plates, through which a cold fluid is passed. It generally used for freezing products such as whole fish, fillets, shrimps, and other seafood products into blocks. In the spray- or immersion-freezing method, the product comes into direct contact with the fluid refrigerant, such as liquid nitrogen. The method is normally used for producing very high value and specialized IQF products. The choice regarding freezing systems will depend upon cost, function, and feasibility because this method of preservation can be expensive.

2.1.3 Preservatives

Various problems are encountered with seafood products even when they are kept in cold storage. Frozen tuna becomes dark brown during cold storage because of oxidation of hemoglobin in the blood and myoglobin (Mb) in the meat. In crustaceans, browning or blackening of frozen shrimps and prawns is also observed. These problems prompted the food industry to look for ways to control quality deterioration in seafood products, and the use of preservatives has been one of the immediate responses.

The addition of antioxidants to products is one of the most widely studied methods for controlling discoloration and lipid oxidation in fish meat and other meat products. Antioxidants have many modes of action which include sequestering catalytic metal ions, decreasing oxygen concentration, quenching singlet oxygen and superoxide anion, decomposing primary oxidation products to non-volatile compounds, preventing first-chain initiation by scavenging initially generated radicals and chain breaking. The chain-breaking mechanism has been studied for several antioxidants (Roginsky & Lissi, 2005). In this mechanism, the antioxidant donates a hydrogen atom to a lipid peroxyl radical and forms

an antioxidant radical, the antioxidant radical subsequently either combines with other lipid peroxyl radical or another antioxidant radical to terminate the reaction.

Several categories of antioxidants can be used for food applications. However, selecting antioxidants for foods is a major concern in the industry because of strict regulations. Generally, the antioxidants involved in food additives must be effective at low doses, must not affect sensory flavor, and must not toxic. Therefore, natural antioxidants are generally preferred for food applications.

3. Lipid oxidation in seafood products

Lipids are one of the important structural and functional components of foods. They provide energy to humans and essential nutrients such as eicosapentaenoic acid; docosahexaenoic acid; and fat-soluble vitamins including, vitamins A, D, E, and K. Lipids are generally defined as "fatty acids and their derivatives, and substances related biosynthetically or functionally to these components" (Christie, 1987). They have been known to significantly affect food quality even though they constitute a minor component of food. Lipids not only impart flavor, odor, texture, and color to foods but also contribute to the feeling of satiety and help in making food products palatable.

However, constant exposure of lipids, particularly unsaturated fatty acids, to air could adversely affect food quality. The susceptibility of lipids to oxidation is one of the main causes of quality deterioration in various types of fresh food products as well as in processed foods. Lipid oxidation is a perennial problem for both the food industry and the consumers. Lipid oxidation has been believed to be one of the factors limiting the shelf life of foods, particularly that of many complex products (Jacobsen, 1999). It is a complex process whereby lipids, particularly polyunsaturated fatty acids, are degraded via free radical formation, causing the deterioration of flavor, texture, color, aroma, taste, consistency, nutritional benefits, and to some extent, the safety of foodstuffs for human consumption. Notably, consumers` preferences for foods are being influenced by such factors. Thus, lipid oxidation is a decisive factor in the useful processing and storage of food products.

Various effects of lipid oxidation on food properties are briefly summarized in Figure 1. Lipid oxidation itself is primarily the formation of reactive compounds like hydroperoxides (HPO) and peroxy radicals. The primary products very often undergo further reactions to form more stable compounds such as hydroxy acids or epoxides. It has been reported that compounds like hydroxy acids can contribute to bitter taste (Grosch et al., 1992). In complex food systems, the interaction of lipid HPO and secondary oxidation products with proteins and other components significantly impact oxidative and flavor stability and texture during processing, cooking, and storage (Erickson, 1992). Oxidized lipids can react with amines, amino acids, and proteins to form brown macromolecular products (Frankel, 1998). Color formation is known to be primarily influenced by the degree of fatty acid unsaturation, water activity, oxygen pressure, and the presence of phenolic compounds (Pan, 2004).

Some of the known factors that promote or inhibit lipid oxidation in foods are shown in Table 1. Metal, metallo-proteins, and enzymes are important factors affecting lipid oxidation in raw materials. Water activity, lipid interactions, proteins, and sugars are important elements affecting the food quality of processed foods.

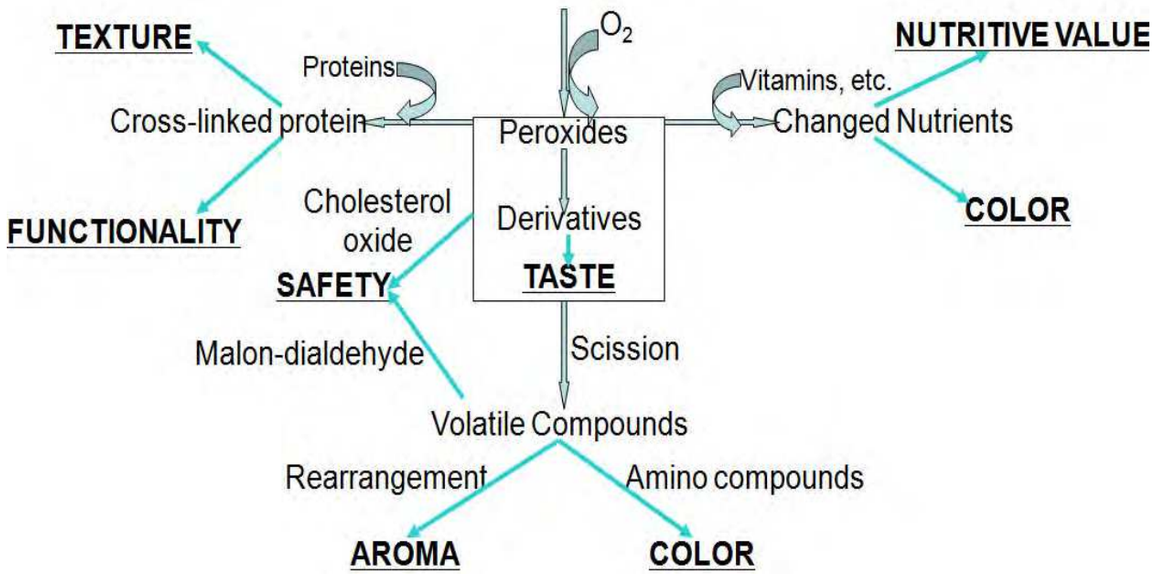


Fig. 1. Reactions of polyunsaturated fatty acids leading to quality and nutritional changes in foods (Erickson, 1992).

Type of lipid fatty acid	Non-polar, polar, fatty acids, sterols, terpenes, chain length, unsaturation, cis-trans isomers, free or bound
Catalysts	Enzymes, heme compounds, trace metals
State and access of oxygen	Triplet, singlet or radical, package
Light	Frequency, intensity, sensitizers
Temperature	Denaturating, non-denaturating
pH	Dissociation, denaturing
Inhibitors	Antioxidants, chelators, enzymes, enzyme inhibition or activation

Table 1. Lipid oxidation factors in food (Frankel, 1998).

3.1 Control of lipid oxidation by natural antioxidants

Fish tissue contains many endogenous antioxidants that can contribute to stabilizing the natural color of fish meat by acting against either lipid oxidation or Mb oxidation in the meat. These antioxidants may act directly or indirectly to inhibit the initiation and propagation steps of lipid oxidation. Antioxidants that interfere with the propagation step by converting free radicals to stable compounds are generally referred to as primary antioxidants, and those interfering with the initiation step are considered as secondary antioxidants or preventive inhibitors. Secondary antioxidants include both oxygen scavengers and chelators.

The endogenous antioxidants found in aquatic food products include the tocopherols (Syvaoja & Salminen, 1985), ubiquinol (Petillo et al., 1998), carotenoids (Miki, 1991), ascorbate, organic acids, glutathione peroxidase (Watanabe et al., 1996), peroxide dismutase (Aksnes & Njaa, 1981), catalase, peroxidases (Kanner & Kinsella, 1983), ferroxidases (Kanner

et al., 1988), nucleotides, peptides, amino acids (Boldyrev et al., 1987), and phospholipids (Ohshima et al., 1993). These natural antioxidants are usually extracted from fruits, vegetables, and other edible materials in nature.

Mushrooms have been known as a potential source of antioxidants. Numerous studies have shown that certain mushroom extracts (ME) have antioxidative activity *in vitro* and *in vivo* (Mau et al., 2002; Jang et al., 2004; Cheung & Cheung, 2005; Elmastas et al., 2007). Active compounds, including ergothioneine (ERT), certain phenolic compounds, and saccharides, have been found in extracts from different mushroom species (Wasser, 2002; Quang et al., 2006; Dubost et al., 2007).

3.2 Prevention of fish meat discoloration and lipid oxidation by dietary supplementation with ME

Mushrooms are widely cultivated because of their known uses and health benefits. However, accumulation of industrial waste has been an issue with the increasing production of mushrooms worldwide. Although spent medium is still underutilized, it is normally used as compost or raw material for extracting soluble sugars (Makishima et al., 2006). Among the mushroom species that are cultivated, *Flammulina velutipes* has been known as a medicinal mushroom and has also been cultured as an edible mushroom on a large scale in Japan and other Asian countries (Wasser, 2002).

Jang et al. (2004) reported the antioxidative properties of the *F. velutipes* extracts against the oxidation of cod liver oil in oil-in-water emulsions. In addition, water extracts from this mushroom have been reported to inhibit the oxidation of oxymyoglobin (MbO_2) isolated from cattle meats (Ashida & Sato, 2005). The activity of phenolic compounds depends on their structure and is relative to the number and location of the hydroxyl (OH) groups involved (Dziedric & Hudson, 1984). Recently, ERT is another potent antioxidant which usually exists in mushrooms. It has been known as a powerful scavenger of hydroxyl radicals (·OH) and an inhibitor of ·OH generation from hydrogen peroxide; ·OH generation is catalyzed by iron or copper ions (Akanmu et al., 1991). Arduini et al. (1990) showed that ferrylmyoglobin (ferrylMb), which was formed when deoxymyoglobin (deoxyMb) and metmyoglobin (metMb) were exposed to hydrogen peroxide, was reduced to metMb in the presence of ERT.

Bao et al. (2008) found that *F. velutipes* contains 300 ug/g ERT and can prevent lipid oxidation as well as stabilize the color of beef and fish meats during low-temperature storage. In addition, Bao et al. (2009a) evaluated the antioxidative activity of hydrophilic extract prepared from solid waste medium obtained during *F. velutipes* cultivation with regard to value-added utilization of the waste for recovery of bioactive compounds. The extract contained 341 ug/mL ERT and was used for stabilizing the lipids and the color of the dark meat of two year-old yellowtail, *Seriola quinqueradiata* through feeding prior to harvest (Bao et al., 2009a).

The DPPH radical scavenging activity of the extracts correlated with their ERT content is shown in Figure 2. In this study, the effective content of ERT was 0.3 ug in the extract in which the DPPH radicals were scavenged by 50% of the original.

Changes in the HPO content of the yellowtail dark muscle during chilled storage are shown in Figure 3. The HPO content in the dark muscle of the control yellowtail increased with

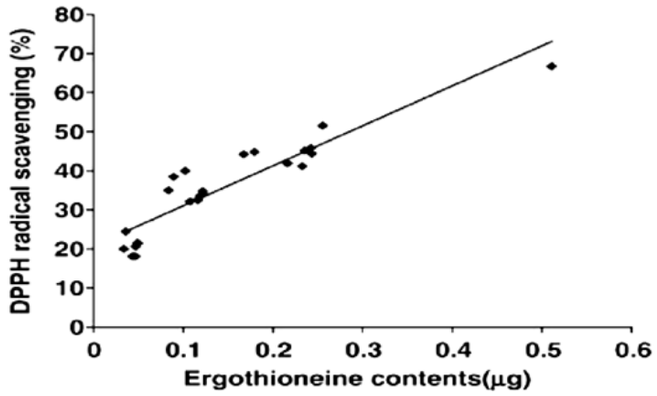


Fig. 2. DPPH radical scavenging activity of the mushroom (*Flammulina velutipes*) extract. Results are presented in terms of mean ± standard deviation (n = 3) (Bao et al., 2009a).

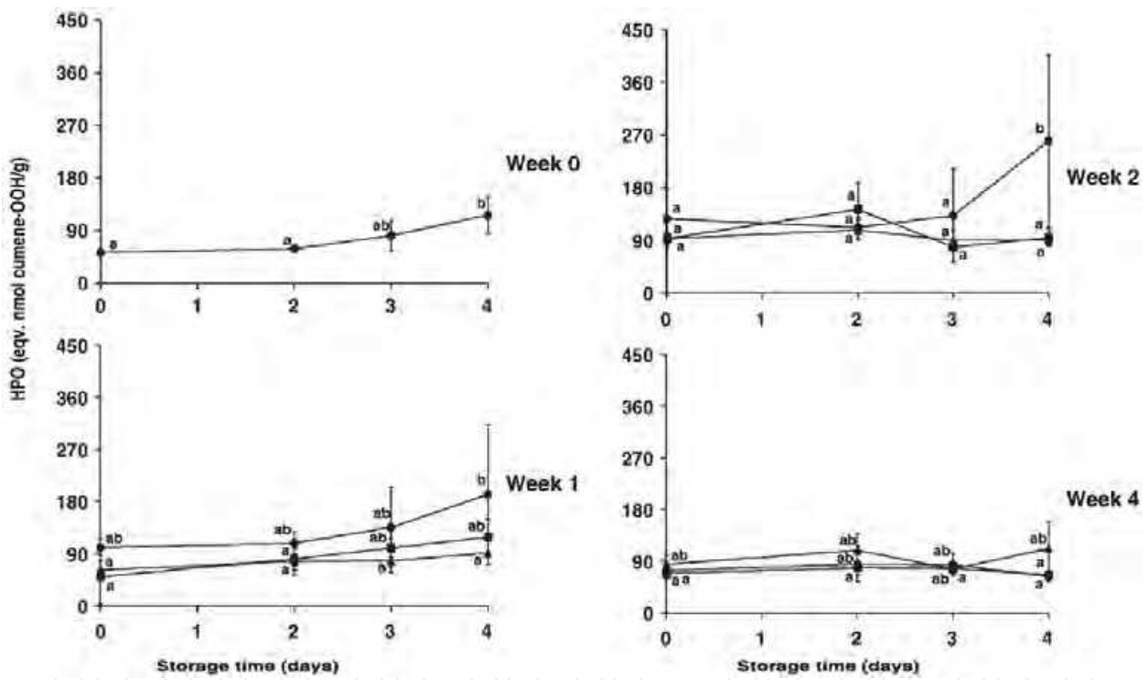


Fig. 3. Changes in the total lipid hydroperoxide level of yellowtail (*Seriola quinqueradiata*) dark muscle during chilled storage. (●), fed a diet without mushroom extract; (■), fed with diet containing 1% concentrated mushroom extract; (▲), fed with diet containing 10% mushroom extract. Data are presented in terms of mean ± standard deviation (n = 5). The values with different superscript letters represent significant difference (P < 0.05) (Bao et al., 2009a).

prolongation of storage time, whereas HPO accumulation in the ME-supplemented fish was significantly suppressed. This effect is believed to be because of the radical scavenging behavior of ERT (Franzoni et al., 2006). ERT is also known to not only protect organs against lipid peroxidation but also conserve endogenous glutathione and α-tocopherol (Deiana et al., 2004). The same study also showed that feeding yellowtails with the ME remarkably delayed metMb formation in the dark muscle during chilled storage, as shown in Figure 4.

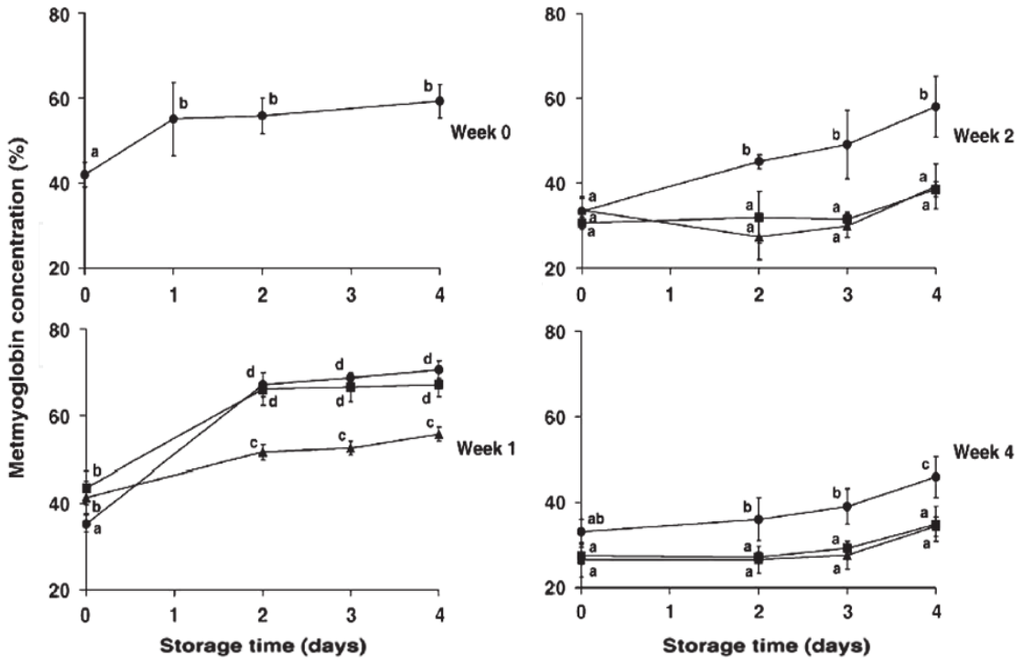
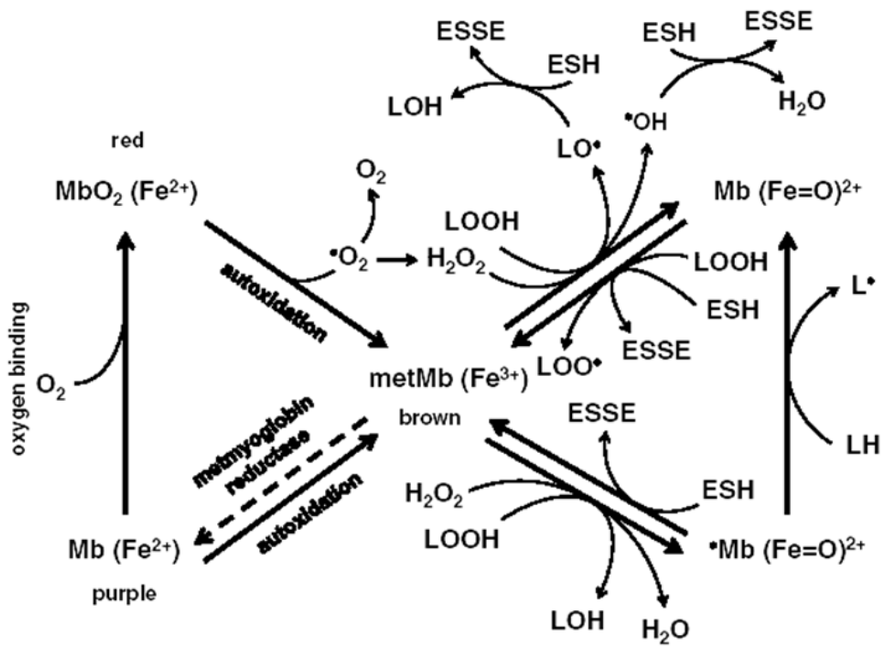


Fig. 4. Changes in the metmyoglobin content of yellowtail (*Seriola quinqueradiata*) dark muscle during chilled storage. (●), fed a diet without mushroom extract; (■), fed with diet containing 1% concentrated mushroom extract; (▲), fed with diet containing 10% mushroom extract. Data are presented in terms of mean ± standard deviation (n = 5). The values with different superscript letters represent significant difference (P <0.05) (Bao et al., 2009a).

Because ERT conserves endogenous antioxidants such as glutathione and α-tocopherol, it enhances the color stability of fish meat during postharvest handling and chilled storage. In addition, discoloration of meats is known to result from interaction between Mb and lipid oxidation (Renerre, 1990). Mb exists in 3 forms in fresh meat: deoxyMb, oxyMb, and metMb. metMB is an undesirable form not only because of its brown color but also because of its catalytic effect during the oxidation of unsaturated lipids (Love & Pearson, 1971). Grunwald & Richards (2006) reported that the reaction between metMb and lipid HPO generates ferrylMb, which can abstract a hydrogen atom from lipid (LH) to form an alkyl radical (L·). In the presence of oxygen, the alkyl radical then generates a peroxyl radical (LOO·), which can in turn abstract a hydrogen atom from another unoxidized lipid, resulting in the production of lipid HPO. The lipid HPOs generated are continuously involved in the redox reactions of Mb to generate radicals. Once underway, the alkoxyl radical (LO·) formed rearranges to generate an epoxyradical (epoxyL·), which reacts with molecular oxygen to form LOO·. The epoxyL· can also react with ferrylMB to form metMB, which continuously plays a role as an intermediate for oxidizing lipids in meat. In this case, the antioxidants that are present in the ME and accumulated in the meat could have acted as chain-breaking antioxidants. The radicals generated in meat from the interaction between Mb and lipid oxidation could be scavenged by the antioxidants, and the ferrylMb produced from this process could be reduced by donating electrons from the thiol groups of the antioxidants (Romero et al., 1992).

The proposed mechanism for ERT-delayed oxidation of lipid and Mb in minced big eye tuna (*Thunnus obesus*) meat to which *F. velutipes* extract has been added is shown in Figure 5.



ESH, ergothioneine; ESSH, dimer of ergothioneine; LO, lipid; LO•, alcoxyl radical, LOOH, lipid hydroperoxide, LOO, Peroxy radical; Mb, myoglobin; $Mb(Fe=O)^{2+}$, ferryl myoglobin; $•Mb(Fe=O)^{2+}$, ferryl myoglobin radical; MbO_2, oxymyoglobin; Mb·, myoglobin radical; metMb, metmyoglobin.

Fig. 5. Proposed mechanism for ERT-delayed oxidation of lipid and myoglobin in minced big eye tuna *(Thunnus obesus)* meat to which *F. velutipes* extract has been added (Bao et al., 2009b).

Thus, the delay in metMb formation in the dark muscle of yellowtail fed with ME might result from both suppression of lipid oxidation and conservation of endogenous antioxidants. The *in vivo* study of Bao et al. (2009a) was the first model to show that dietary supplementation with ME can be used on an industrial scale for preventing discoloration and lipid oxidation in the dark muscle of yellowtail.

4. Melanosis in crustaceans

The development of melanosis or blackspot during the postharvest period of crustaceans is a well-known postmortem phenomenon attributed to the polymerization of phenol into an insoluble black pigment, melanin. Phenol polymerization is mainly initiated by the action of an enzymatic complex called polyphenoloxidase (PPO; also known as tyrosinase). Severe blackspot formations can cause tremendous economic losses because of the high value commanded by these aquatic products in the marketplace (Kim et al., 2002). Its prevention has been a challenge to the industry, especially for food scientists.

Intensive studies on PPO in crustaceans could help understand the mechanisms underlying the development of melanosis in crustaceans; the properties of PPO; their substrates and inhibitors; and the physical, biological and chemical factors that affect each of these parameters. Complete understanding of this phenomenon and the mechanisms underlying it may help provide a scientific approach to prevent melanosis and slow its rate, thus extending the shelf life and acceptability of the product.

4.1 PPO in melanosis development

PPO plays an important physiological role in crustaceans, particularly in the process of sclerotization. The melanization cascade has been reported to be closely associated with the occurrence of factors that stimulate cellular defence by aiding phagocytosis and encapsulation reactions (Cerenius et al., 2008). Thus, melanization is an important immune response in crustaceans, similar to that observed in plants. In plants, the compounds produced as a result of the polymerization of quinones have been reported to exhibit both antibacterial and antifungal activities, whereas in crustaceans, PPO is thought to be involved in wound healing and sclerotization of the cuticle. In live crustaceans, the activation of prophenoloxidase (proPO) to PPO requires proteases and microbial activators such as polysaccharide-binding proteins (García-Carreño et al., 2008). This activation system plays an important role in the primary immune response, cuticle sclerotization, and injury healing in crustaceans (Buda & Shafer 2005; Martínez-Álvarez et al., 2005; José-Pablo et al., 2009). Simple diagram on the induction of the proPO cascade in the invertebrate immunity has been shown in Figure 6.

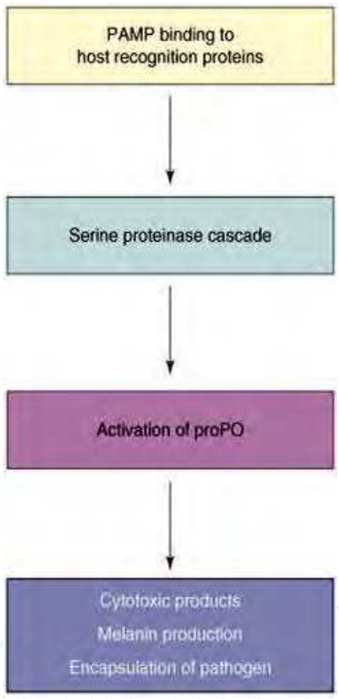


Fig. 6. Induction of the prophenoloxidase (proPO) cascade in the invertebrate immunity. Pathogen-associated molecular patterns (PAMPs; e.g., peptidoglycans, lipopolysaccharides, and β-1,3-glucans) are bound by host recognition proteins. This initiates a serine proteinase cascade that leads to the conversion of zymogenic proPO into catalytically active phenoloxidase and ultimately results in the generation of cytotoxic products and encapsulation of the pathogen (Cerenius et al., 2008).

Postharvest PPO-catalyzed blackening of the shell in crustaceans adversely affects both quality and the consumers' acceptance of these products. Crustaceans such as lobsters, shrimps, and crabs are extremely vulnerable to enzymatic blackening or melanosis. Although the occurrence of melanosis in these aquatic products does not necessarily mean that they are unfit for human consumption, consumers tend to be selective regarding these

products, mainly because browning in the carapace connotes spoilage; thus, melanosis decreases the product's market value.

Enzymatic browning or melanosis takes place in the presence of oxygen when tyrosinase and their polyphenolic substrates are mixed. Tyrosinase catalyzes 2 fundamental reactions: 1) hydroxylation at the *o*-position adjacent to an existing hydroxyl group of phenolic compounds (monophenolase activity) and 2) oxidation of diphenol to *o*-benzoquinones (diphenolase activity).

The subunits of tyrosinase have been reported to differ with respect to chemical, physical and kinetic properties, which were believed to be responsible for the relative affinities of the enzymes for both mono- and diphenolic substrates (Kim et al., 2002). The aforementioned considerations have led to many studies on the molecular mechanism underlying the monophenolase and diphenolase activity of tyrosinase. Many studies have been performed on the monophenolase activity of tyrosinase, based on 3 forms of the enzyme. The monophenolase cycle is shown in Figure 7, wherein monophenol reacts with the oxy form and binds at the axial position of one of the coppers ions of this oxy form. It has been reported that rearrangement through the trigonal bipyramidal intermediate leads to binding of peroxide and thereby generates a coordinated *o*-diphenol that is oxidized to *o*-quinone, resulting in a deoxy form ready for dioxygen binding. In the diphenolase cycle, both the oxy and met forms react with *o*-diphenol, oxidizing it to *o*-quinone. However, monophenol can compete with *o*-

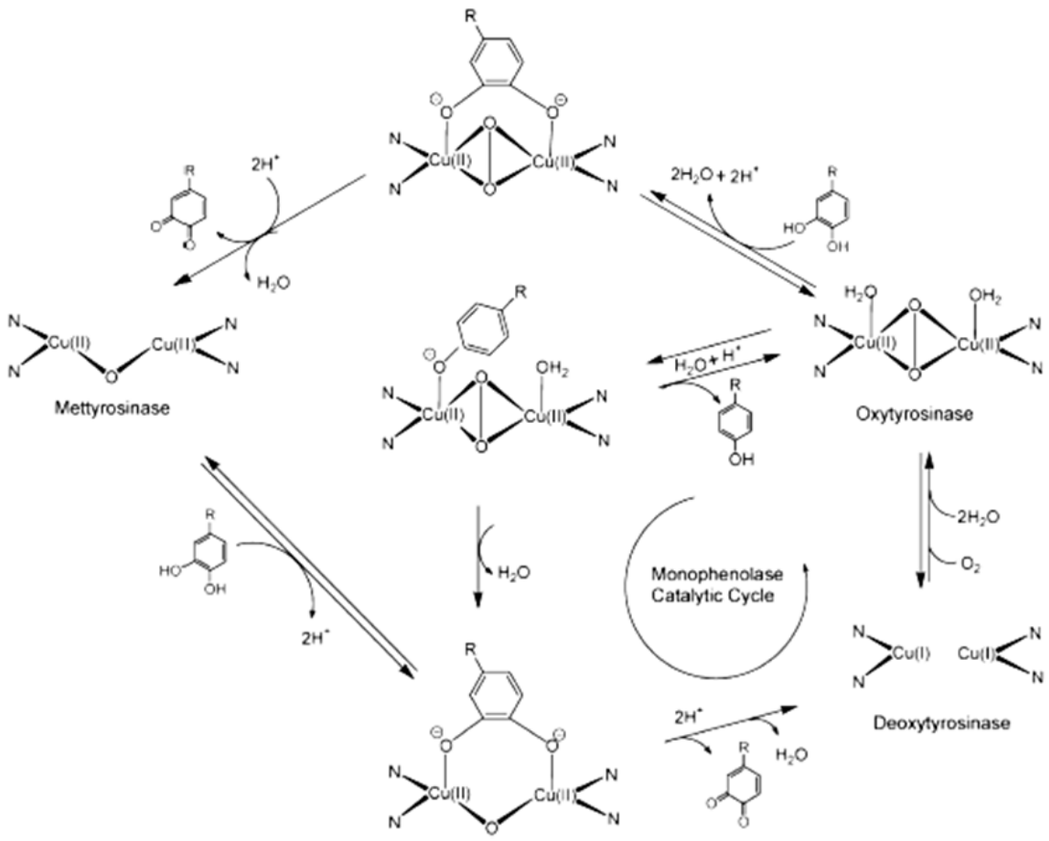


Fig. 7. Catalytic cycles of the hydroxylation of monophenol and oxidation of *o*-dipehol to *o*-quinone by tyrosinase (Kim and Uyama, 2005).

diphenol for binding to the met form site, thereby inhibiting its reduction (Kim & Uyama, 2005). Diphenolases have received much attention because of their high catalytic rate and their association with quinone formation that in turn leads to melanin production.

4.2 Control of melanosis

The fundamental step in melanosis is the transformation of an *o*-diphenol such as 3,4-dihydroxyphenylalenine (L-DOPA) to the corresponding *o*-quinone, which undergoes further oxidation to form brown or black pigments (Martinez & Whitaker, 1995). *o*-Quinones are known to be powerful electrophiles that can be attacked by water, other polyphenols, amino acids, peptides, and proteins, leading to Michael-type products (Mayer et al., 1990).

Many studies have been performed on controlling or inhibiting the activity of PPO in foods, and various mechanisms and techniques have been proposed and developed over the years to mitigate concerns regarding undesirable products of enzyme activity. This involves elimination of one or more of the important components involved in the enzymatic reaction, such as oxygen, copper, substrate, and the enzyme itself. There are also known compounds that react with the product of enzyme activity, thus inhibiting the formation of colored compounds. Many other techniques are also being applied to prevent melanosis in foods, such as processing methods, different types and kinds of inhibitors, and a molecular approach to control PPO, as presented in the following section.

4.2.1 Processing

Traditionally, heating is one of the most popular method to destroy microorganism and to inactivate enzymes. The catalytic activity of PPO is inhibited at temperatures ranging from 70^{o}C to 90^{o}C (Vamos-Vigyoso, 1981). Lee et al. (1988) reported that blanching green beans at temperatures above 82 ^{o}C inactivated its enzymes, including PPO.

Similar to heat treatment, low-temperature treat can also help control enzyme-catalyzed reactions. At low temperatures, the kinetic energy of the reactant molecules decreases, subsequently leading to a decrease in the mobility and reaction necessary for the formation of enzyme-substrate complexes and their products (Kim et al., 2002). Thus, low-temperature preservation and storage are used during distribution and retailing to control the development of melanosis in food products. Temperatures as low as -18^{o}C can be used to inactivate enzymes. However, this changes the physical attributes of the products.

Dehydration can also be used to control enzyme catalyzed reactions that cause browning in foods. Generally, water greatly influences enzyme activity because it acts as a solvent or a reactant (Ashie & Simpson, 1996). Water activity can be controlled by physical drying (i.e., dehydration or freeze drying) or by chemical methods (i.e., addition of water-binding agents such as sugars and salts).

Irradiation is another method used for controlling enzymatic browning in foods. It uses ionizing radiation to inactivate microorganisms as well as enzymes. Irradiation is being increasingly recognized as a method for reducing postharvest losses, maintaining food quality, as well as ensuring the hygienic quality of food products.

The rearrangement or destruction of noncovalent bonds such as hydrogen bonds, hydrophobic interactions, and ionic bonds of the tertiary protein structure can lead to

enzyme denaturation. This can be achieved by high-pressure treatment that reduces molecular spacing and increases interchain reactions. However, this varies depending the enzymes; the nature of the substrate; and the temperature and duration of high-pressure processing (Cheftel, 1992).

Other processing methods that have been developed and used to control the enzymatic browning of food products included the treatment of food with supercritical carbon dioxide, ultrafiltration, or ultrasonication (Kim et al., 2002). Treatment with supercritical carbon dioxide inactivates the enzyme through carbonic acid production, which causes a significant pH reduction. In contrast, ultrafiltration removes PPO during the process, whereas ultrasonication causes severe shear stresses, thereby promoting enzyme denaturation.

4.2.2 Inhibitors

Melanosis can be inhibited by targeting the enzyme, the substrate, and/or the reaction products involved in the process. Inhibition that targets the enzyme includes metal chelators and compounds that consist of carboxylic acids of the benzoic and cinnamic series. These compounds can be competitive owing to their structural similarity with phenolic substrates. Inhibition that targets the substrate can be performed by removing either oxygen or the phenolic substrate from the reaction. Inhibition targeting the products involves compounds that can react with *o*-quinones to produce a colorless addition product.

These PPO inhibitors can be classified into 6 categories that include: 1) reducing agents such as sulfites, ascorbic acid and its analogs, cysteine, and glutathione; 2) chelating agents such as phosphates, EDTA, and organic acids; 3) acidulants such as citric acid and phosphoric acid; 4) enzyme inhibitors such as aromatic carboxylic acids, aliphatic alcohols, anions, peptides, and substituted resorcinols; 5) treatments with enzymes such as oxygenases, *o*-methyltransferase and proteases; and 6) complexing agents such as cyclodexrins (McEvily et al., 1992). To date, there have been many studies on the different compounds used to inhibit melanosis in foods.

4.2.3 Molecular approach

The nature of PPO and its role in the immune system of plants and crustaceans have been extensively reviewed. One possible approach to reduce the activity of PPO and the resultant enzymatic browning reactions is to characterize and inactivate the PPO coding genes. This can be done by generating antisense RNAs specific for PPO.

Antisense genes have been successfully used for altering plant processes and improving crops. It involves blocking gene expression of the plant enzymes involved in a certain process. The process is based on blocking the flow of information from DNA via RNA to protein by introducing an RNA strand complementary to the sequence of the target mRNA. The antisense approach involves the insertion of a gene or a significant part of it into the cell in a reverse orientation. This approach has been used to increase the shelf life of fruits (Fray & Grierson, 1993), and commercial applications of this technology now include alteration of flower color, viral resistance induction, and fruit ripening. It has been reported that the lack of bruising sensitivity in transgenic potatoes without any side effects opens up the possibility of preventing melanosis in wide varieties of food crops and even to crustaceans, without the use of any physical and chemical treatments. Thus, by using this antisense

technology, it is possible to develop fruits, vegetables and crustaceans that are resistant to enzymatic browning.

4.3 Application of ERT-rich edible mushroom (*Flammulina velutipes*) extract for controlling melanosis

As mentioned earlier, many technologies and techniques have been developed for the inhibition of melanosis in food products, and some more new approaches are under study. However, these alternatives must be evaluated in terms of their impact on the overall food quality, effectiveness, cost, and regulatory status. Because of health concerns, only a number of inhibitors have been accepted for food application, based on the basis of government health regulations.

A relatively new approach involves the design and development of another application of ERT-rich edible mushroom (*F. velutipes*) extract for inhibiting melanosis in commercially important crustaceans.

4.3.1 Dietary supplementation

The extract from the edible mushroom *F. velutipes* has been found to significantly inhibit mushroom PPO activity; prevent browning in apples; and delay melanosis in shrimps which usually develops during storage (Jang et al., 2002; 2003). These observations suggest that the mushroom extract contains certain compounds contributing to such actions. In section 3, it has been mentioned that ME containing ERT at the level of 3.03 ± 0.07 mg/mL has been reported to show remarkable DPPH radical scavenging activity and suppress lipid oxidation in bigeye tuna meat (Bao et al., 2008). ME have been reported to stabilize the fresh color of tuna meat during ice storage (Bao et al., 2009a). These results strongly suggest that ERT could be one of the major compounds in the ME that effectively inhibited mushroom PPO activity, apple browning, and melanosis in shrimps in the previous studies (Jang et al., 2002; 2003).

The *in vivo* application of L-ERT as an antioxidant in rats (Deiana et al., 2004) and the effects of feeding fish with ERT-rich ME have been studied; however, before this study, no study had been performed to determine the efficacy of extracts that contains this compound, when fed to crustaceans to control melanosis. Therefore, a study was performed on the use of a hydrophilic extract prepared from the fruiting body of *F. velutipes* for controlling melanosis and lipid oxidation in cultured kuruma shrimps (*Marsupenaeus japonicus*) *in vivo*.

In a study performed by Encarnacion et al. (2010), the hot water ME containing 2.05 ± 0.24 mg/mL ERT remarkably inhibited mushroom PPO. The PPO activity is usually found to be higher in the carapace area during postmortem of crustaceans (José-Pablo et al., 2009) and this study showed that the development of melanosis in the carapace area of the shrimps during ice storage was relatively controlled in the ME-fed group than in the control (Fig. 8). The accumulation of ERT from the ME added in the diet during feeding could have contributed to this effect.

In the same study, the PPO activities of hemolymph from shrimps fed a diet containing the ME was significantly lower than that of the control group, and the expression of the proPO genes in the hemocytes of shrimps fed with the ME was relatively lower than that in the

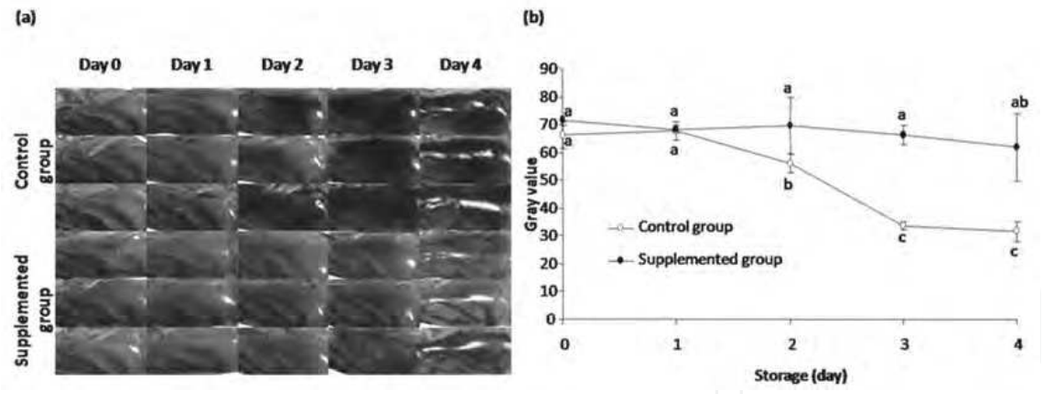


Fig. 8. Development of melanosis (a) and changes in the mean gray values of the carapace area (b) of kuruma shrimps (*Marsupenaeus japonicus*) during ice storage. Results are presented interms of mean±standard deviation (n = 3). The values with different superscript letters represent significant difference between groups for different storage periods (p < 0.05) (Encarnacion et al., 2010).

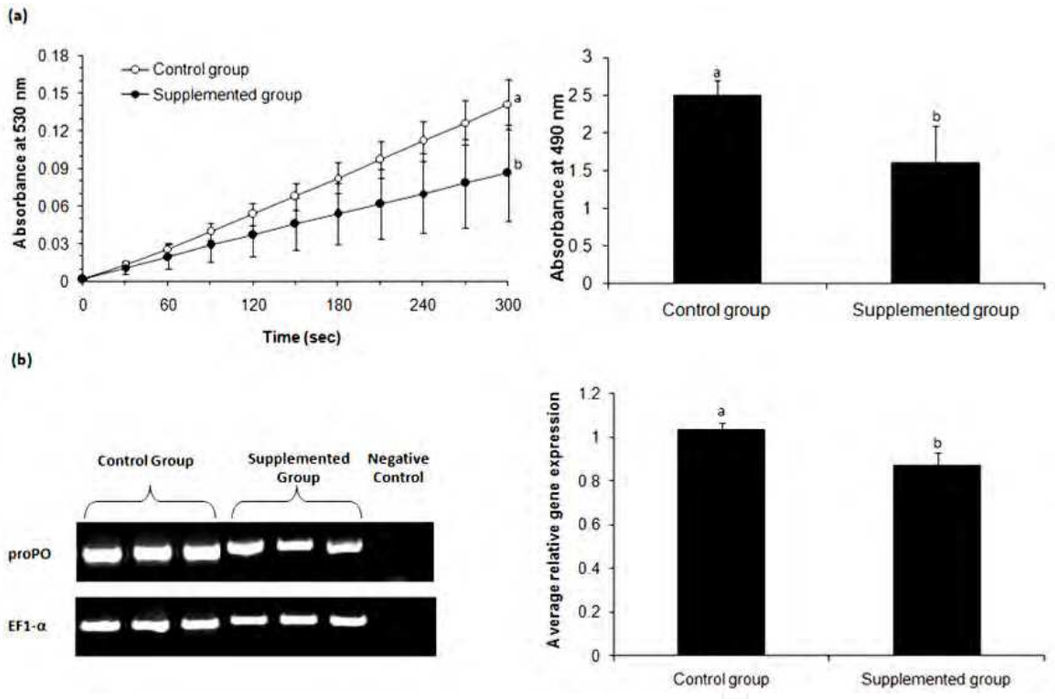


Fig. 9. (a) Enzymatic oxidation of catechol and L-DOPA (b) in the hemolymph of kuruma shrimps (*Marsupenaeus japonicus*) after 7 days of feeding. Results are presented in terms of mean ± standard deviation (n = 4). The values with different superscript letters represent significant difference (p < 0.05) at the end of the reaction period for each assay. (b) Gene expression analysis of prophenoloxidase (proPO) transcripts in the hemocytes of hemolymphs of kuruma shrimp (*M. japonicus*) after 7 days of feeding (representative gel and quantitative analyses of band intensities obtained using the ImageJ software. Elongation factor1-α gene (EF1-α) was used as the standard for computing relative gene expression level for each sample band. Results are presented in terms of mean ± standard deviation (n = 3). The values with different superscript letters represent significant difference (p < 0.05) (Encarnacion et al., 2010).

hemocytes of the control samples, showing that the ME inhibited PPO activation in the hemolymphs of the supplement-fed shrimps (Fig. 9). Because PPO activity mainly depends on the activation of the proPO system, decreasing the expression of proPO genes in hemocytes consequently reduces the activity of PPO.

The results of a study performed by Amparyup et al. (2009) indicated the significant role of the proPO activating system as one of the major immune responses in shrimps and also reported that inhibiting proPO activation in shrimps could cause death. However, the study did not show any significant differences in mortality between the supplement-fed shrimps the control samples. A study by Leclerc et al. (2006) reported that microbial infections in *Drosophila* did not require the activation of the proPO system for survival thus, raising questions regarding the precise function of phenoloxidase activation and its level of participation in the immune defense system of invertebrates because they use 2 broad but interacting categories of defense responses against pathogens, namely, the cellular and hormonal responses.

4.3.2 Immersion technique

Because feeding shrimps with MEs is time consuming and requires tedious technical work, the possibility of immersing live full-grown crustaceans in an ME solution for inhibiting postharvest melanosis was considered. Martínez-Álvarez et al. (2005) reported the efficacy of immersing shrimp in 0.05% hexylresorcinol (HR) for preventing postharvest melanosis. *In vivo* treatment with antimelanosic agents may be more effective than postmortem treatment because of greater absorption of antimelanosic agents through the gills, increased accumulation in the hemolymph, and enhanced distribution throughout the body. Therefore, the effects of *in vivo* treatment with *F. velutipes* ME, sodium ascorbate (ascorbic acid, AA), sodium sulfate (SS), and HR on postharvest melanosis in crustaceans were compared.

The study conducted by Encarnacion et al. (2011a) showed that immersing live *M. japonicus* shrimps in ME effectively inhibited postharvest melanosis in the shrimps (Fig. 10). Moreover, the development of melanosis in the carapace decreased with increase in the concentration of ME in the immersion solution. These effects may be due to greater absorption and accumulation of ERT in the shrimps with increase in the concentration of ME in the immersion solution. At 0.5 % ME concentration in pure seawater, melanosis was effectively inhibited in shrimp samples after 2 days of ice storage; the effect was the same as that obtained with 500 ppm HR and better than obtained with 500 ppm AA.

The study performed by Encarnacion et al. (2011b) also used this technique for the red queen crab, *Chionoecetes japonicus,* and the result clearly showed that treating live crabs with purified seawater containing 1.0% ME effectively prevented melanosis during 4 days of ice storage; and the effect obtained the same as that with 500 ppm SS or with 500 ppm HR solutions (Fig. 11).

ERT accumulation in shrimp tissue may inhibit PPO activity in the carapace during the postharvest period. Because thiols, such as ERT, are powerful nucleophiles that can chelate Zn^{2+} and Cu^{2+} (Hanlon, 1971; Park et al., 2006), the mechanism underlying the inhibitory effect of ERT could be due to its Cu^{2+} chelating activity. The mechanism of ERT could also be attributed to its Cu^{2+} chelating activity; thus, melanosis in the shrimps immersed in the ME solution was inhibited.

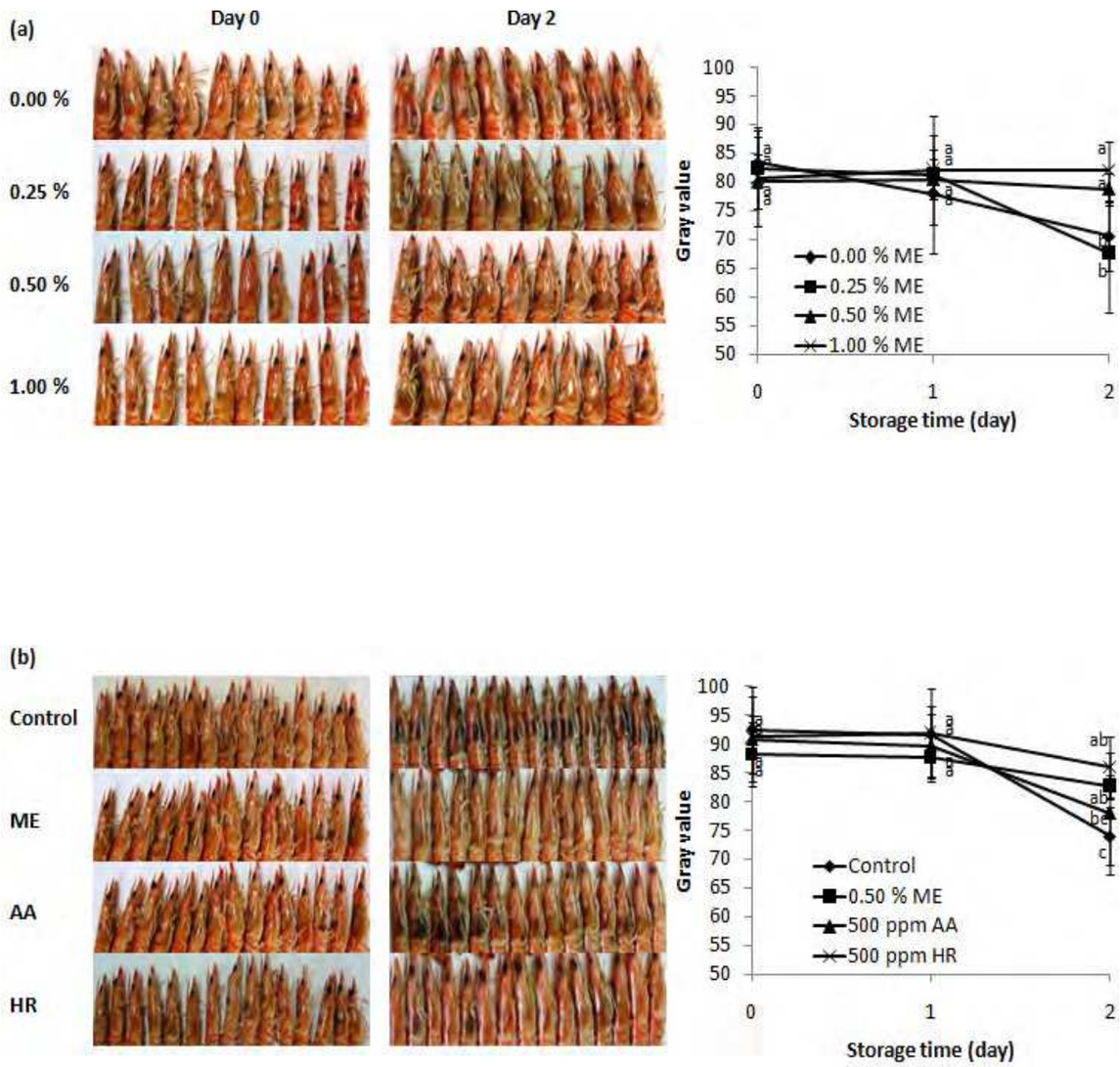


Fig. 10. Development of melanosis and changes in the mean gray value of the carapace of *Marsupenaeus japonicus* shrimps before and after ice storage: (a) dose-dependent effect of ME, (b) comparison with other antimelanosic compounds. Results are presented in terms of mean ± standard deviation (n = 3). The superscript letters above each data point represent statistically significant differences ($p < 0.05$) (Encarnacion et al., 2011a).

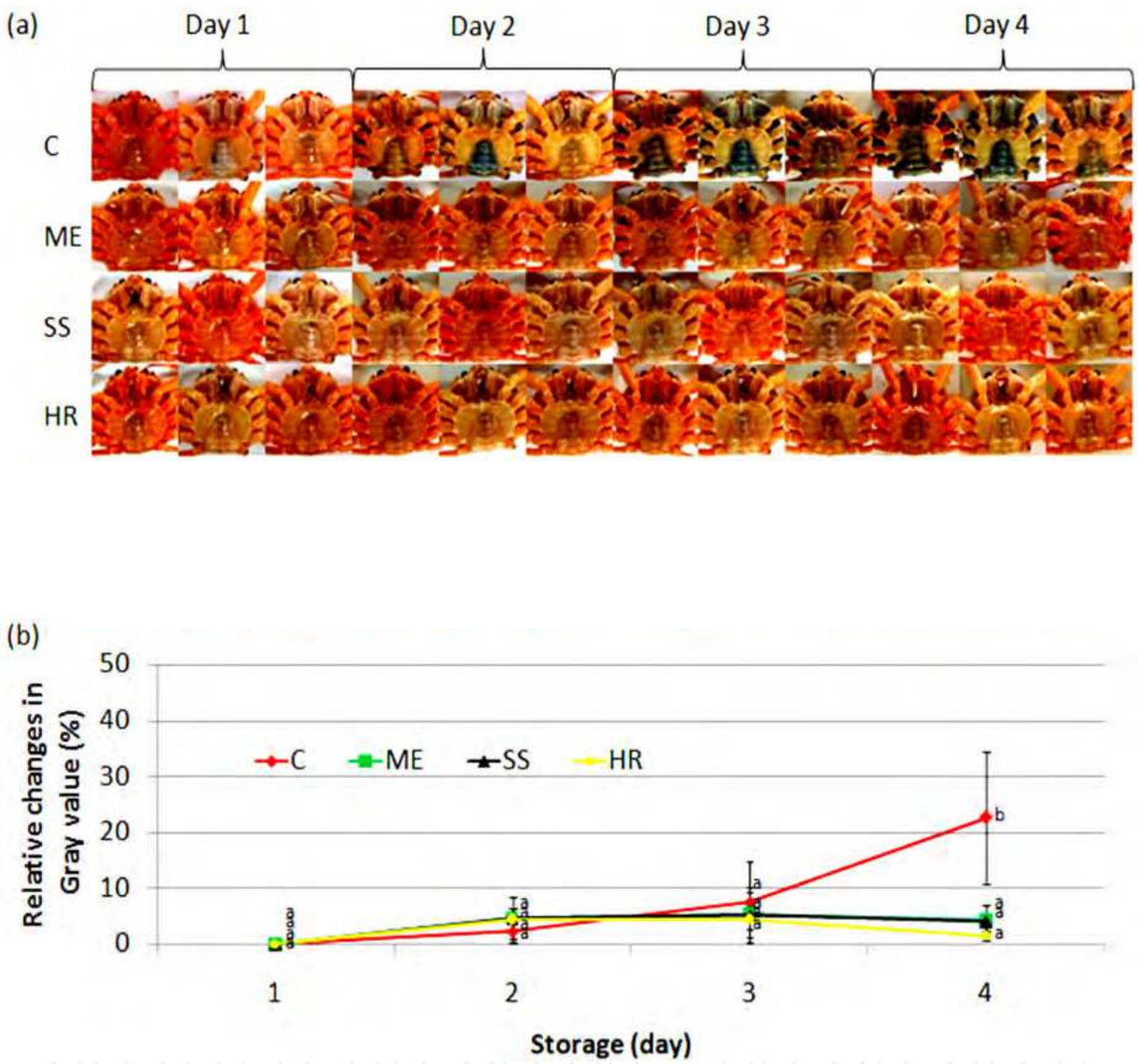


Fig. 11. (a) Digital photographs of the development of melanosis in *Chionoecetes japonicus* crabs pretreated with purified seawater (C), 1.0% mushroom extract (ME), 500 ppm sodium sulphite (SS), and 500 ppm 4-hexyl-1,3-benzenediol (HR) solutions during ice storage. (b) Relative changes in the mean gray value of the carapace of *C. japonicus* during ice storage, analyzed using the ImageJ software. Results are presented in terms of mean (SD) (n = 3). The superscript letters above each data point represent statistically significant differences (p < 0.05) (Encarnacion et al., 2011b).

In addition to inhibiting PPO activity, ME also suppressed proPO gene expression in the hemolymph of *M. japonicus* (Fig. 12) and *C. japonicus* (Fig. 13). The same result was obtained in the feeding trial. Because immersing shrimp in ME significantly reduced PPO activity in

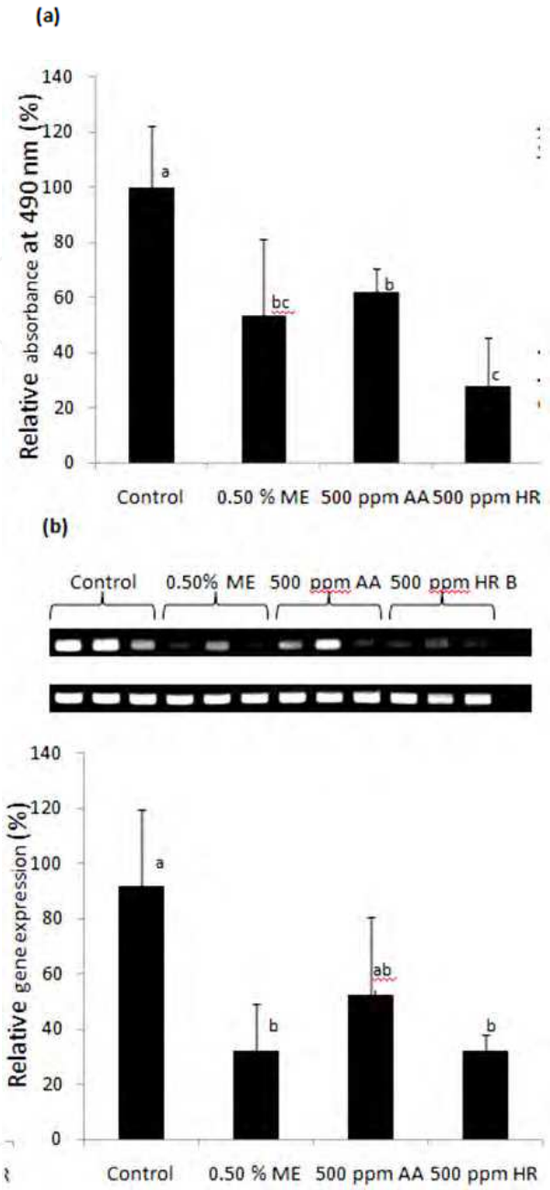


Fig. 12. (a) Enzymatic oxidation of L-DOPA in the hemolymph of kuruma shrimps (*M. japonicus*) immersed in control or treatment solutions. In each graph, the average absorbances of samples are shown relative to the average absorbance of the control (defined as 100%). Results are presented in terms of mean ± SD (n = 5). (b) Gene expression analysis of prophenoloxidase (proPO) transcripts in the hemocytes of *M. japonicus* shrimp immersed in control or treatment solutions. The top and bottom panels show representative gels and quantitative analyses of the band intensities obtained using the ImageJ software, respectively. The expression level of the elongation factor 1α (EF1-α) gene was used as the reference for calculating the relative expression level for each band. B denotes a blank sample (negative control). Results are presented in terms of mean ± SD (n = 3). The superscript letters above each bar represent statistically significant differences ($p < 0.05$) (Encarnacion et al., 2011a).

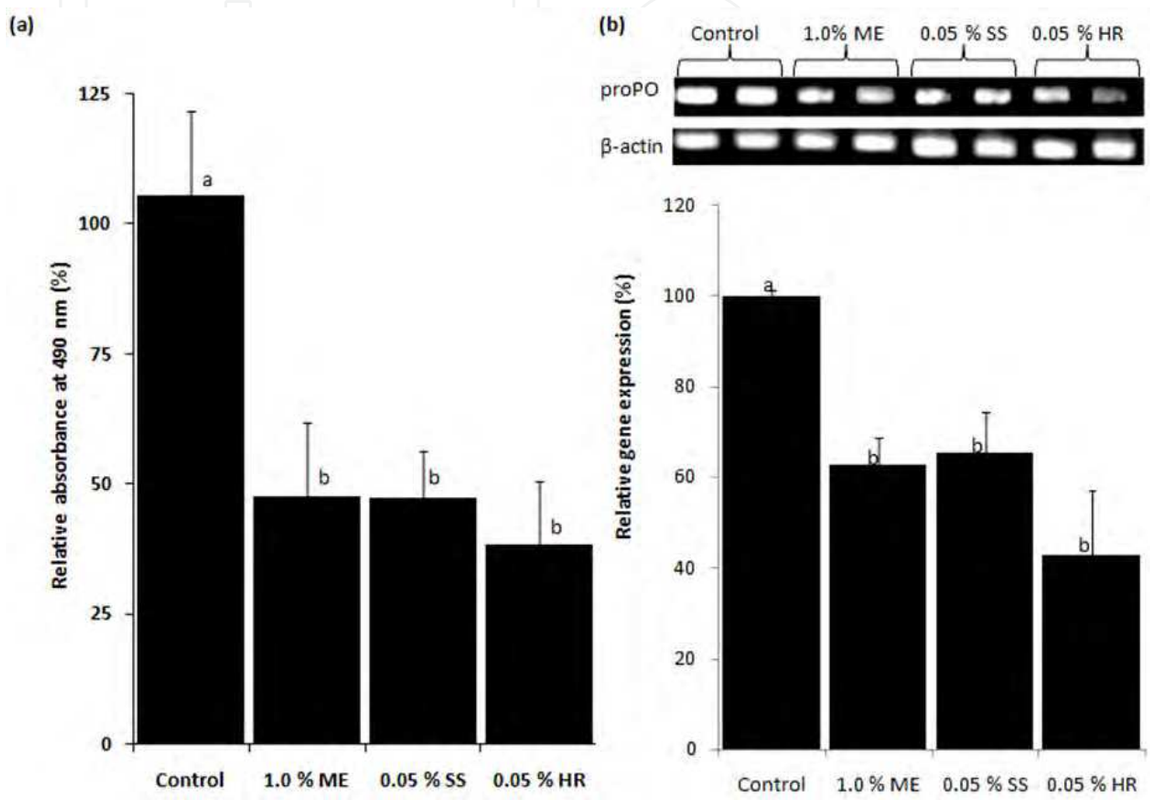


Fig. 13. (a) Enzymatic oxidation of L-DOPA in the hemolymph of red queen crabs (*Chionoecetes japonicus*) immersed in control or treatment solutions. In each graph, the average absorbances of samples are shown relative to the average absorbance of the control (defined as 100%). Results are presented in terms of mean ± SD (n = 5). (b) Gene expression analysis of prophenoloxidase (proPO) in the hemocytes of *C. japonicus* immersed in purified seawater (Control), 1.0% mushroom extract (ME), 500 ppm sodium sulphite (SS), or 500 ppm 4-hexyl-1,3-benzenediol (HR) solutions, performed using reverse transcription–polymerase chain reaction. (b) Quantitative analysis of the band intensities obtained using the ImageJ software. The expression level of the β-actin gene was used as the reference for carculating the relative expression level for each band. Results are presented in terms of mean (SD). The superscript letters above each bar represent statistically significant differences ($p < 0.05$) (Encarnacion et al., 2011b).

the hemolymph, it is possible that the ME containing ERT inhibited PPO activation. The observation that proPO gene expression in the hemocytes of ME-treated shrimps was lower than that in control shrimps supports this hypothesis. Thus, decreasing the expression of proPO reduces the amount that can be proteolytically activated into PPO (Adachi et al., 2003).

The efficacy of ME in inhibiting postharvest melanosis is comparable to the efficacy of existing antimelanosic compounds, such as HR, AA and SS in this regard. HR forms an inactive complex with PPO that is incapable of catalyzing melanosis development (Guererro-Beltran et al., 2005). Therefore, PPO activity significantly decreases in the presence of HR (Encarnacion et al., 2010; 2011). HR possesses antimicrobial activity in addition to its antimelanosic activity. The antimicrobial activity of HR (Martínez-Álvarez et al., 2005) may be related to the decrease in proPO gene expression because invading microorganisms trigger a proteolytic cascade culminating in the release of active PPO (Leclerc et al., 2006). Because AA did not significantly affect proPO gene expression, its mechanism of inhibiting postharvest melanosis is most likely to be due to its reducing power. The slight melanosis that occurred in AA-immersed shrimps after 2 days of ice storage may be due to the oxidation of AA, which prevented it from reducing *o*-quinones, thereby allowing melanin development (Guererro-Beltran et al., 2005). Sulfites are known to inhibit both enzymatic and nonenzymatic browning reactions. Bisulphite is reported to inhibit melanosis by 2 mechanisms: 1) by reacting with intermediate quinones in the melanosis reaction that forms sulfoquinones; and 2) by irreversibly reacting with PPO, causing complete inactivation (Ferrer et al., 1989). Therefore, PPO activity also significantly decreases in the presence of SS. SS and HR posses antimicrobial activities in addition to their antimelanosic activities, SS and HR are also antimicrobial agents. The antimicrobial activity of SS (McFeeters et al., 2004; Martínez-Álvarez et al., 2005) may also be related to the decrease in proPO gene expression.

4.3.3 Mechanism underlying the inhibitory effect of the extract on melanosis

The mushroom hot water ME remarkably inhibited mushroom PPO. Commercial L-ERT had the same effect on mushroom PPO. Mushroom PPO activity remarkably declined with increase in the concentrations of L-ERT (Fig. 14). PPO inhibition by the ME was also depended on L-ERT concentration. These results suggest that extracts containing ERT as one of their active compounds potently inhibit mushroom PPO activity.

In vitro experiments performed by Encarnacion et al. (2010) also showed that the transcript expression of the proPO genes in the HLS was lower than that in the L-ERT- and *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (*p*-APMSF)-treated HLSs (Fig. 15). The PPO activity in the L-ERT-treated HLS was also remarkably low. ERT could have been involved in inhibiting transcriptional factors in the cascade system, leading to a decrease in proPO gene expression. Maeda et al. (2007) reported that in human melanosis, hydroperoxy traxastane-type triterpene decreased the protein levels of PPO and its related proteins in B16 melanoma cells because of inhibition of the transcription factor melanocyte-type isoform of the microphthalmia-associated transcription factor, leading to a decrease in the PPO gene and its related genes. In the case of crustaceans, it is generally believed that the enzyme responsible for the activation of proPO to PPO is a serine protease, or commonly called as proPO-activating enzyme (PPAE). This enzyme is believed to be involved in the final step in

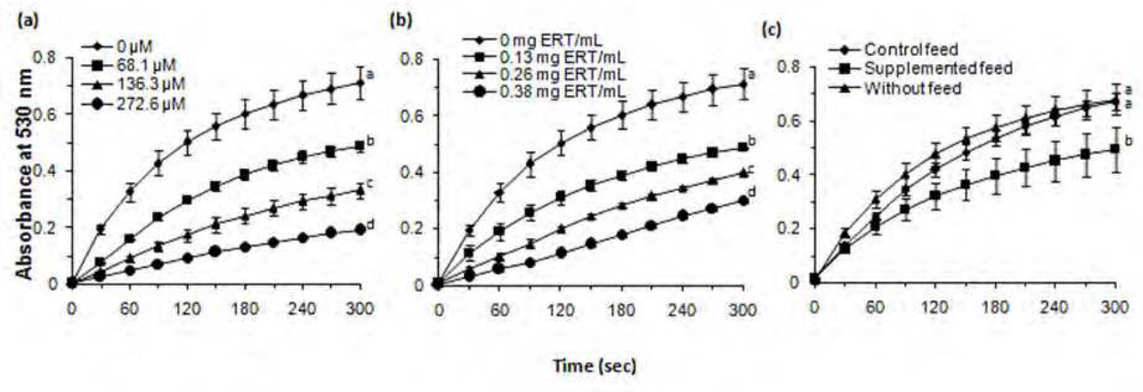


Fig. 14. Inhibitory effects of commercial L-ergothioneine (a), mushroom (*F. velutipes*) extract (b), and residues of diets used in the feeding trial (c) on the activity of mushroom polyphenoloxidase. Results are presented in terms of mean ± standard deviation (n=3). The values with different superscript letters represent significant difference (p<0.05) at the end of the reaction period (Encarnacion et al., 2010).

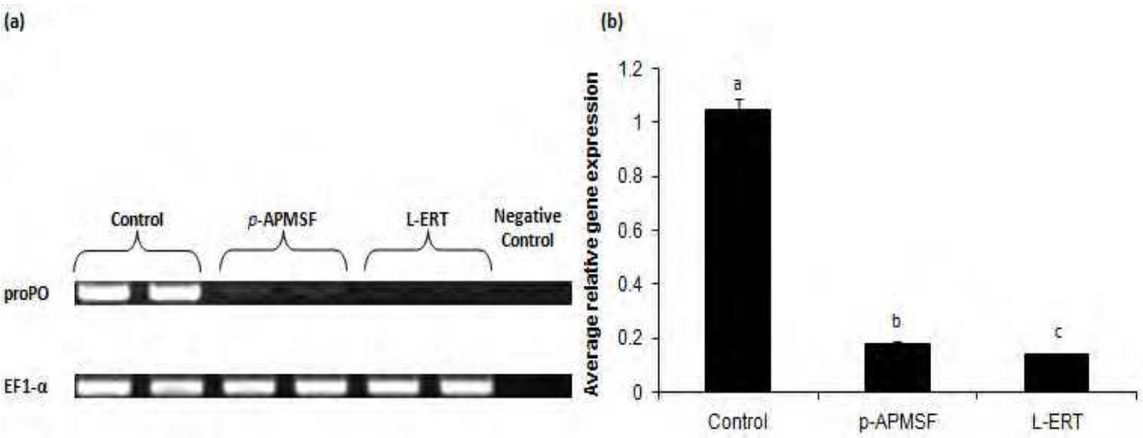


Fig. 15. Inhibition of the activation of the proPO system in hemocyte lysate supernatant by commercial L-ergothioneine (L-ERT) and *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (*p*-APMSF). Polyphenoloxidase activity (a) and peptidase activity (b). 7-amino-4-methylcoumarin (AMC) was used as the standard solution in the peptidase activity assay. The final concentration of each inhibitor in the reaction system was 1 mM. Results are presented in terms of mean ± standard deviation (n = 3). The values with different superscript letters represent significant difference between groups for different storage periods (p < 0.05) (Encarnacion et al., 2010).

the proPO cascade leading to PPO activation and is itself tightly regulated (Buda & Shafer, 2005). The results of the peptidase activity assay indicated that L-ERT inhibited serine protease activity but that this effect was not as strong as that of *p*-APMSF (Fig. 16b). However, the presence of L-ERT could possibly affect the overall activation of the proPO system because it inhibited PPO activity (Fig.16a). At least 4 mechanisms underlying PPAE regulation have been recognized: gene induction, activation by another protease, requirement for noncatalytic serine protease homologues as cofactors, and inactivation by serine protease inhibitors (Wang & Jiang, 2004). Thus, other proPO activating factors such as

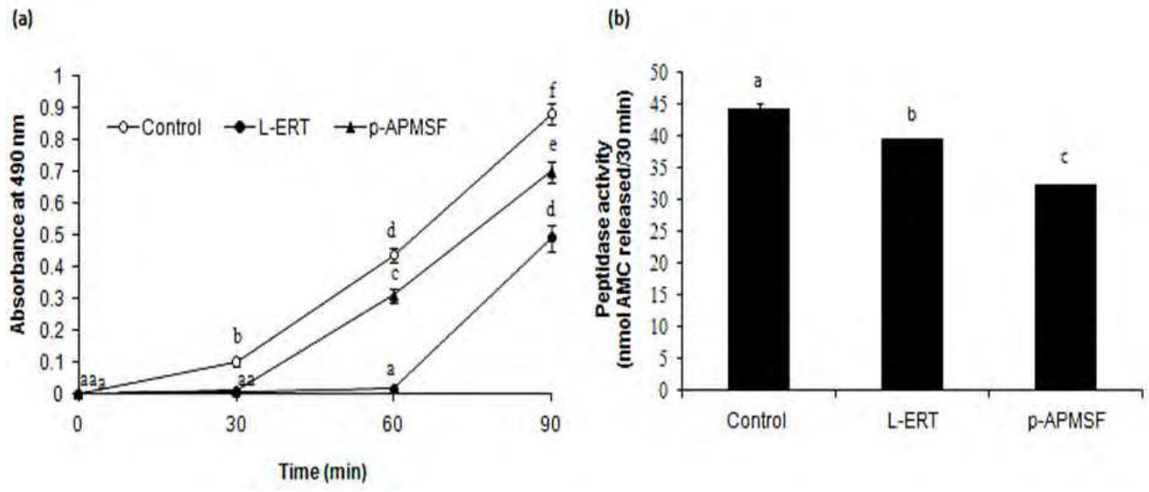


Fig. 16. Gene expression analysis of proPO transcripts in hemocyte lysate supernatant treated with commercial L-ergothioneine (L-ERT) and *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (*p*-APMSF). Representative gel (a) and quantitative analyses of band intensities obtained using the ImageJ software (b). Elongation factor1-α gene (EF1-α) was used as the standard for computing the relative gene expression level for each sample band. The final concentration of the inhibitor in the reaction system was 1mM (Encarnacion et al., 2010).

serine protease homologues and other proteases could have been inhibited by L-ERT. The inhibition of PPO activity in the hemolymphs of the supplement-fed shrimp supports this phenomenon. Moreover, accumulation of ERT in the shrimp muscles by feeding or in the hemolymph by the immersion technique could directly inhibit PPO activity in the carapace during the postharvest period. The thiol (SH) group, present in compounds such as ERT, is a powerful nucleophile, that tends to chelate Zn^{2+} and Cu^{2+} (Park et al., 2006). The latent PPO activity of hemocyanin, a copper-binding protein, in whiteleg shrimp *Penaeus vannamei* has been shown to be involved in postmortem melanosis. The mechanism underlying the inhibitory effect of ERT could also be attributed to its Cu^{2+} chelating activity, leading to the inhibition of melanosis in ME-fed and ME-immersed shrimps and crabs.

5. Summary

Mushroom trimmings are cost-effective and scalable source of ME and contain significant amounts of ERT, which has been proven to have antioxidative and melanosis-inhibiting properties. Yellowtail fish *S. quinqueradiata* that were fed a diet, including ME showed significantly reduced myoglobin and lipid oxidation in dark muscles during chilled storage. The results of the feeding trial are also supported by data from similar trials on other aquacultured fish species such as jack mackerel and rainbow trout (data not shown). Immersion of live *M. japonicus* shrimps and *C. japonicus* crabs in an *F. velutipes* ME solution containing a significant amount of ERT effectively inhibited postharvest melanosis in shrimps and crabs, and this result was comparable to those obtained using melanosis-inhibiting agents such as HR, AA and SS. *In vitro* experiments with exogenous ERT confirmed that it inhibits PPO activity and proPO gene expression in shrimp HLS. Thus, ME containing ERT is a promising natural alternative to the synthetic melanosis-inhibiting

agents used to prevent postharvest melanosis in shrimps and other crustaceans. Furthermore, the use of biowaste as the raw material for extracting and producing antioxidants or of melanosis-inhibiting compounds such as ERT for food and industrial applications could also help reduce the growing problem of agricultural waste.

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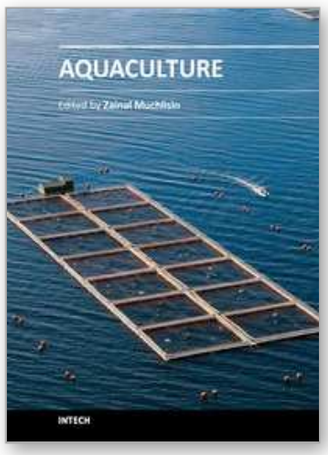

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This book provides an understanding on a large variety of aquaculture related topics. The book is organized in four sections. The first section discusses fish nutrition second section is considers the application of genetic in aquaculture; section three takes a look at current techniques for controlling lipid oxidation and melanosis in Aquaculture products. The last section is focused on culture techniques and management, ,which is the larger part of the book. The book chapters are written by leading experts in their respective areas. Therefore, I am quite confident that this book will be equally useful for students and professionals in aquaculture and biotechnology.

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