10. The biochemistry and control of melanosis in crustaceans though application of bioactive compounds

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Abstract. Crustaceans generally have a limited shelf-life due to the melanosis that affects consumer’s acceptability and, hence, products market value, leading to considerable financial loss. To address such problem, melanosis inhibitors have been used to control the development of black spot in shrimps or crustaceans during handling and storage. Among the melanosis inhibitors, sulphiting agents and 4-hexylresorcinol, ascorbic acid, sodium sulphite and other chemical compounds, have been intensively studied. However, increasing regulatory attention and consumer awareness on the risk associated with chemical additives in food processing have led to the interest in natural additives to prevent melanosis in shrimp. This chapter presents the recent natural additives such as mushroom, tea and lead seed extracts. Inhibition of melanosis in shrimps and other crustaceans has been achieved by using these extracts containing bioactive compounds.

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**Introduction.** The preference of consumers on food products is generally influenced by the quality of food such as its freshness, color, aroma, and safety. Food appearance is normally associated with color and is one of the primary attributes used by consumers to evaluate food quality. Factors such as naturally occurring pigments in foods and pigments due to both enzymatic and non-enzymatic reactions greatly influence the color of foods. Notably, enzymatic browning is one of the most well studied color reactions that affect fruits, vegetables, and seafood products. It is catalyzed by the enzyme polyphenoloxidase (PPO), generally used to refer to tyrosinase (EC 1.14.18.1) and cathecoloxidase (EC 1.10.3.1) (José-Pablo et al 2009).

Many studies on enzymatic browning reactions have been conducted and still remain as one of the most interesting topic in the fields of food science, horticulture, plant and postharvest physiology, microbiology, and most importantly in insect and crustacean physiology due to its diverse impact in these systems (Kim et al 2002).

Enzymatic browning usually reflects detrimental influence but some enzymatic browning are essential to the overall acceptability of foods such as tea and cocoa. It has been reported that black and oolong tea relies on enzymatic browning for color and flavor development likewise the color development of cocoa, which is facilitated by PPO activity during fermentation and drying (Kim et al 2002).

However, enzymatic browning can also bring devastating reactions in fruits, vegetables, and seafoods particularly in crustaceans. It was estimated that over 50% losses in fruit occur due to enzymatic browning reaction (Whitaker & Lee 1995). Severe blackspot formations or melanosis caused by enzymatic reaction in crustaceans can cause tremendous economic losses due to the high value commanded by these aquatic products in the market place (Kim et al 2002). Such losses have prompted considerable interest in understanding and controlling PPO activity in foods.

**Melanosis in crustaceans.** Polyphenoloxidase plays an important physiological role in crustaceans particularly in the process of sclerotization. Melanisation cascade has been reported to be closely associated with the occurrence of factors stimulating cellular defence by aiding phagocytosis and encapsulation reactions (Cerenius et al 2008). Thus, melanisation is an important immune response in crustaceans similar to plants. In plants, the compounds produced as a result of the polymerization of quinones have been reported to exhibit both antibacterial and antifungal activities while in the latter, 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
healing of injuries in crustaceans (Buda & Shafer 2005, Martínez-Álvarez et al 2005, José-Pablo et al 2009). Figure 1 shows the activation of proPO system and cellular immunity.

**Figure 1.** Activation of the crustacean prophenoloxidase (proPO) system and cellular immunity. Microbial polysaccharides and other pathogen-associated molecular patterns (PAMPs) trigger release of proPO system proteins from hemocytes that results in their subsequent activation. A cascade of proteinases (not shown) triggers activation of the proform of prophenoloxidase-activating enzyme (ppA) into active ppA, which cleaves proPO into active PO and results in melanin production. The active ppA directly, or through an intermediate proteinase, activates properoxinectin (proPXN), a myeloperoxidase homologue, into peroxinectin (PXN). Pacifastin negatively regulates both PXN and ppA production. ProPXN lacks the cell adhesion, opsonin and encapsulation-promoting activities of PXN. Both proPXN and PXN, however, possess peroxidase activity. PXN has been shown to associate with an extracellular superoxide dismutase (eSOD) and to an integrin. Because PXN has peroxidase activity and mediates hemocyte-microbe binding (i.e. opsonisation), this protein might produce hypohallic acid in the vicinity of the bound pathogen. An attractive hypothesis is that an active microbe-PXN-dismutase complex is phagocytosed and results in intracellular killing (insert) through the generation of reactive oxygen intermediates (HOCL) by NADPH oxidase. Independently of the proPO-system cascade, plasma hemocyanin (Hcy) 2 may be proteolytically processed by a hemocyte-derived proteinase to a protein expressing PO-activity (Hcy 2-PO)(adapted from Cerenius et al 2008).
Unfortunately, PPO-catalyzed blackening of the shell in crustaceans during post-harvest adversely affects both quality and the consumers’ acceptability 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 necessary mean unfit for human consumption, consumers tend to be selective on these products mainly because browning in the carapace connotes spoilage. Severe melanosis on these expensive and economically valuable products can cause tremendous economic losses due to the high value commanded by these aquatic products in the market place. There have been several cases of aquatic products worth millions of dollars that are reduced markedly or lost completely due to the severity of melanosis (Kim et al 2002).

As such, it is clear that PPO-catalyzed reactions have both beneficial and deteriorative effects. Control of the deteriorative effects of melanosis therefore has been a challenge to the industry, especially for food scientists. The intensive researches on the inhibition of enzymatic browning in fruits and vegetables could provide an understanding of the mechanisms responsible for the development of melanosis in crustaceans, the properties of PPO, their substrates and inhibitors, and the physical, biological and chemical factors which affect each of these parameters. Full understanding of this phenomenon and its mechanisms may provide scientific approach on the prevention of melanosis, slowing its rate, thus extending the shelf life and acceptability of the product.

**Biochemical characteristics of polyphenol oxidase.** Isolations and partial purification of PPO have been done from numerous plant and animal sources but little has been characterized. Structural models for the active site of PPO from *Streptomyces castaneoglobisporus* have been proposed as shown in Figure 2.

Matoba et al (2006) reported that the PPO from *S. castaneoglobisporus*, complexed with a protein, ORF378. The authors attribute the ability of this PPO to act as a monophenolase as due to some of the observed structural differences to other partially characterized PPO from plants and animals.

Polyphenol oxidase exists in immature, mature latent and active isoforms in higher plants and fungi (Kim & Uyama 2005). It has been describe to contain three domains, of which the central domain contains two copper binding sites, and that six histidine residues bind a pair of copper ions its active site which interacts with both molecular oxygen and its phenolic substrate (Jackman et al 1991). Figure 3 shows the domain structures of PPOs from different groups of species. Accordingly, chemical and spectroscopic studies of tyrosinase have demonstrated that the geometric and electronic structures of its binuclear copper active site are very similar to those found in hemocyanin van (Gelder et al 1997).
However, it is clear that PPO from different sources do have distinct characteristics and that not only the amino acid sequences differ, but that there are also some differences at the conserved active site (Mayer 2006). The amino acid sequence of some PPO from fungi, plants, and other organisms has been published (van Gelder et al 1997, Nakamura et al 2000, Wichers et al 2003, Cho et al 2003, Marusek et al 2006, Halaouili et al 2006, Hernandez-Romero et al 2006, Matoba et al 2006). Generally, except for the active site, amino acid sequences show variability among these PPOs.

As such, reports on the molecular weight of PPOs are very diverse and variable assumed to be due to partial proteolysis of the enzyme during isolation and differences in the family of genes coding each type of PPO (Mayer 2006).

**Reaction mechanisms in melanosis formation.** Basically, enzymatic browning or melanosis takes place in the presence of oxygen when PPO and their polyphenolic substrates are mixed. Polyphenol oxidase catalyzes two fundamental reactions: hydroxylation to the $o$-position adjacent to an existing hydroxyl group of the phenolic compounds (monophenolase activity), and oxidation of diphenol to $o$-benzoquinones (diphenolase activity).

The subunits of PPO has been reported to differ with respect to chemical, physical and kinetic properties, which were believed to be responsible for relative affinities of the enzymes for both mono- and diphenolic substrate (Kim et al 2002).
Figure 3. Domain structure of PPOs from different groups of species showing the conserved Cu-binding sites CuA and CuB as well as the other conserved domains. The arrow indicates the known or predicted cleavage site for the generation of the protease resistant fragment from the mature protein (adapted from van Gelder et al 1997).

Figure 4. Catalytic cycles of the hydroxylation of monophenol and oxidation of \( o \)-diphehol to \( o \)-quinone by PPO (adapted from Kim & Uyama 2005).
The aforementioned considerations have led many studies on the molecular mechanism for the monophenolase and diphenolase activity of PPO. There have been numerous studies on the monophenolase activity of PPO based on three forms of the enzymes. Figure 4 shows the monophenolase cycle, where in monophenol reacts with the oxy form and binds with the axial position of one of the coppers of this oxy form. It has been reported that rearrangement through the trigonal bipyramidal intermediate leads to the bound peroxide that generates a coordinated o-diphenol, which is oxidized to o-quinone, resulting in a deoxy from ready for dioxygen binding. In the diphenolase cycle, both the oxy and met form reacts with o-diphenol, oxidizing it to o-quinone. However, monophenol can compete with o-diphenol for binding to the met form site, inhibit its reduction (Kim & Uyama 2005). Diphenolase have received much attention due to their high catalytic rate and their association with the formation of quinones that leads to the production of melanins.

**Control of melanosis.** As discussed above, 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 undergo 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 (Route-Mayer et al 1990).

There have been a lot of studies 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 on the undesirable product of the enzyme activity. This involves elimination of one or more of the important components involve in the enzymatic reaction such as oxygen, copper, substrate and the enzyme itself. There are also known compounds that react with the product of the enzyme activity thus, inhibiting the formation of colored compounds. Still many other techniques are being applied to prevent melanosis in foods that include processing methods, involvement of different types and kinds of inhibitors, and molecular approach to control PPO as presented in the following review.

**Processing.** Traditionally, heating is one of the most popular methods to destroy microorganism and to inactivate enzymes. Generally, the catalytic activity of PPO is inhibited at temperatures ranging from 70-90 °C (Vamos-Vigyozo 1981). Lee et al (1988) reported that blanching of green beans above 82 °C inactivated the activities of the enzymes including PPO.

Similar to heat treatment, low temperature can also control enzyme-catalyzed reactions. At low temperatures, reduction in the kinetic energy of the reactant molecules subsequently results 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 during distribution and retailing are being done to control the development of melanosis in food products. Relative to this, freezing temperatures as low as -18 °C can inactivate enzymes. However, this causes changes on the physical attributes of the products.

Dehydration is also one of the ways to control enzyme catalyzed reactions resulting to browning in foods. Generally, water exerts a vast influence on the activity of enzymes since 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).

Another method of controlling enzymatic browning in foods is irradiation. It uses ionizing radiation to inactivate microorganisms as well as enzymes. It is increasingly recognized as method of reducing postharvest losses, maintaining food quality as well as ensuring 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 by reducing molecular spacing, and increasing interchain reactions. However, this varies on the enzymes, nature of the substrate, the temperature and the duration of high pressure processing (Cheftel 1992).

Other processing methods that have been developed and used in food products to control enzymatic browning include treatment of food with supercritical carbon dioxide, ultrafiltration and ultrasonication (Kim et al 2002). Treatment with supercritical carbon dioxide inactivates the enzyme by the production of carbonic acid causing a significant reduction in pH. Ultrafiltration on the other hand, removes PPO during the process while ultrasonication causes severe shear stresses promoting enzyme denaturation.

**Inhibitors.** Melanosis can be inhibited by targeting the enzyme, the substrate, and/or the reaction products. Inhibition targeting 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 substrate. Inhibition targeting the substrate can be done by removal of either the oxygen or the phenolic substrate from the reaction. Meanwhile, 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 six categories that including: 1) reducing agents such as sulphites, ascorbic acid and 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 alcohol, anions, peptides and substituted resorcinols; 5) enzyme treatments such as oxygenases, o-methyl transferase and proteases; and 6) complexing agents such as cyclodextrins (McEvily et al 1992). To date, there have been a lot of researches on the different compounds used to inhibit melanosis in foods.

**Molecular approach.** Previous reviews in this chapter presented the nature of PPO and its role in the immune system of plants and crustaceans. 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 plant processes alteration and crop improvement. It involves blocking the gene expression of the plant enzymes involved in a certain process. It is based on blocking information flow from DNA via RNA to protein by introduction of an RNA strand complementary to the sequence of the target mRNA. Technically, it involves the insertion of gene or significant part of it into the cell in a reverse orientation. As shown in Figure 5, messenger RNA encoded by this antisense gene undergoes hybridization with that encoded by the endogenous gene, precluding production of the protein product.

![Antisense RNA approach to control gene expression at the transcriptional level](image)

**Figure 5.** Antisense RNA approach to control gene expression at the transcriptional level (adapted from Martinez & Whitaker 1995).
The antisense approach has been applied to increase the shelf life of fruits (Fray & Grierson 1993) and commercial applications of this technology now include alterations of flower color, viral resistance 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. It is then possible to develop fruits, vegetables and crustaceans that are resistant to enzymatic browning with the application of this anti-sense technology.

**Application of ergothioneine-rich edible mushroom (*Flammulina velutipes*) extract to control melanosis.** 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 antimelanotic compounds or inhibitors (Whitaker 1994). Melanosis in crustaceans is normally controlled by means of direct application of various inhibitors such as 4-hexylresorcinol, sulphites, and phosphates (Martínez-Álvarez et al 2005).

However, direct application of synthetic inhibitors to melanosis 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 (Kim et al 2002). Moreover, increased regulatory attention and heightened consumer awareness of the risk associated with synthetic antimelanotic compounds when consumed with the food have created a need for safe and effective alternatives for food application (Gokoglu & Yerlikaya 2008). 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 develop during storage (Jang et al 2002, 2003). 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 (Jang et al 2004, Bao et al 2008). 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 (Bao et al 2008). The extract also stabilized fresh color of the tuna meat during ice storage (Bao et al 2009). 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 (Jang et al 2002, Jang et al 2003).

**Control of melanosis in shrimp by diet supplementation with mushroom fruiting body extract.** In the study of Encarnacion et al (2010), dietary supplementation of the mushroom extract containing ERT effectively inhibited post mortem melanosis in Kuruma shrimp (*Marsupenaeus japonicus*). The mushroom PPO inhibition activities of the commercial ERT, mushroom hot water extract as well as prepared diets used for the feeding experiment are shown in Figure 5. With increasing concentrations of L-ERT, the mushroom PPO activity remarkably declined. The amount of ERT in the mushroom hot water extract used in the feeding trial was 2.05±0.24 mg/mL. Notably, 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 (Ey et al 2007). Thus, results of this study showed that the ERT from the mushroom extract added in the diet was accumulated in the shrimp during feeding.

The proPO activating system is an important non-self recognition system in invertebrates (Amparyup et al 2009). This system has a key role in the primary immune response, cuticle sclerotization and wound healing processes in crustaceans (Amparyup et al 2009, Cerenius & Söderhäll 2004, Söderhäll & Cerenius 1998, Iwanaga & Lee 2005, Charoensapsri et al 2009). It has been reported that two prophenoloxidases in *Penaeus monodon*, PmpRO1 and PmpRO2, expressed in the hemocyte, are important for the survival of *Vibrio harveyi* challenged shrimp *P. monodon* (Amparyup et al 2009). 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 (Encarnacion et al 2010). This was further supported by data in the same study that there was no significant difference on the hemocyte counts 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 (Charoensapsri et al 2009). It has been reported that microbial infections in *Drosophila* did not require the activation of the proPO system for survival (Leclerc et al 2006). 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 (García-Carreño et al 2008). In this study of Encarnacion et al (2010), the PPO activities of hemolymph from shrimp fed the diet containing the mushroom extract was significantly lower than that of the control group. 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 showing that the mushroom extract inhibited PPO activation in the hemolymphs of the supplement fed shrimp.

Because 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 the study of Encarnacion et al (2010) showed that the transcript of proPO genes in the HLS showed lower expression in the L-ERT- and *p*-APMSF-treated HLSs and 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 (Maeda et al 2007). 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 (Buda & Shafer 2005). Based on 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 non-catalytic serine protease homologues as cofactors and inactivation by serine protease inhibitors (Wang & Jiang 2004). 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 (José-Pablo et al 2009). This study showed that the development of melanosis in the carapace area of the shrimp during ice storage was relatively controlled in the mushroom extract-fed group as shown in Figure 8. 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 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$^{2+}$ (Park et al 2006). The direct interaction of L-ERT with the Cu$^{2+}$ ions in pH 7.4 was assessed spectrophotometrically. The effect of increasing CuCl$_2$ concentration on the spectral characteristics of L-ERT is described in the shifts in band 250 to 275 nm and increase in the shoulder between 275 to 300 nm as the result of Cu-L-ERT complex formation. Latent PPO activity of hemocyanin, a copper-binding protein in whiteleg shrimp Penaeus vannamei has 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. The mushroom hot water extract containing ERT can be used as a natural antimelanosic material for food application.

Inhibition of melanosis in crustaceans using mushroom processing waste extract by immersion technique. A concentrated extract of mushroom trimmings from processing waste was also reported to inhibit mushroom PPO activity (Encarnacion et al 2011). Specifically, this showed that immersing live shrimp in ME effectively inhibited postharvest melanosis in M. japonicus shrimp. The development of melanosis in the carapace decreased as the concentration of the ME in the immersing solution increased. These effects may be due to greater absorption and accumulation of ERT in the shrimp as the concentration of ME in the immersing solution increased. At 0.5 % ME concentration in the pure seawater, melanosis was effectively inhibited in shrimp samples after 2 days of ice storage and comparatively the same with the effect of 500 ppm hexyl resorcinol (HR) and better than 500 ppm ascorbic acid (AA).
The efficacy of ME in inhibiting postharvest melanosis as supported by biochemical analysis such as PPO activity and proPO gene expression is comparable to the efficacy of existing antimelanotic compounds, such as HR and AA.

HR forms an inactive complex with PPO that is incapable of catalyzing melanosis formation (Guerrero-Beltran et al 2005). As a result, PPO activity significantly decreased in the presence of HR. In addition to its antimelanotic activity, HR is also an antimicrobial agent. 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). Since AA did not have a significant effect on proPO gene expression, its mechanism of inhibiting postharvest melanosis is most likely due to its reducing power. The slight melanosis that occurred in AA-immersed shrimp after 2 days of ice storage may be due to oxidation of AA that prevented it from reducing o-quinones, thereby allowing the formation of melanin (Guerrero-Beltran et al 2005).

Previously, it was determined that feeding ERT to shrimp prevented postharvest melanosis. This study improves the previous work by showing that immersing shrimp in ME containing ERT is a simpler and less time-consuming technique to prevent postharvest melanosis. ME was extracted from an edible mushroom, a common part of Japanese cuisine, and rinsing the shrimp with pure seawater after immersion renders the samples safe and free from any awful taste and odor. The efficacy of this immersion technique is probably due to absorption of ERT through the gills and its subsequent accumulation in the hemolymph of shrimp.

The mushroom extract was also applied to prevent melanosis in red queen crab, Chionoecetes japonicus. In Japan, the red queen crab is a commercially important crustacean and is considered an expensive delicacy (Nagata et al 2000, Yosho 2000). Economically important crustaceans such as red queen crab command lower prices when blackening begins to manifest in the carapace. Thus, melanosis has a tremendous impact on the market value of this crab. Thus, in the study conducted by Encarnacion et al (2012a), the effects of in vivo treatment with F. velutipes ME, sodium sulphite (SS), and 4-hexyl-1,3-benzenediol or 4-hexyl-resorcinol (HR) on postharvest melanosis formation in the crab were compared. Development of melanosis in crab samples during ice storage showed increased changes in gray values on images of the carapace. After 4 days of storage on ice, blackening in the carapace of the control group was more pronounced than in samples immersed in 1.0% ME solution. Visual inspection showed that immersion of crabs in 1.0% ME solution effectively prevented the
development of melanosis. In addition, graphical analysis illustrated that gray values increased sharply on day 4 in the control samples whereas those of the live crabs treated in 1.0% ME remained unchanged. In addition, the effect of treatment in 1.0% ME solution was similar to that of treatment in each of the 500 ppm SS and 500 ppm HR solutions. These results show that treatment in 1.0% ME solution significantly inhibits the development of postharvest melanosis in this species of crab.

More applications of the mushroom extract to other crustaceans such as black tiger (*Penaeus monodon*) and Pacific white (*Litopenaeus vannamei*) shrimps were done using immersion technique describe in the previous chapters. At 0.5 % ME concentration in the freshwater, melanosis was effectively inhibited in shrimp samples after 3 days of ice storage and comparatively the same with the effect of 500 ppm SS and better than 500 ppm HR. Assimilation of ERT in shrimp tissues may also inhibited PPO activity in the carapace during postharvest. In addition to having an inhibitory effect on PPO activity, ME also suppressed proPO gene expression based on the study conducted by Encarnacion et al (2012b).

The biochemical interventions of ergothioneine-rich mushroom extract from *F. velutipes* inhibited the PPO activity thus, melanosis was effectively controlled in treated shrimp and crab samples comparable with the commercial antimelanosic agents. Thus, the hotwater mushroom extract can be a natural alternative to currently used synthetic antimelanosic compounds in the food industry.

**Application of tea extract to control melanosis in shrimp.** Both green tea and mulberry tea have been reported to possess antioxidant, antimicrobial, ant mutagenic, anticarcinogenic and anti-inflammatory properties (Cabrera et al 2006). The study of Nirmal & Benjakul (2011) investigated the inhibition effect of green tea and mulberry tea extract with and without prior chlorophyll removal on PPO from the cephalothorax of Pacific white shrimp (*L. vannamei*) and the impact of ethanolic green tea extract with prior chlorophyll removal on quality changes of Pacific white shrimp during iced storage.

It was noted that extracts from green tea with and without prior chlorophyll removal showed the higher polyphenoloxidase (PPO) inhibitory activity, compared with mulberry tea extract, at the concentration used (0.1, 0.5 or 1 g L\(^{-1}\)). Additionally, green tea extracts had the higher copper chelating activity, compared with mulberry tea extract. Ethanolic green tea extract with prior chlorophyll removal contained (\(\beta\))-catechin (C), (\(-\))-epicatechin (EC), (\(-\))-epigallocatechin (EGC), (\(-\))-epigallocatechin gallate (EGCg) and (\(-\))-epicatechin gallate (ECG) at the levels of 242, 33.4, 125.6, 140.6 and 25.2 g kg\(^{-1}\) dry extract, respectively. Shrimp treated with 5 g L\(^{-1}\)
ethanolic green tea extract with prior chlorophyll removal possessed lower melanosis, compared with the control, and showed similar score to those treated with sodium metabisulphite. Furthermore, based on their results, ethanolic green tea extract with prior chlorophyll removal had no adverse impact on sensory attributes of treated shrimp.

Inhibition of melanosis formation in shrimp by lead seed extract. The lead tree, *Leucaena leucocephala*, belongs to a tropical and subtropical legume family. It is used as livestock feed because of the tree’s high content of protein, carotenoids, vitamin K, xanthophylls and minerals (Kamada et al 1997). Additionally, seeds and leaves of lead are consumed as human foods (Sahlu et al 1995). In Mexico, the seeds of guaje (*Leucaena esculenta*) are eaten with salt. Green lead seeds are also consumed as a fresh side dish in Thailand, but the mature brown seeds have not yet been exploited. Phenolics, secondary plant compounds found in different parts of *Leucaena* consisted of condensed tannin (Echeverria et al 2002), quercetin and myricetin glycosides (Lowery et al 1984), galallocatechin, epigallocatechin and epicatechin (Erickson et al 2000).

*Leucaena* is known to contain a non-protein amino acid called mimosine (β-(3-hydroxy-4-pyridon-1-yl)-L-alanine) (Lalitha & Kulothungan 2006). The mimosine is chemically similar to dihydroxyphenylalanine with a 3-hydroxy-4-pyridone ring instead of a 3,4-dihydroxyphenyl ring (Soedarjo et al 1994). Mimosine is removed very rapidly from the body via urine (Sahlu et al 1995). Lead (*Leucaena leucocephala*) seed extract was prepared using distilled water as a medium. Total phenolic and mimosine contents in the lead seed extract powder (LSEP) were 17.4 g GAE/100 g and 8.8 g/100 g, respectively. LSEP at different concentrations (0.05%, 0.1%, 0.25%, 0.5%, and 1%, w/v) showed inhibitory activity towards PPO of Pacific white shrimp in a dose dependent manner. When the whole Pacific white shrimp were treated with 0.25% and 0.5% (w/v) LSEP, the shrimp treated with 0.5% LSEP had the lower melanosis score throughout the storage of 12 days and showed a higher score for colour and odour, as well as overall likeness, compared with the control (without treatment) and 1.25% sodium metabisulphite treated samples at day 12. Sodium metabisulphite might inhibit melanosis by reacting with intermediate quinone, forming sulphoquinone, or it can act as a competitive inhibitor (Ferrer et al 1989). Meat of shrimps treated with LSEP at both levels had the increase in mimosine content up to 8 days, suggesting the migration of mimosine into shrimp muscle during extended storage. The presence of mimosine and phenolic compounds in LSEP might retard the melanosis formation in white shrimp by a combined mechanism such as PPO inhibitor, chelation of copper at the active site of PPO, reduction of quinone to hydroquinone, etc.
Mimosine is an analogue of tyrosine (Echeverria et al. 2002). Therefore, 0.5% LSEP can be used as a novel melanosis inhibitor for Pacific white shrimp.

**Conclusion.** Polyphenoloxidase plays an important physiological role in crustaceans particularly in the process of sclerotization. In plants, the compounds produced as a result of the polymerization of quinones has been reported to exhibit both antibacterial and antifungal activities while in the latter, PPO is thought to be involved in wound healing and sclerotization of the cuticle. Unfortunately, PPO-catalyzed blackening of the shell in crustaceans during post-harvest adversely affects both quality and the consumers’ acceptability of these products.

Crustaceans such as lobsters, shrimps and crabs are extremely vulnerable to enzymatic blackening or melanosis. Severe melanosis on these expensive and economically valuable products can cause tremendous economic losses due to the high value commanded by these aquatic products in the marketplace.

Basically, enzymatic browning or melanosis takes place in the presence of oxygen when tyrosinase and their polyphenolic substrates are mixed. Tyrosinase catalyzes two fundamental reactions: hydroxylatation to the \( o \)-position adjacent to an existing hydroxyl group of the phenolic compounds (monophenolase activity), and oxidation of diphenol to \( o \)-benzoquinones (diphenolase activity). Diphenolase have received much attention due to their high catalytic rate and their association with the formation of quinones that leads to the production of melanins.

As such, control of the deteriorative effects of melanosis therefore has been a challenge to the industry, especially for food scientists. Many studies 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.

Melanosis in crustaceans is normally controlled by means of direct application of various inhibitors such as 4-hexylresorcinol, sulphites, ascorbic acid, and phosphates. 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. Moreover, increased regulatory attention and heightened consumer awareness of the risk associated with synthetic antimelanosic compounds when consumed with the food have created a need for safe and effective alternatives for food application. Thus, there have been many studies conducted on the utilization of extracts from natural foods including numerous species of mushrooms as PPO inhibitors.
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 develop during storage as reported in previous studies. As reported in previous researches, the mushroom extract contained 2-thiol-L-histidine-betaine (ergothioneine, ERT) at a level of 3.03 ± 0.07 mg/mL that could be one of the major compounds in the mushroom extract that acted as an antimelanosis agent.

This chapter presented the application of a hydrophilic extract prepared from the fruiting body of *F. velutipes* to control melanosis in various commercially important crustaceans *in vivo*. Moreover, the inhibitory effects of L-ERT on the hemolymph PPO activity and activation of proPO cascade in the hemocyte of commercially important crustaceans have been established *in vitro*. Diet supplementation of *F. velutipes* extract in Kuruma shrimp (*M. japonicus*) for seven days successfully inhibited melanosis in post mortem shrimp. *In vitro* experiments also confirmed that ERT inhibited the activation of proPO cascade in Kuruma shrimp hemocyte. The study suggested that ERT in mushrooms when accumulated in tissues through feeding can be a potential antimelanogenic and antioxidative compound to control melanosis in aquacultured shrimp during post harvest.

However, since feeding shrimp with a mushroom extract as previously described is time-consuming and requires tedious technical work, the possibility of immersing live, full-grown Kurma shrimp (*M. japonicas*), red queen crab (*C. japonicus*), black tiger (*P. monodon*) and Pacific white (*L. vannamei*) in a solution of mushroom extract to inhibit postharvest melanosis was considered. Also, mushroom trimmings were used as a cost-effective and scalable source for making mushroom extract. Immersion of live shrimp in a concentrated aqueous extract of *F. velutipes* mushroom that contains a high concentration of ERT effectively inhibited postharvest melanosis in *M. japonicus* shrimp. The efficacy of immersion in mushroom extract in inhibiting melanosis was similar to that of hexyl resorcinol, sulphite, and ascorbic acid.

Thiol containing compounds like ERT, are known to be powerful nucleophiles that can chelate Zn$^{2+}$ and Cu$^{2+}$. Interestingly, PPOs and hemocyanin-derived PPOs from most of the crustaceans have been characterized as a copper-containing metalloenzyme or tyrosinase-type copper-containing PPO involved in the melanization. The inhibitory mechanism of ERT could be attributed to its Cu$^{2+}$-chelating activity in the active site of the PPO, consequently preventing melanosis formation. It has been demonstrated in the previous chapter the inherent characteristic of L-ERT to form a chelate with the copper ion. It inhibited the PPO by
producing an interaction between the L-ERT and copper ion and the catalytic domain of the PPO. The inhibitory mechanism of ERT in PPO activation may also be either at the transcriptional level, by inhibiting a transcription factor, or at the protein level, by affecting the activity or regulation of proenzyme proteases as manifested with the decease on the proPO gene expression transcript.

It was also reported in this chapter that green tea extract has PPO inhibitory activity. Ethanol was the better extracting medium than water, in which the higher recovery of phenolic compound with PPO inhibitory activity was obtained. When ethanolic green tea extract with prior chlorophyll removal was used to soak Pacific white shrimp (I. vannamei) for melanosis was retarded. It was also noted that ethanolic green tea extract with prior chlorophyll removal did not affect sensory attribute of treated shrimps. The efficacy of the extract was generally better than sodium metabisulphite thus, be used as an alternative melanosis inhibitor or preservative in post-mortem shrimp.

Meanwhile, lead brown seed extract could be used as a natural inhibitor of Pacific white shrimp PPO and could retard the formation of melanosis during iced storage. The efficacy of LSEP in inhibiting PPO and melanosis formation was in a dose dependent manner. Shrimps treated with LSEP had superior sensory properties, compared to the control, after 12 days of iced storage thus, the extract from lead brown seed could serve also as an alternative processing aid for controlling melanosis in shrimp or other crustaceans during post mortem handling or storage.

Generally, the biochemical interventions of natural extracts containing bioactive compounds inhibited the PPO activity thus, melanosis was effectively controlled in treated shrimp and crab samples comparable with the commercial antimelanosic agents. Thus, these natural extracts can be alternatives to currently used synthetic antimelanosic compounds in the food industry.

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References

5. Amparyup P., Charoensapsri W., Tassanakajon A, 2009 Two prophenoloxidases are important for the survival of Vibrio harveyi challenged shrimp Penaeus monodon. Dev Comp Immunol 33: 247-256.
30. Guererro-Beltran J., Swanson B., Barbosa-Cánovas G, 2005 Inhibition of polyphenoloxidase in mango puree with 4-hexylresorcinol, cysteine and ascorbic acid. LWT-Food Sci Tech 38: 625-630.