Inhibition of Polyphenoloxidase and Melanosis in Pacific White Shrimp (*Litopenaeus vannamei*) by Phenolic Compounds

Nilesh Prakash Nirmal

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Food Science and Technology

Prince of Songkla University

2011

Thesis Title Inhibition of Polyphenoloxidase and Melanosis in Pacific White Shrimp (*Litopenaeus vannamei*) by Phenolic Compounds

Author Mr.Nilesh Prakash Nirmal

Major Program Food Science and Technology

Major Advisor:

.....

(Prof. Dr.Soottawat Benjakul)

Examining Committee:

.....Chairperson

(Assoc. Prof. Dr.Aran H-Kittikun)

.....Committee

(Prof. Dr.Soottawat Benjakul)

.....Committee

(Assoc. Prof. Dr.Prapaporn Utarabhand)

.....Committee

(Assoc. Prof. Dr.Jirawat Yongsawatdigul)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Doctor of Philosophy Degree in Food Science and Technology.

.....

(Prof. Dr.Amornrat Phongdara)

Dean of Graduate School

Thesis Title	Inhibition of Polyphenoloxidase and Melanosis in Pacific White Shrimp (*Litopenaeus vannamei*) by Phenolic Compounds
Author	Mr.Nilesh Prakash Nirmal
Major Program	Food Science and Technology
Academic Year	2010

ABSTRACT

Effects of ferulic acid and catechin on polyphenoloxidase (PPO) from Pacific white shrimp *(Litopenaeus vannamei)* were evaluated. Both compounds showed PPO inhibitory activity in a dose dependent manner ($P < 0.05$). When whole shrimp were treated with ferulic acid solution (1 or 2%) or catechin solution (0.05 or 0.1 %) and stored in ice for 10 days, the increase in psychrotrophic bacteria count (PBC), pH, total volatile base (TVB) content and thiobarbituric acid reactive substances (TBARS) were retarded, in comparison with the control and those treated with 1.25% sodium metabisulfite (SMS) ($P < 0.05$). After 10 days of storage, shrimp treated with 2% ferulic acid or 0.1% catechin had the lower melanosis score and higher likeness, compared with the control and SMS treated shrimp ($P < 0.05$).

Shrimp subjected to freeze-thawing with different thawing methods and various cycles showed the increase in melanosis during subsequent refrigerated storage (4^{o}C) up to 4 days. Melanosis score was lower in shrimp thawed at 4^{o}C, compared with that found in samples thawed at room temperature or using tap water ($P < 0.05$). Shrimp treated with catechin (0.05, 0.1 and 0.2% (w/v)) or ferulic acid (1, 2 and 3% (w/v)) and subjected to freeze-thawing with various cycles had lower melanosis and quality changes during the subsequent refrigerated storage of 4 days, compared with the control ($P < 0.05$). Thus, either catechin or ferulic acid could be used as the potential additive to lower melanosis of shrimp with prior freeze-thawing.

Green tea extract (GTE) was used as a natural source of catechin with PPO inhibitory activity. GTE with and without prior chlorophyll removal showed the higher PPO inhibitory activity, compared with mulberry tea extract. Additionally, GTE had the higher antioxidant activity, compared to mulberry tea extract ($P < 0.05$). Whole shrimp treated with GTE with prior chlorophyll removal at concentrations of

0.5 and 1 % (w/v) and stored in ice for 12 days lowered PBC, lipid oxidation, and melanosis formation, compared with the control and shrimp treated with 1.25 % SMS ($P < 0.05$). Furthermore, GTE with prior chlorophyll removal had no adverse impact on sensory attributes of treated shrimp. When GTE (0.1%) was used in combination with ascorbic acid (AA; 0.005 or 0.1%), the greater PPO inhibitory activity was achieved as compared to GTE alone ($P < 0.05$). Shrimp treated with 0.1 % GTE in combination with AA (0.005 or 0.01%) (GTE + AA) and stored in iced had the lowered quality changes, in comparison with the control and those treated with 1.25 % SMS ($P < 0.05$). Shrimp without treatment stored under modified atmosphere packaging (MAP) had lowered microbial and chemical changes, in comparison with shrimp stored in air (control) ($P < 0.05$). When shrimp were treated with GTE in combination with AA and stored under MAP, higher inhibition on melanosis formation and microbial growth was obtained, compared with other treatments ($P < 0.05$). Therefore, shrimp treated with GTE in combination with AA prior to MAP had the lowest losses in quality during refrigerated storage.

Lead (*Leucaena leucocephala*) brown seed extract was studied for PPO inhibitory activity. Lead seed extract powder (LSEP) was prepared using distilled water as a medium. LSEP (0.05, 0.1, 0.25, 0.5, and 1 %, w/v) showed PPO inhibitory activity in a dose dependent manner. When the whole shrimp were treated with 0.25 and 0.5 % (w/v) LSEP, the shrimp treated with 0.5 % LSEP had the lower melanosis score and showed a higher likeness, compared with the control and 1.25 % SMS treated samples at day 12 of iced storage ($P < 0.05$). Thus, LSEP can be used as a novel melanosis inhibitor for Pacific white shrimp.

Biochemical properties of PPO from cephalothorax of Pacific white shrimp were investigated. PPO showed the maximal activity using L-β-(3, 4 dihydroxylphenyl) alanine (L-DOPA) as a substrate at pH 6 and 55^{o}C. PPO was stable over a pH range of 5-10 but was unstable at a temperature greater than 60^{o}C. Based on the activity staining with L-DOPA, the apparent molecular weight of PPO was 210 kDa. The Michaelis constant (K_m) of PPO for the oxidation of L-DOPA was 2.43 mM. Trypsin, copper acetate, and sodium dodecyl sulfate (SDS) were unable to activate PPO, suggesting that the enzyme was in the active form.

Inhibition kinetics and mode of catechin, ferulic acid and mimosine towards PPO from cephalothorax of Pacific white shrimp were investigated. Catechin and mimosine showed mixed type reversible inhibition with K_i value of 1.4 and 3.7 mM, respectively. Inhibition kinetic study of ferulic acid exhibited non-competitive reversible inhibition on PPO with K_i value of 37 mM. With increasing concentrations, catechin or ferulic acid or mimosine had higher copper (Cu^{2+}) reduction and copper chelating capacity ($P < 0.05$). Addition of catechin or ferulic acid or mimosine, into browning reaction could prevent dopachrome formation by inactivation of PPO or by binding with browning product.

Therefore, phenolic compounds including catechin, ferulic acid and mimosine could be a safe natural substitute for the synthetic chemical used in shrimp processing industry to lower melanosis and quality losses of shrimp during extended iced or refrigerated storage.

CONTENTS

CONTENTS (Continued)

Chapter **Page**

CONTENTS (Continued)

Chapter **Page**

CONTENTS (Continued)

Chapter **Page**

CONTENTS (Continued)

Chapter **Page**

CONTENTS (Continued)

Chapter **Page**

CONTENTS (Continued)

Chapter **Page**

CONTENTS (Continued)

Chapter **Page**

CONTENTS (Continued)

Chapter **Page**

CONTENTS (Continued)

Chapter **Page**

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Pacific white shrimp (*Litopenaeus vannamei*) accounts for 90% of the global aquaculture shrimp production. Thailand is the world's leading shrimp-farming country and has become the top supplier of farmed shrimp to the United States and Japan (Wyban, 2007). Pacific white shrimp (*Litopenaeus vannamei*) is an important commercial species primarily cultured in Thailand and have become the essential income generator of the country. Despite their delicacy, shrimp are highly perishable with the limited shelf-life, mainly associated with melanosis (discoloration) and microbial spoilage (Gokoglu and Yerlikaya, 2008). Free amino acids and other soluble non-nitrogenous substances in shrimp serve as digestible nutrient for microbial growth (Zeng *et al.*, 2005). Spoilage microorganisms contribute to the loss of essential fatty acids and proteins, production of biogenic amines and formation of off-odors (Mastromatteo *et al.*, 2010). Melanosis is triggered by a biochemical mechanism which oxidizes phenols to quinones by polyphenol oxidase (PPO) (Kim *et al.*, 2000). This is followed by non-enzymatic polymerization and autooxidation of the quinones, giving rise to dark pigments of high molecular weight (Benjakul *et al.*, 2005). Although melanosis (black spots) seems to be harmless to consumers, it drastically reduces the product's market value, consumer's acceptability and hence causing the considerable financial loss (Montero *et al.*, 2001a). PPO is synthesized as a zymogen (proPPO) in crustaceans and can be activated by protease cascade triggered by bacterial cell wall components including lipopolysaccharides, peptidoglycans and 1, 3-β-glucans (Encarnacion *et al*., 2010). Melanosis and quality changes of shrimp were retarded during frozen storage, but continued in defrosted shrimp (Lopez-Caballero *et al.*, 2007). Apart from melanosis, lipid oxidation is another deteriorative reaction causing the unacceptability of shrimp and shrimp products. Fish lipids are susceptible to oxidation owing to the high levels of polyunsaturated fatty acid; this can be initiated by autoxidation, enzymatic reaction

induced by lipoxygenase, peroxidase and microbial enzymes. Lipid oxidation is associated with physicochemical changes, rancidity and off-flavor in fish meat (Bak *et al.*, 1999).

To maintain the quality and to avoid melanosis of shrimp or other crustaceans, melanosis inhibitiors such as sulfite and 4-hexylresorcinol have been widely used (Martinez-Alverez *et al.*, 2008a; Montero *et al.*, 2001b). However, the increases in regulatory attention and consumer awareness of the risk associated with chemical additives in food product have created a need for a safe and effective additive (McEvily *et al.*, 1991). Therefore, safe compounds from natural origin such as ascorbic acid, kojic acid (Chen *et al*, 1991a); ficin (Taoukis *et al*., 1990), enokitake extract (Jang *et al.*, 2003); dodecyl gallate (Kubo *et al.*, 2003) and oxalic acid (Son *et al.*, 2000) have been used as the substitutes of sulfiting agents. Recently, it has been reported that the grape seed extract could inhibit the melanosis in shrimp (*Parapenaeus longirostris*) (Gokoglu and Yerlikaya, 2008). Encarnacion et al. (2010) found that the dietary supplement of mushroom extract (*Flammulina velutipes*) in kuruma shrimp (*Marsupenaeus japonicus*) could reduce post mortem development of melanosis.

In addition to melanosis inhibition, plant phenolic compounds may act as antimicrobial and antioxidant, which could retard the microbial and chemical spoilage of shrimp. Nevertheless, a little information regarding the use of single phenolic compound or plant extracts on melanosis prevention as well as shelf-life extension of white shrimp during post-mortem storage has been reported. The information gained can provide the important and potential approach for shrimp processor to maintain the prime quality of white shrimp during handling and storage, thereby lowering the loss in market value.

1.2 Review of Literature

1.2.1 Polyphenoloxidase (PPO)

Polyphenoloxidase (PPO) or tyrosinase (monophenol, L-dopa: oxygen oxidoreductase, EC 1.14.18.1) is a bi-functional, copper containing enzyme widely distributed throughout the phylogenetic scale (Garcia-Molina *et al*., 2007). It catalyzes the hydroxylation of monophenol to *o*-diphenols and their subsequent oxidation to *o*-quinones by molecular oxygen (Likhitwitayawuid, 2008; Garcia-Molina *et al*., 2007; Decker and Tuczek, 2000). In all cases, PPO is associated with dark pigmentation in the organism and has a protective function (Martinez and Whitaker, 1995). Different PPO obtained from several biological sources has similar structural and fuctional characteristics (Garcia-Molina *et al*., 2005). PPO plays an essential role in physiological functions, particularly for the sclerotization of the cuticle of crustaceans such as shrimp and lobster. Sclerotization is the hardening of the chitin shell after molting, which is part of the growing phase for the organism (Terwilliger, 1999). The highly reactive *o*-quinones cross-link with histidyl residues of cuticular proteins and chitin, resulting in hardening of the exoskeleton (Xu *et al.,* 1997). A second function of polyphenoloxidase is immunity and self-recognition. The *o*-quinones are involved in the melanin synthesis, a compound with antimicrobial, antiviral properties and thus PPO is important components of the innate immune response in crustaceans (Figure 1) (Terwilliger and Ryan, 2006).

1.2.1.1 Distribution (localization) of PPO in crustaceans:

PPO is mainly present in the cuticle, specifically on the internal surface, inside chromatophore and hemolymph of crustacean's blood as zymogen form (proPPO) (Gimenez *et al*., 2010). This enzyme has also been found in hepatopancrease, where it is synthesized (Yang *et al.*, 1993). The activation of proPPO to PPO requires proteases and microbial activators such as polysaccharide binding proteins (Figure 1) (Encarnacion *et al*., 2010). Pro-PPO activating enzyme is serine protease that exhibits trypsin like activity (Williams *et al*., 2003). PPO is localized in the carapace of the cephalothorax, in the caudal zone and in the cuticle of

the abdomen, mainly where the cuticle segments are joined and where the cuticle is connected to the pleopods (Benjakul *et al*., 2005). PPO from different species, body location, sex, molting stage and tissue might have different activity levels as well as varying properties (Ferrer *et al*., 1989).

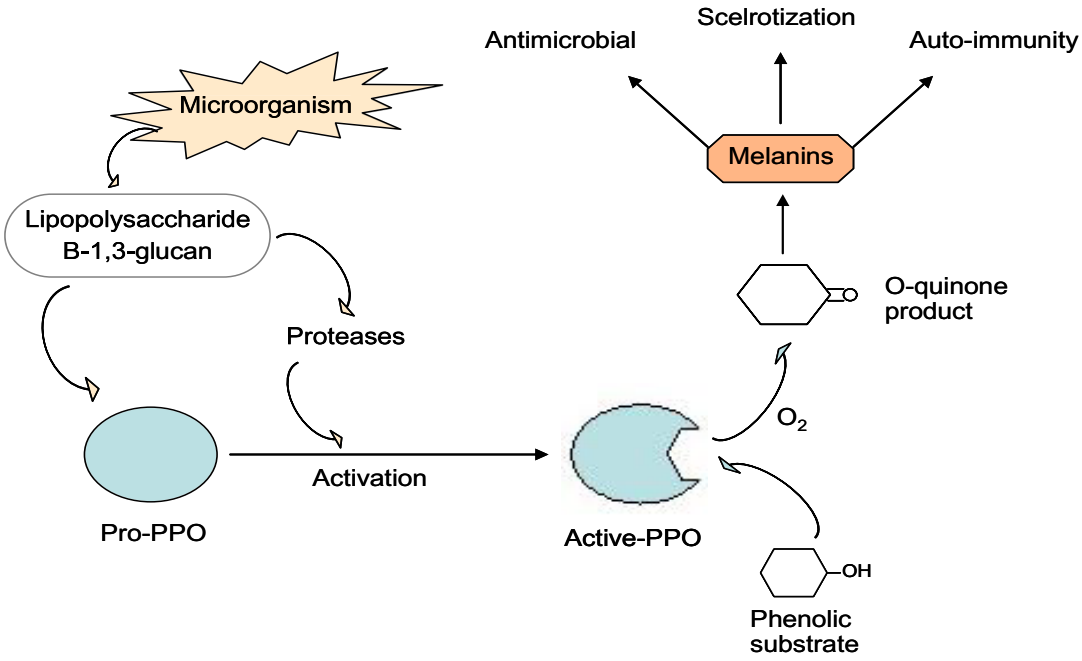

Figure 1. Schematic diagram of the activation of PPO by microorganism.
Source: Modified from Kim *et al*. (2000)

PPO is distributed in many parts of shrimp with different levels of activity (Montero *et al*., 2001a; Zamorano *et al*., 2009). PPO also localizes in the surface membrane covering the muscle and in the hemolymph (Ogawa *et al.*, 1984). Norway lobster (*Nephrops norvegicus*) develops melanosis mainly on cephalothorax due to the high concentration of PPO (Martinez-Alvarez *et al.*, 2008b). When the activity of partly purified PPO from different body portions of deep water pink shrimp was determined, the greatest PPO activity was found in the carapace (Figure 2), followed by the abdomen exoskeleton, cephalothorax and pleopods and telson (Zamorano *et al.*, 2009). The muscle and the pereopods and maxillipeds showed no detectable PPO activity.

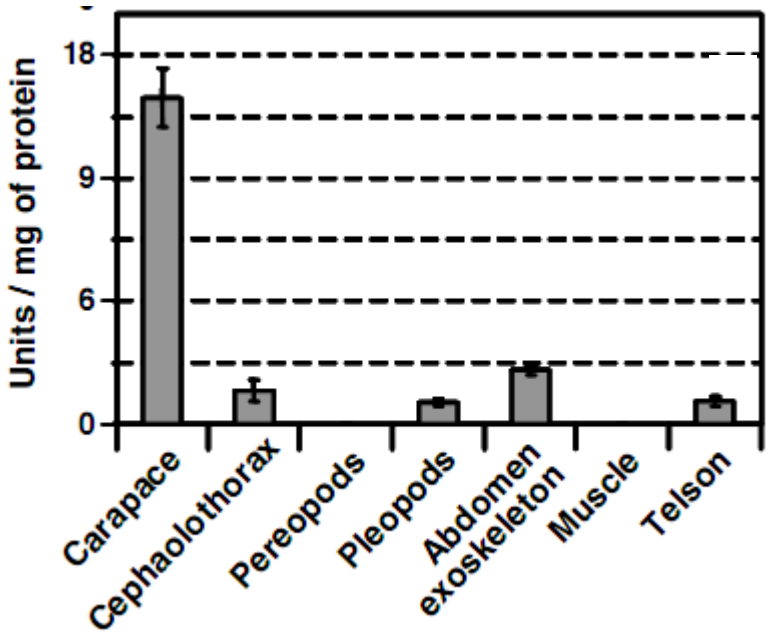

Figure 2. PPO activity of partially purified extract using 40–70% ammonium sulfate from different organs of deepwater pink shrimp.

Source: Modified from Zamorano *et al*. (2009)

Hemocyanain (Hc) which is closely resemble with the PPO in their sequence and in their active site can also acquire PPO activity through proteolytic cleavage in its terminal amino end by serine proteases (Martinez-Alvarez *et al.*, 2008a). Hc is found in hemolymph as well as in cuticle (Adachi *et al.*, 2005).

1.2.1.2 Molecular structure

The active site of tyrosinase consists of two copper atoms and three states, 'met', 'deoxy', and 'oxy' as shown in Figure 3 (Himmelwright *et al*., 1980). Oxy-tyrosinase has two tetragonal Cu (II) atoms. Each Cu atom is coordinated by three N-His ligands, consisting of a weak axial and two strong equatorial bonding. The exogenous oxygen molecule (O_2) is bound to this site as peroxide and bridges the two Cu ions. Met-tyrosinase has a tetragonal bi-cupric structure with endogenous oxygen bridge (Solomon *et al.,* 1996). De-oxytyrosinase has a bi-cuprous structure Cu (I)–Cu (I). No oxygen bridge is present in this structure. Two-electron reduction to the de-oxy site followed by binding of molecular oxygen regenerates oxy-tyrosinase (Sanchez-Ferrer *et al.*, 1995).

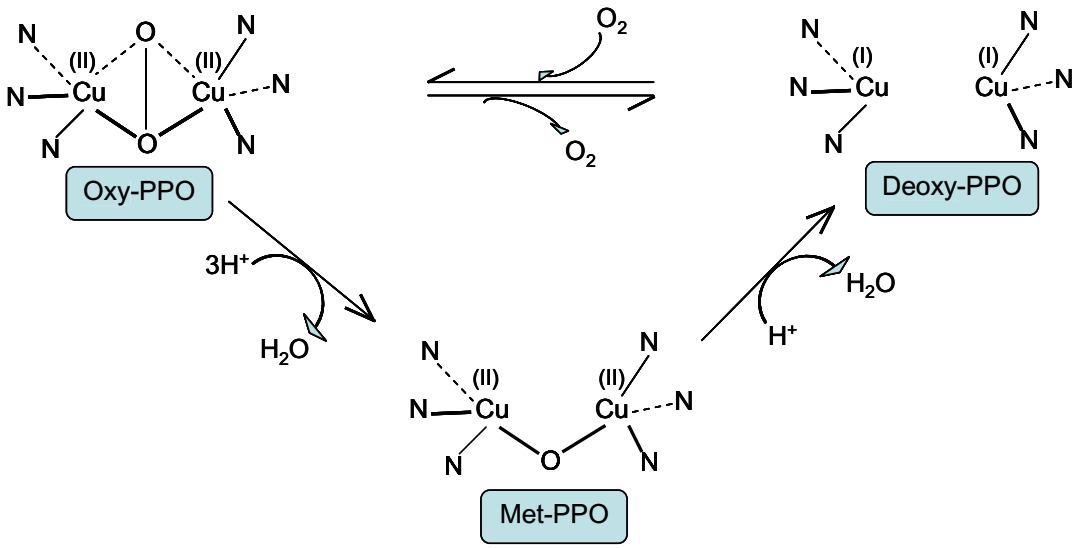

Figure 3. Three states of tyrosinase.
Source: Modified from Himmelwright *et al*. (1980)

Decker and Tuczek (2000) reported that type-3 copper proteins from *Limulus polyphemus* hemocyanin containing a binuclear copper active site coordinated by six histidines residues contributed from the four helices of the α-bundle. Both CuA and CuB are coordinated by N_ε of histidine residues, which are His173, His177, His204 to CuA, and His324, His328, His364 to CuB (Figure 4). The phenyl ring of the substrate is almost perpendicular to Cu-O_2-Cu plane, with close contacts of its hydroxyl group to CuA and of its *ortho*-position to one of the two oxygen atoms of the dioxygen ligand.

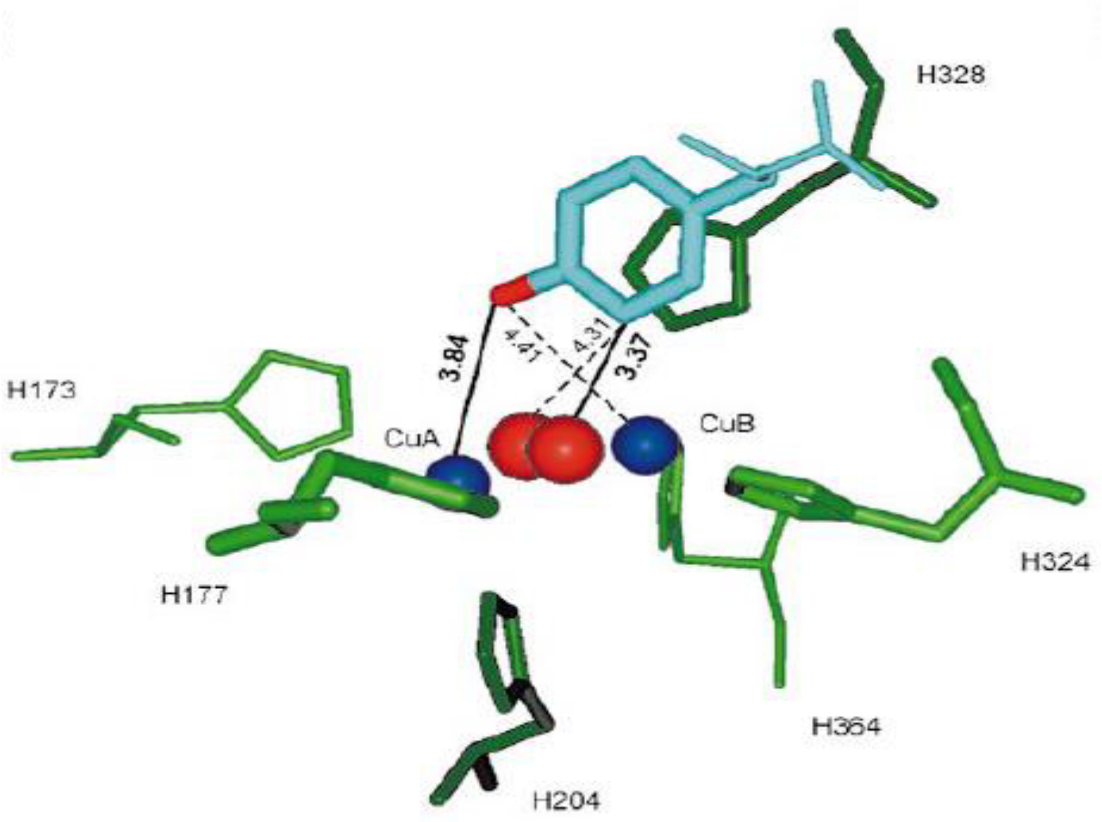

Figure 4. X-ray crystallography structure of *Limulus polyphemus* hemocyanin oxy form. Structure shows the two copper atoms (dark blue), the dioxygen (red), histidines (green) and the tyrosine substrate (cyan). Distances are given in A^{o}.

Source: Decker and Tuczek (2000)

1.2.1.3 Enzyme mechanisms

PPO catalyzes two basic reactions, in the presence of molecular oxygen, including *o*-hydroxylation of monophenols to give *o*-diphenols (Monophenol oxidase, cresolase activity) and the further oxidation of *o*-diphenols to *o*-quinones (Diphenoloxidase, catecholase activity) (Garcia-Molina *et al.*, 2005).

1.2.1.3.1 Monophenol oxidase

It catalyzes the hydroxylation of monophenols to diphenols. As shown in Figure 5, the monophenolase activity begins with the binding of the substrate

monophenol to one of the Cu atoms of the oxygenated form (oxy-tyrosinase) to generate Oxy-tyrosine (Rodriguez-Lopez *et al*., 1992). Then, *o*-hydroxylation of the monophenol by the bound peroxide occurs, and an enzyme-coordinated *o*-diphenol structure (Met-D) is formed. It should be noted that monophenol can react with oxy-tyrosinase, but not with met-tyrosinase, to form the product *o*-quinone. Monophenolase activity shows a characteristic lag period. This may be due to the fact that tyrosinase in the resting form contains 15 % oxy sites, which is the only form that can react with monophenol substrates (Likhitwitayawuid, 2008).

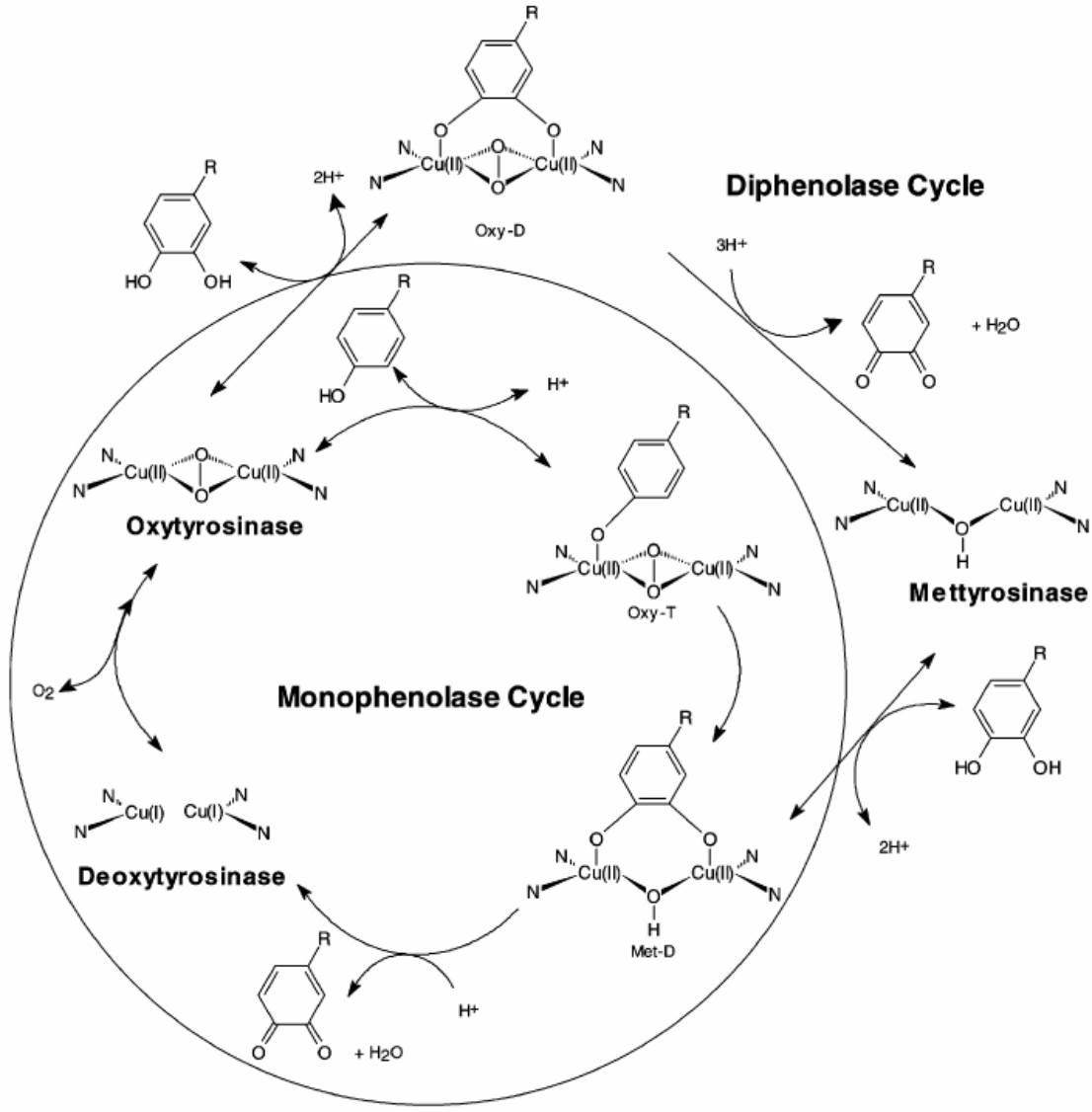

Figure 5. Mechanism for monophenolase and diphenolase activity of tyrosinase.
Source: Likhitwitayawuid (2008)

1.2.1.3.2 Diphenol oxidase

It catalyzes the oxidation of the di-phenol to quinones. The reaction of oxidation of diphenol to quinone is shown in Figure 5. This reaction receives more attention because of its faster rate than the monophenol oxidase and its association with the formation of quinones, which polymerize with amino acids / protein or self polymerize non-enzymetically to form melanin (Figure. 6) (Garcia-Molina *et al.,* 2005). The *o*-diphenol can react with both the oxy and the met forms to produce *o*-quinone. The reaction of diphenol with met-tyrosinase converts the enzyme to the de-oxy form, bringing it into the monophenolase cycle (Solomon *et al.*, 1996). When quinones are formed and undergo polymerization with protein or amino acids or self polymerized, they form a melanoid compound (dark brown color) (Satoh *et al.*, 1999).

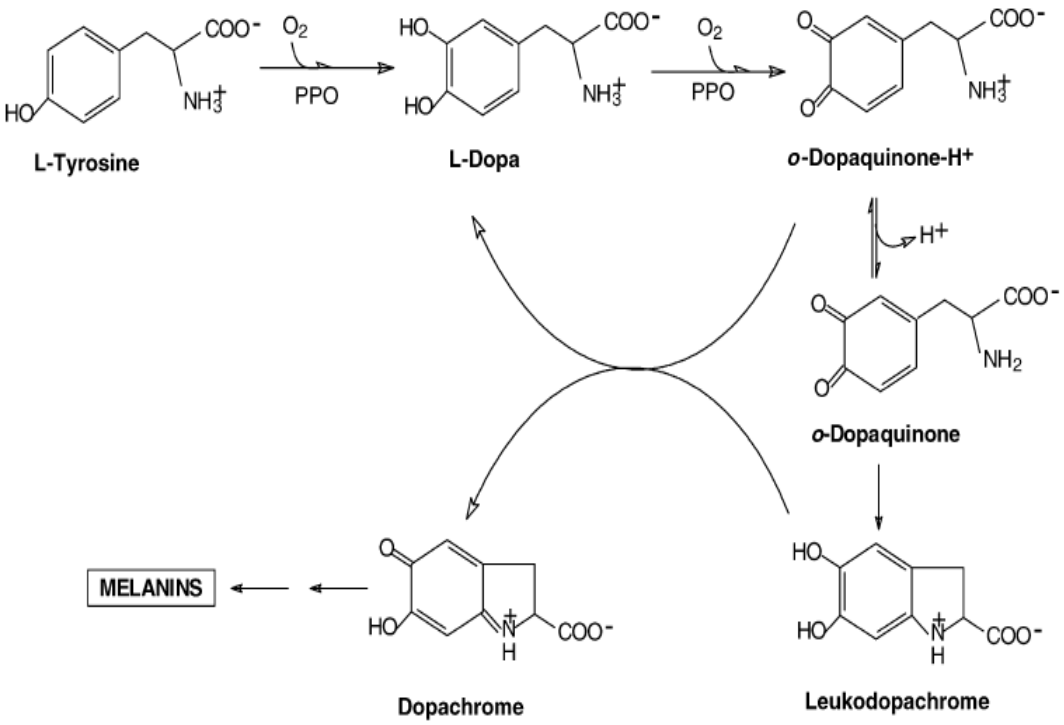

Figure 6. Melanin biosynthesis from tyrosine.

Source: Garcia-Molina *et al.* (2005)

1.2.1.4 Characteristics of PPO from crustaceans

1.2.1.4.1 Molecular weight

PPO from different shrimp comprise the different iso-forms with varying molecular weights (Chen *et al.,* 1991a). Also, the molecular weight of PPO varied with the molting stage (Ferrer *et al.,* 1989). PPO from the kuruma prawn cephalothorax had the molecular weight of 160 kDa (Benjakul *et al.*, 2005). Antarctic krill catechol oxidases had the molecular weights of 75 and 83 kDa (Ohshima and Nagayama, 1980). For PPO from shrimp (*P. setiferus*), its molecular weight was 30 kDaA (Simpson *et al.,* 1987). The molecular weights of pink shrimp PPO were 30 and 35 kDa, while those of white shrimp were 20 and 25 kDa (Chen *et al.,* 1997). The apparent molecular weight of proPPO and PPO from hemolymph of crab *Charybdis japonica* was 69.5 and 64.5 kDa, respectively (Liu *et al.*, 2006). Zamorano *et al*. (2009) reported that PPO with the molecular weight of 500 kDa and 200 kDa were found in deep water pink shrimp (*Parapenaeus longirostris*). PPO from viscera and carapace extracts of cephalothorax of Norway lobster (*Nephrops norvegicus*) had apparent molecular weight about 200- 220 kDa as determined by activity staining using L-tyrosine and 4-tert-butyl-catechol as substrates (Gimenz *et al.*, 2010).

1.2.1.4.2 pH optima and stability

The pH profile for PPO isolated from different crustaceans varies with species. In the case of shrimp (*Penaeus setiferus*), PPO was active in the pH range of 6.5– 9 (Simpson *et al.*, 1988). For *Penaeus monodon* PPO, the maximum activity was reported at pH 6.0 (Rolle *et al.*, 1991). Lobster (*Panulirus argus*) PPO had the optimal pH of 6–6.5 (Ali *et al.*, 1994). The maximal activity of PPO from the kuruma prawn cephalothorax (Benjakul *et al*., 2005) and Antractic krill (Ohshima and Nagayama 1980) was observed at pH 6.5. Montero *et al*. (2001a) reported that PPO from imperial tiger prawn (*Penaeus japonicus*) was most active at pH 5 and 8. Zamorano *et al*. (2009) found that PPO from deep water pink shrimp (*Parapenaeus longirostris*) had the highest activity at pH 4.5. PPO from the carapace of shrimp (*P. setiferus*) showed the maximal activity at pH 7.5 (Simpson *et al.,* 1987). PPO from hemolymph of crab *Charybdis japonica* showed optimum pH at 6 (Liu *et al*., 2006). PPO from

carapace and viscera of Norway lobster (*Nephrops norvegicus*) had optimum pH at 7 and 8, respectively (Gimenez *et al.*, 2010).

In general, the conformational change at the active site of enzyme due to dramatic pH changes may cause the significant loss in enzyme activity. PPO activity markedly decreased in either acidic or alkaline pH ranges (Whitaker, 1995). Montero *et al.* (2001a) reported that PPO activity of imperial tiger prawn (*Penaeus japonicus*) was decreased at pH below 5. PPO is not stable at acidic pH. PPO from lobster (*Panulirus argus*) (Ali *et al.*, 1994) and white shrimp (*Penaeus setiferus*) (Simpson *et al*., 1987) was unstable below pH 5. Nevertheless, PPO from pink shrimp (*Penaeus duorarum*) exhibited very high stability between pH 6 and 12, with maximum activity at pH 8–9 (Simpson *et al.,* 1988). PPO from the kuruma prawn cephalothorax was stable over a wide pH range (3–10) with the remaining activity above 90% (Benjakul *et al.*, 2005). PPO from deep water pink shrimp (*Parapenaeus longirostris*) was most stable at pH 4.5 and 9.0 (Zamorano *et al*., 2009). PPO from carapace and viscera of Norway lobster (*Nephrops norvegicus*) was stable over neutral to alkaline pH range (Gimenz *et al.*, 2010). Thus, the pH stability of PPO varies with species.

1.2.1.4.3 Temperature optima and stability

The optimal temperature of PPO has been reported to be varied, depending on species and habitat temperature. Montero *et al*. (2001a) found that PPO activity from imperial tiger prawn increased by 20% for every 10^{o}C increment to reach plateaus at 40-45^{o}C. The maximum activity was noticeable at 40–45^{o}C for PPO extracted from lobster (*Panulirus argus*) (Ali *et al.*, 1994) or different shrimp including *Penaeus duorarum* (Simpson *et al*., 1988), *Penaeus monodon* (Rolle *et al.*, 1991) and *Penaeus setiferus* (Simpson *et al*., 1987). The optimal temperature of PPO from the kuruma prawn cephalothorax was 35^{o}C (Benjakul *et al*., 2005). PPO from hemolymph of crab *Charybdis japonica* showed optimum activity at 40^{o}C (Liu *et al*., 2006). However, PPO activity from Norway lobster (*Nephrops norvegicus*) (Gimenz *et al*., 2010) and deep water pink shrimp (*Parapenaeus longirostris*) (Zamorano *et al*., 2009) was continuously increased up to 60^{o}C.

PPO of shrimp (*Penaeus duorarum* and *Penaeus monodon*) was unstable at temperatures over 30–35^{o}C (Rolle *et al.*, 1991; Simpson *et al.*, 1988). For PPO of *Penaeus setiferus*, the upper limit of stability was 50^{o}C (Simpson *et al.*, 1987), while PPO of lobster (*Panulirus argus*) had the stability at 40^{o}C (Ali *et al*., 1994). Even within the same species, there are differences depending on the state of activation of enzyme (Ferrer *et al.,* 1989). PPO from Kuruma prawn was stable up to 40^{o}C and slightly lost its stability at 50^{o}C (Benjakul *et al*., 2005). Montero *et al.* (2001a) reported that thermal stability of PPO from imperial tiger prawn was considerably reduced when the enzyme extract was subjected to heating at temperatures up to 35^{o}C. PPO from deep water pink shrimp had the stability at 30–35^{o}C (Zamorano *et al*., 2009).

1.2.2 Melanosis and factors influencing melanosis in crustaceans

Melanosis starts in refrigerated and frozen crustaceans within just a few hours of capture (Montero *et al*., 2004). Melanosis or black spot formation affects the quality of these products and consumers acceptability (Kim *et al.*, 2000). Melanosis is first detected in the head and then down spreads to the other tissues during chilled storage (Zamorano *et al*., 2009). The black spot on shrimp begin to form on the head and proceed down the shrimp, forming black lines just under the shell that outline the sections of the tails (Gokoglu and Yerlikaya, 2007). Melanosis is triggered by a biochemical mechanism, which oxidizes phenols to quinones by PPO. This is followed by non-enzymatic polymerization and autooxidation of the quinones, giving rise to pigments of high molecular weight and very dark or black coloring (Benjakul *et al.*, 2005). PPO is most commonly found in the cephalothorax of prawn and shrimp (Montero *et al*., 2001a). PPO remains active under refrigeration, iced and thawed products (Montero *et al*., 2001b). Melanosis spreads rapidly and hence places a limit on the shel life of crustaceans (Montero *et al*., 2001b).

There are various factors influencing the melanosis in crustaceans such as species, method of capture, metal ion, protease and some chemicals.

1.2.2.1 Species

Melanosis is associated with PPO activity. PPO activity varies with species. PPO from pink shrimp was more active than that from white shrimp (Madero and Finne, 1982; Simpson *et al*., 1987). In chilled shrimp, the rate of spread of melanosis differs among various species. This could be related to differences in levels of substrate or levels of enzyme concentration or enzymatic activity in each species (Simpson *et al*., 1987; Montero *et al*., 2001a). The spread of melanosis in pink shrimp (*Penaeus duorarum*) is faster than in white shrimp (*Penaeus setiferus*) (Simpson *et al*., 1987). Melanosis develops very rapidly in deepwater pink shrimp (Martinez-Alvarez *et al*., 2008a).

1.2.2.2 Method of capture and season

Capture and rough handling of the catch and other 'traumatic' events seem to trigger a defense mechanism in shellfish involving the activation of PPO, resulting ultimately in increased black spot (Kim *et al.*, 2000). Lobster and shrimp can be induced to form melanin by injuring them while alive (Ogawa, 1987). Danish Norway lobster processors have reported an annually recurring rise in black spot-related problems around September each year. Annual drop in catch quality was truly related to changes in PPO activity (Bartolo and Birk, 1998). Nevertheless, hemocyanin derived PPO could be responsible for the rapid formation of black spots in broken clawed legs, parapods or carapace during postmortem handling (Gimenz *et al*., 2010).

1.2.2.3 Metal ion

The role of copper in catalysis of oxidation of monophenols and *o*-diphenols was elucidated. Some Metal ions such as Cu^{2+}, Zn^{2+}, and Mg^{2+} have a significant effect on PPO activity. Liu *et al*. (2006) reported that PPO activity from crab (*Charybdis japonica*) was strongly inhibited by Cu^{2+}, Zn^{2+}, and Mg^{2+}. Nevertheless, Cu^{2+} had an obvious recovery effect on the activity of EDTA-pretreated PPO, but the other metal ions did not have such an effect. Simpson *et al*. (1987) reported that PPO activity from shrimp (*Penaeus setiferus*) increased with the addition of the copper, whereas Benjakul *et al*.(2005) found that PPO isolated from

the kuruma prawn cephalothorax might be in the active form, which did not require copper ion for PPO activation. The increase in copper ion might cause the conformational change of enzyme by affecting the ionic interaction stabilizing the structure of enzyme. Therefore, the copper dependency of PPO depended on species, tissue, and other intrinsic factors determining the activity. The activity of lobster PPO was stimulated by the addition of copper but inhibited by EDTA (Opoku-Gyamfua *et al*., 1992).

1.2.2.4 Protease and some chemicals

In crustaceans, PPO is localized in the cuticle and hemolymph as a zymogen or proPPO form, which can be activated by protease, fatty acids and lipids, laminarin (1,3- glucan) acetone, alcohol and sodium dodecyl sulfate (Ferrer *et al*., 1989). Activation of proPPO from the hemolymph of the lobster (*Homarus americanas*) could be achieved by treatment with an anionic or cationic detergent but not with nonionic detergents. In addition, phospholipids, especially lysolecithin proved to be the most potent activators of proPPO (Sugumaran and Nellaiappan, 1991). The activation causes the formation of melanins, which possess antimicrobial properties. However, Benjakul *et al*. (2005) found that sodium dodecyl sulfate and methanol showed no influence on PPO activity from kuruma prawn cephalothorax. Trypsin had no effect on PPO activity from kuruma prawn cephalothorax (Benjakul *et al*., 2005) and shrimp (*Penaeus setiferus*) (Simpson *et al*., 1987), suggesting that the activated PPO might be present in the extract. However, Norway lobster proPPO was activated by the addition of trypsin (Yan and Taylor, 1991). In addition, Zn-serine protease, Zn-thiol protease and thiol protease were found to activate proPPO from Norway lobster (Zotos and Taylor, 1997). Gollas-Galvan *et al.* (1999) reported that proPPO purified from blood cell of brown shrimp (*Penaeus californiensis*) is a 114-kDa monomeric protein, which can be hydrolyzed by proteinases, producing a 107-kDa active PPO.

During post-mortem storage of crustaceans, proPPO can be also activated into PPO by the action of proteolytic enzymes leaching from the digestive tract. Moreover; protein hydrolysis by these proteases originates substrates for active PPO (Ali *et al.*, 1994). Lee *et al*. (2000) showed that the 36-kDa LGBP

(lipopolysaccharide- and β-1,3-glucan-binding protein) plays a role in the activation of the proPO activating system in freshwater crayfish (*Pacifastacus leniusculus*). Garcia-Carreno et al. (2008) reported that hemocyanin (Hc) from whiteleg shrimp (*Penaeus vannamei*) was converted to HcPPO by SDS treatment.

1.2.3 Melanosis/ PPO inhibitors

Many studies have focused on various techniques and mechanisms to control these undesirable PPO activities, thereby controlling melanosis. These techniques attempt to eliminate one or more of essential components (oxygen, enzyme, copper, or substrate) from the reaction (Gokoglu and Yerlikaya, 2008). Enzymatic browning can be inhibited by targeting the enzyme, the substrates (oxygen and polyphenols) or the products of the reaction. Melanosis inhibitors can be classified into different groups according to their mode of action.

1.2.3.1 Acidulants

pH of the chemical has the tendency to affect ionizable groups of protein by reduction or oxidation; it can affect enzyme and substrate by changing ionization state and breakdown of structural conformation, respectively. These groups must be in the appropriate ionic form in order to maintain the conformation of the active site, to bind substrates, or to catalyze the enzymatic reaction (Segel, 1976). Generally acidulants maintain the pH below that required for optimum catalytic activity of an enzyme. Acidulants are often used in combination with other antibrowning agents. Acidulants such as citric, malic, and phosphoric acids are capable of lowering the pH (≈ 3) of a system, thus rendering PPO inactive (Richardson and Hyslop, 1985).

Citric acid is the one of the most widely used acid in the food industry. Ascorbic acid or erythroboric acid and their neutral salts have also been used (Montero *et al.*, 2001a). Citric acid exerts inhibition on PPO by reducing the pH as well as by chelating the copper at the enzyme-active site. Benner *et al*. (1994) reported that brown shrimp *(Penaeus aztecus*) treated with L-lactic acid in combination with 4-hexylresorcinol (0.0025 %) had the decrease in melanosis.

1.2.3.2 Chelating agents

PPO possess metal ion at their active site for the functional activity. These metal ions are participated in enzyme reaction. Removal of these metal ion by chelating agents make enzyme inactive. Chelating agents are able to form complex with PPO activators such as copper and iron ions, through an unshared pair of electrons in their molecular structures (Kim *et al*., 2000).

The well known chelating agent is EDTA (ethylenediamine tetra acetic acid). Chelators used in the food industry include sorbic acid, polycarboxylic acid (citric, malic, tartaric, oxalic and succinic acids), polyphosphate (triphosphate and pyrophosphate), macromolecules (porphyrins, proteins) and EDTA. Kojic acid has potential applicability in the prevention of melanosis in both plant and seafood products (Chen *et al*., 1991a). The phenolic derivatives of benzoic acid appear to act as chelating agents of copper (Montero *et al.,* 2001a). Sodium benzoate was more effective in lowering the melanosis formation of prawns (*Penaeus japonicus*) than ascorbic acid or citric acid during storage at 4^{o}C for 8 days (Montero *et al*., 2001b). Opoku-Gyamfua *et al*. (1992) reported that EDTA inhibited PPO from lobster (*Homarus americanus*). PPO from crab *Charybdis japonica* was totally inhibited by phenylthiourea and was extremely sensitive to EDTA or diethyldithiocarbamate (DETC) (Liu *et al.*, 2006)

1.2.3.3 Reducing agents

The most widely used chemicals in preventing enzymatic browning are reducing agents such as sulfiting agent, ascorbic acid, cysteine and glutathione. Reducing agents prevent enzymatic browning either by reducing *o*-quinones to colorless diphenols, or by reacting irreversibly with *o*-quinones to form stable colorless products (Ferrer *et al.,* 1989) (Figure 7). Ascorbic acid is highly water-soluble, which is acidic and moderately strong reducing compound. Ascorbic acid also acts as an oxygen scavenger for the removal of molecular oxygen in PPO reactions. Walker (1977) reported that PPO inhibition by ascorbic acid and cysteine has been attributed to the reduction of enzymatically formed *o*-quinones to their precursor diphenols. The inhibition of melanosis by sulfhydryl compounds, such as cysteine and glutathione is thought to be due to the formation of colorless thiol-

conjugated *o*-quinones (Benjakul *et al*., 2006). Cysteine-quinone adducts were proved to be the competitive inhibitors of PPO (Kim *et al*., 2000). Arias *et al*. (2007) reported that ascorbic acid (AA) prevents browning by two different mechanisms. In the absence of PPO subtstatres AA inactivates PPO irreversibly and in the presence of PPO substrates, it reduces quinone back to hydroquinone.

Sulfites serve a multifunctional role in foods. They possess antimicrobial activity and inhibit both enzymatic and non-enzymatic browning reaction (Januario and Dykes, 2005). Sulfiting agents (sulfur dioxide, SO_2; sulfite, SO_3; hydrogen sulfite, HSO_3; metabisulfite, S_2O_5) are the most widely applied reagents for the control of browning in the food industry (Gokoglu and Yerlikaya, 2008). Bisulfite (HSO_3^-) is a competitive inhibitor of PPO by binding a sulfhydryl group on the PPO active site (Madero and Finne, 1982). Inhibition on the PPO catalyzed melanosis in lobster was accomplished by bisulfite via its reaction with intermediate quinones forming sulfoquinones, and via its complete inactivation of PPO (Ferrer *et al*., 1989). Martinez-Alvarez *et al*. (2005a) reported that prawns (*Marsupenaeus japonicus*) treated with sulfite-based solution had the lowest melanosis score up to 8 days. Figure 7 depict the inhibition of brown color formation by reducing agents which convert reactive *o*-quinone to diphenol. Gomez-Guillen *et al*. (2005) used sodium metabisulfite (6.2 to 50 gKg^{-1}) to prevent melanosis in fresh deep water pink shrimp (*Parapenaeus longirostris*) by immersion method for 1 h. Marinez-Alvarez *et al*. (2008b) reported that Norwegian lobsters (*Nephrops norvegicus*) dusted with sulfites had retarded formation of black spot for at least 7 days during chilled storage. Rotllant *et al*. (2002) reported that the shrimp (*Aristeus anteunatus*) treated with increasing concnentration of HQ-Bacterol F containing 40 % sodium metabisulfite could lower black spot formation up to 27 h but increased the residual SO_2 in th tissue. Prawn (*Marsupenaeus japonicus*) treated with sulfites showed initially better protection to lower melanosis and quality losses; however 4-HR was more effective at the end of storage (Martinez-Alvarez *et al*., 2005a). Ascorbic acid and sodium metabisulfite inhibited the activity of both polyphenoloxidase and hemocyanin from deepwater pink shrimp (*Parapenaeus longirostris*) (Martinez-Alvarez *et al*., 2008a).

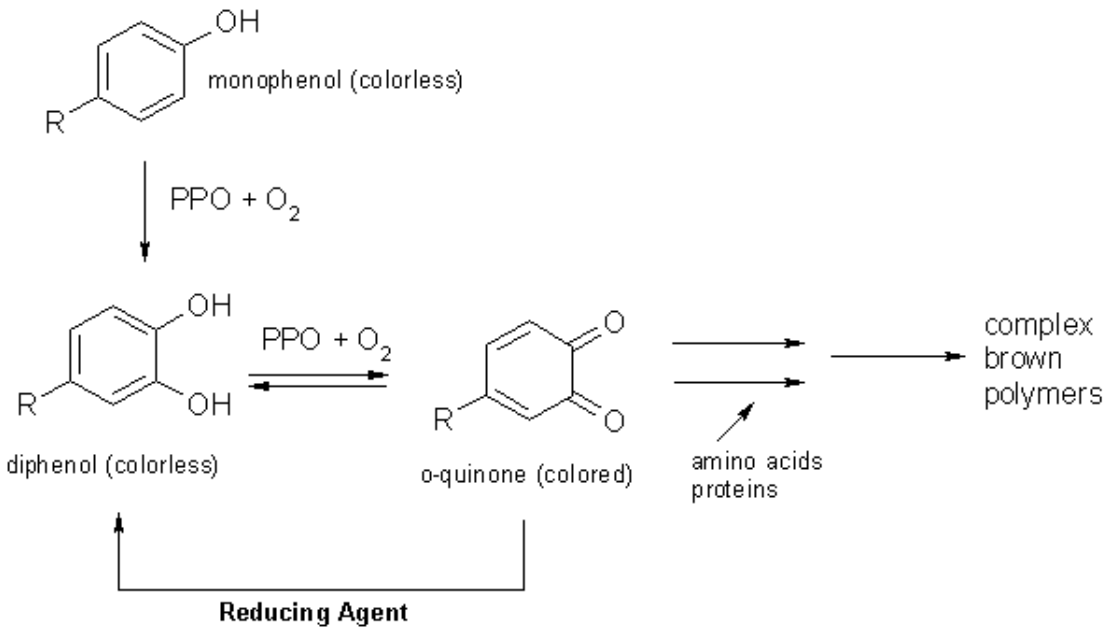

Figure 7. Inhibition of brown color polymers by reducing agent.
Source: Kim *et al.* (2000)

The FDA has proposed the maximum residual sulfur dioxide levels for certain foods. Shrimp products having residual sulfite levels above 100 ppm are considered adulterated, since these levels are considered unsafe. It has been necessary to search for alternatives that show effective inhibitory effect on melanosis but are devoid of health concerns to consumers (Chen *et al*., 1991a).

1.2.3.4 PPO inhibitor (4-Hexylresorcinol)

4-Hexylresorcinol (4-HR) has several advantages over sulfites in food, including its specific mode of inhibitory action (Figure 8), its lower level required for effectiveness, its inability to bleach preformed pigments, and its chemical stability (McEvily *et al*., 1992). 4-HR acts as an enzyme-competitive inhibitor due to structural resemblance to phenolic substrates (McEvily *et al.,* 1991). 4-HR (100 mg/kg) showed a marked ability to inhibit or slow down melanosis in shrimp (*Parapenaeus longirostris*), compared with the sodium metabisulfite (1 g/kg) (Guandalini *et al*., 1998).

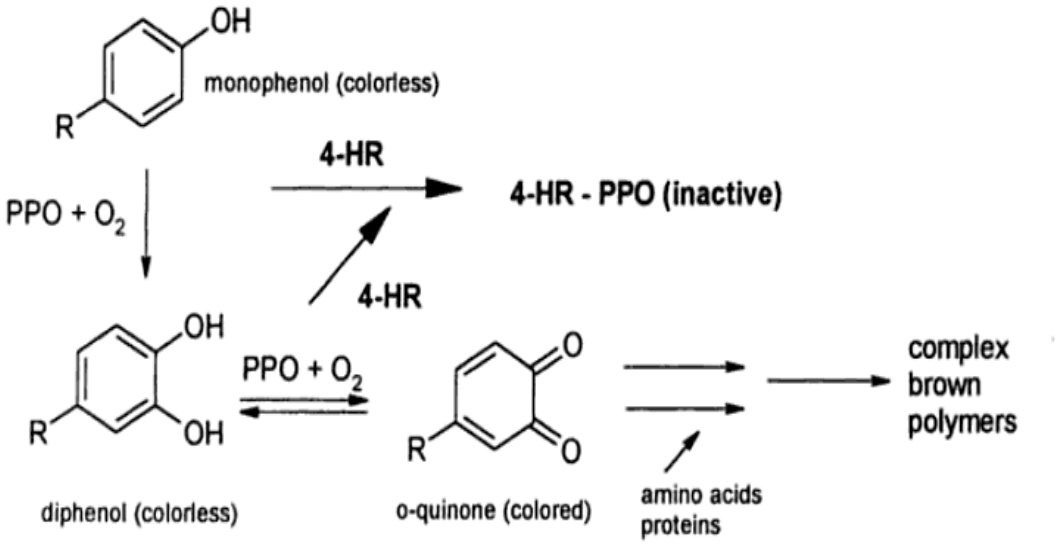

Figure 8. Inhibitory effect of 4-hexylresorcinol on PPO

Source: Lambrecht (1995)

McEvily *et al*. (1991) reported that dipping shrimps, brown shrimp (*Penaeus aztecus*) and pink shrimp (*Penaeus duorarum*), into 50 ppm 4-HR in sea water with subsequent storage on crushed ice inhibited black spot formation up to 14 days. Lambrecht (1995) reported that the headless brown shrimp (*Penaeus aztecus*) dipped in 4-HR for 1 min controlled black spot formation for a longer period of time than the control or those treated with 1.25 % sodium metabisulfite. 4-HR alone or in combination with ascorbic or citric acid, was effective as inhibitors of melanosis and microbial spoilage in prawns (*Penaeus japonicus*) (Montero *et al.*, 2001b). Montero *et al*. (2006) studied inhibition of melanosis in pink shrimp (*Parapenaeus longirostris*) treated by immersion and dusting method with various concentration (0.0025 to 5 g of inhibitor per 100 g of shrimp) of 4-HR during chilled storage of 12 days. Melanosis inhibition increased with inhibitor concentration. PPO activity of Norway lobster (*Nephrops norvegicus*) was inhibited with a formulation containing 4-hexylresorcinol (0.05 and 0.1%) in combination with organic acids and chelating agents (Martinez-Alvarez *et al*., 2007)**.** Montero *et al*. (2001b) reported that 4-HR in combination with ascorbic or citric acid showed most efffectvie melanosis inhibition in prawns (*Penaeus japonicus*) stored at 4^{o}C for 8 days. Shrimp (*Penaeus Japonicus*) treated

with 1 % citirc or lactic acid in combination with sodium metabisulfite (0.3%) showed lowered melanosis formation, compared with the shrimp treated with acids alone (Gokoglu, 2004). Incubation of mushroom PPO with 4-HR decreased PPO activity effectively, due to the high affinity of 4-HR for PPO (Arias *et al*., 2007). 4-HR did not inhibit the PPO activity of hemocyanin from deepwater pink shrimp (*Parapenaeus longirostris*) (Martinez-Alvarez *et al*., 2008a).

1.2.3.5 Miscellaneous

Chemical and natural compounds have been reported to lower the enzymatic browning in fruits, vegetables and seafoods. Inorganic halides are known as the inhibitors of PPO. NaF was the most potent inhibitor, followed by NaCl, NaBr and NaI (Janovitz-Klapp *et al.*, 1990). Sodium salts of four n-alkyl xanthate compounds, $C_2H_5OCS_2Na$ (I), $C_3H_7OCS_2Na$ (II), $C_4H_9OCS_2Na$ (III), and $C_6H_{13}OCS_2Na$ (IV) exhibited the inhibitory activity towards cresolase and catecholase of mushroom tyrosinase (Saboury *et al.,* 2007). Amino acids (cysteine, glutathione and histidine), peptides or proteins can also inhibit browning reaction by reducing quinone or directly react with PPO (McEvily *et al.*, 1992). Melanosis of pink shrimp (*Penaeus duorarum*) was inhibited by treatment with solution of ficin, a sulfhydryl protease (Taoukis *et al.*, 1990). Richard-Forget *et al.* (1998) reported that crude papaya extract containing some ‘quinone-trapping’ substances, identified as cysteine and a dipeptide cysteine-glutamic acid, which shows the competitive inhibitor towards PPO.

Honey has been shown to inhibit enzymatic browning. Vela *et al*. (2006) studied thirty-six Spanish honeys of different floral origin (nectars and honeydews) and found that honeydew honeys showed higher antioxidant capacities and ability to inhibit enzymatic browning in apple homogenate than nectar honeys. Jeon and Zhao (2005) found that fresh cut apples dip in 10 % honey for 30 min had antibrowning effect, however vacuum impregnation (75 mmHg for 15 min) with 10 % honey was more effective in prevention of browning discoloration.

Maillard reaction products (MRP) have also been known to inhibit PPO from apple and mushroom (Maillard *et al*., 2007). Cheriot *et al*. (2007) reported that MRP from a preheated cysteine-derived compound and a carbonyl component,

especially hydroxymethylfurfural (HMF), furfural and benzaldehyde, exhibited a stronger inhibitory potency towards PPO of eggplant, apple, and mushroom. Matmaroh *et al*. (2006) had found that the inhibitory activity of MRPs towards browning in back tiger shrimp, induced by PPO, was most likely due to their reducing power as well as copper chelating property.

Mimosine inhibited mammalian tyrosinase competitively because of its structural similarity to the substrate, L-DOPA and its tendency to chelate cupric ion (Hashiguchi and Takahashi, 1977). Cabanes *et al.* (1987) reported that L-mimosine was a slow binding inhibitor of mushroom tyrosinase for oxidation L-DOPA. Mimosine inhibited polyphenoloxidase from *S. rolfsii* competitively and decreased the specific activity by 87 % (Serrano *et al.*, 1983). This indicated that mimosine most likely inhibit PPO by binding the active site of the PPO. A number of naturally occurring tyrosinase inhibitors consist of a phenol structure or of metal chelating agents (Fadimatou *et al*., 2010). The inhibition of metal-dependent enzymes by L-mimosine was related to its metal chelating ability (Stunzi *et al.*, 1980). Mimosine and kojic acid was reported to be a standard inhibitor for mushroom tyrosinase with competitive type inhibition (Matsumoto-Akanuma *et al.*, 2011; Fadimatou *et al*., 2010; Sabudak *et al*., 2006). Mimosine inhibited both monophenol and diphenoloxidase activity from European spiny lobster (*Palinurus elephas*) competitively (Brack *et al.*, 2008).

A competitive and mixed type inhibition occurred for mushrrom PPO depending on the phenolic substrates studied (Chen *et al.*, 1991b). Kojic acid showed a mixed type inhibition towards PPO from white shrimp, grass prawn and lobster (Chen *et al*., 1991b). Dodecyl gallate was found as a mixed-type inhibitor for mushroom tyrosinase, when L-DOPA was used as a substrate (Kubo *et al.*, 2000). Inhibition constant K_i value of dodecyl gallate on mushroom tyrosinase was 0.636 mM (Kubo *et al.*, 2003). Benjakul *et al.* (2006) reported that cysteine and glutathione showed competitive inhibition toward kuruma prawn PPO with K_i values of 0.45-0.46 mM. Ozagrel was a reversible mixed type inhibitor of diphenoloxidase activity of mushrrom tyrosinase with KS1, KS2, Ki1 and Ki2 was 2.21, 3.89, 0.454 and 0.799 mM, respectively (Li *et al.*, 2009).

1.2.4 Changes in quality of shrimp during post mortem storage

Storage of fish and shellfish in crushed iced or liquid ice (chilling or super chilling) has been the routine practice of preserving fish on board and at shore (Lakshmanan *et al.*, 2002). However, freezing technology has been widely used to stored crustaceans for export (Lopez-caballero *et al*., 2007). Although freezing is an effective method of preserving foods, some deterioration in frozen food quality occur during storage. The extent of quality loss is dependent upon many factors, including storage temperature, rate of freezing and thawing, temperature fluctuations, freeze-thaw abuse during storage (Srinivasan *et al.*, 1997). The impact of iced and frozen storage on shrimp quality has been dependent on the length of storage (Erickson *et al*., 2007).

1.2.4.1 Microbiological changes during storage

Microorganisms are the major cause of spoilage of most seafood products by formation of amines, sulfides, alcohols, aldehydes, ketones, and organic acids with unpleasant and unacceptable off-flavors (Gram and Dalgaard, 2002). The high content of free amino acids and other soluble non-nitrogenous substances, can serve as easily digestible nutrients for microbial growth (Zeng *et al.*, 2005). However the specific spoilage organism (SSO) are not the same in every case and the microbial flora isolated from seafoods differs considerably from one study to another, depending on the species of fish, their environment, the mode of capture, the type of fish product as well as the climatic and storage conditions (Gram and Dalgaard, 2002). Generally, pseudomonas, H_2S- producing bacteria and lactic acid bacteria (LAB) are predominant in spoiled fish flora, while *Enterobacteriaceae,* a gram negative bacteria is also frequently present (Sallam, 2007).

On iced storage, the total bacterial load was reduced to one log from initial load in fresh fish/shrimp due to cold shock (Lakshmanan *et al.*, 2002). Zeng *et al*. (2005) had found that shrimp (*Pandalus borealis*) stored in liquid ice slowed down microbial growth as compared to shrimp stored in flake ice or brine mixed ice. Black tiger prawns (*Marsupenaeus japonicus*) treated with the formulation containing 0.1 % 4-HR in combination with organic acids (citric, ascorbic, and acetic acids), EDTA and

disodium dihydrogen pyrophosphate (PPi) had the lower microbial growth (total bacteria count, H2S-producing bacteria, LAB, enterobacteria, and pseudomonas) as compared to control and sulfite-based treated prawn during chilled storage of 13 days (Martinez-Alvarez *et al.*, 2005a). Lopez-caballero *et al.* (2007) reported that thawed deep water pink shrimp (*Parapenaeus longirostris*) treated with different formulations containing 4-HR (0.05 and 0.1 %) in combination with organic acids and chelating agents, showed lowered total bacterial count in comparison with those treated with a commercial formula based on sulfites, and a mixture of gluconic acid and commercial sulfites during 14 days of chilled storage. Mastromatteo *et al*. (2010) reported that a slight antimicrobial effect was observed in ready-to-eat peeled shrimp (*Palaemon serratus*) when coated with thymol essential oil (500, 1000 and 1500 ppm) and effect was concentration dependent. Fresh white shrimp (*Penaeus vannamei*) coated with antimicrobial film prepared from catfish skin gelatin incorporation with potassium sorbate and sodium tripolyphosphate could retard microbial growth and prolong shelf life for up to 10 days of iced storage (Jiang *et al*., 2011).

1.2.4.2 Chemical changes during storage

Chemical changes in seafoods are mostly related with the microbial load (Gram and Dalgaard, 2002). Quality of fish and shellfish are generally accessed by the changes in lipid oxidation (PV, TBARS**,** etc), total volatile base (TVB), trimethylamine (TMA), K-value, and pH**.** Lipid oxidation in fish meat can be initiated by autoxidation, photosensitized oxidation, or an enzymatic reaction involving lipoxygenase, peroxidase and microbial enzymes (Bak *et al.*, 1999). The volatile base found in the shrimp more likely indicated that an autolytic process was involved during postmortem handling. Adenosine and adenosine monophosphate (AMP) deaminase might play a major role in this process after capture and transportation (Lopez-caballero *et al.*, 2007). TMA is produced by some bacteria capable of using trimethylamine oxide (TMAO) in anaerobic respiration (Gram and Dalgaard, 2002). K-value has been used as the freshness index in fish and shellfish. During postmortem handling and storage, endogenous enzymes break down adenosine triphosphate to different derivatives such as adenosine diphosphate, adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR), and

finally hypoxanthine (Hx) (Yamagata and Low, 1995). The increase in pH was associated with the accumulation of basic compounds, mainly resulted from microbial action (Lopez-caballero *et al.*, 2007). In general, a K-value of 60% has been realized as the rejection limit. TMA acceptability limit for shrimp was reported to be 5 mg N/100g and TVB content of 40 mg N/100g has been used as the freshness borderline (Uchiyama and Kakuda, 1984; Mendes *et al*., 2002).

Thepnuan *et al*. (2008) found that the shrimp (*Litopenaeus vannamei*) pretreated with 2 % pyrophosphate and 0.25 % 4-HR and stored under MAP showed the lower TBARS value throughout 12 days of storage. Marination of deep water pink shrimp (*Parapenaeus longirostris*) with rosemary extract lowered TBA value by 2.7 times than the control at the end of storage day at 1 ± 0.5^{o}C (Cadun *et al*., 2008). Lopez-caballero *et al.* (2007) reported that thawed deep water pink shrimp (*Parapenaeus longirostris*) treated with different formulations containing 4-HR in combination with organic acids and chelating agents, a commercial formula based on sulfites, and a mixture of gluconic acid and commercial sulfites, did not show any noticeable differences for TVB and TMA during 14 days of chilled storage. The control banana shrimp (*Penaeus merhuiensis*) and those treated with sodium bisulfite had similar K-values (43%) at the last day (day 6) of iced storage (Yamagata and Low, 1995).

1.2.4.3 Physical and Sensorial changes during storage

Super chilling of muscle food can result in partial freezing, which may lead to negative changes such as decrease in water holding capacity (WHC) and shear force (Zeng *et al.*, 2005). Over the extended storage time, the breakdown of structural protein in shrimp tissue may also cause loss of physical integrity (Rutherford *et al.*, 2007). Generally, the softening of shrimp or fish muscle is associated with proteolysis caused by endogenous or microbial proteinases and collagenase (Benjakul *et al.*, 1997). Lopez-caballero *et al.* (2007) found that WHC was slightly decreased for thawed deep water pink shrimp (*Parapenaeus longirostris*) treated with different formulations containing 4-HR in combination with organic acids and chelating agents throughout storage period. Treatment with sulfite based formulations showed the increase in shear strength at the end of storage (14 day). Thepnuan *et al.* (2008)

reported that whole Pacific white shrimp (*Litopenaeus vannamei*) treated with pyrophosphate and 4-HR and packaged under MAP-2 (80% CO_2, 20% N_2) had the highest shear force within the first 6 days of storage. However, no marked difference in WHC was observed within different treatments throughout storage.

Erickson *et al*. (2007) studied sensory attribute for both fresh and frozen commercially available shrimp and found that only appearance could uniquely differentiate a specific type of frozen shrimp. Fresh shrimp were characterized as being sweeter and juicier than frozen shrimp. Zeng *et al*. (2005) reported that shrimp stored in liquid ice at -1.5^{o}C had highest sensory scores than shrimp stored at +1.5^{o}C and in ice at +1.5^{o}C and in brine mixed with ice at -1.5^{o}C. In general, the loss in sensory properties, especially on appearance, is associated with the melanosis. Refrigeration alone does not prevent, but slow down black spot formation, because the PPO remains active during refrigeration, storage on ice, and post freeze-thawing (Montero *et al.*, 2004). During storage, proPPO can be activated into PPO by the action of proteolytic enzymes, which may produce substrates for active PPO by protein hydrolysis (Ali *et al.*, 1994). Marination of deep water pink shrimp (*Parapenaeus longirostris*) with rosemary extract had lowered the sensorial quality loss compared with the control at the end of storage day at 1 ± 0.5^{o}C (Cadun *et al*., 2008).

1.2.5 Modified atmosphere packaging (MAP)

Modified atmosphere packaging (MAP) is the removal and/or replacement of the atmosphere surrounding the product before sealing in vapor-barrier materials. MAP can be vacuum packaging (VP), which removes most of the air before the product is enclosed in barrier materials (McMillin, 2008). The combination of CO_2, N_2 and O_2 is generally used in MAP (Rutherford *et al*., 2007). CO_2 is the most important gas used in MAP, because of its bacteriostatic properties (Lopez-Caballero *et al*., 2002). CO_2 is highly soluble in water and fat, and the solubility increases greatly with decreasing temperature. The solubility of CO_2 leads to dissolved CO_2 in the food product and form carbonic acid (Sivertsvik *et al*., 2002), according to the following equation:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

Thus, the resultant decrease in pH can alter bacterial cell membrane, affecting nutrient uptake and absorption, and can inhibit intracellular enzyme activity (Rutherford *et al*., 2007). It has been suggested that atmosphere poor in oxygen and rich in CO_2 could act as a coadjuvent in delaying melanosis and chemical changes (Goncalves *et al*., 2003). Nitrogen (N_2), an inert and tasteless gas, is mostly used as filler gas in MAP. The use of low level of oxygen (O_2) in MAP is generally recommended to avoid lipid oxidation and growth of aerobic spoilage bacteria (Martinez-Alvarez *et al.*, 2005b). The ratio between the volume of gas and volume of food product (G/P ratio) is usually 2:1 or 3:1. This high G/P ratio is also necessary to prevent package collapse because of the CO_2 solubility in wet foods (Sivertsvik *et al*., 2002).

Deepwater pink shrimp (*Parapenaeus longirostris*) packed under MAP (40% CO_2 / 30% O_2 / 30% N_2 and 45% CO_2 / 5% O_2 / 50% N_2) and stored at 2^oC for 9 days had lowered bacterial count (total bacteria count, H_2S-producing microorganism and enterobacteria) and biochemical indexes (TVB and TMA) as compared to air-packed or iced stored shrimp (Lopez-Caballero *et al*., 2002). Deepwater pink shrimp (*Parapenaeus longirostris*) treated with sulfite and packed under 45% CO_2 / 5% O_2 / 50% N_2 had lowered black spot formation in comparison with the treated shrimp packed under 40% CO_2 / 30% O_2 / 30% N_2 on day 7 of iced storage (Goncalves *et al*., 2003). Deep water pink shrimp (*Parapenaeus longirostris*) treated with 0.1 % 4-HR and kept in MAP (53% CO_2 / 7% O_2 / 40% N_2) showed less melanosis than shrimp treated with 4-HR and kept in ice (Martinez-Alvarez *et al*., 2005b). Lu (2009) reported that whole or decapitated Chinese shrimp (*Fenneropenaeus chinensis*) stored under MAP (40% CO_2 / 30% O_2 / 30% N_2) and 100% CO_2 after soaking with bactericide (1 g/L 4-HR, 500 IU/mL nisin and 5 g/L sodium dehydroacetate) had increased shelf life up to 13 and 17 day as compared to the control (up to 9 days) at 2^oC. Thepnuan *et al*. (2008) found that the whole and decapitated white shrimp (*Litopenaeus vannamei*) pretreated with 2% pyrophosphate and 0.25% 4-hexylresorcinol and stored under MAP (80% CO_2 / 10% O_2 / 10% N_2 or 80% CO_2 / 20% N_2) showed the lowered microbiological (mesophilic, psychrophilic, lactic acid

bacteria, coliforms and *E.coli*); chemical deteriorations (TVB, TMA, TBARS values) and lower melanosis score throughout the storage of 12 days at 4°C, compared with those without treatment. Martinez-Alvarez *et al*. (2005b) reported that shrimp (*Parapenaeus longirostris*) treated with 4 % sulfites in combination with MAP (53% CO_2 / 7% O_2 / 40% N_2) totally inhibited darkening during 20 days of storage at 2°C. Nevertheless, the application of atmosphere in shrimps without antimelanosis agents did not inhibit melanosis (Martinez-Alvarez *et al*., 2005b). Mastromatteo *et al*. (2010) reported that thymol coated (1000 ppm) ready-to-eat peeled shrimp (*Palaemon serratus*) and kept under MAP (5%O_2 : 95%CO_2) had lowered microbial growth and sensorial quality loss, thereby increasing the shelf life up to 14 days, compared to sample in air (5 days).

1.2.6 Phenolic compounds

Phenolic compounds are naturally occurring secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants (Figure 9) (Ryan and Robards, 1998).

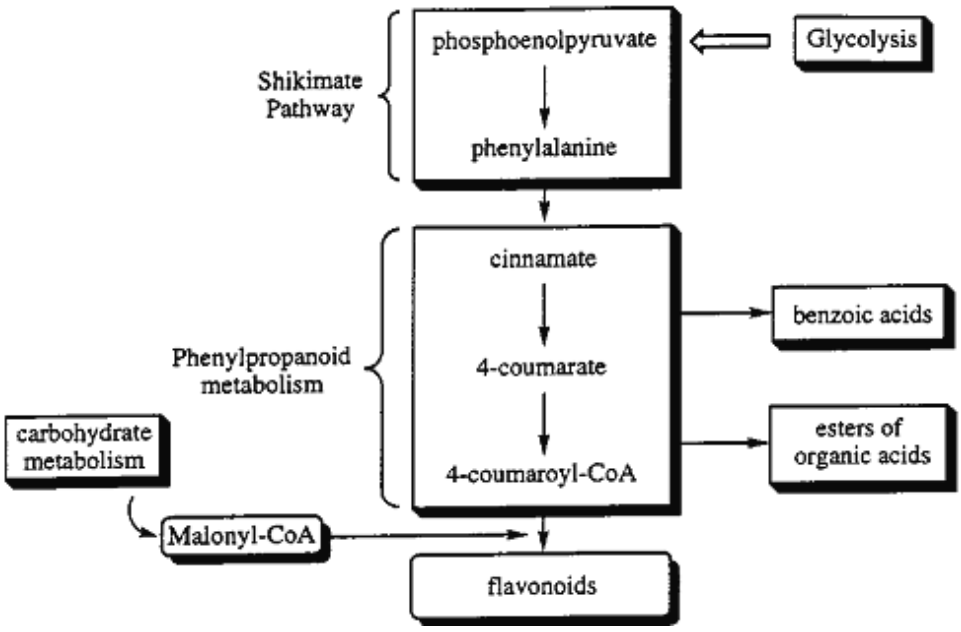

Figure 9. Metabolic pathways for the formation of phenolic compounds.
Source: Ryan and Robards (1998)

They are present in fruits, vegetables, leaves, nuts, seeds, flowers, and barks (Sellappan *et al.*, 2002). These compounds are of considerable physiological and morphological importance in plants (Balasundram *et al.*, 2006).

Chemically, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl groups, and range from simple phenolic molecules to highly polymerized compounds (Dykes and Rooney, 2007). Most naturally occurring phenolic compounds are present as conjugates with saccharides (mostly mono and disaccharides), linked to one or more of the phenolic groups by acid-labile hemiacetal bond (Ryan and Robards, 1998) and may also occur as functional derivatives such as esters and methyl esters (Balasundram *et al.*, 2006). Despite the structural diversity, the wide range of phenolic compounds are often referred to as polyphenols and basically be categorized into several classes as shown in Table 1 (Ryan and Robards, 1998; Naczk and Shahidi, 2004; Balasundram *et al.*, 2006; Dykes and Rooney, 2007).

Table 1. Classes of phenolic compounds in plants

Classes	**Structure**
Simple phenolics, benzoquinones	C_6
Hydroxybenzoic acids	C_6-C_1
Acethophenones, phenylacetic acids	C_6-C_2
Hydroxycinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	C_6-C_3
Napthoquinones	C_6-C_4
Xanthones	C_6-C_1-C_6
Stilbenes, anthraquinones	C_6-C_2-C_6
Flavonoids, isoflavonoids	C_6-C_3-C_6
Lignans, neolignans	$(C_6$-$C_3)_2$
Biflavonoids	$(C_6$-C_3-$C_6)_2$
Lignins	$(C_6$-$C_3)_n$
Condensed tannins (proantocyanidins or flavolans)	$(C_6$-C_3-$C_6)_n$

Source: Balasundram *et al*. (2006)

Phenolic acids consist of two subclasses, hydroxybenzoic acids and hydroxycinnnamic acids. Hydroxybenzoic acids (C6-C1) include gallic, *p*-hydroxybenzoic, vanillic, syringic and protocatecguic acids. The hydroxycinnamic acids have C6-C3 structure and include coumaric, caffeic, ferulic, and sinapic acids (Dykes and Rooney, 2007). Ferulic acid (4-nydroxy-3-methoxycinnamic acid) is ubiquitous phenolic acid in plant kingdom (Figure 10) (Ou *et al.*, 2007). Ferulic acid can be found in the different plant sources at various levels: 0.5 % of wheat bran, 0.9 % of sugar beet pulp, 5 % of corn kernel (Ou and Kwok, 2004; Zhao *et al.*, 2005), 1.5-2.8 % of rice bran oil (Taniguchi *et al*., 1999) and 1.36-2.58 % of sugarcane bagasse (Ou *et al*., 2007).

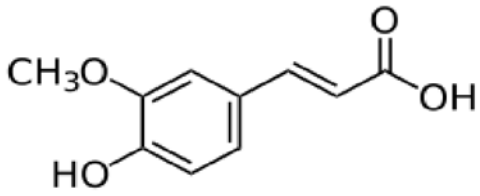

Figure 10. Structure of ferulic acid.
Source: Balasundram *et al*. (2006)

The flavonoids are a large class of compounds, ubiquitous in plants, and usually occurring as glycosides (Rice-Evans *et al.*, 1997). Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C_6-C_3-C_6 configuration. They contain several phenolic hydroxyl functional groups attached to ring structures, designated as A, B and C (Balasundram *et al*., 2006). Variation in substitution pattern to ring C result in flavanols (e.g. catechin), lacking the 2,3-double bond and the 4-one structure (Figure11) (Rice-Evans *et al.*, 1997). Green tea is the most abundant source of polyphenols, mostly in the form of gallic acid and catechin derivatives. Green tea catechins are structurally flavanols. The four major catechins in green tea are (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epicatechin (EC) (Figure 12) (Cabrera *et al*., 2006). In general, these four major caetchin derivatives together constitute 30 % of the dry

leaf weight (Gupta *et al*., 2002). Among these, EGCG represents approximately 59 % of the total catechins (Cabrera *et al*., 2006).

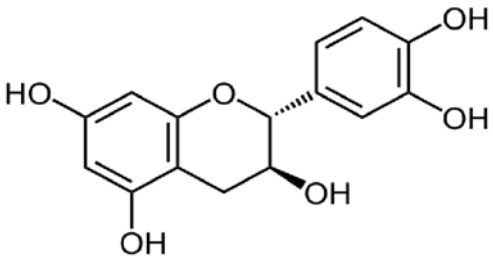

Figure 11. Structure of catechin.
Source: Wang and Helliwell (2000).

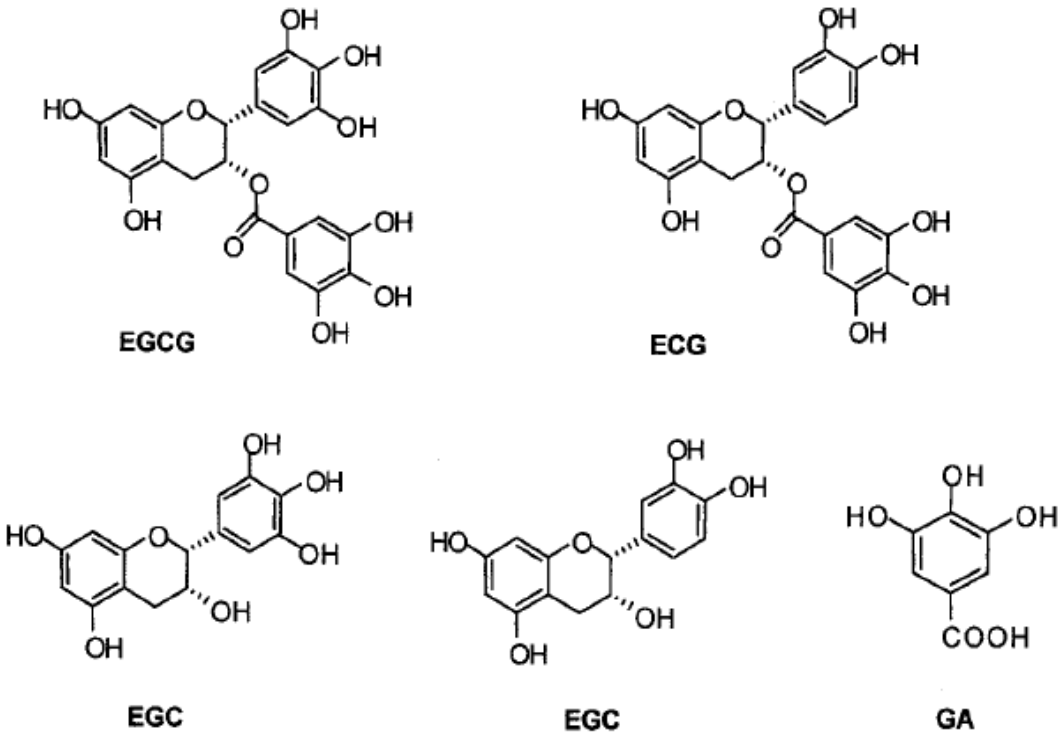

Figure 12. Chemical structure of gallic acid and the four major catechins in green tea. GA, gallic acid; EGCG, (-)-epigallocatechin-3-gallate; EGC, (-)-epigallocatechin; ECG, (-)-epicatechin-3- gallate; EC, (-)-epicatechin.
Source: Cabrera *et al.* (2006)

Tannins, the relatively high molecular weight compounds, which constitute as the third important group of phenolics may be subdivided into hydrolysable tannin, which is ester of gallic acid and condensed tannins, are polymers of polyhydroxy flavanol monomers (Naczk and Shahidi, 2004).

The functionality of phenolic compounds is expressed through their action as an inhibitor or an activator for a large variety of mammalian enzyme system and as metal chelators as well as scavengers of free radicals (Pringent, 2005; Rice-Evans *et al.*, 1997; Shahidi *et al.*, 2007). Phenolic compounds have been associated with the health benefits derived from consuming high levels of fruits and vegetables, because of their antioxidant activity (Balasundram *et al.*, 2006; Sellappan *et al.*, 2002) and could therefore be a natural source of antioxidants.

1.2.6.1 Extraction of phenolic compounds

The structural diversity of phenolic compounds varies from simple to highly polymerized substances that include varying proportions of phenolics, distributed randomly in plants at the tissue, cellular and subcellular levels. They may also exist as complexes with carbohydrates, proteins and other plant components (Luthria *et al.*, 2006). Extraction of phenolic compounds in plant materials is therefore influenced by their chemical nature, the extraction method employed, sample particle size, storage time and conditions as well as the presence of interfering substances (Naczk and Shahidi, 2004). Therefore, phenolic extracts of plant materials are always mixture of different classes of phenolics that are soluble in the solvent system used. Solubility of phenolic compounds is governed by the type of solvent (polarity) used, degree of polymerization of phenolics, as well as interaction of phenolics with other food constituents and formation of insoluble complexes (Robbins, 2003). Therefore, there is no uniform or completely satisfactory procedure that is suitable for extraction of all phenolics or a specific class of phenolic substances in plant materials (Luthria *et al.*, 2006). Methanol, ethanol, acetone, water, ethyl acetate and, to a lesser extent, propanol, dimethyl formamide, and their combinations are frequently used for the extraction of phenolics (Naczk and Shahidi, 2004). In some cases, mechanical means to enhance molecular interaction between the phenolic compounds and solvent are employed (Robbins, 2003). Shaking, stirring, vortex

mixing and sonication are four commonly used extraction techniques (Luthria *et al.*, 2006).

Row and Jin (2006) reported the recovery of catechin compounds from Korean tea by solvent extraction. The optimum extraction condition was 80^{o}C for 40 min in pure water. The extract was partitioned with water/chloroform (1:1 v/v), which was suited to remove caffeine impurity from the extract. The resulting extract was further partitioned with water/ethyl acetate (1:1 v/v) to deeply purify the catechin compounds. Torre *et al.* (2008) investigated the release of ferulic acid from corn cobs by alkaline hydrolysis. Optimum hydrolysis condition for hydroxy-cinnamic acids content was 0.5 N NaOH and solid/liquid ratio of 0.084 for 6 h. Perva-Uzunalic *et al*. (2006) studied the extaction efficiency of caetchins and caffeine from green tea leaves using different solvents (acetone, ethanol, methanol, acetonitrile, and water) at different temperature and times. Optimal condition for extraction of caetchin with water was obtained at 80^{o}C for 20 min. Rusak *et al.* (2008) extracted phenolics from bagged and loose leaves of white and green tea by distilled water (80^{o}C), distilled water (80^{o}C) with 5 ml of lemon juice and aqueous ethanol (10, 40 and 70 %). Addition of lemon juice could enhance the extraction of phenolic from white tea and aqueous ethanol (40 %) was most effective in the extraction of catechins. Extracction of ferulic acid and vanillin from flax shives, wheat bran and corn bran were carried out by non-pressurised alkaline hydrolysis (0.5 M NaOH) and pressurized solvents (0.5 M NaOH, water, ethanol and ammonia) (Buranov and Mazza, 2009).

1.2.6.2 Identification and characterization of phenolic compounds

In the last twenty years, the analytical technique that has dominated the separation and characterization of phenolic compounds is HPLC with reverse phase (RP) column technology (Robbins, 2003). There is no single wavelength appropriate to monitor all phenolics since they display the absorbance maxima at different wavelengths (Herrera and Luque de Castro, 2005). Other less common means of detection, coupled to LC are through electrochemical detection (EC), fluorescence (F), and MS (Robbins, 2003).

Phenolic compounds from strawberries were detected by liquid chromatography with photodiode array UV detection. The mobile phase consisted of

methanol-water (10-90, v/v) and methanol-water (50:50, v/v) both adjusted to pH 3 with acetic acid and different gradients were tested on the Hypersil ODS column. Most benzoic acid derivatives show the maximum absorbance close to 254 nm. However, gallic acid, salicylic and syringic acid have their maxima absorbance at 275, 310 and 280 nm, respectively. Chlorogenic, caffeic and *p*-coumaric acid have the maximum absorbance close to 325 nm, and ferulic acid close to 310 nm (Herrera and Luque de Castro, 2005). Ma *et al*. (2009) detected seven phenolic compounds of two families including cinnamic acids and benzoic acid from citrus peel extract using C-18 reverse phase column and elution was performed with the mobile phase consisting of 4 % (v/v) acetic acid in water: 100 % methanol (80:20, v/v) at solvent flow rate of 1 mL/min. Charrouf *et al*. (2007) studied the separation and characterization of phenolic compounds in argan fruit pulp using liquid chromatography (LC) negative electrospray ionization (ESI) mass spectroscopy (MS/MS). A C-18 (50 x 2.1 mm, i.d 3.5 μm) was used for the separation and gradient elution was performed with water/0.05% acetic acid and acetonitrile at a flow rate of 600 μL/min. Sixteen compounds were identified, mainly flavonoid glycosides and flavonoid aglycons. Parejo *et al*. (2004) separated and characterized phenolic compounds in Fennel (*Foeniculum vulgare*) using liquid chromatography-negative ionization mass spectrometry. The fennel waste was extracted by water for 15 min and then chromatographed on sephadex LH-20 to collect seven fractions, each of them subjected to LC-MS analysis and forty-two phenolic compounds were identified.

Weisz *et al*. (2009) extracted eleven phenolic compounds from defatted sunflower (*Helianthus annuus* L.) kernels and shells and characterized by HPLC with diode array and electrospray ionization (ESI) mass spectrometric detection in negative mode. The column used was 150mm x 3.0mm inner diameter, 4 μm particle size, C18 Hydro-Synergi. The mobile phase consisted of 2 % (v/v) acetic acid in water (eluent A) and of 0.5 % acetic acid in water and acetonitrile (50:50, v/v, eluent B) with flow rate of 0.4 mL/min. UV-Visable spectra were recorded in the range of 200-600 nm. Five phenolic compounds, namely gallic acid, procyanidin B2, (-)-galllocatechin, (-)-epicatechin, and (-)-epicatechin-3-gallate were identified from 50 % ethanol extract of litchi (*Litchi sinensis* Sonn.) seeds, after application of reverse phase high performance liquid chromatography, coupled to a diode array detector and

electrospray ionization mass spectra (Prasad *et al*., 2009). The samples were eluted with gradient system consisting of solvent A (2 % acetic acid, v/v) and solvent B (acetonitrile:methanol, 10:15, v/v), with a flow rate of 1 mL/min from C-18 column (250 x 4.6 mm, 5 μm particle size). UV-visible absorption spectra were recorded from 200 to 600 nm during HPLC analysis.

1.2.6.3 Antioxidant activity of phenolic compounds

The chemical activity of phenols in terms of their reducing properties as hydrogen or electron donating agents predicts their potential for action as antioxidants (Rice-Evans *et al.*, 1997). Radical-scavenging activity (1,1-diphenyl-2-picrylhydrazyl method) and total antioxidant activity (phosphomolybdenum method) of grape (*Vitis vinifera*) seed extracts of acetone:water:acetic acid (90:9.5:0.5) and methanol:water:acetic acid (90:9.5:0.5) were determined at 25 and 50 ppm concentration. It was found that acetone:water:acetic acid (90:9.5:0.5) extract showed the higher radical scavenger than methanol:water:acetic acid (90:9.5:0.5) extract (Jayaprakasha *et al.*, 2003). Negi *et al.* (2005) successively extracted seabuckthorn (*Hippophae rhamnoides* L.) seeds with chloroform, ethyl acetate, acetone and methanol using soxhelt extractor for 8 h each. The reducing power and antioxidant activities of natural crude methanol extract evaluated using 1,1-diphenyl-2-picrylhydrazine and liposome model system were the highest.

Shahidi *et al*. (2007) evaluated the antioxidant activity in hazelnut kernel (*Corylus avellana* L.) and hazelnut byproduct ethanol extract. Extracts of hazelnut byproducts (skin, hard shell, green leafy cover, and tree leaf) exhibited the stronger antioxidant activities than hazelnut kernel at all concentrations tested. Hazelnut extracts showed different antioxidative efficacies, related to the presence of phenolic compounds. Among samples, extracts of hazelnut skin, in general, showed superior antioxidative efficacy and higher phenolic content as compared to other extracts. Prasad *et al.* (2009) reported that 50 % ethanol extract of *Litchi sinensis* Sonn. seeds showed total antioxidant capacity, scavenging the 1,1-diphenyl-2-picryl hydrazyl radical and inhibitory activity against lipid peroxidation, at all concentrations tested (25- 100 μg/mL) and it was comparable to the activity of the synthetic antioxidant, butylated hydroxyl toluene. Yerlikaya *et al.* (2009) reported that

natural grape seed extract or garlic extract (6 %) in batter coating of shrimp was able to lower lipid oxidation during frozen storage.

Green tea extract prepared with hot water showed high antioxidant activity for an oil-in-water emulsion at pH 5.5 during prolonged storage (40 days). Myricetin showed the higher contribution to the antioxidant activity than EGCG and ECG (Roedig-Penman and Gordon, 1997). The Tungting oolong tea (Taiwanese tea) together with Chinese green tea effectively suppressed the prooxidant activities of the dark meat and skin of blue sprat assessed by peroxide value and carbonyl value during refrigeration (Seto *et al.*, 2005). The antioxidant activity of Taiwanese tea extract showed positive correlation with the total catechins, especially with that of EGCG but a weak correlation with the contents of total polyphenols (Seto *et al.*, 2005). Based on TEAC, FRAP and hypochlorite scavenging activity, the odrer of phenolics was: procyanidin dimmer > flavanol > flavonol > hydroxycinnamic acids > simple phenolic acids (Soobrattee *et al*., 2005). Gallic acid and rosmarinic acid were the most potent antioxidants among the simple phenolic and hydroxycinnamic acids (Soobrattee *et al.,* 2005). Theaflavins in black tea and caetchins in green tea are equally effective antioxidants, when tested in Cu^{2+} mediated LDL oxidation (Leuang *et al.*, 2001).The presence of ferulic acid or similar phenolic compounds reduced the free radical damage in neural cell (Kanski *et al*., 2002). Ferulic acid showed high scavenging activity for hydrogen peroxide, superoxide, hydroxyl radical and nitrogen dioxide radicals dut to the resonance stabilized phenoxy radical (Ou and Kwok, 2004).

1.2.6.4 Antimicrobial activity of phenolic compounds

Phenolic compounds might disrupt the cell wall of microorganism by forming complex with proteins in cell wall and make lyses of cell wall (Chanthachum and Beuchat, 1997). The treatment with 5 % kiam wood extract containing tannic acid retarded the rate of growth of *Listeria monocytogenes*, aerobic mesophiles and psychrophilic micro-organism naturally present on cabbage (Chanthachum and Beuchat, 1997). Among chloroform, ethylacetate, and n-butanol extracts of artichoke (*Cynara scolymus* L.) leaf extracts, the n-butanol fraction exhibited the most significant antimicrobial activities against seven bacteria species, four yeasts, and four molds. The minimum inhibitory concentrations of these compounds were between 50

and 200 µg/mL (Zhu *et al.*, 2004). Jayaprakasha *et al*. (2003) found that the gram positive bacteria were completely inhibited at 850-1000 ppm, while gram negative bacteria were inhibited at 1250-1500 ppm concentration of grape (*Vitis vinifera*) seed extract. Study on antibacterial activity of seabuckthorn (*Hippophae rhamnoides* L.) seeds extracted with chloroform, ethyl acetate, acetone and methanol using soxhelt extractor for 8 h each revealed that methanol extracts showed the maximum antibacterial activity (Negi *et al*., 2005). The MIC values, with respect to methanol extract for *Bacillus cereus, Bacillus coagulans, Bacillus subtilis, Listeria monocytogenes, Yersinia enterocolitica*, were found to be 200, 300, 300, 300, and 350 ppm, respectively. The phenolic compounds extracted from the table olive from Portugal (100mg/mL) showed antimicrobial activity against gram-positive, gram-negative and fungi (Pereira *et al.*, 2006).

Finnish berry extracts containing flavonoids and phenolic acids inhibited the growth of gram-negative but not gram-positive bacteria. The extract from sea buckthorn berry and blackcurrant showed the least inhibitory activity against gram-negative bacteria. Cloudberry, raspberry and strawberry extracts were strong inhibitors of *Salmonella* (Puupponen-Pimia *et al*., 2001). Aqueous extract of tea (*Camellia sinensis*) containing epigallocatechin, epigallocatechin gallate and epicatechin gallate inhibited wide range of pathogenic bacteria including methicillin-resistant *Staphylococcus aureus* (Yam *et al*., 1997). In black tea extracts, theaflavin and its gallates are antibacterial active components (Yam *et al*., 1997). Aqueous extract of pu-erh tea (2 mg/mL) containing caffeine and epicatechin showed potential antimicrobial effect on gram-positive bacteria than that of gram-negative bacteria (Wu *et al*., 2007). The ethanolic extract of green tea significantly inhibited the spoilage microflora and certain pathogens of acidulant treated mutton (pH 3.8) for up to 4 days at 25 ± 2^{o}C and 85 ± 5 % RH (Kumudavally *et al.*, 2008). The antimicrobial mechanism of ferulic acid was attributed to its inhibition of arylamine *N*-acetyltransferase in the bacteria (Lo and Chung, 1999).

1.2.6.5 Antibrowning activity of phenolic compounds

Aromatic carboxylic acids of cinnamic acid and its analogues, *p*-coumaric, ferulic, and sinapic acids are competitive inhibitors of PPO (Kim *et al.*,

2000). Some phenolic compounds inhibit PPO activity by interacting with active site of the enzyme (Janovitz-klapp *et al.*, 1990). Furthermore, phenolic compounds could interact with protein or enzymes via hydrogen bond or hydrophobic interaction (Prigent, 2005). Chen *et al*. (1991a) found that kojic acid showed a mixed-type inhibition for white shrimp, grass prawn, and lobster polyphenoloxidase. Among the various phenolic acids tested, kojic acid showed the highest inhibitory effect on browning in apple slices (Son *et al.*, 2001). Cuminaldehyde (*p*-isopropylbenzaldehyde) was identified as potent mushroom tyrosinase inhibitor (ID_{50} =7.7 μg/mL) in cumin (Kubo and Kinst-Hori, 1998). Kubo *et al*. (2003) reported that 1.55 mM dodecyl gallate exhibited the inhibitory activity towards mushroom tyrosinase, in which 50 % activity loss was obtained. Prasad *et al*. (2009) identified gallic acid, procyanidin B2, (-)-gallocatechin, (-) - epicatechin, and (-)-epicatechin-3-gallate in extract of *Litchi sinensis* Sonn. seeds. This litchi seed extract showed inhibitory activity of tyrosinase in a concentration dependent manner (25-100 μg/mL). Gokoglu and Yerlikaya (2008) found that shrimp (*Parapenaeus longirostris*) treated with ethanol extract of grape seed (*Vitis vinifera* sp.) at concentration of 1.5 % had the lowered the melanosis formation during storage at 4^{o}C for 3 days. Stilbenes are C6-C2-C6 compounds, found in nature as monomers and oligomers. They possess inhibitory activity towards mushroom tyrosinase (Likhitwitayawuid, 2008). Jang *et al*. (2003) reported that shrimp (*Trachypenaeus curvirostris*) treated with 70% acetone extract of enokitake mushroom (*Flammulina velutipes*) (2.5 g wet enokitake/mL, test sample) had the delayed darkening at 24^{o}C for 20 h as compared to control. The hot water extract prepared from fruiting body of the edible mushroom (*Flammulina velutipes*) contained ergothioneine (ERT) at a level of 2.05 mg/mL. The dietary supplement of mushroom extract in kuruma shrimp (*Marsupenaeus japonicus*) could reduce post mortem development of melanosis by suppressing PPO activity in the hemolymphs of the shrimp (Encarnacion *et al.*, 2010).

1.2.7 Mimosine

Mimosine is a non-protein amino acid called (β-(3-hydroxy-4-pyridon-1-yl)-L-alanine) (Lalitha and Kulothungan, 2006). Mimosine is an analogue of the tyrosine (Figure 13) (Echeverria *et al*, 2002).

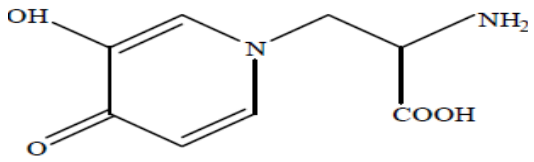

Figure 13. Structure of Mimosine.
Source: Champanerkar *et al*. (2010)

Mimosine is highly concentrated in the seed and foliage of two legume genera, Mimosa and *Leucaena* (Serrano *et al*., 1983). Lead tree, *Leucaena leucocephala*, is belonging to a tropical and subtropical legume family. It has been used as livestock feed because of their high content of protein, carotenoids, vitamin k, xanthophylls and minerals (Kamada *et al.*, 1997). Phenolics found in different parts of *Leucaena* were condensed tannin (Echeverria *et al.*, 2002), quercetin and myricetin glycosides (Lowery *et al.*, 1984), gallocatechin, epigallocatechin and epicatechin (Erickson *et al*., 2000). Additionally, seeds and leaves of lead are consumed as human foods (Sahlu *et al*., 1995). The seeds of guaje (*L. esculenta*) are eaten with salt in Mexico.

Mimosine is a water soluble amino acid (Shiroma and Hongo, 1981). Different parts of the *Leucaena* contain varying amounts of mimosine, 2-10 % of dry leaf, 2-5 % of dry seed (Lalitha *et al*., 1993) and 1-1.5 % of root (Soedarjo *et al*., 1994). It is less soluble in methanol and ethanol, insoluble in other organic solvent, and soluble in dilute acid and base (Champanerkar *et al*., 2010). The concentration of mimosine is higher in the seeds than in other parts of the plant, second only to the immature tender leaves (Chanchay and Poosaran, 2009). Mimosine could be removed by prolonged soaking of leaves or seeds in water at 30^{o}C (Puchala *et al.*, 1995). About

90 % of mimosine could be extracted by soaking the leaves in freshwater for 36 h (Chanchay and Poosaran, 2009). Kamada *et al*. (1997) extracted mimosine from brown ripe seed powder of *L. leucocephala* by using boiling water for 1 h with a seed powder/ water ratio of 1:7 (g/mL). The equivalent amount of ethanol was added to precipitate impurities before the solution was cooled and then subjected to filtration. The filtrate were stored at freezer (-18^{o}C) for 8 h and precipitated impurities were removed by centrifugation. The remaining supernatant were concentrated by rotary evaporator and used as crude mimosine (Kamada *et al*., 1997). Drying leaves of *L. leucocephala* at 60^{o}C for 24 h and soaking in water for 72 h at room temperature could remove 94.7 % of mimosine (Chanchay and Poosaran, 2009). The fresh leaves of *L. leucocephala* and dry seed powder were mixed with boiling water with seed powder/ water ratio of 1:10 (g /mL) for 5 min. After cooling, an equal amount of 0.2 M HCl was added and mimosine was extracted by homogenization and centrifugation at 15000 rpm for 20 min (Lalitha *et al*., 1993). The decolorisation of extract was carried out by boiling with activated charcoal for 15 min. The solution was cooled and filtered.

The mimosine was extracted from *Mimosa pudica* Linn. whole plant powder using 1 % HCl in water (Champanerkar *et al.*, 2010). Whole plant powder (25 mg) was mixed with 10 mL of methanol and shaken for 60 min at room temperature. After filtration of mixture by using Whatmann No. 41 filter paper, residue was collected and treated with 1 % HCl (10 mL). The solution was vortex for 3 min and kept at room temperature for overnight. The content was filtered and clear supernatant were used as crude mimosine extract (Champanerkar *et al.*, 2010). Mimosine was extracted by griding the plant (*L. leucocephala*) tissue in 0.1N HCl for 1 min at 5^{o}C (Vestena *et al*., 2001). Many of the extraction procedure reported in the literature were carried out by using boiling water and in the presence of HCl. However, the heating process and used of HCl degraded native mimosine in to 3-hydroxy-4-(1H) pyridine (DHP), a degradative product (Lalitha *et al.*, 1993).

1.3 Objectives

1. To determine the inhibitory effect of ferulic acid (FA) or catechin on Pacific white shrimp (*Litopenaeus vannamei*) polyphenoloxidase (PPO) and the impact of FA or catechin treatment on melanosis inhibition and quality changes of shrimp during iced storage.
2. To study the effect of multiple freeze-thaw cycles on PPO activity and melanosis in white shrimp during extended refrigerated storage and the prevention of melanosis by ferulic acid and catechin.
3. To study the PPO inhibitory activity of extracts from green tea and lead seed and to identify the compounds exhibiting PPO inhibition in both extracts.
4. To investigate the synergetic effect of green tea extract with ascorbic acid on inhibition of PPO and melanosis and quality changes of Pacific white shrimp during storage in ice and under modified atmosphere packaging (MAP).
5. To determine the biochemical properties of polyphenoloxidase from cephalothorax of Pacific white shrimp.
6. To investigate the inhibition mechanism of catechin, ferulic acid and mimosine on PPO from cephalothorax of Pacific white shrimp.

CHAPTER 2

EFFECTS OF FERULIC ACID ON INHIBITION OF POLYPHENOLOXIDASE AND QUALITY CHANGES OF PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*) DURING ICED STORAGE

2.1 Abstract

Effects of ferulic acid (FA) on polyphenoloxidase (PPO) and the quality changes of Pacific white shrimp *(Litopenaeus vannamei)* during iced storage of 10 days were investigated. Both FA and oxygenated FA (OFA) with different concentrations (0.1, 0.5, 1 and 2 % (w/v)) showed PPO inhibitory activity in the dose dependent manner. FA was generally more effective in PPO inhibition than was OFA. Based on activity staining, white shrimp PPO with an apparent molecular weight of 210 kDa was inhibited by FA. When whole shrimp were treated with FA solution with concentrations of 1 or 2% and stored in ice for up to 10 days, the increase in psychrotrophic and mesophilic bacterial count were retarded, in comparison with the control and those treated with 1.25% sodium metabisulfite (SMS). The coincidental lower rates of increase in pH and total volatile base content were obtained. Additionally, shrimp treated with 2% FA possessed the lowest peroxide value and thiobarbituric acid reactive substances (TBARS) value during the storage. After 10 days of storage, shrimp treated with 2% FA had the lower melanosis score and higher score for color, flavor and overall likeness, compared with the control and SMS treated shrimp ($P < 0.05$).

2.2 Introduction

Pacific white shrimp (*Litopenaeus vannamei*) accounts for 90% of the global aquaculture shrimp production. Thailand is the world's leading shrimp-farming country and has become the top supplier of farmed shrimp to the United States and

Japan (Wyban, 2007). Despite their delicacy, shrimp are highly perishable with the limited shelf-life, mainly associated with melanosis (discoloration) and microbial spoilage (Gokoglu and Yerlikaya, 2008). Melanosis is triggered by a biochemical mechanism which oxidizes phenols to quinones by polyphenoloxidase (PPO) (Kim *et al.*, 2000). This is followed by non-enzymatic polymerization and autooxidation of the quinones, giving rise to dark pigments of high molecular weight (Benjakul *et al.*, 2005a). Although melanosis (black spots) seems to be harmless to consumers, it drastically reduces the product's market value, consumer's acceptability and hence occasioning considerable financial loss (Montero *et al.*, 2001b). Lopez-Caballero *et al*. (2007) reported that melanosis and spoilage were retarded during frozen storage, but continued in defrosted shrimp. Apart from melanosis, lipid oxidation is another deteriorative reaction causing the unacceptability of fish and shrimp products. Fish lipids are susceptible to oxidation owing to the high levels of polyunsaturated fatty acid; this can be initiated by autoxidation, enzymatic reaction like lipoxygenase, peroxidase and microbial enzymes. Lipid oxidation causes physicochemical changes, rancidity and off-flavors in fish meat (Bak *et al*., 1999).

To extend the shelf-life of shrimp or crustaceans, melanosis inhibitors have been used. Sulfites and their derivatives are widely used as PPO inhibitor by preventing the polymerization of quinones, combining irreversibly with them, and forming colorless compounds (Montero *et al*., 2001b). However, sulfiting agents are known to produce allergic reactions and serious disturbances in asthmatic subjects (DeWitt, 1998). Therefore safe compounds from natural origin such as ascorbic acid, kojic acid (Chen *et al*, 1991a), ficin (Taoukis *et al*., 1990), citric acid (Montero *et al*., 2001b), dodecyl gallate (Kubo *et al*., 2003) and oxalic acid (Son *et al*., 2000) have been used as the substitutes of sulfiting agents.

Plant phenolic compounds are another promising agents possessing antimicrobial and antioxidant activities (Chanthachum and Beuchat, 1997; Souza *et al*., 2008). Plant phenolic compounds such as flavonoid compounds, tocopherols, coumarins and cinnamic acid derivatives have an antioxidative effect (Jayaprakash *et al.*, 2001). Recently, it has been reported that the grape seed extract could inhibit the melanosis in shrimp (*Parapenaeus longirostris*) (Gokoglu and Yerlikaya, 2008). From our previous study, different phenolic compounds including ferulic acid exhibited the

different inhibitory effects on the white shrimp PPO (data not shown). In addition to melanosis inhibition, plant phenolic compounds may act as antimicrobial and antioxidant, which could retard the microbial and chemical spoilage of shrimp. Nevertheless, no information regarding the use of selected phenolic compound on melanosis prevention as well as shelf-life extension of white shrimp has been reported. The aim of this study was to investigate inhibition effect of ferulic acid on PPO, the formation of melanosis as well as chemical, microbial and sensorial changes of Pacific white shrimp (*Litopenaeus vannamei*) during iced storage.

2.3 Materials and Methods

2.3.1 Chemicals

L-β-(3, 4 dihydroxylphenyl) alanine (L-DOPA), Brij-35, ferulic acid, malonaldehyde bis (dimethyl acetal), thiobarbituric acid (TBA), ferrous chloride and ammonium thiocyanate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), sodium metabisulfite, ammonium sulfate, methanol, ethanol, chloroform, anhydrous sodium sulfate and standard plate count agar (PCA) were obtained from Merck (Darmstadt, Germany). Sodium dedocyl sulfate (SDS), *N*, *N, N′ N′*-tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA).

2.3.2 Shrimp collection and preparation

Pacific white shrimp (*Litopenaeus vannamei*) with the size of 55-60 shrimp/kg were purchased from the dock in Hat Yai, Songkhla, Thailand. The shrimp were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, shrimp were washed in cold water and stored in ice until used (not more than 5 h).

2.3.3 Preparation of PPO extract from the cephalothoraxes of Pacific white shrimp

The cephalothoraxes of twenty shrimp were separated, pooled and powderised by grinding with liquid nitrogen in a Waring blender. The powder obtained was kept in polyethylene bag and stored at -20 °C for not more than 2 weeks. The isolation of PPO was carried out according to the method of Simpson *et al*. (1987) with a slight modification. The powder (50 g) was mixed with 150 mL of the extracting buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 1.0 M NaCl and 0.2% Brij 35). The mixture was stirred continuously at 4^{o}C for 30 min, followed by centrifugation at 8000 x g at 4^{o}C for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). Solid ammonium sulfate was added into the supernatant to obtain 40% saturation and allowed to stand at 4^{o}C for 30 min. The precipitate was collected by centrifugation at 12,500 x g at 4^{o}C for 30 min using a refrigerated centrifuge. The pellet obtained was dissolved in a minimum volume of 0.05 M sodium phosphate buffer, pH 7.2 and dialyzed against 15 volumes of the same buffer at 4^{o}C with three changes of dialysis buffer. The insoluble materials were removed by centrifugation at 3000 x g at 4^{o}C for 30 min and the supernatant was used as "crude PPO extract".

2.3.4 Measurement of PPO activity

PPO activity was assayed using L-DOPA as a substrate according to the method of Simpson *et al*. (1987) with a slight modification. The assay system consisted of 100 μL of crude PPO extract, 600 μL of 15 mM L-DOPA in deionized water, 400 μL of 0.05 M phosphate buffer, pH 6.0 and 100 μL of deionized water. The PPO activity was determined for 3 min at 45^{o}C by monitoring the formation of dopachrome at 475 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of PPO activity was defined as an increase in the absorbance at 475 nm by 0.001/min. Activity was expressed as unit/mL. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture and the deionized water was used instead.

2.3.5 Effect of ferulic acid on the inhibition of Pacific white shrimp PPO

2.3.5.1 Preparation of ferulic acid (FA) and oxygenated ferulic acid (OFA) solutions

Ferulic acid solutions with different concentrations (0.2, 1, 2 and 4%, w/v; 50 mL) were adjusted to pH 8 by 6 N NaOH and stirred for 15 min at room temperature (26-28^{o}C). Thereafter, the pH of the solution was adjusted to 7 by using 6 N HCl and referred to as 'ferulic acid solution' (FA).

Oxygenated ferulic acid solution (OFA) was prepared by the modified method of Balange and Benjakul (2009). Ferulic acid solutions at different concentrations (0.2, 1, 2 and 4%, w/v; 50 mL) were adjusted to pH 8 using 6 N NaOH or 6 N HCl. The prepared solution was placed in a temperature-controlled water bath (40^{o}C) and subjected to oxygenation for 1 h by bubbling the solution with oxygen (TTS Gas Agency, Hat Yai, Thailand) to convert the FA to quinone. After being oxygenated for 1 h, the solution was then adjusted to pH 7 by using 6 N HCl and was referred to as 'oxygenated ferulic acid solution' (OFA).

2.3.5.2 Inhibitory effect of FA and OFA on PPO activity

FA or OFA with different concentrations (0.2, 1, 2 and 4% w/v) (100 µL) were mixed with crude PPO extract (100 µL) to obtain the final concentrations of 0.1, 0.5, 1 and 2% (w/v), respectively. This reaction mixture was incubated for 30 min at room temperature. Then, the assay buffer (400 µL) was added. To initiate the reaction, 600 µL of pre-incubated 15 mM L-DOPA (45^{o}C) were added. The reaction was conducted at 45^{o}C and the absorbance at 475 nm was monitored for 3 min. The control was run in the same manner, except deionized water was used instead of FA or OFA. Residual activity was determined and expressed as the activity relative to the control (without FA or OFA) as follows:

$$\text{Relative activity (\%)} = \frac{B}{A} \text{ X } 100$$

where A: PPO activity of control; B: PPO activity in the presence of FA or OFA.

2.3.6 SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and PPO activity staining

Protein patterns of PPO crude extract were analyzed by SDS–PAGE according to the method of Laemmli (1970). The extract was mixed with the sample buffer containing 1.5 M ME at a ratio of 1:1 (v/v). The samples (25 μg protein) were loaded onto the polyacrylamide gel made of 7.5% running gel and 5% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, one of two identical gels was immersed in a McIlvaine buffer (0.2 M sodium phosphate and 0.1 M sodium citrate), pH 6.5, containing 15 mM L-DOPA for 25 min at 25^{o}C. The activity zone appeared as the dark band. Another gel was stained by 0.125% Coomassie Brilliant Blue R-250 and de-stained in 25% methanol and 10% acetic acid. To estimate the molecular weight of PPO, the markers including myosin from rabbit muscle (200 kDa), -galactosidase from *E. coli* (116 kDa), phosphorylase b from rabbit muscle (97 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase from bovine liver (55 kDa), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (36 kDa), and carbonic anhydrase from bovine erythrocytes (29 kDa) were used.

To study the inhibitory effect of FA towards PPO, crude PPO extract was incubated with FA (1, 2 and 4%) at a ratio of 1:1 (v/v) for 30 min at room temperature prior to loading onto polyacrylamide gel, followed by activity staining as previously described.

2.3.7 Protein Determination

Protein concentration was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as the standard.

2.3.8 Effect of FA on the quality of Pacific white shrimp during iced storage

2.3.8.1 Preparation of shrimps treated with FA

Whole Pacific white shrimp were immersed in FA solution (1 and 2%) at a shrimp / solution ratio of (1:2, w/v) at 4°C for 15 min. Another portion of shrimp was soaked in sodium metabisulfite (1.25%) at a ratio of 1:2 (w/v) (Kim *et al.*, 2000) for 1 min at 4°C. Treated shrimps were drained on the screen for 3 min at 4°C. Shrimp without any treatment were used as the control. All samples were stored in polystyrene box containing ice using a shrimp/ice ratio of 1:2 (w/w). To maintain shrimp/ice ratio, the molten ice was removed and the same amount of ice was added. Samples (25 shrimp) were taken for each treatment every 2 days up to 10 days for chemical, microbiological, sensorial analysis and melanosis determination.

2.3.8.2 Microbiological analysis

Microbiological analysis was performed following the method of Thepnuan *et al*. (2008) with some modifications. Five whole Pacific white shrimp were collected aseptically and used as the composite sample. Ground sample (without peeling) (25 g) was placed in a stomacher bag containing 225 mL of 0.85 % saline water. After mixing for 1 min in a Stomacher blender (M400, Seward, UK), appropriate dilutions were prepared for the determination of total viable counts (TVC) using plate count agar (PCA) containing 0.5% NaCl by using spread plate method. Mesophilic and psychrotrophic bacterial counts were determined by incubating plates at 35°C for 2 days and 4°C for 7 days, respectively.

2.3.8.3 Chemical analyses

2.3.8.3.1 pH measurement

pH measurement was performed by the method of Lopez-Caballero *et al*. (2007) with a slight modification. Shrimp meat (2 g) was homogenized with 10 volumes of deionized water for 1 min and the homogenate was kept at room temperature for 5 min. The pH was determined using a pH-meter (Sartorious North America, Edgewood, NY, USA).

2.3.8.3.2 Determination of total volatile base and trimethylamine contents

Total volatile base (TVB) and trimethylamine (TMA) contents in shrimp meat were determined using the Conway micro-diffusion method (Conway and Byrne, 1936). Sample (2 g) was extracted with 8 mL of 4% (w/v) trichloroacetic acid (TCA) solution. The mixture was homogenized at 8,000 rpm for 1 min using PT 2100 homogenizer (Kinematica AG, CH-6014, Littau/Luzern, Switzerland). The homogenate was kept at room temperature for 30 min. The homogenate was filtered through Whatman No.41 filter paper (Schleicher & Schuell, Maidstone, England). The filtrate was collected and the final volume was adjusted to 10 mL using 4% TCA. The inner ring solution (1 mL) and filtrate (1 mL) were added to inner ring and outer ring of the Conway unit, respectively. One mL of saturated K_2CO_3 solution was then added into outer ring. The Conway unit was closed and the solution was mixed slowly. The mixture was incubated at 37°C for 60 min and the inner ring solution was titrated with 0.02 N HCl using micro-burette until green color turned into pink. For blank, TCA solution (4%) was used instead of sample extract. TMA content was determined in the same manner as TVB but 1 mL of 10% formaldehyde was added to the filtrate to fix ammonia present in the sample. The amounts of TVB and TMA were calculated and results were expressed as mg N/ 100 g shrimp meat.

2.3.8.3.3 Determination of peroxide value

Lipid was extracted by the method of Bligh and Dyer (1959). Sample (25 g) was homogenized with 200 mL of a chloroform: methanol: distilled water mixture (50:100:50) at the speed of 9,500 rpm for 2 min at 4^{o}C using an IKA Labortechnik homogenizer (Selangor, Malaysia). The homogenate was added with 50 mL of chloroform and homogenized at 9,500 rpm for 1 min. Then, 25 mL of distilled water were added and homogenized again for 30 sec. The homogenate was centrifuged at 3,000 rpm at 4^{o}C for 15 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The chloroform phase was drained off into the 125 mL erlenmeyer flask containing about 2-5 g of anhydrous sodium sulfate, shaken very well, and decanted into a round-bottom flask through a Whatman No.4 filter paper (Schleicher & Schuell, Maidstone, England). The solvent

was evaporated at 25^{o}C using an EYELA N-100 rotary evaporator (Tokyo, Japan) and the residual solvent were removed by flushing nitrogen.

Peroxide value was determined spectrophotometrically by ferric thiocyanate method (Wu *et al*., 2003) with a slight modification. Extracted lipids (5 µg) were added with 2.395 mL of 75 % ethanol and the mixture was vortexed until completely dissolved. Thereafter, 50 µL of 30% ammonium thiocyanate, and 50 µL of 20 mM ferrous chloride solution in 3.5% HCl were added. The mixture was mixed thoroughly and was allowed to stand for 3 min. The absorbance was read at 500 nm and peroxide value (PV) was expressed as A_{500}.

2.3.8.3.4 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS in the samples was determined as described by Benjakul and Bauer (2001) with some modifications. Ground shrimp meat (1 g) was mixed with 9 mL of a solution containing 0.375% TBA, 15% TCA and 0.25 N HCl. The mixture was heated in boiling water for 10 min, followed by cooling with the running water. The mixture was centrifuged at 4000 x g for 20 min (MIKRO20, Hettich Zentrifugan, Germany). The supernatant was collected and the absorbance was read at 532 nm using a UV- 160 spectrophotometer. TBARS value was calculated from the standard curve of malonaldehyde (0 to 2 ppm) and expressed as mg malonaldehyde /kg shrimp meat.

2.3.8.4 Melanosis assessment

Melanosis or blackening of Pacific white shrimp was evaluated through visual inspection by six trained panelists using 10-point scoring test (Montero *et al*., 2001b). Panelists were asked to give the melanosis score (0 to 10), where 0 = absent; 2 = slight (up to 20% of shrimps' surface affected); 4 = moderate (20 to 40% of shrimps' surface affected); 6 = notable (40 to 60% of shrimps' surface affected); 8 = severe (60 to 80% of shrimps' surface affected); 10 = extremely heavy (80 to 100% of shrimps' surface affected).

2.3.8.5 Sensory evaluation

At day 0 and day 10 of storage, whole shrimp without and with different treatments were placed on a stainless steel tray, covered with an aluminum foil and steamed for 5 min. The cooked samples were evaluated by 30 panelists from the Department of Food Technology with the ages of 25-35, using the 9-point hedonic scale, where 9: like extremely; 7: like moderately; 5: neither like or nor dislike; 3: dislike moderately; 1: dislike extremely (Meilgaard *et al*., 1990). Panelists were regular consumers of shrimp and had no allergyes to shrimp. All panelists were asked to evaluate for color, odor, taste, flavor and overall likeness. Samples were presented unpeeled in plates coded with three-digit random numbers.

2.3.9 Statistical analyses

All experiments were performed in triplicate and a completely randomised design (CRD) was used. Analysis of variance (ANOVA) was performed and means comparisons were done by Duncan's multiple range tests (Steel and Torrie, 1980). For pair comparison, T-test was used. Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA).

2.4 Results and Discussion

2.4.1 Effect of ferulic acid on the inhibition of PPO

The effect of ferulic acid (FA) and oxygenated FA (OFA), on the inhibition of PPO activity is shown in Figure 14. For both FA and OFA, the increased inhibition of PPO was observed with increasing concentrations ($P < 0.05$). At the same concentration used, FA exhibited a higher inhibitory activity towards PPO than did OFA counterpart ($P < 0.05$). Nevertheless, similar inhibitory activity was noticeable at 2% ($P > 0.05$). Quinone prevalent in OFA might loss the reducing power, in which DOPA quinone formed induced by PPO could be reduced to DOPA. Quinone has been reported to cross-link the proteins or enzymes (Balange and Benjakul, 2009). Furthermore, phenolic compounds (reduced form) could interact

with protein or enzymes via hydrogen bond or hydrophobic interaction (Prigent, 2005). Some phenolic compounds inhibit PPO activity by interacting with active site of the enzyme (Janovitz-Klapp *et al*, 1990). Among the various phenolic acids tested, kojic acid showed the highest inhibitory effect on browning in apple slices (Son *et al*., 2001). The prawn (*Penaeus japonicus*) treated with kojic acid had no melanosis up to 5 days at 4^{o}C (Montero *et al*., 2001b). The result suggested that FA was more effective in PPO inhibition in comparison with OFA. This indicated the role of hydroxyl group in PPO inhibition, possibly via its ability of electron donating to intermediate quinone or its role in cross-linking PPO via hydrogen bond, which caused the loss in PPO activity. FA might inhibit PPO by acting as a competitive inhibitor. Aromatic carboxylic acids of cinnamic acid and its analogues, *p*-coumaric, ferulic, and sinapic acids are competitive inhibitors of PPO due to their structural similarities to its phenolic substrates (Kim *et al*., 2000).

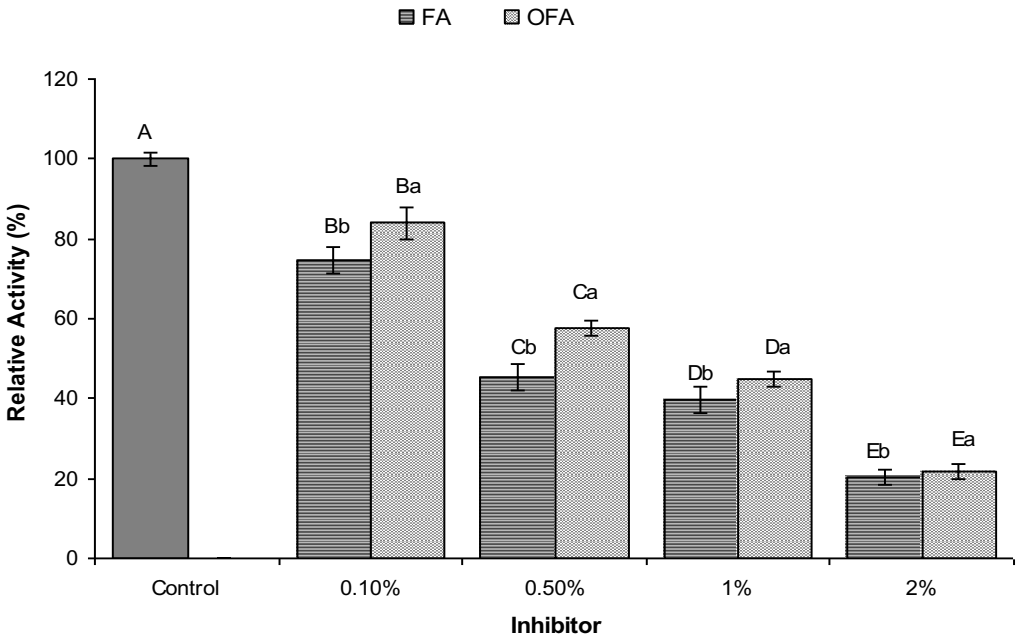

Figure 14. Effect of ferulic acid (FA) and oxygenated ferulic acid (OFA) at different levels on the inhibiton of polyphenoloxidase from the cephalothoraxes of Pacific white shrimp. Bars represent the standard deviation from triplicate determinations. Different capital letters on the bars within the same FA or OFA together with the control indicate the significant differences ($P < 0.05$). The different letters on the bars within the same level of FA and OFA indicate significant differences ($P < 0.05$).

The activity staining of PPO from the Pacific white shrimp cephalothoraxes in the absence or presence of FA is shown in Figure 15. Without FA, PPO was able to induce the oxidation of DOPA to DOPA-quinone and the intermediate products subsequently underwent polymerization to melanin. One activity zone (Lane-A) was observed as dark brown color at the apparent molecular weight of 210 kDa. In the presence of FA at levels of 0.5, 1 and 2 %, activity band intensity decreased as the concentrations increased (Lanes B C & D). These results were in accordance with *in-vitro* PPO inhibitory activity of FA (Figure 14). These results reconfirmed the inhibitory effect of FA on white shrimp PPO. PPO from different shrimp had the different iso-forms with varying molecular weights (Chen *et al*., 1991c). PPO from the kuruma prawn cephalothoraxes had the molecular weight of 160 kDa (Benjakul *et al*., 2005a). The molecular weights of pink shrimp PPO were 30 and 35 kDa, while those of white shrimp were 20 and 25 kDa (Chen *et al.*, 1997). Zamorano *et al*. (2009) reported that PPO from deep water pink shrimp (*Parapenaeus longirostris*) had the molecular weights of 500 and 200 kDa.

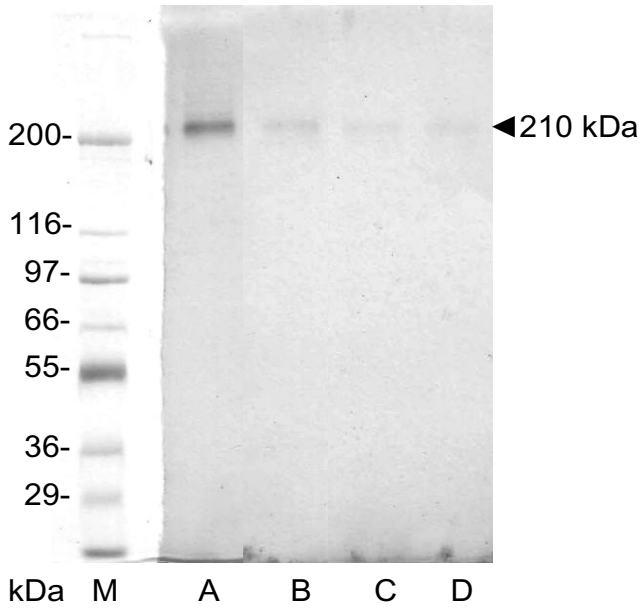

Figure 15. Activity staining of polyphenoloxidase from the cephalothoraxes of Pacific white shrimp in the absence or presence of FA at different levels. M: molecular weight marker; A: PPO crude extract; B: PPO crude extract with 0.5% FA; C: PPO crude extract with 1% FA; D: PPO crude extract with 2% FA.

2.4.2 Effect of FA on the microbiological changes of Pacific white shrimp during iced storage

Changes in psychrotrophic and mesophilic bacterial count of Pacific white shrimp during iced storage as influenced by FA treatment are shown in Figure 16A and 16B, respectively. In general, psychrotrophic bacterial count increased continuously in the control (sample without treatment) throughout the storage of 10 days ($P < 0.05$). After treatment with sodium metabisulfite (SMS), 1% FA or 2% FA, the lower psychrotrophic bacterial count was obtained, in comparison with the control ($P < 0.05$). At the same storage time, the lowest psychrotrophic bacterial count was found in shrimp treated with 2 % FA ($P < 0.05$). At the end of storage (day 10), psychrotrophic bacterial count of the control, those treated with SMS, 1% FA and 2% FA were 4.6, 4.3, 4.2 and 3.9 log CFU g^{-1}, respectively. The result indicated the antimicrobial activity of FA towards psychrotrophic bacteria in white shrimp during iced storage. FA might disrupt the cell wall of microorganism by forming complex with proteins in cell wall and make lyses of cell wall (Chanthachum and Beuchat, 1997).

Changes in mesophilic bacterial count of Pacific white shrimp without and with treatments during iced storage are depicted in Figure 16B. The decrease in mesophilic bacterial count was observed in white shrimp after the treatments with either SMS or FA ($P < 0.05$). During storage, mesophilic bacterial count increased continuously up to day 4. Thereafter, a slight increase in mesophilic bacterial count was found until the end of storage (day 10). The increase in mesophilic bacterial count within the first 4 days was due to the tolerance to cold condition of those microorganisms up to the certain limit. Zeng *et al*. (2005) reported that low temperature inhibited the micro-flora. If the cooling is not ensured for example when ice has melted in the chilling system, the micro-flora may be more active in the spoilage process. At day 4 of storage, the control, shrimp treated with SMS, 1% FA or 2% FA had mesophilic bacterial count of 5.9, 5.1, 5.1 and 4.7 log CFU g^{-1}, respectively. After 4 days of storage, some mesophilic bacteria could not tolerate to the cold temperature as indicated by the lower count.

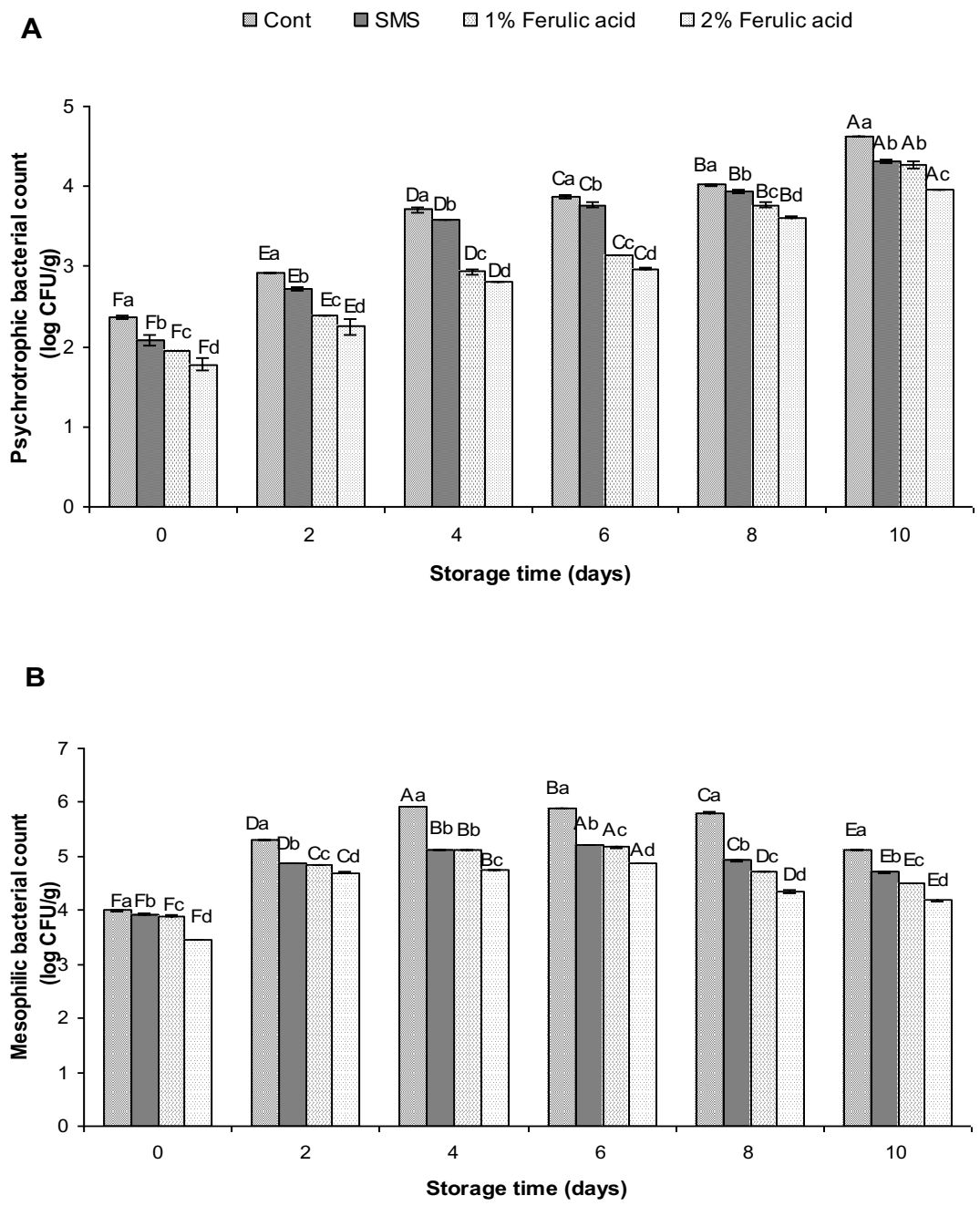

Figure 16. Psychrotrophic (A) and mesophilic (B) bacterial count of Pacific white shrimp treated with FA at different levels during 10 days of iced storage. Bars represent the standard deviation from triplicate determinations. Different capital letters on the bars within the same treatment indicate the significant differences ($P < 0.05$). The different letters on the bars within the same storage time indicate significant differences ($P < 0.05$). Cont.: control; SMS: sodium metabisulfite.

At the end of iced storage, the control, those treated with SMS, 1% FA and 2% FA had mesophilic bacterial count of 5.1, 4.7, 4.5 and 4.2 log $CFUg^{-1}$, respectively. Antimicrobial activity of phenolic compounds present in herbaceous and woody plants has not been fully defined (Scalbert, 1991). Chanthachum and Beuchat (1997) reported that treatment with 5% kiam wood extract retarded the rate of growth of *Listeria monocytogenes*, aerobic mesophiles and psychrotrophic micro-organism naturally present on cabbage.

2.4.3. Effect of FA on the chemical changes of Pacific white shrimp during iced storage

2.4.3.1 pH

Changes in pH of the Pacific white shrimp with and without different treatments during iced storage are shown in Figure 17A. pH of the fresh Pacific white shrimp at day 0 was 6.62. As the storage time increased, pH of all shrimps increased ($P < 0.05$). The increase in pH was different among the treatments. Among all samples, those treated with 2% FA had the lowest pH within the first 4 days of storage. During 6-10 days of storage, shrimp treated with 1% or 2% FA showed the lowest pH, compared with the control and those treated with SMS ($P < 0.05$). The increase in pH was associated with the accumulation of basic compounds, mainly resulted from the microbial action (Lopez-Caballero *et al*., 2007). The increases in pH value were more rapid in the shrimp (*Pandalus borealis*) stored in ice at 1.5^{o}C and reached a final pH of 8.26 as compared to sample stored in liquid ice (pH 7.98) (Zeng *et al*., 2005). Goncalves *et al*. (2003) reported that deepwater pink shrimp (*Parapenaeus longirostris*) was considered unacceptable at pH values of 7.56, 7.64 and 7.55 for air packed shrimp, ice stored shrimp and modified atmosphere packed shrimp, respectively. Shamshad *et al*. (1990) reported that *Penaeus merguiensis* was not acceptable when the pH was greater than 7.6. The lower increase in pH of shrimp treated with 1% or 2% FA was in accordance with the lower microbial count (Figure 16). These results suggested that FA might play a role in retarding microbial growth, in which the spoilage or decomposition could be lowered.

2.4.3.2 TVB and TMA contents

TVB contents of Pacific white shrimp without and with different treatments are shown in Figure 17B. The initial TVB content of Pacific white shrimp for the control (8.01 mgN/100g shrimp meat) was slightly higher than those of shrimps treated with SMS, 1%FA and 2%FA (7.64-7.66 mg N / 100g shrimp meat). During treatment, some basic decomposed compounds might be leached out to some extent. This might lead to the lower pH of treated shrimps. The increase in TVB content of the control and shrimps treated with SMS was observed as storage time increased ($P < 0.05$). Nevertheless, shrimp treated with 1 or 2 % FA had the constant TVB content up to 4 days of iced storage ($P > 0.05$). After 4 days of storage, TVB content of shrimp treated with FA also increased but at the lower rate, compared with that found in the control. At the last day of iced storage, shrimp treated with 2 % FA had the lowest TVB content (8.35 mg N/100g), compared to other treatments ($P < 0.05$). The lowest value correlated with the lower microbial counts found in FA treated shrimp. The lower TVB content of Pacific white shrimp treated with 1 or 2% FA might be owing to the inhibitory effect of ferulic acid against microbes and proteolytic enzymes. Lopez-Caballero *et al*. (2007) reported that the total base content of all deepwater pink shrimp (*Parapenaeus longirostris*) treated with resorcinol had the decreased TVB content.

At the beginning of iced storage, there was no TMA detected up to day 6 of iced storage for all samples (data not shown). TMA content of 0.14 mg/100g shrimp meat was found after 6 days of iced storage in the control and SMS treated shrimp. However no TMA was found in the shrimp treated with 1 or 2% of FA even at day 10 of iced storage. Lopez-Caballero *et al*. (2007) reported TMA-N level of 3.4 mg N /100g in deep water pink shrimp (*Parapenaeus longirostris*) after 14 days of iced storage. Zeng *et al*. (2005) showed that the initial TMA value of the shrimp (*Pandalus borealis*) was 0.5 mg N/100g at the beginning of storage and the acceptability limit for shrimp was reported to be 5 mg N /100g.

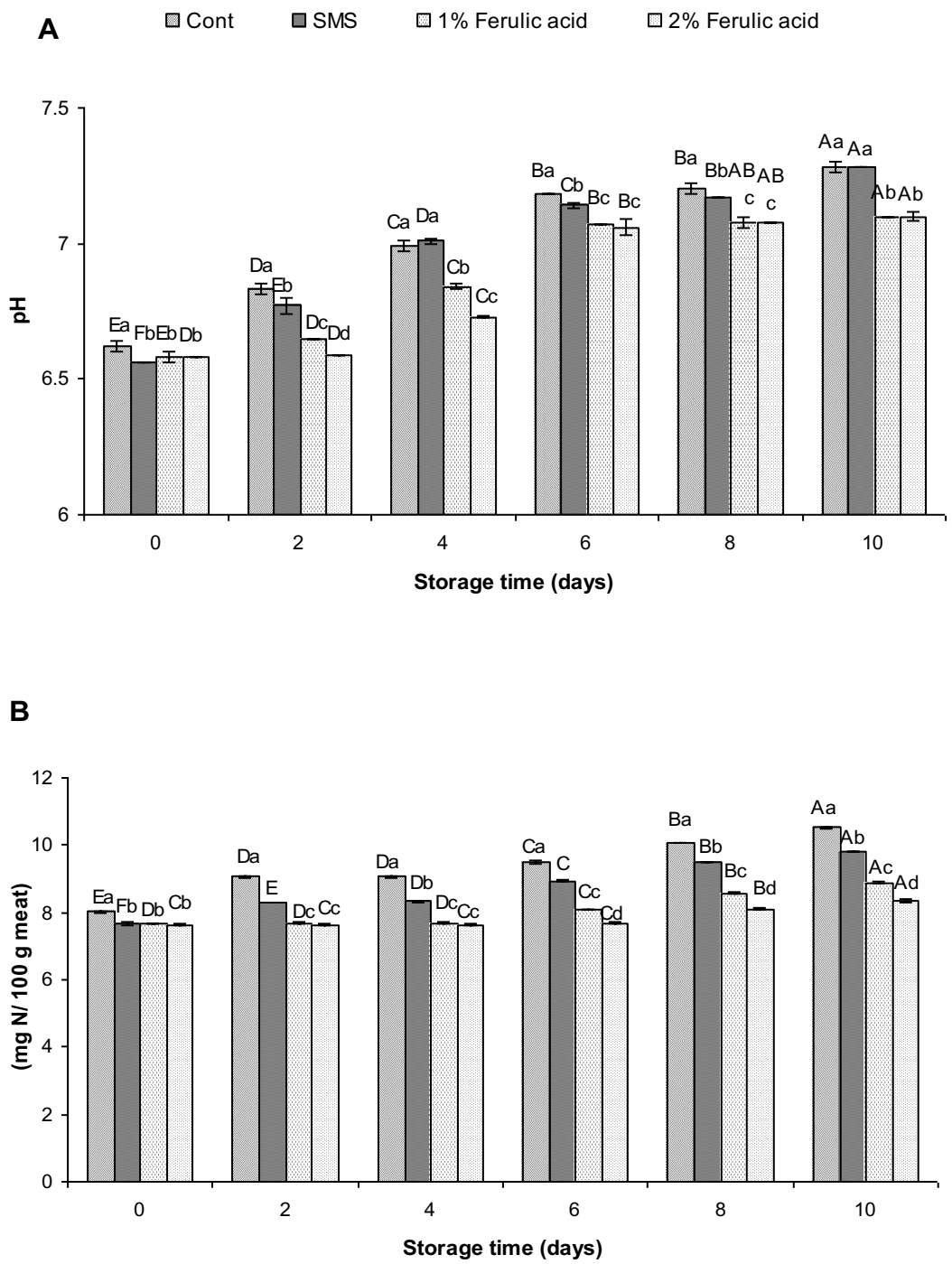

Figure 17. pH (A) and total volatile base (B) content of Pacific white shrimp treated with FA at different levels during 10 days of iced storage. Bars represent the standard deviation (n=3). Key: see Figure 16 caption.

2.4.3.3 Peroxide value and thiobarbituric acid reactive substances (TBARS) Value

Peroxide value (PV) and TBARS value of Pacific white shrimp without and with different treatments during iced storage are depicted in Figure 18. PV of all samples increased as the storage time increased ($P < 0.05$). The increase in PV suggested that fatty acids in shrimp muscle underwent oxidation during storage, in which hydroperoxide or peroxide were formed. Abstraction of hydrogen from fatty acid double bond produces fatty acid free radicals, which further react with oxygen to produce fatty acid hydroperoxide (Benjakul *et al*., 2005b). However, a slight decrease in PV was observed in all samples at day 4 of iced storage, except for SMS treated samples. The decrease in PV at day 4 was more likely due to the decomposition of hydroperoxide formed to the secondary products such as aldehydes. Nevertheless, shrimp treated with FA had the lower PV, compared to control and those treated with SMS, throughout the storage ($P < 0.05$) (Figure 18A). This indicated the antioxidative activity of FA and its preventive effects on lipid oxidation was dose dependent. In shrimp treated with 1 or 2% FA, free radicals formed might be scavenged by FA via hydrogen or electron donating mechanism. As a result, the radicals underwent lipid peroxidation to a lower level. Among all samples, shrimp treated with 2% FA showed the lowest PV ($P < 0.05$).

For TBARS value, the control sample had the continuous increase in TBARS value after 2 days of storage up to 8 days ($P < 0.05$). Thereafter a slight decrease in TBARS in all samples, except SMS treated sample, was noticed at day 10 ($P < 0.05$). Among all samples, 2% FA treated shrimp showed the lower TBARS level, compared to other treatments at all storage times ($P < 0.05$) (Figure 18B). TBARS values of 1% FA treated shrimp were lower than SMS treated shrimp at all storage times ($P < 0.05$). The increase in TBARS values were generally in accordance with the increase in PV (Figure 18A). These results revealed that Pacific white shrimp treated with 2% FA had the higher stability towards lipid oxidation than other samples. As a result, lipid oxidation in shrimp muscle could be effectively retarded. Polyphenols-rich-leaf extract from Amazonian plant acted as powerful antioxidant in human LDL protein by lowering TBARS levels (Souza *et al*., 2008). Lipid peroxidation in fish meat can be initiated by autoxidation, photosensitized oxidation

or by means of enzymatic reaction like lipoxygenase, peroxidase and microbial enzyme (Decker and Hultin, 1990). Tissue membrane of crustacean contains highly polyunsaturated fatty acid and the damage of tissues during processing can induce lipid oxidation (Morrissey *et al.*, 1998). Lipid oxidation produces off-flavors during peeling and may also be detectable in the peeled product (Bak *et al*., 1999).

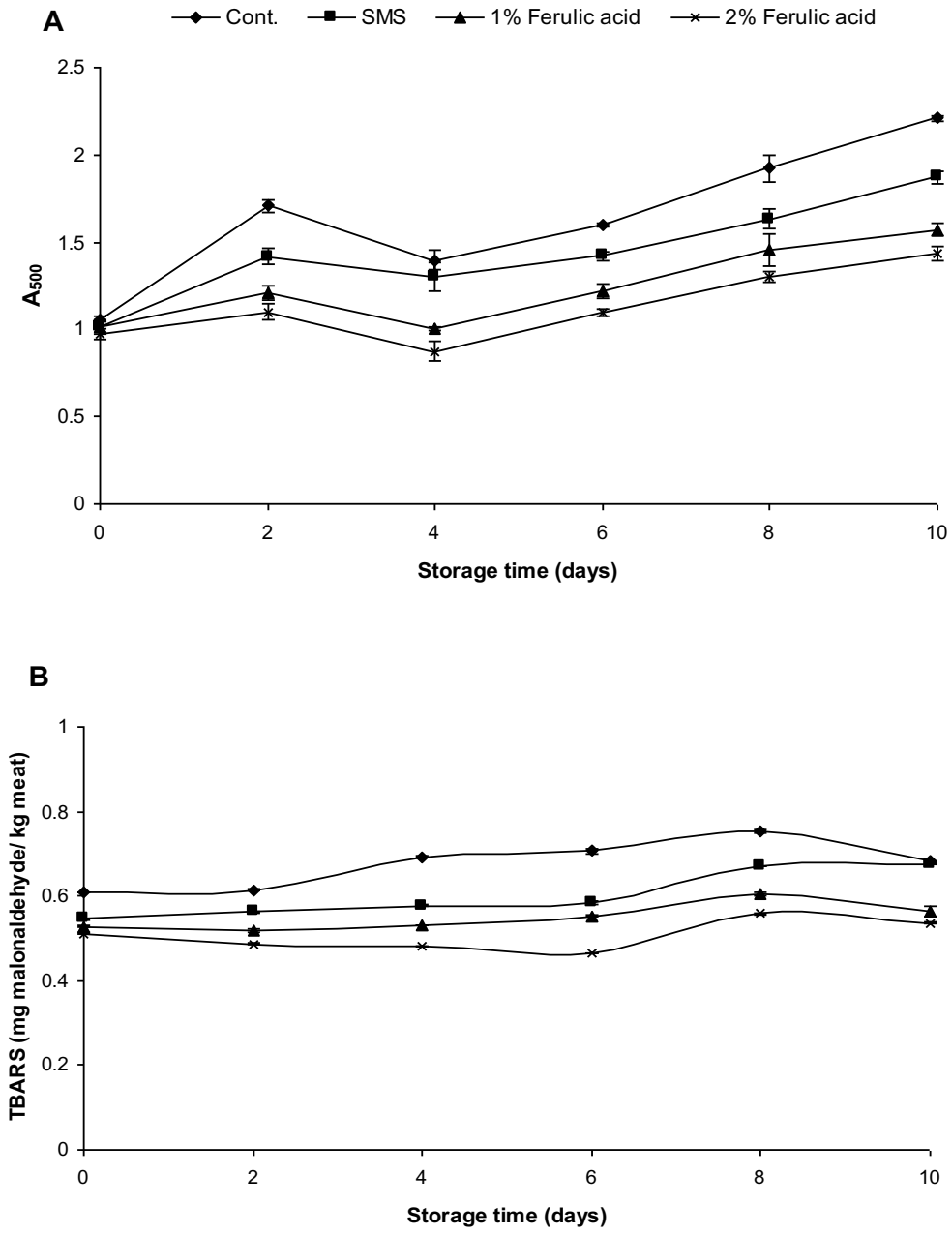

Figure 18. PV (A) and TBARS (B) values of Pacific white shrimp treated with FA at different levels during 10 days of iced storage. Bars represent the standard deviation (n=3). Key: see Figure 16 caption.

2.4.4 Effect of FA on melanosis of Pacific white shrimp during iced storage

Melanosis score of Pacific white shrimp without and with treatments of SMS, 1% or 2% FA during iced storage is illustrated in Figure 19. At day 0, all samples had no melanosis (score=0). When the storage time increased, melanosis score in the control increased ($P < 0.05$). However, no melanosis was noticeable in samples treated with SMS or 2% FA in the first 2 days of storage. At day 2, SMS treated shrimp had the lower melanosis score than 1% FA treated shrimp ($P < 0.05$). Thereafter, treatment of shrimp with 1% FA showed the higher effectiveness in lowering melanosis, when compared with SMS treatment. During the first 2 days of storage, SMS at 1.25 % used in this study was more effective in melanosis prevention than 1% FA. However, SMS might not be stable during the extended storage, in which sulfur dioxide formed could be liberated. Therefore, the required concentration of SMS is dependent on the length of time the melanosis must be inhibited. It has been reported that sulfites do not irreversibly inhibit browning (Taylor *et al.*, 1986). Additionally, the mechanism of melanosis inhibition between SMS and ferulic acid could be different, leading to the varying in melanosis inhibition. Bisulfite might inhibit melanosis by reacting with intermediate quinone, forming sulfoquinone or it can act as a competitive inhibitor (Ferrer *et al*., 1989). During 4-10 days of storage, the formation of melanosis was lowest in the sample treated with 2% FA, followed by those treated with 1% FA and with SMS, respectively. Shrimp treated with 2% FA showed the best appearance as compared to others at the last day of storage (day 10), while the severe melanosis was found in the control samples. The retardation of melanosis formation of FA treated shrimp was coincidental with PPO inhibitory activity of FA (Figure 14 and 15). Montero *et al*. (2004) reported that melanosis inhibition of deepwater pink shrimp (*Parapenaeus Iongirostris*) increased with increasing 4-hexylresorcinol concentration.

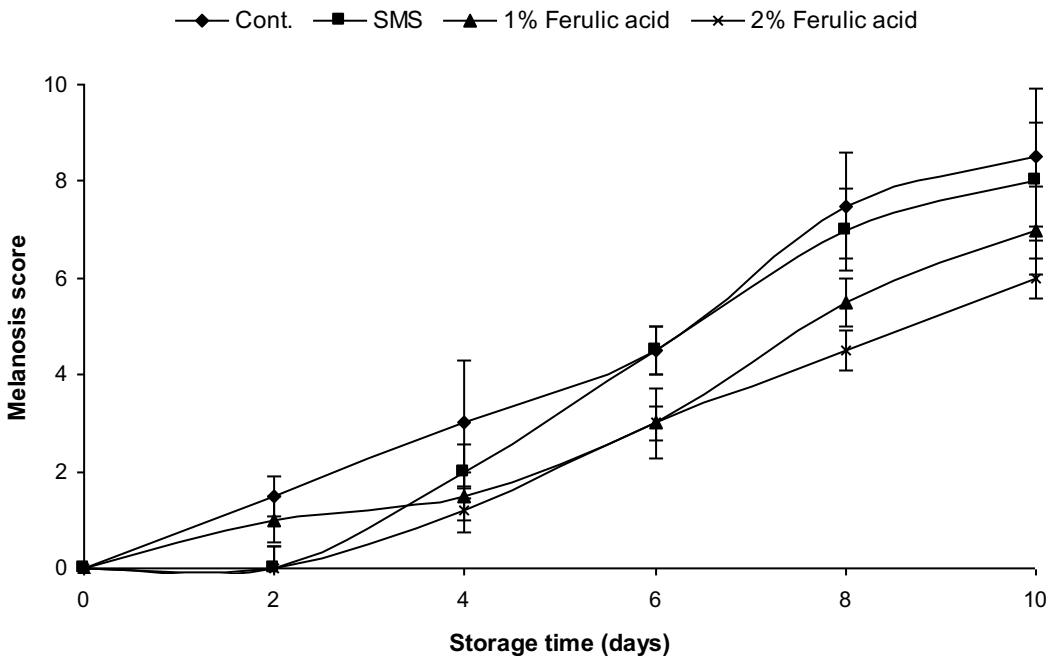

Figure 19. Melanosis Score of Pacific white shrimp treated with FA at different levels during 10 days of iced storage. Bars represent the standard deviation (n=3). Key: see Figure 16 caption. where, 0=absent; 2= slight (up to 20% of shrimps' surface affected); 4= moderate (20 to 40% of shrimps' surface affected); 6=notable (40 to 60% of shrimps' surface affected); 8= severe (60 to 80% of shrimps' surface affected); 10=extremely heavy (80 to 100% of shrimps' surface affected).

2.4.5 Effect of FA on sensory properties of Pacific white shrimp during iced storage

Changes in sensory properties of Pacific white shrimp without and with different treatments during iced storage are presented in the Table 2. Color, odor, taste, flavor and overall likeness of shrimp were evaluated at day 0 and 10 of iced storage. At day 0, no differences in likeness for all attributes were observed among all samples ($P > 0.05$). After storage of 10 days, the decreases in likeness for all attributes in all samples were noticeable ($P < 0.05$). At day 10, the higher scores for color, flavor an overall likeness were found in shrimp treated with 1 and 2 % FA, compared with the control and that treated with SMS ($P < 0.05$). Nevertheless, no differences in odor likeness were found among all samples ($P > 0.05$). The higher scores for color and overall likeness were in agreement with the lower melanosis in samples treated with

FA, particularly at a level of 2 %. The higher flavor and taste likeness scores of samples treated with FA or SMS were mostly associated with the lower microbial load in those samples, in comparison with the control. Therefore, the treatment of Pacific white shrimp with FA could improve the sensory property of treated shrimp after extended storage, which was most likely associated with the lowered melanosis.

Table 2. Effect of FA treatment on likeness score of Pacific white shrimp before and after 10 days of iced storage

Storage time (days)	Treat-ments	Color	Odor	Taste	Flavor	Overall
0	Cont.	9.0±0.64 aA	8.0±1.22 aA	8.0±0.94 aA	8.0±1.04 aA	9.0±0.90 aA
	SMS	9.0±0.82 aA	8.0±1.20 aA	8.0±0.98 aA	8.0±1.36 aA	9.0±1.02 aA
	1% FA	9.0±0.80 aA	8.5±0.90 aA	8.0±1.16 aA	8.5±1.04 aA	9.0±0.87 aA
	2% FA	9.0±0.50 aA	8.5±0.93 aA	8.5±1.13 aA	8.5±0.87 aA	9.0±0.82 aA
10	Cont.	3.5±1.75 cB	6.0±1.09 aB	5.0±1.14 bB	5.0±1.24 cB	5.0±0.71 bB
	SMS	4.0±1.81 cB	6.0±0.96 aB	5.5±1.43 abB	5.5±1.36 bcB	5.0±0.80 bB
	1% FA	5.5±1.69 bB	7.0±0.83 aB	6.5±1.26 aB	6.0±1.22 abB	6.5±0.89 aB
	2% FA	7.0±1.35 aB	7.0±0.79 aB	6.5±1.30 aB	6.5±1.32 aB	7.0±0.73 aB

* Different capital letters in the same column within the same treatment indicate the significant differences ($P < 0.05$). The different letters in the same column within the same storage time indicate significant differences ($P < 0.05$). Values are mean ± standard deviation (n=30). Cont.: control; SMS: sodium metabisulfite; 1% FA: 1% ferulic acid; 2%FA: 2% ferulic acid.

2.5 Conclusions

Ferulic acid could be used as the promising agent for melanosis prevention in Pacific white shrimp during iced storage. The efficacy was in dose dependent manner. Apart from prevention of melanosis, treatment of shrimp with ferulic acid could retard lipid oxidation and microbial growth. Moreover, shrimp treated with ferulic acid had superior sensory properties to the control after 10 days of iced storage. This led to the extended shelf-life of white shrimp. From the consumer health point of view, the use of natural additives like ferulic acid or other plant phenolic compound can be a safer means to maintain the quality of shrimp, compared to synthetic additives.

CHAPTER 3

MELANOSIS AND QUALITY CHANGES OF PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*) TREATED WITH CATECHIN DURING ICED STORAGE

3.1 Abstract

Melanosis, microbiological, chemical and physical changes of Pacific white shrimp (*Litopenaeus vannamei*) treated with catechin were monitored during iced storage of 10 days. Whole shrimp treated with catechin solution (0.05 or 0.1 %) had the retarded growth of psychrotrophic bacteria and spoilage microorganisms including H_2S-producing bacteria and enterobacteriaceae throughout the storage in comparison with the control and those treated with 1.25% sodium metabisilfite (SMS) ($P < 0.05$). The lower increases in pH and total volatile base (TVB) content were obtained in the shrimp treated with catechin solution at both levels, compared with other samples ($P < 0.05$). Lipid oxidation, loss in freshness and melanosis were lowered by catechin treatment. In general, the efficacy of catechin in lowering melanosis and quality losses increased with increasing levels used. Additionally, catechin (0.01, 0.05 and 0.1% (w/v)) showed inhibitory activity towards polyphenoloxidase (PPO) of Pacific white shrimp in the dose-dependent manner. Therefore, catechin can be used as a promising melanosis inhibitor as well as antimicrobial and antioxidant in ice-stored shrimp.

3.2 Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is an important commercial species primarily cultured in Thailand and accounts for 90% of the global aquaculture shrimp production. Shrimp constitutes 18-20 of Thai nion s sales and exports are also expected to rise up to 400,000 tons in 2008 with an increase in value up to $ 2.3 billion in the United States (Wyban, 2007). Shrimp is a very perishable

product, and postmortem changes occur rapidly, compared with fish (Zeng *et al*., 2005). Rapid microbial spoilage during postmortem storage is a serious problem in shrimp processing (Gokoglu and Yerlikaya, 2008). Free amino acids and other soluble non-nitrogenous substances in shrimp serve as digestible nutrients for microbial growth (Zeng *et al.*, 2005). Shrimp generally has the limited shelf-life due to the formation of black spots (melanosis). Even though the presence of black spots seems to be harmless to consumers, it drastically reduces the product s market value and consumer s acceptability, leading to considerable financial loss (Montero *et al*., 2001b). Melanosis is triggered by a biochemical mechanism which oxidizes phenols to quinones by PPO. This is followed by non-enzymatic polymerization and autooxidation of the quinones, giving rise to pigments of high molecular weight and very dark or black coloring (Benjakul *et al*., 2005a). Apart from melanosis and microbial spoilage, lipid oxidation associated with physicochemical changes and off-flavors (Decker and Hultin, 1990) and loss in freshness are companied with the lower quality, causing market loss of shrimps.

Many studies have focused on preventing melanosis or inhibiting PPO over the years through different techniques. Reducing agents such as sulfiting agents and their derivatives are the most widely used chemicals for the control of melanosis or browning in food industry (Gokoglu and Yerlikaya, 2008). However, the increases in regulatory attention and consumer s awareness of the risk associated with sulfited food products have created a need for a safe effective sulfite alternative for food processing (McEvily *et al*., 1991). Plant phenolics have paid increasing attention as the potential natural additives with antioxidant and antimicrobial activities (Banerjee, 2006; Pereira *et al*., 2006). Jayaprakasha *et al*. (2001) demonstrated that plant phenolic compounds such as tocopherols, flavonoid compounds, cinnamic acid derivatives and coumarins exhibited an antioxidant effect in per-oxidation model system. Recently, it has been reported that enokitake extract (Jang *et al*., 2003) and grape seed extract (Gokoglu and Yerlikaya, 2008) could inhibit the melanosis in shrimp.

Among natural extracts, especially from plants, catechin from tea has been intensively studied as an excellent antioxidant (Banerjee, 2006). Due to the similarity of catechin to PPO substrate, it might act as PPO inhibitor, which could

prevent melanosis in shrimp. Additionally, it might function as both antioxidant and antimicrobial, which can maintain the quality of shrimp during the storage. However, no information regarding the use of catechin as the natural additive to prevent the melanosis or extend the shelf-life of shrimp has been reported. The aim of this study was to investigate the inhibition of melanosis and quality changes of Pacific white shrimps treated with catechin during iced storage.

3.3 Materials and Methods

3.3.1 Chemicals

L- -(3, 4 dihydroxylphenyl) alanine (L-DOPA), Brij-35, (±)-catechin hydrate, malonaldehyde bis (dimethyl acetal), thiobarbituric acid (TBA) and anion exchange resin-AG (R) 1-X4-400 mesh Cl-form were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), perchloric acid (PCA), hydrochloric acid, potassium hydroxide, ammonium hydroxide, sodium chloride, ammonium sulfate, standard plate count agar, triple sugar iron agar (IA), and Eosin methylene blue agar (EMB) were obtained from Merck (Darmstadt, Germany).

3.3.2 Shrimp collection and preparation

Pacific white shrimp (*Litopenaeus vannamei*) with the size of 55-60 shrimp/kg were purchased from the dock in Songkhla, Thailand. The shrimp were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, shrimp were washed in cold water and stored in ice until used (not more than 5 h). Three different lots of shrimp were used for the entire study.

3.3.3 Effect of catechin treatment on the quality of Pacific white shrimp during iced storage

3.3.3.1 Preparation of shrimps

Whole Pacific white shrimp were immersed in catechin solution at different concentrations (0.05 and 0.1%) with a shrimp / solution ratio of 1:2 (w/v) at 4^{o}C for 15 min. Another portion of shrimp was soaked in 1.25% sodium metabisulfite at a ratio of 1:2 (w/v) for 1 min at 4^{o}C (Kim *et al*., 2000). Treated shrimp were drained on the screen for 3 min at 4^{o}C. Shrimp without any treatment were used as the control. All samples were stored in polystyrene box containing crushed ice with a shrimp/ice ratio of 1:2 (w/w). To maintain shrimp/ice ratio, the molten ice was removed and the same amount of ice was added every day. Samples (25 shrimp) were randomly taken for each treatment every 2 days up to 10 days for microbiological, chemical and physical analyses. Melanosis was also determined.

3.3.3.2 Microbiological analyses

Microbiological analyses were performed by spread plate method (Sallam, 2007). Five whole Pacific white shrimp were collected aseptically and used as the composite sample. Ground sample (without peeling) (25 g) was placed in a stomacher bag containing 225 mL of 0.85 % saline water. After mixing for 1 min in a stomacher blender (Stomacher M400, Seward Ltd, Worthington, England), further serial dilutions were prepared from this homogenate using 0.85 % saline water as diluent. The appropriate dilutions were used for microbiological analyses.

3.3.3.2.1 Psychrotrophic bacterial count

Psychrotrophic bacterial count was determined by inoculating 0.1 mL of appropriate dilution of homogenate on plate count agar, containing 0.5% NaCl by a spread plate method. Then the plates were incubated at 4^{o}C for 10 days.

3.3.3.2.2 Hydrogen sulfide producing bacteria

H_2S- producing bacteria were grown on triple sugar iron agar by spread plate method using 0.1 mL of appropriate dilution of homogenate. Plates were incubated at 25^{o}C for 3 days. Black colonies, due to precipitation of ferrous sulfide on this medium, were count.

3.3.3.2.3 Enterobacteriaceae count

For the determination of enterobacteriaceae count, 0.1 mL of appropriate dilution of homogenate was plated on EMB agar and incubated at 37^{o}C for 24 h.

3.3.3.3 Chemical analyses

3.3.3.3.1 pH measurement

pH measurement was performed by the method described by Lopez-Caballero *et al*. (2005) with a slight modification. Shrimp meat (2 g) was homogenized with 10 volumes of deionized water for 1 min using PT 2100 homogenizer (Kinematica AG, CH-6014, Littau/Luzern, Switzerland). The homogenate was kept at room temperature for 5 min. The pH was determined using a pH-meter (Sartorious North America, Edgewood, NY, USA).

3.3.3.3.2 Determination of total volatile base content

Total volatile base (TVB) content in shrimp meat was determined using the Conway micro-diffusion method (Conway and Byrne, 1936). TVB content was calculated and expressed as mg N/ 100 g shrimp meat.

3.3.3.3.3 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS in the samples was determined following the method of Benjakul and Bauer (2001) with some modifications. Ground shrimp meat (1 g) was mixed with 9 mL of a solution containing 0.375% TBA, 15% TCA and 0.25 N HCl. The mixture was heated in boiling water for 10 min, followed by cooling with the running water. The mixture was centrifuged at 4000 x g for 20 min (MIKRO20, Hettich Zentrifugan, Germany). The supernatant was collected and the absorbance was read at 532 nm using a UV- 160 spectrophotometer (Shimadzu, Kyoto, Japan). TBARS was calculated from the standard curve of malonaldehyde (0 - 2 ppm) and expressed as mg malonaldehyde / kg shrimp meat.

3.3.3.3.4 Determination of K-value

K-value was determined using anion-exchange column chromatography (Uchiyama and Kakuda, 1984). Ground sample (1 g) was subjected to a serial extraction using 10% PCA and 5% PCA. Final extract (2 mL) was adjusted pH to 9.4 using 0.5 N NH_4OH. The prepared extract (pH 9.4) was loaded onto anion exchange column (Resin-AG (R) 1-X4-400 mesh Cl-form). The column was rinzed using deionized water (20 mL). The elution was performed using 45 mL of solution-A (0.001N HCl). The eluate was collected and the volume was made up to 50 mL using solution-A. Thereafter, the column was eluted with 45 mL of solution-B (0.01 N HCl containing 0.6 NaCl). The resulting eluate was made up to 50 mL using solution-B. Both eluates were read at 250 nm using the corresponding eluent (solution A or B) as blank. K-value was calculated as follows:

$$\text{K-value (\%)} = \frac{A}{A + B} \times 100$$

where A: A_{250} of eluate A representing the amount of inosine (HxR) and hypoxanthine (Hx); B: A_{250} of eluate B representing the amount of ATP, ADP, AMP and IMP

3.3.3.4 Physical analyses

3.3.3.4.1 Determination of shear force

Shear force of shrimp meat without and with treatment, was measured using the TA-XT2i texture analyzer (Stable Micro Systems, Surrey, England) equipped with a Warner-Bratzler shear apparatus (Brauer *et al*., 2003). The operating parameters consisted of a cross head speed of 10 mm/s and a 25 kg load cell. The shear force, perpendicular to the axis of muscle fibers, was measured at the second segment of shrimp. Five samples were determined for each treatment. The peak of the shear force profile was regarded as the shear force value and expressed in Newton (N).

3.3.3.4.2 Melanosis assessment

Melanosis or blackening of Pacific white shrimp was evaluated through visual inspection by six trained panelists using 10-point scoring test (Montero *et al.*, 2001b). Panelists were asked to give the melanosis score (0 to 10), where 0 = absent;

2 slight (up to 20 of shrimps surface affected) 4 moderate (20 to 40% of shrimps surface affected) 6 notable (40 to 60 of shrimps surface affected) 8 severe (60 to 80 of shrimps surface affected) 10 extremely heavy (80 to 100 of shrimps surface affected).

3.3.4 Preparation of PPO extract from the cephalothoraxes of Pacific white shrimp

The cephalothoraxes of twenty shrimp were separated, pooled and powderized by grinding with liquid nitrogen in a Waring blender. The powder obtained was kept in polyethylene bag and stored at -20 °C for not more than 2 weeks. The extraction of PPO was carried out according to the method of Simpson *et al*. (1987) with a slight modification. The powder (50 g) was mixed with 150 mL of the extracting buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 1.0 M NaCl and 0.2% Brij 35). The mixture was stirred continuously at 4^{o}C for 30 min, followed by centrifugation at 8000 x g at 4^{o}C for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). Solid ammonium sulfate was added into the supernatant to obtain 40% saturation and the mixture was allowed to stand at 4^{o}C for 30 min. The mixture was then subjected to centrifugation at 12,500 x g at 4^{o}C for 30 min using a refrigerated centrifuge. The pellet obtained was dissolved in a minimum volume of 0.05 M sodium phosphate buffer, pH 7.2 and dialyzed against 15 volumes of the same buffer at 4^{o}C with three changes of dialysis buffer. The insoluble materials were removed by centrifugation at 3000 x g at 4^{o}C for 30 min and the supernatant was used as crude PPO extract .

3.3.5 Effect of catechin on inhibition of Pacific white shrimp PPO

3.3.5.1 Preparation of catechin solutions

Catechin was mixed with distilled water to obtain the different final concentrations (0.02, 0.1 and 0.2%, w/v). The mixtures (20 mL) were adjusted to pH 9 by 6 N NaOH and stirred for 15 min at room temperature (26-28^{o}C) to completely

dissolve catechin. Thereafter, the pH of the solution was adjusted to 7 by using 6 N HCl and referred to as catechin solution .

3.3.5.2 Inhibitory effect of catechin solution on PPO activity

Catechin solutions with different concentrations (0.02, 0.1 and 0.2%, w/v) (100 L) were mixed with crude PPO extract (100 L) to obtain the final concentrations of 0.01, 0.05 and 0.1% (w/v), respectively. The mixtures were incubated for 30 min at room temperature. Then, the assay buffer (400 L) was added. To initiate the reaction, 600 L of preincubated 15 mM L-DOPA (45^{o}C) were added. The reaction was conducted at 45^{o}C and the absorbance at 475 nm was monitored every min up to 3 min. The control was run in the same manner, except the deionized water was used instead of catechin solution. The blank was prepared for each catechin solution by using distilled water instead of L-DOPA.

3.3.6 Statistical analyses

All experiments were performed in triplicate and a completely randomized design (CRD) was used. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan s multiple range tests (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA).

3.4 Results and Discussion

3.4.1 Effect of catechin treatment on microbiological changes of Pacific white shrimp during iced storage

Changes in psychrotrophic bacterial count, H_2S producing bacteria and enterobacteriaceae count of Pacific white shrimp treated with 0.05 or 0.1% catechin during iced storage in comparison with the control and those treated with 1.25% SMS are shown in Figure 20.

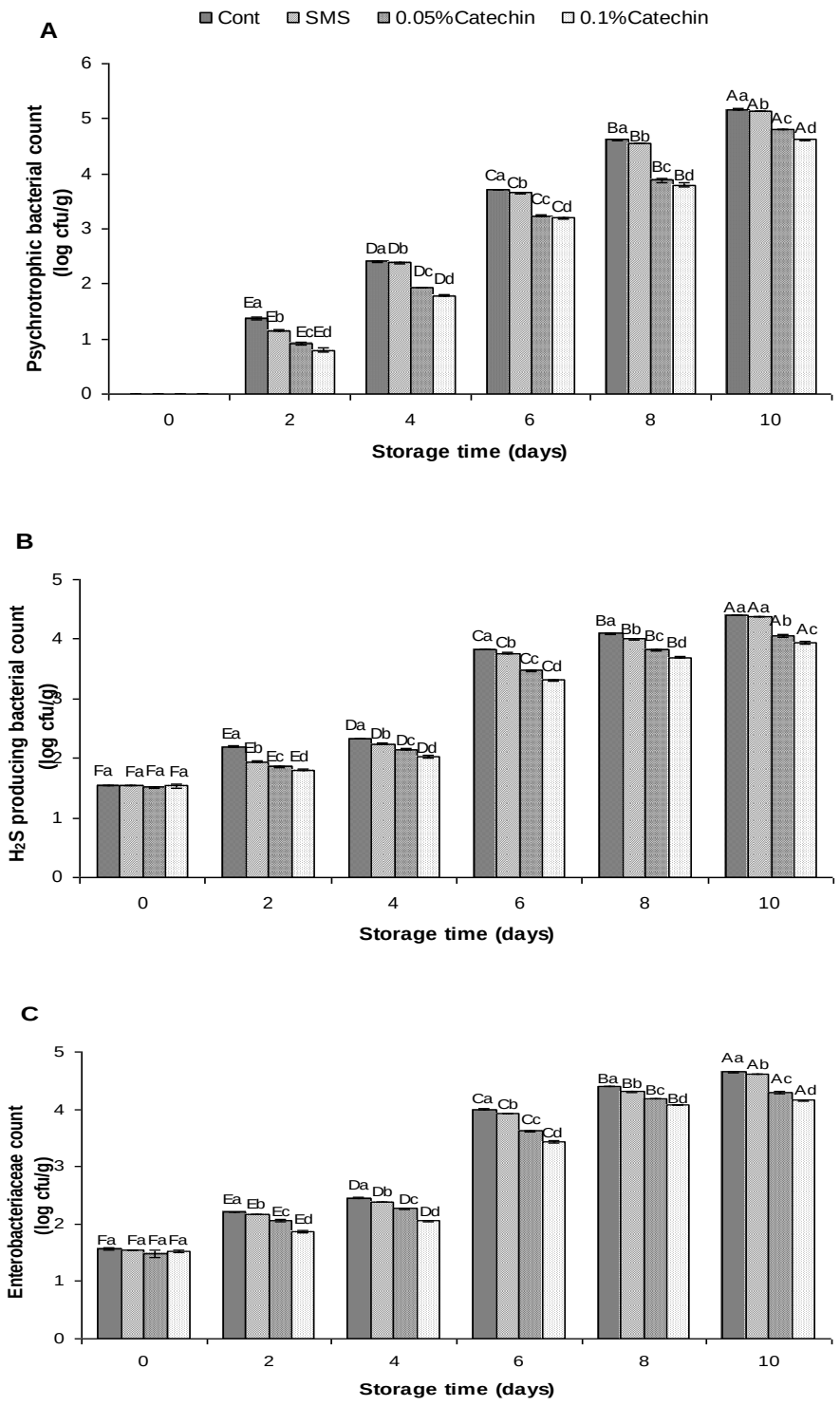

Figure 20. Psychrotrophic (**A**), H_2S-producing bacteria (**B**) and Enterobacteriaceae (**C**) count of Pacific white shrimp treated with catechin at different levels. Bars represent the standard deviation (n = 3). Different capital letters on the bars within the same treatment indicate the significant differences ($P < 0.05$). The different letters on the bars within the same storage time indicate significant differences ($P < 0.05$).

No psychrotrophic bacteria were found in all samples at day 0. The continuous increases in psychrotrophic bacterial count in all samples were noticeable with increasing storage time up to 10 days ($P < 0.05$). During 2-10 days of storage, samples treated with sodium metabisulfite (SMS), 0.05% or 0.1% catechin had the lower psychrotrophic bacterial count, in comparison with the control ($P < 0.05$) (Figure 20A). At the same storage time, the lowest psychrotrophic bacterial count was found in shrimp treated with 0.1 % catechin ($P < 0.05$). During 4-10 days of storage, SMS treatment did not exhibit the inhibition towards the growth of psychrotrophic bacteria as compared to catechin treatment. However, there is significant difference in bacterial count as compared to control. At the end of storage (day 10), psychrotrophic bacterial count of the control, those treated with SMS, 0.05% catechin and 0.1% catechin were 5.17, 5.13, 4.81 and 4.61 log CFU g^{-1}, respectively. The result indicated the antimicrobial activity of catechin towards psychrotrophic bacteria in Pacific white shrimp during iced storage. The antimicrobial activity was dependent on the concentration used. The treatment with 5% kiam wood extract containing tannic acid retarded the rate of growth of *Listeria monocytogenes*, aerobic mesophiles and psychrotrophic micro-organism naturally present on cabbage (Chanthachum and Beuchat, 1997). Treatments with 2% sodium acetate or 2% sodium lactate had little or no effect in reduction of the growth of psychrotrophic bacteria in shrimp over 12 days of storage at 4^{o}C (Zhuang *et al*., 1996).

Changes in H_2S producing bacterial count of Pacific white shrimp without and with different treatments during iced storage are depicted in Figure 20B. During 10 days of iced storage, H_2S producing bacterial count was less in shrimp treated with SMS as compared to control up to 8 days; however no difference between both samples was observed at the end of storage day ($P > 0.05$). Shrimp treated with 0.1% catechin contained the lowest H_2S producing bacteria throughout the storage ($P < 0.05$), followed by shrimp treated with 0.05% catechin. At the end of iced storage, the control, those treated with SMS, 0.05% catechin and 0.1% catechin had H_2S producing bacterial count of 4.40, 4.37, 4.05 and 3.93 log $CFUg^{-1}$, respectively. Deepwater pink shrimp (*Parapenaeus longirostris*) stored under chilled condition showed 4.0 log $CFUg^{-1}$ of H_2S producing bacteria after 15 days (Mendes *et al*., 2002). Phenolic compounds might disrupt the cell wall of microorganism by forming

complex with proteins in cell wall and make lyses of cell wall (Chanthachum and Beuchat, 1997). Leaf extract of artichoke (*Cynara scolymus* L.) exhibited the most significant antimicrobial activities against seven bacteria species including gram positive and negative species (Zhu *et al*., 2004). Generally, specific spoilage organisms like H_2S producing bacteria and enterobacteriaceae are mostly predominant in spoilage of fish and fish products, causing off-flavors and rejection (Sallam, 2007). Thus, catechin was shown to retard the growth of spoilage bacteria, which were able to produce H_2S.

Enterobacteriaceae count of Pacific white shrimp without and with treatments during iced storage of 10 days illustrated in Figure 20C. In general, enterobacteriaceae count of all samples increased throughout the storage of 10 days ($P < 0.05$). During the storage, lowered count was observed in shrimp treated with SMS, 0.05% catechin and 0.1% catechin as compared to that of the control ($P < 0.05$). Treatment of shrimp with 0.1% catechin was more effective in lowering enterobacteriaceae count, followed by treatment with 0.05% catechin ($P < 0.05$). SMS treatment showed a little impact on inhibition of enterobacteriaceae. The control shrimp, those treated with SMS, 0.05% catechin and 0.1% catechin had enterobacteriaceae count of 4.64, 4.62, 4.29 and 4.15 log $CFUg^{-1}$, respectively, at the end of iced storage. Pink shrimp treated with sulfites had enterobacteriaceae count of 5 log CFU g^{-1} at day 9 of chilled storage (Lopez-Caballero *et al*., 2002). Sodium meatbisulfite (SMS) has been reported as an antimicrobial due to its release of sulfur dioxide, which can pass across cell membrane and disrupt the normal metabolic activity of bacteria (DiPersio *et al*., 2004). In this study, sodium metabisulfite (1.25% SMS) exhibited the lower efficiency in retardation the growth of enterobacteriaceae. Sulfur dioxide derived from SMS might be evaporated during the extended storage or could be dissolved with molten ice. This might lead to the lower amount of SMS remaining in the sample. Catechin at either 0.05% or 0.1% showed the stronger inhibitory activity against spoilage bacteria than did SMS. As a result, the spoilage caused by microorganism could be retarded by catechin treatment.

3.4.2 Effect of catechin treatment on chemical changes of Pacific white shrimp during iced storage

3.4.2.1 pH

Changes in pH of Pacific white shrimp with and without different treatments during iced storage are shown in Figure 21A. Fresh Pacific white shrimp had the pH of 6.44. As the storage time increased, pH of all shrimps increased ($P < 0.05$). Within the first two days of storage, no differences in pH were noticeable among all samples ($P > 0.05$). After two days of storage, those treated with 0.1% catechin had the lowest pH, followed by those treated with 0.05% catechin and SMS, respectively ($P < 0.05$). At the end of storage, the control, shrimp treated with SMS, 0.05% catechin and 0.1% catechin had pHs of 7.55, 7.38, 7.30 and 7.28, respectively. The increase in pH was associated with the accumulation of basic compounds, mainly resulted from the microbial action (Lopez-Caballero *et al*., 2007). The lower increase in pH of shrimp treated with 0.05% or 0.1% catechin was in accordance with the lower microbial count (Figure 20). Shrimp, *Penaeus merguiensis*, was not acceptable when the pH was greater than 7.6 (Shamshad *et al*., 1990). The increases in pH value were more rapid in the shrimp (*Pandalus borealis*) stored in ice at 1.5^{o}C and reached a final pH of 8.26 as compared to sample stored in liquid ice (pH-7.98) (Zeng *et al*., 2005).

3.4.2.2 TVB contents

TVB contents of Pacific white shrimp without and with different treatments are depicted in Figure 21B. Continuous increase in TVB content was observed in all samples, but the rate of increase in TVB content varied with treatments ($P < 0.05$). The initial TVB content of Pacific white shrimp for all treatments was 7.2-7.4 mg N / 100g shrimp meat. Volatile base compound found in the shrimp more likely indicated that autolytic process was involved during post-mortem handling. Adenosine and adenosine monophosphate (AMP) deaminase might play a major role in this process after capture and transportation (Lopez-Caballero *et al*., 2007).

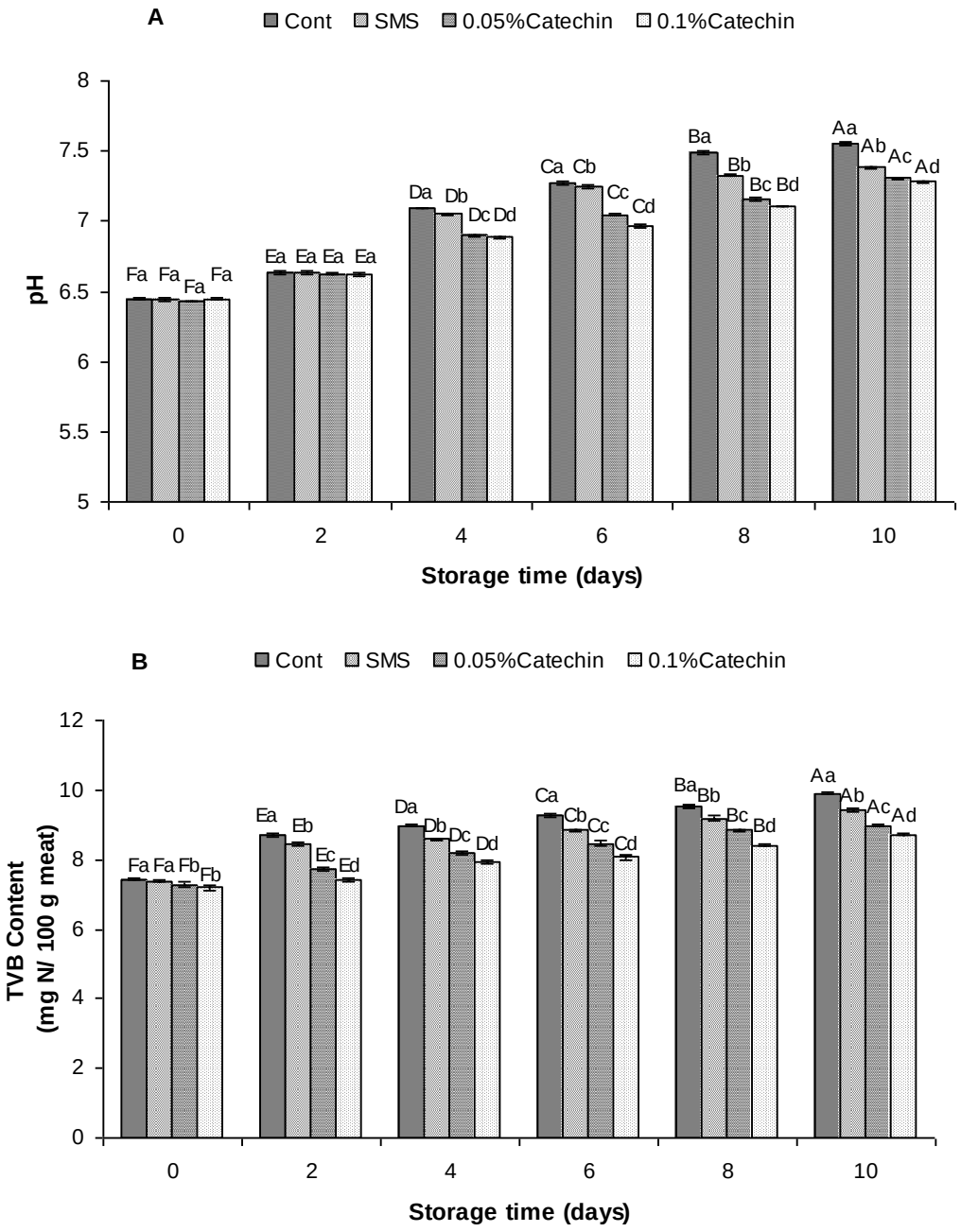

Figure 21. pH (**A**) and total volatile base (**B**) content of Pacific white shrimp treated with catechin at different levels. Bars represent the standard deviation (n=3). Key: see Figure 20 caption.

The lowered rate of increase in TVB content of shrimp treated with 0.05 or 0.1% catechin was observed, as compared to the control and shrimps treated

with SMS, when the storage time increased ($P < 0.05$). The lowest TVB content correlated with the lowest microbial counts found in 0.1% catechin treated shrimp (Figure 20). Moreover, the lower TVB content was coincidental with the lower pH of Pacific white shrimp (Figure 21A). At the last day of iced storage, shrimp treated with 0.1 % catechin had the lowest TVB content (8.70 mg N/100g), compared to other treatments ($P < 0.05$). Total volatile base content of deepwater pink shrimp (*Parapenaeus longirostris*) treated with resorcinol had the lowered TVB content (35 mg N/ 100g) as compared with other treatments and the control (Lopez-Caballero *et al*., 2007). TVB content of 40 mg N/ 100g has been used as freshness borderline (Mendes *et al*., 2002).

3.4.2.3 Thiobarbituric acid reactive substances (TBARS)

TBARS values of the Pacific white shrimp without and with treatments during iced storage are illustrated in Figure 22. TBARS value of the control sample increased continuously throughout iced storage ($P < 0.05$). The lower increase in TBARS was noticeable within the first 2 days. At all storage times, shrimp treated with 0.1% catechin showed the lower TBARS value, compared to other treatments ($P < 0.05$), except at day 2. Similar TBARS value of SMS treated shrimp and shrimp treated with 0.05% catechin was found during 4- 6 days of storage ($P > 0.05$). Thereafter, a higher TBARS value was found in the former, indicating that lipid oxidation took place at a higher extent in SMS treated sample. Generally, shrimps treated with catechin had the lower TBARS value throughout the storage ($P < 0.05$). Results revealed that Pacific white shrimp treated with 0.1% catechin had the higher stability towards lipid oxidation than other samples. Catechin at a high level most likely showed the strong antioxidative effect in shrimp muscle. Catechin has been reported to have antioxidant activity including radical scavenging activity (Banerjee, 2006). Lipid per-oxidation in fish meat can be initiated by autoxidation, photosensitized oxidation or by means of enzymatic reaction associated with lipoxygenase, peroxidase and microbial enzyme (Decker and Hultin, 1990). In lipid oxidation, unstable hydroperoxide is form and decomposes readily to shorter chain hydrocarbon such as aldehydes; those final products can be detected as TBARS (Benjakul *et al*., 2005b).

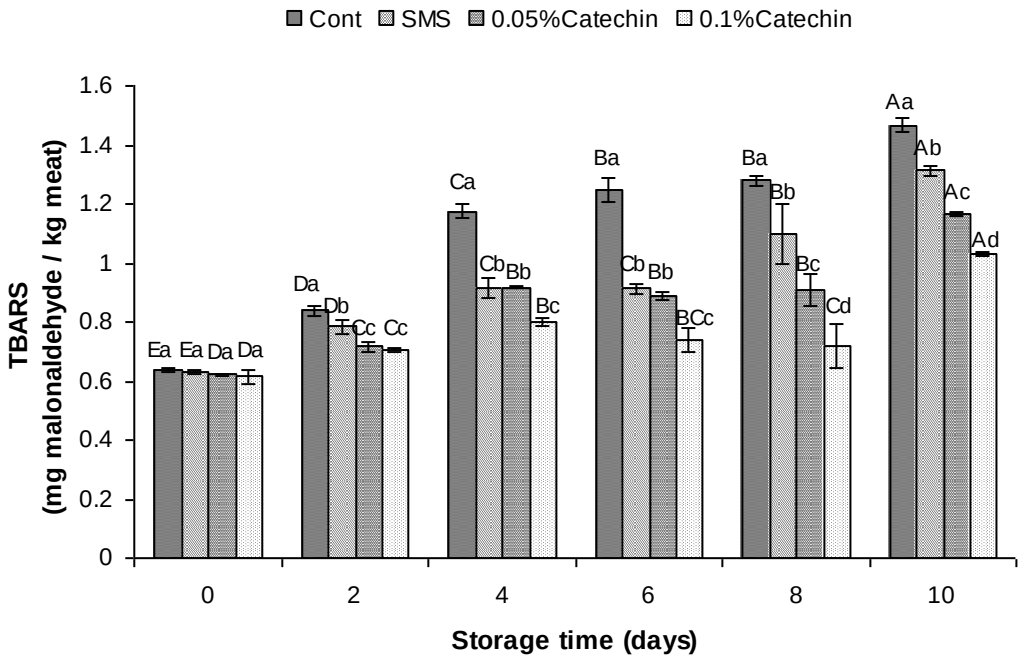

Figure 22. TBARS values of Pacific white shrimp treated with catechin at different levels. Bars represent the standard deviation (n=3). Key: see Figure 20 caption.

Catechin and its derivatives effectively inhibited the lipoxygenase activity in mackerel muscle (Banerjee, 2006). Therefore lipid oxidation in shrimp treated with catechin could be prevented to some degree during extended storage.

3.4.2.4 K-value

K-value, of Pacific white shrimp without and with different treatments during iced storage is depicted in Figure 23. K-value has been used as the freshness index in fish and shellfish (Yamagata and Low, 1995). The control and all treated samples had K-value of approximately 20% at day 0. K value of *Penaeus japonicus* was reported to be approximately 20% at the initial storage time (Matsumoto and Yamanaka, 1990). Deepwater pink shrimp (*Parapenaeus longirostris*) had K-value of 9 % at the beginning of chilled storage (2^{o}C) and reaches 40% at day 10 of storage (Mendes *et al.*, 2002).

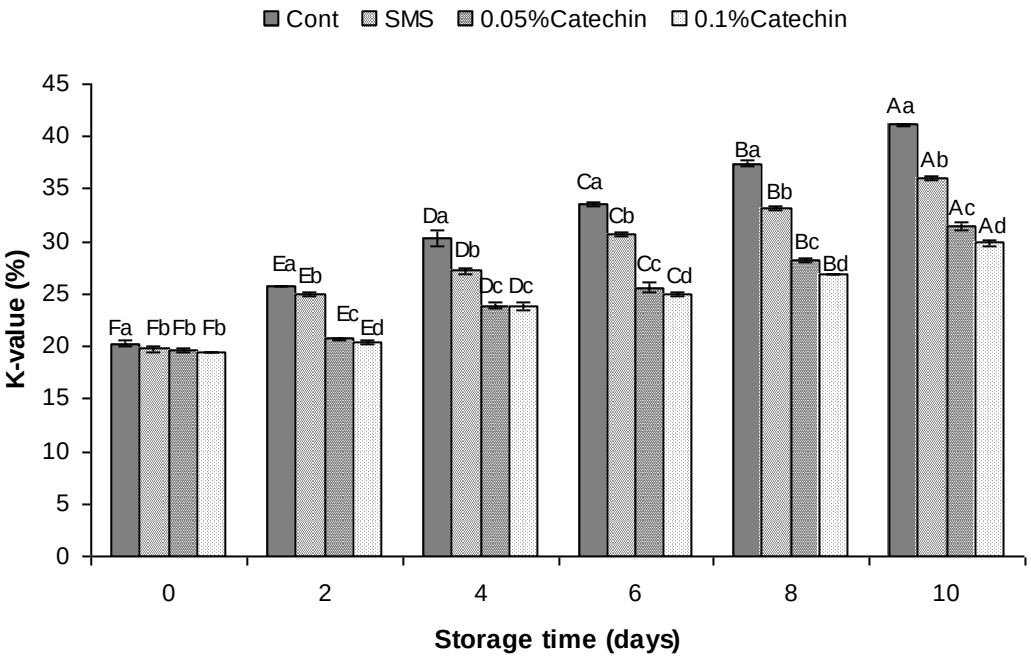

Figure 23. K-values of Pacific white shrimp treated with catechin at different levels. Bars represent the standard deviation (n=3). Key: see Figure 20 caption.

Continuous increases in K-value were found in the control shrimp and all treated samples during the storage ($P < 0.05$). It was noticed that shrimp treated with catechin had the lower rate of increase in K-value as compared to that found in the control shrimp and those treated with SMS ($P < 0.05$). The control banana shrimp (*Penaeus merguiensis*) and those treated with sodium bisulfite had similar K-value (43%) at the last day of iced storage (Yamagata and Low, 1995). Catechin at levels of 0.05% or 0.1% showed the similar effect towards K-value within the first 4 days of storage. Thereafter, 0.1 % catechin exhibited the greater impact on the retardation of the increase in K-value. At the end of storage, shrimp treated with 0.1% catechin had the lowest K-value (29%), compared to those of other treatments (35 - 41%). During post-mortem handling and storage, endogenous enzymes break down adenosine triphosphate (ATP) to different derivatives such as adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR), and finally hypoxanthine (Hx) (Yamagata and Low, 1995). In general, K-value of 20% or lower indicates a very good quality fish, while 60% has been realized as the rejection limit (Uchiyama and Kakuda, 1984). From the result, catechin might inhibit the ATP

degradative enzymes via cross-linking mechanism. Catechin might bind or cross link those enzymes, leading to the lower rate of ATP degradation. It can be inferred that catechin treatment could retard the losses in freshness of white shrimp during storage.

3.4.3 Effect of catechin treatment on physical changes of Pacific white shrimp during iced storage

3.4.3.1 Shear force

Figure 24 illustrates the shear force of muscle of Pacific white shrimp without and with different treatments during iced storage. At day 0 of storage, all samples showed the similar shear force (18.90-18.94 N) ($P > 0.05$). As the storage time increased, different shear forces were obtained among the samples ($P < 0.05$). The control shrimp and those treated with SMS had similar shear force ($P > 0.05$). Shrimp treated with catechin possessed the higher shear force ($P < 0.05$). Higher shear force was found in shrimp treated with 0.1% catechin, compared with those treated with 0.05% catechin, after 10 days of storage ($P < 0.05$). These results revealed that muscle softening of shrimps during the extended iced storage could be lowered by catechin treatment. Catechin and epigallocatechin gallate (EGCG) from green tea could inhibit the action of collagenase against collagen (Madhan *et al*., 2007). Generally, the softening of shrimp or fish muscle is associated with proteolysis caused by endogenous or microbial proteinases and collagenase (Benjakul *et al*., 1997). The higher shear force of shrimp treated with catechin was coincidental with the lower microbial load (Figure 20). These spoilage microorganisms mostly produce proteinases (Shamshad *et al*., 1990), which are capable of hydrolyzing muscle proteins. Inhibitory activity of catechin towards those microorganisms ultimately decreased the degradation of muscle proteins of shrimp including collagen. Additionally, catechin might inactivate those proteinases, leading to the lower decreases in shear force. Shear strength of deepwater pink shrimp (*Parapenaeus longirostris*) treated with 4-hxylresorcinol had the lowest value as compared to those treated with commercial sulfite, gluconic acid + commercial sulfite formulation and the control (Lopez-Caballero *et al*., 2007).

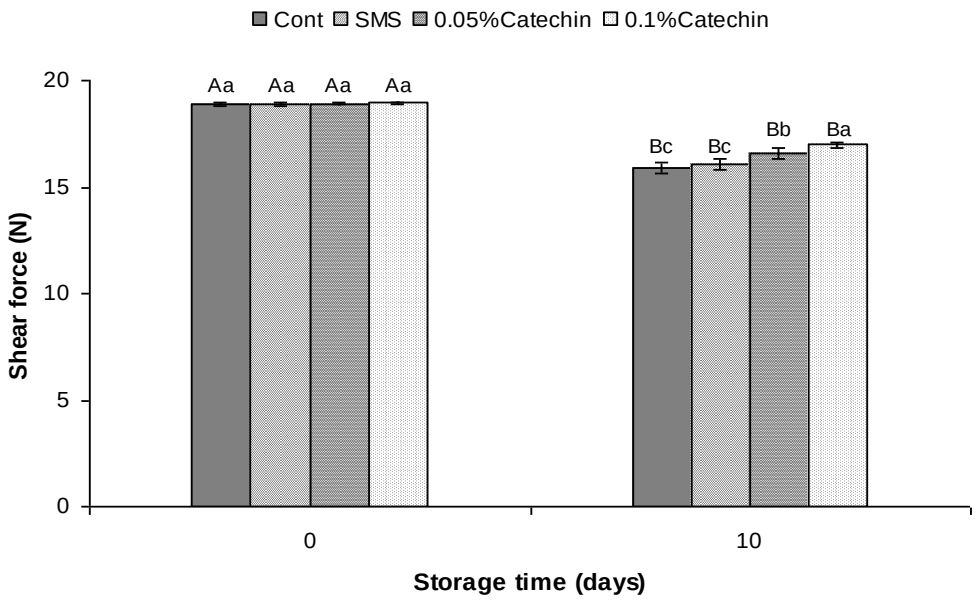

Figure 24. Shear force of Pacific white shrimp treated with catechin at different levels. Bars represent the standard deviation (n=3). Key: see Figure 20 caption.

3.4.3.2 Melanosis score

Melanosis score of Pacific white shrimp without and with treatments of SMS, 0.05% catechin or 0.1% catechin during iced storage is illustrated in Figure 25. At day 0, all samples had no melanosis (score=0). When the storage time increased, melanosis score in the control increased continuously ($P < 0.05$). However, no melanosis was noticeable in sample treated with 0.05% or 0.1% catechin within the first 2 days of storage. Shrimp treated with 0.05% and 0.1% catechin had no difference in melanosis score at day 4 ($P > 0.05$). Nevertheless, during 4-10 days of storage, the formation of melanosis was lower in sample treated with 0.1% catechin, than those treated with 0.05% catechin ($P < 0.05$). After 4 days of storage, shrimp treated with SMS had severe melanosis like control shrimp up to 10 days ($P > 0.05$). Melanosis formation of Pacific white shrimp without and with treatment on day 10 of iced storage is shown in Figure 26. Shrimp (*Parapenaeus Iongirostris*) treated with 1.5% of grape seed extract and stored at 4^{o}C had the best melanosis score (score 6) as compared to other treatment (Gokoglu and Yerlikaya, 2008). Shrimp (*T. curvirostris*) immersed in 2.5 g wet enokitake extract / mL of 0.9% KCl for 10 min had no melanosis up to 20 h at 24^{o}C (Jang *et al*., 2003).

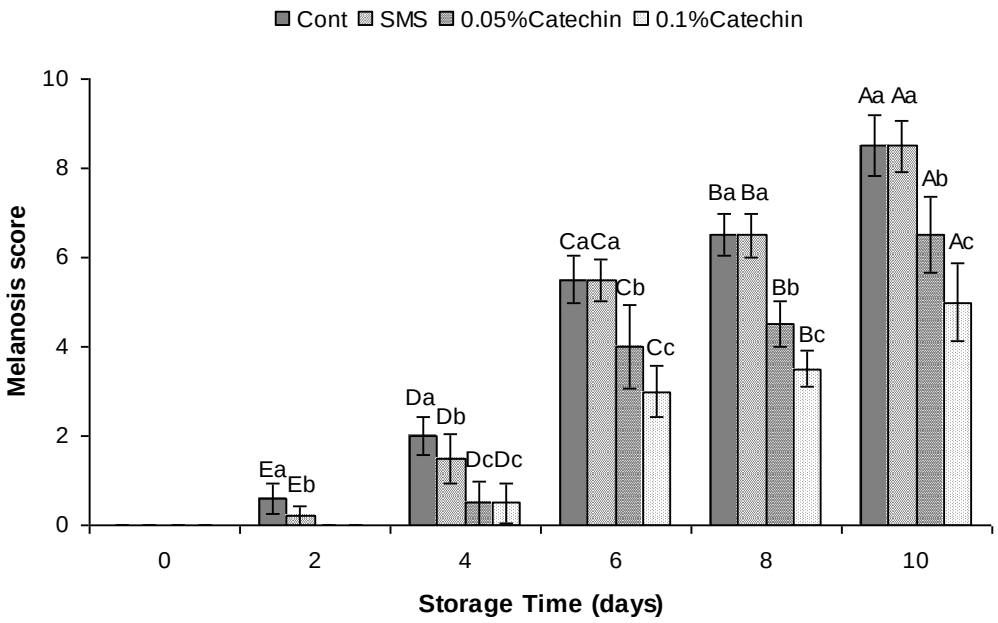

Figure 25. Melanosis score of Pacific white shrimp treated with catechin at different levels during 10 days of iced storage. Bars represent the standard deviation (n = 3). where, 0=absent; 2= slight (up to 20% of shrimps surface affected) 4 moderate (20 to 40 of shrimps surface affected) 6 notable (40 to 60 of shrimps surface affected) 8 severe (60 to 80 of shrimps surface affected) 10 extremely heavy (80 to 100 of shrimps surface affected).

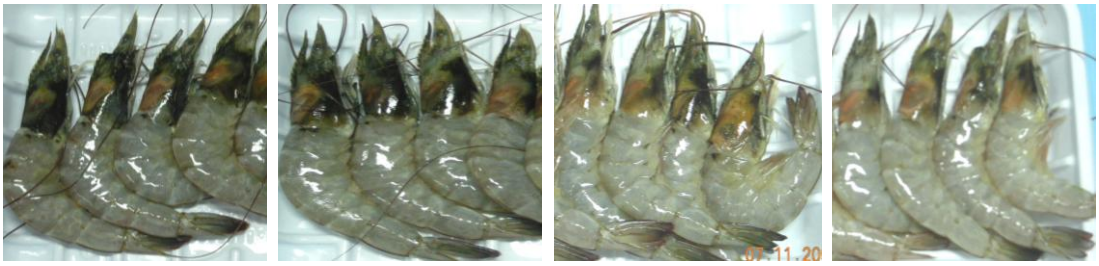

Figure 26. Photograph of Pacific white shrimp without and with treatment on day 10 of iced storage**.**

Melanosis is a phenomenon, in which brown color is developed by the enzymatic reaction mediated by polyphenoloxidase (Benjakul *et al.*, 2005a). Melanosis can occur in shrimp and crustaceans, leading to the lower market value (Montero *et al.*, 2001). Based on the result of melanosis, Pacific white shrimp treated

with catechin had the negligible blackening within the first 3 or 4 days and the formation of melanosis was lower than untreated and those treated with sodium metabisulfite during the extended iced storage.

3.4.4 Effect of catechin on PPO inhibition

Effect of catechin solution at different concetrations on inhibition of PPO from Pacific white shrimp is shown in Figure 27. Catechin showed PPO inhibitory activity in the dose-dependent manner ($P < 0.05$). At catechin levels of 0.05 and 0.1%, almost 90% inhibition was observed.

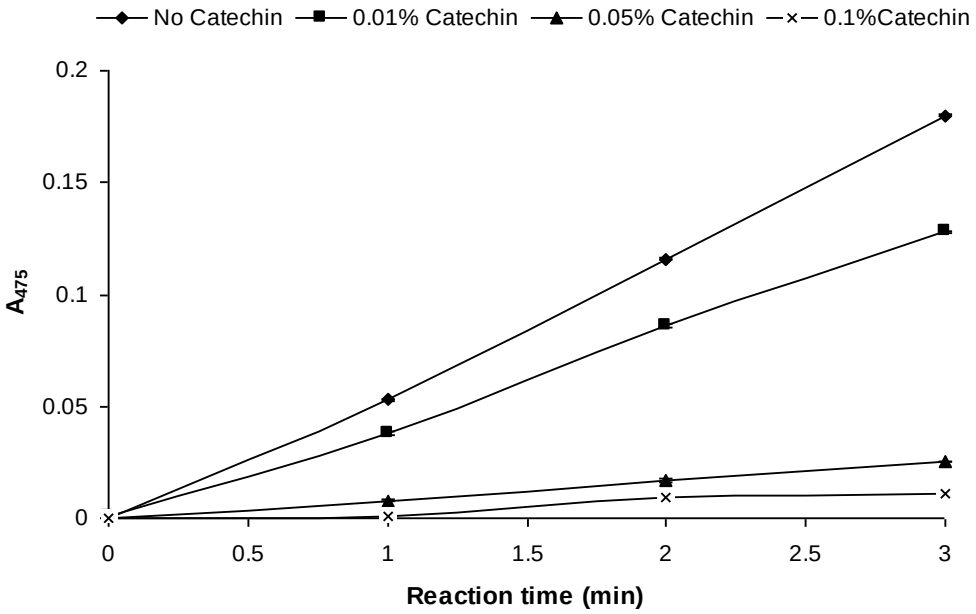

Figure 27. Effect of catechin at different levels on the activity of polyphenoloxidase from the cephalothoraxes of Pacific white shrimp. The decrease in A_{475} indicates the inhibition of DOPA-chrome formation induced by PPO.

Catechin probably acted as a competitive inhibitor for PPO, because of its structural similarity to L-DOPA, a substrate for PPO. Aromatic carboxylic acids of cinnamic acid and its analogues, *p*-coumaric, ferulic, and sinapic acids are competitive inhibitors of PPO (Simpson *et al*., 1987). Some phenolic compounds

inhibit PPO activity by interacting with active site of the enzyme (Janovitz-klapp *et al*., 1990). Furthermore, phenolic compounds could interact with protein or enzymes via hydrogen bond or hydrophobic interaction (Prigent, 2005). Thus, Pacific white shrimp PPO might undergo aggregation, losing its activity, in the presence of catechin. Kubo *et al*. (2003) reported that 1.55 mM of dodecyl gallate was the inhibitory concentration, leading to 50% activity lost of mushroom tyrosinase.

Furthermore, hydroxyl group of catechin might involve in reduction of DOPA-chrome to DOPA, possibly via its ability of electron donating to intermediate quinone, DOPA-chrome. Those actions could be associated with the lowered blackening caused by PPO in Pacific white shrimps during the extended iced storage (Fig. 25). The result reconfirmed that catechin was effective in PPO inhibition especially at high concentration. This contributed to the retardation of melanosis in catechin treated shrimps.

3.5 Conclusions

Catechin could be used as the natural promising agent for melanosis prevention in Pacific white shrimp during iced storage. Apart from prevention of melanosis, treatment of shrimp with catechin could retard microbial growth and lipid oxidation and was able to maintain the freshness. This led to the extended shelf-life of white shrimp. From the consumer health point of view, the use of natural additives like catechin can be a safer means to maximize the storage stability of shrimp.

CHAPTER 4

EFFECT OF CATECHIN AND FERULIC ACID ON MELANOSIS AND QUALITY OF PACIFIC WHITE SHRIMP SUBJECTED TO PRIOR FREEZE-THAWING DURING REFRIGERATED STORAGE

4.1 Abstract

Melanosis of Pacific white shrimp (*Litopenaeus vannamei*) subjected to freeze-thawing with different thawing methods and various cycles were monitored during subsequent refrigerated storage (4^{o}C) up to 4 days. Melanosis score was lower in Pacific white shrimp thawed at 4^{o}C, compared with that found in samples thawed at room temperature or using tap water. Polyphenoloxidase (PPO) activity increased as freeze-thaw cycles increased ($P < 0.05$). Enhanced PPO activity was most likely associated with increased melanosis. Pacific white shrimp treated with catechin (0.05, 0.1 and 0.2% (w/v)) or ferulic acid (1, 2 and 3% (w/v)) and subjected to freeze-thawing with various cycles showed the retarded melanosis during the subsequent refrigerated storage of 4 days, compared with the control ($P < 0.05$). Treatment of shrimp with both phenolic compounds could impede the growth of psychrotrophic bacteria and the spoilage as evidenced by the lowered psychrotrophic bacteria count and total volatile base content (TVB). Sample treated with 0.2% catechin or 3% ferulic acid also exhibited the retarded lipid oxidation during the subsequent refrigerated storage, compared with the control ($P < 0.05$). Thus, either catechin or ferulic acid could be used as the potential additive to lower melanosis of shrimp with prior freeze-thawing.

4.2 Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is an economically important species in Thailand with high market value and have become the essential income generator of the country (Sriket *et al*., 2007). This high-value crustacean is

very perishable and the frozen storage is therefore an important preservation method used for shrimp processing industry. Despite microbial spoilage being effectively terminated, quality deterioration e.g., texture, flavor, and color, still occurs during frozen storage (Sriket *et al*., 2007). Repeated freeze-thawing is a common practice in retail shop, restaurant or home (Boonsumrej *et al*., 2007). During thawing, foods are damaged and quality loss is enhanced, depending upon storage temperature, rate of freezing and thawing, temperature fluctuations, and freeze-thaw abuse during the storage (Srinivasan *et al*, 1997a). Slow thawing process of large seafood portion at 4^{o}C is recommended for retail trade (Diaz-Tenorio *et al*., 2007).

Frozen storage can effectively retard physicochemical changes of shrimp, however black spot formation (melanosis) could take place after thawing (Diaz-Tenorio *et al*., 2007). As a consequence, it drastically reduces the product market value, leading to the considerable financial loss (Kim *et al*., 2000). Melanosis is triggered by a biochemical mechanism which oxidizes phenols to quinones by polyphenoloxidase (PPO). This is followed by non-enzymatic polymerization and auto-oxidation of the quinones, giving rise to dark pigments of high molecular weight (Benjakul *et al*., 2005a). During freezing and thawing, the inactive PPO stored in hemocytes and digestive gland are easily released and activated, and in the presence of suitable substrates and oxygen, melanosis develops more rapidly (Diaz-Tenorio *et al*., 2007). To alleviate or retard melanosis, several melanosis inhibitors including sulfiting agent (Gomez-Guillen *et al*., 2005) and 4-hexylresorcinol (Montero *et al*., 2006) have been used in conjunction with iced storage. However, increasing regulatory attention and awareness of consumers against synthetic additive in food processing have led to the interest in natural additives to prevent melanosis in shrimp. Grape seed extract (Gokoglu and Yerlikaya, 2008) and enokitake extract (Jang *et al*., 2003) could inhibit the melanosis in shrimp during iced storage. Recently, plant phenolic compounds including ferulic acid and catechin were found as the effective additives to retard the melanosis and maintain the quality of Pacific white shrimp during the extended iced storage (Nirmal and Benjakul, 2009a, 2009b). Ferulic acid is belongs to hydroxycinnamic acid, whereas catechin belongs to flavanols class of phenolic compounds. Aromatic carboxylic acids of cinnamic acid and its analogues, *p*-coumaric, ferulic, and sinapic acids are competitive inhibitors of PPO (Kim *et al*.,

2000). These ferulic acid and catechin are easily available from plant source like maize, and green tea etc.

Freeze-thawing might inactivate PPO in kuruma prawn (Adachi *et al*., 2001), however PPO activity was increased in white leg shrimp after freeze-thawing (Diaz-Tenorio *et al*., 2007). Nevertheless, the impact of multiple freeze-thawing on PPO and melanosis in Pacific white shrimp has not been reported. To lower the quality losses caused by the enhanced melanosis after repeated freeze-thawing, phenolic compounds might be used to prevent such a change. The objectives of this study were to evaluate the effect of thawing methods and freeze-thaw cycles on PPO and melanosis in Pacific white shrimp and to study the impact of catechin and ferulic acid at different concentrations on melanosis and quality changes of freeze-thawed shrimp during subsequent refrigerated storage.

4.3 Materials and Methods

4.3.1 Chemicals

L- -(3, 4 dihydroxylphenyl) alanine (L-DOPA), azocasein, Brij-35, catechin, ferulic acid, malonaldehyde bis (dimethyl acetal) and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), ammonium sulfate, potassium carbonate and standard plate count agar (PCA) were obtained from Merck (Darmstadt, Germany). Sodium dedocyl sulfate (SDS), *N*, *N*, *N* *N* -tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA).

4.3.2 Shrimp collection and preparation

Fresh Pacific white shrimp (*Litopenaeus vannamei*) with the size of 55-60 shrimp/kg were purchased from the dock in Hat Yai, Songkhla, Thailand. The shrimp were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h.

Upon arrival, shrimp were washed in cold water and stored in ice until used (not more than 3 h).

4.3.3 Effect of different thawing methods and multiple freeze-thaw cycles on PPO activity and melanosis of Pacific white shrimp

4.3.3.1 Freeze-thawing of Pacific white shrimp

Whole Pacific white shrimp were packed in polyethylene bags (twenty shrimps per bag), heat-sealed and frozen at -20^{o}C using an air-blast freezer (Patkol Co. Ltd., Bangkok, Thailand) for 24 h. These frozen shrimp were thawed using three different methods: 1) leaving at refrigerated temperature (4^{o}C) for 6 h (Srinivasan *et al.*, 1997a), 2) leaving at room temperature (28-30^{o}C) for 45 min (Lopkulkiaert *et al*., 2009), and 3) using running tap water (27-28^{o}C) for 30 min (Sriket *et al*., 2007) until the core temperature of second segment of shrimp reached 0-2^{o}C. Thawed samples were referred to as one freeze-thaw cycle . Thawed samples were again frozen for 24 h, followed by thawing using different methods up to 3 and 5 cycles. Sample without freeze-thawing was referred to as zero freeze-thaw cycle . All samples obtained were subjected to measurement of PPO and protease activities as well as melanosis score assessment.

4.3.3.1.1 Preparation of PPO extract from the cephalothoraxes of Pacific white shrimp

The cephalothoraxes of twenty shrimp with different freeze-thaw cycles (0, 1, 3 and 5 cycles) and different thawing methods were separated and powderized by grinding with liquid nitrogen in a waring blender (AY46, Moulinex, China). PPO was extracted from the prepared powder according to the method of Simpson *et al*. (1987) with a slight modification. The powder (50 g) was mixed with 150 mL of the extracting buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 1.0 M NaCl and 0.2% Brij 35). The mixture was stirred continuously at 4^{o}C for 30 min, followed by centrifugation at 8,000 x g at 4^{o}C for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). Solid ammonium sulfate was added into the supernatant to obtain 40% saturation and the

mixture was allowed to stand at 4°C for 30 min. The precipitate was collected by centrifugation at 12,500 x g at 4°C for 30 min using a refrigerated centrifuge. The pellet obtained was dissolved in a minimum volume of 0.05 M sodium phosphate buffer, pH 7.2 and dialyzed against 15 volumes of the same buffer at 4°C with three changes of dialysis buffer. The insoluble materials were removed by centrifugation at 3,000 x g at 4°C for 30 min and the supernatant was used as crude PPO extract .

4.3.3.1.2 Measurement of PPO activity

PPO activity was assayed using L-DOPA as a substrate according to the method of Simpson *et al.* (1987) with a slight modification. The assay system consisted of 100 L of crude PPO extract, 600 L of 15 mM L-DOPA in deionized water, 400 L of 0.05 M phosphate buffer, pH 6.0 and 100 L of deionized water. The reaction mixture was incubated for 3 min at 45°C and the formation of dopachrome was monitored by measuring the absorbance at 475 nm (A_{475}) using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of PPO activity was defined as an increase in A_{475} by 0.001 /min. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture and the deionized water was used instead.

4.3.3.1.3 SDS-PAGE and activity staining of PPO

Protein patterns of PPO crude extract were analyzed by SDS–PAGE according to the method of Laemmli (1970). The extract was mixed with the sample buffer containing 1.5 M ME at a ratio of 1:1 (v/v). The samples (15 L) were loaded onto the polyacrylamide gel made of 7.5% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, one of two identical gels was immersed in a McIlvaine buffer (0.2 M sodium phosphate and 0.1 M sodium citrate), pH 6.5, containing 15 mM L-DOPA for 25 min at 25°C. PPO activity zone appeared as the dark band. Another gel was stained by 0.125% Coomassie Brilliant Blue R-250 and de-stained in 25% methanol and 10% acetic acid. To estimate the molecular weight of PPO, the markers including myosin from rabbit muscle (200 kDa), -galactosidase from *E. coli* (116 kDa), phosphorylase

b from rabbit muscle (97 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase from bovine liver (55 kDa), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (36 kDa), and carbonic anhydrase from bovine erythrocytes (29 kDa) were used.

4.3.4 Effect of different thawing methods and multiple freeze-thaw cycles on protease activity of Pacific white shrimp

4.3.4.1 Preparation of protease extract from the cephalothoraxes of Pacific white shrimp

Cephalothoraxes powder (20 g) of twenty shrimp from each treatment was mixed with 2 volumes of 0.01 M sodium phosphate buffer (pH 7.6) (Brauer *et al.*, 2003). The mixture was homogenized for 5 min using a homogenizer (IKA labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was stirred for 30 min at 4^{o}C, followed by centrifugation at 10,000 x g for 30 min at 4^{o}C using a refrigerated centrifuge. The supernatant was referred to as crude protease extract .

4.3.4.2 Determination of protease activity

Protease activity of crude protease extract was determined using azocasein as a substrate according to the method of Muhila-Almazan and Garcia-Carreno (2002). To initiate the reaction, 10 L of the extract was added into assay mixtures containing 500 L of 1% azocasein in 50 mM Tris-HCl buffer, pH 8 at 25^{o}C. The reaction was terminated after 10 min by the addition of 500 L of 20 % trichloroacetic acid (TCA) and the mixture was allowed to stand for 15 min at 4°C to precipitate unhydrolyzed protein substrate, followed by centrifuging at 7,000 x g for 5 min. The supernatant was separated and the absorbance at 366 nm (A_{366}) of the released dye was recorded. A blank was prepared in the same manner, except the extract was added after addition of 20 % TCA.

One unit of protease was expressed as the change in A_{366} per min under the condition used and the specific activity was expressed as unit per mg of protein.

4.3.5 Effect of different thawing methods and multiple freeze-thaw cycles on melanosis formation of Pacific white shrimp

Whole Pacific white shrimp subjected to different freeze-thaw cycles with various thawing methods were drained for 5 min at 4^{o}C. The samples (20 shrimp) were placed on a polystyrene tray (20 x 20 cm^{2}) and covered with shrink film (Tesco Lotus, Hat Yai, Thailand). All samples were kept at 4^{o}C. Melanosis or blackening of Pacific shrimp was evaluated through visual inspection by ten trained panelists using 10-point scoring test (Montero *et al*, 2001b) every day up to 4 days. Panelists were asked to give the melanosis score (0 to 10), where 0 = absent; 2 = slight (up to 20 of shrimps surface affected) 4 moderate (20 to 40 of shrimps surface affected) 6 notable (40 to 60 of shrimps surface affected) 8 = severe (60 to 80 of shrimps surface affected) 10 extremely heavy (80 to 100 of shrimps surface affected).

4.3.6 Effect of catechin and ferulic aicd on melanosis inhibition and the quality changes of Pacific white shrimp with prior freeze-thawing during the refrigerated storage

4.3.6.1 Preparation of catehcin and ferulic acid solutions

Catechin solutions with different concentrations (0.05, 0.1 and 0.2%) were adjusted to pH 8 by 6 N NaOH and stirred for 15 min at room temperature (26-28^{o}C). Thereafter, the pH of the solution was adjusted to 7 using 6 N HCl and referred to as catechin solution . Ferulic acid solution with different concentrations (1, 2 and 3%) was also prepared in the same manner.

4.3.6.2 Preparation of shrimps treated with catechin and ferulic acid

Whole Pacific white shrimp were immersed in the solution of catechin (0.05, 0.1 and 0.2%) or ferulic acid (1, 2 and 3%) using the shrimp/solution ratio of 1:2 (w/v) for 15 min. The treated shrimp were drained on the screen for 3 min at 4^{o}C. Shrimp without any treatment were used as the control. The samples were placed on

polystyrene tray (20 x 20 cm^2) and covered with shrink film. All samples were then stored at 4^oC and melanosis was evaluated every day up to 8 days.

Another portion of samples were packed in polyethylene bag (20 shrimp / bag) and subjected to freeze-thawing with different cycles (1, 3 and 5 cycles) using thawing method by leaving the frozen samples at 4^oC as described previously. The samples were drained for 5 min at 4^oC. The samples were placed on tray, covered with shrink film and stored at 4^oC and melanosis and quality changes were monitored every day for totally 4 days.

4.3.6.3 Determination of psychrotrophic bacterial count (PSC)

Psychrotrophic bacterial count was determined following the method of Thepnuan *et al*. (2008) with some modifications. Five whole Pacific white shrimp were collected aseptically and used as the composite sample. Ground sample without peeling (25 g) was placed in a stomacher bag containing 225 mL of 0.85 % saline water. After mixing for 1 min in a Stomacher blender (M400, Seward, UK), appropriate dilutions were prepared. Determination of psychrotrophic bacterial count was conducted using plate count agar (PCA) containing 0.5% NaCl by spread plate method at 4^oC for 7 days.

4.3.6.4 Determination of total volatile base (TVB)

Total volatile base (TVB) contents in shrimp meat were determined using the Conway micro-diffusion method (Conway and Byrne, 1936). Sample (2 g) was extracted with 8 mL of 4% (w/v) trichloroacetic acid (TCA) solution. The mixture was homogenized at 8,000 rpm for 1 min using a PT 2100 homogenizer (Kinematica AG, CH-6014, Littau/Luzern, Switzerland). The homogenate was kept at room temperature for 30 min. The homogenate was filtered through Whatman No.41 filter paper (Schleicher & Schuell, Maidstone, England). The filtrate was collected and the final volume was adjusted to 10 mL using 4% TCA. The inner ring solution (1 mL) and filtrate (1 mL) were added to inner ring and outer ring of the Conway unit, respectively. One mL of saturated K_2CO_3 solution was then added into outer ring. The Conway unit was closed and the solution was mixed slowly. The mixture was incubated at 37°C for 60 min and the inner ring solution was titrated with 0.02 N HCl

using a micro-burette until green color turned into pink. For the blank, TCA solution (4%) was used instead of sample extract. The amounts of TVB were calculated and results were expressed as mg N/ 100 g shrimp meat.

4.3.6.5 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS in the samples was determined as described by Benjakul and Bauer (2001) with some modifications. Ground shrimp meat (1 g) was mixed with 9 mL of a 0.25 N HCl solution containing 0.375% TBA and 15% TCA. The mixture was heated in boiling water for 10 min, followed by cooling with the running water. The mixture was centrifuged at 4,000 x g for 20 min (MIKRO20, Hettich Zentrifugan, Germany). The supernatant was collected and the absorbance was read at 532 nm using a UV- 160 spectrophotometer. TBARS was calculated from the standard curve of malonaldehyde (0 to 2 ppm) and expressed as mg malonaldehyde /kg shrimp meat.

4.3.7 Statistical analyses

All experiments were performed in triplicate and a completely randomized design (CRD) was used. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan s multiple range tests (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA).

4.4 Results and Discussion

4.4.1 Effect of different thawing methods and multiple freeze-thaw cycles on PPO activity and melanosis of Pacific white shrimp

The effect of different thawing methods and freeze-thaw cycles on PPO activity of Pacific white shrimp cephalothoraxes is shown in Figure 28. For the same thawing method, the increase in PPO activity was observed with increasing freeze-thaw cycles ($P < 0.05$). Nevertheless, no differences in activity were noticeable

between the fresh (without freeze-thawing) and those subjected to thawing at 4°C with one freeze-thaw cycle ($P > 0.05$).

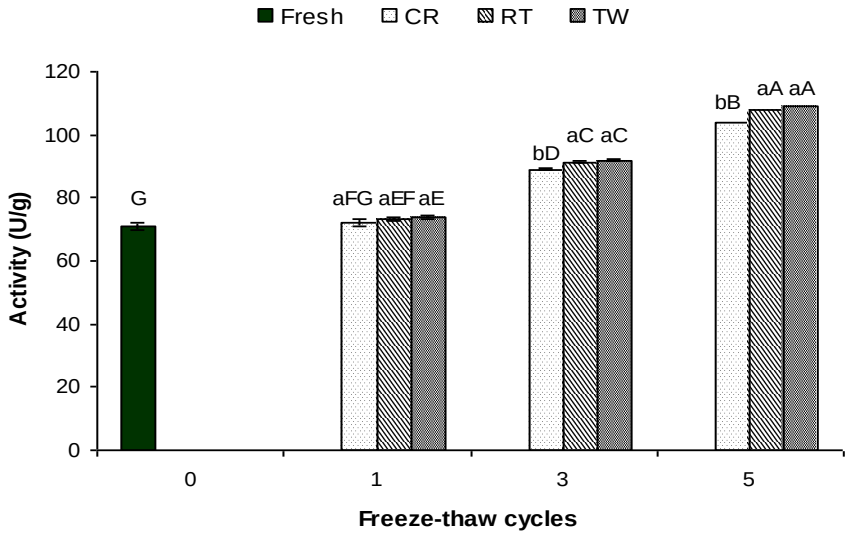

Figure 28. Polyphenoloxidase activity of Pacific white shrimp subjected to freeze-thawing using different thawing methods with various freeze-thaw cycles. CR: thawing at 4°C; RT: thawing at room temperature; TW: thawing using running tap water. Bars represent the standard deviation from triplicate determinations. Different capital letters on the bars within the same thawing method together with the control indicate the significant differences ($P < 0.05$). The different letters on the bars within the same freeze-thaw cycle indicate significant differences ($P < 0.05$).

During freezing and thawing, the inactive form of PPO stored in hemocytes, the digestive gland and chromatophores are easily released and activated (Diaz-Tenorio *et al*., 2007). For the same freeze-thaw cycle, thawing at 4°C resulted in the lower PPO activity, compared to other thawing methods ($P < 0.05$). Diaz-Tenorio *et al*. (2007) recommended cryogenic freezing and thawing at 4°C of white leg shrimp (*Litopenaeus vannamei*) for marketing purpose. With one freeze-thaw cycle, thawing at room temperature and by using running tap water caused the increase in PPO activity, compared with that found in fresh sample. Both thawing methods might contribute to more rapid increase in temperature than thawing at 4°C, which is a slow thawing process in low-temperature environment (Boonsumrej *et al*., 2007). It has been known that repeated melting and reformation of ice crystals causes the damage to cell membrane and organelles (Sriket *et al*., 2007). The release of more

or less PPO was dependent on number of freeze-thaw cycles. Srinivasan *et al.* (1997b) reported that the protein denatruation and destabilization was dependent on thawing rate or method. Therefore, an increase in PPO activity was governed by the number of freeze-thaw cycles.

Activity staining of PPO from the cephalothoraxes of Pacific white shrimp subjected to multiple freeze-thaw cycles by leaving the sample at 4^{o}C is shown in Figure 29. PPO in crude extracts from cepahalothoraxes of white shrimp without and with multiple freeze-thaw cycles appeared as dark brown color with the apparent molecular weight of 210 kDa.

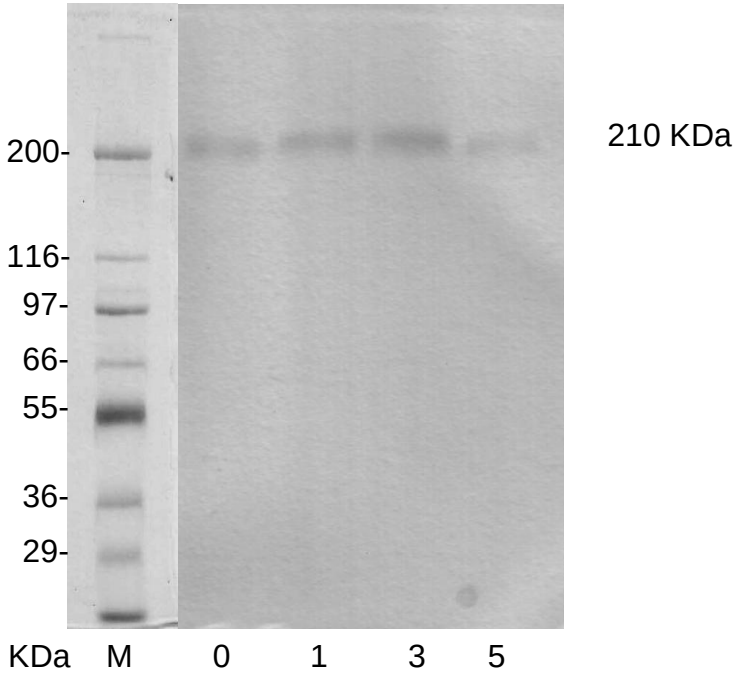

Figure 29. Activity staining of polyphenoloxidase of white shrimp subjected to freeze-thawing with different cycles. Frozen shrimp were thawed by leaving the sample at 4^{o}C for 6 h. Numbers designate freeze-thaw cycles. M: molecular weight marker.

PPO was able to induce the oxidation of DOPA to DOPA-quinone and the intermediate products subsequently underwent polymerization to melanin (Benjakul *et al*., 2005a). PPO band intensity increased when one and three freeze-thaw cycles were applied. The result was in accordance with the increase in PPO activity of sample subjected to freeze-thawing for one and three cycles (Figure 28). The partially purified PPO extract from deepwater pink shrimp (*Parapenaeus*

longirostris) after 3 cycles of freezing-thawing had similar electrophoretic mobility, compared with that found in PPO extract without freeze-thawing. However, the increase in band intensity was found (Zamorano *et al*., 2009). With five freeze-thaw cycles, PPO band was found to be less intense, which was not correlated with *in vitro* PPO activity. After being subjected to five freeze-thaw cycles, active PPO might be susceptible to the conformational changes in the presence of sample buffer used for electrophoresis. However, Zamorano *et al*. (2009) suggested that active PPO from deepwater pink shrimp (*Parapenaeus longirostris*) formed the aggregates corresponding to 212 kDa that survived from SDS treatment. Benjakul *et al*. (2005a) reported that PPO from the kuruma prawn cephalothoraxes had molecular weight of 160 kDa.

Protease activity of crude protease extract from the cephalothoraxes of shrimp without and with freeze-thawing was similar, regardless of freeze-thaw cycles ($P > 0.05$) (data not shown). Wang *et al*. (1992) reported that the proteases are one of the main factors influencing phenolase activation. For all samples, protease activity was found in the range of 4.7- 5.76 units/g cephalothorax powder. Muhila-Almazan and Garcia-Carreno (2002) reported that trypsin and chymotrypsin are the most abundant proteolytic enzymes in the midgut gland of shrimp. However, there were no significant changes in total protease activity of Pacific white shrimp after being subjected to multiple freeze-thaw cycles. Wang *et al*. (1992) found two thiol proteases and one metal dependent serine protease from Norway lobster (*Nephrops norvegicus*) head. Nevertheless, only a serine protease was involved in the phenolase activation process in Norway lobster.

Melanosis score of Pacific white shrimp subjected to different thawing methods and freeze-thaw cycles during the subsequent refrigerated storage up to 4 days is shown in Figure 30. Shrimp with different thawing methods had the different rate in melanosis development during the extended storage. Shrimp without freeze-thawing had melanosis score of 4 at day 4. Melanosis score of white shrimp increased as the number of freeze-thaw cycles increased for all samples with different thawing methods ($P < 0.05$). However, the increase in melanosis was more pronounced in shrimp thawed at room temperature and using tap water than that subjected to thawing at 4^{o}C. These results were in accordance with *in vitro* PPO activity (Figure 28).

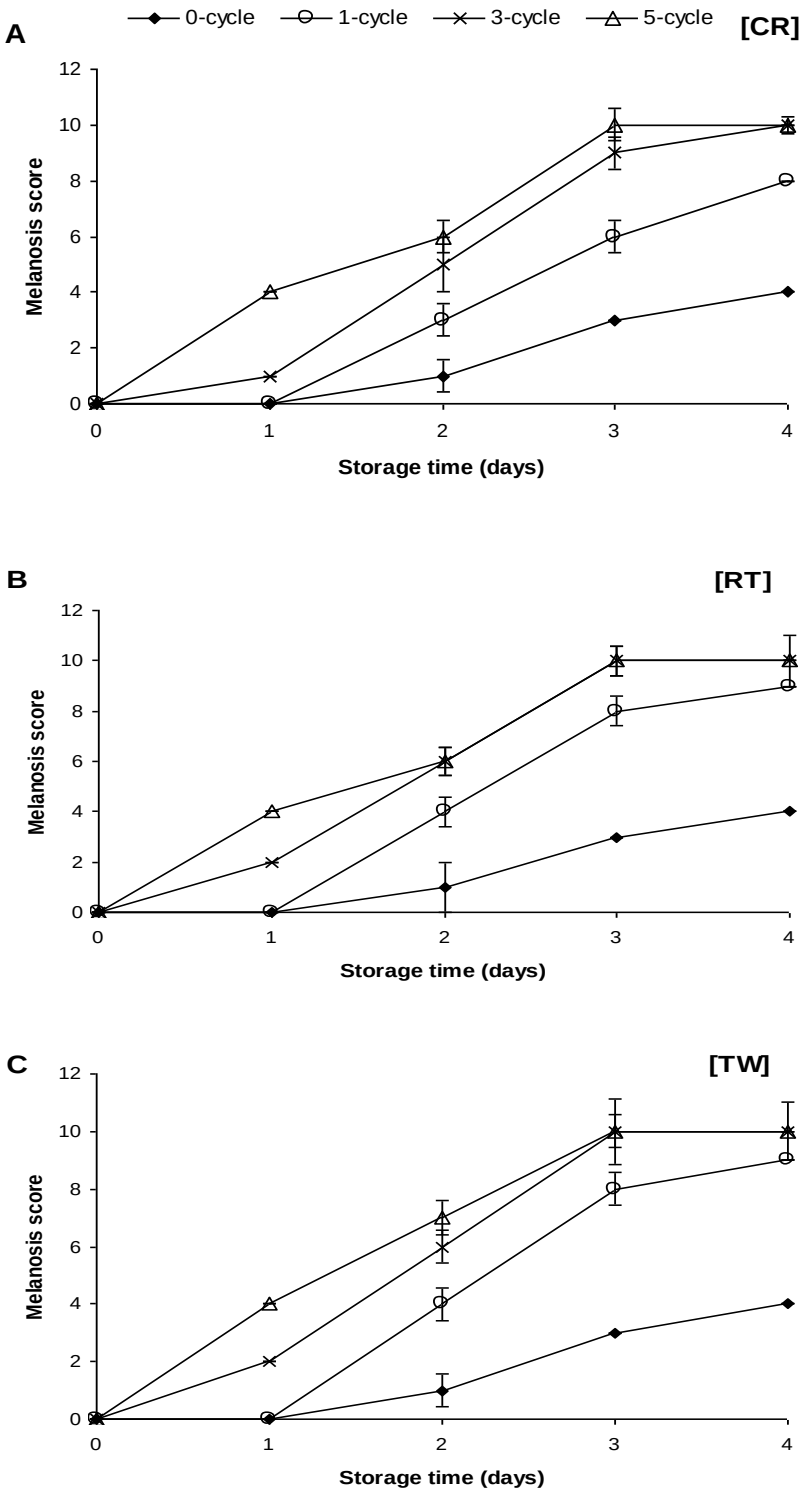

Figure 30. Melanosis score of shrimp subjected to freeze-thawing using different thawing methods with various freeze-thaw cycles during the subsequent refrigerated storage. Bars represent the standard deviation (n=3). CR: thawing at 4^{o}C; RT: thawing at room temperature; TW: thawing using running tap water.

With 1 or 3 freeze-thaw cycles, melanosis score was lower in shrimp thawed at 4^{o}C than those thawed at room temperature and using tap water. However, with 5 freeze-thaw cycles, similar melanosis score of white shrimp was observed for all samples ($P > 0.05$). These results were in agreement with Diaz-Tenorio *et al*. (2007) who found that freezing and thawing significantly increased the rate of melanosis of white leg shrimp stored at 4^{o}C. Shrimp with multiple freeze-thaw cycles (1, 3 and 5 cycles) had the scores of 8-10, compared with that of shrimp without freeze-thawing at day 4 of refrigerated storage ($P < 0.05$). Melanosis is a phenomenon, in which brown color is developed by the enzymatic reaction mediated by PPO (Benjakul *et al*., 2005a). Therefore, melanosis in refrigerated Pacific white shrimp was governed by prior freeze-thawing as well as the storage time.

4.4.2 Effect of catechin and ferulic acid on melanosis and the quality changes of Pacific white shrimp with prior freeze-thawing during the extended refrigerated storage

4.4.2.1 Changes in melanosis

Melanosis score of shrimp treated without and with catechin and ferulic acid at different levels during 8 days of refrigerated storage is shown in Figure 31. At day 0 and 1, no melanosis was detected in all samples (score=0). When the storage time increased, melanosis score in all samples increased ($P < 0.05$). However, the increasing rate of melanosis varied with treatments. The intensity of melanosis formation depends on the substrate, enzyme concentration and activity (Simpson *et al.*, 1987). No melanosis was observed in samples treated with catechin and ferulic acid at all levels used within the first 2 days of storage. Melanosis was retarded in the shrimp with the treatment of catechin or ferulic acid in the concentration dependent manner. Shrimp treated with 0.1 or 0.2 % catechin or 2 or 3 % ferulic acid showed the lower melanosis, compared to others at the last day of storage (day 8), while the severe melanosis was found in the control samples. Gokoglu and Yerlikaya (2008) found that shrimp (*Parapenaeus longirostris*) treated with ethanol extract of grape seed (*Vitis vinifera* sp.) at a concentration of 1.5 % had the lowered melanosis formation during

storage at 4^{o}C for 3 days. Therefore, treatment of shrimp with 0.2 % catehcin or 3 % ferulic acid could lower melanosis during the extended refrigerated storage.

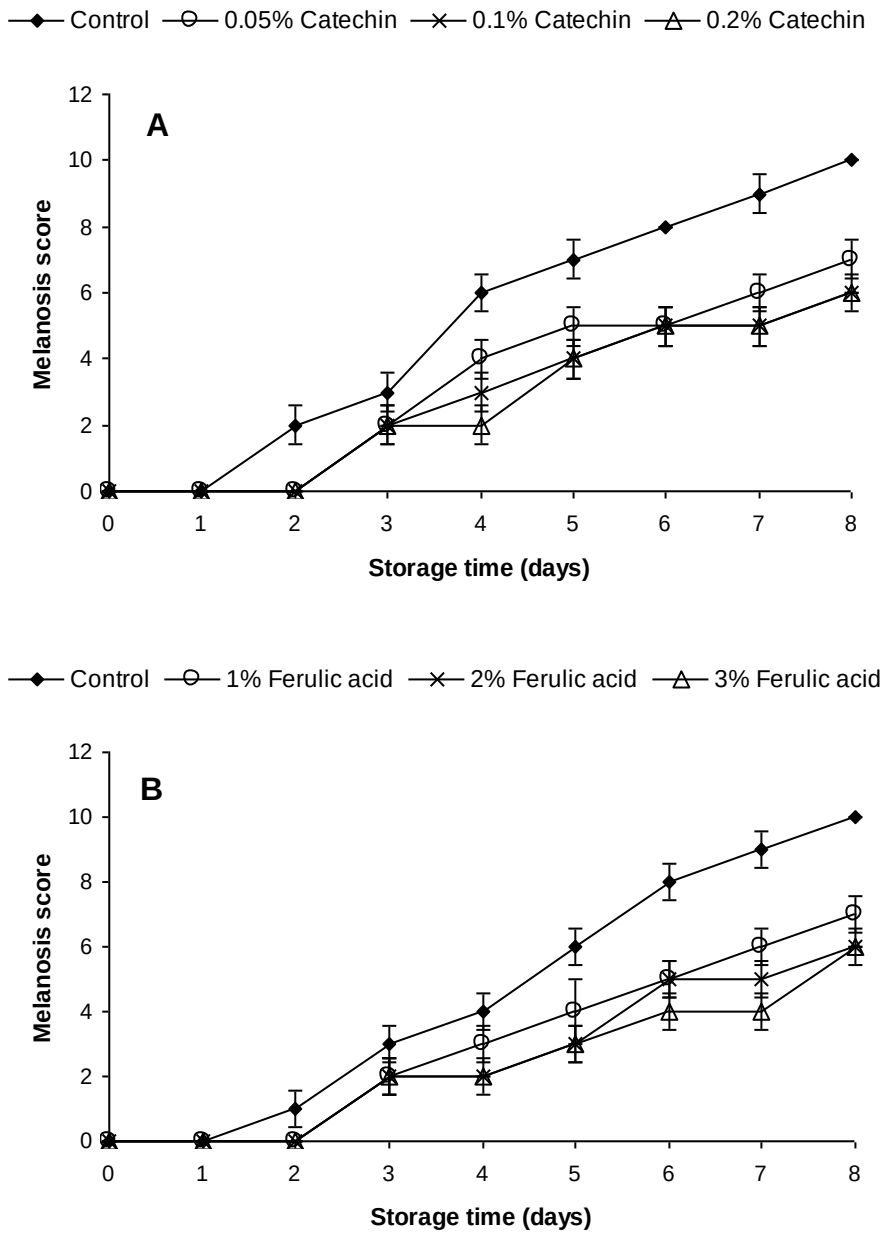

Figure 31. Melanosis score of shrimp treated with catechin (A) and ferulic acid (B) at different levels during 8 days of refrigerated storage. Bars represent the standard deviation from triplicate determinations.

Table 3 shows the melanosis score of Pacific white shrimp treated without and with catechin and ferulic acid at different levels and subjected to freeze-thawing with various cycles during the subsequent refrigerated storage of 4 days. In general, there was no melanosis observed immediately after freeze-thawing (day 0) for all samples. Melanosis score of shrimp increased as the subsequent storage time and the number of freeze-thawing cycles increased ($P < 0.05$). At the same storage time, the increase in melanosis score was lowered in shrimp treated with catehin and ferulic acid, compared with the control ($P < 0.05$). Melanosis was found in shrimp treated with 2 or 3% ferulic acid at day 2, regardless of freeze-thaw cycles. However, as the number of freeze-thawing cycles and subsequent storage time increased, catechin, even with an increasing level, had low inhibitory effect towards melanosis formation ($P > 0.05$). Nevertheless, ferulic acid at levels of 2 or 3% lowered melanosis formation more effectively ($P < 0.05$). Ferulic acid at 2 or 3% might penetrate in shrimp to a higher extent, compared with catechin at levels of 0.1 or 0.2% during soaking. Moreover, ferulic acid has the lower molecular weight (194 kDa), compared with catechin (290 kDa). As a result, ferulic acid could migrate into shrimp with ease. Shrimp treated with ferulic acid at levels of 2 and 3% and subjected to 5 freeze-thawing cycles had melanosis score of 8 and 6, respectively at day 4 of subsequent refrigerated storage, which was similar to the score found in the control (without treatment) at day 2 of storage when the same freeze-thawing cycle was applied.

Therefore, the multiple freeze-thawing could enhance melanosis formation of Pacific white shrimp during subsequent refrigerated storage, compared with the shrimp without freeze-thawing. Shrimp treated with catechin (0.1 or 0.2%) and ferulic acid (2 or 3%) prior to freeze-thawing with different cycles had the lowered melanosis during subsequent refrigerated storage. It was noted that the latter was more effective in prevention of melanosis in Pacific white shrimp with prior freeze-thawing during the subsequent refrigerated storage.

Table 3. Melanosis score of Pacific white shrimp treated without and with catechin and ferulic acid at different levels and subjected to freeze-thawing at various cycles during the subsequent refrigerated storage.

Freeze-thaw cycle	Storage time (days) at 4°C	Catechin (%)				Ferulic acid (%)			
		0	**0.05**	**0.1**	**0.2**	**0**	**1**	**2**	**3**
I	**0**	0 ± 0.0 E	0 ± 0.0 D	0 ± 0.0 D	0 ± 0.0 D	0 ± 0.0 D	0 ± 0.0 D	0 ± 0.0 C	0 ± 0.0 C
	1	2 ± 0.5 aD	1 ± 0.5 bD	1 ± 0.5 bD	0 ± 0.0 bD	0 ± 0.0 D	0 ± 0.0 D	0 ± 0.0 C	0 ± 0.0 C
	2	4 ± 1.0 aC	3 ± 0.5 bC	2 ± 0.0 bC	2 ± 0.5 bC	3 ± 0.5 aC	2 ± 0.5 bC	2 ± 0.0 bB	2 ± 0.5 bB
	3	6 ± 0.5 aB	4 ± 0.5 bB	4 ± 0.5 bB	3 ± 0.5 bB	6 ± 0.5 aB	3 ± 0.5 bB	2 ± 0.5 bB	2 ± 0.5 bB
	4	10 ± 0.0 aA	8 ± 0.5 bA	7 ± 0.5 cA	6 ± 0.5 dA	9 ± 0.5 aA	5 ± 0.5 bA	4 ± 0.5 bcA	3 ± 0.5 cA
III	**0**	0 ± 0.0 E	0 ± 0.0 E	0 ± 0.0 E	0 ± 0.0 E	0 ± 0.0 E	0 ± 0.0 D	0 ± 0.0 C	0 ± 0.0 C
	1	3 ± 0.5 aD	3 ± 0.0 aD	2 ± 0.5 bD	2 ± 0.5 bD	2 ± 0.5 aD	0 ± 0.0 bD	0 ± 0.0 bC	0 ± 0.0 bC
	2	5 ± 0.5 aC	4 ± 0.5 aC	4 ± 0.5 aC	4 ± 0.5 aC	5 ± 0.5 aC	4 ± 0.5 abC	4 ± 0.5 abB	3 ± 0.5 bB
	3	8 ± 0.5 aB	6 ± 0.5 bB	5 ± 0.5 bB	5 ± 0.5 bB	8 ± 0.5 aB	5 ± 0.5 bB	4 ± 0.5 cB	3 ± 0.0 cB
	4	10 ± 0.0 aA	10 ± 0.0 aA	9 ± 0.5 bA	8 ± 0.5 cA	10 ± 0.5 aA	7 ± 0.5 bA	6 ± 0.5 bcA	5 ± 0.5 cA
V	**0**	0 ± 0.0 D	0 ± 0.0 E	0 ± 0.0 E	0 ± 0.0 E	0 ± 0.0 D	0 ± 0.0 E	0 ± 0.0 C	0 ± 0.0 D
	1	4 ± 0.5 aC	3 ± 0.5 aD	3 ± 0.5 aD	3 ± 0.5 aD	4 ± 0.5 aC	2 ± 0.5 bD	0 ± 0.0 cC	0 ± 0.0 cD
	2	7 ± 0.5 aB	7 ± 0.5 aC	6 ± 0.5 abC	5 ± 0.5 bC	7 ± 0.5 aB	5 ± 0.5 bC	4 ± 0.5 bcB	3 ± 0.5 cC
	3	10 ± 0.5 aA	8 ± 0.5 bB	7 ± 0.5 bB	7 ± 0.5 bB	10 ± 0.5 aA	6 ± 1.0 bB	4 ± 0.0 cB	4 ± 0.5 cB
	4	10 ± 0.0 A	10 ± 0.0 A	10 ± 0.0 A	10 ± 0.0 A	10 ± 0.0 aA	10 ± 0.5 aA	8 ± 0.5 bA	6 ± 0.5 cA

Different capital letters in the same column within the same freeze-thaw cycle indicate the significant differences ($P < 0.05$); Different letters in the same row within the same phenolic compound indicate significant differences ($P < 0.05$). Value are mean ± standard deviation (n=10).

4.4.2.2 Changes in psychrotrophic bacterial count

Psychrotrophic bacterial count (PSC) of Pacific white shrimp treated without and with 0.2% catechin or 3% ferulic acid and subjected to freeze-thawing with various cycles during the subsequent refrigerated storage of 4 days is shown in Table 4. PSC increased continuously in the control (without freeze-thawing) throughout the storage of 4 days ($P < 0.05$). No PSC was found in all samples at day 0, suggesting that no psychrotrophic bacteria were found as the normal flora in the fresh shrimp. At the end of storage (day 4), PSC of the control, those treated with 0.2 % catechin or 3 % ferulic acid without prior freeze-thawing were 4.93, 4.56, and 3.93 log CFU/g, respectively. The result indicated the antimicrobial activity of both compounds in treated shrimp during the storage. Lopez-Caballero *et al*. (2007) reported that microbial spoilage was retarded during frozen storage, but continued in defrosted shrimp. It was noted that PSC was detectable at day 2 for the samples with multiple freeze thaw cycles. However, PSC was found at day 3 for the sample treated with 3% ferulic acid and subjected to freeze-thawing for 1 or 3 cycles. As the storage time increased, PSC for all samples increased ($P < 0.05$). However, the increase in PSC was lowered in shrimp treated with 3 % ferulic acid. Treatment of shrimp with 0.2 % catechin also exhibited the inhibition towards psychrotrophic bacteria in shrimp. Phenolic compounds might disrupt the cell wall of microorganism by forming complex with proteins in cell wall and make lyses of cell wall (Chanthachum and Beuchat, 1997). From the result, the lowest PSC was found in samples with 1 freeze-thaw cycle, compared to those with 3 and 5 freeze-thaw cycles ($P < 0.05$). It was postulated that the nutrient released after repeated freeze-thawing could promote the growth of psychrotrophic bacteria in shrimp stored in ice. Regardless of freeze-thaw cycles, treatment of catechin or freulic acid could retard the growth of PSC during the subsequent storage. Therefore, the treatment of ferulic acid or catechin might show the inhibitory effect on the growth of psychrotrophic bacteria in shrimp subjected to freeze-thawing.

Table 4. Psychrotrophic bacterial count (PSC), total volatile base (TVB) and thiobarbituric acid reactive substances (TBARS) of shrimp treated without and with 0.2% catechin or 3% ferulic acid and subjected to freeze-thawing at various cycles during the subsequent refrigerated storage.

Freeze-thaw cycles	Storage time (days) at 4°C	Psychrotrophic bacterial count (PSC) log CFU g^{-1}			Total volatile base (TVB) mg N/ 100 g meat			Thiobarbituric acid reactive substances (TBARS) mg malanoaldehyde/ Kg meat		
		Control	0.2% catechin	3% ferulic acid	Control	0.2% catechin	3% ferulic acid	Control	0.2% catechin	3% ferulic acid
0	0	NA	NA	NA	4.11 ± 0.13 aE	4.10 ± 0.14 aD	4.11 ± 0.13 aD	0.73 ± 0.06 aE	0.77 ± 0.01 aE	0.74 ± 0.05 aD
	1	2.88 ± 0.03 aD	2.39 ± 0.02 bD	1.88 ± 0.04 cD	4.76 ± 0.23 aD	4.28 ± 0.14 bD	4.31 ± 0.14 bD	1.15 ± 0.05 aD	1.02 ± 0.03 bD	0.96 ± 0.06 bC
	2	3.66 ± 0.04 aC	2.96 ± 0.03 bC	2.14 ± 0.03 cC	5.42 ± 0.13 aC	5.12 ± 0.12 abC	4.99 ± 0.21 bC	1.47 ± 0.07 aC	1.34 ± 0.02 aC	1.12 ± 0.16 bB
	3	4.21 ± 0.08 aB	3.85 ± 0.04 bB	2.95 ± 0.02 cB	6.05 ± 0.15 aB	5.55 ± 0.04 bB	5.48 ± 0.15 bB	1.66 ± 0.03 aB	1.47 ± 0.01 bB	1.42 ± 0.04 bA
	4	4.93 ± 0.02 aA	4.56 ± 0.05 bA	3.93 ± 0.12 cA	6.90 ± 0.13 aA	6.22 ± 0.03 bA	6.11 ± 0.20 bA	1.77 ± 0.01 aA	1.58 ± 0.01 bA	1.58 ± 0.02 bA
I	0	NA	NA	NA	4.22 ± 0.02 aE	4.09 ± 0.15 aE	4.18 ± 0.01 aE	1.35 ± 0.00 aC	1.12 ± 0.01 bC	0.98 ± 0.02 cD
	1	NA	NA	NA	5.04 ± 0.02 aD	4.54 ± 0.12 bD	4.43 ± 0.00 bD	1.43 ± 0.06 aC	1.18 ± 0.02 bC	1.10 ± 0.02 bC
	2	1.93 ± 0.02 aC	1.13 ± 0.13 bC	NA	5.60 ± 0.01 aC	5.29 ± 0.02 bC	5.10 ± 0.18 bC	1.55 ± 0.06 aB	1.32 ± 0.04 bAB	1.28 ± 0.03 bB
	3	2.53 ± 0.05 aB	2.21 ± 0.01 bB	1.55 ± 0.06 cB	6.14 ± 0.22 aB	5.73 ± 0.12 bB	5.59 ± 0.22 bB	1.57 ± 0.04 aAB	1.28 ± 0.07 bB	1.22 ± 0.08 bB
	4	2.88 ± 0.03 aA	2.36 ± 0.10 bA	1.84 ± 0.06 cA	7.15 ± 0.12 aA	6.31 ± 0.05 bA	6.22 ± 0.05 bA	1.67 ± 0.05 aA	1.38 ± 0.01 bA	1.37 ± 0.02 bA
III	0	NA	NA	NA	4.59 ± 0.14 aE	4.29 ± 0.14 bE	4.28 ± 0.14 bD	1.41 ± 0.03 aD	1.17 ± 0.06 bCD	1.08 ± 0.03 bD
	1	NA	NA	NA	5.32 ± 0.02 aD	5.01 ± 0.02 bD	4.96 ± 0.06 bC	1.52 ± 0.05 aC	1.23 ± 0.01 bC	1.19 ± 0.05 bB
	2	1.99 ± 0.02 aC	1.34 ± 0.03 bB	NA	5.77 ± 0.15 aC	5.28 ± 0.01 bC	5.14 ± 0.17 bC	1.63 ± 0.04 aB	1.36 ± 0.02 bB	1.36 ± 0.00 bA
	3	2.85 ± 0.03 aB	2.44 ± 0.03 bA	1.87 ± 0.02 cB	6.63 ± 0.14 aB	5.79 ± 0.12 bB	5.75 ± 0.11 bB	1.32 ± 0.08 aD	1.13 ± 0.08 bD	1.12 ± 0.07 bBC
	4	3.11 ± 0.03 aA	2.44 ± 0.12 bA	2.14 ± 0.13 cA	7.46 ± 0.16 aA	6.50 ± 0.13 bA	6.41 ± 0.05 bA	1.77 ± 0.01 aA	1.48 ± 0.01 bA	1.43 ± 0.02 cA
V	0	NA	NA	NA	4.86 ± 0.05 aE	4.48 ± 0.02 bE	4.46 ± 0.01 bE	1.49 ± 0.02 aC	1.24 ± 0.00 bCD	1.17 ± 0.02 cB
	1	NA	NA	NA	5.61 ± 0.03 aD	5.11 ± 0.13 bD	5.09 ± 0.14 bD	1.62 ± 0.02 aB	1.26 ± 0.04 bC	1.24 ± 0.04 bB
	2	2.13 ± 0.01 aC	1.81 ± 0.03 bC	1.15 ± 0.15 cB	6.20 ± 0.08 aC	5.55 ± 0.04 bC	5.38 ± 0.13 bC	1.70 ± 0.07 aB	1.39 ± 0.03 bB	1.38 ± 0.05 bA
	3	3.14 ± 0.01 aB	2.76 ± 0.01 bB	2.23 ± 0.02 cA	7.09 ± 0.13 aB	6.28 ± 0.03 bB	6.22 ± 0.01 bB	1.22 ± 0.07 aD	1.20 ± 0.02 aD	1.15 ± 0.09 aB
	4	3.32 ± 0.02 aA	2.92 ± 0.03 bA	2.34 ± 0.04 cA	7.93 ± 0.12 aA	6.67 ± 0.05 bA	6.51 ± 0.15 bA	1.93 ± 0.01 aA	1.48 ± 0.02 bA	1.45 ± 0.01 bA

Different capital letters in the same column within the same freeze-thaw cycle indicate the significant differences ($P < 0.05$); Different letters in the same row within the same parameter indicate significant differences ($P < 0.05$). Value are mean ± standard deviation (n=3). NA: Not detectable

4.4.2.3 Changes in TVB content

TVB content of Pacific white shrimp treated without and with 0.2% catechin or 3% ferulic acid and subjected to freeze-thawing with various cycles during the subsequent storage at 4^{o}C for 4 days is shown in Table 4. After freeze-thawing, the slight increase in TVB content was observed as freeze-thaw cycles increased, regardless of treatment. TVB content increased continuously in all samples throughout the storage of 4 days ($P < 0.05$). However, the increase in TVB content was lowered in the sample treated with 0.2% catechin or 3% ferulic acid, compared to that found in the control (without treatment) during the storage ($P < 0.05$). Increase in TVB content was observed as the number of freeze-thaw cycles increased ($P < 0.05$). Despite the increase in freeze-thaw cycles, the increase in TVB content was lowered in shrimp treated with 0.2 % catechin or 3 % ferulic acid, compared to the control ($P < 0.05$). In general, TVB content was in accordance with PSC. The lower TVB content of Pacific white shrimp treated with 0.2% catechin or 3% ferulic acid might be owing to the inhibitory effect of catechin and ferulic acid against microbes, especially spoilage bacteria. Boonsumrej *et al*. (2007) reported that TVB value for tiger shrimp (*Penaeus monodon*) with 0-4 freeze-thaw cycles was 10.2-14.6 mg N/100g sample. According to Thai standard, the freshness limit for frozen shrimp is 30 mg N/100g meat (TIS, 1986).

4.4.2.4 Changes in TBARS

Table 4 shows TBARS value of Pacific white shrimp treated without and with 0.2% catechin or 3% ferulic acid and subjected to freeze-thawing with various cycles during the subsequent refrigerated storage of 4 days. TBARS value of all samples without freeze-thawing at day 0 was found to be 0.73-0.77 mg malonaldehyde/ kg meat ($P > 0.05$). With increasing freeze-thaw cycles, the increase in TBARS value was noticeable, especially with the control (without treatment). However, those treated with 0.2 % catechin or 3 % ferulic acid had the lower TBARS value than the control. Repeated freeze-thawing could disrupt the organelles associated with the release of prooxidants as well as reactant (Boonsumrej *et al*., 2007). This led to the enhanced lipid oxidation in the shrimp subjected to freeze-thawing with multiple cycles. When lipid oxidation occurs, unstable hydroperoxide is

formed and decomposes readily to shorter chain hydrocarbon such as aldehydes; those final products can be detected as TBARS (Benjakul *et al*., 2005b). At the end of storage (day 4), TBARS value for shrimp treated with 0.2% catechin or 3% ferulic acid was lower than that observed in the control, regardless of freeze-thaw cycles ($P < 0.05$). These results revealed that Pacific white shrimp treated with 0.2% catechin or 3% ferulic acid were more stable towards lipid oxidation. Recently, Maqsood and Benjakul (2009) reported that both catechin and ferulic acid exhibited the radical scavenging activity, reducing power and metal chelating activity in dose dependent manner. Thus both compounds could retard the lipid oxidation in shrimp subjected to freeze-thawing, in which the reactants and prooxidants could be more available for reaction. Radicals formed could be scavenged and free metal ions could be chelated. As a consequence the lipid oxidation was lowered in freeze-thawed shrimp.

4.5 Conclusions

As the number of freeze-thaw cycles increased, melanosis of Pacific white shrimp increased. However, thawing at the refrigerated condition (4^{o}C) was suggested to lower melanosis in shrimp. Catechin and ferulic acid could be used as the promising agent for melanosis prevention in Pacific white shrimp with prior freeze-thawing during the subsequent storage. The efficacy was in dose dependent manner. Apart from prevention of melanosis, treatment of shrimp with catechin or ferulic acid could retard the growth of psychrotrophic microorganism and lipid oxidation. From the commercial point of view, the use of natural additives including catechin or ferulic acid can be the promising safe additive to control the melanosis of shrimp, especially those with prior freeze-thawing.

CHAPTER 5

USE OF TEA EXTRACTS FOR INHIBITION OF POLYPHENOLOXIDASE AND RETARDATION OF QUALITY LOSS OF PACIFIC WHITE SHRIMP DURING ICED STORGAE

5.1 Abstract

Green tea and mulberry tea powder with and without prior chlorophyll removal were extracted with water and 80% ethanol. Extraction yield and total phenolic content of green tea extract were higher than those of mulberry tea extract, regardless of extraction media ($P < 0.05$). 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 (0.01, 0.05 or 0.1%). Additionally, green tea extracts had the higher reducing power, 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activities and copper chelating activity, compared with mulberry tea extract ($P < 0.05$). Ethanolic green tea extract with prior chlorophyll removal contained (+)-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 mg/g dry extract, respectively. Whole white shrimp (*Ltiopenaeus vannamei*) treated with ethanolic green tea extract with prior chlorophyll removal at concentrations of 0.5 and 1 % (w/v) and stored in ice for up to 12 days had the lower psychrotrophic bacterial count and lipid oxidation, compared with the control and shrimp treated with 1.25 % sodium metabisulfite ($P < 0.05$). Shrimp treated with 0.5 % ethanolic green tea extract with prior chlorophyll removal possessed the lower melanosis, compared with the control, and showed similar score to those treated with 1.25% sodium metabisulfite ($P > 0.05$). Furthermore, ethanolic green tea extract with prior chlorophyll removal had no adverse impact on sensory attributes of treated shrimp.

5.2 Introduction

Crustaceans are widely consumed all over the world because of their delicacy and nutritional value. Shrimp and shrimp products of Thailand are well known for their long-standing excellent reputation worldwide, owing to the outstanding quality, freshness, variety and taste (Rattanasatheirn *et al*., 2008). Pacific white shrimp (*Litopenaeus vannamei*) is an important commercial species primarily cultured in Thailand and accounts for 90 % of global aquacultured shrimp production (Nirmal and Benjakul, 2009a). Generally, shelf-life of fresh shrimp is very short because of melanosis and microbial deterioration (Martinez-Alverez *et al*., 2005b). Melanosis is caused by the action of polyphenoloxidase (PPO), which oxidizes phenols to quinone. This colorless quinone subsequently undergoes polymerization, giving rise to black high molecular weight pigment (Benjakul *et al*., 2005a). Melanosis (discoloration) in shrimp drastically reduces the products market value, leading to considerable financial loss (Martinez-Alverez *et al*., 2005b). Apart from melanosis, lipid oxidation is another deteriorative reaction causing the unacceptability of shrimp. Autoxidation, and the enzymatic reaction mediated by lipoxygenase, peroxidase and microbial enzymes take place during post-mortem storage of fish and shellfish (Nirmal and Benjakul, 2009a).

To maintain the quality and to avoid melanosis of shrimp or other crustaceans, sulfite and 4-hexylresorcinol have been widely used (Martinez-Alverez *et al*., 2008b; Montero *et al*., 2001b). However, the increases in regulatory attention and consumer awareness of the risk associated with chemical additives in food product have created a need for a safe and effective alternative for food processing (McEvily *et al*., 1991b). Nowadays, natural antioxidant and antimicrobial compounds, especially of plant origin, have been paid increasing attention as food additives. Inhibition of melanosis in shrimp was achieved by using grape seed extract (Gokoglu and Yerlikaya, 2008) and enokitake extract (Jang *et al*., 2003). Recently, Prasad *et al*. (2009) reported that 50 % ethanol extract of litchi (*Litchi sinensis* Sonn.) seeds had showed the highest antioxidant capacity and inhibitory activity towards mushroom tyrosinase. Recently, catechin was reported to exhibit an inhibitory activity towards polyphenoloxidase of Pacific white shrimp (Nirmal and Benjakul, 2009b). Among

plant, green tea is the major source of catechin, which acts as free radical-scavenger and metal chelator (Farhoosh *et al*., 2007).

Both green tea and mulberry tea have been reported to possess antioxidant, antimicrobial, antimutagenic, anticarcinogenic and anti-inflammatory properties (Cabrera *et al*., 2006). However, there is no available information regarding the use of tea extract to inhibit PPO and to extend the shelf-life of shrimp. The aim of this study were to investigate 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 (*Litopenaeus vannamei*) and to study the impact of ethanolic green tea extract with prior chlorophyll removal on quality changes of Pacific white shrimp during iced storage.

5.3 Materials and Methods

5.3.1 Chemicals and materials

L- -(3, 4 dihydroxylphenyl) alanine (L-DOPA), Brij-35, malonaldehyde bis (dimethyl acetal), thiobarbituric acid (TBA), 2,2-diphenyl-1-picryl hydrazyl (DPPH), tetramethylmurexide (TMM), and standard catechin and its derivatives including (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCg) and (-)-epicatechin gallate (ECG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), ethanol, chloroform, ammonium sulfate, folin-ciocalteu, and plate count agar were obtained from Merck (Darmstadt, Germany). Potassium ferricyanide, and ferric chloride were procured from Fluka Chemical Co. (Buchs, Switzerland). Copper sulfate and sodium metabisulfite were purchased from Fisher Scientific (Loughborough, Leicestershire LE11 5RG, UK). Dry green tea (*Camellia sinensis* L.), and mulberry tea (*Morus alba* L.) cultivated in Chiang Rai province, Northern Thailand were purchased from a local market in Hat Yai, Songkhla, Thailand.

5.3.2 Preparation of tea extracts

Dry green tea and mulberry tea leaves were ground into fine powder using a blender (Philips, Dezheng Road South, Guangzhou, China) and sieved through a stainless steel sieve of 80 mesh size. To prepare water extract, the powder (2 g) was mixed with 80 mL of hot distilled water (80^{o}C) for 20 min with continuous stirring. For ethanolic extract, tea powder (2 g) was extracted with 80 mL of 80 % ethanol at 40^{o}C for 2 h (Perva-Uzunalic *et al*., 2006). The extracts were filtered through Whatman filter paper No.1 (Schleicher & Schuell, Maidstone, England).

Another portion of green tea and mulberry tea powder were subjected to chlorophyll removal to lower the green color of extract caused by the chlorophyll. Tea powder was mixed with chloroform (1:20 (w/v)) and the mixture was stirred for 30 min, followed by filtration using whatman filter paper No.1 (Vovk *et al*., 2005; Row and Jin, 2006). Green tea and mulberry tea powder with chlorophyll removal were subjected to extraction with water and 80% ethanol as mentioned above. Aliquots of each tea extract without and with prior chlorophyll removal were subjected for determination of total phenolic and total chlorophyll contents.

Water extract and ethanolic extract were concentrated at 50^{o}C for 45 min and 35^{o}C for 25 min, respectively, using a rotary evaporator (EYELA N-100, Tokyo, Japan). The concentrated sample was dried in hot air oven at 60^{o}C for 12 h, until the solvent was completely evaporated. The extract powder obtained was subjected to analyses.

5.3.3 Analyses of tea extracts

5.3.3.1 Determination of total phenolic and total chlorophyll contents

Total phenolic content in the extracts was determined with Folin-Ciocalteu reagent according to the method of Slinkkard and Singleton (1997). Appropriately diluted teas extracts with or without prior chlorophyll removal (1 mL) were added with 0.2 mL of two-fold diluted Folin-Ciocalteu reagent and mixed thoroughly. After 3 min, 3 mL of 2 % sodium carbonate solution were added. After standing for 30 min at room temperature, the absorbance was measured at 760 nm

using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The concentration of total phenolic compound in tea extracts was calculated from the standard curve of catechin with the range of 0.01-0.1 mg/mL and expressed as mg catechin/g tea powder.

Total chlorophyll content was determined spectrophotometrically according to the method of the AOAC (1990). Extract without and with prior chlorophyll removal was dehydrated with anhydrous sodium sulfate. Immediately, the pigments were quantified spectrophotometrically at 660 and 642 nm. For the blank, water and 80% ethanol were used instead of extracts. Total chlorophyll content (TCC) was calculated using the following equation:

TCC (g/g of tea powder) 7.12 (A_{660}) + 16.8 (A_{642})

5.3.3.2 Determination of PPO inhibitory activity

5.3.3.2.1. Preparation of PPO extract from the cephalothoraxes of Pacific white shrimp

Pacific white shrimp with the size of 55-60 shrimp/kg were purchased from a supplier in Hat Yai, Songkhla province, Thailand. Shrimp were freshly caught and completely free of additives. The shrimp were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, shrimp were washed in cold water and stored in ice until used (not more than 5 h). The cephalothoraxes of twenty shrimp were separated, pooled and powderized by grinding with liquid nitrogen in a waring blender (AY46, Moulinex, China). The powder obtained was kept in polyethylene bag and stored at -20 °C for not more than 2 weeks. The isolation of PPO from the cephalothoraxes of white shrimp was carried out according to the method of Nirmal and Benjakul (2009a) and the supernatant was used as crude PPO extract .

5.3.3.2.2 Assay of inhibition

PPO activity was assayed using L-DOPA as a substrate according to the method of Nirmal and Benjakul (2009b). The extracts dissolved in distilled water at different concentrations (0.02, 0.1, and 0.2% w/v) (100 L) were mixed with crude

PPO extract (100 L) to obtain the final concentrations of 0.01, 0.05, and 0.1 % (w/v), respectively. The reaction mixtures were incubated for 30 min at room temperature. Then, the assay buffer (400 L, 0.05 M phosphate buffer, pH 6.0) was added. To initiate the reaction, 600 L of pre-incubated 15 mM L-DOPA (45^{o}C) were added and mixed thoroughly. The reaction was conducted at 45^{o}C and the absorbance at 475 nm was monitored for 3 min. The control was run in the same manner, except deionized water was used instead of the extract. One unit of PPO activity was defined as an increase in the absorbance at 475nm by 0.001 /min. Residual activity was calculated and the inhibitory activity was expressed as percentage inhibition as follows:

$$\text{Inhibition (\%)} = \frac{A - B}{A} \text{ X } 100$$

where A: PPO activity of control; B: PPO activity in the presence of tea extract.

5.3.3.3 Determination of reducing power

Reducing power of tea extracts was determined as described by Negi *et al*. (2005). Tea extracts (0.1, 0.5 and 1 mg) were mixed with 1.0 mL of 2.0 M phosphate buffer, pH 6.6 and 1 mL of 1 % potassium ferricyanide. The mixture was incubated at 50^{o}C for 20 min; 1 mL of 10 % trichloroacetic acid was added, and the mixture was centrifuged at 2,000 x g for 10 min using a centrifuge (MIKRO20, Hettich Zentrifugan, Germany). The upper layer of the solution (1 mL) was mixed with distilled water (1 mL) and 0.1 % ferric chloride (0.2 mL) and the absorbance was read at 700 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Increase in the absorbance indicates the increase in reducing power.

5.3.3.4 Determination of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity of tea extracts was determined according to the method of Maqsood and Benjakul (2010) with a slight modification. The extracts (1.5 mL) with the concentrations of 0.1, 0.5 and 1 mg/mL were added with 1.5 mL of 0.15 mM 2,2-diphenyl-1-picryl hydrazyl (DPPH) in 95 % ethanol. The reaction mixture was allowed to stand for 30 min at room temperature in dark and the

absorbance was measured at 517 nm using a spectrophotometer. The sample blank at each concentration was prepared in the same manner except that ethanol was used instead of DPPH solution. A standard curve using catechin in the range of 1-10 M were prepared and the activity was expressed as mole catechin e uivalents (CE)/mL.

5.3.3.5 Determination of copper chelating activity

The copper binding capacity of tea extracts was determined according to the method of Wettasinghe and Shahidi (2002) with a slight modification. One milliliter of copper sulfate solution (1 mM in 10 mM hexamine-HCl buffer containing 10 mM KCl (pH 5.0)) was mixed with 1 mL of extracts or standards, prepared in the same buffer. The mixture was allowed to stand at room temperature for 10 min, followed by the addition of 0.1 mL of a 1 mM tetramethylmurexide (prepared in the same buffer). The final concentration of extracts and standards was 0.01, 0.05 and 0.1 % (based on dry weight). Absorbance of the reaction mixtures was recorded at 460 and 530 nm and the ratio of A_{460} to $A_{530\ nm}$ was calculated. These absorbance ratios were then converted to corresponding free Cu^{2+} concentrations using a standard curve of free Cu^{2+} concentration (50- 400 M) *vs* absorbance ratio. The difference between the total Cu^{2+} and the free Cu^{2+} concentrations indicated the concentration of chelated Cu^{2+}. Copper chelating activity was calculated using the following equation:

$$\text{Copper chelation activity (\%)} = \frac{\text{Concentration of chelated } Cu^{2+}}{\text{Concentration of total } Cu^{2+}} \text{ X } 100$$

5.3.3.6 HPLC analysis of green tea extract

Reverse phase high performance liquid chromatography was performed to analyze phenolic compounds present in the ethanolic green tea extract powder with prior chlorophyll removal rendering the highest PPO inhibitory activity and antioxidant activity. The separation module consisted of an Agilent 1100 series HPLC (Agilent, Herrenberger Str. 130, 71034 Boblingen, Deutschland, Germany) equipped with a C8 size column (Zorbax Eclipse XDB C8 4.6 x 150 mm, 5 m, Herrenberger Str. 130, 71034 Boblingen, Deutschland, Germany) and a diode array detector

(Rheodyne, USA). The samples was eluted with a gradient system consisting of solvent A (water-acetonitril-85% phosphoric acid, 95.45:4.5:0.05, v/v/v) and solvent B (water-acetonitril-85% phosphoric acid, 49.95:50:0.05, v/v/v), used as the mobile phase, with a flow rate of 1 mL/min. The temperature of the column was maintained at 25^{o}C and the injection volume was 20 µL. The gradient system started at 90 % solvent A and 10% solvent B and was maintained for 5 min, then increased to 30 % solvent B within 3 min. This condition was maintained for 2 min followed by an increase of solvent B to 80 % in 5 min. The final conditions were held for an additional 5 min (Yoshida *et al*., 1999). The peaks of the phenolic compounds were monitored at 280 nm.

For calibration, standard stock solutions (1000 mg/L) were diluted with methanol to obtain the concentration levels of 500 and 800 mg/L. The standard solutions were then injected into column and the elution was performed in the same manner with the samples. Individual compound was quantified using a calibration curve of the corresponding standard compound.

5.3.4 Preparation of shrimp treated with ethanolic extract from green tea with prior chlorophyll removal

Ethanolic extract powder from green tea with prior chlorophyll removal was prepared as previously described. Whole shrimp were immersed in the extract solution (0.5, and 1 %) using a shrimp/solution ratio of 1:2 (w/v) at 4^{o}C for 15 min. Extract solution was prepared by dissolving 0.5 and 1 g of the extract powder in 100 mL of distilled water. Another portion of shrimp was soaked in 1.25 % sodium metabisulfite dissolved in distilled water at a ratio of 1:2 (w/v) for 1 min at 4^{o}C (Kim *et al*., 2000). Treated shrimp were drained on the screen for 3 min at 4^{o}C. Shrimp without any treatment were used as the control. All samples were stored in polystyrene box containing ice using a shrimp/ice ratio of 1:2 (w/w). To maintain shrimp/ice ratio, the molten ice was removed and the same amount of ice was added. Samples (20 shrimp) were taken for each treatment every 2 days up to 12 days for determination of psychrotrophic bacterial count, lipid oxidation and melanosis.

5.3.4.1. Melanosis assessment

Melanosis or blackening of Pacific white shrimp was evaluated through visual inspection by six trained panelists using 10-point scoring test (Montero *et al*., 2001b). Samples (10 shrimp) were selected randomly immediately after with and without treatment. The selected fixed samples were evaluated for melanosis score, throughout the storage time. Panelists were asked to give the melanosis score (0 to 10), where 0 absent 2 slight (up to 20 of shrimps surface affected) 4 moderate (20 to 40 of shrimps surface affected) 6 notable (40 to 60 of shrimps surface affected) 8 severe (60 to 80 of shrimps surface affected) 10 extremely heavy (80 to 100 of shrimps surface affected).

5.3.4.2 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS in the samples was determined as described by Nirmal and Benjakul (2010a). Ground shrimp meat (1 g) was mixed with 9 mL of a solution containing 0.375 % TBA, 15 % TCA and 0.25 N HCl. The mixture was heated in boiling water for 10 min, followed by cooling with the running water. The mixture was centrifuged at 4,000 x g for 20 min (MIKRO20, Hettich Zentrifugan, Germany). The supernatant was collected and the absorbance was read at 532 nm using a spectrophotometer. TBARS value was calculated from the standard curve of malonaldehyde (0 to 2 ppm) and expressed as mg malonaldehyde /kg meat.

5.3.4.3 Determination of psychrotrophic bacterial count (PBC)

Five whole Pacific white shrimp were collected aseptically and used as the composite sample (Nirmal and Benjakul, 2009a). Ground whole sample containing cephalothorax (25 g) was placed in a stomacher bag containing 225 mL of 0.85 % saline water and mixed for 1 min in a stomacher blender (M400, Seward, UK). The psychrotrophic bacterial count was determined by inoculating 0.1 mL of an appropriate dilution of homogenate on plate count agar containing 0.5 % NaCl and a spread plate method was used. Then the plates were incubated at 4^{o}C for 7 days.

5.3.4.4 Sensory evaluation

Whole Pacific white shrimp without treatments, shrimp treated with 1.25 % SMS, shrimp treated with 0.5 and 1 % ethanolic green tea extract with prior chlorophyll removal were used for sensory analysis. The samples were placed on a stainless steel tray, covered with aluminium foil and steamed for 5 min. The cooked samples were evaluated by 30 panelists, the graduate students in Food Science and Technology program, Department of Food Technology, Prince of Songkla University with the age of 23 – 25 years. Panelists were acquainted with shrimp consumption and had no allergies to shrimp. Nine-point hedonic scale were used to evaluate samples, where 9 = like extremely; 7 = like moderately; 5 = neither like or nor dislike; 3 = dislike moderately; 1 = dislike extremely (Meilgaard *et al.*, 1990). All panelists were asked to evaluate for color, odor, taste, flavor and overall likeness. Samples were presented unpeeled in plates coded with random three-digit numbers.

5.3.5 Statistical analyses

All analyses were performed in triplicate and a completely randomized design (CRD) was used. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan s multiple range tests. For pair comparison T-test was used (Steel and Torrie, 1980). *P* values less than 0.05 were considered statistically significant. Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA).

5.4 Result and Discussion

5.4.1 Characteristics and PPO inhibitory activity of green tea and mulberry tea extracts prepared under different conditions

5.4.1.1 Yield, total phenolic content and total chlorophyll content

Extraction yield, total phenolic and total chlorophyll contents of water and ethanolic extracts from green tea and mulberry tea with and without prior chlorophyll removal are shown in Table 5.

Table 5. Extraction yield, total phenolic content and total chlorophyll content of water and ethanolic extracts from green tea and mulberry tea with and without prior chlorophyll removal.

Extraction media	Pretreatment	Extraction Yield (%)		Total phenolic content (mg catechin/ g tea powder)		Total chlorophyll content (µg chlorophyll/ g tea powder)	
		Green tea	Mulberry tea	Green tea	Mulberry tea	Green tea	Mulberry tea
Distilled water	Without PCR*	27.73 ± 0.7 c	23.86 ± 0.9 a	118.4 ± 2.0 c	15.2 ± 1.2 b	6.25 ± 0.19 b	10.68 ± 0.14 c
	With PCR	20.93 ± 1.0 d	16.00 ± 0.4 b	93.0 ± 1.8 d	7.5 ± 0.2 d	1.54 ± 0.10 c	3.23 ± 0.08 d
80% ethanol	Without PCR	31.53 ± 0.5 a	15.46 ± 0.4 b	185.2 ± 4.3 a	16.1 ± 1.9 a	39.41 ± 0.41 a	41.27 ± 0.22 a
	With PCR	23.07 ± 1.0 b	10.53 ± 0.6 c	137.1 ± 0.6 b	11.4 ± 0.1 c	5.19 ± 0.12 b	22.70 ± 0.44 b

For each treatment, means within the same column followed by different letters are significantly different at $P < 0.05$. Values are mean ± standard deviation (n = 3).

* PCR: prior chlorophyll removal

In general, extraction yield and total phenolic content of green tea was higher than those of mulberry tea extract when both extraction media were used ($P < 0.05$). For green tea, 80 % ethanol rendered the extract with higher yield and total phenolic content, compared with water ($P < 0.05$). On the other hand, water resulted in the greater extraction yield but lower total phenolic content for mulberry tea extract than ethanol ($P < 0.05$). This could be attributed to different affinities of the extraction solvent with tea constituents due to the differences in polarity of solvent and compounds in tea powder (Moure *et al*.., 2001). The extracts of green tea and mulberry tea with prior chlorophyll removal had the lower yield and total phenolic content, compared with those without prior chlorophyll removal, regardless of extraction media ($P < 0.05$). The use of chloroform to remove chlorophyll from tea powders not only removed chlorophyll, but also caffeine and other components from tea powder. This more likely resulted in the loss of some phenolic compounds. In general, the more partitioning of catechin to extraction medium was achieved, the more recovery of catechin was obtained for Korean tea (Row and Jin, 2006).

Total chlorophyll content of ethanolic extracts from both green tea and mulberry tea was higher than water extracts ($P < 0.05$). Ethanol (80 %) might possess the appropriate polarity or affinities for chlorophyll extraction. Total chlorophyll content of mulberry tea extract was much greater than that of green tea extract for both extraction media ($P < 0.05$). Tea powders with prior chloroform treatment yielded the extract with lowered chlorophyll content. However, some chlorophyll was still found in the extract from tea powder with prior chlorophyll removal.

5.4.1.2 PPO inhibitory activity

PPO inhibitory activity of different extracts from green tea and mulberry tea expressed as percentage inhibition is represented in Table 6. All tea extracts showed PPO inhibitory activity in a concentration-dependent manner. The result was in agreement with Soysal (2008) who reported that the inhibitory effect of green tea extract towards apple PPO increased with increasing extract concentration. At the same concentration and extraction medium used, green tea extract exhibited higher inhibitory activity towards PPO than did mulberry tea extract ($P < 0.05$). When the concentration of 0.1 % was used, green tea extract with prior chlorophyll removal

showed the higher inhibitory activity against PPO than those without prior chlorophyll removal, regardless of extraction media used ($P < 0.05$). At 0.1 % concentration, water and ethanolic extracts of green tea with and without prior chlorophyll removal had no difference in PPO inhibition ($P > 0.05$). Commercial catechin showed the highest PPO inhibitory activity ($P < 0.05$). The main flavonoids present in green tea include catechins (flavan-3-ols) (Cabrera *et al*., 2006). However, mulberry tea contain major flavonol glycosides including, quercetin 3-(6-malonylglucoside), rutin, isoquercitrin, astragalin (Katsube *et al*, 2006). Catechin probably acted as a competitive inhibitor for PPO because of its structural similarity to substrate for PPO (Nirmal and Benjakul, 2009b).

Table 6. Percent inhibition towards Pacific white shrimp PPO of water and ethanolic extracts from green tea and mulberry tea with and without prior chlorophyll removal

Samples	Extraction media	Pretreatment	Extract concentration (mg/mL)		
			0.1	0.5	1
Catechin			29.94 ± 2.6 a	68.51 ± 2.4 a	86.42 ± 0.9 a
Green tea	Distilled water	Without PCR	18.27 ± 3.8 b	33.84 ± 0.4 c	52.60 ± 2.5 c
		With PCR	17.93 ± 0.9 b	37.62 ± 0.5 b	56.06 ± 1.6 b
	80% ethanol	Without PCR	15.34 ± 1.5 c	33.03 ± 1.1 c	52.92 ± 0.6 c
		With PCR	15.98 ± 1.5 c	38.25 ± 1.5 b	56.62 ± 1.8 b
Mulberry tea	Distilled water	Without PCR	14.13 ± 1.8 d	29.99 ± 1.0 d	34.89 ± 0.6 d
		With PCR	12.15 ± 1.5 e	27.02 ± 0.8 e	32.23 ± 0.9 e
	80% ethanol	Without PCR	14.83 ± 1.5 d	27.14 ± 0.6 e	34.30 ± 1.8 d
		With PCR	12.23 ± 0.4 e	26.58 ± 1.3 e	31.55 ± 1.0 e

Means within the same column followed by different letters are significantly different at $P < 0.05$. Values are mean ± standard deviation (n = 3).

The result indicated that green tea and mulberry tea extracts could inhibit PPO from cephalothoraxes of Pacific white shrimp to some extent. The inhibitory activity could be enhanced by further fractionation of the extract to obtain catechin rich fraction. Higher total phenolic content (Table 5) in tea extract was in accordance with the higher PPO inhibitory activity. Tea extract, especially green tea extract, containing phenolic compound could be used as the natural inhibitor for PPO from Pacific white shrimp. Ethanolic green tea extract with prior chlorophyll removal was more applicable for further use due to the higher extraction yield and total

phenolic content, compared with the water extract. Furthermore, the extract had the low greenness since chlorophyll was removed prior to extraction.

5.4.2 Antioxidant activities of green tea and mulberry tea extracts prepared under different conditions

5.4.2.1 Reducing power

Reducing power of water and ethanolic extracts with and without prior chlorophyll removal is shown in Figure 32. Reducing power of extracts increased with increasing concentrations ($P < 0.05$). For the same tea, no difference in reducing power was noticeable between the extract with and without prior chlorophyll removal, when the same extraction medium was used ($P > 0.05$). Green tea extract showed much higher reducing power, compared to mulberry tea extract for both extraction media used ($P < 0.05$). Reducing power was higher when 80 % ethanol was used as extracting medium ($P < 0.05$). These results were in agreement with the total phenolic content of both extracts, which was higher when 80 % ethanol was used for extraction (Table 5). Farhoosh *et al*. (2007) found that ethyl acetate extract of green tea possessed the greater reducing power than did water and methanolic extracts. This result suggested that all extracts were capable of donating electron. These extracts might be used to lower melanosis by reducing DOPA-quinone to DOPA. As a consequence, quinone cannot be further convert to brown pigment and melanosis can be retarded. A variety of plants extracts including green tea extract have been reported to have reducing power and act as antioxidant for many food systems (Roedig-Penman and Gordon, 1997; Negi *et al*., 2005). Prior chlorophyll removal had no impact on reducing power of extracts prepared using the same extracting medium. Chlorophyll might show the similar reducing power to other reducing agents, especially catechin and its derivatives.

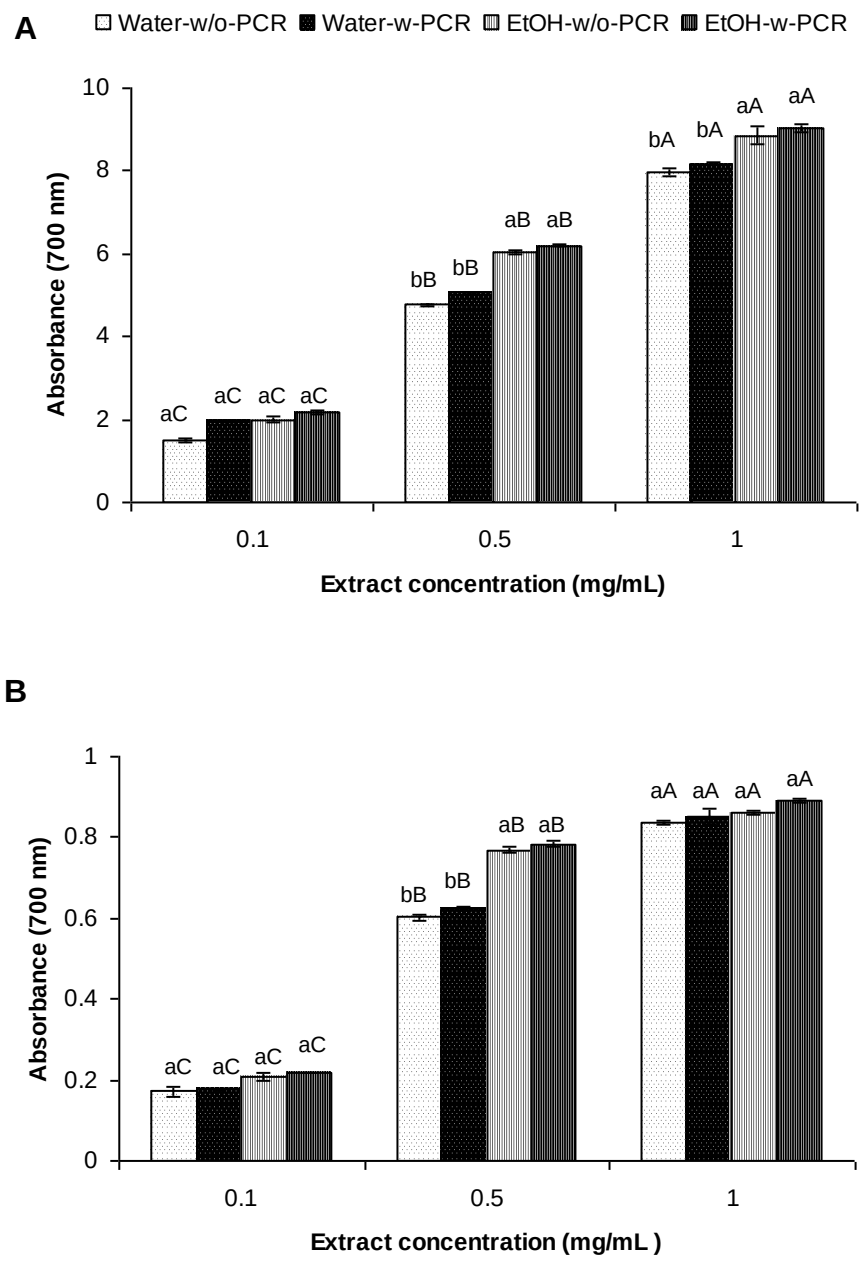

Figure 32. Reducing power of water and ethanolic extract from green tea (A) and mulberry tea (B) with and without prior chlorophyll removal. Bars represents the standard deviation (n=3). Different letters within the same concentration denote the significant differences ($P < 0.05$). Different capital letters within the same extract denote the significant difference ($P < 0.05$). Water-w/o-PCR: water extract without prior chlorophyll removal; Water-w-PCR: water extract with prior chlorophyll removal; EtOH-w/o-PCR: ethanolic extract without prior chlorophyll removal; EtOH-w-PCR: ethanolic extract with prior chlorophyll removal.

5.4.2.2 DPPH radical-scavenging activity

DPPH radical-scavenging activity of water and ethanolic extracts from green tea and mulberry tea with and without prior chlorophyll removal is depicted in Figure 33.

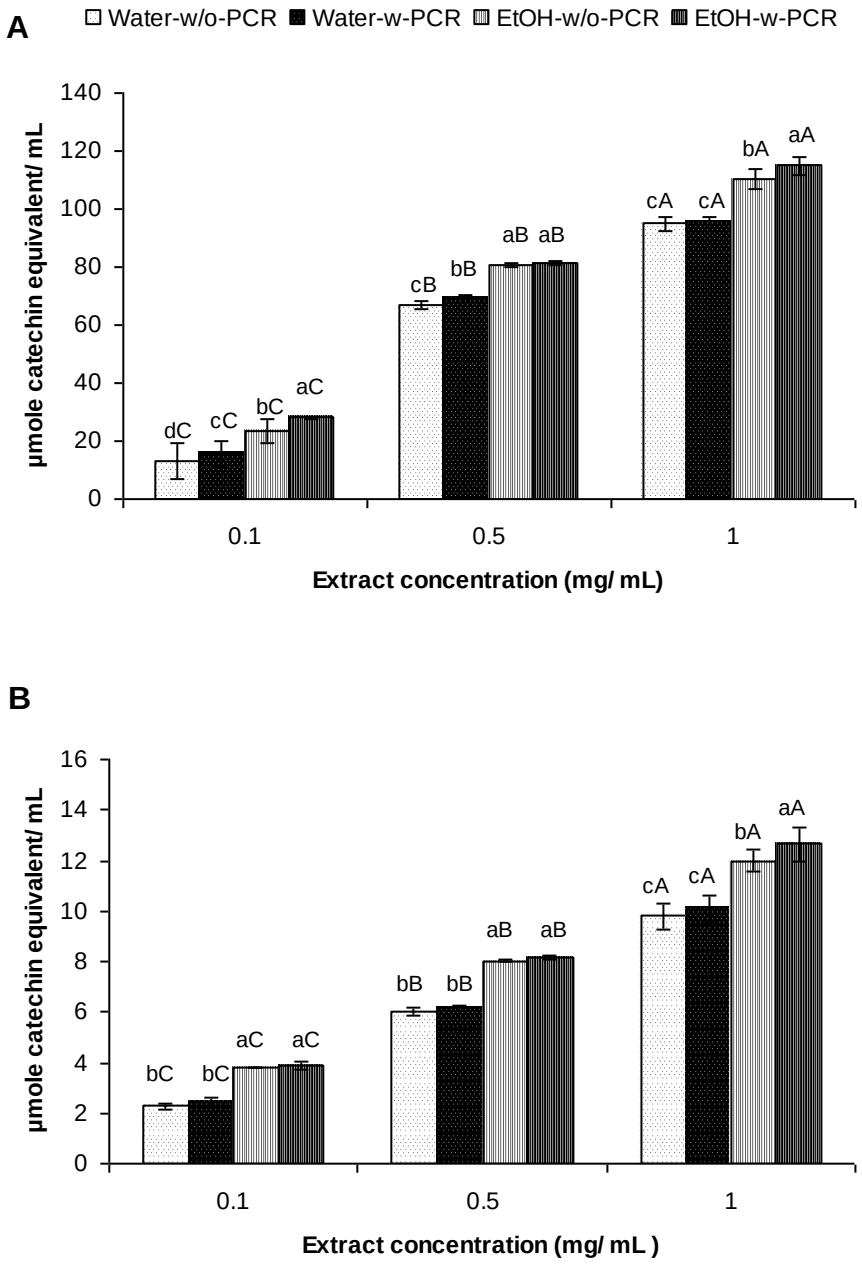

Figure 33. DPPH radical scavenging activity of water and ethanolic extract from green tea (A) and mulberry tea (B) with and without prior chlorophyll removal. Bars represent the standard deviation (n=3). Key: see Figure 32 caption.

DPPH is used as a free radical to evaluate antioxidative activity of some natural sources, and the degree of its discoloration is attributed to hydrogen donating ability of test compounds, which is indicative of their scavenging potential (Shimada *et al*., 1992). DPPH radical scavenging activity of all extracts increased when the concentration increased ($P < 0.05$). DPPH radical scavenging activity of ethanolic extract was higher than that of water extract ($P < 0.05$). However, no difference in DPPH radical scavenging activity was found between extracts, prepared from powder with and without prior chlorophyll removal ($P > 0.05$), when the same extracting medium was used. These results were coincidental with those of reducing power (Figure 32). The result indicated that green tea extracts were the potential free radical scavengers, which reacted with radicals by donating their hydrogen and acted as primary antioxidants. Yen and Chen (1995) found that the pouching tea had the highest DPPH radical scavenging activity, followed by green tea, oolong tea and black tea, respectively.

5.4.2.3 Copper chelating activity

Table 7 shows copper chelating activity of water and ethanolic extracts of green tea and mulberry tea with and without prior chlorophyll removal. Tea extracts with prior chlorophyll removal exhibited the higher copper chelating activity, compared with those without prior chlorophyll removal at all concentrations used ($P < 0.05$), except for ethanolic green tea extract at a level of 0.1 %, where there was no difference in copper chelating activity between those with and without prior chlorophyll removal ($P > 0.05$). Green tea extract showed the higher copper chelating activity, compared with mulberry tea extract ($P < 0.05$). Commercial catechin had the lowest copper chelating activity, compared with both tea extracts with and without chlorophyll removal, regardless of extracting medium ($P < 0.05$). Copper chelating activity of ethanolic green tea extract was higher than that of water extract ($P < 0.05$). Similar result was found for mulberry extract when a concentration of 0.1 % was used. The ortho 3, 4- dihydroxy substitution in the B ring of flavonoids was shown to be important for Cu^{2+}-chelate formation (Brown *et al*., 1998). The greater chelating activity of green tea extract with prior chlorophyll removal indicated that extracts

might become more concentrated and phenolic compounds were able to form the complex with copper ion more effectively.

Table 7. Copper chelating activity (%) of water and ethanolic extracts from green tea and mulberry tea with and without prior chlorophyll removal

Samples	Extraction media	Pretreatment	Extract concentration (mg/mL)		
			0.1	0.5	1
Catechin			21.66 ± 0.47 e	35.54 ± 0.18 h	46.53 ± 0.66 h
Green tea	Distilled water	Without PCR	22.70 ± 0.53 d	51.93 ± 0.53 d	60.71 ± 0.38 c
		With PCR	25.23 ± 0.44 c	54.66 ± 0.32 c	63.93 ± 0.45 b
	80% ethanol	Without PCR	29.44 ± 0.28 b	64.50 ± 0.50 b	73.81 ± 0.30 a
		With PCR	31.91 ± 0.16 a	66.45 ± 0.40 a	74.13 ± 0.64 a
Mulberry tea	Distilled water	Without PCR	22.10 ± 0.38 ed	38.25 ± 0.57 g	53.21 ± 0.31 g
		With PCR	24.21 ± 0.28 c	40.94 ± 0.39 f	55.30 ± 0.37 e
	80% ethanol	Without PCR	23.35 ± 0.38 d	38.07 ± 0.16 g	54.30 ± 0.41 f
		With PCR	24.71 ± 0.45 c	42.23 ± 0.45 e	56.34 ± 0.52 d

Means within the same column followed by different letters are significantly different at $P < 0.05$. Values are mean ± standard deviation (n = 3).

The ortho hydroxy substitution basically at 3 and 4 position of B-ring of flavanol increased the copper chelating activity (Brown *et al*., 1998). Due to copper chelating activity of extracts, they could inhibit PPO by chelating Cu (II) in the active site of PPO, leading to the lowered PPO activity. The capacity of phenolic compounds for chelating metals is strongly dependent on the number of hydroxylic groups in ortho position (Maqsood and Benjakul, 2010). PPO is one of the metalloproteins with two copper atoms in the active site (Jang *et al*, 2003). The copper chelating activity of extracts was in accordance with their PPO inhibitory activity (Table 6).

5.4.3 Identification of phenolic compound in green tea extract

Ethanolic extract of green tea with prior chlorophyll removal showing the highest inhibitory activity towards PPO was subjected for identification and quantification. Five catechin compounds namely, (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCg), and epicatechin gallate (ECG) were found in the ethanolic green tea extract at the levels

of 242, 33.4, 125.6, 140.6 and 25.2 mg/ g extract powder, respectively (Table 8). These result indicated that (+)-catechin was the major phenolic compound, followed by EGCg and EGC, respectively. Cabrera *et al*. (2006) reported that four major catechins in green tea were (-)-epigallocatechin gallate (EGCg), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epicatechin (EC).

Table 8. Content of catechin and its derivatives in ethanolic extract powder from green tea with prior chlorophyll removal

Sample	Compounds	Retention time (min)	Concentration (mg/g extract powder)
Green tea extract	Catechin	8.2	242 ± 0.64
	Epicatechin	10.8	33.4 ± 0.46
	Epigallocatechin	6.1	125.6 ± 0.10
	Epigallocatechin gallate	11.2	140.6 ± 0.13
	Epicatechin gallate	14.7	25.2 ± 2.57

Values are mean ± standard deviation (n = 3).

The epimeriztion of catechin and its derivatives might occur during drying process. Moreover, during hot air drying of extract, the degradation of some catechins possibly took place to some degree. Perva-Uzunalic *et al*. (2006) found that the major catechins content in the water extract was decreased as the temperature and time of extraction increased due to their degradation. The removal of chlorophyll prior to extraction could concentrate catechin and its derivatives in the resulting tea extract. With chloroform treatment for chlorophyll removal, caffeine and related impurities were removed from green tea powder (Row and Jin, 2006).

5.4.4 Effect of ethanolic green tea extract treatment on melanosis and quality of Pacific white shrimp during iced storage

5.4.4.1 Melanosis

Figure 34 illustrates the melanosis score of Pacific white shrimp treated without and with ethanolic green tea extract with prior chlorophyll removal. Shrimp were immerse in ethanolic green tea extract for 15 min, which was sufficient time to lower the melanosis formation in Pacific white shrimp during iced storage, compared

to 20 or 25 min ($P > 0.05$) (data not shown). The extract used was light pale yellow in color, due to the removal of chlorophyll. It had no negative effect on the color or appearance of treated shrimp. There was no melanosis found at day 0 for all samples. As the storage time increased, continuous increase in melanosis score was obtained for the control ($P < 0.05$). At day 2, shrimp treated with 1.25 % sodium metabisulfite or ethanolic green tea extract showed negligible melanosis score (score 0). After 4 days of storage in ice, shrimp treated with 0.5 % ethanolic green tea extract and 1.25 % sodium metabisulfite showed the lowered melanosis score, compared with those treated with 1 % ethanolic green tea extract ($P < 0.05$). No difference in melanosis between shrimp treated with 0.5 % ethanolic green tea extract and 1.25 % sodium metabisulfite throughout the storage of 12 days in ice ($P > 0.05$).

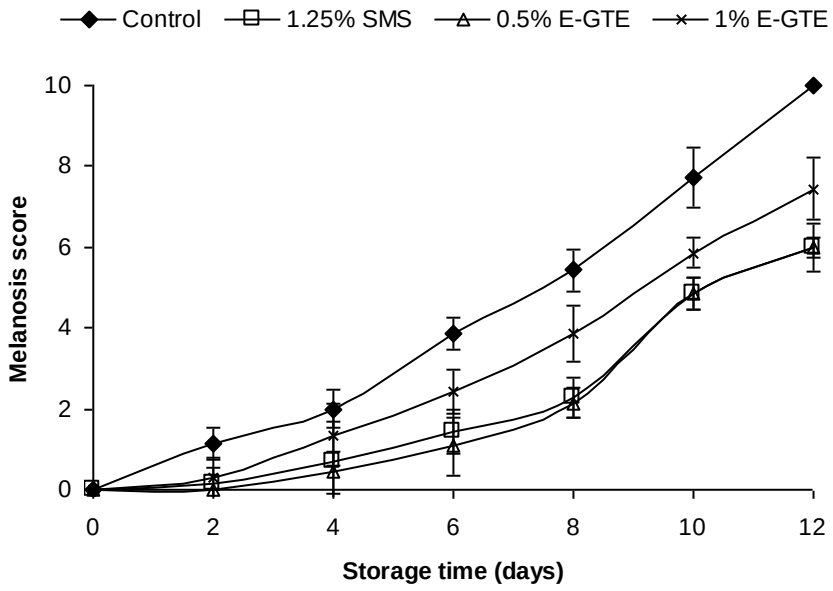

Figure 34. Melanosis score of Pacific white shrimp treated with ethanolic green tea extract with prior chlorophyll removal at different levels during 12 days of iced storage. Bars represents the standard deviation (n=3). 1.25% SMS: 1.25 % sodium metabisulfite; 0.5% E-GTE: 0.5 % ethanolic green tea extract; 1% E-GTE: 1 % ethanolic green tea extract.

Photographs of melanosis formation of Pacific white shrimp without and with different treatments at day 12 of iced storage are shown in Figure 35.

Samples in the photograph are representative of the same sample, which were evaluated for melanosis score throughout the iced storage of 12 days. Soysal (2008) reported that the green tea extract at 15 mg/mL was effectively retarded the browning of apple slices. Bisulfite might inhibit melanosis by reacting with intermediate quinone, forming sulfoquinone (Ferrer *et al*., 1989). Green tea extract at a level of 1 % was less effective in controlling the formation of melanosis in Pacific white shrimp, as compared to the extract at 0.5 %, during iced storage. Phenolic compounds in green tea extract at high level might undergo polymerization, leading to the fewer amounts of free catechin or its derivatives available for melanosis inhibition. Additionally, phenolic compounds in the extract at high level might cross-link the proteinaceous tissue of shrimp, where PPO was localized. As a result, the penetration of active catechin and its derivatives to inactivate PPO became lowered. Furthermore, the extract in the treated shrimp could be leached out by the molten ice to some degree during the extended storage.

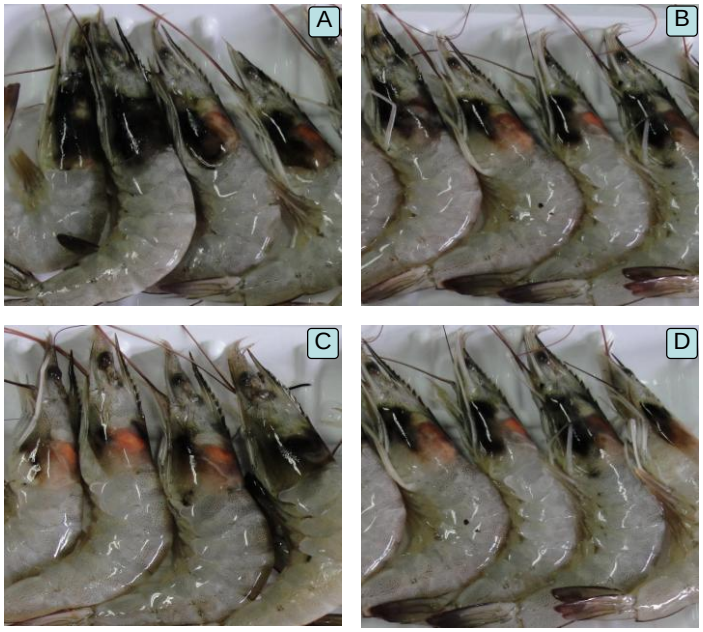

Figure 35. Photographs of Pacific white shrimp treated without and with ethanolic green tea extract with prior chlorophyll removal after 12 days of iced storage. A: Control; B: 1.25 % sodium metabisulfite; C: 0.5 % ethanolic green tea extract; D: 1 % ethanolic green tea extract.

The result revealed that ethanolic green tea extract at the level of 0.5 % had the inhibitory effect on melanosis formation of Pacific white shrimp during iced storage. Since the extract exhibited both copper chelating activity as well as reducing power, the inhibition of melanosis of the extract was more likely due to the combined effect between PPO inhibition as well as the reduction of quinone formed. As a result, the melanosis could be retarded.

5.4.4.2 Lipid oxidation

Thiobarbituric acid reactive substances (TBARS) value of Pacific white shrimp treated without and with ethanolic green tea extract with prior chlorophyll removal during iced storage is shown in Figure 36. Generally, the increase in TBARS was found within the first 2 days ($P < 0.05$). Thereafter, the continuous decrease was observed during day 4 – 8 ($P < 0.05$). However, during day 8 – 12, the marked increase was noticeable ($P < 0.05$). Higher TBARS value was observed in the control, compared with shrimp treated with ethanolic green tea extract and those treated with sodium metabisulfite during iced storage of 12 days ($P < 0.05$). At day 12 of storage, shrimp treated with 1 % ethanolic green tea extract showed the lowest TBARS values ($P < 0.05$), followed by those treated with 0.5 % extract and 1.25 % sodium metabisulfite, respectively. Unstable hydroperoxide is formed during lipid oxidation and decomposes readily to shorter chain hydrocarbon such as aldehyde, which can be detected as TBARS (Benjakul *et al*., 2005b). The decrease in TBARS value during 2 – 8 days of storage was most likely because of leaching out effect of those secondary products by molten ice during iced storage. The addition of green tea extract retarded the oxidation in mackerel patties during refrigerated (4^{o}C) and illuminated storage (Tang *et al*., 2001).

Overall, shrimp treated with green tea extract at both levels, had the lowered lipid oxidation, especially after 8 days of storage, compared to shrimp treated with 1.25% sodium metabisulfite and the control ($P < 0.05$). Retardation of lipid oxidation of green tea extract treated shrimp was attributed to its radical-scavenging activity (Figure 33). Additionally, the extract might chelate the metal pro-oxidants in the shrimp muscle. As a result, propagation stage could be impeded and rancidity could be prevented.

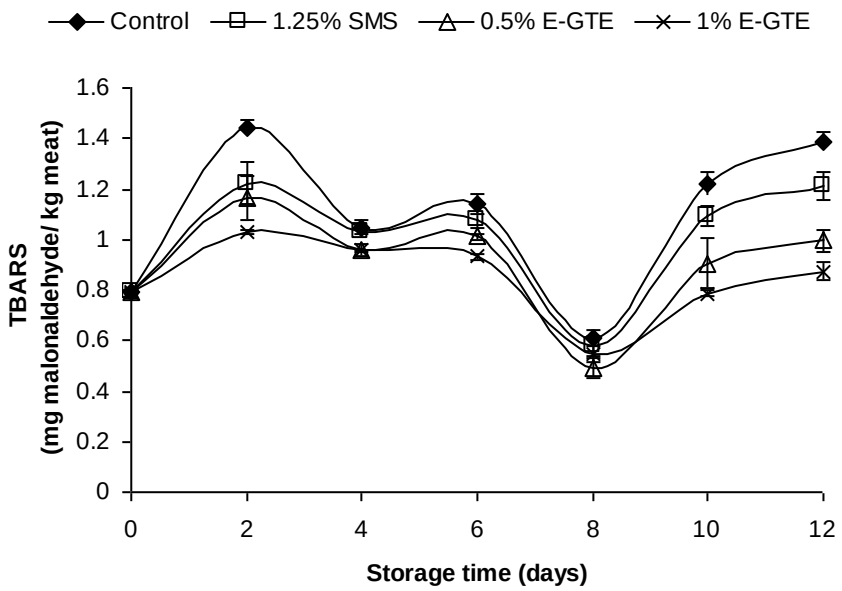

Figure 36. Thiobarbituric acid reactive substances of Pacific white shrimp treated with ethanolic green tea extract with prior chlorophyll removal at different levels during 12 days of iced storage. Bars represents the standard deviation (n=3). Key: see the caption for Figure 34

5.4.4.3 Psychrotrophic bacterial count

Psychrotrophic bacterial count (PBC) of Pacific white shrimp treated without and with ethanolic green tea extract with prior chlorophyll removal during iced storage is shown in Figure 37. PBC in all samples increased with increasing storage time ($P < 0.05$). However, the higher PBC was found in the control, compared to shrimp treated with sodium metabisulfite and ethanolic green tea extract ($P < 0.05$). Shrimp treated with both 0.5 % and 1 % ethanolic green tea extract showed the lower PBC after 4 days of storage in ice, compared with those treated with 1.25 % sodium metabisulfite ($P < 0.05$). Therefore, sodium metabisulfite exhibited the lower efficacy in controlling psychrotrophic bacteria in comparison with ethanolic green tea extract during the extended iced storage. Sulfur dioxide derived from sodium metabisulfite might be evaporated during extended storage or could be dissolved with molten ice, which leads to the lower amount of sodium metabisulfite remaining in the sample (Nirmal and Benjakul, 2009b).

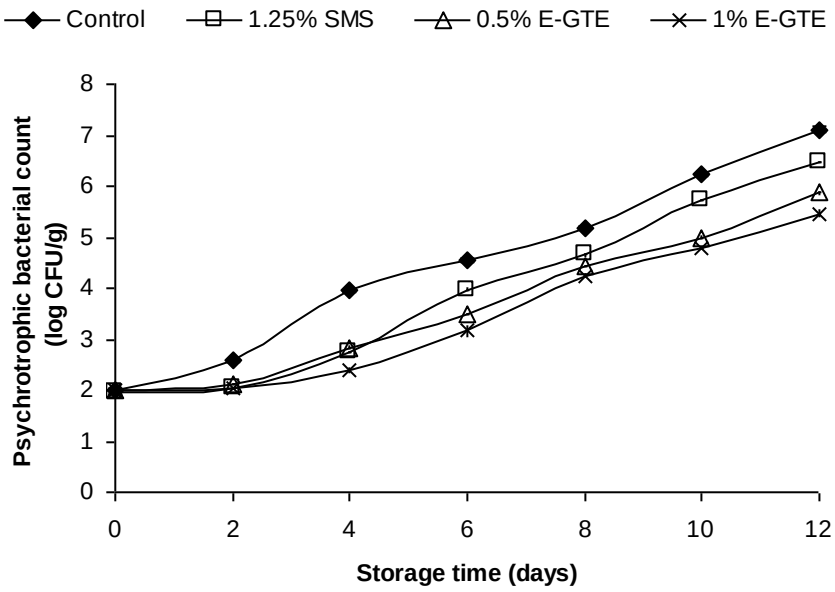

Figure 37. Psychrotrophic bacterial count of Pacific white shrimp treated with ethanolic green tea extract with prior chlorophyll removal at different levels during 12 days of iced storage. Bars represents the standard deviation (n=3). Key: see the caption for Figure 34.

Phenolic compound might form complexes with proteins in the cell wall of microorganism, causing lysis of cell wall (Chanthachum and Beuchat, 1997). Furthermore, phenolics, especially catechin and its derivatives in the extract, might chelate some metal ions required for microbial growth. Therefore, microbial growth in shrimp could be retarded to some degree by the treatment using ethanolic green tea extract. Kumudavally *et al*. (2008) reported that sheep mutton treated with the ethanolic extract of green tea had the lowered count of spoilage microorganisms and increased shelf life at ambient storage condition for up to 4 days.

5.4.4.4 Sensory properties

Likeness score of Pacific white shrimp treated with ethanolic green tea extract with prior chlorophyll removal in comparison with that of the control and those treated with 1.25% SMS at day 0 of iced storage is shown in Figure 38. All samples had the score higher than 7.0 for all attributes tested and no differences in likeness were found between all treatments ($P > 0.05$). Therefore, the treatment of

shrimp with ethanolic green tea extract with prior chlorophyll removal had no impact on color, taste or flavor and overall likeness of treated shrimp. Since the chlorophyll in green tea was removed, the extract obtained had the light straw-yellow in color. Additionally, caffeine was also removed by dechlorophyllization (Row and Jin 2006). Therefore, there might be less probability of ethanolic green tea extract with prior chlorophyll removal to adversely affect the color, taste or flavor of the shrimp treated with the extract.

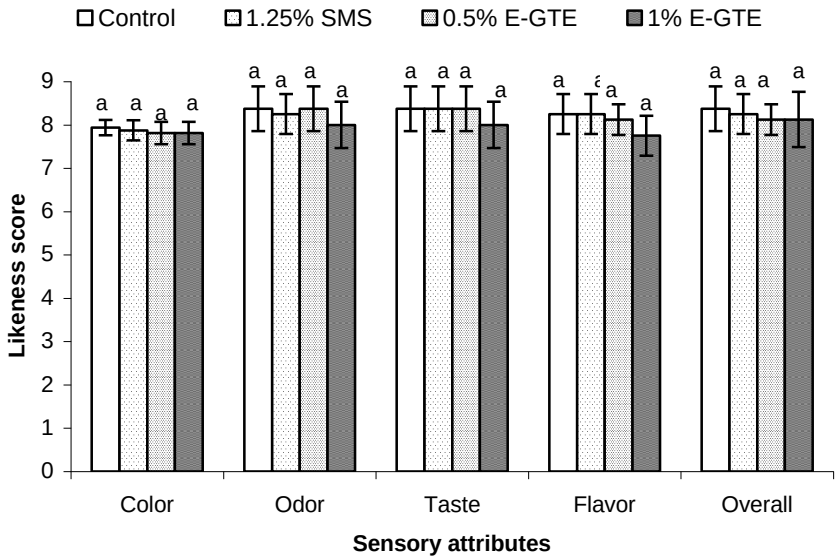

Figure 38. Likeness score of Pacific white shrimp treated without and with ethanolic green tea extract with prior chlorophyll removal at day 0 of iced storage. Values are mean ± standard deviation (n=30). Different letters within the same attribute denote the significant differences ($P < 0.05$). Key: see the caption for Figure 34.

5.5 Conclusions

Green tea extract showed the higher PPO inhibitory activity and antioxidative activity, compared with mulberry tea extract. Ethanol was the better extracting medium than water, in which the higher recovery of phenolic compound with PPO inhibitory activity and antioxidative activity was obtained. When ethanolic

green tea extract with prior chlorophyll removal was used to soak Pacific white shrimp for 15 min, melanosis, microbial growth, and lipid oxidation were retarded. Moreover, ethanolic green tea extract with prior chlorophyll removal did not affect sensory attribute of treated shrimp. Efficacy of the extract was generally higher than sodium metabisulfite. Therefore, ethanolic green tea extract could be used as an alternative melanosis inhibitor or preservative in postmortem shrimp.

CHAPTER 6

EFFECT OF GREEN TEA EXTRACT IN COMBINATION WITH ASCORBIC ACID ON THE RETARDATION OF MELANOSIS AND QUALITY CHANGES OF PACIFIC WHITE SHRIMP DURING ICED STORGAE

6.1 Abstract

Melanosis and quality changes of Pacific white shrimp (*Litopenaeus vannamei*) treated with 0.1 % green tea extract (GTE) in combination with ascorbic acid (AA) at different levels (0, 0.005 and 0.01 %) were monitored during iced storage of 12 days. Based on *in vitro* study, 0.1 % GTE inhibited polyphenoloxidase (PPO) from cephalothorax of Pacific white shrimp by 60.2 %. Nevertheless, 0.1 % GTE in combination with 0.01 % AA exhibited the greater PPO inhibitory activity (93.0 %) ($P < 0.05$). When shrimp treated with 0.1 % GTE in combination with AA (0.005 or 0.01%) (GTE + AA), the increase in psychrotrophic bacteria and spoilage microorganisms including H_2S- producing bacteria and enterobacteriaceae were retarded to a higher extent, in comparison with the control and those treated with 1.25 % sodium metabisulfite (SMS) ($P < 0.05$). The coincidental lowered rate of increase in pH, total volatile base (TVB) content and thiobarbituric acid reactive substances (TBARS) were obtained in the shrimp treated with GTE + AA ($P < 0.05$). Additionally, shrimp treated with GTE + AA had the lower melanosis score but higher score for color, odor, taste, flavor and overall likeness, compared with the control and those treated with SMS ($P < 0.05$). Generally, AA at levels of 0.005 and 0.01 % showed a similar synergist effect with GTE on both melanosis inhibition as well as retardation of quality loss of shrimp.

6.2 Introduction

Shrimp is the leading seafood consumed in many countries over the world because of their delicacy. Thailand is the world s leading shrimp-farming

country and has become the top supplier of farmed shrimp to the United States (Wyban 2007). This high value crustacean is very perishable associated with microbiological, chemical, and physical changes during postmortem storage (Nirmal and Benjakul 2010a). Melanosis is a major cause of deleterious changes in the organoleptic properties, resulting in shorter shelf-life, poor quality, as well as financial loss (Montero *et al*., 2006). Melanosis (black spot) is a phenomenon in which phenols oxidize to quinone by a biochemical mechanism, caused by polyphenoloxidase (PPO). This colorless quinone subsequently undergoes non-enzymatic polymerization, giving rise to black high molecular weight pigment. Although the presence of black spot on shrimp is not dangerous to human health, it drastically reduces the consumer s acceptability and the market value because of their appearance (Kim *et al*., 2000). Apart from melanosis, microbiological changes and lipid oxidation occurred in shrimp during the iced storage (Nirmal and Benjakul 2009a). Chilling or refrigerating does not prevent, but only slow down the development of black spot. PPO still remains active during refrigeration, storage on ice and post freeze-thawing (Montero *et al*., 2004).

To alleviate or retard melanosis during iced and refrigerated storage, several PPO inhibitors including sulfiting agent and 4-hexylresorcinol, alone or in combination with other chemicals, have been used (Gomez-Guillen *et al.*, 2005; Thepnuan *et al*., 2008). Single inhibitor at high level may be required to effectively inhibit PPO or melanosis (Montero *et al*., 2004). As a consequence, most PPO inhibitors can be used at a lower concentration when other synergists such as acids, metal chelator etc. are combined (Montero *et al*. 2006). However, sulfiting agents are known to produce allergic reaction in some group of population (DeWitt, 1998) and 4-hexyl resorcinol is still costly for application. Recently, catechin was found as the effective additive to retard the melanosis and maintain the quality of Pacific white shrimp during iced storage (Nirmal and Benjakul 2009b).

Green tea is the major source of catechins and has been known as an excellent antioxidant. These compounds are effective free radical-scavengers as well as metal chelator (Farhoosh *et al*., 2007). Therefore, green tea extract containing catechin could be used as the natural anti-melanosis, especially in conjunction with some synergists, thereby reducing the development of melanosis and preventing the

lipid oxidation in shrimp during the extended storage. The objectives of study were to investigate the effect of green tea extract in combination with other additives on inhibition of PPO from Pacific white shrimp and to study the impact of immersion time of shrimp in selected additives on melanosis formation and quality changes of shrimp during iced storage.

6.3 Materials and Methods

6.3.1 Chemicals and green tea

L- -(3, 4 dihydroxylphenyl) alanine (L-DOPA), Brij-35, malonaldehyde bis (dimethyl acetal), and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), L-ascorbic acid, citric acid, sodium pyrophosphate, sodium chloride, ammonium sulfate, standard plate count agar, triple sugar iron agar (IA), and Eosin methylene blue agar (EMB) were obtained from Merck (Darmstadt, Germany). Green tea (*Camellia sinensis* L.) cultivated in Chiang Rai province, Northern Thailand was purchased from a local market of Hat Yai, Songkhla, Thailand.

6.3.2 Shrimp collection and preparation

Pacific white shrimp (*Litopenaeus vannamei*) with the size of 55-60 shrimp/kg were purchased from a supplier in Songkhla, Thailand. The shrimp were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, shrimp were washed in cold water and stored in ice until used (not more than 3 h).

6.3.3 Preparation of PPO extract from the cephalothoraxes of Pacific white shrimp

The cephalothoraxes of twenty shrimp were separated, pooled and powdered by grinding with liquid nitrogen in a Waring blender (AY46, Moulinex,

Guangdong, China). The powder obtained was kept in polyethylene bag and stored at -20 °C for not more than 2 weeks. The extraction of PPO from the powdered cephalothoraxes of white shrimp was carried out according to the method of Nirmal and Benjakul (2009a) and the crude PPO extract was used for PPO inhibition study.

6.3.4 Effect of green tea extract and its synergists on inhibition of PPO from Pacific white shrimp

6.3.4.1 Preparation of green tea extract

Dry green tea leaves were ground into fine powder using a blender (Philips, Dezheng Road South, Guangzhou, China) and sieved through a stainless steel sieve of 80 mesh size. Green tea powder was treated with chloroform using a powder/ solvent ratio of 1:20 (w/v) to remove chlorophyll (Row and Jin 2006). The mixture was stirred for 30 min, followed by filtration using whatman filter paper No.1 (Schleicher & Schuell, Maidstone, England). To prepare the green tea extract, the de-chlorophyllized green tea powder (2 g) was mixed with 80 mL of 80 % (w/v) ethanol at 40^{o}C for 2 h with continuous stirring (Perva-Uzunalic *et al*., 2006). The extract was filtered through whatman filter paper No.1. The filtrate was concentrated by a rotary evaporator (EYELA N-100, Tokyo, Japan). The concentrated sample was dried in hot air oven at 60^{o}C for 12 h. The dry extract contained 566.8 mg catechin/ g dry extract as determined by high performance liquid chromatography (Yoshida *et al*., 1999). The extract was kept in a polythene bag and placed in a dessicator in the dark at 4^{o}C until use.

6.3.4.2 Inhibitory effect of green tea extract and its synergists on PPO activity

PPO activity was assayed using L-DOPA as a substrate according to the method of Nirmal and Benjakul (2009b). GTE was dissolved in distilled water to obtain the concentration of 0.2 % (w/v). GTE solution (100 L) was mixed with crude PPO extract (100 L). The mixture was incubated for 30 min at room temperature. Thereafter, the assay buffer (400 L, 0.05 M phosphate buffer, pH 6.0) was added. To initiate the reaction, 600 L of preincubated 15 mM L-DOPA (45^{o}C) were added. The

reaction was conducted at 45^{o}C and the absorbance at 475 nm was monitored for 3 min. The control was run in the same manner, except the deionized water was used instead of GTE solution. The sample blank was prepared by using distilled water instead of L-DOPA. One unit of PPO activity was defined as an increase in the absorbance at 475nm by 0.001 /min. Residual activity was calculated and the inhibitory activity was expressed as percentage inhibition as follows:

$$\text{Inhibition (\%)} = \frac{A - B}{A} \text{ X } 100$$

where A: PPO activity of the control; B: PPO activity in the presence of inhibitor.

Different synergists with various final concentrations including ascorbic acid (0.005 & 0.01 %), citric acid (0.05 & 0.1 %), sodium pyrophosphate (1 %) and sodium chloride (1 %) alone or in combination with 0.1 % GTE were determined for PPO inhibitory activity. GTE in combination with the selected synergist showing the highest PPO inhibitory activity was used for further study.

6.3.5 Effect of different immersion time of white shrimp in green tea extract in combination with ascorbic acid on melanosis during iced storage

Whole Pacific white shrimp were immersed in 0.1 % GTE in the presence of AA at different levels (0, 0.005 and 0.01 %) for different times (5, 15, 30 and 60 min) at 4^{o}C using a shrimp/ solution ratio of 1:2 (w/v). Treated shrimp were drained on the screen for 3 min at 4^{o}C. Shrimp without any treatment were used as the control. All samples were stored in polystyrene box containing ice using a shrimp/ice ratio of 1:2 (w/w). To maintain shrimp/ice ratio, the molten ice was removed and the same amount of ice was added. Melanosis of all samples was evaluated every 2 days up to 12 days.

6.3.6 Effect of green tea extract in combination with ascorbic acid on the quality of Pacific white shrimp during iced storage

6.3.6.1 Treatment of shrimp

Whole Pacific white shrimp were treated in different solutions including 0.1 % GTE mixed without and with AA (0.005 and 0.01 %) for 15 min, following by draining. Sample treated with 1.25 % sodium metabisulfite (SMS) at a ratio of 1:2 (w/v) for 1 min at 4^{o}C (Kim *et al*. 2000) was also prepared. Sample without treatment was used as the control. Samples (25 shrimp) were randomly taken for each treatment every 2 days up to 12 days for microbiological, chemical and physical analyses. Melanosis was also determined.

6.3.6.2 Microbiological analyses

Five whole Pacific white shrimp were collected aseptically and used as the composite sample. Microbiological analyses including (i) psychrotrophic bacterial counts (ii) H_2S- producing bacteria and (iii) *Enterobacteriaceae* count were performed as per the method of Nirmal and Benjakul (2009b).

6.3.6.3 Chemical analyses

6.3.6.3.1 pH measurement

pH measurement was conducted following the method described by Nirmal and Benjakul (2009a).

6.3.6.3.2 Determination of total volatile base content

Total volatile base (TVB) content in shrimp meat was determined using the Conway micro-diffusion method (Conway and Byrne 1936).

6.3.6.3.3Determination of thiobarbituric acid reactive substances (TBARS)

TBARS value in the samples was determined as per the method of Nirmal and Benjakul (2010a).

6.3.6.4 Melanosis assessment

Melanosis or blackening of Pacific white shrimp was evaluated through visual inspection by six trained panelists using 10-point scoring (Montero *et al*.,

2001b). Panelists were asked to give the melanosis score (0 to 10), where 0 = absent; 2 slight (up to 20 of shrimps surface affected) 4 moderate (20 to 40 of shrimps surface affected) 6 notable (40 to 60 of shrimps surface affected); 8 = severe (60 to 80 of shrimps surface affected) 10 extremely heavy (80 to 100 of shrimps surface affected).

6.3.6.5 Sensory evaluation

At day 0 and 12 of storage, whole shrimp without and with different treatments were placed on a stainless steel tray, covered with aluminium foil and steamed for 5 min. The cooked samples were evaluated by 30 panelists, the graduate students in Food Science and Technology program, Department of Food Technology, Prince of Songkla University with the age of 23 – 25 years. Panelists were acquainted with shrimp consumption and had no allergies to shrimp. Nine-point hedonic scale were used to evaluate samples, where 9 = like extremely; 7 = like moderately; 5 = neither like or nor dislike; 3 = dislike moderately; 1 = dislike extremely (Meilgaard *et al*., 1990). All panelists were asked to evaluate for color, odor, taste, flavor and overall likeness. Samples were presented unpeeled in plates coded with random three-digit numbers.

6.3.6.6. Statistical analyses

All experiments were performed in triplicate and a completely randomized design (CRD) was used. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan s multiple range tests (Steel and Torrie 1980). Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

6.4 Results and Discussion

6.4.1 Effect of green tea extract without and with different additives on PPO inhibition

The effect of green tea extract (GTE) alone and in combination with other additives on the inhibition of PPO from the cephalothorax of Pacific white shrimp is shown in Figure 39. GTE (0.1 %) in combination with and without additives including ascorbic acid (AA), citric acid (CA), sodium pyrophosphate (SPP) or sodium chloride (NaCl) showed the inhibitory effect towards PPO. GTE (0.1 %) alone inhibited PPO from cephalothorax of Pacific white shrimp by 61.2 %. AA at concentrations of 0.005 and 0.01 % showed 47.1 and 61.9 % inhibition towards PPO, respectively. CA at concentrations of 0.05 and 0.1 % exhibited 25.4 and 60.2 % PPO inhibition, respectively. AA acts as strong reducing agent and oxygen scavenger, whereas CA exerts its inhibitory effect on PPO by lowering the pH as well as chelating the copper at the active site of the enzyme (Kim *et al*., 2000). Lower inhibitory activity of 1 % SPP and 1 % NaCl was observed, in which 25.0 and 32.7 % inhibition towards PPO was obtained, respectively. Pyrophosphates can act as a metal chelating agent and sodium chloride is known to lower PPO activity (Kim *et al*., 2000). At the concentrations used, AA (0.005 and 0.01 %) or CA (0.05 and 0.1 %) had the higher inhibitory activity towards PPO than did 1 % SPP or 1 % NaCl ($P < 0.05$). For both AA and CA, their inhibitory activity increased with increasing concentrations.

When 0.1 % GTE was used in combination with 0.01 % AA or 0.1 % CA, PPO inhibitory activity of 0.1 % GTE increased from 61.2 % to 93.0 %. AA and CA might act synergistically with GTE by reducing quinone to dihydroxy phenyl alanine or might chelate with copper at the active site of PPO. However, 0.1 % GTE in combination with 1 % SPP or 1 % NaCl had slightly lower inhibitory effect, compared with GTE alone ($P < 0.05$). High concentration of SPP or NaCl might be required to enhance the combination effect with GTE towards PPO inhibition. From *in vitro* study, GTE in combination with AA at varying concentrations either 0.005 or 0.01 % exhibited the profound inhibitory effect on PPO. Although CA (0.1 %)

showed the equivalent inhibitory effect on PPO to AA (0.01 %), the latter was used at 10-fold lower amount. This indicated the higher efficacy of AA, compared with CA as the synergist with GTE.

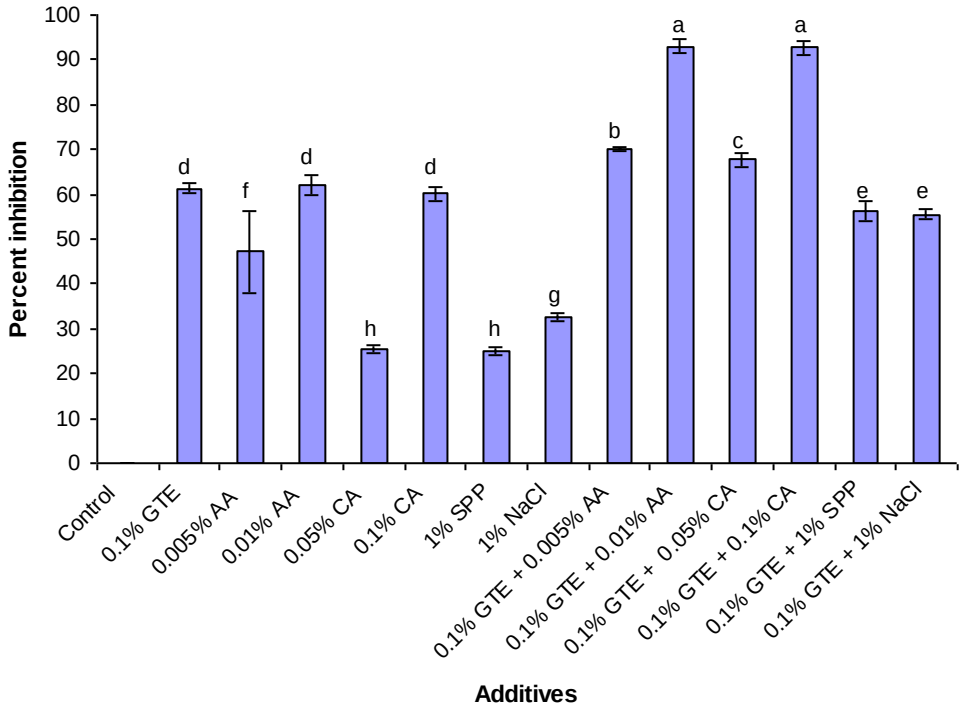

Figure 39. Effect of green tea extract and other additives alone or combined on the inhibition of PPO from the cephalothorax of Pacific white shrimp. Bars represent the standard deviation (n = 3). Different letters on the bars indicate significant differences ($P < 0.05$). GTE: green tea extract; AA: ascorbic acid; CA: citric acid; SPP: sodium pyrophosphate; NaCl: sodium chloride.

Table 9 represents the melanosis score of Pacific white shrimp soaked with 0.1 % GTE without and with AA (0.005 and 0.01 %) for different times during iced storage of 12 days. At the day 0, there was no melanosis found for all samples, regardless of treatment and soaking time ($P > 0.05$). Melanosis score of all samples increased as the storage time increased ($P < 0.05$). At day 2 of iced storage, melanosis was detectable in the control and shrimp treated with 0.1 % GTE alone and 0.1 % GTE + 0.005 % AA with the immersion time of 5 min.

Table 9. Melanosis score of Pacific white shrimp treated with 0.1 % GTE or 0.1 % GTE + AA (0.005 or 0.01 %) using different immersion times during 12 days of iced storage

Immersion time (min)	Treatment	Storage time (days)						
		0	2	4	6	8	10	12
	Control	0 ± 0.0 aG	1.6 ± 0.3 aF	2.8 ± 0.3 aE	4.3 ± 0.6 aD	6.6 ± 0.6 aC	8.3 ± 0.5 aB	10 ± 0.0 aA
5	0.1% GTE	0 ± 0.0 aG	1 ± 0.0 bF	1.6 ± 0.2 bE	2.6 ± 0.3 bD	4 ± 0.0 bcC	5.6 ± 0.6 bB	6.6 ± 0.6 bA
	0.1% GTE + 0.005% AA	0 ± 0.0 aF	0.8 ± 0.3 bE	1 ± 0.0 cE	2 ± 0.0 cdD	3.3 ± 0.3 cdeC	4.6 ± 0.5 cdeB	6 ± 0.0 bcdA
	0.1% GTE + 0.01% AA	0 ± 0.0 aF	0 ± 0.0 cF	0.5 ± 0.0 cdE	2 ± 0.0 cdD	3.1 ± 0.3 deC	4.6 ± 0.6 cdeB	6 ± 0.0 bcdA
15	0.1% GTE	0 ± 0.0 aF	0 ± 0.0 cF	0.6 ± 0.3 cdE	2.3 ± 0.6 bcD	4 ± 0.0 bcC	5.5 ± 0.5 bcB	6.5 ± 0.5 bcA
	0.1% GTE + 0.005% AA	0 ± 0.0 aF	0 ± 0.0 cF	0.6 ± 0.5 cdE	1.6 ± 0.3 dD	2.6 ± 0.5 eC	4.5 ± 0.5 deB	5.3 ± 0.6 deA
	0.1% GTE + 0.01% AA	0 ± 0.0 aF	0 ± 0.0 cF	0.6 ± 0.6 cdE	1.3 ± 0.3 dD	2.8 ± 0.3 eC	4.3 ± 0.3 eB	5 ± 0.0 eA
30	0.1% GTE	0 ± 0.0 aF	0 ± 0.0 cF	0.6 ± 0.3 cdE	1.6 ± 0.3 dD	3.6 ± 0.6 bcdC	5.3 ± 0.3 bcdB	6.5 ± 0.5 bcA
	0.1% GTE + 0.005% AA	0 ± 0.0 aF	0 ± 0.0 cF	0.5 ± 0.0 cdE	1.3 ± 0.3 dD	3.3 ± 0.2 cdeC	4.6 ± 0.3 cdeB	5.6 ± 0.3 deA
	0.1% GTE + 0.01% AA	0 ± 0.0 aF	0 ± 0.0 cF	0.6 ± 0.3 cdE	1.3 ± 0.6 dD	3 ± 0.0 deC	4.6 ± 0.3 cdeB	5.3 ± 0.3 deA
60	0.1% GTE	0 ± 0.0 aF	0 ± 0.0 cF	1 ± 0.0 cE	2.6 ± 0.3 bD	4.3 ± 0.3 bC	5.6 ± 0.6 bB	6.6 ± 0.6 bA
	0.1% GTE + 0.005% AA	0 ± 0.0 aF	0 ± 0.0 cF	0.3 ± 0.3 eE	1.3 ± 0.3 dD	3.3 ± 0.3 cdeC	5.3 ± 0.6 bcdB	6 ± 0.0 bcdA
	0.1% GTE + 0.01% AA	0 ± 0.0 aF	0 ± 0.0 cF	0.5 ± 0.0 cdE	1.5 ± 0.0 dD	3.6 ± 0.6 bcdC	5 ± 0.0 bcdeB	5.8 ± 0.3 cdA

Different letters in the same column indicate significant differences ($P < 0.05$); different capital letters in the same row indicate significant differences ($P < 0.05$). Value are mean ± standard deviation (n=3).

When shrimp soaked in 0.1 % GTE alone or 0.1 % GTE + AA (0.005 or 0.01 %) for longer time (15, 30 or 60 min), melanosis was observed on day 4 of iced storage. Immersion time of 5 min might be not sufficient to allow GTE as well as AA to penetrate through carapace or cuticle of the shrimp. Shrimp soaked with 0.1 % GTE + 0.01 % AA for 15 min had the lowest melanosis score during 8 - 12 days of storage, compared with other treatments ($P < 0.05$). Montero *et al*. (2001a) reported that prawns (*Penaeus japonicus*) soaked in 0.5 % 4-hexylresorcinol (4-HR) in combination with 0.5 % AA or 0.5 % CA for 120 min had the lowest melanosis formation during iced storage of 8 days. On the other hand, when deep water pink shrimp (*Parapenaeus longiritris*) was immersed in 4-HR (0.1 or 0.25 %) in combination with 0.5 % AA or 0.5 % CA for 60 min, both acids did not increase melanosis inhibition of shrimp during chilled storage of 12 days, compared with 4-HR alone (Montero *et al*., 2004). For this study, Pacific white shrimp soaked for 15 or 30 min in 0.1 % GTE + 0.005 % AA or 0.1 % GTE + 0.01 % AA had slightly higher retardation of melanosis, compared with those treated with 0.1 % GTE alone ($P < 0.05$). Thus, AA exhibited the combination effect on melanosis inhibition with GTE

6.4.2 Effect of green tea extract without and with ascorbic acid on microbial changes of Pacific white shrimp during iced storage

Changes in psychrotrophic bacterial count (PBC) of Pacific white shrimp during iced storage as influenced by the treatment using GTE or GTE +AA are presented in Figure 40A. At day 0 of storage, PBC was 2.5 log CFU / g for all samples, regardless of treatments. As the storage time increased, a continuous increase in PBC was observed in all samples ($P < 0.05$). However, the lower rate of increase was observed in the samples treated with 0.1 % GTE alone or 0.1 % GTE + AA (0.005 or 0.01 %) in comparison with the control and those treated with SMS ($P < 0.05$). Pacific white shrimp treated with 0.1 % catechin had the lowered PBC during iced storage of 10 days (Nirmal and Benjakul, 2009b). Fresh or frozen shellfish are considered to be satisfactory if the aerobic plate count (APC) at 35^{o}C is not more than 5 log CFU/g (National Research Council, 1985).

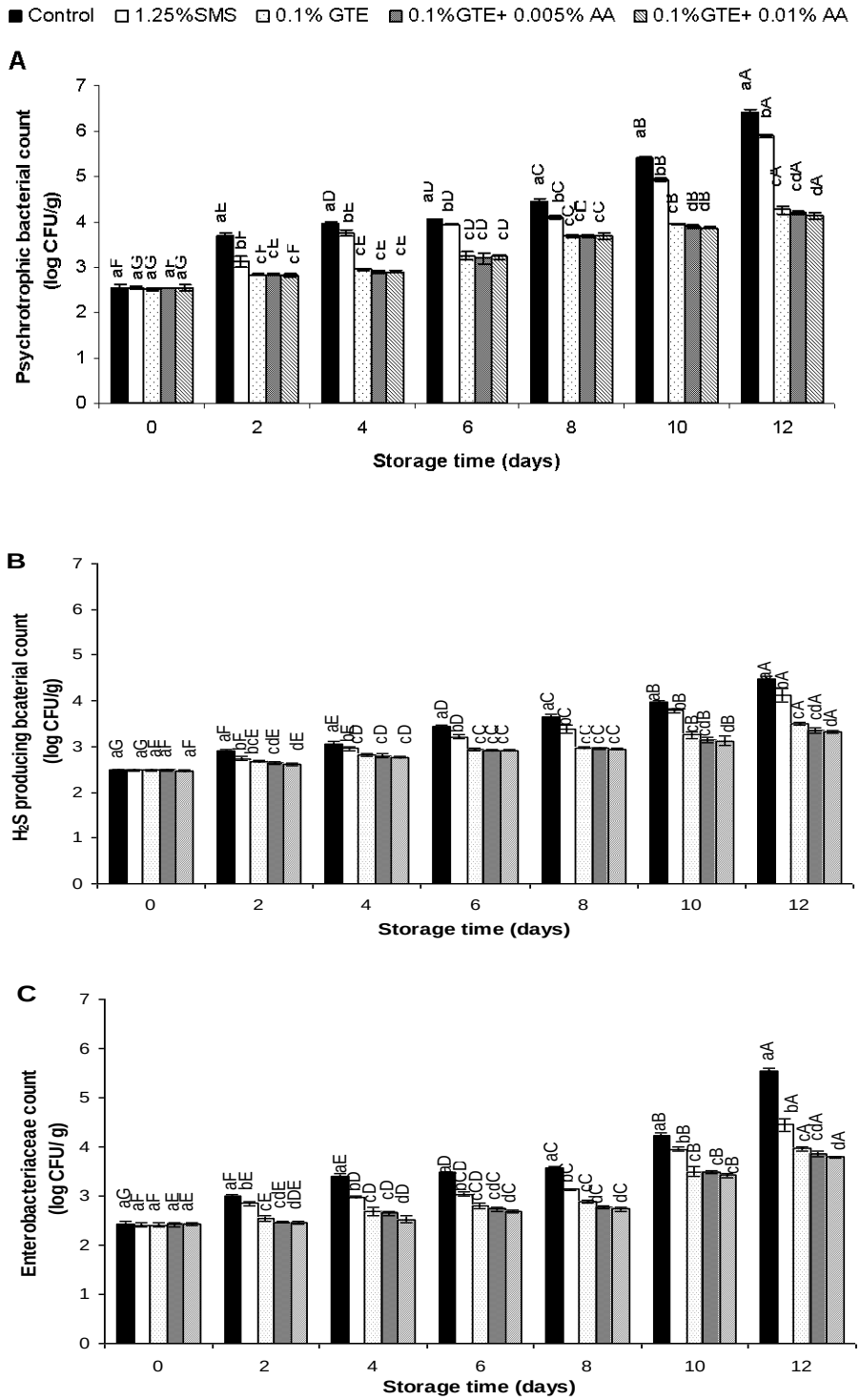

Figure 40. Psychrotrophic bacterial count (A), H_2S producing bacterial count (B), and enterobacteriaceae count (C) of Pacific white shrimp treated with 0.1 % GTE or 0.1 % GTE + AA (0.005 or 0.01 %) during 12 days of iced storage. Bars represent the standard deviation (n = 3). Different capital letters on the bars within the same treatment indicate the significant differences ($P < 0.05$). The different letters on the bars within the same storage time indicate significant differences ($P < 0.05$). SMS: sodium metabisulfite; GTE: green tea extract; AA: ascorbic acid

During 10-12 days of storage, the lowest PBC was found in the shrimp treated with GTE +AA, compared to those treated with GTE alone ($P < 0.05$). The control shrimp and those treated with SMS, 0.1% GTE alone and 0.1 % GTE + 0.01 % AA had PBC of 6.40, 5.89, 4.26 and 4.13 log CFU/g, respectively, at the end of iced storage. Result suggested that the GTE in combination with AA could retard the increase in PBC. Banon *et al*. (2007) reported that ascorbate and green tea extract increased the shelf life of beef patties by delaying the microbial spoilage.

H_2S producing bacterial count of Pacific white shrimp treated without and with GTE or GTE + AA was monitored during iced storage as shown in Figure 40B. The initial load for all samples was 2.47 log CFU/g. H_2S producing bacterial count generally increased in all samples throughout the storage of 12 days ($P < 0.05$). Nevertheless, the increase in H_2S producing bacterial count was lowered in shrimp treated with GTE or GTE + AA, compared with the control and those treated with SMS ($P < 0.05$). At the end of storage, H_2S- producing bacterial count was found to be 4.47 and 3.32 log CFU/g for the control and those treated with 0.1 % GTE + 0.01 % AA respectively. Thus, AA might exhibit the combination effect with GTE on inhibition of H_2S producing bacteria. Montero *et al*. (2001a) found that prawn (*Penaeus japonicus*) treated with 4-HR in combination with ascorbic acid had the lower total viable count as compared to those treated with 4-HR alone. However, H_2S-producing microorganisms were not detected during 10 days of iced storage. Epigallocatechin gallate (EGCG, 48 µg/mL) inhibited the increase of methicillin-resistant *Staphylococcus aureus* (MRSA) and the addition of AA (32 µg/mL) enhanced the antibacterial activity of EGCG (Hatano *et al*., 2008). GTE, which was the source of catechin, could inhibit some microorganisms in shrimp. Moreover, the efficacy of GTE to retard microbial growth was increased, when GTE was used in combination with AA.

At day 0, enterobacteriaceae count for all samples was 2.41 log CFU/g (Figure 40C). Generally, the increase in enterobactriaceae count was found in all samples as the storage time increased ($P < 0.05$). However, shrimp treated with GTE alone or GTE + AA had the lower count, in comparison with the control or those treated with SMS ($P < 0.05$). This result was in agreement with Nirmal and Benjakul (2009b) who reported that Pacific white shrimp treated with 0.1 % catechin had the

lower enterobacteriaceae count as compared to the control after 10 days of storage in ice. At day 12 of storage, shrimp treated with 0.1 % GTE + 0.01 % AA showed the lowest enterobacteriaceae count, compared with other treatments ($P < 0.05$). The maximum limit for enterobacteriaceae in food product including fishery products is 4 log CFU/g (IFST, 1999). The result reconfirmed that AA play a synergistic role in inhibition of microorganisms with GTE in Pacific white shrimp during iced storage.

Overall, shrimp treated with 0.1 % GTE had the lowered PBC, H_2S-producing bacterial count and enterobacteriaceae count, compared with those treated with 1.25 % SMS. Phenolic compound, especially catechin and derivatives in green tea extract, might form complex with protein of cell wall of microorganism, causing lyses of cell wall. Additionally, AA was found to show the combination effect with GTE in retardation of the bacterial growth of shrimp during iced storage. AA was effective in retaining the epigallocatechin gallate (EGCG) in the stable form, thereby increasing antibacterial activity of EGCG (Hatano *et al*., 2008).

6.4.3 Effect of green tea extract without and with ascorbic acid on chemical changes of Pacific white shrimp during iced storage

Figure 41A depicted the changes in pH of Pacific white shrimp treated with GTE alone or GTE +AA during iced storage. The pH value at day 0 of all samples was 6.54. It was noted that the soaking solution containing AA with pH of 3.87 did not alter the pH of the treated shrimp. This value was in accordance with pH value reported for fresh Pacific white shrimp (Nirmal and Benjakul 2009a, 2009b). During the iced storage of 12 days, the continuous increase in pH value was observed for the control and reached the value of 7.42 at the end of the storage ($P < 0.05$). An increase in the pH value of shrimp during storage was the result of the accumulation of basic compounds generated from both endogenous and microbial enzymatic actions (Lopez-Caballero *et al*., 2007). The increase in pH value was lowered in shrimp treated with GTE and GTE + AA during iced storage ($P < 0.05$). Shrimp treated with SMS showed the higher pH value, compared with those treated with GTE or GTE + AA after 6 days of storage ($P < 0.05$). The lowered increasing rate of pH observed in, shrimp treated with GTE or GTE + AA was in accordance with the lowered microbial

load of shrimp treated with GTE or GTE + AA during iced storage (Figure 40). Total volatile base content in all samples at the beginning of storage was 6.96 mg N/ 100 g (Figure 41B). The TVB content of fresh deepwater pink shrimp (*Parapenaeus longirostris*) was reported to be 30 mg N/ 100g (Lopez-Caballero *et al*., 2007).

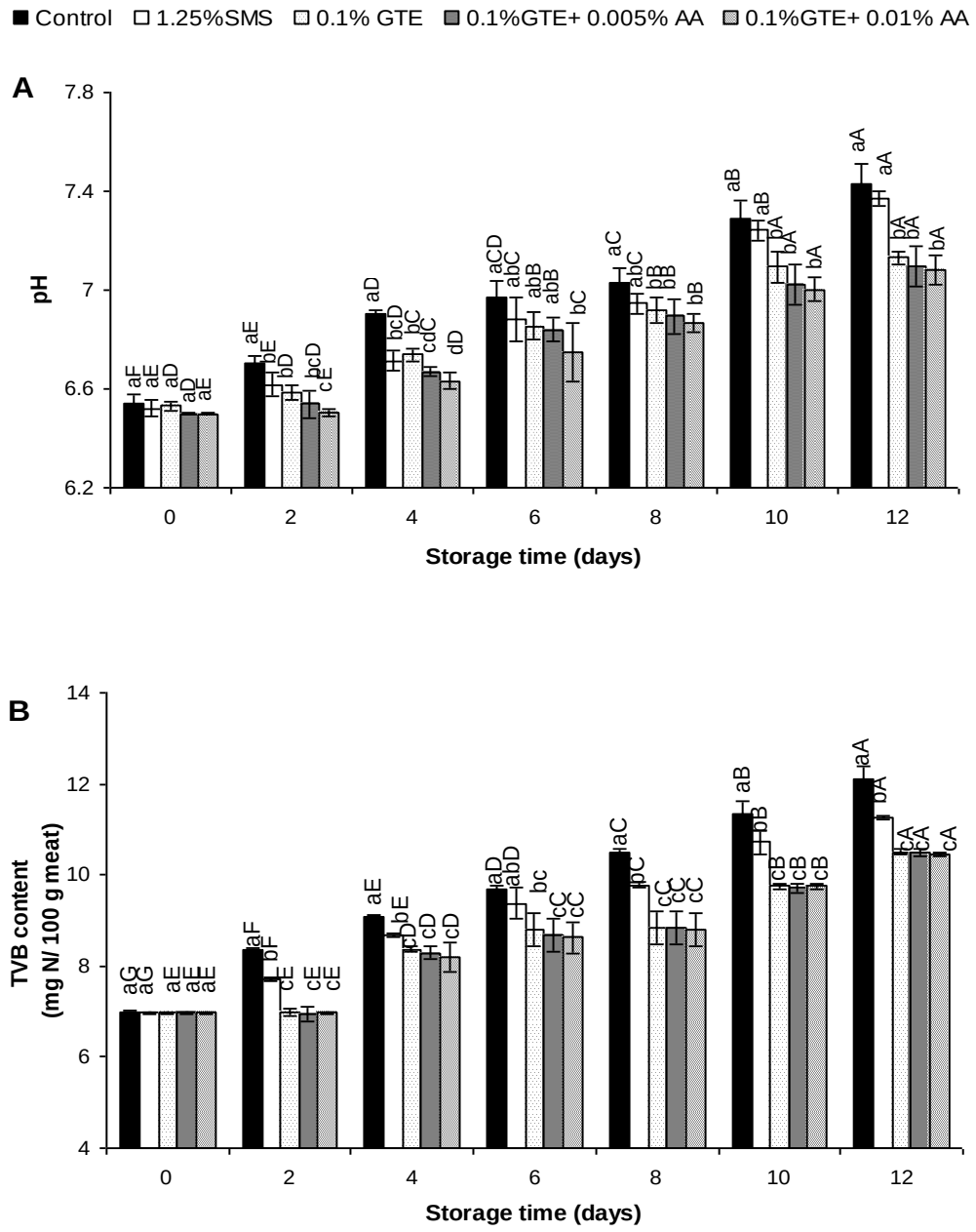

Figure 41. pH (A) and total volatile base content (B) of Pacific white shrimp treated with 0.1 % GTE or 0.1 % GTE + AA (0.005 or 0.01 %) during 12 days of iced storage. Bars represent the standard deviation (n=3). Key: see Figure 40 caption.

This difference in the initial TVB value might be attributed to the different culture habitat, feed and body composition of the animal. TVB content of all samples increased with increasing storage time ($P < 0.05$). However, the increase in TVB content was lower in the shrimp treated with GTE or GTE + AA in comparison with the control and those treated with SMS throughout the storage of 12 days ($P < 0.05$). The lower increase in TVB content of shrimp treated with GTE + AA was concomitant with the lower microbial load (Figure 40) and the lower increase in pH (Figure 41A). The lower TVB content of Pacific white shrimp treated with ferulic acid (1 or 2 %) was reported to be due to the inhibitory activity of ferulic acid against microbes and proteolytic enzymes (Nirmal and Benjakul 2009a). According to Thai standard, the TVB limit for frozen shrimp is 30 mg N/ 100 g meat (TIS, 1986). The result suggested that GTE had antimicrobial effect in shrimp during iced storage. Catechin or derivatives in GTE could crosslink adenosine monophosphate deaminase, responsible for the formation of volatile base in crustacean. This more likely resulted in the lowered formation of TVB in treated shrimp. Moreover, AA could stabilize the catechin or derivatives in GTE by preventing oxidation of catechin and enhancing its effect against microbes or proteolytic enzyme.

Thiobarbituric acid reactive substances (TBARS) value of Pacific white shrimp without and with treatment of GTE or GTE +AA during iced storage is shown in Figure 42. In general, TBARS value of all samples increased with increasing storage time ($P < 0.05$). When lipid oxidations take place, unstable hydroperoxide is formed and decomposes readily to shorter chain hydrocarbons such as aldehydes, which can be detected as TBARS (Benjakul *et al*. 2005b). Shrimp treated with GTE showed the lower TBARS during 4-12 days, compared with the control and those treated with SMS ($P < 0.05$). From the result, AA had no combination effect on antioxidative activity of GTE, regardless of concentrations used. AA has been known to be oxygen scavenger as well as reducing agent (Niki, 1991). It has been reported that catechin (0.1 %) showed a strong antioxidative effect in Pacific white shrimp muscle and lowered lipid oxidation during iced storage (Nirmal and Benjakul, 2009b). Majchrzak *et al*. (2004) reported that incorporation of AA up to 30 mg into 100 mL of green tea extract solution showed the linear increase in total antioxidant activity of green tea extract. Hence, lipid oxidation in shrimp treated with

GTE could be prevented to some degree without the incorporation of AA during extended storage.

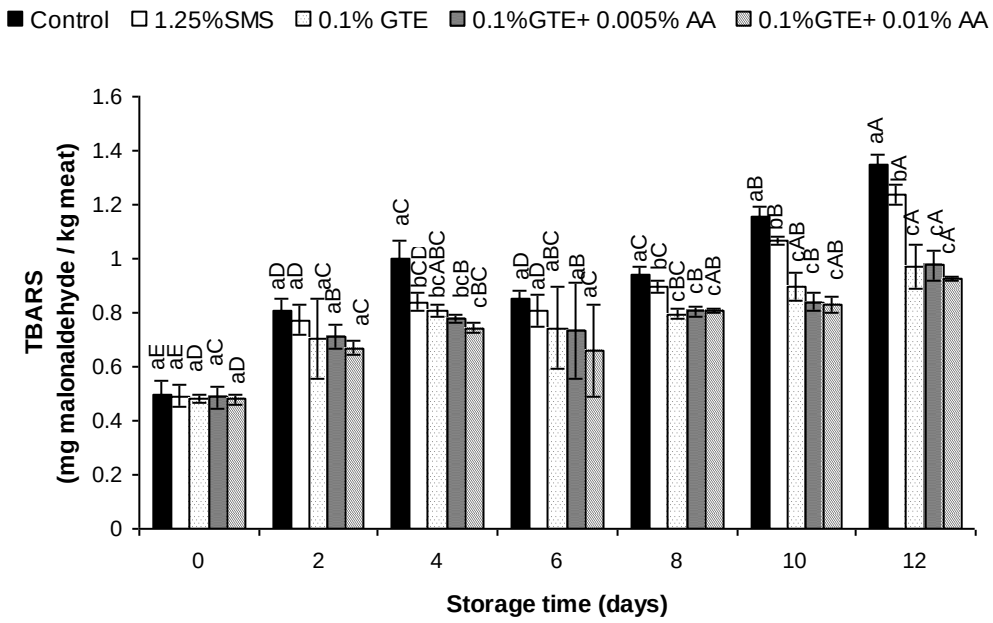

Figure 42. TBARS of Pacific white shrimp treated with 0.1 % GTE or 0.1 % GTE + AA (0.005 or 0.01 %) during 12 days of iced storage. Bars represent the standard deviation (n=3). Key: see Figure 40 caption.

6.4.4 Effect of green tea extract without and with ascorbic acid on melanosis of Pacific white shrimp during iced storage

Melanosis score of Pacific white shrimp treated with GTE or GTE +AA during iced storage is represented in Figure 43. No negative effect of GTE or GTE + AA treatments on color or appearance of resulting shrimp was found. At day 0, there was no melanosis score for all samples. During the storage, the increase in melanosis score was observed in all samples ($P < 0.05$). However, the increase in melanosis score was lowered in shrimp treated with GTE or GTE + AA in comparison with the control and SMS treated samples. SMS treatment showed the effectiveness in preventing melanosis comparable to GTE within the first 8 days of storage ($P > 0.05$).

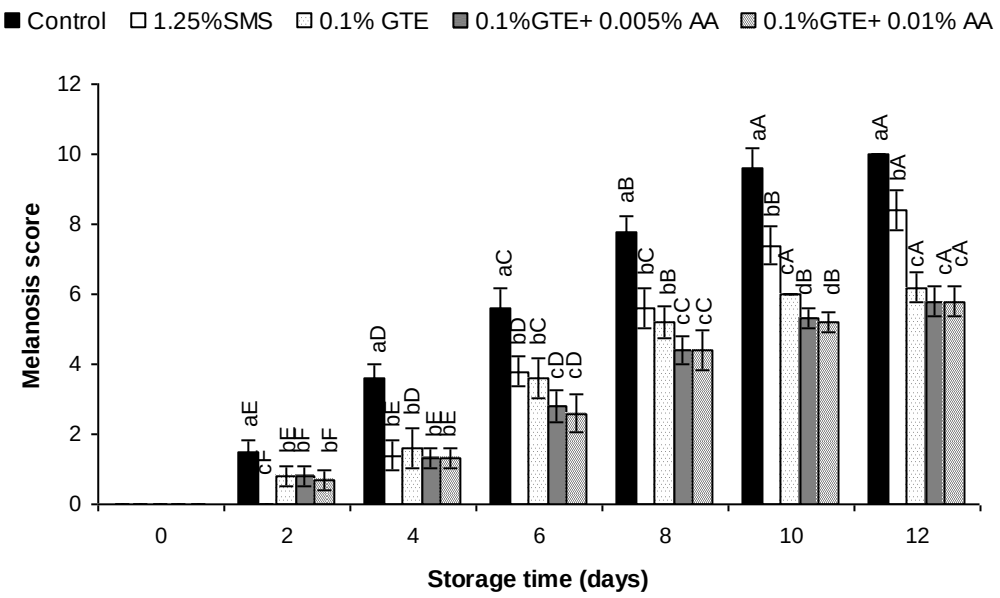

Figure 43. Melanosis score of Pacific white shrimp treated with 0.1 % GTE or 0.1 % GTE + AA (0.005 or 0.01 %) during 12 days of iced storage. Bars represent the standard deviation (n=3). Key: see Figure 40 caption.

However, the lower melanosis inhibitory activity was observed during 10 -12 days of storage. When GTE was used in combination with AA, the lower melanosis score was found up to 10 days, in comparison with the use of GTE alone. The higher inhibition of melanosis by GTE in conjunction with AA was in agreement with the greater inhibition of PPO for *in vitro* study (Figure 39). Phenolic compound could act as reducing agent, metal chelator or oxygen scavenger, which rendered PPO inactive, thereby retarding the melanosis formation. AA could provide hydrogen molecule to catechin or could directly involve in the reduction of quinone back to hydroquinone.

6.4.5 Effect of green tea extract without and with ascorbic acid on sensory properties of Pacific white shrimp stored in ice

Likeness score for Pacific white shrimp treated with 0.1 % GTE or 0.1 % GTE + AA (0.005 or 0.01 %) is shown in Table 10.

Table 10. Likeness score of Pacific white shrimp treated with 0.1 % GTE or 0.1 % GTE + AA (0.005 or 0.01 %) before and after 12 days of iced storage

Storage time (days)	Treatments	Color	Odor	Taste	Flavor	Overall
0	Cont.	7.4 ± 1.34 aA	7.3 ± 0.74 aA	7.5 ± 1.16 aA	7.4 ± 1.08 aA	7.7 ± 0.57 aA
	1.25% SMS	7.3 ± 0.84 aA	7.4 ± 0.87 aA	7.3 ± 0.99 aA	7.3 ± 1.09 aA	7.6 ± 1.01 aA
	0.1% GTE	7.2 ± 1.31 aA	7.5 ± 0.64 aA	7.8 ± 0.86 aA	7.4 ± 0.85 aA	7.6 ± 0.75 aA
	0.1% GTE + 0.005% AA	7.5 ± 0.85 aA	7.5 ± 0.63 aA	7.6 ± 1.15 aA	7.6 ± 1.01 aA	8.0 ± 0.55 aA
	0.1% GTE + 0.01% AA	7.5 ± 1.22 aA	7.6 ± 0.49 aA	7.7 ± 0.61 aA	7.4 ± 0.85 aA	8.0 ± 0.55 aA
12	Cont.	4.9 ± 0.82 cB	5.5 ± 0.51 cB	5.3 ± 0.92 cB	5.2 ± 0.89 bB	5.5 ± 0.65 cB
	1.25% SMS	5.4 ± 0.51 cB	6.1 ± 0.77 bB	5.6 ± 0.75 bcB	5.5 ± 0.85 bB	5.5 ± 0.65 cB
	0.1% GTE	6.4 ± 0.51 bB	6.5 ± 0.51 bB	6.0 ± 0.91 bB	5.8 ± 0.69 bB	6.2 ± 0.72 bB
	0.1% GTE + 0.005% AA	6.7 ± 0.46 abB	6.9 ± 0.47 abB	6.6 ± 0.74 aB	6.6 ± 0.84 aB	6.8 ± 0.69 aB
	0.1% GTE + 0.01% AA	7.1 ± 0.66 aA	7.1 ± 0.66 aA	6.6 ± 0.75 aB	6.6 ± 0.84 aB	6.9 ± 0.82 aB

Different capital letters in the same column within the same treatment indicate the significant differences ($p<0.05$). The different letters in the same column within the same storage time indicate significant differences ($p<0.05$). Values are mean $\pm$ standard deviation ($n=3$). Cont.: control; SMS: sodium metabisulfite; GTE: green tea extract; AA: ascorbic acid.

At day 0, all samples had the score higher than 7.0 for all attributes tested and no differences in likeness were found between all treatments ($P > 0.05$). Therefore, the treatment of shrimp with GTE or GTE + AA had no impact on color, taste or flavor of treated shrimp. Since the chlorophyll in green tea was removed, the extract obtained had the light straw-yellow in color. Additionally, caffeine was also removed by dechlorophyllization (Row and Jin, 2006). Therefore, there might be less probability of GTE to adversely affect the color, taste or flavor of the shrimp treated with GTE or GTE + AA. At the end of storage time, the decreases in likeness for all attributes were observed for all samples ($P < 0.05$). Shrimp treated with GTE + AA showed the higher likeness score for all attributes, in comparison with other treatments ($P < 0.05$). The higher likeness score for the color was in agreement with lowered melanosis in the sample treated with GTE + AA (Figure 43). The higher odor, taste and flavor of shrimp treated with GTE + AA was probably attributed to the lower spoilage of sample as evidenced by the lower bacterial count, TVB content as well as TBARS values compared with the control and those treated with GTE alone or SMS. Nirmal and Benjakul (2009a) reported that shrimp treated with 2 % ferulic acid had higher score for color, taste and flavor likeness after 10 days of iced storage, compared with the control and those treated with 1.25 % sodium metabisulfite. Thus, the treatment of Pacific white shrimp using GTE + AA yielded the shrimp with the higher sensory property when stored for a long time in ice.

6.5 Conclusions

GTE showed the increase in PPO inhibitory activity, when AA was combined. Soaking time of 5 min was sufficient to lower melanosis in shrimp treated with 0.1 % GTE + AA (0.005 or 0.01 %). Treated shrimp had the retardation in melanosis formation, microbial growth, and lipid oxidation. Moreover, shrimp treated with GTE + AA had the superior sensory properties to the control sample and sample treated with GTE alone or SMS. Thus, green tea extract (0.1 %) in combination with ascorbic acid (0.005 %) could be used to treat Pacific white shrimp in order to retard melanosis as well as to extend the shelf-life of shrimp stored in ice.

CHAPTER 7

RETARDATION OF QUALITY CHANGES OF PACIFIC WHITE SHRIMP BY GREEN TEA EXTRACT TREATMENT AND MODIFIED ATMOSPHERE PACKAGING DURING REFRIGERATED STORAGE

7.1 Abstract

The effect of modified atmosphere packaging (MAP) on the quality changes of Pacific white shrimp (*Litopenaeus vannamei*) treated with or without green tea extract (1 g/L; GTE) in combination with or without ascorbic acid (0.05 g/L; AA) during refrigerated storage of 10 days was investigated. Shrimp without treatment stored under MAP had lowered psychrotrophic bacteria, enterobacteriaceae and H_2S- producing bacteria count ($P < 0.05$) but similar lactic acid bacteria count ($P > 0.05$), in comparison with shrimp stored in air (control). The coincidental lowered rate of increase in pH, total volatile base (TVB) content and thiobarbituric acid reactive substances (TBARS) were obtained in shrimp stored under MAP ($P < 0.05$). However, MAP slightly lowered melanosis formation and improved likeness score to some extent. When shrimp were treated with GTE and stored under MAP, the lower microbiological and chemical changes as well as the lowest melanosis formation was observed, compared to shrimp kept under MAP without treatment and the control ($P < 0.05$). GTE treatment in combination with MAP could retard chemical changes and melanosis formation, regardless of AA incorporation ($P > 0.05$). Nevertheless, GTE in combination with AA had higher inhibition on microbial growth and yielded the shrimp with higher likeness, compared with the other treatments ($P < 0.05$). Therefore, shrimp treated with GTE in combination with AA prior to MAP had the lowest losses in quality during refrigerated storage.

7.2 Introduction

Shrimp have high demand in many countries including Japan, the United States and Europe because of their delicacy (Paquotte *et al*., 1998). Thailand is the world s leading shrimp-farming country and has become the top supplier of farmed shrimp. Pacific white shrimp (*Litopenaeus vannamei*) is an important commercial species primarily cultured in Thailand (Nirmal and Benjakul, 2009a). Shrimp and shrimp products of Thailand are well known for their long-standing excellent reputation worldwide, owing to the outstanding quality, freshness, variety and taste (Rattanasatheirn *et al*., 2008). Deterioration of shrimp is associated with microbiological, chemical, and physical changes during postmortem storage (Nirmal and Benjakul, 2010a). Seafood spoilage microorganisms contribute to the loss of essential fatty acids and proteins, production of biogenic amines and formation of off-odours (Mastromatteo *et al*., 2010). Free amino acids and other soluble non-nitrogenous substances in shrimp serve as digestible nutrient for microbial growth (Zeng *et al*., 2005). Shrimp generally have the limited shelf life due to formation of black spots (melanosis). Although melanosis is harmless to consumers it reduces the consumer s acceptability, leading to loss of market value (irmal and Benjakul, 2009b). Melanosis is a biochemical process, in which phenols are oxidized to quinone by polyphenoloxidase (PPO).

Several studies have been conducted to extend the shelf life of shrimp, especially by lowering melanosis using natural extract or phenolic compounds (Nirmal and Benjakul, 2009a, 2009b, 2010b; Gokoglu and Yerlikaya, 2008). The use of natural additives like catechin and ferulic acid could be safer to maintain the quality of shrimp, compared to synthetic additives (Nirmal and Benjakul, 2010a). Green tea is the major source of catehcin and has been reported to have antioxidant and antibacterial activities, anti-inflammatory, antimutagenic, antidiabetic, and antibacterial (Cabrera *et al*., 2006). Green tea extract was found to retard melanosis as well as extend the shelf life of shrimp stored in ice (Nirmal and Benjakul, 2011a). Recently, natural extract such as plant extract e.g. rosemary extract (Cadun *et al*., 2008), green tea extract (Kumudavally *et al*., 2008) have been used as the natural safe additives. Modified atmosphere packaging (MAP) of food has gained considerable

popularity as a modern method for packaging (Soldatou *et al*., 2009; Fernandez *et al*., 2009). MAP was able to act as coadjuvant with chilled storage in delaying melanosis and microorganism growth in crustaceans (Martinez-Alvarez *et al*., 2005b). Packaging is another important hurdle, which can preserve foods but MAP is still questionable to ensure quality and safety of the foods (Sivertsvik *et al*., 2002). Such a limited success of MAP has led to the processing or treatment of meat or seafood product prior to packaging (Mastromatteo *et al*., 2010; Soldatou *et al*., 2009; Fernandez *et al*., 2009).

Recently, Nirmal and Benjakul (2010b) reported that green tea extract in combination with ascorbic acid showed synergistic effect on melanosis inhibition as well as retardation of quality loss of Pacific white shrimp stored in ice. The use of green tea extract treatment prior to MAP might increase the hurdles for microbial growth, thereby retarding quality changes of shrimp more effectively. Therefore, the aim of this study was to investigate the combined effect of green tea extract in the presence and absence of ascorbic acid with MAP on the retardation of quality changes of Pacific white shrimp stored at refrigerated temperature during 10 days.

7.3 Materials and Methods

7.3.1 Chemicals and green tea

Malonaldehyde bis (dimethyl acetal) and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), L-ascorbic acid, sodium chloride, standard plate count agar, triple sugar iron agar (IA), man rogosa sharpe broth (MRS), and eosin methylene blue agar (EMB) were obtained from Merck (Darmstadt, Germany). Green tea (*Camellia sinensis* L.) cultivated in Chiang Rai province, Northern Thailand was purchased as green tea powder from a local market of Hat Yai, Songkhla, Thailand.

7.3.2 Shrimp

Pacific white shrimp (*Litopenaeus vannamei*) with the size of 55-60 shrimp/kg were purchased from a supplier in Hat Yai, Songkhla province, Thailand. Shrimp were freshly caught and completely free of additives. The shrimp were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, shrimp were washed in cold water and stored in ice until used (not longer than 5 h).

7.3.3 Combined effect of green tea extract and MAP on the quality of Pacific white shrimp during the refrigerated storage

7.3.3.1 Preparation of green tea extract (GTE)

GTE was prepared according to the method of Nirmal and Benjakul (2011a). Green tea powder was treated with chloroform using a powder/ solvent ratio of 1:20 (w/v) to remove chlorophyll. The mixture was stirred for 30 min, followed by filtration using a Whatman filter paper No.1 (Schleicher & Schuell, Maidstone, England). To prepare GTE, the de-chlorophyllized green tea powder (2 g) was mixed with 80 mL of 80 % ethanol at 40^{o}C for 2 h with continuous stirring. The extract was filtered through a Whatman filter paper No.1. The filtrate was concentrated by a rotary evaporator (EYELA N-100, Tokyo, Japan). The concentrated sample was dried in hot air oven at 60^{o}C for 12 h. GTE powder was kept in a polyethylene bag and placed in a dessicator in the dark at 4^{o}C until use.

7.3.3.2 Sample preparation

Whole Pacific white shrimp were soaked with 1 g GTE /L solution containing no AA and 0.05 g AA /L at a shrimp/solution ratio of 1:2 (w/v) for 15 min at 4^{o}C (Nirmal and Benjakul, 2010b). After treatment, shrimp were drained for 3 min at 4^{o}C. Then six shrimp of each treatment were placed on a polystyrene tray. The tray containing samples were inserted in nylon/LLDPE bag (29 x 21 cm^2) (Asian Foams, HatYai, Thailand) with the thickness of 80µm and gas permeability for CO_2, N_2 and O_2: 0.17, 0.01 and 0.04 m^3 mm/cm^2 s cmHg at 25^{o}C, 1 atm pressure, respectively. The

bags containing with samples were packed using a shrimp/gas ratio of 1:3 (w/v) using a Henkovac type 1000 (Tecnovac, Italy). A gas mixture containing 50% CO_2 / 5% O_2 / 45% N_2 was used (Lopez-caballero *et al*., 2002). Shrimp without treatment stored in air or under MAP were also prepared. All treatments are listed as follows:

1) Control: Shrimp packed in air.
2) MAP: Shrimp packed under MAP.
3) G+M: Shrimp treated with GTE prior to MAP.
4) G+A+M: Shrimp treated with GTE plus AA prior to MAP.

All samples were stored at 4^{o}C. Two trays (6 shrimp/tray) of each sample were randomly taken and used as composite sample for microbiological and chemical analyses every 2 days up to 10 days. Total number of trays used was 12 for each treatment.

To assess melanosis, another two trays (12 shrimp/tray) for each treatment were used as the fixed sample, in which melanosis was assessed every 2 days until the end of storage time (10 days).

For sensory evaluation, the samples were taken at day 0 and 10. Totally 5 trays were used for each treatment.

7.3.3.3. Microbiological analyses

Composite samples were collected aseptically. The samples without peeling (25g) were placed in a stomacher bag containing 225 mL of 0.85 % saline water. After mixing for 1 min in a Stomacher blender (M400, Seward, UK), further serial dilution was done using the same diluent. Thereafter, 0.1 mL of appropriate dilution was used for microbiological analysis by spread plate method. The media and the conditions used were: plate count agar incubated at 4^{o}C for 10 days for psychrotrophic bacterial counts; triple sugar iron agar incubated at 25^{o}C for 3 days for H_2S- producing bacteria and eosin methylene blue agar incubated at 37^{o}C for 24 h for *Enterobacteriaceae* count (Nirmal and Benjakul, 2009b). Lactic acid bacteria (LAB) count was also determined using Man Rogosa Sharpe (MRS) agar after 3 days of incubation at 37^{o}C (Thepnuan *et al*., 2008).

7.3.3.4 Chemical analyses

7.3.3.4.1. pH measurement

pH measurement was conducted following the method described by Nirmal and Benjakul (2009a).

7.3.3.4.2 Determination of total volatile base content

Total volatile base (TVB) content in shrimp meat was determined using the Conway micro-diffusion method (Conway and Byrne, 1933).

7.3.3.4.3 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS value in the samples was determined as per the method of Benjakul and Bauer (2001) as modified by Nirmal and Benjakul (2010a).

7.3.3.5 Melanosis assessment

Melanosis or blackening of Pacific white shrimp was evaluated through visual inspection by six trained panelists using 10-point scoring (Montero *et al*., 2001b). Panelists were asked to give the melanosis score (0 to 10), where 0 = absent; 2 slight (up to 20 of shrimps surface affected); 4 = moderate (20 to 40% of shrimps surface affected) 6 notable (40 to 60 of shrimps surface affected) 8 severe (60 to 80 of shrimps surface affected) 10 extremely heavy (80 to 100 of shrimps surface affected).

7.3.3.6 Sensory evaluation

At day 0 and 10 of storage, the control, shrimp stored under MAP with or without prior treatments with GTE solution in the presence and absence of AA were placed on a stainless steel tray, covered with an aluminum foil and steamed for 5 min. The cooked samples were evaluated by 30 panelists from the Department of Food Technology with the ages of 25-35, using the 9-point hedonic scale, where 9: like extremely; 7: like moderately; 5: neither like or nor dislike; 3: dislike moderately; 1: dislike extremely (Meilgaard *et al*., 1990). Panelists were regular consumers of shrimp and had no allergies to shrimp. All panelists were asked to evaluate for color,

odor, taste, flavor and overall likeness. Samples were presented unpeeled in plates coded with three-digit random numbers.

7.3.4 Statistical analyses

All experiments were performed in triplicate and a completely randomized design was used. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan s multiple range tests. For pair comparison, T-test was used. Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

7.4 Results and Discussion

7.4.1 Combined effect of GTE with or without AA treatment and MAP on microbiological changes of Pacific white shrimp during refrigerated storage

Psychrotrophic bacteria, enterobacteriaceae, H_2S producing bacteria and lactic acid bacteria counts of Pacific white shrimp treated without and with GTE solution containing no AA or 0.05g AA /L and stored under MAP during 10 days of refrigerated storage in comparison with the control are shown in Figure 44.

At day 0 of storage, psychrotrophic bacteria count (PBC) of all samples was 2.4 log cfu/g (Figure 44A). In general, PBC of the control (stored in air) increased continuously as the storage time increased ($P < 0.05$). Increase in PBC was lowered when shrimp were packed under modified atmosphere ($P < 0.05$). The retardation of microbial growth was mainly attributed to CO_2. CO_2 has been known to extend the lag phase and reduce the growth rate of microorganisms during the logarithmic phase (Lopez-caballero *et al*., 2002). Most of the spoilage microorganism found inside the body of fresh shrimp included *Pseudomonas, Achromobacter, Flavobacterium* and *Micrococcus* (Lu, 2009). Bacterial flora associated with Indian white shrimp (*Penaeus indicus*) stored in dry ice were *Aeromonas, Pseudomonas, Vibrio, Flavobacterium* and *Serratia* (Jeyasekaran *et al*., 2006). The Gram-negative psychrotrophic bacteria are the major group of microorganism responsible for

spoilage of iced stored fish and shellfish (Gram and Huss, 1996). Shrimp treated with GTE prior to MAP had lowered PBC, compared to the control and those stored under MAP without prior treatment ($P < 0.05$). This result suggested the antibacterial activity of GTE. MAP in combination with bactericides increased the shelf life of Chinese shrimp (*Fenneropenaeus chinensis*) stored at 2^{o}C (Lu, 2009). Nirmal and Benjakul (2011a) reported that Pacific white shrimp treated with GTE (5 and 10 g / L) had lowered PBC during 12 days of iced storage in comparison with the control. AA (0.05 g/L) had no synergistic effect with GTE (1 g/L) on lowering PBC. Shrimp treated with GTE in combination with and without AA prior to MAP had similar PBC at day 10 of refrigerated storage ($P > 0.05$). At day 10 of storage, PBC of the control, sample stored under MAP, sample treated with GTE in the absence and presence of AA and stored under MAP were 5.97, 4.46, 3.98 and 3.94 log cfu/g, respectively. Shrimp treated with GTE or AA alone showed the slightly higher PBC than those stored under MAP (without GTE and AA treatment) during 10 days of refrigerated storage (data not shown). Therefore, treatment of shrimp with GTE prior to MAP could retard the growth of psychrotrophic bacteria more effectively, compared with MAP alone.

Enterobacteriaceae count of 2.5 log cfu/g was found in all samples at day 0 of storage (Figure 44B). The spoilage potential of enterobacteriaceae must be considered especially in the case of polluted water or delay in chilling of fish after capture (Sallam, 2007). As the storage time increased, a continuous increase in enterobacteriaceae count was observed in all samples ($P < 0.05$). However, the increase in enterobacteriaceae count was lowered in shrimp stored under MAP, compared with control ($P < 0.05$). Enterobacteriaceae are a family of gram negative bacteria, including many pathogenic organisms such as *Salmonella* and *Escherichia coli*. In general, gram negative bacteria are generally more sensitive to CO_2 than gram positive bacteria (McMillin, 2008). Treatment of shrimp with GTE prior to MAP retarded the growth of enterobacteriaceae more effectively than the use of MAP alone throughout the refrigerated storage of 10 days ($P < 0.05$). Catechin (1 g/L) treated Pacific white shrimp had the lower enterobaceriacae count, compared with the control during 10 days of iced storage (Nirmal and Benjakul, 2009b). Thus, GTE, which is the major source of catechin (Nirmal and Benjakul, 2011a), could be used for shrimp

treatment to lower enterobaceriaceae count. Shrimp treated with GTE in combination with AA prior to MAP had the lowest enterobacteriacae count compared with other samples ($P < 0.05$).

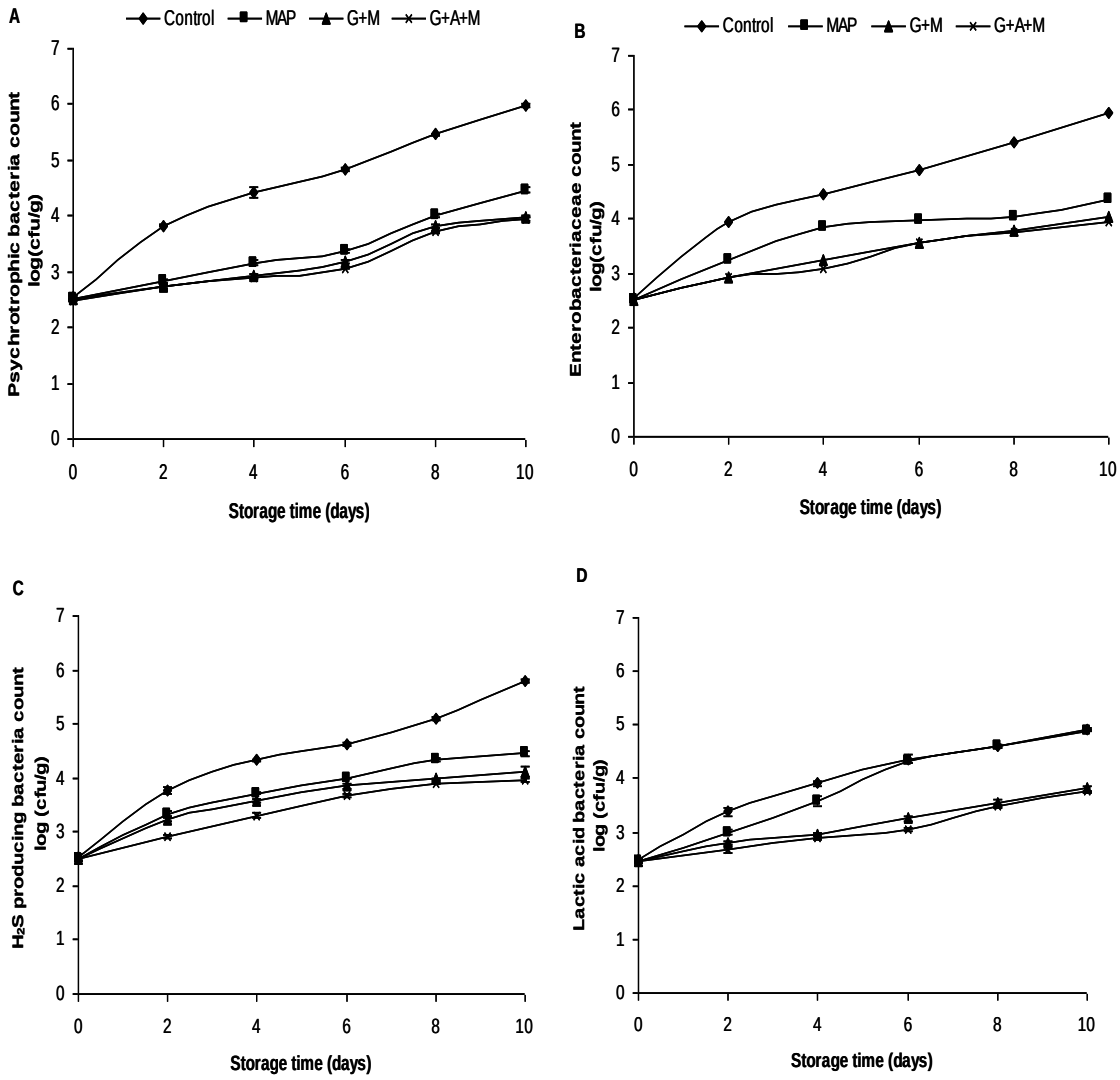

Figure 44. Psychrotropic bacteria (A), enterobacteriaceae (B), H_2S producing bacteria (C) and lactic acid bacteria (D) count of Pacific white shrimp without and with GTE treatment in the absence or presence of AA during 10 days of storage under MAP at 4^{o}C. Bars represent the standard deviation (n = 3). MAP: modified atmosphere packaging; G+M: GTE + MAP; G+A+M: GTE + AA + MAP.

This result was in agreement with Nirmal and Benjakul (2010b) who reported that Pacific white shrimp treated with GTE (1 g/L) + AA (0.1 g/L) had the

lowest enterobacteriacae count, compared with the control and those treated with GTE during iced storage ($P < 0.05$). Therefore, treatment of shrimp with GTE in combination with AA prior to MAP could lower the increase in enterobacteriaceae count during refrigerated storage of 10 days.

H_2S producing bacteria count of shrimp at day 0 was 2.5 log cfu/g for all samples (Figure 44C). In general, a continuous increase in H_2S producing bacteria count was found in all samples, but the control showed the higher increase throughout the storage ($P < 0.05$). When shrimp were stored under MAP, the increase in H_2S producing bacteria was lowered, compared with those kept in air (control) ($P < 0.05$). H_2S producing bacteria are mainly belonging to *Pseudomonas* spp. and *Shewanella putrefaciens* (Sallam, 2007). CO_2-packing of marine fish inhibits the development of *Pseudomonas* and *S. putrefaciens* (Gram and Huss, 1996). *Pseudomonas* spp., *S. putrefaciens* and few other gram negative psychrotropic organisms were dominant in proteinaceous foods stored aerobically at chilled temperatures (Gram *et al*., 2002). Shrimp treated with GTE prior to MAP had lower H_2S producing bacteria count, compared to the control and those kept under MAP without treatment ($P < 0.05$). This result was in accordance with Nirmal and Benjakul (2009b) who found that Pacific white shrimp treated with catechin (1 g/L) had lowered H_2S producing bacteria count, compared with the control and shrimp treated with sodium metabisulfite during iced storage of 10 days. When shrimp treated with GTE in combination with AA prior to MAP, the lowest H_2S producing bacteria count was obtained, compared with other samples ($P < 0.05$). GTE might show synergistic effect with AA on inhibition of H_2S producing bacteria in Pacific white shrimp. Thus, the efficacy of MAP in retarding H_2S producing bacteria was increased, when shrimp treated with GTE in combination with AA prior to MAP.

The initial lactic acid bacteria (LAB) count for all samples was 2.49 log cfu/g (Figure 44D). Generally, the increase in LAB count was found in all samples as the storage time increased ($P < 0.05$). When shrimp without any treatment were stored under MAP, slight inhibition of LAB was found up to day 4 ($P < 0.05$). Thereafter similar LAB count was obtained compared to the control ($P > 0.05$). Oxygen (5 %) used in MAP might be available to lower the growth of LAB up to day 4. Subsequently, facultative anaerobic condition developed might favor the growth of

LAB. Fresh water fish with CO_2 and vacuum packing had gram positive organism, mainly LAB, as dominant spoilage microorganism (Gram and Huss, 1996). However, LAB count for all samples was lower than the other bacterial count determined in this study during refrigerated storage. LAB counts for the control, shrimp treated with GTE in combination without and with AA and stored under MAP were 4.90, 3.81 and 3.75 log cfu/g at day 10 of the refrigerated storage, respectively. LAB inhibits growth of other bacteria due to the formation of lactic acid and bacteriocins (Gram and Dalgaard, 2002). However, the low number of LAB in all samples might be not sufficient to produce enough amounts of antimicrobial agents. Therefore, LAB did not have any inhibition effect on other bacteria as evidenced by the higher PBC, enterobacriaceae and H_2S producing bacteria counts. Nevertheless, shrimp treated with GTE in combination with or without AA prior to MAP had lower LAB count compared with the control and those kept under MAP without prior treatment ($P < 0.05$). This result reconfirmed the inhibitory effect of GTE against bacteria including LAB. Inhibitory activity of GTE was more pronounced when AA was incorporated.

Overall, shrimp stored under MAP had lower psychrotrophic, enterobacteriaceae, H_2S- producing and lactic acid bacteria counts when compared with the control. High carbon dioxide (50%) and lower oxygen (5%) levels used in MAP were the major factors contributing to the inhibition of most gram negative aerobic bacteria during extended storage. Moreover, the enhanced inhibition of psychrotrophic, enterobacteriaceae, H_2S- producing and lactic acid bacteria was observed when shrimp were treated with GTE together with AA prior to MAP. Catechin and its derivative in GTE might form complex with protein of cell wall of microorganisms, causing lyses of cell wall or might chelate some metal ions required for microbial growth (Nirmal and Benjakul, 2011a). Catechin inhibits growth of *Escherichia coli*, in a dose dependent manner (Chunmei *et al*., 2010). In addition, GTE in combination with AA prior to MAP showed increased inhibitory effect against microbial growth in shrimp. AA was effective in increasing antibacterial activity of epigallocatechin gallate, by retaining its stable form (Hatano *et al*., 2008). Hence, the increases in hurdles could increase the prevention effect on microbial growth in shrimp stored at refrigerated temperature.

7.4.2 Combined effect of GTE with or without AA treatment and MAP on chemical changes of Pacific white shrimp during refrigerated storage

7.4.2.1 pH

The combined effect of GTE treatment with or without AA and MAP on the pH change of Pacific white shrimp during refrigerated storage is shown in Figure 45. The initial pH value for all samples was 6.59. Generally, pH of all samples increased when the storage time increased ($P < 0.05$). The increase in pH value of shrimp during the iced or refrigerated storage was the result of accumulation of basic compounds generated from both autolytic processes by endogenous enzymes and microbial enzymatic actions (Nirmal and Benjakul, 2009b).

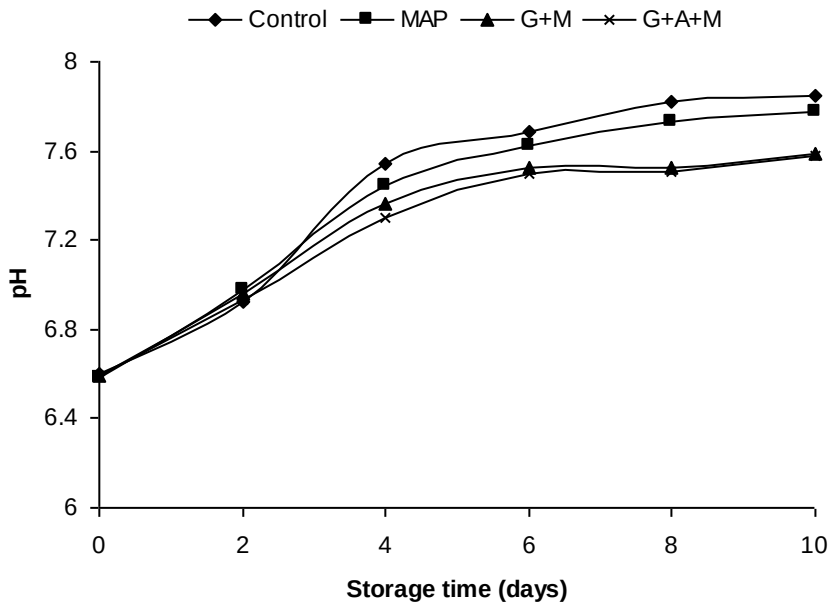

Figure 45. pH of Pacific white shrimp without and with GTE treatment in the absence or presence of AA during 10 days of storage under MAP at 4^{o}C. Bars represent the standard deviation (n=3). Key: see Figure 44 caption.

The similar pH value was observed for all samples at day 2 of refrigerated storage ($P > 0.05$). After day 2, the increase in pH value was lower in shrimp stored under MAP, compared with the control (stored in air) ($P < 0.05$). The

increase in pH value was lowered in the shrimp treated with GTE with or without AA prior to MAP, compared with that of sample stored under MAP without GTE treatment ($P < 0.05$). This result was in agreement with the lower microbial growth in shrimp treated with GTE with or without AA prior to MAP (Figure 44). Nirmal and Benjakul (2010b) reported that lowered increasing rate of pH was observed in the Pacific white shrimp treated with GTE with or without AA, compared with the control and those treated with sodium metabisulfite. Shrimp (*Penaeus merguiensis*) was not acceptable when the pH was greater than 7.6 (Shamshad *et al*., 1990). Thus, the lowered increase in pH value was more likely related with the lower microbial growth in the sample during the refrigerated storage.

7.4.2.2 Total volatile base (TVB) content

Figure 46 depicted the TVB content of Pacific white shrimp as affected by the GTE treatment with or without AA and MAP during refrigerated storage. At day 0, TVB content of all samples was found between 12.07 and 12.19 mg N / 100 g meat. As the storage time increased, a continuous increase in TVB content was observed in all samples ($P < 0.05$), but the increasing rate varied with treatments. *Aeromonas* spp., enterobaceriaceae, *P. phosphoreum*, *Shewanella putrifaciens* and *Vibrio* spp. were capable of reducing TMAO to TMA, and decarboxylating amino acid to biogenic amines (Gram and Dalgaard, 2002; Lopez- Caballero *et al*., 2002). The increase in TVB content was lowered in shrimp kept under MAP in comparison with those stored in air ($P < 0.05$). When shrimp treated with GTE with or without AA prior to MAP, the lowest TVB content was observed, compared with the control and shrimp kept under MAP without prior GTE treatment ($P < 0.05$). At day 10, sample treated with GTE in combination with AA prior to MAP showed the lowest TVB content ($P < 0.05$). Catechin or derivatives in GTE might cross-link adenosine monophosphate deaminase, responsible for the formation of volatile bases in crustacean (Nirmal and Benjakul, 2010b). TVB content correlated well with microbial load (Figure 44) and pH (Figure 45) in the corresponding samples. Thus, inhibitory effect of GTE against microbial growth could retard or lower the production of microbial degradation products. Pacific white shrimp treated with catechin (1 g/ L) had the lowest TVB content at day 10 of iced storage, compared with the control and

those treated with sodium metabisulfite (Nirmal and Benjakul, 2009b). According to Thai standard, TVB limit for fresh and frozen shrimp is 30 mg N/ 100 g meat (TIS, 1986). All sample kept for 10 day still had TVB below the limit.

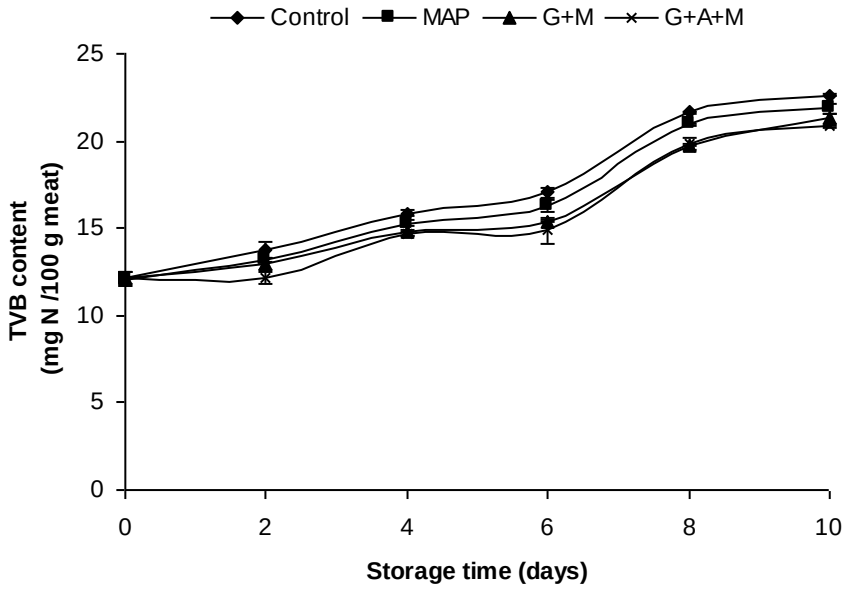

Figure 46. Total volatile base (TVB) content of Pacific white without and with GTE treatment in the absence or presence of AA during 10 days of storage under MAP at 4^{o}C. Bars represent the standard deviation (n=3). Key: see Figure 44 caption.

7.4.2.3 Thiobarbituric Acid Reactive Substances (TBARS)

TBARS values of Pacific white shrimp stored under MAP with or without prior treatment with GTE in the absence and presence of AA during refrigerated storage are presented in Figure 47. At the beginning of storage, TBARS value of all samples was found in the range of 0.54-0.56 mg malanoaldehyde/ Kg meat. In general, TBARS value of all samples increased up to day 4 ($P < 0.05$). The abrupt decrease was obtained at day 6, followed by the gradual increase in TBARS value up to 10 days of storage ($P < 0.05$). The decreased TBARS value at day 6 was more likely due to the losses in the secondary lipid oxidation products previously formed within the first 4 days. Lipid oxidation is one of the deteriorative reaction causing the unacceptability of fish and shrimp product; this can be initiated by

autoxidation and enzymatic reaction involving lipoxygenase, peroxidase and microbial enzymes (Nirmal and Benjakul, 2009a; Sallam, 2007). The increase in TBARS value was lowered in shrimp stored under MAP in comparison with the control ($P < 0.05$). Shrimp treated with GTE, regardless of AA, prior to MAP showed the lower TBARS values than those kept under MAP without GTE treatment and the control ($P < 0.05$). Psychrotrophic bacteria, mainly *Pseudomonas* species produce lipase and phospholipase causing an increase in free fatty acid (Koka and Weimer, 2001). These free fatty acids are highly susceptible to oxidation and form unstable lipid hydroperoxide. This hydroperoxide readily decomposes to shorter chain hydrocarbon such as aldehydes, these final product can be detected as TBARS (Benjakul *et al*., 2005b).

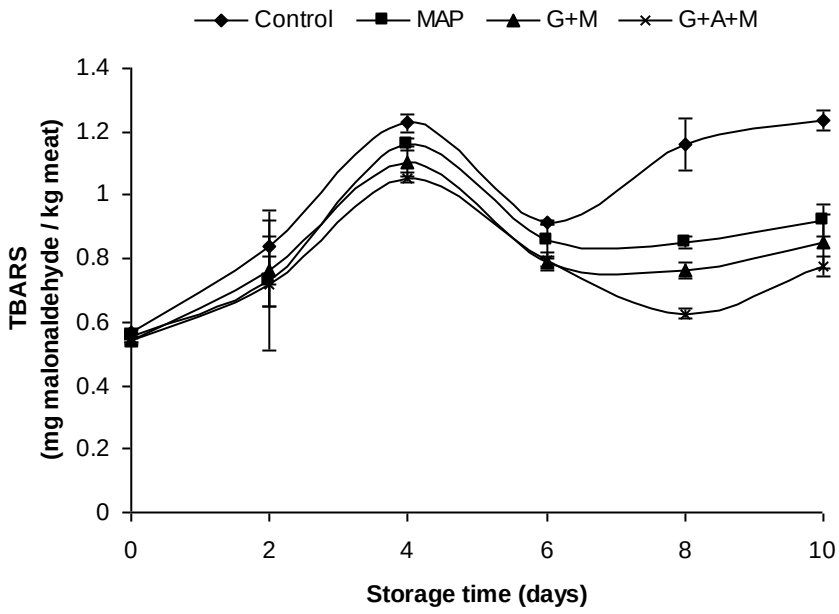

Figure 47. Thiobarbituric acid reactive substances (TBARS) of Pacific white shrimp without and with GTE treatment in the absence or presence of AA during 10 days of storage under MAP at 4^{o}C. Bars represent standard deviation (n=3). Key: see Figure 44 caption.

Lowering in lipid oxidation was in accordance with the lower microbial growth of shrimp treated with GTE solution containing no AA and 0.05 g AA/L prior to MAP (Figure 44). Nirmal and Benjakul (2009b) reported that catechin (1 g/ L) showed a strong antioxidative effect in shrimp muscle during extended iced

storage. GTE containing catechin and its derivative showed high reducing power, DPPH radical scavenging activity and metal chelating activity (Nirmal and Benjakul, 2011a). Lower TBARS value in shrimp stored under MAP was related with the lowered oxygen level (5 %) in the package and lower microbial count. However, treatment with GTE, regardless of AA incorporation, prior to MAP could increase the efficacy in retarding lipid oxidation, more likely owing to the antioxidative and antimicrobial effect of GTE. Addition of AA in combination with GTE did not increase the antioxidative activity of GTE.

7.4.3 Combined effect of GTE with or without AA treatment and MAP on melanosis of Pacific white shrimp during refrigerated storage

Melanosis score of Pacific white shrimp stored under different conditions is shown in Figure 48. No melanosis was observed in all samples at day 0 of storage. During the extended storage, melanosis was more pronounced in the control ($P < 0.05$). In general, melanosis score was increased for all samples as the storage time increased ($P < 0.05$). The lower melanosis was observed in shrimp stored under MAP without prior GTE treatment, up to 4 days of storage in comparison with the control ($P < 0.05$). Thereafter, similar melanosis was found between shrimp stored under MAP and the control during 6 and 10 days of storage ($P > 0.05$). The application of controlled atmosphere with high CO_2 and low O_2 in pink shrimp (*Parapenaeus longirostris*) without antimelanosis agent did not inhibit melanosis (Martinez-Alvarez *et al*., 2005b). Shrimp treated with GTE prior to MAP had lowered melanosis score in comparison with shrimp stored under MAP (without GTE treatment) or the control, regardless of AA incorporation ($P < 0.05$). Therefore, AA at a level of 0.05 g/L might be not sufficient to lower melanosis of shrimp treated with GTE during extended storage. PPO was synthesized as a zymogen (proPPO) in crustaceans, which was activated by protease cascade triggered by bacterial cell wall components including lipopolysaccharides, peptidoglycans and 1, 3- -glucans (Encarnacion *et al*., 2010). Therefore, the retardation of microbial growth by MAP or MAP in combination with GTE or AA treatment might be related with the lowered melanosis of white shrimp.

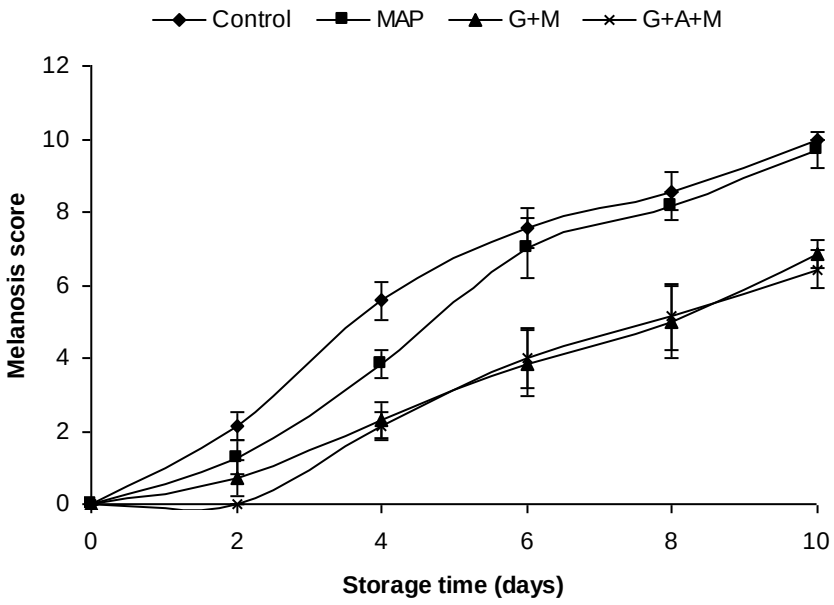

Figure 48. Melanosis score of Pacific white shrimp without and with GTE treatment in the absence or presence of AA during 10 days of storage under MAP at 4^{o}C. Bars represent the standard deviation (n=3). Key: see Figure 44 caption.

The acceptable melanosis score for shrimp was less than 8 (Otwell *et al*., 1992). Control shrimp or shrimp stored under MAP had shelf life of 6 days, whereas shrimp treated with GTE or GTE +AA prior to MAP was acceptable up to 10 days. The inhibition of melanosis in Pacific white shrimp treated with GTE was plausibly due to the combined effect between PPO inhibition as well as the reduction of quinone formed during extended iced storage (Nirmal and Benjakul, 2011a). Catechin probably acted as a competitive inhibitor for PPO because of its structural similarity to substrate for PPO (Nirmal and Benjakul, 2009b).

7.4.4 Combined effect of GTE with or without AA treatment and MAP on Sensory properties of Pacific white shrimp during refrigerated storage

Table 11 represents the likeness score at day 0 and 10 of Pacific white shrimp without and with GTE treatment in the absence and presence of AA and stored under MAP, in comparison with the control. At day 0, all samples had the score

higher than 7.0 for all attributes tested and no differences in likeness were found between all treatments ($P > 0.05$). This result suggested that treatment of Pacific white shrimp with GTE with prior chlorophyll removal had no impact on color, taste or flavor of treated shrimp. Chlorophyll and caffeine in green tea were removed by chloroform treatment prior to extraction (Nirmal and Benjakul, 2010b).

Table 11. Likeness score of Pacific white shrimp without and with GTE treatment in the absence or presence of AA before and after 10 days of storage under MAP at 4°C.

Storage time (days)	Treat-ments	Color	Odor	Taste	Flavor	Overall
0	Control	7.8 ± 0.69 aA	7.5 ± 0.51 aA	7.6 ± 0.64 aA	7.5 ± 0.51 aA	7.8 ± 0.42 aA
	MAP	7.6 ± 0.74 aA	7.3 ± 0.49 aA	7.4 ± 0.64 aA	7.6 ± 0.64 aA	7.6 ± 0.51 aA
	G+M	7.6 ± 0.51 aA	7.4 ± 0.51 aA	7.6 ± 0.51 aA	7.5 ± 0.75 aA	7.6 ± 0.64 aA
	G+A+M	7.7 ± 0.61 aA	7.3 ± 0.49 aA	7.5 ± 0.51 aA	7.6 ± 0.64 aA	7.7 ± 0.46 aA
10	Control	5.3 ± 0.61 bB	5.1 ± 0.47 cB	4.8 ± 1.10 cB	4.8 ± 0.86 cB	4.8 ± 0.89 cB
	MAP	5.2 ± 1.18 bB	5.2 ± 1.00 cB	5.1 ± 0.99 cB	5.2 ± 0.89 cB	5.2 ± 0.57 cB
	G+M	5.9 ± 0.73 aB	6.1 ± 0.77 bB	5.8 ± 0.77 bB	5.9 ± 0.73 bB	5.9 ± 0.82 bB
	G+A+M	6.3 ± 0.49 aA	6.6 ± 0.51 aA	6.6 ± 0.75 aB	6.4 ± 0.75 aB	6.6 ± 0.63 aB

Different capital letters in the same column within the same treatment indicate the significant differences ($P < 0.05$). The different letters in the same column within the same storage time indicate significant differences ($P < 0.05$). Values are means ± standard deviation (n=3). MAP: modified atmosphere packaging; G+M: GTE + MAP; G+A+M: GTE + AA + MAP.

The extract obtained had the light straw-yellow in color. Therefore, the treatment of shrimp with GTE did not affect the color, taste or flavor of the shrimp. At the end of storage, the decreases in likeness for all attributes were observed for all samples ($P < 0.05$). The higher color, odor, taste, flavor and overall likeness scores were found in shrimp treated with GTE incorporation with AA prior to MAP, followed by those treated with GTE prior to MAP. H_2S producing bacteria, enterobacteriacea, Aeromonadaceae, and *Pseudomonas* were the dominant microbial flora observed in freshwater prawn (*Macrobrachium rosenbergii* de Man) during 19 days of ice storage (Lalitha and Surendran, 2006). It is more likely that H_2S producing bacteria and enterobacteriacea could contribute more to the spoilage of shrimp stored in air; however, specific spoilage bacteria were not identified in the present study. Sample was considered as acceptable when the score was higher than 5. At day 10 of storage, the control sample and sample stored under MAP had the overall likeness score of 4.8 and 5.2, respectively. Thus, those samples were considered as

unacceptable or marginally acceptabe, respectively. For the sample treated with GTE/ascorbic acid and stored under MAP, the overall likeness score was 6.6. This was more likely the result of the lowered spoilage of sample as indicated by the lower increase in microbial load (Figure 44), chemical changes (Figure 45, 46 and 47) and melanosis formation (Figure 48), compared with the control and sample stored under MAP ($P < 0.05$).

7.5 Conclusions

MAP of Pacific white shrimp effectively retarded the growth of psychrotrophic bacteria, enterobacteriaceae and H_2S- producing bacteria, but could not inhibit lactic acid bacteria. Coincidentally, chemical changes were lowered. However, MAP was not able to inhibit melanosis. When shrimp were treated with GTE prior to MAP, microbial changes, chemical changes and melanosis formation were retarded. Based on melanosis score limit, shrimp treated with GTE and ascorbic acid prior to MAP were still acceptable within 10 days of storage. Treatment of GTE in conjunction with AA could improve sensory property of shrimp during refrigerated storage. Treatment of shrimp with GTE in combination with AA was recommended prior to MAP to maintain the quality of shrimp during refrigerated storage.

CHAPTER 8

INHIBITION OF MELANOSIS FORMATION IN PACIFIC WHITE SHRIMP BY THE EXTRACT OF LEAD (*LEUCAENA LEUCOCEPHALA*) SEED

8.1 Abstract

Extract of lead (*Leucaena leucocephala*) seed was prepared using distilled water as a medium. Extraction yield of 26.16 g / 100 g of seed was obtained after extraction at room temperature for 12 h. 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 polyphenoloxidase (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 color, odor and overall likeness, compared with the control (without treatment) and 1.25 % sodium metabisulfite treated samples at day 12 ($P < 0.05$). Meat of shrimp 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. Therefore, 0.5 % LSEP can be used as a novel melanosis inhibitor for Pacific white shrimp.

8.2 Introduction

Melanosis or blackening is a problem occurring in crustaceans during post-mortem storage. Melanosis is induced by a biochemical process, in which phenols is oxidized to quinones by polyphenoloxidase (PPO). This colorless quinone can undergo further oxidation to brown melanin or participate in polymerization

reaction with functional groups of protein to form cross-linked polymer (Benjakul *et al.*, 2005a). Shrimp generally has the limited shelf-life due to the melanosis. Even though the blackening seems to be harmless to consumers, it drastically reduces the consumer s acceptability and products market value, leading to considerable financial loss. Pacific white shrimp (*Litopenaeus vannamei*) is an important commercial species primarily cultured in Thailand and have become an essential income generator of the country (Nirmal and Benjakul, 2009b). This high value crustacean is very perishable and iced storage is routinely used to preserve the quality of shrimp. However, during refrigerated or iced storage, melanosis still takes place since PPO remains active under these conditions (Nirmal and Benjakul, 2010a).

Melanosis inhibitors have been used to control the development of black spot in shrimps or crustaceans during handling and storage. Among the melanosis inhibitors, sulfiting agents and 4-hexylresorcinol alone or in combination with other chemical compounds have been intensively studied (Martinez-Alverez *et al.*, 2008b; Montero *et al*., 2001b). However, increasing regulatory attention and consumer awareness of the risk associated with chemical additives in food processing have led to the interest in natural additives to prevent melanosis in shrimp (Nirmal and Benjakul, 2010b). Plant phenolic compounds including catechin (Nirmal and Benjakul, 2009b) and ferulic acid (Nirmal and Benjakul, 2009a) could delay the melanosis formation and extend the shelf life of Pacific white shrimp during iced storage. Inhibition of melanosis in shrimp has also been achieved by using green tea extract (Nirmal and Benjakul, 2011a), grape seed extract (Gokoglu and Yerlikaya, 2008), and enokitake extract (Jang *et al*., 2003). Recently, Encarnacion *et al*. (2010) reported that dietary supplement of mushroom extract (*Flammulina velutipes*) in kuruma shrimp (*Marsupenaeus japonicus*) could delay post mortem development of melanosis.

Lead tree, *Leucaena leucocephala*, is belonging to a tropical and subtropical legume family. It has been used as livestock feed because of their 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). The seeds of guaje (*L. esculenta*) are eaten with salt in Mexico. Green lead seeds are also consumed as fresh side dish in Thailand, but the mature brown seeds

have not been exploited. Phenolics, plant secondary compounds found in different parts of *Leucaena* were condensed tannin (Echeverria *et al*., 2002), quercetin and myricetin glycosides (Lowery *et al*., 1984), gallocatechin, epigallocatechin and epicatechin (Erickson *et al*., 2000). This plant contains a non-protein amino acid called mimosine, (-(3-hydroxy-4-pyridon-1-yl)-L-alanine) (Lalitha and 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 the urine (Sahlu *et al*., 1995). Nevertheless, no information regarding the use of lead seed extract in inhibiting PPO or preventing melanosis formation in Pacific white shrimp has been reported. The aim of this study was to investigate the inhibitory effect of brown lead seed extract on PPO and the formation of melanosis in Pacific white shrimp stored in ice.

8.3 Materials and Methods

8.3.1 Chemicals and lead seeds

L- -(3, 4 dihydroxylphenyl) alanine (L-DOPA), Brij-35, and L-mimosine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), orthophosphoric acid, ammonium sulfate, Folin-Ciocalteu, -nitroaniline and sodium nitrite were obtained from Merck (Darmstadt, Germany). Brown lead (*L. leucocephala*) seeds were collected from the trees in Prince of Songkla University, Hat Yai, Thailand.

8.3.2 Preparation of lead seed extract powder (LSEP)

Dry brown lead seeds were ground into fine powder using a blender (Philips, Dezheng Road South, Guangzhou, China) and sieved through a stainless steel sieve of 40 mesh to remove the outer shells of seeds. The powder (2 g) was mixed with 40 mL of distilled water at room temperature (28 - 30^{o}C) for 12 h with continuous stirring. The extract was centrifuged at 8000 x g for 20 min at 20^{o}C using

a Beckman Coulter centrifuge (Avanti J-E Centrifuge, Fullerton, CA, USA). Supernatant was collected and freeze dried using a Model Coolsafe 55, Scanvac freeze dryer (Coolsafe, Lynge, Denmark). The powder obtained was referred to as lead seed extract powder LSEP and was subjected to analyses and was also used for the shrimp treatment.

8.3.2.1 Total phenolic content

Total phenolic content in LSEP was determined using Folin-Ciocalteu reagent according to the method of Slinkkard and Singleton (1997). LSEP (5 mg) was dissolved in 10 mL of distilled water. Appropriately diluted LSEP solution (1 mL) was added with 0.2 mL of two-fold diluted Folin-Ciocalteu reagent and mixed thoroughly. After 3 min, 3 mL of 2 % sodium carbonate solution were added. After standing for 30 min at room temperature, an absorbance was measured at 760 nm using a UV- 1601 spectrophotometer (Shimadzu, Kyoto, Japan). The concentration of total phenolic compounds in LSEP was calculated from the standard curve of gallic acid with the range of 0-0.05 mg / mL and expressed as g GAE / 100 g LSEP.

8.3.2.2 Determination of mimosine content

8.3.2.2.1 Spectrophotometric method

Mimosine content in LSEP was determined spectrophotometrically according to the method of Lalitha *et al*. (1993). LSEP (5 mg) was dissolved in 10 mL of distilled water. Appropriately diluted aliquot of the LSEP solution (3.5 mL) was mixed with 1 mL of sodium phosphate buffer (0.25 M, pH 7). Then 0.5 mL of diazotized -nitroaniline reagent was added to the reaction mixture and mixed well. Reaction mixture was incubated at room temperature for 15 min and the colour developed was measured spectrophotometrically at 400 nm. Diazotized -nitroaniline reagent was prepared freshly by mixing e ual volume of -nitroaniline solution (0.05 % in 0.033 M H_3PO_4) and sodium nitrite solution (0.1 % in distilled water).

The concentration of mimosine in LSEP was calculated from the standard curve of mimosine with the range of 2 -10 µM and expressed as g mimosine / 100 g LSEP.

8.3.2.2.2 High-performance liquid chromatography (HPLC)

Mimosine content in LSEP was also determined by HPLC as per the method of Soedarjo *et al*. (1994). The separation system consisted of an Agilent 1100 series HPLC equipped with hypersil ODS 4.0*250 mm, 5 µm column (Agilent, Boblingen, Germany) and a UV detector (Rheodyne, Cotati, CA, USA). The temperature of the column was maintained at 25^{o}C and the injection volume was 20 µL. Mimosine was eluted by using 0.2 % orthophosphoric acid (v/v) at a flow rate of 1 mL/min and detected at 280 nm.

Standard stock solutions (1000 mg / L) were diluted with 0.1 N HCl to obtain the concentrations of 50 - 500 mg / L. Standard solutions were then injected into the column and the elution was performed in the same manner with the samples. Mimosine in LSEP solution was quantified using a standard curve.

8.2.3 Effect of LSEP on the inhibition of Pacific white shrimp PPO

8.3.3.1 Preparation of PPO extract from the cephalothoraxes of Pacific white shrimp

Pacific white shrimp (*Litopenaeus vannamei*) with the size of 55-60 shrimp/kg were purchased from a supplier in Hat Yai, Songkhla, Thailand. The shrimp were freshly caught and completely free of additives. The shrimp were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, shrimp were washed in cold water and stored in ice until use (not more than 5 h).

The cephalothoraxes of twenty shrimp were separated, pooled and powderized by grinding with liquid nitrogen in a Waring blender (AY46, Moulinex, Guangdong, China). The powder obtained was kept in polyethylene bag and stored at -20°C, not more than 2 weeks. The isolation of PPO was carried out according to the method of irmal and Benjakul (2009a) and the supernatant was used as crude PPO extract .

8.3.3.2 PPO inhibitory activity of LSEP

LSEP was dissolved in distilled water at different concentrations (0.1, 0.2, 0.5, 1, and 2 % w/v). LSEP solutions (100 L) were mixed with crude PPO extract (100 L) to obtain the final concentrations of 0.05, 0.1, 0.25, 0.5 and 1 % (w/v). This reaction mixture was left for 30 min at room temperature. Then, the assay buffer (400 L, 0.05 M phosphate buffer, pH 6.0) was added. To initiate the reaction, 600 L of pre-incubated 15 mM L-DOPA (45^{o}C) were added. The reaction was conducted at 45^{o}C and the absorbance at 475 nm was monitored for 3 min. Control was run in the same manner, except deionized water was used instead of LSEP. One unit of PPO activity was defined as an increase in the absorbance at 475 nm by 0.001 per min (Nirmal and Benjakul, 2009b). Residual activity was calculated and the inhibitory activity was expressed as percentage inhibition as follows:

$$\text{Inhibition (\%)} = \frac{A - B}{A} \times 100$$

where A: PPO activity of control; B: PPO activity in the presence of LSEP.

8.3.4 Effect of LSEP on melanosis, mimosine content and sensory property of Pacific white shrimp

8.3.4.1 Treatment of Pacific white shrimp with LSEP

Whole shrimp were immersed in LSEP solution (0.25 and 0.5 %, w/v) using a shrimp/solution ratio of 1:2 (w/v) at 4^{o}C for 30 min. When shrimp was soaked with LSEP for different immersion time (10, 20, 30, and 40 min), lowest melanosis score was observed with 30 min immersion time (P<0.05) (data not shown). Another portion of shrimp was soaked in 1.25 % sodium metabisulfite (SMS) at a ratio of 1:2 (w/v) for 1 min at 4^{o}C (Kim *et al*., 2000) and used as a positive control. Treated shrimp were drained on the screen for 3 min at 4^{o}C. Shrimp without any treatment were used as the control. All samples were stored in polystyrene box containing ice using a shrimp/ice ratio of 1:2 (w/w). To maintain shrimp/ice ratio, the molten ice was removed and the same amount of ice was added. Samples (15 shrimp) were taken for each treatment every 2 days up to 12 days for determination of mimosine residue in

the shrimp muscle and for melanosis evaluation. Sensory evaluation was carried out at day 0 and day 12.

8.3.4.2 Melanosis assessment

Melanosis of Pacific white shrimp was evaluated through visual inspection by six trained panelists using 10-point scoring (Nirmal and Benjakul, 2009b). Panelists were asked to give the melanosis score (0 to 10), where 0 = absent; 2 slight (up to 20 of shrimps surface affected) 4 moderate (20 to 40 of shrimps surface affected) 6 notable (40 to 60 of shrimps surface affected) 8 severe (60 to 80 of shrimps surface affected) 10 extremely heavy (80 to 100 of shrimps surface affected).

8.3.4.3 Determination of mimosine content in the muscle of white shrimp

To determine the mimosine content in the muscle of shrimp, the extraction of mimosine was carried out by the method of Kamada *et al*. (1997) with a slight modification. One gram of ground sample was homogenized in 6 mL of 0.1 N HCl solution using an IKA Labortechnik homogenizer (Selangor, Malaysia). Thereafter, 4.8 mL of 15 % trichloroacetic acid (TCA) solution were added to the homogenate. The solution was allowed to stand for 20 min, followed by centrifugation at 10,000 x g for 20 min at 4^{o}C. The supernatant solution was then filtered through a Whatman filter paper no.1 (Schleicher & Schuell, Maidstone, England). Mimosine content was analyzed by spectrophotometric method as described previously and expressed as g / 100 g meat.

8.3.4.4 Sensory evaluation

Samples were placed on a stainless steel tray, covered with aluminium foil and steamed for 5 min. The cooked samples were evaluated by 30 panelists, the graduate students in Food Science and Technology program, Department of Food Technology, Prince of Songkla University, Thailand. Nine-point hedonic scales were used to score sample, where 9 = like extremely; 7 = like moderately; 5 = neither like or nor dislike; 3 = dislike moderately; 1 = dislike extremely (Meilgaard *et al*., 1990). Panelists were familiar with shrimp consumption and had no allergies to shrimp. All

panelists were asked to evaluate for color, odor, taste, flavor and overall likeness. Peeled samples were presented in plates coded with random three-digit numbers.

8.3.5 Statistical analyses

All analyses were performed in triplicate and a completely randomized design (CRD) was used. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan s multiple range tests. For pair comparison, T-test was used (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

8.4 Results and Discussions

8.4.1 Characteristic and PPO inhibitory activity of LSEP

8.4.1.1 Extraction yield, total phenolic and mimosine contents

Lead seed extract powder (LSEP) prepared using distilled water as a medium had the yield of 26.1 g LSEP / 100 g dry seed powder. Water soluble components in LSEP might be proteins, phenolic compounds, mimosine, etc. Total phenolic content in LSEP was 17.4 g GAE / 100 g. Total phenolics and tannin in shoot tip of *Leucaena leucocephala* grown in Thailand were 405 and 60.6 mg / 100 g dry matter, respectively (Chanwitheesuk *et al*., 2005). Phenolics distributed non-uniformly at the tissue, cellular and subcellular levels (Naczk and Shahidi, 2004). Moreover, phenolic content in the plant varied with different parts and different culture habit.

Mimosine content in LSEP was 7.8 g / 100 g when analysed using a spectrophotometric method. Different parts of the *Leucaena* contain varying amounts of mimosine, 2-10 % of dry leaf, 2-5 % of dry seed (Lalitha *et al*., 1993) and 1-1.5 % of root (Soedarjo *et al*., 1994). In this study, mimosine of brown seed extract powder was 8.8 g / 100 g (dry weight basis) when determined by high performance liquid chromatography (Figure 49). Spectrophotometeric method claimed to be a rapid, sensitive and specific for routine mimosine determination (Lalitha *et al*., 1993).

However, HPLC method was used to reconfirm and compared the result with spectrophotometric method. Mimosine concentration determined by spectrophotometric and HPLC method was 0.078 and 0.088 g /g LSEP (P >0.05). *L. leucocephala* leaves and seeds had high content of mimosine (10 - 40 g/ kg dry matter) (Puchala *et al*., 1996). Mimosine in seed (3.46 % of dry matter) was 6-fold higher than that found in leaves (0.56 % of dry matter) (Kamada *et al*., 1997). Mimosine could be removed by prolonged soaking of leaves or seeds in water at 30^{o}C (Puchala *et al*., 1995). Kamada *et al*. (1997) recovered 1.4 g of crude mimosine from 100 g of *Leucaena* seed water extract treated with ethanol to removed impurities. The result suggested that water could be used to extract mimosine from the brown lead seed powder and spectrophotometric method could be used as rapid and sensitive method for detection of mimosine in the sample.

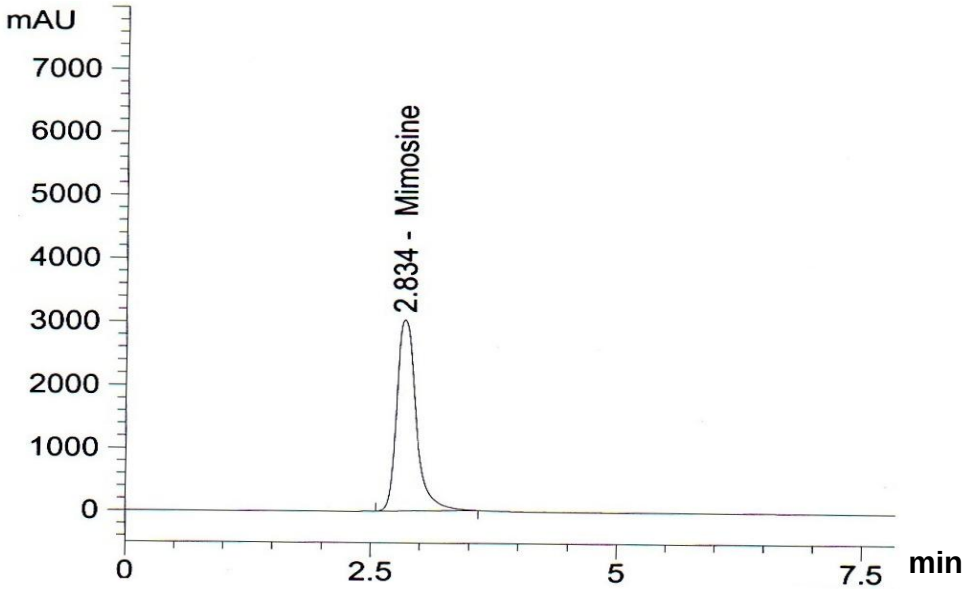

Figure 49. HPLC chromatogram of mimosine from LSEP.

8.4.1.2 PPO inhibitory activity

Table 12 represents the inhibitory activity of LSEP and mimosine towards the PPO from Pacific white shrimp. Inhibitory activity towards PPO of both LSEP and mimosine increased with increasing concentrations used (P < 0.05). However, at the same level, mimosine showed the higher inhibitory activity than

LSEP ($P < 0.05$). Mimosine content in LSEP was 8.8 g /100g of LSEP as measured by HPLC method. Therefore, 0.0044 and 0.0088 g mimosine was present in 0.05 and 0.1 % LSEP and so on, respectively. At the concentration of 0.25 %, LSEP and mimosine showed 55.1 and 79.7 % PPO inhibition, respectively. PPO activity of 69.3 % was inhibited by LSEP, when concentration of 0.5 % was used, whereas the complete inhibition was obtained with the same concentration of mimosine.

Table 12. Percent inhibition of PPO from Pacific white shrimp by LSEP at different concentrations

Concentrations (%)	Inhibition (%)	
	Mimosine	LSEP
0	0	0
0.05	32.77 ± 0.69 dA	15.18 ± 2.92 eB
0.1	47.60 ± 0.80 cA	27.49 ± 1.07 dB
0.25	79.77 ± 0.74 bA	55.15 ± 1.64 cB
0.5	100 aA	69.37 ± 1.10 bB
1.0	100 aA	84.56 ± 4.90 aB

Different letters in the column indicate significant differences ($P < 0.05$). The different capital letters in the row indicate significant different ($P < 0.05$). Values are mean ± standard deviation (n = 3). LSEP: Lead seed extract powder.

Mimosine most probably acted as a competitive inhibitor of PPO, because of its structural similarity with dihydroxyphenylalanine (L-DOPA) (Soedarjo *et al*., 1994), which is substrate for melanosis reaction. Prasad *et al*. (2009) reported the antityrosinase activity from litchi (*Litchi sinesis* Sonn.) seed extract powder. Mimosine also had the metal chelating ability (Puchala *et al*., 1996), which could chelate copper ion at the active site of PPO. The lower PPO inhibitory activity of LSEP was due to the lower level of mimosine in LSEP, compared with mimosine ($P < 0.05$). LSEP contained not only mimosine, but also other water soluble components. As a consequence, mimosine was diluted by other compounds. Nevertheless, some phenolic compounds in LSEP might act as PPO inhibitors. Total phenolic content in 0.05 and 0.1% LSEP was 0.0087 and 0.0174g GAE and so on, respectively. However, the presence of mimosine and total phenolic in LSEP might not be sufficient to compel the PPO inhibitory activity with pure mimosine, at the same concentration ($P < 0.05$). Recently, catechin (Nirmal and Benjakul, 2009b) and ferulic acid (Nirmal

and Benjakul, 2009a) were shown to inhibit PPO from Pacific white shrimp in a dose dependent manner. Therefore LSEP could serve as an alternative inhibitor for PPO from Pacific white shrimp.

8.4.2 Effect of LSEP treatment on melanosis of Pacific white shrimp during iced storage

Melanosis score of Pacific white shrimp treated without and with LSEP at levels of 0.25 and 0.5 % during iced storage is shown in Figure 50. At day 0, there was no melanosis formation in all samples. As the storage time increased, a continuous increase in melanosis score was observed for the control ($P < 0.05$). However, the increase in melanosis score was lowered in the sample treated with 1.25 % SMS and LSEP (0.25 and 0.5 %).

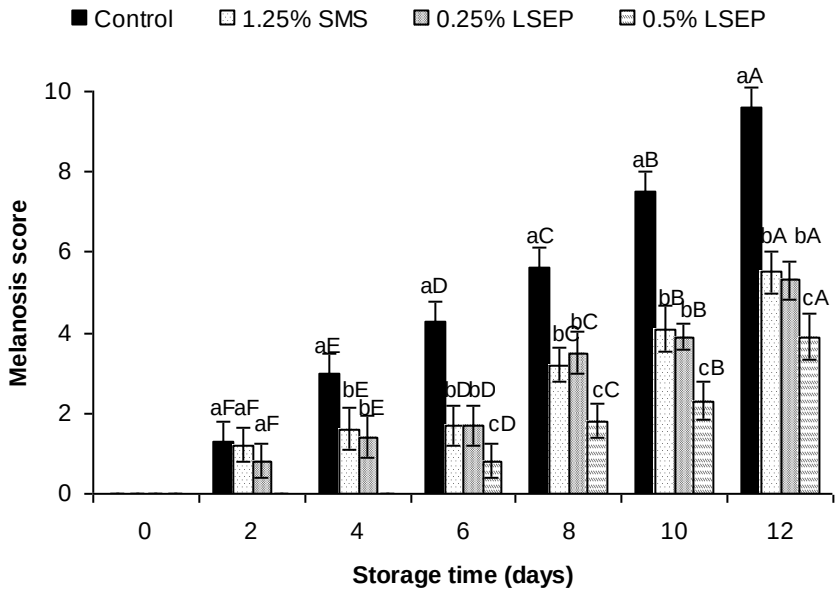

Figure 50. Melanosis score of Pacific white shrimp without and with treatment of LSEP during 12 days of iced storage. Bars represent standard deviation (n=3). Different capital letters on the bars within the same treatment indicate significant difference ($P < 0.05$). The different letters on the bars within the same storage time indicate significant difference ($P < 0.05$). 1.25% SMS: 1.25 % sodium metabisulfite; 0.25% LSEP: 0.25% lead seed extract powder; 0.5% LSEP: 0.5% lead seed extract powder.

Sodium metabisulfite might inhibit melanosis by reacting with intermediate quinone, forming sulfoquinone or it can act as a competitive inhibitor (Ferrer *et al.*, 1989). At day 2, there was no significant difference in melanosis score between all samples, except those treated with 0.5 % LSEP, where no melanosis formation was noticeable. For the samples treated with 0.5 % LSEP, there were no melanosis formation up to 4 day and the black spots were slightly formed at day 6 of iced storage. In general, similar melanosis score was found between samples treated with 1.25 % SMS and 0.25 % LSEP throughout the storage time of 12 days in ice ($P > 0.05$). Presence of mimosine and phenolic compounds in LSEP might retard the melanosis formation in white shrimp by the 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 the tyrosine (Echeverria *et al*., 2002). Lowering of melanosis formation in shrimp treated with LSEP correlated well with the *in vitro* PPO inhibitory activity (Table 12). Retardation of melanosis in Pacific white shrimp treated with LSEP was in a dose dependent manner. Figure 51 represents the photograph of Pacific white shrimp treated without and with 0.25 and 0.5 % LSEP and those treated with 1.25 % SMS after 12 days of iced storage. Overall, shrimp treated with 0.5 % LSEP had the lowered melanosis formation, compared to other treatments during 12 days of iced storage ($P < 0.05$).

Since mimosine was shown to be an active PPO inhibitor for Pacific white shrimp, the content of mimosine in shrimp meat was monitored during iced storage (Figure 52). At day 0, no mimosine was detected in the muscle of both samples treated with 0.25 and 0.5 % LSEP. As the storage time increased, mimosine content in shrimp meat increased up to day 8 of storage ($P < 0.05$). After LSEP treatment of shrimp, mimosine was mainly retained at the shell or outer portion of cephalothorax. During extended iced storage, the mimosine most probably penetrated into muscle. Lower content of mimosine was detected in shrimp treated with 0.25 % LSEP, compared with 0.5 % LSEP during the first 6 days of iced storage ($P < 0.05$). Thereafter, no significant difference was observed in mimosine content in shrimp treated with LSEP at both concentrations ($P > 0.05$).

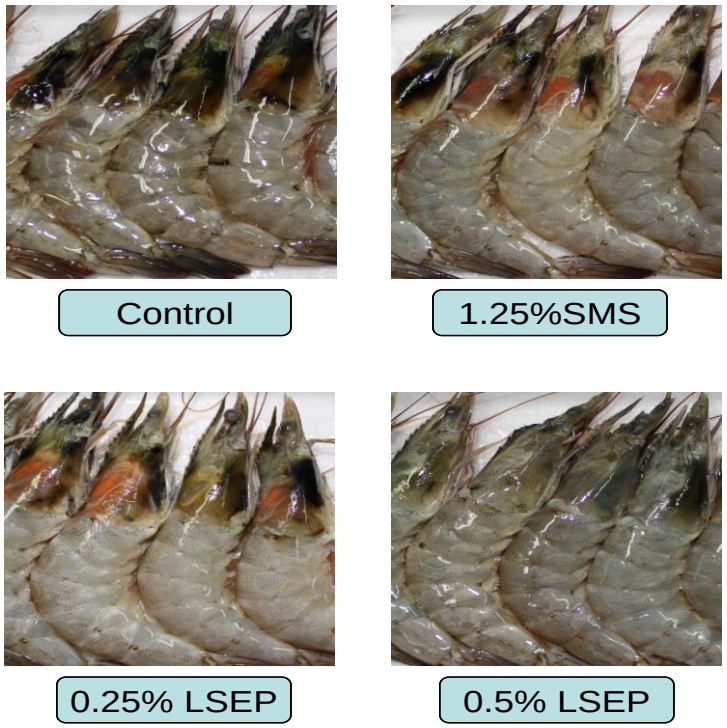

Figure 51. Photographs of Pacific white shrimp without and with treatment of LSEP at different concentrations after 12 days of iced storage. Key: see the caption for Figure 50.

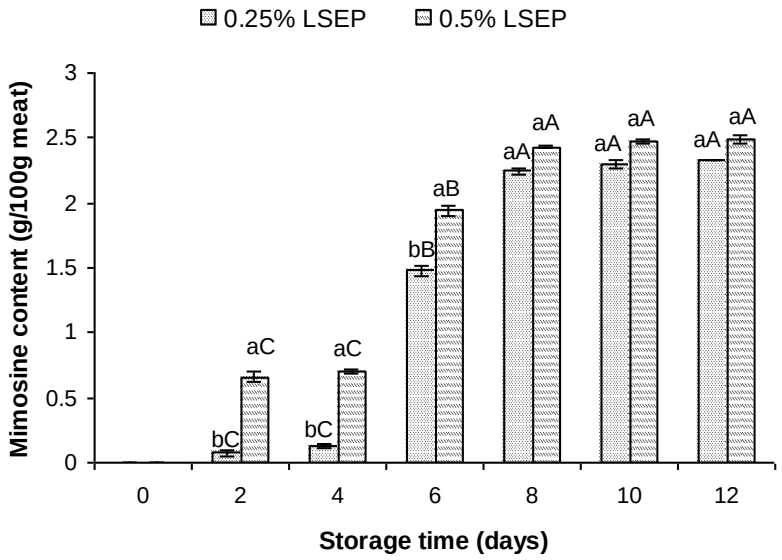

Figure 52. Mimosine content in the muscle of white shrimp treated with 0.25 and 0.5 % LSEP during 12 days of iced storage. Bars represnts standard deviation (n=3). Key: see the caption for Figure 50.

The high content of mimosine in muscle after day 6 of iced strogae was observed. This might be due to the interfering of tyrosine with mimosine measurement. Free tyrosine and tyramine contents in Taiwanese black tiger shrimp (*Penaeus monodon*) were increased during the chilled storage (Rolle *et al*., 1991). This result suggested that phenolic compounds or other substances from LSEP were involved in lowering melanosis particularly after 8 days of iced storage.

8.4.3 Effect of LSEP treatment on sensory properties of Pacific white shrimp during iced storage

Sensory properties of peeled Pacific white shrimp treated without and with LSEP at day 0 and 12 of iced storage are shown in Table 13. At day 0, no differences in likeness for all attributes were observed among all samples ($P > 0.05$). Results indicated that LSEP treatment did not had any negative effect on sensorial properties of white shrimp. After 12 days of iced storage, the decreases in likeness for all attributes in all samples were noted ($P < 0.05$). However, the decrease in likeness score was lowered in the sample treated with 0.5 % LSEP, compared with other treatments and control ($P < 0.05$). There was no difference in color and odor likeness of shrimp treated with 0.5 % LSEP between day 0 and 12 ($P > 0.05$). Although some black spots were formed at some extent, mainly on shell and carapace, only meat with negligible black spot was used for evaluation. This led to no changes in color likeness. Shrimp treated with 0.25 % LSEP and 1.25 % SMS had the similar color and overall likeness at day 12 ($P > 0.05$). For the taste and flavor likeness, there was no difference in all samples at day 12 of iced storage ($P > 0.05$). Higher color, odor and overall likeness score was generally noticeable in shrimp treated with 0.5 % LSEP. Phenolic compounds and mimosine in LSEP most likely contributed to maintaining the quality of shrimp during extended iced storage. Treatment of shrimp with phenolic compounds could retard the growth of psychrotrophic bacteria and lipid oxidation during refrigerated storage (Nirmal and Benjakul, 2010a). Therefore, the treatment of Pacific white shrimp with LSEP could retard the loss in the sensory properties of shrimps during iced storage.

Table 13. Likeness score of Pacific white shrimp treated with 0.25 and 0.5 % LSEP at day 0 and 12 of iced storage

Storage time (days)	Treatments	Color	Odor	Taste	Flavor	Overall
0	Control	7.15 ± 0.55 aA	7.07 ± 0.64 aA	7.30 ± 0.75 aA	7.15 ± 0.68 aA	7.15 ± 0.37 aA
	1.25% SMS	7.15 ± 0.89 aA	6.92 ± 0.49 aA	7.07 ± 0.75 aA	6.92 ± 0.75 aA	7.00 ± 0.00 aA
	0.25% LSEP	7.07 ± 0.75 aA	7.00 ± 0.81 aA	7.00 ± 0.81 aA	7.15 ± 0.98 aA	7.15 ± 0.68 aA
	0.5% LSEP	6.84 ± 0.55 aA	7.15 ± 0.55 aA	7.07 ± 0.49 aA	7.07 ± 0.49 aA	7.07 ± 0.27 aA
12	Control	4.30 ± 0.48 cB	5.38 ± 0.50 cB	5.61 ± 0.50 aB	5.53 ± 0.51 abB	5.00 ± 0.40 bB
	1.25% SMS	5.38 ± 0.65 bB	5.53 ± 0.51 cB	5.84 ± 0.37 aB	5.76 ± 0.43 aB	5.46 ± 0.51 bB
	0.25% LSEP	5.69 ± 0.63 bB	6.23 ± 0.43 bB	5.76 ± 0.59 aB	6.00 ± 0.57 aB	5.53 ± 0.60 bB
	0.5% LSEP	6.92 ± 0.49 aA	7.07 ± 0.27 aA	6.15 ± 0.37 aB	6.23 ± 0.43 aB	6.38 ± 0.50 aB

Different capital letters in the same column within the same treatment indicate the significant differences ($P < 0.05$). The different letters in the same column within the same storage time indicate significant differences ($P < 0.05$). Values are mean $\pm$ standard deviation (n=3). SMS: sodium metabisulphite; LSEP: Lead seed extract powder.

8.5 Conclusions

Lead brown seed extract could be used as the natural inhibitor of Pacific white shrimp PPO and could retard the formation of melanosis during iced storage. Efficacy of LSEP in inhibiting PPO and melanosis formation was in a dose dependent manner. Shrimp treated with 0.5 % LSEP had superior sensory properties, compared to the control after 12 days of iced storage. Therefore, the extract from lead brown seed could serve as an alternative processing aid for controlling melanosis in shrimp or other crustacean during post mortem handling or storage.

CHAPTER 9

BIOCHEMICAL PROPERTIES OF POLYPHENOLOXIDASE FROM CEPHALOTHORAX OF PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*)

9.1 Abstract

Polyphenoloxidase (PPO) from cephalothorax of Pacific white shrimp was partially purified by ammonium sulfate precipitation (0-40 % saturation) and DEAE-Sepahcel anion exchange chromatography with the purification fold of 83.8. PPO showed the maximal activity using L- -(3, 4 dihydroxylphenyl) alanine (L-DOPA) as a substrate at pH 6 and 55^{o}C. PPO was stable over a pH range of 5-10 but unstable at a temperature greater than 60^{o}C. Based on the activity staining with L-DOPA, the apparent molecular weight of PPO was 210 kDa. The Michaelis constant (K_m) of PPO for the oxidation of L-DOPA was 2.43 mM. Trypsin, copper acetate, and sodium dodecyl sulfate (SDS) were unable to activate PPO, suggesting that the enzyme was in the active form. Cysteine, ethylenediamine tetraacetic acid (EDTA), and *p*-amino benzoic acid (PABA) showed PPO inhibitory activity in a dose dependent manner. At the same concentration used (1 and 10 mM), cysteine exhibited higher inhibitory effect towards PPO.

9.2 Introduction

Polyphenoloxidase (PPO) is known as phenolase, tyrosinase and catechol oxidase. PPO is a copper-containing metalloenzyme, which catalyzes two basic reactions, in the presence of molecular oxygen. Those include the *o*-hydroxylation of monophenols to give *o*-diphenols (Monophenol oxidase, cresolase activity, EC 1.14.18.1) and the subsequent oxidation of *o*-diphenols to *o*-quinones (Diphenoloxidase, catecholase activity, EC 1.10.3.1) (Garcia-Molina *et al*., 2005). PPO in crustaceans is distributed mainly in the carapace, followed by abdomen

exoskeleton, cephalothorax, pleopods and telson (Zamorano *et al*., 2009). PPO is most commonly found in the cephalothorax of prawn and shrimp (Montero *et al*., 2001b). PPO is involved in the black spot formation in crustacean during post mortem storage. Black spot formation (melanosis) is one of the problems that occur in crustaceans. It drastically reduces the consumer acceptability and the product s market value (Montero *et al.,* 2001b). The intensity of melanosis formation in the crustacean varies with species, due to the differences in substrate and enzyme concentration (Benjakul *et al*., 2005a).

Therefore, a better understanding of biochemical properties and kinetic of PPO is needed to control and inhibit its action. PPO from cephalothoraxes of various crustaceans such as pink shrimp (*Penaeus duorarum*: Simpson *et al*., 1988; Chen *et al*. 1997; *Parapenaeus longirostris*: Zamorano *et al*., 20009), white shrimp (*Penaeus striferus*: Simpson *et al*., 1988; Chen *et al*., 1997), prawn (*Penaeus japonicus*: Montero *et al*., 2001a; Benjakul *et al*., 2005a) and lobster (*Nephrops norvegicus*: Yan *et al*., 1990; *Homarus Americanus*: Opoku-Gyamfua and Simpson 1993) have been purified and characterized. PPO from different crustaceans showed different molecular weight, optimum pH, thermal stability and kinetic parameters.

Pacific white shrimp (*Litopenaeus vannamei*) is an important commercial species primarily cultured in Thailand and accounts for 90% of the global aquaculture shrimp production (Nirmal and Benjakul 2009a). This high value shrimp is very perishable and susceptible to black spot formation during post mortem handling and storage. However, there is no information on biochemical properties of PPO from the cephalothorax of Pacific white shrimp cultured in Thailand. The aim of this study was to determine the biochemical properties of PPO from Pacific white shrimp and to elucidate the effects of some chemicals on PPO activity.

9.3 Materials and Methods

9.3.1 Chemicals

L- -(3, 4 dihydroxylphenyl) alanine (L-DOPA), Brij-35, phenilmethanesulfonyl fluoride (PMSF), bovine pancreatic trypsin (501.25 Unit/mg),

p-amino benzoic acid and cysteine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dedocyl sulfate (SDS), ammonium sulfate and ethylene diamine tetra acetic acid (EDTA) were obtained from Merck (Darmstadt, Germany). Coomassie Blue R-250 and *N, N, N N* -tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). High-molecular-weight markers and DEAE-Sephacel were purchased from GE Helathcare UK Limited (Buckinghamshire, UK).

9.3.2 Shrimp collection and preparation

Pacific white shrimp (*Litopenaeus vannamei*) with the size of 55-60 shrimp/kg were purchased from a supplier in Songkhla, Thailand. The shrimp, freshly caught and completely free of additives, were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, shrimp were washed in cold water and stored in ice until used (not more than 3 h). The cephalothoraxes of shrimp were separated, pooled and powdered by grinding with liquid nitrogen in a Waring blender (AY46, Moulinex, Guangdong, China). The powder obtained was kept in polyethylene bag and stored at -20 °C for not more than 2 weeks.

9.3.3 Extraction and partial purification of PPO from cephalothorax of Pacific white shrimp

The extraction of PPO from the powdered cephalothoraxes of Pacific white shrimp was carried out according to the method of Nirmal and Benjakul (2009b) with a slight modification. The powder (25 g) was mixed with 75 mL of the extracting buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 1.0 M NaCl and 0.2% Brij 35). PMSF at 1 mg/mL was also added to the extracting buffer to prevent proteolysis by indigenous proteases. The mixture was stirred continuously at 4^{o}C for 30 min, followed by centrifugation at 8000 x g at 4^{o}C for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). Solid ammonium sulfate was added into the supernatant to obtain 40%

saturation and allowed to stand at 4^{o}C for 30 min. The precipitates formed were collected by centrifugation at 12,000 x g for 30 min at 4°C. The pellets were dissolved in a minimum volume of extracting buffer and dialyzed with 50 volumes of cold extracting buffer with three changes overnight. The insoluble materials were removed by centrifugation at 3000 x g at 4^{o}C for 30 min.

Ammonium sulfate fraction was applied onto DEAE-Sephacel column (1.6 x 16 cm), previously equilibrated with 0.05M phosphate buffer, pH 7.2 (Chen *et al*. 1991a). The column was then washed with the same phosphate buffer until A_{280} was below 0.05. PPO was eluted with a linear gradient of 0 to 1.2 M NaCl in 0.05 M phosphate buffer (pH 7.2) at a flow rate of 0.5 mL/min. Fractions of 1.5 mL were collected and those with PPO activity were pooled. The pooled fractions were dialyzed with 50 volumes of 0.05 M phosphate buffer (pH 7.2) with two changes within 12 h. The protein concentration was determined according to the method of Bradford (1976).

9.3.4 Measurement of PPO activity

The assay system consisted of 100 L of PPO solution, 600 L of 15 mM L-DOPA in deionised water, 400 L of 0.05 M phosphate buffer, pH 6.0 and 100 L of deionized water (Nirmal and Benjakul 2009a). The PPO activity was determined for 3 min at 45^{o}C by monitoring the formation of dopachrome at 475 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of PPO activity was defined as an increase in the absorbance at 475 nm by 0.001 /min. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture and the deionized water was used instead.

9.3.5 pH and temperature profiles of PPO

The activity of PPO in DEAE-Sephacel fraction was assayed at different pHs (2.0-10.0) at 45^{o}C using 15 mM L-DOPA as a substrate. Different assay buffers at pH 2.0 - 7.0 (McIlvaine buffer; 0.2 M sodium phosphate and 0.1 M sodium citrate) and pH 8.0-10.0 (0.1M phosphate-borate buffer) were used. For temperature

profile study, the assay was performed at different temperatures (25 - 60°C) for 3 min at pH 6.0. The activity was measured as previously described. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture and the deionized water was used instead.

9.3.6 pH and thermal stability of PPO

To study pH stability of PPO in DEAE-Sephacel fraction, the 100 µL of fraction were mixed with 100 µL of McIlvaine buffer or 0.1 M phosphate-borate buffer at various pHs (2 -10) and incubated at room temperature for 30 min (Benjakul *et al*., 2005a). Residual activity was then determined using 15 mM L-DOPA as a substrate at pH 6.0 and 55^{o}C for 3 min. For thermal stability, 100 µL of DEAE-Sephacel fraction containing PPO were incubated at different temperatures (25-60°C) for 30 min. The sample was cooled rapidly in the iced water (Benjakul *et al*., 2005a). The residual activity was then assayed at pH 6.0 and 55^{o}C for 3 min as previously described and expressed as the activity relative to the control (without incubated PPO).

9.3.7 Kinetic study of PPO

Kinetic of PPO in DEAE-Sephacel fraction was determined as per the method of Opoku-Gyamfua *et al*. (1992) with a slight modification. L-DOPA with the concentration range of 2 - 20 mM was used to study the kinetic of PPO in DEAE-Sephacel fraction at pH 6. The assay was conducted for 3 min at 55^{o}C and absorbance was monitored at 475 nm. The reaction velocity (V_{max}) and Michaelis constants (K_m) were calculated from Lineweaver-Burk plots (Lineweaver and Burk 1934).

9.3.8 PPO activity staining

DEAE-Sephacel fraction containing PPO from cephalothoraxes of Pacific white shrimp was subjected to activity staining as per the method of Nirmal and Benjakul (2009a). The fraction was mixed with the sample buffer containing SDS at a ratio of 1:1 (v/v). The sample (3 µg protein) was loaded onto the PAGE made of

7.5 % separating gel and 4 % stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, one of two identical gels was immersed in a 0.05 M sodium phosphate buffer (pH 6.0) containing 15 mM L-DOPA for 25 min at 25^{o}C. The activity zone appeared as the brown band. Another gel was stained with 0.125% Coomassie Brilliant Blue R-250 and destained in 25% methanol and 10% acetic acid. To estimate the molecular weight of PPO, the markers including myosin from rabbit muscle (200kDa), -galactosidase from *E. coli* (116kDa), phosphorylase b from rabbit muscle (97kDa), bovine serum albumin (66kDa), glutamic dehydrogenase from bovine liver (55kDa), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (36kDa), and carbonic anhydrase from bovine erythrocytes (29kDa) were used.

9.3.9 Effect of trypsin and copper on PPO activity

Effects of trypsin and copper on PPO in DEAE-Sephacel fraction were determined using various concentrations of trypsin and copper acetate as per the method of Opoku-Gyamfua *et al*. (1992) with a slight modification. To 100 µL of fraction containing PPO, 100 µL of trypsin solution was added to obtain the final concentration of 20, 40, 60 and 100 µg/mL. The mixture was incubated at room temperature for 30 min prior to PPO activity assay as described previously. Blank was prepared using the deionized water instead of the L-DOPA solution for each mixture of PPO and trypsin. To study the influence of copper on PPO activity, copper acetate with various final concentrations (0.01, 0.05, 0.1 and 0.5 mM) was used and residual PPO activity was measured as described above.

9.3.10 Effect of sodium dodecyl sulphate (SDS) and inhibitors on PPO activity

The influence of SDS and inhibitors (cysteine, EDTA and PABA) on the activity of PPO in DEAE-Sephacel fraction was determined as described by Benjakul *et al*. (2005a) with a slight modification. To 100 µL of fraction, 100 µL of SDS was added to obtain the various final concentrations (0.05, 0.1 and 0.5 %). The

mixtures were incubated at room temperature for 30 min and the residual activity of PPO was assayed using 15 mM L-DOPA as a substrate at 55^{o}C. Absorbance at 475 nm was recorded up to 3 min against the blank, in which the deionised water was used instead of the L-DOPA solution.

Cysteine, EDTA or PABA (100 µL) was added to 100 µL of DEAE-Sephacel fraction to obtain the final concentrations of 1 and 10 mM. The mixtures were incubated at room temperature for 30 min before PPO activity assay as previously described.

9.3.11 Statistical analyses

All analyses were performed in triplicate and a completely randomized design (CRD) was used. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan s multiple range tests (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA).

9.4 Results and Discussion

9.4.1 Extraction and partial purification of PPO from cephalothorax of Pacific white shrimp

Table 14 represents the purification steps of PPO from cephalothorax of Pacific white shrimp. PPO from cephalothorax of Pacific white shrimp was extracted and fractionated using 40% ammonium sulfate (AS) saturation. After AS fractionation, 2.7-fold purity was achieved. Simpson *et al*. (1987) reported that AS (40% saturation) fractionated PPO from cephalothorax of white shrimp (*Penaeus setiferus*) with 2.4-fold purity. A 1.6-fold increase in purity was reported for the PPO from cephalothorax of kuruma prawn (*Penaeus japonicus*) with 40% AS fractionation (Benjakul *et al*. 2005a). AS fraction was further purified using DEAE-Sephacel anion exchange chromatography and the purity of 83.8 fold was obtained. During DEAE-

Sephacel chromatography, most of the unbound positively charged proteins were removed.

Table 14. Summary of purification of PPO on DEAE-Sephacel anion exchange chromatography

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	49099.5	1396.8	35.1	1	100
AS fractionation	40537	423.5	95.7	2.7	82.5
DEAE-Sephacel column	36062	12.3	2943.8	83.8	73.4

At the end of partial purification through AS fractionation followed by DEAE-Sephacel chromatography, PPO with the yield of 73.4% was recovered. PPO from cephalothorax of white shrimp was purified to 65.6-fold with affinity chromatography (Simpson *et al*. 1987). PPO from cephalothorax of Taiwanese black tiger shrimp (*Penaeus monodon*) with purity of 58-fold was prepared using Phenyl sepharose CL-4B column (Rolle *et al*. 1991). PPO from cephalothorax of pink (*Penaeus duorarum*) and white (*Penaeus setiferus*) shrimp were purified with to 64 and 45 fold using Phenyl sepharose CL-4B chromatography, respectively (Chen *et al*. 1997). This result suggested that DEAE-Sephacel anion exchange chromatography of AS fraction was effective in purification of PPO from cephalothorax of Pacific white shrimp.

9.4.2 pH and temperature profile of PPO from cephalothorax of Pacific white shrimp

The maximal activity of PPO in DEAE-Sephacel fraction from cephalothorax of Pacific white shrimp was observed at pH 6 (Figure 53A). The result was in accordance with Rolle *et al*. (1991) who reported that maximal activity of PPO from cephalothorax of Taiwanese black tiger shrimp was found at pH 6. PPO from carapace and viscera of Norway lobster (*Nephrops norvegicus*) had optimum pH at 7 and 8, respectively (Gimenez *et al*., 2010). The maximum activity of PPO from

cephalothorax of kuruma prawn (*Penaeus japonicus*) was obtained at pH 6.5 (Benjakul *et al*., 2005a).

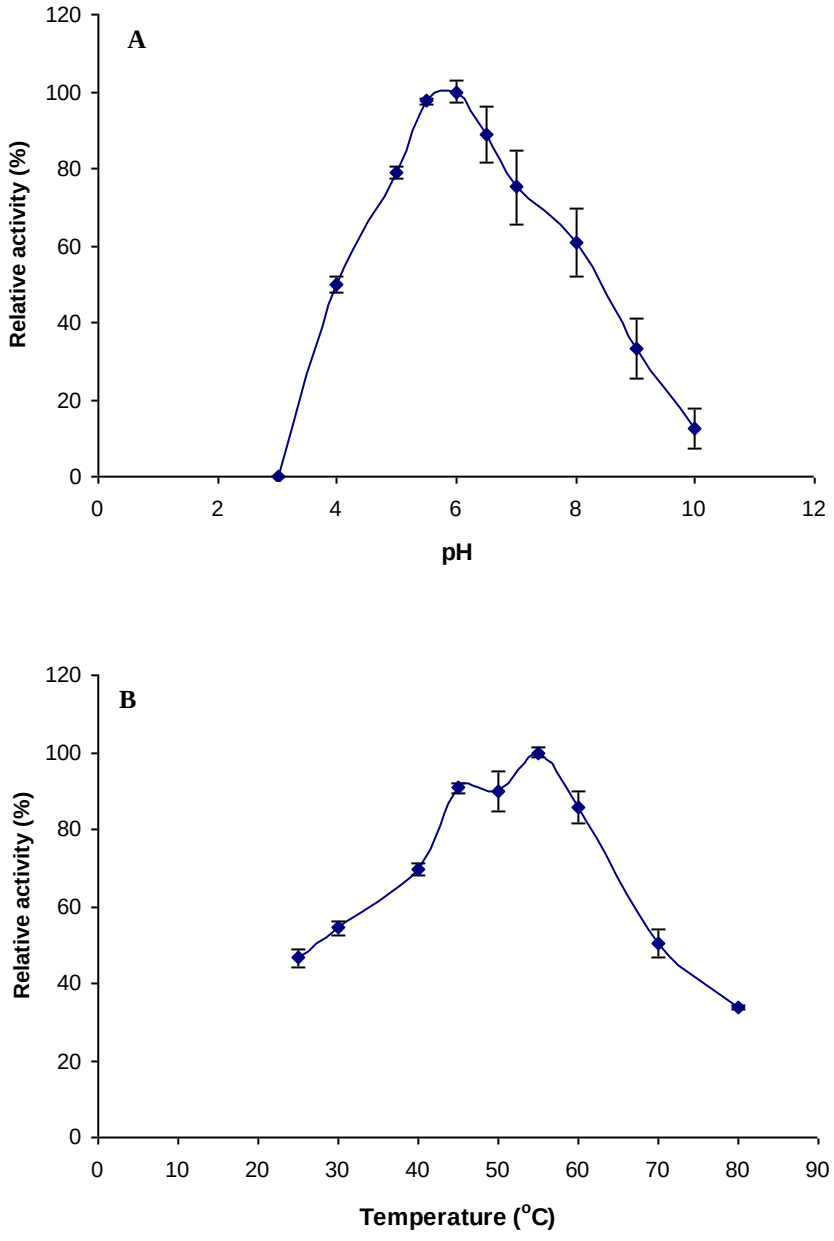

Figure 53. pH (A) and temperature (B) profiles of DEAE-Sephacel fraction containing PPO from cephalothorax of Pacific white shrimp. Bars represents standard deviation (n=3).

Nevertheless, partially purified carapace PPO from deepwater pink shrimp (*Parapenaeus longirostris*) showed a maximum activity at pH 4.5 (Zamorano

et al., 2009). PPO from the cephalothorax of white shrimp showed the maximum activity at pH 7.5 (Simpson *et al*., 1987). The optimal pH of PPO for different crustaceans varied with the species and anatomical location (Benjakul *et al*., 2005a). PPO activity was markedly decreased in either acidic or alkaline pH range. At extreme acidic or alkaline pH conditions, unfolding of enzyme might occur, due to disruption of electrostatic bonds stabilizing enzyme molecules, thereby making PPO inactive.

DEAE-Sephacel fraction containing PPO from cephalothorax of Pacific white shrimp had the optimal temperature at 55^{o}C (Figure 53B). Montero *et al*. (2001a) reported that PPO from carapace of imperial tiger prawn (*Penaeus japonicus*) cultured in Spain showed the maximum activity at 55^{o}C. However, PPO from cephalothorax of kuruma prawn cultured in Japan showed the maximum activity at 35^{o}C (Benjakul *et al*., 2005a). PPO activity from Norway lobster (Gimenz *et al*., 2010) and deep water pink shrimp (Zamorano *et al*., 2009) was continuously increased up to 60^{o}C. The maximum activity was noticeable at 40–45^{o}C for PPO extracted from different shrimps including pink shrimp (Simpson *et al*., 1988), Taiwanese black tiger shrimp (Rolle *et al*., 1991) and white shrimp (Simpson *et al*., 1987). The variation in optimal temperature of different crustaceans was most probably related to their habitat temperature. Further increase in temperature above 55^{o}C resulted in the decrease in PPO activity, more likely due to the thermal denaturation of PPO.

9.4.3 pH and temperature stability of PPO from cephalothorax of Pacific white shrimp

The pH stability study of PPO in DEAE-Sephacel fraction revealed that PPO from cephalothorax of Pacific white shrimp was stable over pH range of 5-10 with remaining activity more than 80 % (Figure 54A). PPO was found to be more stable in neutral and alkaline pH range. However, PPO was unstable at pH below 5. Remaining activities of 43 and 25% were observed at pH 4 and 3, respectively. PPO from kuruma prawn was reported to be stable over pH range of 3-10 (Benjakul *et al*., 2005a). Nevertheless, PPO from pink shrimp (Simpson *et al*., 1988) and deep water

pink shrimp (Zamorano *et al*., 2009) had high stability between pH 6 - 12 and pH 4.5-9.0, respectively. PPO from imperial tiger prawn (Montero *et al*., 2001b) and white shrimp (Simpson *et al*., 1987) was unstable below pH 5.

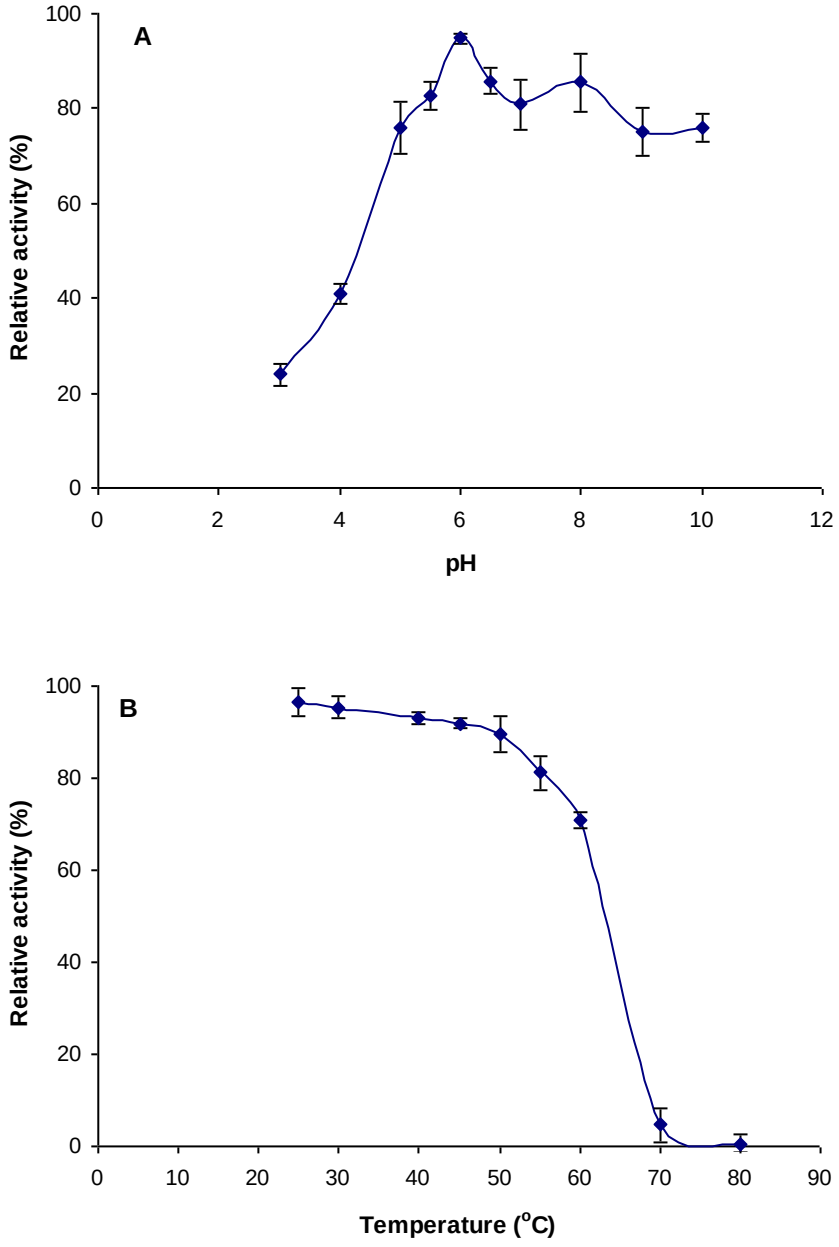

Figure 54. pH (A) and temperature (B) stability of DEAE-Sephacel fraction containing PPO from cephalothorax of Pacific white shrimp. Bars represents standard deviation (n=3).

PPO from carapace and viscera of Norway lobster was stable over neutral to alkaline pH range (Gimenz *et al*., 2010). Stability of Pacific white shrimp PPO at alkaline pH suggested that the conformation of PPO active site was not affected. Thermal stability of DEAE-Sephacel fraction containing PPO from cephalothorax of Pacific white shrimp is presented in Figure 54B. PPO was stable up to 60^{o}C for 30 min with more than 70 % remaining activity. The result suggested that temperature below 60^{o}C did not affect the three dimensional structure of PPO. At temperature above 60^{o}C, a sharp decrease in PPO activity was observed. PPO more likely underwent thermal denaturation at high temperatures. PPO of white shrimp (Simpson *et al*., 1987) and kuruma prawn (Benjakul *et al*., 2005a) had the stability up to 50^{o}C. PPO of pink shrimp (Simpson *et al*., 1988), Taiwanese black tiger shrimp (Rolle *et al*., 1991) and deepwater pink shrimp (Zamorano *et al*., 2009) was unstable at temperatures over 30–35^{o}C. PPO from Pacific white shrimp cultured in Thailand, a tropical country, was more stable than shrimp from temperate water.

9.4.4 Kinetic and molecular weight of PPO from cephalothorax of Pacific white shrimp

The Michaelis constant (K_m) for the oxidation of L-DOPA by PPO in DEAE-Sephacel fraction was 2.43 mM, which was similar to K_m for oxidation of DL-DOPA by PPO from white shrimp (K_m 2.8 mM) (Simpson *et al*.,1988). K_m values reflect the affinity of enzymes for their substrates. K_m values of 1.6 and 1.85 mM were obtained using DL-DOPA as a substrate for PPO from pink shrimp (Simpson *et al*.,1988) and deep water pink shrimp (Zamorano *et al*., 2009), respectively. Lower K_m value indicates the higher catalytic efficiency of the enzyme towards substrate. The K_m values for oxidation of L-DOPA by white shrimp (Chen *et al*., 1991b) and kuruma prawn (Benjakul *et al*., 2006) PPO were 3.48 and 0.26 mM, respectively. PPO from viscera of Norway lobster showed a higher catalytic affinity for catechol than PPO from carapace with apparent K_m values of 5.97 and 19.40 mM, repectively (Gimenez *et al*., 2010). V_{max} for the oxidation of L-DOPA by PPO in DEAE-Sephacel fraction was 61 x 10^3 U/mg PPO. Simpson *et al*. (1988) reported that V_{max} for PPO from the cephalothorax of pink and white shrimp was 5.6 x 10^3 and 3.1 x 10^3 U/mg

PPO, respectively. The result suggested that Pacific white shrimp PPO had higher reaction rate of L-DOPA oxidation than pink and white shrimp PPO. The differences in K_m and V_{max} of PPO from different species were plausibly owing to the differences in moulting stage, method of capture, handling and storage conditions (Rolle *et al*., 1991).

The activity staining of DEAE-Sephacel fraction containing PPO from cephalothorax of Pacific white shrimp is illustrated in Figure 55. Based on activity staining, apparent molecular weight of PPO was 210 kDa. This result reconfirmed our previous report (Nirmal and Benjakul 2009a), where PPO from the crude extract of Pacific white shrimp cephalothorax showed molecular weight of 210 kDa. PPO from viscera and carapace extracts of cephalothorax of Norway lobster had apparent molecular weight of 200- 220 kDa as determined by activity staining using L-tyrosine and 4-tert-butyl-catechol as substrates (Gimenez *et al*., 2010). Zamorano *et al*. (2009) studied the electrophoretic mobility of PPO from deepwater pink shrimp using non-reducing SDS-PAGE, followed by staining with DOPA and found the activity band with molecular weight of 200 kDa.

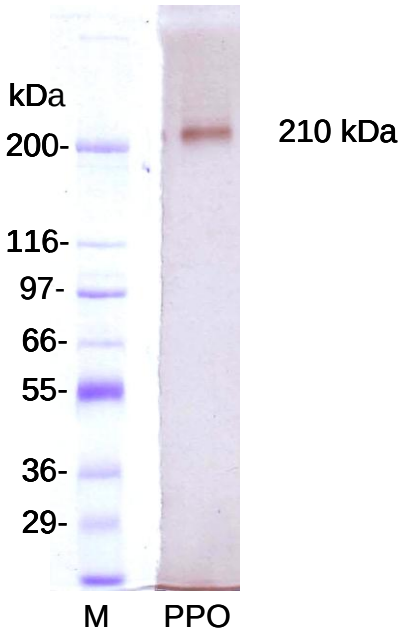

Figure 55. Activity staining of DEAE-sephacel fraction containing PPO from cephalothorax of Pacific white shrimp. M: markers.

From activity staining, PPO from the kuruma prawn cephalothorax showed activity band with molecular weight of 160 kDa (Benjakul *et al*., 2005a). PPO from different species of crustaceans comprised the different isoforms with varying molecular weights (Chen *et al*., 1991a).

9.4.5 Effect of some chemicals on the activity of PPO from cephalothorax of Pacific white shrimp

The effect of trypsin and copper acetate at different concentrations on PPO in DEAE-Sephacel fraction is shown in Table 15. Increasing concentration of trypsin up to 80 µg/ mL had no impact on PPO activity ($P > 0.05$). This result was in agreement with Simpson *et al*. (1987), Benjakul *et al*. (2005a) and Zamorano *et al*. (2009) who found that trypsin had no effect on PPO from white shrimp, kuruma prawn and deepwater pink shrimp, respectively. However, slight increase in PPO activity was observed in the presence of trypsin at the concentration of 100 µg/ mL. Trypsin had slight effect on the conversion of hemocyanin (Hc) of white leg shrimp (*Penaeus vannamei*) to HcPPO (Garcia-Carreno *et al*., 2008). Opoku-Gyamfua *et al*. (1992) reported that PPO activity from lobster was increased with increasing concentration of trypsin up to 20 µg/ mL.

Table 15. Effect of trypsin and copper acetate at different concentrations on relative activity of PPO in DEAE-Sephacel fraction

Chemicals	Concentrations	Relative activity (%)
Control (without chemicals)	--	100 a
Trypsin	20 µg /mL	100 ± 0.11 a
	40 µg /mL	100 ± 0.38 a
	60 µg /mL	102 ± 2.46 a
	100 µg /mL	104 ± 1.39 b
Copper acetate	0.01 mM	100 ± 0.0 a
	0.05 mM	100 ± 0.0 a
	0.1 mM	95.7 ± 0.63 b
	0.5 mM	84.8 ± 0.78 c

Different letters in the same column indicate the significant difference ($P < 0.05$).

Trypsin could activate ProPPO to PPO by cleaving propeptide from proPPO, thereby enhancing its activity (Benjakul *et al*., 2005a). However, PPO in DEAE-Sepahcel fraction more likely existed as active form. Copper ion at the concentrations of 0.01 and 0.05 mM had no effect on PPO in DEAE-Sephacel fraction ($P > 0.05$). Nevertheless, copper ion at higher concentrations (0.1 and 0.5 mM) decreased PPO activity ($P < 0.05$). The result was in accordance with Benjakul *et al*. (2005a) who reported the decrease in activity of PPO from kuruma prawn with increasing concentrations of copper ion. On the other hand, PPO from white shrimp (Simpson *et al*., 1987) and lobster (Opoku-Gyamfua *et al*., 1992) had the increased activity with increasing concentrations of copper ion. The increase in copper ion might cause the conformational change of enzyme by affecting the ionic interaction stabilizing the structure of enzyme (Benjakul *et al*., 2005a). It was noted that PPO activity from Pacific white shrimp was decreased by the higher concentration of copper acetate. In general, trypsin and copper ion have been reported to be activators for PPO from different sources. The result suggested that PPO in DEAE-Sepahcel fraction was in active state and did not require trypsin or copper ion as activators. Also, the structure or conformation of PPO from Pacific white shrimp might be different from PPO from other species. Therefore activation of PPO by trypsin or copper ion depends on the species and other intrinsic factors determining the activity.

The effect of SDS at different concentrations on PPO in DEAE-Sephacel fraction is presented in Figure 56. SDS at 0.05 and 0.1 % had no influence on PPO activity. However, SDS at a concentration of 0.5 % showed inhibitory effect on PPO from Pacific white shrimp. The results are supported by Adachi *et al*. (1999) who observed the inactivation of PPO from the hemocyte of kuruma prawn (*Penaeus japonicus*) by SDS. Nevertheless, Garcia-Carreno *et al*. (2008) reported that hemocyanin (Hc) from white leg shrimp was converted to HcPPO by SDS treatment. PPO from cephalothorax of kuruma prawn was not affected by 0.05 % SDS (Benjakul *et al*., 2005a). These results reconfirm that PPO in DEAE-Sepahcel fraction more likely existed in active state, which was not affected by SDS up to 0.1%. However, at a concentration of 0.5 % SDS might disturb the hydrophobic interaction in PPO, thereby lowering PPO activity.

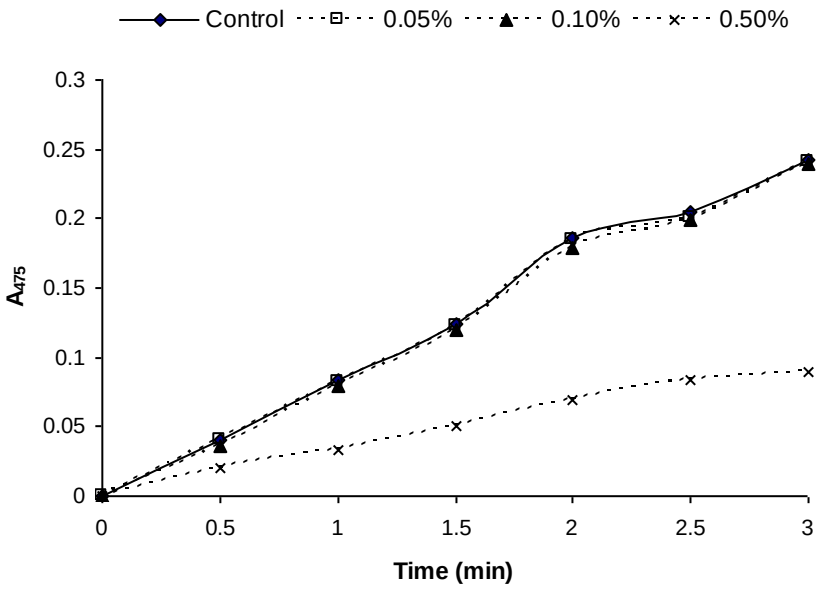

Figure 56. Effect of sodium dodecyl sulphate on activity of PPO from DEAE-Sephacel fraction. The decrease in A_{475} indicates the inhibition of DOPA-chrome formation by PPO.

Table 16 shows the effect of cysteine, EDTA and PABA on PPO in DEAE-Sephacel fraction. In general, increasing concentration of cysteine, EDTA and PABA resulted in the increase in PPO inhibition ($P < 0.05$). Cysteine at the concentration used showed higher inhibitory effect towards PPO, compared with EDTA and PABA. At the concentration of 10 mM, cysteine totally inhibited PPO activity, whereas EDTA and PABA showed 22.41 and 58.60 % inhibition towards PPO, respectively. The results were in accordance with Opoku-Gyamfua *et al*. (1992) who reported that cysteine, PABA and EDTA inhibited PPO from lobster.

Table 16. Effect of cysteine, EDTA and PABA at different concentrations on % inhibition of PPO in DEAE-Sephacel fraction

Inhibitors	Concentration (mM)	
	1	10
Cysteine	91.77 ± 0.17 b	100 ± 0.0 a
EDTA	14.00 ± 0.44 b	22.41 ± 0.83 a
PABA	14.66 ± 0.77 b	58.60 ± 1.01 a

Different letters in the same row indicate the significant difference ($P < 0.05$).

However, oxidation of DOPA by pink and white shrimp PPO was not inhibited by cysteine (Simpson *et al*., 1988). The inhibition of PPO activity by EDTA might relate to their metal chelating capability, thus making Cu^{2+} unavailable at the active site of enzyme. PABA could compete with the DOPA in binding the active site of PPO. Cysteine containing sulfur group might be involved in the PPO inhibition. Cysteine exhibited competitive type inhibition on PPO from kuruma prawn (Benjakul *et al*., 2006) and mulberry (Arslan *et al*., 2004). Thiol reagents might interact with copper at the active site of PPO, leading to the loss of the activity (Benjakul *et al*., 2006). Cysteine can react with *o*-quinones by forming cysteinyl adduct, the colorless compound (Richard-Forget *et al*., 1992). Therefore, oxidation of L-DOPA by Pacific white shrimp PPO was inhibited by cysteine due to the various inhibitory mechanism of cysteine towards PPO.

9.5 Conclusions

PPO from cephalohtorax of Pacific white shrimp was partially purified by DEAE-Sephacel chromatography with 83.8-fold purity. PPO from Pacific white shrimp with apparent molecular weight of 210 kDa showed optimal pH and temperature at 6 and 55^{o}C, respectively. The isolated PPO was stable in neutral and alkaline pH range. PPO in DEAE-Sepahcel fraction with K_m of 2.43 mM might exist in the active form, which did not require any activators e.g. trypsin, copper ion or SDS. Cysteine exhibited higher inhibitory activity towards PPO, compared with EDTA and PABA. Therefore, higher catalytic activity and stability of PPO from Pacific white shrimp could be responsible for the rapid melanosis formation in Pacific white shrimp, especially at the cephalothorax portion during postmortem handling and storage.

CHAPTER 10

INHIBITION MODE OF CATECHIN AND FERULIC ACID ON POLYPHENOLOXIDASE FROM CEPHALOTHORAX OF PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*)

10.1 Abstract

Inhibition kinetics and mode of catechin and ferulic acid towards polyphenoloxidase (PPO) from cephalothorax of Pacific white shrimp were investigated. Catechin or ferulic acid inhibited quinone formation catalyzed by PPO in a dose dependent manner. Catechin showed mixed type reversible inhibition with K_i value of 1.4 mM, whereas ferulic acid exhibited non-competitive reversible inhibition with K_i value of 37 mM. With increasing concentrations, both catechin and ferulic acid had higher copper (Cu^{2+}) reduction and copper chelating capacity ($P < 0.05$). Catechin or ferulic acid could react with browning reaction and thereby prevent dopachrome formation. Thus, catechin or ferulic acid could inhibit melanosis in Pacific white shrimp with different modes of inhibition towards PPO.

10.2 Introduction

Melanosis (blackening) is one of the problems that occur in crustaceans during post mortem handling and storage. Melanosis seems to be harmless to consumers, but it drastically reduces the consumer acceptability and market value (Benjakul *et al*., 2006). This undesirable melanosis in crustacean is induced by a biochemical mechanism catalyzed by polyphenoloxidase (PPO), also known as phenoloxidase, tyrosinase and phenolase (Benjakul *et al*., 2005a). The active site of PPO consists of two copper atoms with three states, met , deoxy and oxy (Rodriguez-Lopez *et al*., 1992). PPO is a bifunctional enzyme, which catalyzes two basic reactions, in the presence of molecular oxygen, the *o*-hydroxylation of monophenols to give *o*-diphenols (Monophenol oxidase, EC 1.14.18.1) and the

subsequent oxidation of *o*-diphenols to *o*-quinones (Diphenoloxidase, EC 1.10.3.1) (Garcia-Molina *et al*., 2005). The intensity of melanosis formation in the crustacean varies with the species, most likely due to the differences in the presence of substrate and enzyme concentration (Benjakul *et al*., 2005a).

Melanosis formation can be controlled by the reduction of *o*-quinones to diphenols; removal of oxygen, chelation of copper ion at active site of PPO and uses of inhibitors (Kim *et al*., 2000). To retard or alleviate melanosis in crustaceans, sulfite derivatives and 4-hexylresorcinol have been intensively used (Martinez-Alverez *et al*., 2008b; Montero *et al*., 2001b). Kojic acid also showed inhibitory activity towards crustaceans PPO (Chen *et al*., 1991b). However, the increasing regulatory attention and awareness of consumers against synthetic additives, especially sulfiting agents, in shrimp and shrimp products have led to the interest in natural additives to prevent melanosis in shrimp (Nirmal and Benjakul, 2010a).

Pacific white shrimp (*Litopenaeus vannamei*) is an important commercial species primarily cultured in Thailand and accounts for 90% of the global aquaculture shrimp production (Nirmal and Benjakul, 2009b). Shelf-life of shrimp is limited due to melanosis formation, especially during the extended storage (Nirmal and Benjakul, 2010b). Recently, plant phenolic compounds including ferulic acid (Nirmal and Benjakul, 2009a) and catechin (Nirmal and Benjakul, 2009b) could be used as effective melanosis inhibitor in Pacific white shrimp. Therefore, it was interested to study the inhibitory action of phenolic compounds in Pacific white shrimp and its products to control melanosis formation. However, there is no information on the inhibitory mechanism of phenolic compounds on PPO from the cephalothorax of Pacific white shrimp cultured in Thailand. This study was undertaken to elucidate the inhibition kinetics and mode of catechin and ferulic acid towards PPO from Pacific white shrimp.

10.3 Materials and Methods

10.3.1 Chemicals

L- -(3, 4 dihydroxylphenyl) alanine (L-DOPA), Brij-35, bathocuproine disulfonic acid, tetramethylmurexide (TMM), catechin and ferulic acid were

purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dedocyl sulfate (SDS), ammonium sulfate, and cupric sulfate were obtained from Merck (Darmstadt, Germany). Coomassie Blue R-250 and *N, N, N N* -tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). High-molecular-weight markers and DEAE-Sephacel were purchased from GE Healthcare UK Limited (Buckinghamshire, UK). All chemical used were analytical grade.

10.3.2 Shrimp collection and preparation

Pacific white shrimp (*Litopenaeus vannamei*) with the size of 55-60 shrimp/kg were purchased from a supplier in Songkhla, Thailand. The shrimp, freshly caught and completely free of additives, were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, shrimp were washed in cold water and stored in ice until used (not more than 3 h). The cephalothoraxes of shrimp were separated, pooled and powdered by grinding with liquid nitrogen in a Waring blender (AY46, Moulinex, Guangdong, China). The powder obtained was kept in polyethylene bag and stored at -20 °C until used.

10.3.3 Extraction and partial purification of PPO from the cephalothoraxes of Pacific white shrimp

The extraction of PPO from the powdered cephalothoraxes of Pacific white shrimp was carried out according to the method of Nirmal and Benjakul (2009a). The powder (50 g) was mixed with 150 mL of the extracting buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 1.0 M NaCl and 0.2% Brij 35). The mixture was stirred continuously at 4^{o}C for 30 min, followed by centrifugation at 8000 x g at 4^{o}C for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). Solid ammonium sulfate was added into the supernatant to obtain 40% saturation and allowed to stand at 4^{o}C for 30 min. The precipitate was collected by centrifugation at 12,500 x g at 4^{o}C for 30 min using a refrigerated centrifuge. The pellet obtained was dissolved in a minimum volume of

0.05 M sodium phosphate buffer, pH 7.2 and dialysed against 50 volumes of the same buffer at 4^{o}C with three changes of dialysis buffer. The insoluble materials were removed by centrifugation at 3000 x g at 4^{o}C for 30 min.

Ammonium sulfate fraction was applied onto DEAE-Sephacel column (1.6 x 16 cm), previously equilibrated with 0.05M phosphate buffer, pH 7.2 (Chen *et al*., 1991b). The column was then washed with the same phosphate buffer until A_{280} was below 0.05. PPO was eluted with a linear gradient of 0 to 1.2 M NaCl in 0.05 M phosphate buffer (pH 7.2) at a flow rate of 0.5 mL/min. Fractions of 1.5 mL were collected and those with PPO activity were pooled. The pooled fractions were dialyzed with 50 volumes of 0.05 M phosphate buffer (pH 7.2) with two changes within 12 h. PPO from cephalothorax of Pacific white shrimp was purified to 83.7 fold after being chromatographed using DEAE-Sephacel column.

10.3.4 Measurement of PPO activity

PPO activity was assayed using L-DOPA as a substrate according to the method of Nirmal and Benjakul (2009a) with a slight modification. The assay system consisted of 100 L of PPO solution, 600 L of 15 mM L-DOPA in deionized water, 400 L of 0.05 M phosphate buffer (pH 6.0) and 100 L of deionized water. The PPO activity was determined for 3 min at 55^{o}C by monitoring the formation of dopachrome at 475 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of PPO activity was defined as an increase in the absorbance at 475 nm by 0.001 /min. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture and the deionized water was used instead.

10.3.5 Inhibition mode of catechin and ferulic acid on PPO from cephalothorax of Pacific white shrimp

10.3.5.1 Inhibitory effect of catechin and ferulic acid on PPO

To study the inhibitory effect of catechin and ferulic acid towards PPO, DEAE-Sephacel fraction containing PPO at a level of 686.9 U/mL was incubated with

catechin or ferulic acid at a ratio of 1:1 (v/v) to obtain the final concentrations of 3 and 6 mM or 50 and 100 mM, respectively. The mixtures were allowed to stand for 30 min at room temperature prior to loading onto polyacrylamide gel, followed by electrophoresis and activity staining, respectively (Nirmal and Benjakul 2009a).

10.3.5.2 Inhibition kinetics of catechin and ferulic acid on PPO

DEAE-Sephacel fraction (100 µL) was mixed with catechin or ferulic acid solution (100 µL) to obtain final concentrations of 0.5, 1.0 and 1.5 mM or 5, 10 and 20 mM, respectively. The mixtures were incubated for 5 min at room temperature (25^{o}C). To initiate the reaction, 1000 µL of L-DOPA in 0.05 M sodium phosphate buffer (pH 6.0) were added. At each concentration of catechin or ferulic acid, L-DOPA with seven different concentrations (0.5– 5 mM) was used as the substrate. The reaction was incubated for 3 min at 55^{o}C and the absorbance at 475 nm was measured using a UV-1800 spectrophotometer. The Michaelis constant (K_m) for PPO was determined by Lineweaver–Burk plots (Lineweaver and Burk, 1934) and K_i value was obtained from Dixon plot (Dixon, 1953).

10.3.5.3 Copper (II) reduction capability of catechin and ferulic acid

Reduction capability of catechin or ferulic acid on cupric copper to cuprous copper was determined as per the method of Chen *et al*. (1991a). One millilitre of catechin solution (0-1.5 mM) or ferulic acid solution (0 - 20 mM) was mixed with 0.5 mL of 0.4 mM cupric sulphate. The mixtures were allowed to stand for 10 min at room temperature. Then 0.5-mL aliquot of 4 mM aqueous bathocuproine disulfonic acid was added. The reaction mixture was incubated at room temperature for 20 min and the absorbance at 483 nm was measured. Since bathocuproine disulfonic acid could interact with Cu^{+} to form a red-color complex having an optimal absorption at 483 nm, the reduction capability of catechin or ferulic acid was determined from the measurement of the absorbance at this wavelength. Blank was prepared in the same manner except deionized water was used instead of catechin or ferulic acid solution.

10.3.5.4 Copper chelating activity of catechin and ferulic acid

Copper chelating activity of catechin (0.5, 1.0 and 1.5 mM) or ferulic acid (5, 10 and 20 mM) was determined according to the method of Wettasinghe and Shahidi (2002) as modified by Nirmal and Benjakul (2011a).

10.3.5.5 Effect of catechin and ferulic acid on browning reaction

The reaction mixture containing 100 µL of DEAE-Sephacel fraction, 400 µL assay buffer and 600 µL of L-DOPA was incubated at 25^{o}C for 3 min. To the reaction mixture, 100 µL of catechin (12 mM) or ferulic acid (200 mM) was added immediately and mixed thoroughly. Final concentration of catechin and ferulic acid in the reaction mixture was 1 mM and 17 mM. Red color developed was monitored by measuring the absorbance at 475 nm up to 5 min at 25^{o}C. For the control, deionized water (100 µL) was added instead of catechin or ferulic acid solution. Decrease in absorbance at 475 nm indicates the reduction of *o*-quinone to phenols or the formation of quinone-phenolic acid complex (Benjakul *et al*., 2006).

10.3.6 Statistical analyses

All analyses were performed in triplicate and a completely randomized design (CRD) was used. Two way analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan s multiple range tests (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA).

10.4 Results and Discussion

10.4.1 Inhibitory effect of catechin and ferulic acid on PPO from cephalothorax of Pacific white shrimp

Activity staining of PPO of DEAE-Sephacel fraction from cephalothorax of Pacific white shrimp in the absence and the presence of catechin or ferulic acid at different concentrations is shown in Figure 57. Catechin and ferulic acid showed inhibitory effect towards PPO from Pacific white shrimp in a dose dependent manner. The decrease in activity band intensity was observed as the higher levels of both compounds were used.

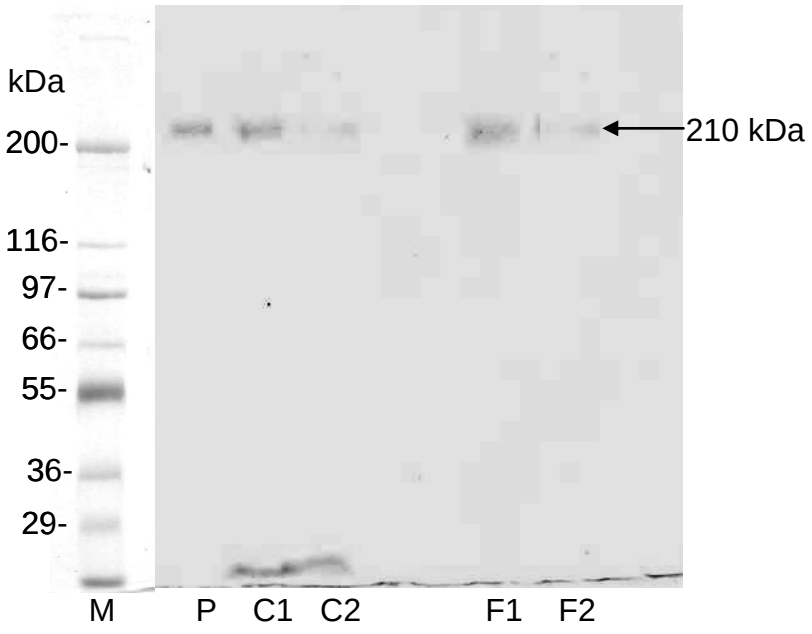

Figure 57. Activity staining of polyphenoloxidase of DEAE-Sephacel fraction from cephalothorax of Pacific white shrimp in the absence and presence of catechin or ferulic acid at different concentrations. M: molecular weight marker; P: DEAE-Sephacel fraction; C1 and C2: DEAE-Sephacel fraction with 3 and 6 mM catechin, respectively; F1 and F2: DEAE-Sephacel fraction with 50 and 100 mM ferulic acid, respectively.

This result was in agreement with our previous report, in which catechin (Nirmal and Benjakul, 2009b) and ferulic acid (Nirmal and Benjakul, 2009a) exhibited dose dependent inhibition of PPO from Pacific white shrimp. Catechin solution concentrations (3 and 6 mM) used in this study was equal to the 0.1 and 0.2 % solution, respectively. Whereas, ferulic acid solution at 50 and 100 mM concentrations were equal to 1 and 2 % solution, respectively. Based on activity staining, it was reconfirmed that PPO with molecular weight of 210 kDa was present in DEAE-Sephacel fraction. PPO from viscera and carapace extracts of cephalothorax of Norway lobster (*Nephrops norvegicus*) had apparent molecular weight about 200-220 kDa as determined by activity staining using L-tyrosine and 4-tert-butyl-catechol as substrates (Gimenez *et al*., 2010). PPO from cephalothorax of Pacific white shrimp could not use L-tyrosine as a substrate (data not shown), suggesting the lack of monophenoloxidase activity.

Ferulic acid is hydroxycinnamic acid, whereas catechin belongs to flavanol class (Nirmal and Benjakul, 2010a). Phenolic compounds are well known antioxidant bearing reducing power and metal chelating capacity (Shahidi *et al*., 2007). Both catechin and ferulic acid could inhibit PPO, plausibly due to the combined effects, e.g. metal chelation or reduction of quinone or competitive inhibition, etc.

10.4.2 Inhibition kinetics of catechin and ferulic acid towards PPO

The Michaelis constant (K_m) for the oxidation of L-DOPA by PPO in DEAE-Sephacel fraction was 2.43 mM, which was similar to K_m for oxidation of DL-DOPA by white shrimp (*Panaeus setiferus*,) (K_m 2.8 mM) (Simpson *et al*., 1988). Lower K_m (1.6 mM) of PPO from pink shrimp (*Panaeus duorarum*) using DL-DOPA as substrate was reported by Simpson *et al*. (1988). The K_m value for oxidation of L-DOPA by white shrimp PPO was 3.48 mM (Chen *et al*., 1991b). The K_m value of 0.26 mM was found for oxidation of L-DOPA by kuruma prawn (*Penaeus japonicus*) (Benjakul *et al*., 2006). K_m values reflect the affinity of enzymes for their substrates. Higher K_m value indicates the lower catalytic efficiency of the enzyme towards substrate (Liu *et al*., 2006).

Inhibition kinetics of catechin and ferulic acid towards PPO from cephalothorax of Pacific white shrimp were elucidated by Lineweaver-Burk Plots as shown in Figure 58.

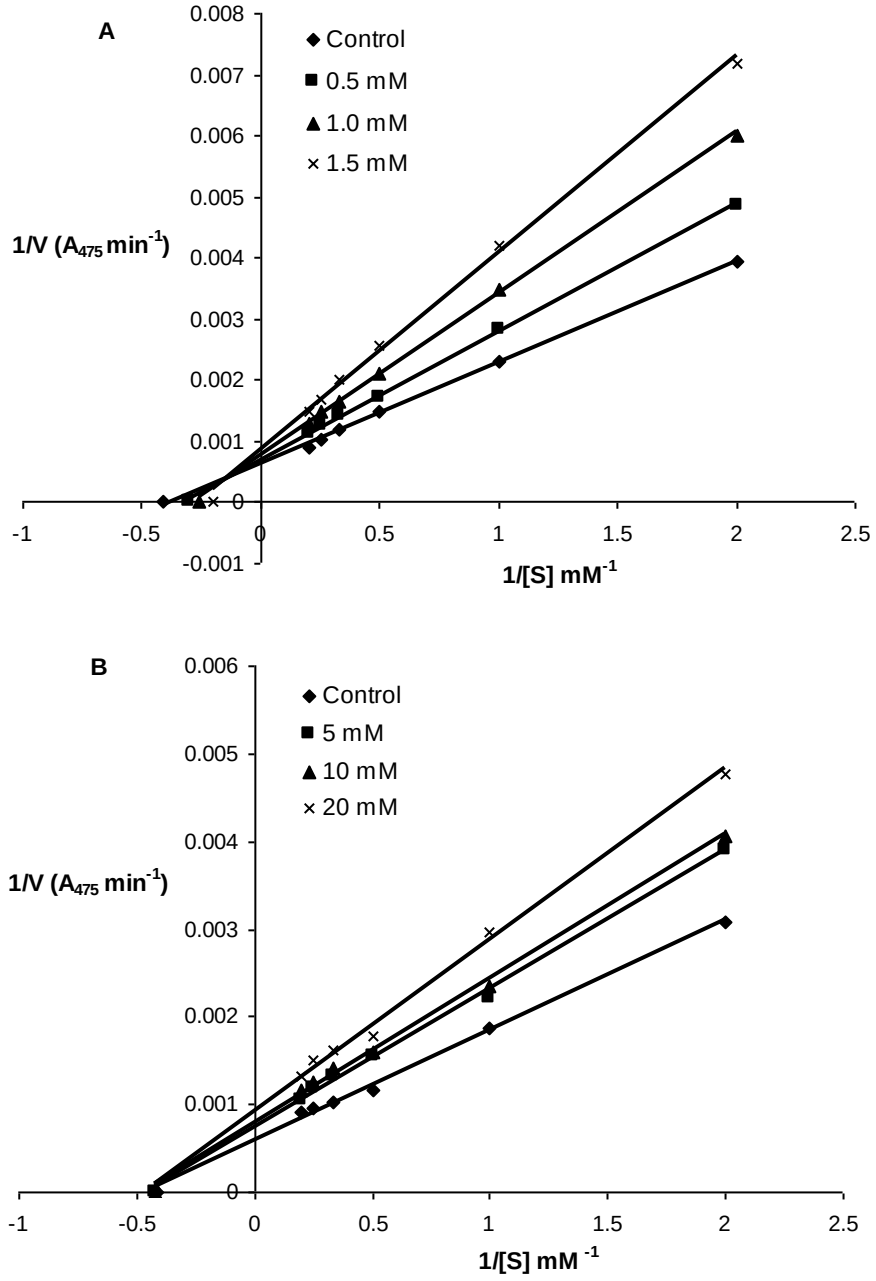

Figure 58. Lineaweaver-Burk plots of polyphenoloxidase from cephalothorax of Pacific white shrimp in the absence and presence of catechin (A) and ferulic acid (B) at different concentrations. L-DOPA at levels of 0.5- 5 mM were used as substrate.

Catechin at different concentrations affected both K_m and V_{max} value of PPO (Figure 58A). When ferulic acid at different concentrations was incorporated, only V_{max} was affected, whilst K_m remained unchanged (Figure 58B). Since K_m value increased and V_{max} value decreased with increasing catechin concentrations, the inhibitory mode of catechin was found to be mixed type. Results indicated that catechin could bind to both enzyme and enzyme-substrate complex, but with different affinities. The inhibitory action of ferulic acid towards PPO was non-competitive as evidenced by the decrease in V_{max} and unchanged K_m value with increasing ferulic acid concentrations. Ferulic acid more likely had identical affinities for both enzyme and enzyme-substrate complex. Kojic acid showed a mixed type inhibition towards PPO from white shrimp, grass prawn and lobster (Chen *et al*., 1991b). Dodecyl gallate was found as a mixed-type inhibitor for mushroom tyrosinase, when L-DOPA was used as a substrate (Kubo *et al*., 2000). K_i values of catechin and ferulic acid obtained from Dixon plots were 1.4 and 37 mM, respectively. The result suggested that catechin showed the higher affinity for binding PPO, compared to ferulic acid. Inhibition constant K_i value of dodecyl gallate on mushroom tyrosinase was 0.636 mM (Kubo *et al*., 2003).

The plots of residual enzyme activity vs. the concentrations of enzyme in the presence of different concentrations of catechin or ferulic acid showed straight lines, which passed through the origin (data not shown). Increasing concentration of catechin or ferulic acid resulted in the decrease in slope of the lines, suggesting their reversible inhibition towards PPO. Increased concentration of dodecyl gallate (Kubo *et al*., 2003) and ozagrel (Li *et al*., 2009) resulted in the decrease in the slope of the lines, revealing their reversible inhibition towards mushroom PPO. Therefore, catechin and ferulic acid showed mixed type and non-competitive reversible inhibition towards PPO in DEAE-Sephacel fraction of cephalothorax from Pacific white shrimp, respectively. Protein contaminants in ammonium sulfate fraction more likely interefered with inhibition kinetic of catechin and ferulic acid towards PPO. Therefore, partially purified PPO (DEAE-Sephacel fracction) was used in this study.

10.4.3 Copper reduction capability of catechin and ferulic acid

Copper reduction capability of catechin and ferulic acid at different concentrations is illustrated in Figure 59. Copper reduction capability of catechin or ferulic acid increased with increasing concentrations as indicated by increase in A_{483}.

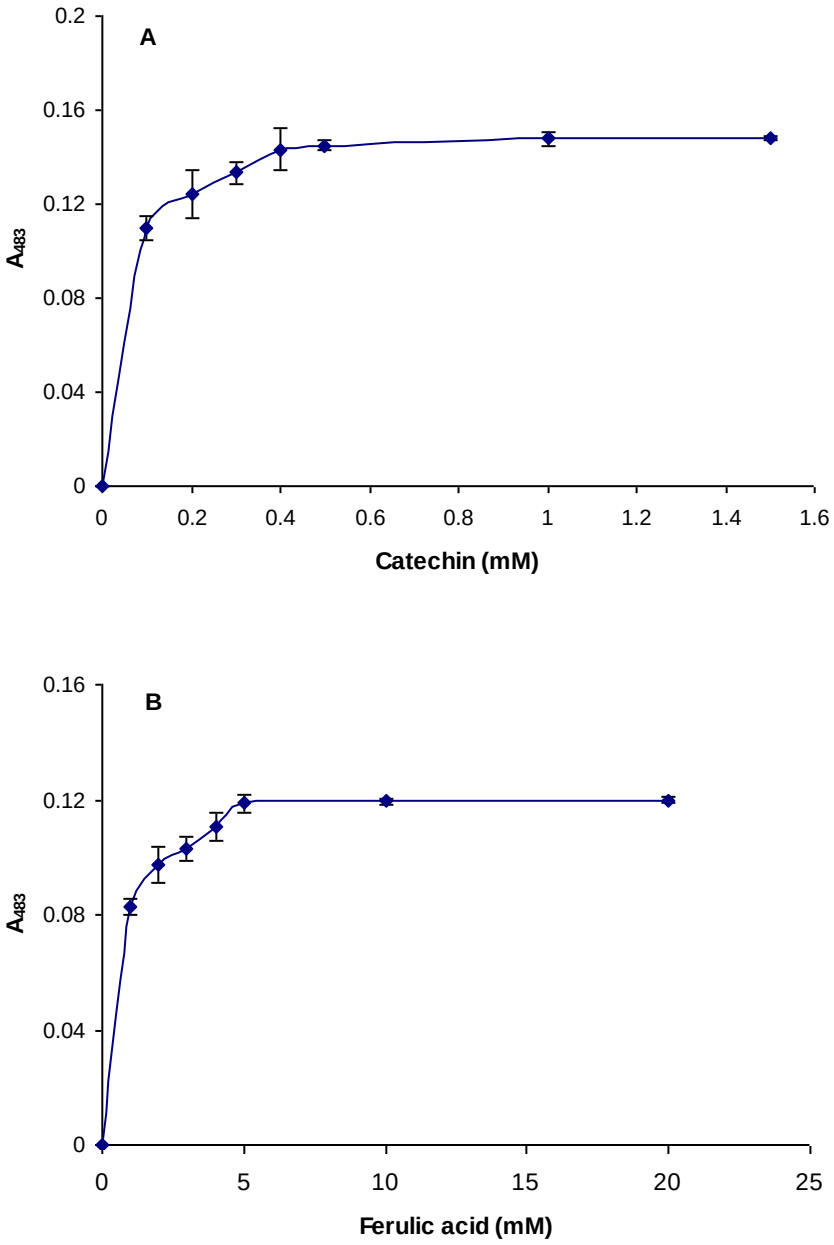

Figure 59. Copper reduction capability of catechin (A) and ferulic acid (B) at different concentrations. The reaction mixture was incubated at 25°C for 20 min and A_{483} was measured. Bars represent standard deviation (n=3).

Increased A_{483} indicated the formation of Cu^{+}, where Cu^{2+} was reduced to Cu^{+}. Nevertheless, the plateau was obtained when catechin and ferulic acid reached the concentration of 0.4 mM and 5 mM, respectively. Catechin and ferulic acid are well known phenolic compounds having reducing power (Maqsood and Benjakul, 2010). Green tea extract containing catechin had reducing power (electron donation) (Nirmal and Benjakul, 2011a). Chen *et al*. (1991a) reported that reduction of Cu^{2+} to Cu^{+} was increased with increasing kojic acid concentration up to 0.28 mM. PPO exists in three different types of isoforms namely oxy-PPO [Cu (II) Cu (II) O_2], met-PPO [Cu (II) Cu (II)] and deoxy-PPO [Cu (I) Cu (I)] (Likhitwitayawuid, 2008). Reduction of Cu^{2+} to Cu^{+} at the active site of PPO, could convert PPO into deoxy form, which may have the altered rate of enzymatic reaction. Ascorbic acid can reduce met-tyrosinase into deoxy-tyrosinase, thereby modifying the enzymatic turnover (Rodriguez-Lopez *et al*., 1992). This result suggested that catechin or ferulic acid could lower or slow down the dopachrome formation by reducing met-PPO [Cu (II) Cu (II)] to deoxy-PPO [Cu (I) Cu (I)].

10.4.4 Copper chelating activity of catechin and feurlic acid

Table 17 shows copper chelating activity of catechin or ferulic acid at different concentrations. Copper chelating activity of catechin or ferulic acid increased with increasing concentrations ($P < 0.05$).

Table 17. Copper chelating activity of catechin and ferulic acid at different concentrations

Inhibitor	Concentration (mM)	Chelating activity (%)
Catechin	0.5	54.7 ± 0.37 f
	1	60.3 ± 0.28 e
	1.5	64.9 ± 0.63 d
Ferulic acid	5	89.0 ± 1.48 c
	10	92.1 ± 0.34 b
	20	94.5 ± 0.15 a

Different letters in the same column indicate the significant difference ($P < 0.05$).

It was noted that 94.5% chelating activity was obtained when 20 mM ferulic acid was used and 64.9% chelating activity was found as catechin at 1.5 mM was incorporated. The copper chelating activity of catechin or ferulic acid was in accordance with their PPO inhibitory activity (Figure 58). Plant phenolic compounds had capacity of chelating metal ion, which is dependent on the number of hydroxyl group in ortho position (Wettasinghe and Shahidi, 2002). Green tea extract containing catechin was able to inhibit PPO by chelating Cu^{2+} in the active site of PPO, leading to lower the PPO activity (Nirmal and Benjakul, 2011a). Therefore, copper chelating capacity of catechin or ferulic acid was one of inhibitory modes involved in PPO inhibition.

10.4.5 Effect of catechin and ferulic acid on browning reaction

A_{475} of dopachrome formation of the browning reaction in the presence of catechin or ferulic acid into the prior incubated assay mixture is shown in Figure 60. In general, continuous increase in dopachorme formation was observed in the control as the reaction time increased. The increasing rate of A_{475} was decreased when catechin or ferulic acid were added, suggesting that both compounds might form a complex with dopaquinone. As a result, the complexes formed might have the shifted absorbance maxima from 475 nm. Catechin (Nirmal and Benjakul, 2009b) and ferulic acid (Nirmal and Benjakul, 2009a) could prevent the blackening in Pacific white shrimp by converting quinone to diphenol. The addition of kojic acid into the reaction mixture containing PPO from lobster and DL-DOPA as substrate caused the change of the color from red-brown to violet (Chen *et al*., 1991a). This result was concomitant with the inhibitory activity staining of catechin and ferulic acid (Figure 57), where the activity bands became smaller in the presence of both compounds.

The result indicated that formation of red-brown color compound from L-DOPA induced by PPO was retarded by catechin or ferulic acid and pale yellow color was observed instead. Catechin or ferulic acid might reduce quinone back to diphenol or react with dopachrome to form pale yellow color complex.

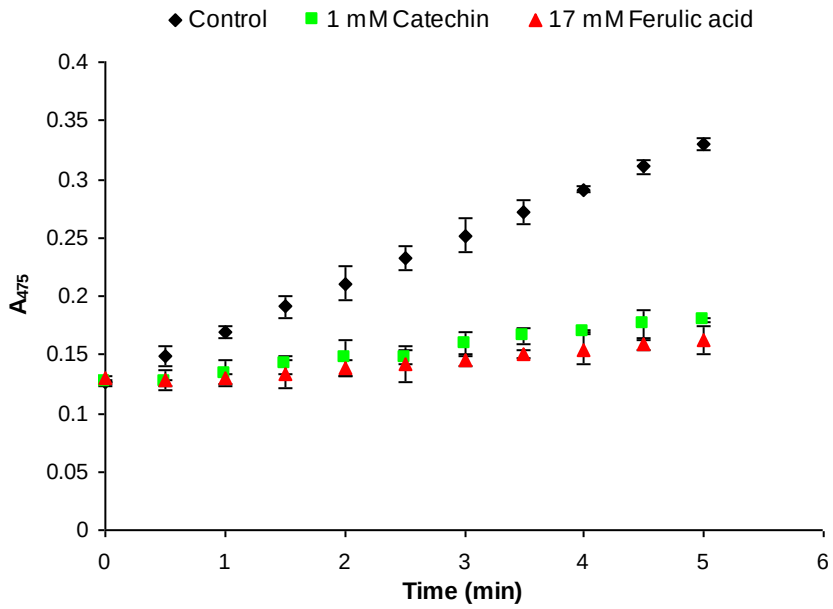

Figure 60. Absorbance of dopachrome formed at different times after the addition of catechin (1 mM) or ferulic acid (17 mM). The assay mixture was incubated at 25°C for 3 min prior to addition of catechin or ferulic acid. A_{475} was monitored for another 5 min at 25°C. Bars represent standard deviation (n=3).

10.5 Conclusions

Catechin or ferulic acid showed dose dependent inhibitory activity towards PPO from cephalothorax of Pacific white shrimp with different inhibition kinetics. Catechin and ferulic acid exhibited mixed type and non-competitive reversible inhibition on PPO, respectively. Both phenolic compounds showed copper (Cu^{2+}) reduction and chelating capability. Catechin or ferulic acid also affected the browning product by reduction of quinone to diphenol or by formation of yellow color complex. Thus, catechin or ferulic acid could be used as natural PPO inhibitors, which could prevent melanosis formation in Pacific white shrimp.

CHAPTER 11

INHIBITORY EFFECT OF MIMOSINE ON POLYPHENOLOXIDASE FROM CEPHALOTHORAXES OF PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*)

11.1 Abstract

The inhibitory effect of mimosine on polyphenoloxidase (PPO) from cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*) was studied. Mimosine showed inhibitory activity towards PPO from white shrimp as evidenced by the decrease in activity staining band appear as compare to control with apparent molecular weight of 210 kDa. Inhibition kinetic study revealed that mimosine exhibited the mixed type reversible inhibition on PPO from white shrimp with K_i value of 3.7 mM. Mimosine showed copper (Cu^{2+}) reduction and chelating capacity in a dose dependent manner. Mimosine could react with browning reaction, thereby rendering lower red-brown color formation. Therefore, mimosine could inhibit PPO by different modes of inhibition and could be used to prevent melanosis formation in Pacific white shrimp.

11.2 Introduction

Black spot formation (melanosis) is one of the serious problems occurring in crustaceans during post-mortem handling and storage. Melanosis in shrimp drastically reduces the consumer acceptability and the product s market value, leading to considerable financial loss (Nirmal and Benjakul, 2011a). Melanosis is triggered by a biochemical mechanism which oxidizes phenols to quinones by polyphenoloxidase. Polyphenoloxidase (PPO) is also known as phenolase, tyrosinase and catechol oxidase and is involved in vertebrate pigmentation and browning of fruits and vegetables (Espin *et al*., 1998). PPO is a bifunctional, copper-containing enzyme which catalyzes two basic reactions, in the presence of molecular oxygen.

Those include *o*-hydroxylation of monophenols to give *o*-diphenols (Monophenol oxidase, EC 1.14.18.1) and the subsequent oxidation of *o*-diphenols to *o*-quinones (Diphenoloxidase, EC 1.10.3.1) (Garcia-Molina *et al*., 2005). PPO exists in three different types of isoforms namely oxy-PPO [Cu (II) Cu (II) O_2], met-PPO [Cu (II) Cu (II)] and deoxy-PPO [Cu (I) Cu (I)] (Likhitwitayawuid, 2008).

The intensity of melanosis formation in the crustacean is varying with species, most likely due to the differences in substrate and enzyme concentration (Benjakul *et al*., 2005a). Many studies have focused on either inhibiting or preventing PPO activity by eliminating one or more of the essential components, e.g. enzyme, oxygen, copper from the reaction (Gokoglu and Yerlikaya, 2008). To control the undesirable browning in crustaceans, sulfite derivatives and 4-hexylresorcinol have been intensively used. Owing to the strict regulation for the use of sulfiting agents and high price of commercial PPO inhibitor, the interest in natural additives for retardation of melanosis in shrimp has become increasing (Nirmal and Benjakul, 2010a). Recently, plant phenolic compounds including grape seed extract (Gokoglu and Yerlikaya, 2008), ergothioneine from mushroom extract (Encarnacion *et al*., 2010) and green tea extract (Nirmal and Benjakul, 2011a) were found as the effective additive to retard melanosis in shrimp. Benjakul *et al*. (2006) reported that amino acid like cysteine or glutathione had inhibitory effect on PPO from kuruma prawn (*Penaeus japonicus*).

Pacific white shrimp (*Litopenaeus vannamei*) accounts for 90% of the global aquaculture shrimp production (Nirmal and Benjakul, 2009a). Thailand is the worlds leading shrimp farming country and has become the top supplier of farmed shrimp to the USA (Nirmal and Benjakul, 2010b). Shrimp is a very perishable product and generally has the limited shelf-life due to the formation of black spots (Nirmal and Benjakul, 2009b). Recently, lead (*Leucaena leucocephala*) seed extract has been reported to prevent melanosis formation in Pacific white shrimp during iced storage (Nirmal and Benjakul, 2011b). *L. leucocephala* contains a non-protein amino acid called mimosine, (-(3-hydroxy-4-pyridon-1-yl)-L-alanine) (Lalitha and Kulothungan, 2006). Therefore, better understanding in kinetics and mode of inhibition towards PPO of Pacific white shrimp should pave a way for melanosis control in shrimp and its products. However, there is no information on the inhibitory mechanism of

mimosine towards PPO from Pacific white shrimp cultured in Thailand. The objective of this study was to elucidate the inhibition mechanism of mimosine towards PPO from Pacific white shrimp.

11.3 Materials and Methods

11.3.1 Chemicals

L- -(3, 4 dihydroxylphenyl) alanine (L-DOPA), Brij-35, bathocuproine disulfonic acid, tetramethylmurexide (TMM), and L-mimosine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dedocyl sulfate (SDS), ammonium sulfate, and cupric sulfate were obtained from Merck (Darmstadt, Germany). Coomassie Blue R-250 and *N, N, N N* -tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). High-molecular-weight markers and DEAE-Sephacel were purchased from GE Healthcare UK Limited (Buckinghamshire, UK).

11.3.2 Shrimp collection and preparation

Pacific white shrimp (*Litopenaeus vannamei*) with the size of 55-60 shrimp/kg were purchased from a supplier in Songkhla, Thailand. The shrimp were freshly caught and completely free of additives, were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, shrimp were washed in cold water and stored in ice until used (not more than 3 h). The cephalothoraxes of shrimp were separated, pooled and powdered by grinding with liquid nitrogen in a Waring blender (AY46, Moulinex, Guangdong, China). The powder obtained was kept in polyethylene bag and stored at -20°C for not more than 2 weeks.

11.3.3 Extraction of PPO

Cephalothorax powder (50 g) was mixed with 150 mL of 0.05 M sodium phosphate buffer (pH 7.2) containing 1.0 M NaCl, 0.2% Brij 35, and 1 mg/mL of Phenilmethanesulfonyl fluoride (PMSF). The mixture was stirred continuously at 4^{o}C for 30 min, followed by centrifugation at 8000 x g at 4^{o}C for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). Solid ammonium sulfate was added into the supernatant to obtain 40% saturation (Nirmal and Benjakul, 2009a). The precipitate was collected by centrifugation at 12,500 x g at 4^{o}C for 30 min using a refrigerated centrifuge. The pellet obtained was dissolved in a minimum volume of 0.05 M sodium phosphate buffer, pH 7.2 and dialyzed against 50 volumes of the same buffer at 4^{o}C with three changes of dialysis buffer. The insoluble materials were removed by centrifugation at 3000 x g at 4^{o}C for 30 min.

11.3.4 DEAE-Sephacel column chromatography of PPO

The clear ammonium sulfate fraction was applied onto DEAE-Sephacel column (1.6 x 16 cm), previously equilibrated with 0.05M phosphate buffer, pH 7.2 (Chen *et al*., 1991b). The column was then washed with the same phosphate buffer until A_{280} was lower than 0.05. PPO was eluted with a linear gradient of 0 to 1.2 M NaCl in 0.05 M phosphate buffer (pH 7.2) at a flow rate of 0.5 mL/min. Fractions of 1.5 mL were collected and those with PPO activity were pooled. The pooled fractions were dialyzed with 50 volumes of 0.05 M phosphate buffer (pH 7.2) with two changes within 12 h. PPO from cephalothorax of Pacific white shrimp was purified to 83.7 fold after being chromatographed using DEAE-Sephacel column.

11.3.5 PPO activity assay

PPO activity was assayed using L-DOPA as a substrate according to the method of Nirmal and Benjakul (2009a) with a slight modification. The assay system consisted of 100 L of PPO solution, 600 L of 15 mM L-DOPA in deionized

water, 400 L of 0.05 M phosphate buffer (pH 6.0) and 100 L of deionized water. The PPO activity was determined for 3 min at 55^{o}C by monitoring the formation of dopachrome at 475 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of PPO activity was defined as an increase in the absorbance at 475 nm by 0.001 /min. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture and the deionized water was used instead.

11.3.6 Effect of mimosine on PPO activity

To study the inhibitory effect of mimosine towards PPO, DEAE-Sephacel fraction with PPO activity of 686.9 U/mL was incubated with mimosine at a ratio of 1:1 (v/v) to obtain the final concentration of 5 and 10 mM. The mixtures were allowed to stand for 30 min at room temperature prior to loading onto polyacrylamide gel, followed by electrophoresis and activity staining, respectively (Nirmal and Benjakul 2009a).

11.3.7 Inhibition kinetics of mimosine on PPO

DEAE-Sephacel fraction (100 µL) was mixed with mimosine solution (100 µL) to obtain final concentrations of 0.5, 2.5 and 5.0 mM. The mixture was incubated for 5 min at room temperature (25^{o}C). To initiate the reaction, 1000 µL of L-DOPA in 0.05 M sodium phosphate buffer (pH 6.0) were added. At each concentration of mimosine, L-DOPA with seven different concentrations (0.5– 5 mM) was used as the substrate. The reaction mixtures were incubated for 3 min at 55^{o}C and the absorbance at 475 nm was measured using a UV-1800 spectrophotometer. The Michaelis constant (K_m) for PPO was determined by Lineweaver–Burk plots (Lineweaver and Burk, 1934) and K_i value was obtained from Dixon plot (Dixon, 1953).

11.3.8 Determination of copper (II) reduction capacity of mimosine

Reduction capacity of mimosine on cupric copper to cuprous copper was determined as per the method of Chen *et al*. (1991a). One milliliter of mimosine solution (0-5.0 mM) was mixed with 0.5 mL of 0.4 mM cupric sulfate. The mixtures were allowed to stand for 10 min at room temperature. Then 0.5 mL aliquot of 4 mM aqueous bathocuproine disulfonic acid was added. The reaction mixtures were incubated at room temperature for 20 min and the absorbance at 483 nm was measured. Since bathocuproine disulfonic acid could interact with Cu^{+} to form a red-color complex having an optimal absorption at 483 nm, the reduction capability of mimosine was determined from the measurement of the absorbance at this wavelength. Blank was prepared in the same manner except deionized water was used instead of mimosine solution.

11.3.9 Determination of copper chelating capacity of mimosine

Copper chelating capacity of mimosine (0.5, 2.5 and 5.0 mM) was determined according to the method of Wettasinghe and Shahidi (2002) as modified by Nirmal and Benjakul (2011a).

11.3.10 Effect of mimosine on browning reaction

The reaction mixture containing 100 µL of DEAE-Sephacel fraction, 400 µL assay buffer and 600 µL of L-DOPA was incubated at 25^{o}C for 3 min. To the reaction mixtures, 100 µL of mimosine (10 and 20 mM) were added immediately and mixed thoroughly. Final concentration of mimosine in the reaction mixture was 0.8 and 1.6 mM. Red-brown color developed was monitored by measuring the absorbance at 475 nm up to 5 min at 25^{o}C. For the control, deionized water (100 µL) was added instead of mimosine solution. Decrease in absorbance at 475 nm indicates the reduction of *o*-quinone to phenols or formation of quinone-mimosine complex (Benjakul *et al*., 2006).

11.3.11 Statistical analyses

All analyses were performed in triplicate and a completely randomized design (CRD) was used. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan s multiple range tests (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA).

11.4 Results and Discussion

11.4.1 Effect of mimosine on PPO from cephalothorax of Pacific white shrimp

Activity staining of DEAE-Sephacel fraction containing polyphenoloxidase from cephalothorax of Pacific white shrimp in the absence and presence of mimosine at the concentrations of 5 and 10 mM is illustrated in Figure 61.

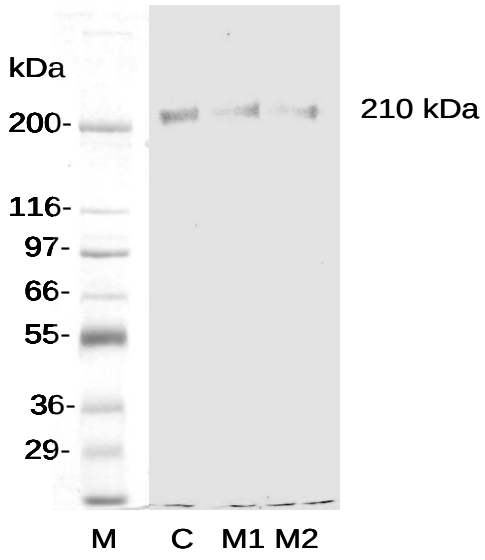

Figure 61. Activity staining of DEAE-Sephacel fraction containing polyphenoloxidase from cephalothorax of Pacific white shrimp in the absence and presence of mimosine at different concentrations. M: molecular weight marker; C: DEAE-Sephacel fraction; M1 and M2: DEAE-Sephacel fraction with 5 and 10 mM mimosine, respectively.

Mimosine showed inhibitory effect on PPO from cephalothorax of Pacific white shrimp as indicated by the lowered band intensity, as compared to that of control (without mimosine). Inhibitory activity of mimosine towards PPO was in a dose dependent manner. Nirmal and Benjakul (2011b) reported that lead (*Leucaena leucocephala*) seed extract powder containing mimosine had inhibitory effect on PPO from Pacific white shrimp. 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 most probably inhibit the PPO activity by competing with the substrate in binding with active site of PPO. Based on activity staining, PPO had molecular weight of 210 kDa. This result reconfirmed our previous report (Nirmal and Benjakul, 2009a) where PPO from cephalothorax of Pacific white shrimp showed molecular weight of 210 kDa. Zamorano *et al*. (2009) studied the electrophoretic mobility of PPO from deepwater pink shrimp (*Parapenaeus longirostris*) on non-reducing SDS-PAGE followed by staining with DOPA and found the activity band with molecular weight of 200 kDa. The viscera and carapace extracts from cephalothorax of Norway lobster (*Nephrops norvegicus*) showed both mono and diphenoloxidase activity when activity staining was carried out using L-tyrosine and 4-tert-butyl-catechol as substrates. Apparent molecular weight of PPO was 200- 220 kDa (Gimenez *et al*., 2010). PPO from cephalothorax of Pacific white shrimp could not use L-tyrosine as a substrate (data not shown), suggesting that lack of monophenoloxidase activity of PPO. *Leucaena leucocephala* seed extract powder containing mimosine and phenolic compounds could chelate a copper ion at the active site of PPO, thus inactivating PPO (Nirmal and Benjakul, 2011b).

11.4.2 Mode of PPO inhibition by mimosine

From the Lineweaver-Burk plots, the Michaelis constant (K_m) for the oxidation of L-DOPA by PPO in DEAE-Sephacel fraction was 2.43 mM. Km values reflect the affinity of enzymes for their substrates. The K_m observed for Pacific white shrimp PPO was approximately equal to that reported for the oxidation of DL-DOPA (K_m 2.8 mM) by *Panaeus setiferus* PPO (Simpson *et al*., 1988). The K_m value of 0.26 mM was found for oxidation of L-DOPA by PPO from kuruma prawn (*Penaeus*

japonicus) (Benjakul *et al*., 2006). The K_m value for oxidation of L-DOPA by *Charybdis japonica* PPO was 3.41 mM (Liu *et al*., 2006). Higher K_m value indicates the lower catalytic efficiency of the enzyme towards substrate (Liu *et al*., 2006). Variations in enzyme preparation and assay methods can be associated with differences in K_m values (Chen *et al*., 1991b).

Inhibition kinetics of mimosine towards PPO from cephalothorax of Pacific white shrimp was studied from Lineweaver-Burk plot as shown in Figure 62. Mimosine at different concentrations affected both K_m and V_{max} value of PPO. Since K_m value increased and V_{max} value decreased with increasing mimosine concentrations, the inhibitory mode of mimosine was found to be a mixed type.

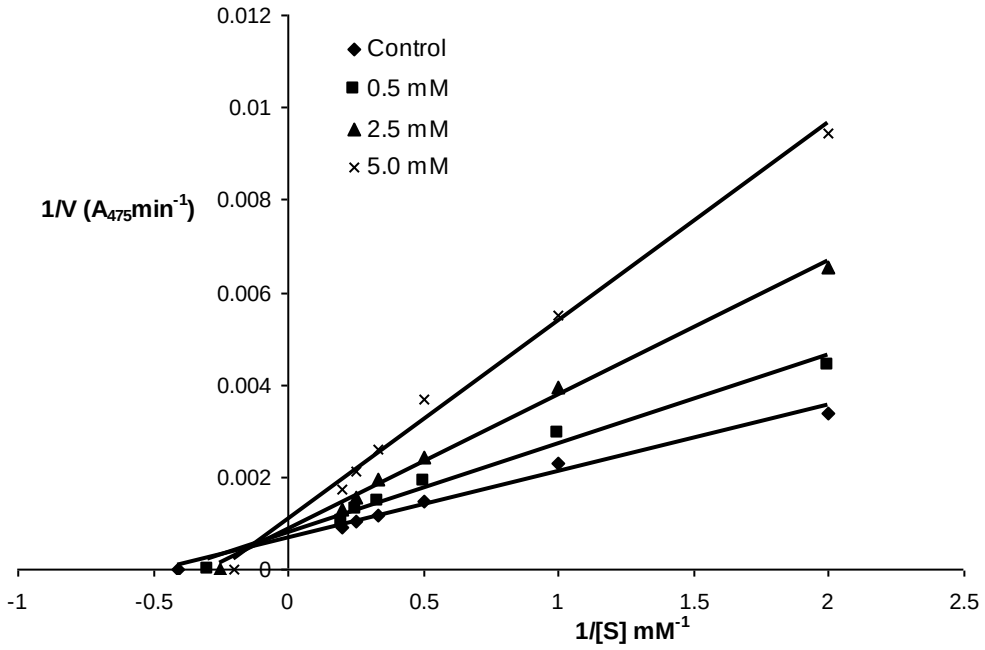

Figure 62. Lineweaver- Burk plot of polyphenoloxidase in DEAE-Sephacel fraction from cephalothorax of Pacific white shrimp in the absence and presence of mimosine at different concentrations. L-DOPA at levels of 0.5- 5 mM were used as substrate.

Results indicated that mimosine could bind with both enzyme and enzyme-substrate complex, but with different affinities. Mimosine and kojic acid was reported to be a standard inhibitor for mushroom tyrosinase with competitive type

inhibition (Fadimatou *et al*., 2010; Sabudak *et al*., 2006). Kojic acid showed a mixed type inhibition towards white shrimp, grass prawn and lobster PPO (Chen *et al*, 1991b). K_i value of mimosine obtained from Dixon plots was 3.7 mM. Inhibition constant K_i value of dodecyl gallate on mushroom tyrosinase was 0.636 mM (Kubo *et al*., 2003). Benjakul *et al*. (2006) reported that cysteine and glutathione showed competitive inhibition toward kuruma prawn PPO with K_i values of 0.45-0.46 mM. The plots of residual enzyme activity vs. the concentrations of enzyme in the presence of different concentrations of mimosine showed straight lines, which passed through the origin (data not shown). Mimosine at higher concentrations resulted in the decrease in the slope of line, suggesting its reversible inhibition towards PPO. The decrease in the slope of the lines with increasing concentration of dodecyl gallate indicated the reversible inhibition (Kubo *et al*., 2003). Therefore, mimosine showed mixed type reversible inhibition on PPO from cephalothorax of Pacific white shrimp.

11.4.3 Copper reduction capacity of mimosine

Copper reduction capacity of mimosine at different concentrations is shown in Figure 63. Increases in the absorbance at 483 nm indicate the formation of Cu^+. Increased absorbance was observed as the concentration of mimosine increased up to 0.5 mM. Thereafter, no changes in absorbance were found, indicating that all Cu^{2+} ions were reduced to Cu^+. With increasing kojic acid concentration up to 0.28 mM, the copper reduction capacity of kojic acid increased (Chen *et al*., 1991a). PPO at met-[Cu^{2+} Cu^{2+}] form is reduced by reductant to deoxy-PPO [Cu^+ Cu^+], which then interacts with oxygen to form oxy-PPO [Cu^{2+} Cu^{2+} O_2]. Oxy-PPO is highly active isoform, capable of catalyzing mono and diphenols (Chen *et al*., 1991a). Copper in the active site of PPO is primarily involved in the browning reaction (Benjakul *et al*., 2006). Reduction of Cu^{2+} to Cu^+ at the active site of PPO by mimosine could convert PPO in to deoxy form. Ascorbic acid can reduce met-tyrosinase into deoxy-tyrosinase, which modifies the enzymatic turnover (Rodriguez-Lopez *et al*., 1992). This result indicated that mimosine could lower or slow down the dopachrome formation by reducing met-[Cu^{2+} Cu^{2+}] to deoxy-PPO [Cu^+ Cu^+].

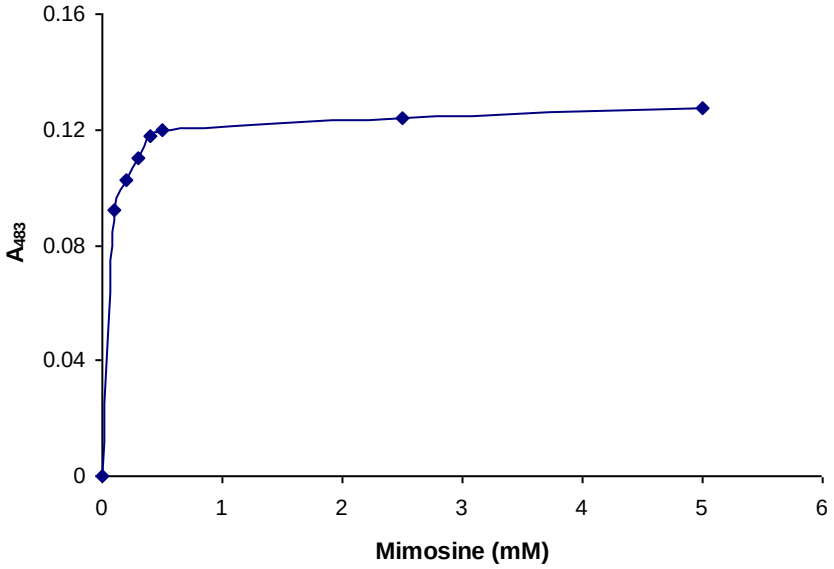

Figure 63. Copper reduction capacity of mimosine at different concentrations. The reaction mixture was incubated at 25^{o}C for 20 min. Absorbance was measured at 483 nm.

11.4.4 Copper chelating capacity of mimosine

Copper chelating activity of mimosine at different concentrations is shown in Table 18. Mimosine showed copper chelating activity at all concentrations tested. Copper chelating activity of mimosine was increased as the concentrations increased ($P < 0.05$). Copper chelating activity of 97.2 % was observed in the presence of 5 mM mimosine. The copper chelating activity of mimosine was in accordance with their PPO inhibitory activity (Figure 61).

Table 18. Copper chelating activity of mimosine at different concentrations

Mimosine (mM)	Chelating activity (%)
0.5	90.4 ± 0.48 c
2.5	95.5 ± 0.10 b
5.0	97.2 ± 0.15 a

Different letters in the same column indicates the significant difference ($P < 0.05$)

The inhibition of metal-dependent enzymes by L-mimosine was related to its chelating ability (Stunzi *et al*., 1980). Two alternative donor centres in the L-mimosine, (CO, O^-) maltol like and (COO^-, NH_2) -aminocarboxylate site contribute to copper chelating behaviour of L-mimosine (Chruscinska *et al*., 1999). At acidic condition L-mimosine deprotonated at -keto enolate site and at the carboxylic group, which favors copper chelation, whereas at basic conditions deprotonation of ammonium groups dominant for copper chelation. The *N*-nitroso and *N*-hydroxy group of hydroxylamine were suggested to be essential for the tyrosinase inhibitory activity, probably due to copper chelating ability (Shiino *et al*., 2001). Cysteine and glutathione might interact with copper at the active site of kuruma prawn PPO, leading to loss of the activity (Benjakul *et al*., 2006). Therefore, copper chelating capacity of mimosine is one of the important inhibitory mechanisms involved in inhibition of PPO from Pacific white shrimp.

11.4.5 Effect of mimosine on browning reaction

A_{475} representing dopachrome formation without and with addition of mimosine into the prior incubated assay mixture is illustrated in Figure 64. In general, continuous increase in dopachorme formation with time was observed in the control. The formation of dopachrome was retarded in the presence of mimosine. Lower rate of increase in A_{475} indicated that mimosine might chelate copper at the active site of PPO, rendering inactivation of PPO. Additionally mimosine could form a complex with dopaquinone, in which yellow complex with the maximum absorbance at 440 nm was observed (data not shown). This result was concomitant with the inhibition kinetics, where mimosine showed mixed type inhibition (Figure 62). Cysteine or glutathione could lower the dopachrom formation by reduction of quinone or formation of the cysteinyl adducts (Benjakul *et al*., 2006). The result indicated that formation of red-brown color compound in the reaction mixture through the action of PPO and L-DOPA was lowered by mimosine. Therefore, mimosine could inhibit dopachrom formation by reacting with PPO and PPO-DOPA complex. Furthermore, mimosine might chelate the copper ion at the active site of PPO or react with dopachrome to form yellow color complex.

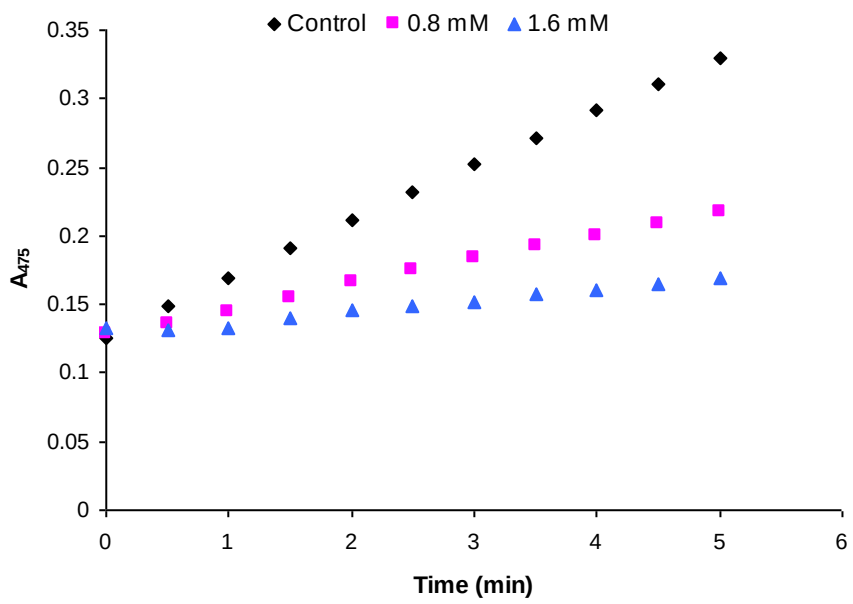

Figure 64. Absorbance of dopachrome after the addition of mimosine at different reaction times. The assay mixture was incubated at 25°C for 3 min prior to addition of mimosine. The absorbance at 475 nm was monitored for another 5 min at 25°C.

11.5 Conclusions

Mimosine showed dose dependent inhibitory activity towards PPO from cephalothorax of Pacific white shrimp. Mimosine exhibited mixed type reversible inhibition on PPO from white shrimp. Mimosine showed copper (Cu^{2+}) reduction and chelating capacity. Mimosine could impede the formation of browning product, by chelating copper at the active site of PPO or by formation of yellow color complex with PPO-DOPA. Thus mimosine could be used as a potential natural plant source to inhibit PPO and subsequently prevent melanosis formation in Pacific white shrimp.

CHAPTER 12

SUMMARY AND FUTURE WORKS

12.1 Summary

1. Ferulic acid or catechin could be used as the promising replacer of synthetic chemical such as SMS for melanosis prevention in shrimp during iced storage. Apart from prevention of melanosis, treatment of shrimps with ferulic acid or catechin could retard lipid oxidation, microbial growth and was able to maintain the freshness.

2. Repeated freeze-thawing increased melanosis of white shrimp. Thawing at the refrigerated condition (4^{o}C) was suggested to lower melanosis in shrimp. Catechin and ferulic acid could be used as the promising agent for melanosis prevention in shrimp with prior freeze-thawing during the subsequent storage.

3. GTE extracted with 80 % ethanol showed the higher PPO inhibitory activity and antioxidative activity, compared with mulberry tea extract. When ethanolic GTE with prior chlorophyll removal was used to soak Pacific white shrimp for 15 min, melanosis, microbial growth, and lipid oxidation were retarded. Efficacy of the extract was generally higher than SMS. Therefore, ethanolic GTE could be used as an alternative melanosis inhibitor or preservative in postmortem shrimp.

4. GTE showed the increase in PPO inhibitory activity, when AA was combined. Soaking time of 5 min was sufficient to lower melanosis in shrimp treated with 0.1 % GTE + AA (0.005 or 0.01 %). Treated shrimp had the lower quality loss during postmortem iced storage.

5. When shrimp were treated with GTE prior to MAP, microbial changes, chemical changes and melanosis formation were retarded more effectively than shrimp stored only under MAP. Treatment of GTE in conjunction with AA prior to MAP could improve sensory property of shrimp during refrigerated storage.

6. Lead brown seed extract could be used as the natural inhibitor of shrimp PPO and could retard the formation of melanosis during iced storage.

Therefore, the extract from lead brown seed could serve as an alternative processing aid for controlling melanosis in shrimp during postmortem handling or storage.

7. Partially purified PPO from cephalohtorax of shrimp showed optimal pH and temperature at 6 and 55^{o}C, respectively. Partially purified PPO with apparent molecular weight of 210 kDa might exist in the active form, which did not require any activators e.g. trypsin, copper ion or SDS. PPO with the higher catalytic activity and stability could be responsible for the rapid melanosis formation in shrimp, especially at the cephalothorax portion during postmortem handling and storage.

8. Catechin or mimosine and ferulic acid exhibited mixed type and non-competitive reversible inhibition on PPO, respectively. These phenolic compounds showed copper (Cu^{2+}) reduction and chelating capability. Catechin or ferulic acid or mimosine also affected the browning reaction by formation of yellow color complex. Thus, catechin or mimosine or ferulic acid could be used as natural PPO inhibitors, which could prevent melanosis formation in Pacific white shrimp.

12.2 Future works

1. Phenolic compounds from different sources should be screened for PPO inhibitory activity and ability to retard melanosis and quality losses during iced or refrigerated storage.

2. Different catechin derivatives can be studied for their PPO and melanosis inhibition.

3. Effect of the selected phenolic compounds in combination with some PPO inhibitors on precooked shrimp should be investigated.

4. Treatment of plant extracts before harvesting of shrimp should be conducted to lower melanosis formation in shrimp or other crustaceans.

REFERENCES

Adachi, K., Endo, H., Watanabe, T., Nishioka, T. and Hirata, T. 2005. Hemocyanin in the exoskeleton of crustaceans: enzymatic properties and immunolocalization. Pigment Cell Res. 18: 136-143.

Adachi, K., Hirata, T., Nagai, K. and Sakaguchi, M. 2001. Hemocyanin a most likely inducer of black spots in kuruma prawn *Penaeus japonicus* during storage. J. Food Sci. 66: 1130-1136.

Adachi, K., Hirata, T., Nagai, K., Fujisawa, S., Kinoshita, M. and Sakaguchi, M. 1999. Purification and characterization of prophelnoxidase from kuruma prawn *Penaeus japonicus*. Fish Sci. 65: 919-925.

Ali, M.T, Gleeson,R. A., Wei, C. I., and Marshall, M. R. 1994. Activation mechanisms of pro-phenoloxidase on melanosis development in Florida spiny lobster (*Panulirus argus*) cuticle. J. Food Sci. 59: 1024–1030.

AOAC. 1990. Chlorophyll in plants. In *Official Methods of Analysis of the AOAC*; Helrich, K., Ed.; AOAC: Arlington, VA, 1990; pp 62−63.

Arias, E., Gonzalez, J., Peiro, J., M., Oria, R. and Lopez-Buesa, P. 2007. Browning prevention by ascorbic acid and 4-hexylresorcinol: different mechanisms of action on polyphenoloxidase in the presence and in the absence of substrates. J. Food Sci. 72: C464-C470.

Arslan, O., Erzengin, M., Sinan, S. and Ozensoy, O. 2004. Purification of mulberry (*Morus alba* L.) polyphenoloxidase by affinity chromatography and investigation of its kinetic and electrophoretic properties. Food Chem. 88: 479-484.

Bak, L. S., Andersen, A. B, Andersen, E. M., and Bertelsen, G. 1999. Effect of modified atmosphere packaging on oxidative changes in frozen stored cold water shrimp (*Pandalus borealis*). Food Chem. 64: 169-175.

Balange, A. and Benjakul, S. 2009. Enhancement of gel strength of bigeye snapper (*Priacanthus tayenus*) surimi using oxidised phenolic compounds. Food Chem. 113: 61–70.

Balasundram, N., Sundram, K. and Samman, S. 2006. Phenolic compound in plants and agri-industrial by-products: antioxidant activity, occurrence, and potential uses. Food Chem. 99: 191-203.

Banerjee, S. 2006. Inhibition of mackerel (*Scomber scombrus*) muscle lipoxygenase by green tea polyphenols. Food Res. Int. 39: 486-491.

Banon, S., Diaz, P., Rodriguez, M., Garrido, M. D. and Price, A. 2007. Ascorbate, green tea and grape seed extracts increase the shelf life of low sulphite beef patties. Meat Sci. 77: 626-633.

Bartolo, I. and Birk, E. O. 1998. Some factors affecting Norway lobster (*Nephrops norvegicus*) cuticle polyphenol oxidase activity and blackspot development. Int. J. Food Sci. Technol. 33: 329–336.

Benjakul, S. and Bauer, F. 2001. Biochemical and physicochemical changes in catfish (*Silurus glanis Linne*) muscle as influenced by different freeze-thaw cycles. Food Chem. 72: 207–217.

Benjakul, S., Seymour, T. S., Morrissey, M. T., and An, H. 1997. Physiochemical changes in Pacific whiting muscle proteins during iced storage. J. Food Sci. 62: 729-733.

Benjakul, S., Visessanguan, W. and Tanaka, M. 2005a. Properties of phenoloxidase isolated from the cephalothorax of kuruma prawn (*Penaeus japonicus*). J. Food Biochem. 29: 470-485.

Benjakul, S., Visessanguan, W. and Tanaka, M. 2006. Inhibitory effect of cysteine and glutathione on phenoloxidase from kuruma prawn (*Penaeus japonicus*). Food Chem. 98: 158-163.

Benjakul, S., Visessanguan, W., Phongkanpai, V. and Tanaka, M. 2005b. Antioxidative activity of caramelisation products and their preventive effect on lipid oxidation in fish mince. Food Chem. 90: 231-239.

Benner, R.A., Migit, R., Finne, G. and Acuff, G.R. 1994. Lactic acid/melanosis inhibitors to improve shelf life of brown shrimp (*Penaeus aztecus*). J. Food Sci. 59: 242-245, 250.

Bligh, E. G. and Dyer, W. J. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-931.

Boonsumrej, S., Chaiwanichsiri, S., Tantratian, S., Suzuki, T., and Takai, R. 2007 Effects of freezing and thawing on the quality changes of tiger shrimp (*Penaeus monodon*) frozen by air-blast and cryogenic freezing. J. Food Eng. 80: 292-299.

Brack, A., Hellmann, N. and Decker, H. 2008. Kinetics properties of hexameric tyrosinase from the crustacean *Palinurus elephas*. Photochem. Photobiol. 84: 692-699.

Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microorganism quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-254.

Brauer, J. M. E., Leyva, J. A. S., Alvarado, L. B. and Sandez, O. R . 2003. Effect of dietry protein on muscle collagen, collagenase and shear force of farmed white shrimp (*Litopenaeus vannamei*). Eur. Food Res. Technol. 217: 277–80.

Brown, J. E., Khodr, H., Hider, R. C. and Rice-Evans, C. A. 1998. Structural dependence of flavonoid interaction with Cu^{2+} ions: implications for their antioxidant properties. Biochemical J. 330: 1173-1178.

Buranov, A. U. and Mazza, Z. 2009. Extraction and purification of ferulic acid from flax shives, wheat and corn bran by alkaline hydrolysis and pressurized solvents. Food Chem. 115: 1542-1548.

Cabanes, J., Garcia-Canovas, F., Tudela, J., Lozano, J. A. and Garcia-Carmona, F. 1987. L-mimosine, a slow binding inhibitor of mushroom tyrosinase. Phytochem. 26: 917-919.

Cabrera, C., Artacho, R. and Gimenez, R. 2006. Beneficial effects of green tea- a review. J. Am. College Nutr. 25: 79-99.

Cadun, A., Kisla, D. and Cakh, S. 2008. Marination of deep-water pink shrimp with rosemary extract and the determination of its shelf-life. Food Chem. 109: 81-87.

Champanerkar, P. A., Vaidya, V. V., Shailajan, S. and Menon, S. N. 2010. A sensitive rapid and validated liquid chromatography tandem mass spectrometry (LC-MS-MS) method for determination of mimosine in *Mimosa pudica* Linn. Natural Sci. 7: 713-717.

Chanchay, N. and Poosaran, N. 2009. The reduction of mimosine and tannin contents in leaves of *Leucaena leucocephala*. As. J. Food Ag-Ind. ISSN 1906-3040: S137-S144.

Chang, T. S. 2009. An updated review of tyrosinase inhibitors. Int. J. Mol. Sci. 10: 2440-2475.

Chanthachum, S. and Beuchat, L. R. 1997. Inhibitory effect of kiam (*Cotylelobium Ianceotatum* craih) wood extract on gram positive food-born pathogens and spoilage micro-organisms. Food Microbiol. 14: 603-608.

Chanwitheesuk, A. Teerawutgulrag, A. and Rakariyatham, N. 2005. Screening of antioxidant activity and antioxidant compounds of some edible plants of Thailand. Food Chem. 92: 491-497.

Charrouf, Z., Hilali, M., Jauregui, O., Soufiaoui, M. and Guillaume, D. 2007. Separation and characterization of phenolic compounds in argan fruit pulp using liquid chromatography negative electrospray ionization tandem mass spectroscopy. Food Chem. 100: 1398-1401.

Chen, J. S., Charest, D. J., Marshall, M. R. and Wei, C. I. 1997. Comparison of two treatment methods on the purification of shrimp polyphenol oxidase. J. Sci. Food Agric. 75: 12-18.

Chen, J. S., Rolle, R. S., Marshall, M. R. and Wei, C. I. 1991c. Comparison of phenoloxidase acticity from Florida spiny lobster and Western Australian lobster. J. Food Sci. 56: 154–160.

Chen, J. S., Wei, C. I. and Marshall, M. R. 1991b. Inhibition mechanism of kojic acid on polyphenol oxidase. J. Agric. Food Chem. 39: 1897-1901.

Chen, J. S., Wei, C. I., Rolle, R. S., Otwell, W. S., Balaban, M. O. and Marshall, M. R. 1991a. Inhibitory effect of kojic acid on some plant and crustacean polyphenoloxidases. J. Agric. Food Chem. 39: 1396–1401.

Cheriot, S., Billaud, C., Maillard, M. N. and Nicolas, J. 2007. Inhibition of polyphenoloxidase activity by mixtures of heated cysteine derivatives with carbonyl compounds. Mol. Nutr. Food Res. 51: 395-403.

Chruscinska, E., Garribba, E., Micera, G. and Panzanelli, A. 1999. $_{L}$-Mimosine, an amino acid with maltol-type binding properties towards copper (II), oxovanadium (IV) and other metal ions. J. Inorg. Biochem. 75: 225-232.

Chunmei, D., Jiabo, W., Weijun, K., Cheng, P. and Xiaohe, X. 2010. Investigation of antimicrobial activity of catechin on *Escherichia coli* growth by microcalorimetry. Env. Toxicol. Pharmacol. 30, 284-288.

Conway, E. J. and Byrne, A. 1936. An absorption apparatus for the micro-determination of certain volatile substances I. The micro-determination of ammonia. J. Biochem. 27: 419-429.

Decker, H. and Tuczek, F. 2000. Tyrosinase/catecholoxidase activity of hemocyanins: structural basis and molecular mechanism. Trends Biochem. Sci. 25: 392-396.

DeWitt, B. J. 1998. An improved methodology for the estimation of sulfur dioxide in shrimp. Resumen Internacional. 48: 3463–3464.

Diaz-Tenorio, L. M., Garcia-Carreno, F. L. and Pacheco-Aguilar, R. 2007. Comparison of freezing and thawing treatments on muscle properties of whiteleg shrimp (*Litopenaeus vannamei*). J. Food Biochem. 31: 563-576.

DiPersio, P. A., Kendall, P. A. and Sofos, J. N. 2004. Inactivation of *Listeria monocytogenes* during drying and storage of peach slices treated with acidic or sodium metabisulfite solutions. Food Microbiol. 21: 641-648.

Dixon, M. 1953. The determination of enzyme inhibitor constants. Biochem. J. 55: 170-171.

Dykes, L. and Rooney, L. W. 2007. Phenolic compounds in cereal grains and their health benefits. Cereal Foods World. 52: 105-111.

Echeverria, V., Belmar, R., Ly, J. and Santos-Ricalde, R. H. 2002. Effect of *Leucaena leucocephala* leaf meal treated with acetic acid or sodium hydroxide on apparent digestibility and nitrogen retention in pig diets. Animal Feed Sci. Technol. 101: 151-159.

Encarnacion, A. B., Fagutao, F., Hirono, I., Ushio, H. and Oshima, T. 2010. Effects of ergothioneine from mushrooms (*Flammulina velutipes*) on melanosis and lipid oxidation of kuruma shrimp (*Marsupenaeus japonicus*). J. Agric. Food Chem. 58: 2577-2585.

Erickson, A. J., Ramsewak, R. S., Smucker, A. J. and Nair, M. G. 2000. Nitrification inhibitors from the roots of *Leucaena leucocephala.* J. Agric. Food Chem. 48: 6174-6177.

Erickson, M. C., Bulgarelli, M. A., Resurreccion, A. V. A., Vendetti, R. A. and Gates, K. A. 2007. Sensory dofferentiation of shrimp using trained descriptive analysis panel. LWT-Food Sci. Technol. 40: 1774-1783.

Espin, J. C., Garcia-Ruiz, P. A., Tudela, J. and Garcia-Canovas, F. 1998. Study of stereospecificity in mushroom tyrosinase. Biochem. J. 331: 547-551.

Fadimatou, Momeni, J., Kimbu, S. F., Sondengam, B. L., Khan, M. T. H, Choudhary, M. I. and Rahman, A. U. 2010. Potent inhibitors of tyrosinase activity from citrullus colocynthis Schrad. (Cucurbitaceae). Acta Pharm. Sci. 52: 328-334.

Farhoosh, R., Golmovahhed, G. A. and Khodaparast, M. H. H. 2007. Antioxidant activity of various extracts of old leaves and black tea wastes (*Camellia sinensis* L.). Food Chem. 100: 231-236.

Fernandez, K., Aspe, E. and Roeckel, M. 2009. Shelf life extension on fillets of Atlantic Salmon (*Salmo salar*) using natural additives, superchilling and modified atmosphere packaging. Food Cont. 20: 1036-1042.

Ferrer, O.J., Koburger, J.A., Otwell, W.S., Gleeson, R.A., Simpson, B.K., and Marshall, M.R. 1989. Phenoloxidase from the cuticle of Florida spiny lobster (*Panulirus argus*): mode of activation and characterization. J. Food Sci. 54: 63-67.

Garcia-Carreno, F. L., Cota, K. and Toro, M. A .N. 2008. Phenoloxidase activity of hemocyanin in whiteleg shrimp *Penaeus vannamei*: conversion, characterization of catalytic properties, and role in postmortem melanosis. J. Agric. Food Chem. 56: 6454-6459.

Garcia-Molina, F., Munoz, J. L., Garcia-Ruiz, P. A., Rodriguez-Lopez J. N., Garcia-Canovas, F. and Tudela J. 2007. A further step in the kinetic characterisation of the tyrosinase enzymatic system. J. Math. Chem. 41: 393-406.

Garcia-Molina, F., Penalver, M. J., Rodriguez-Lopez J. N., Garcia-Canovas, F. and Tudela J. 2005. Enzymatic method with polyphenol oxidase for the determination of cysteine and N-acetylcysteine. J. Agric. Food Chem. 53: 6183-6189.

Gimenez, B. Martinez-Alvarez, O., Montero, P. and Gomez-Guillen, M. C. 2010. Characterization of phenoloxidase activity of carapace and viscera from cephalothorax of Norway lobster (*Nephrops norvegicus*), LWT- Food Sci. Technol. 43: 1240-1245.

Gokoglu**,** N. 2004. The effect of organic acid treatments on the melanosis inhibition and shelf-life in shrimp (*Penaeus Japonicus*). Acta Alimentaria. 33: 191-199.

Gokoglu**,** N. and Yerlikaya, P. 2008. Inhibition effects of grape seed extracts on melanosis formation in shrimp (*Parapenaeus longirostris*) Int. J. Food Sci. Technol. 43: 1004–1008.

Gollas-Galvan, T., Hernandez-Lopez, J. and Vargas-Albores, F. 1999. Prophenoloxidase from brown shrimp (*Penaeus californiensis*) hemocytes. Comp. Biochem. Physiol. 122B: 77-82.

Gomez-Guillen, M.C, Martinez-Alvarez, O., Llamas, A. and Montero, P. 2005. Melanosis inhibition and SO_2 residual levels in shrimps (*Parapenaeus longirostris*) after different sulfite-based treatments. J. Sci. Food Agric. 85: 1143–1148.

Goncalves, A. C., Lopez-Caballero, M. E. and Nunes, M. L. 2003. Quality changes of deepwater pink shrimp (*Parapenaeus longirostris*) packed in modified atmosphere. J. Food Sci. 68: 2586-2590.

Gram, L. and Dalgaard, P. 2002. Fish spoilage bacteria-problems and solutions. Curr. Opinion Biotech. 13: 262-266.

Gram, L. and Huss, H. H. 1996. Microbiological spoilage of fish and fish products. International. J. Food Microbiol. 33: 121-137.

Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B. and Givskov, M. 2002. Food spoilage interactions between food spoilage bacteria. Int. J. Food Microbiol. 78: 79-97.

Guandalini, E., Ioppolo, A., Mantovani, A., Stachini, P. and Giovannini, C. 1998. 4-hexylresorcinol as inhibitor of shrimp melanosis: efficacy and residue studies; evaluation of possible toxic effect in a human intestinal *in vitro* model (Caco-2); preliminary safety assessment. Food Add. Contam. 15: 171-180.

Gupta, S., Saha, B. and Giri, A.K. 2002. Comperative antimutagenic and anticlastogenic effects of green tea and black tea: a review. Mutt. Res. 512: 37-65.

Hashiguchi, H. and Takahashi, H. 1977. Inhibition of two copper containing enzymes, tyrosinase and dopamine β-hydroxylase, by L-mimosine. Mol. Phramacol. 13: 362-367.

Hatano, T., Tsugawa, M., Kusuda, M., Taniguchi, S., Yoshida, T., Shiota, S. and Tsuchiya, T. 2008. Enhancement of antibacterial effects of epigallocatechin gallate, using ascorbic acid. Phytochem. 69: 3111-3116.

Herrera, M. C. and Luque de Castro, M. D. 2005. Ultrasound-assisted extraction of phenolic compounds from strawberries prior to liquid chromatographic separation and photo diode array ultraviolet detection. J. Chromatogr. A. 1100: 1-7.

Himmelwright, R.S., Eickman, N.C., Lubien, C.D., Lerch, K. and Solomon, E.I. 1980. Chemical and spectroscopic studies of the binuclear copper active site of *Neurospora* tyrosinase. Comparison to hemocyanins. J. Am. Chem. Soc. 102: 7339-7344.

IFST. 1999. Development and use of microbiological criteria for foods, pp 79. Inst. Food Sci. Technol. London.

Jang, M. S., Sanada, A., Ushio, H., Tanaka, M. and Ohshima, T. 2003. Inhibitory effect of enokitake extract on melanosis of shrimp. Fish. Sci. 69: 379-384.

Janovitz-klapp, A.H., Richard, F. C., Goupy, P. M. and Nicolas, J. J. 1990. Inhibiton studies on apple polyphenol oxidase. J. Agric. Food Chem. 38: 926-931.

Januario, F., E., S. and Dykes, G. A. 2005. Effect of sodium metabisulfite and storage temperature on the survival of *Vibrio cholerae* on prawn s (*Penaeus monodon*). World J. Microbiol. Biotechnol.21: 1017-1020.

Jayaprakash, G. K., Singh, R. P. and Sakariah, K.K. 2001. Antioxidant activity of grape seed (*Vitis vinifera*) extract on peroxidation model *in vitro*. Food Chem. 73: 285-290.

Jayaprakasha, G. K., Selvi, T. and Sakariah, K. K. 2003. Antibacterial and antioxidant activities of grape seed extracts. Food Res. Int. 36: 117-122.

Jeon, M. and Zhao, Y. 2005. Honey in combination with vacuum impregnation to prevent enzymatic browning of fresh-cut apples. Int. J Food Sci. Nutr. 56: 165-176.

Jeyasekaran, G., Ganesan, P., Anandaraj, R., Shakila, R. J. and Sukumar, D. 2006. Quantitative and qualitative studies on the bacteriological quality of Indian white shrimp (*Penaeus indicus*) stored in dry ice. Food Microbiol. 23: 526-533.

Jiang, M., Liu, S. and Wang, Y. 2011. Effects of antimicrobial coating from catfish skin gelatin on quality and shelf life of fresh white shrimp (*Penaeus vannamei*). J. Food Sci. 76: M204-M209.

Kamada, Y., Oshiro, N., Oku, H., Hongo, F. and Chinen, I. 1997. Mimosine toxicity in broiler chicks fed *Leucaena leucocephala* seed powder. Animal Sci. Technol. 68: 121-130.

Kanski, J., Aksenova, M., Stoyanova, A. and Butterfield, D. A. 2002. Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems *in vitro*: structure activity studies. J Nutr. Biochem. 13: 273-281.

Katsube, T., Imawaka, N., Kawano, Y., Yamazaki, Y., Shiwaku, K. and Yamane, Y. 2006. Antioxidant flavonol glycosides in mulberry (*Morus alba* L.) leaves isolated based on LDL antioxidant activity. Food Chem. 97: 25-31.

Kaufmann, B. and Christen, P. 2002. Recent extraction techniques for natural products: Microwave-assisted extraction and pressurized solvent extraction. Phytochem. Anal. 13: 105-113.

Kim, J., Marshall, M.R. and Wei, C. 2000. Polyphenoloxidase. *In* Seafood enzyme utilization and influence on post harvest seafood quality. (Haard, N.F., Simpson, B.K., eds) P. 271 -315. Marcel Dekker. New York.

Koka, R. and Weimer, B. C. 2001. Influence of growth conditions on heat stable phospholipase activity in *Pseudomonas*. J. Dairy Res. 68: 109-116.

Kubo, I. and Kinst-Hori, I. 1998. Tyrosinase inhibitor from cumin. J. Agric. Food Chem.46: 5338-5341.

Kubo, I., Chen, Q-X., and Nihei, K-I. 2003. Molecular design of antibrowning agents, antioxidative tyrosinase inhibitors. Food Chem. 81: 241–247.

Kubo, I., Kinst-Hori, I., Kubo, Y., Yamagiwa, Y., Kamikawa, T. and Haraguchi, H. 2000. Molecular design of antibrowning agents. J. Agric. Food Chem. 48: 1393-1399.

Kumudavally, K. V., Phanindrakumar, H. S., Tabassum, A., Radhakrishna, K. and Bawa, A. S. 2008. Green tea- a potential preservative for extending the shelf-life of fresh mutton at ambient temperature (25 ± 2^{o}C). Food Chem. 107: 426-433.

Laemmli, U.K. 1970. Cleavage of structure proteins during the assembly of the head of bacteriophage T_4. Nat. 277: 680-685.

Lakshmanan, R., Shakila, R. J., and Jeyasekaran, G. 2002. Survival of amine forming bacteria during the ice storage of fish and shrimp. Food Microbiol. 19:617-625.

Lalitha, K. and Kulothungan, S. R. 2006. Selective determination of mimosine and its dihydroxypyridinyl derivative in plant systems. Amino Acids. 31: 279-287.

Lalitha, K. V. and Surendran, P. K. 2006. Microbiological changes in farm reared freshwater prawn (Macrobrachium rosenbergii de Man) in ice. Food Cont. 17: 802-807.

Lalitha, K., Vargheese, C. M. and Balasubramanian, N. 1993. Spectrophotometric determination of mimosine and 3-nydroxy-4-(1H)-pyridone- The toxic principles of *Leucaena leucocephala*. Anal. Biochem. 213: 57-62.

Lambrecht, H.S. 1995. Sulfite substitutes for the prevention of enzymatic browning in foods. *In* Enzymatic Browning and Its Prevention. (Lee, C.Y. and Whitaker, J.R., eds). P. 313-323. American Chemistry Society. Washington, DC.

Lee, S. Y., Wang, R. and Sorderhall, K. 2000. A lipopolysaccharide- and β-1,3-glucan-binding protein from hemocytes of the freshwater crayfish *Pacifastacus leniusculus* purification, characterization, and cDNA cloning. J. Biol. Chem. 275: 1337-1343.

Leuang, L. K., Su, Y., Chen, R., Zhang, Z., Huang, Y. and Chen, Z. Y. 2001. Theaflavins in black tea and caetchins in green tea are equally effective antioxidants. J. Nutr. 2248-2251.

Li, S. B., Xue, Y., Lv, X. Y., Nie, H. L., Zhu, L. M., Zhang, H. T., Qiu, T. and Zhou, L. M. 2009. *In vitro* effect of ozagrel on mushroom tyrosinase. Protein J. 28: 182-188.

Likhitwitayawuid, K. 2008. Stilbenes with tyrosinase inhibitory activity. Curr. Sci. 94: 44-52.

Lineweaver, H. and Burk, D. 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56: 658-666.

Liu, G., Yang, L., Fan, T., Cong, R., Tang, Z., Sun, W. Meng, X. and Zhu, L. 2006. Purification and characterization of phenoloxidase from crab *Charybdis japonica*. Fish. Shellfish. Immun. 20: 47-57.

Lo, H. H. and Chung, J. G. 1999. The effects of plant phenolics, caffeic acid, chlorogenic acid and ferulic acid on arylamine N-acetyltransferase activities in human gastrointestinal microflora. Anticancer Res. 19: 133-139.

Lopez-Caballero, M. E., Goncalves, A. and Nunes, M. L. 2002. Effect of CO_2/O_2-containing modified atmospheres on packed deepwater pink shrimp (*Parapenaeus longirostris*). Eur. Food Res. Technol. 214: 192-197.

Lopez-Caballero, M. E., Martinez-Alvarez, O. Gomez-Guillen, M. C. and Montero, P. 2007. Quality of thawed deepwater pink shrimp (*Parapenaeus longirostris*) treated with melanosis-inhibiting formulations during chilled storage. Int. J. Food Sci. Technol. 42: 1029–1038.

Lopkulkiaert, W., Prapatsornwattana, K. and Rungsardthong, V. 2009. Effects of sodium bicarbonate containing traces of citric acid in combination with sodium chloride on yield and some properties of white shrimp (Penaeus vannamei) frozen by shelf freezing, air-blast and cryogenic freezing. LWT-Food Sci. Technol. 42: 768-776.

Lowery, J. B., Cook, N. and Wilson, R. D. 1984. Flavonol glycoside distribution in cultivars and hybrids of *Leucaena leucocephala*. J. Sci. Food Agric. 35: 401-407.

Lowry, O. H., Rosebrough, N. J., Fan, A. L. and Randall, R. J. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 256-275.

Lu, S. 2009. Effects of bactericides and modified atmosphere packagning on shelf-life of Chinese shrimp (*Fenneropenaeus chinensis*). LWT-Food Sci. Technol. 42: 286-291.

Luthria, D. L., Mukhopadhyay, S. and Kwansa, A. L. 2006. A systematic approach for extraction of phenolic compounds using parsley (*Petrpselinum crispum*) flakes as a model substrate. J. Sci. Food Agric. 86: 1350-1358.

Ma, Y. Q., Chen, J. C., Liu, D. H. and Ye X. Q. 2009. Simultaneous extraction of phenolic compounds of citrus peel extracts: effect of ultrasound. Ultrasonics Sonochem. 16: 57-62.

Ma, Y., Ye, X., Hao, Y., Xu, G., Xu, G. and Liu, D. 2008. Ultrasound-assisted extraction of hesperidin from Penggan (*Citrus reticulata*) peel. Ultrasonics Sonochem. 15: 227-232.

Madero, C.F. and Finne, G. 1982. Properties of polyphenoloxidase isolated from gulf shrimp. Proc Seventh Annual Trop Subtrop Fish Technol Conf Americas. 7: 328-339.

Madhan, B.; Krishnamoorthy, G.; Rao, J. R. and Nair, B.U. 2007. Role of green tea polyphenolos in the inhibition of collagenolytic activity by collagenase. Int. J. Biol. Macromole. 41: 16-22.

Maillard, M. N., Billaud, C., Chow, Y. N., Ordonaud, C. and Nicolas, J. 2007. Free radical scavenging, inhibiton of polyphenoloxidase activity and copper chelating properties of model Maillard systems. LWT-Food Sci. Tenol. 40: 1434-1444.

Majchrzak, D., Mitter, S. and Elmadfa, I. 2004. The effect of ascorbic acid on total antioxidant activity of black and green teas. Food Chem. 88: 447-451.

Maqsood, S. and Benjakul, S. 2010. Comparative studies of four different phenolic compounds on *in vitro* antioxidative activity and the preventive effect on lipid oxidation of fish oil emulsion and fish mince. Food Chem. 119: 123-132.

Martinez, M. V. and Whitaker J. R. 1995. The biochemistry and control of enzymatic control. Trends Food Sci. Technol. 6: 195-200.

Martinez-Alvarez**,** O., Gomez-Guillen, C. And Montero, P. 2008a. Presence of hemocyanin with diphenoloxidase activity in deepwater pink shrimp (*Parapenaeus longirostris*) post mortem. Food Chem. 107: 1450–1460.

Martinez-Alvarez**,** O., Gomez-Guillen, C. and Montero, P. 2008b. Chemical and microbial quality indexed of Norwegian lobsters (*Nephrops norvegicus*) dusted with sulphites. Int. J. Food Sci. Technol. 43: 1099-1110.

Martinez-Alvarez, O., Lopez-Caballero, M. E., Montero, P. and Gomez-Guillen M. C. 2005a. A 4-hexylresorcinol based formulation to prevent melanosis and microbial growth in chilled tiger prawns (*Marsupenaeus japonicus*) from aquaculture. J. Food Sci. 70: M415-M422.

Martinez-Alvarez, O., Lopez-Caballero, M.E., Montero, P. and Gomez-Guileen, M. C. 2007. Spraying of 4-hexylresorcinol based formulations to prevent enzymatic browning in Norway lobsters (*Nephrops norvegicus*) during chilled storage. Food Chem. 100: 147-155.

Martinez-Alvarez, O., Montero, P. and Gomez-Guillen, M.C. 2005b. Controlled atmosphere as coadjuvant to chilled storage for prevention of melanosis in shrimps (*Parapenaeus longirostris*). Eur. Food Res. Technol. 220:125-130.

Mastromatteo, M., Danza, A., Conte, A., Muratore, G. and Nobile, M.A. D. 2010. Shelf life of ready to use peeled shrimps as affected by thymol essential oil and modified atmosphere packaging. Int. J. Food. Microbiol. 144: 250-256.

Matmaroh, K., Benjakul, S. and Tanaka, M. 2006. Effect of reactant concentration on the maillard reaction in a fructose-glycine model system and the inhibition of black tiger shrimp polyphenoloxidase. Food Chem. 98: 1-8.

Matsumoto, M. and Yamanaka, H. 1990. Post-mortem biochemical changes in the muscle of kuruma prawn during storage and evaluation of the freshness. Nippon Suisan Gakkaishi. 56: 1145–1149.

Matsumoto-Akanuma, A., Akanuma, S., Motoi, M., Yamagishi, A. and Ohno, N. 2011. Partial purification and chararcterization of polyphenoloxidase from culinary-medicinal royal sun mushroom (the Himematsutake), Agaricus brasiliensis S. Wasser et al. (Agaricomycetideae). DOI: 10.1615/Int. J. Med. Mushr. V13.i1.90.

McEvily, A. J., Iyengar, R. and Otwell, S. 1991b. Sulfite alternative prevents shrimp melanosis. Food Technol. 45: 80-86.

McEvily, A.J., Iyengar, R. and Gross, A. 1991a. Compositions and methods for inhibiting browning in foods using resorcinol derivatives. U.S. patent 5,059,438.

McEvily, A.J., Iyengar, R. and Otwell, W.S. 1992. Inhibition of enzymatic browning in foods and beverages. Crit. Rev. Food Sci. Nutr. 32: 253-273.

McMillin, K. W. 2008. Where is MAP going? A review and future potential of modified atmosphere packaging for meat. Meat Sci. 80: 43-65.

Meilgaard, M., Civille, G. V. and Carr, B.T. 1990. *Sensory Evaluation Techniques* (pp. 231-263). CRC Press, Inc, Florida, USA.

Mendes, R., Huidobro, A., and Caballero, E. L. 2002. Indole levels in deepwater pink shrimp (*Parapenaeus longirostris*) from the Portuguese coast. Effects of temperature abuse. Eur. Food Res. Technol. 214: 125-130.

Montero, P., Avalos, A. and Perez-Mateos, M. 2001a. Characterization of polyphenoloxidase of prawns (*Penaeus japonicus*) alternatives to inhibition: additives and high-pressure treatment. Food Chem. 75: 317–324.

Montero, P., Lopez-Caballero. M..E. and Perez-Mateos, M. 2001b. The effect of inhibitors and high pressure treatment to prevent melanosis and microbial growth on chilled prawns (*Penaeus japonicus*). J. Food Sci. 66: 1201-1206.

Montero, P., Martinez-Alvarez, O. and Gomez-Guillen, M. C. 2004. Effectiveness of onboard application of 4-hexylresocinol in inhibiting melanosis in shrimp (*Parapenaeus longirostris*). J. Food Sci. 69: C643-C647.

Montero, P., Martinez-Alvarez, O., Zamorano, J. P., Alique, R. and Gomez-Guillen, M.C. 2006. Melanosis inhibition and 4-hexylresorcinol residual levels in deep water pink shrimp (*Parapenaeus longirostris*) following various treatments. Eur. Food Res. Technol. 223: 16-21.

Morrissey, P.A., Sheehy, P. J. A., Galvin, K., Kerry, J. P. and Buckley, D. J. 1998. Lipid stability in meat and meat products. Meat Sci. 49: S73-S86.

Moure, A., Cruz, J. M., Franco, D., Dominguez, J. M., Sineiro, J., Dominguez, H., Nunez, M. J. and Parajo, J. C. 2001. Natural antioxidants from residual sources. Food Chemi. 72: 145-171.

Muhila-Almazan, A. and Garcia-Carreno, F. L. 2002. Influence of molting and starvation on the synthesis of proteolytic enzymes in the midgut gland of the white shrimp *Penaeus vannamei*. Com. Biochem. Physiol. Part B.133: 383-394.

Naczk, M. and Shahidi, F. 2004. Extraction and analysis of phenolics in food. J. Chromatogr. A. 1054: 95-111.

National Research Council.1985. An evaluation of the role of microbiological criteria for food and food ingredients, pp 184-307. National Academy press, Washington DC, USA.

Negi, P. S., Chauhan, A. S., Sadia, G. A., Rohinishree, Y. S. and Ramteke, R. S. 2005. Antioxidant and antibacterial activities of various seabuckthorn (*Hippophae rhamnoides L*.) seed extracts. Food Chem. 92: 119-124.

Niki, E. 1991. Action of ascorbic acid ass a scavenger of active and stable oxygen radicals. Am. J. Clin. Nutri. 54: 1119S-1124S.

Nirmal, N. P. and Benjakul, S. 2009a. Effect of ferulic acid on inhibition of polyphenoloxidase and quality changes of Pacific white shrimp (*Litopenaeus vannamei*) during iced storage. Food Chem. 116: 323-331.

Nirmal, N. P. and Benjakul, S. 2009b. Melanosis and quality changes of Pacific white shrimp (*Litopenaeus vannamei)* treated with catechin during iced storage. J. Agric. Food Chem. 57: 3578-3586.

Nirmal, N. P. and Benjakul, S. 2010a. Effect of catechin and ferulic acid on melanosis and quality of Pacific white shrimp subjected to freeze-thawing prior refrigerated storage. Food Cont. 21: 1263-1271.

Nirmal, N. P. and Benjakul, S. 2010b. Effect of green tea extract in combination with ascorbic acid on the retardation of melanosis and quality changes of Pacific white shrimp during iced storage. Food Biopro. Technol. DOI 10.1007/s11947-010-0483-5.

Nirmal, N. P. and Benjakul, S. 2011a. Use of tea extracts for inhibition of polyphenoloxidase and retardation of quality loss of Pacific white shrimp during iced storage. LWT-Food Sci.Technol. 44: 924-932.

Nirmal, N. P. and Benjakul, S. 2011b. Inhibition of melanosis formation in Pacific white shrimp by the extract of lead (*Leucaena leucocephala*) seed. Food Chem. 128: 427-432.

Ogawa, M. 1987. Black spot occurrence in lobster and shrimp. Infofish Marketing Digest. 1: 43.

Ogawa, M., Perdigao, N.B., Santiago, M.E.de. and kozuma, T.T. 1984. On physiological aspects of black spot appearance in shrimp. Bull. Jap. Soc. Sci. Fish. 50: 1763–1769.

Opoku-Gyamfua, A. and Simpson, B. K. 1993. Purification and characterization of three poylphenol oxidase isozymes from lobster (*Homarus Americanus*). J. Food Biochem. 16: 291-306.

Opoku-Gyamfua, A., Simpson, B. K. and Squires E. J. 1992. Comparative studies on the polyphenol oxidase fraction from lobster and tyrosinase. J. Agric. Food Chem. 40: 772-775.

Oshima, T. and Nagayama, F. 1980. Purification and properties of catechol oxidase from the Antarctic krill. Bull. Jap. Soc. Sci. Fish. 46: 1035.

Otwell W.S., Iyengar R. and Mcevily A. J. 1992. Inhibition of shrimp melanosis by 4-hexylresorcinol. J. Aqua. Food Prod. Technol. 1, 53-65.

Ou, S. Y. and Kwok, K. C. 2004. Ferulic acid: pharmaceutical functions, preparation and applications in foods. J. Sci. Food. Agric. 84: 1261-1269.

Ou, S., Luo, Y., Xue, F., Huang, C., Zhang, N. and Liu, Z. 2007. Separation and purification of ferulic acid in alkaline –hydrolysate from sugarcane bagasse by activated charcoal adsorption/anion macroporous resin exchange chromatography. J. Food Eng. 78: 1298-1304.

Paquotte, P., Chim, L., Martin, J. L. M., Lemos, E., Stern, M. and Tosta, G., 1998. Intensive culture of shrimp *Penaeus vannamei* in floating cages: zootechnical, economics and environmental aspects. Aquacul. 164: 151-166.

Parejo, I., Jauregui, O., Sanchez-Rabaneda, F., Viladomat, F., Bastida, J. and Codina, C. 2004. Separation and characterization of phenolic compounds in Fennel (*Foeniculum vulgare*) using liquid chromatography-negative ionization tendem mass spectrometry. J. Agric. Food Chem. 52: 3679-3687.

Pereira, J. A., Pereira, A. P. G., Ferreira, I. C. F. R., Valentao, P., Andrade, P. B., Seabra, R., Estevinho, L. and Bento, A. 2006. Table olives from portugal: phenolic ocmpounds, antioxidant potential and antimicrobial activity. J. Agric. Food Chem. 54: 8425-8431.

Perva-Uzunalic, A., Skerget, M., Knez, Z. Weinreich, B., Otto, F. and Gruner, S. 2006. Extraction of active ingredients from green tea (*Camellia sinensis*): extraction efficiency of major caetchins and caffeine. Food Chem. 96: 597-605.

Prasad, K.N., Yang, B., Yang, S., Chen, Y., Zhao, M., Ashraf, M. and Jiang, Y. 2009. Identification of phenolic compounds and appraisal of antioxidant and antityrosinase activities from litchi (*Litchi sinensis* Sonn.) seeds. Food Chem. 116: 1-7.

Prigent**,** S. 2005. Interactions of phenolic compounds with globular proteins and their effects on food related functional properties. Ph. D. thesis, Wageningen University, The Netherlands.

Puchala, R., Davis, J. J. and Sahlu, T. 1996. Determination of mimosine and 3,4-dihydroxypyridine in milk and plasma of goats. J. Chrom. B: Biomed. Appl. 685: 375-378.

Puchala, R., Sahlu, T., Davis, J. J. and Hart, S. P. 1995. Influence of mineral supplementation on 2, 3-dihydroxypyridine toxicity in Angora goats. Animal Feed Sci. Technol. 55: 253-262.

Puupponen-Pimia, R., Nohynek, L., Meier, C., Kahkonen, M., Heinonen, M., Hopia, A. and Oksman-Caldentey, K. M. 2001. Antimicrobial properties of phenolic compounds from berries. J. Appl. Microbiol. 90: 494-507.

Rattanasatheirn, N., Benjakul, S., Visessanguan, W. and Kijroongrojana, k. 2008. Properties, translucence, and microstructure of Pacific white shrimp treated with mixed phosphates as affected by freshness and deveining. J. Food Sci. 73: S31-S40.

Rice-Evans, C. A., Miller, N. J. and Paganga, G. 1997. Antioxidant properties of phenolic compounds. Trends Plant Sci. 2: 152-159.

Richard-Forget, F. C., Goupy, P. M. and Nicolas, J. J. 1992. Cysteine as an inhibitor of enzymatic browning. 2. Kinteic studies. J. Agric. Food Chem. 40: 2108-2113.

Richard-Forget, F., Cerny, M. Fayad, N. Saunier, T. and Varoquaux P. 1998. Isolation and characterization of a 'quinone-trapping' substance from a crude *Carica papaya* protein preparation. Int. J. Food Sci. Technol. 33: 285-296.

Richardson, T. and Hyslop, D.B. 1985. Enzymes. *In* O.R. Fennema, ed. *Food Chemistry*, p. 371-476. New York, Marcel Dekker.

Robbins, R. J. 2003. Phenolic acids in foods: an overview of analytical methodology. J. Agric. Food Chem. 51: 2866-2887.

Rodriguez-Lopez, J. N., Tudela, J., Varon, R., Garcia-Carmona, F. and Garcia-Canovas, F. 1992. Analysis of a kinetic model for melanin biosynthesis pathway. J. Biol. Chem. 261: 3801-3810.

Roedig-Penman, A. and Gordon, M. H. 1997. Antioxidant properties of catechins and green tea extracts in model food emulsions. J. Agric. Food Chem. 45: 4267-4270.

Rolle, R.S. Guizani, N., Chen. J.S., Marshall, M.R., Yang, J S. and Wei, C.I. 1991. Purification and characterization of phenoloxidase from Taiwanese black tiger shrimp (*Penaeus monodon*). J. Food Biochem. 15: 17-32.

Rotllant, G., Arnau, F., Garcia, J. A., Garcia, N., Rodriguez, M. and Sarda, F. 2002. Effect of metabisulfite treatments and freezing on melanosis inhibition in rose shrimp *Aristeus antennatus* (Risso, 1816). Food Sci. Tech. Int. 8: 243-247.

Row, K. H. and Jin, Y. 2006. Recovery of catechin compounds from Korean tea by solvent extraction. Biores. Technol. 97: 790–793.

Rusak, G., Komes, D., Likic, S., Horzic, D. and Kovac, M. 2008. Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and solvent used. Food Chem. 110: 852-858.

Rutherford, T. J., Marshall, D. L., Andrews, L. S., Coggins, P. C., Schilling, M. W. and Gerard, P. 2007. Combined effect of packaging atmosphere and storage temperature on growth of *Listeria monocytogenes* on ready to eat shrimp. Food Microbiol. 24: 703-710.

Ryan, D. and Robards, K. 1998. Phenolic compounds in olives. Analyst. 123: 31R-44R.

Saboury, A. A., Alijanianzadeh, M. and Mansoori-Torshizi H. 2007. The role of alkyl chain length in the inhibitory effect n-alkyl xanthates on mushroom tyrosinase activities. Acta Biochim. Polonica. 54: 183-192.

Sabudak, T., Khan, M. T. H., Choudhary, M. I. and Oksuz, S. 2006. Potent tyrosinase inhibitors from *Trifolium balansae*. Natural Prod. Res. 20: 665-670.

Sahlu, T., Puchala, R., Reis, P. J., Davis, J. J., Tesfai, K., Fernandez, J. M. and Millamena, A. A. 1995. Technical note: tissue residues of mimosine and 2, 3-dihdroxypyridine after intravenous infusion in goats. J. Animal Sci. 73: 172-176.

Sallam**,** K. I. 2007. Antimicrobial and antioxidant effects of sodium acetate, sodium lactate, and sodium citrate in refrigerated sliced salmon. Food Cont. 18: 566–575.

Sánchez-Ferrer, A., Rodríguez-López, J. N., García-Cánovas, F. and García-Carmona, F. 1995. Tyrosinase: a comprehesive review of its mechanism. Biochim. Biophys. Acta. 1247: 1–11.

Satoh, D., Horii, A., Ochiai, M. and Ashida, M. 1999. Prophenoloxidase-activating enzyme of the silkworm, *Bombyx mori*, purification, characterization, and cDNA cloning. J. Biol. Chem. 274, 7441–7453.

Scalbert, A. 1991. Antimicrobial properties of tannins. Phytochem. 30: 3875-3883.

Segel, I.H. 1976. *Biochemical calculations*. p. 273-277. New York, John Wiley and Sons

Sellappan, S., Akoh, C. C. and Krewer, G. 2002. Phenolic compound and antioxidant capacity of Georgia grown blueberries and blackbaerries. J. Agric. Food Chem. 50: 2432-2438.

Serrano, E. P., Ilag, L. L., and Mendoza, E. M. T. 1983. Biochemical mechanism of mimosine toxicity to *Sclerotium rolfsii* Sacc. Aust. J. Biol. Sci. 36: 445-454.

Seto, Y., Lin, C. C., Endo, Y. and Fujimoto, K. 2005. Retardation of lipid oxidation in blue sprat by hot water tea extracts. J. Sci. Food Agric. 85: 1119-1124.

Shahidi, F., Alasalvar, C. and Liyana-Pathirana, C. M. 2007. Antioxidant phytochemicals in hazelnut kaernel (*Corylus avellana* L.) and hazelnut byproducts. J. Agric. Food Chem. 55: 1212-1220.

Shamshad, S. I., Nisa, K. U., Riaz, M., Zuberi, R. and Qadri, R. B. 1990. Shelf life of shrimp (*Penaeus merguiensis*) stored at different temperatures. J. Food Sci. 55: 1201-1205.

Shiino, M., Watanabe, Y. and Umezawa, K. 2001. Synthesis of *N*-substituted *N*-nitrosohydroxylamines as inhibitors of mushroom tyrosinase. Bioorg. Med. Chem. 9: 1233-1240.

Shimada, K., Fujikawa, K., Yahara, R. and Nakamura, T. 1992. Antioxidative properties of xanthan on autoxidation of soybean oil in cycodextrin emulsion. J. Agric. Food Chem. 40: 945-948.

Shiroma, S. and Hongo, F. 1981. Performances of layers as affected by Leucaena Leucocephala (Lam.) de Wit feeding. Sci. Bull. Coll. Agr. Unv. Ryukyus, 28: 215-225.

Simpson, B. K., Marshall, M. R. and Otwell, W. S. 1987. Phenoloxidase from shrimp (*Penaues setiferus*): Purification and some properties. J. Agric. Food Chem. 35: 918–921.

Simpson, B.K., Marshall, M.R. and Otwell, W.S. 1988. Phenoloxidase from pink and white shrimp: Kinetic and other properties. J. Food Biochem. 12: 205-217.

Sivertsvik, M., Jeksrud, W. K. and Rosnes, J. T. 2002. A review of modified atmosphere packaging of fish and fishery product- significance of microbial growth, activities and safety. Int. J. Food Sci. Technol. 37: 107-127.

Slinkkard, K. and Singleton, V. L. 1997. Total phenol analyses: automation and comparison with manual methods. Am. J. Enol. Viticulture. 28: 49–55.

Soedarjo, M., Hemscheidt, T. K. and Borthakur, D. 1994. Mimosine, a toxin present in leguminous trees (*Leucaena spp.*), induces a mimosine-degrading enzyme activity in some *Rhizobium* strains. Appl. Environ. Microbiol. 60: 4268-4272.

Soldatou, N., Nerantzaki, A., Kontominas, M. G. and Savvaidis, I. N. 2009. Physicochemical and microbiological changes of “Soulaki”- a greek delicacy lamb meat product: Evaluation of shelf-life using microbial, color and lipid oxidation parameters. Food Chem. 113: 36-42.

Solomon, E. I., Sundaram, U. M. and Machonkin, T. E. 1996. Multicopper oxidases and oxygenases. Chem. Rev. 96: 2563– 2605.

Son, S. M., Moon, K. D. and Lee, C. Y. 2000. Kinetic study of oxalic acid inhibition on enzymatic browning. J. Agric. Food Chem. 48: 2071-2074.

Son, S. M., Moon, K. D. and Lee, C. Y. 2001. Inhibitory effect of various antibrowning agents on apple slices. Food Chem. 73: 23-30.

Soobrattee, M. A., Neerhgheen, V. S., Luximon-Ramma, A., Aruoma, O. I. and Bahorun, T. 2005. Phenolic as potential antioxidant therapeutic agents: mechanism and actions. Mutt. Res. 579: 200-213.

Souza, J. N. S., Silva, E. M., Loir, A., Rees, J. F., Rogez, H., and Larondelle, Y. 2008. Antioxidant capacity of four polypheol-rich Amazonian plant extracts: A correlation study using chemical and biological *in vitro* assays. Food Chem. 106: 331-339.

Soysal, C. 2008. Effects of green tea extract on golden delicious apple polyphenoloxidase and its browning. J. Food Biochem. 33: 134-148.

Sriket, P., Benjakul, S., Visessanguan, W. and Kijroongrojana, K. 2007. Comparative studies on the effect of the freeze thawing process on the physicochemical properties and microstructures of black tiger shrimp (*Penaeus monodon*) and white shrimp (*Penaeus vannamei*) muscle. Food Chem. 104: 113-121.

Srinivasan, S., Xiong, Y. L., Suzanne, Blanchard, P., and Tidwell J. H. 1997a. Physicochemical changes in prawns (*Machrobrachium rosenbergii*) subjected to multiple freeze-thaw cycles. J. Food Sci. 62: 123-127.

Srinivasan, S., Xiong, Y., L. and Blanchard, S., P. 1997b. Effects of freezing and thawing methods and storage time on thermal properties of freshwater prawns (*Macrobrachium rosenbergii*). J. Sci. Food Agric. 75: 37-44.

Steel, R. G. D. and Torrie, J. H. 1980. Principles and Procedures of Statistics: A Biometrical Approach (2nd Ed.). McGraw-Hill Inc., New York.

Stunzi, H., Haris, R. L. N, Perrin, D. D. and Teitei, T. 1980. Stability constants for metal complexation by isomers of mimosine and related compouns. Austr. J. Chem. 33: 2207-2220.

Sugumaran, M. and Nellaiappan, K. 1991. Lysolecithin-A potent activator of prophenoloxidase from the hemolymph of the lobster (*Homarus americanas*). Biochem. Biophys. Res. Comm. 176, 1371-1376.

Tang, S., Kerry, J. P., Sheehan, D., Buckley, D. J. and Morrissey, P. A. 2001. Antioxidative effect of added tea catechins on susceptibility of cooked red meat, poultry and fish patties to lipid oxidation. Food Res. Int. 34: 651–657.

Taniguchi, H., Hosoda, A., Tsuno, T., Maruta, Y. and Nomura, E. 1999. Preparation of ferulic acid and its application for the synthesis of cancer chemopreventive agents. Anticancer Res. 19: 3757-3761.

Taoukis, P. S., Labuza, T. P., Lillemo, J. H. and Lin, S. W. 1990. Inhibition of shrimp melanosis (black spot) by ficin. LWT- Food Sci. Technol. 23: 52-54.

Taylor, S. L., Higley, N. A. and Bush R. K. 1986. Sulfites in food: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity, and hypersensitivity. Ad. Food Res. 30: 1-76.

Terwilliger, N. B. 1999. Hemolymph proteins and molting in crustaceans and insects. Am. Zool. 39: 589-599.

Terwilliger, N. B. and Ryan, M. C. 2006. Functional and phylogenetic analyses of phenoloxidase from brachyuran (*Cancer magister*) and branchiopod (*Artemia franciscana, Triopslongicaudatus*) crustaceans. Biol. Bull. 210: 38-50.

Thepnuan, R., Benjakul, S. and Visessanguan, W. 2008. Effect of pyrophosphate and 4 hexylresorcinol pretreatment on quality of refrigerated white shrimp

(*Litopenaeus vannamei*) kept under modified atmosphere packaging. J. Food Sci. 73: S124-S133.

TIS. 1986. Thai industrial standard for quick-frozen shrimps or prawns, pp 115-2529. Bangkok, Thailand.

Torre, P., Aliakbarian, B., Rivas, B., Domnguez, J. M. and Converti, A. 2008. Release of ferulic acid from corn cobs by alkaline hydrolysis. Biochem. Eng. J. 40: 500-506.

Uchiyama, H. and Kakuda K. 1984. A simple and rapid method for measuring k-value, a fish freshness index. Bull. Jap. Soc. Sci. Fish. 50: 263-267.

Uchiyama, H. and Kakuda, K. 1984. A simple and rapid method for measuring k-value, a fish freshness index. Bull. Jap. Soc. Sci. Fish. 50: 263-267.

Vela, L., Lorenzo, C. and Perez, R. A. 2006. Antioxidant capacity of Spanish honeys and its correlation with polyphenol content and other physicochemical properties. J. Sci. Food Agric. 87: 1069-1075.

Vestena, S., Fett-Neto, A. G., Duarte, R. C. and Ferreira, A. G. 2001. Regulation of mimosine accumulation in *Leucaena leucocephala* seedlings. Plant Sci. 161: 597-604.

Vovk, I., Simonovska, B. and Vuorela, H. 2005. Separation of eight selected flavan-3-ols on cellulose thin-layer chromatographic plates. J. Chrom. A. 1077: 188-194.

Walker, J.R.L. 1977. Enzymatic browning in foods. Its chemistry and control. Food Technol NZ. 12: 19-25.

Wang, H. and Helliwell, K. 2000. Epimerisation of catechins in green tea infusions. Food Chem. 70: 337-344.

Wang, J., Sun B G, Cao Y P, Tian Y, and Li, X H. 2008. Optimisation of ultrasound-assisted extraction of phenolic compounds from wheat bran. Food Chem. 106: 804–810.

Wang, Z., Taylor, K., D., A. and Yan, X. 1992. Studies on the protease activities in Norway lobster (*Nephrops norvegicus*) and their role in the phenolase activation process. Food Chem. 45: 111-116.

Weisz, G. M., Kammerer, D. R. and Carle, R. 2009. Identification and quantification of phenolic compounds from sunflower (*Helianthus annuus* L.) kernels and shells by HPLC-DAD/ESI-MS. Food Chem. 115: 758-765.

Wettasinghe, M. and Shahidi, F. 2002. Iron (II) chealtion activity of extracts of borage and evening primrose meals. Food Res. Int. 35: 65-71.

Whitaker, J.R. 1995. Polyphenol oxidase. *In* Food Enzymes, Structure and Mechanism (Wong, D. W. S. ed.). p. 271-303. Chapman & Hall. New York.

Williams, H. G., Davidson, G. W. and Mamo, J. C. 2003. Heat induced activation of polyphenoloxidase in western rock lobster (*Panulirus cygnus*) hemolymph: implication for heat processing. J. Food Sci. 68: 1928-1932.

Wu, H.C., Chen, H. M. and Shiau, C. U. 2003. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). Food Res. Int. 36: 949-957.

Wu, S. C., Yen, G. C., Wang, B. S., Chiu, C. K., Yen, W. J., Chang, L. W. and Duh, P. D. 2007. Antimutagenic and antimicrobial activities of pu-erh tea. LWT- Food Sci. Technol. 40: 506-512.

Wyban, J. 2007. Thailand white shrimp Revolution. Global Aquaculture Advocate, May/june, 56-58.

Xu, R., X. Huang, T. L. Hopkins, and K. J. Kramer. 1997. Catecholamine and histidyl protein cross linked structures in sclerotized insect cuticle. Insect Biochem. Mol. Biol. 27: 101-108.

Yam, T. S., Shah, S. and Hamilton-Miller, J. M. T. 1997. Microbiological activity of whole and fractionated crude extracts of tea (*Camellia sinensis*) and of tea components. FEMS Microbiol. Lett. 152: 169-174.

Yamagata, M and Low, L. K. 1995. Banana shrimp, *Penaeus merguiensis,* quality changes during iced and frozen storage. J Food Sci. 60: 721-726.

Yan, X. and Taylor, K.D.A. 1991. Studies of the mechanism of phenolase activation in Norway lobster (*Nephrops norvegicus*). Food Chem. 41: 11-21.

Yan, X. Taylor, K. D. A. and Hanson, S. W. 1990. Phenolase in Norway lobster (*Nephrops norvegicus*): activation and purification. Food Chem 36: 19-30.

Yang, J. S., Perng, F. S. and Marshell, M. R. 1993. Immunohistochemical localization of phenoloxidase in hepatopancreases, muscle and epidermis of grass shrimp (*Penaeus monodon* F.). J. Food Biochem. 17: 115-124.

Yen, G., C. and Chen, H., Y. 1995. Antioxidant activity of various tea extracts in relation to their antimutagenicity. J. Agric. Food Chem. 43: 27-32.

Yerlikaya, P., Gokoglu, N. and Topuz, O. K. 2009. Use of natural plant extract in batter coating of shrimp and their effects on the quality of shrimp during frozen storage. J. Food Proc. Preser. 34: 127-138.

Yoshida, Y., Kiso, M. and Goto, T. 1999. Efficiency of the extraction of catechins from green tea. Food Chem. 67: 429-433.

Zamorano, J. P., Martinez-Alvaerz, O., Montero, P., and Gomez-Guillen, M. C. 2009. Characterisation and tissue distribution of polyphenol oxidase of deepwater pink shrimp (*Parapenaeus longirostris*). Food Chem. 112: 104-111.

Zeng, Q. Z., Thorarinsdottir, K. A. and Olafsdottir G. 2005. Quality changes of shrimp (*Pandalus borealis*) stored under different cooling conditions. J. Food Sci. 70: S459-S466.

Zhao, Z., Egashira, Y. and Sanada, H. 2005. Phenolic antioxidants richly contained in corn bran are slightly bioavailable in rats. J. Agric. Food Chem. 53: 5030-5035.

Zhu, X., Zhang, H. and LO, R. 2004. Phenolic compounds from the leaf extract of artichoke (*Cynara scolymus* L.) and their antimicrobial activities. J. Agric. Food Chem. 52: 7272-7278.

Zhuang, R.-Y., Huang,Y.-W. and Beuchat, L. R. 1996. Quality changes during refrigerated storage of packaged shrimp and catfish fillets treated with sodium acetate, sodium lactate or propyl gallate. J. Food Sci. 61: 241–244, 261.

Zotos, A. and Taylor, K. D.A. 1997. Studies on the roles and identification of the small molecule factors on phenolase activation in Norway lobster (*Nephrops norvegicus*). Food Chem. 60: 469- 478.

VITAE

Name Mr.Nilesh Prakash Nirmal

Student ID 5111030015

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science in Chemistry	University of Pune India	2000
Master of Science in Biochemistry	University of Pune India	2002

Scholarship Awards during Enrolment

Graduate Scholarship granted by the Graduate School, Prince of Songkla University, Hat Yai, Thailand.

List of Publication and Proceedings

Publications

1. Nirmal, N. P. and Benjakul, S. 2009. Effect of ferulic acid on inhibition of polyphenoloxidase and quality changes of Pacific white shrimp (*Litopenaeus vannamei*) during iced storage. Food Chem. 116: 323-331.
2. Nirmal, N. P. and Benjakul, S. 2009. Melanosis and quality changes of Pacific white shrimp (*Litopenaeus vannamei)* treated with catechin during iced storage. J. Agric. Food Chem. 57:3578-3586.
3. Nirmal, N. P. and Benjakul, S. 2010. Effect of catechin and ferulic acid on melanosis and quality of Pacific white shrimp subjected to prior freeze-thawing during refrigerated storage. Food Cont. 21: 1263-1271.
4. Nirmal, N. P. and Benjakul, S. 2010. Effect of green tea extract in combination with ascorbic acid on the retardation of melanosis and quality changes of Pacific white shrimp during iced storage. Food Bioproc. Technol. DOI 10.1007/s11947-010-0483-5.

5. Nirmal, N. P. and Benjakul, S. 2011. Use of green tea extracts for inhibition of polyphenoloxidase and retardation of quality loss of Pacific white shrimp during iced storage. LWT- Food Sci. Technol. 44: 924-932.
6. Nirmal, N. P. and Benjakul, S. 2011. Inhibition of melanosis formation in Pacific white shrimp by the extract of lead (*Leucaena leucocaphala*) seed. Food Chem. 128: 427-432.
7. Nirmal, N. P. and Benjakul, S. 2011. Retardation of quality changes of Pacific white shrimp by green tea extract treatment and modified atmosphere packaging during refrigerated storage. Int. J. Food Microbiol. (Manuscript under review).
8. Nirmal, N. P. and Benjakul, S. 2011. Inhibition mechanism of caetchin and ferulic acid on polyphenoloxidase from cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*). Food Chem. (manuscript under review).
9. Nirmal, N. P. and Benjakul, S. 2011. Inhibitory effect of mimosine on polyphenoloxidase from cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*). J. Agric. Food Chem. (manuscript under review).
10. Nirmal, N. P. and Benjakul, S. 2011. Biochemical properties of polyphenoloxidase from cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*). Biotechnol. Bioproc. Eng. (submitted)

Proceedings

1. Benjakul, S. and Nirmal, N. P. 2009. Effect of ferulic acid on inhibition of polyphenoloxidase and quality changes of Pacific white shrimp (*Litopenaeus vannamei*) during iced storage. 60th Pacific Fisheries Technologist Annual Conference, Portland, Oregon, USA. February 22-25, 2009 (Invited oral presentation).
2. Benjakul, S., Nirmal, N. P. and Eun, J. B. 2009. Melanosis of Pacific white shrimp and it s prevention using phenolic compound. 76th Annual meeting of Korean Society of Food Science and Technology (KoSFoST) and International Symposium with the theme of -global Food Safety 2009 at Daejeon Convention Center in Daejeon, South Korea. May 27-29, (Invited oral presentation).

3. Nirmal, N. P. and Benjakul, S. 2009. Effects of catechin and ferulic aicd on the quality of Pacific white shrimp during iced storage. Food Innovation Asia Conference, 11th Agro-industrial conference, BITEC, Bangkok, Thailand; June 18-19, (Poster presentation).
4. Nirmal, N. P. and Benjakul, S. 2010. Melanosis, polyphenoloxidase activity and quality changes of Pacific white shrimp as affected by prior freeze-thawing and phenolic treatment. Food Innovation Asia Conference, 12th Agro-industrial conference, BITEC, Bangkok, Thailand; June 17-18, (Poster presentation).
5. Nirmal, N. P. and Benjakul, S. 2010. Synergetic effect of green tea extract with ascorbic acid on the retardation of melanosis and quality changes of Pacific white shrimp during iced storage. Bioscience for the future 2010, merge of 7th IMT-GT UNINET and the 3rd Joint International PSU-UNS Conference, Prince of Songkla Unviersity, Hat Yai, Thailand. October 7-8, (Oral presentation).
6. Nirmal, N. P. and Benjakul, S. 2010. Antioxidative and Anti-polyphenoloxidase activities of green and mulberry tea towards melanosis inhibition in Pacific white shrimp. International Conference on Agriculture and Agro-Industry (ICCAAI 2010), *Food, Health and Trade*, Mae Fah Luang University, Chiang Rai, Thailand; November 19-20, (Poster presentation).
7. Nirmal, N. P. and Benjakul, S. 2010. Inhibition of polyphenoloxidase and retardation of quality loss of Pacific white shrimp by green tea extract during iced storage. International Conference on Food Research (ICFR-2010), under theme of *Sustainable and Quality Food for All*, hosted by Universiti Putra Malaysia, Malaysian Institute of Food Technology and ILSI, Putrajaya, Malaysia. November 21-22, (Oral presentation).
8. Nirmal, N. P. and Benjakul, S. 2011. Effect of green extract treatment and modified atmosphere packaging on the retardation of quality changes of Pacific white shrimp during refrigerated storage. Pacific Fisheries Technologists 62nd Annual Meeting, under theme of *Head to tail, the whole story*, Vancouver, BC, Canada, February 13-16, (Poster presentation).