Heated Sulfur-Containing Compounds: Properties and the Uses for Shelf-Life Extension of Pacific White Shrimp (*Litopenaeus vannamei*) Stored in Ice

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ชื่อวิทยานิพนธ์ สารประกอบซัลเฟอร์ที่ผ่านการให้ความร้อน: สมบัติและการใช้สำหรับยืดอายุการเก็บรักษากุ้งขาวในน้ำแข็ง

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บทคัดย่อ

จากการศึกษาสมบัติบางประการของเอนไซม์พอลิฟีนอลออกซิเดสจากหัวกุ้งขาว พบว่าเอนไซม์พอลิฟีนอลออกซิเดสมีกิจกรรมสูงสุดเมื่อใช้ DOPA (3, 4-dihydroxy phenylalanine) เป็นสับสเตรตที่อุณหภูมิ 45 องศาเซลเซียสและพีเอช 6.0 เอนไซม์ดังกล่าวมีความคงตัวในช่วงพีเอช 5.0-7.0 และไม่คงตัวที่อุณหภูมิสูงกว่า 50 องศาเซลเซียส เอนไซม์พอลิฟีนอลออกซิเดสจากหัวกุ้งขาวมีน้ำหนักโมเลกุลเท่ากับ 180 กิโลดัลตัน เมื่อตรวจสอบโดยแอคติวิตี้สเตนนิ่งโดยใช้ DOPA เป็นสับสเตรท สารประกอบซัลเฟอร์โดยเฉพาะอย่างยิ่งซิสเตอีนและกลูตาไธโอนที่ผ่านการให้ความร้อนในสภาวะด่างสามารถยับยั้งกิจกรรมของเอนไซม์พอลิฟีนอลออกซิเดสจากหัวกุ้งขาวได้ โดยขึ้นอยู่กับความเข้มข้นที่ใช้

ซิสเตอีนที่ผ่านการให้ความร้อนที่อุณหภูมิ 100 องศาเซลเซียสในสภาวะที่เป็นด่าง (พีเอช 10) สามารถยับยั้งกิจกรรมของเอนไซม์พอลิฟีนอลออกซิเดสได้มากกว่าไกลซีน เมทไธโอนีนและผลิตภัณฑ์จากปฏิกิริยาเมลลาร์ดจากระบบจำลองกลูโคส/ซิสเตอีนที่ผ่านการให้ความร้อนในสภาวะด่าง ซิสเตอีนที่ผ่านการให้ความร้อนในสภาวะด่างมีความสามารถในการจับกับทองแดง ความสามารถในการรีดิวซ์ และความสามารถในการจับอนุมูลอิสระสูงกว่าสารประกอบซัลเฟอร์อื่นๆ จากการศึกษาผลของซิสเตอีนที่ผ่านการให้ความร้อนในสภาวะที่เป็นด่าง(ระดับความเข้มข้น 0, 20 และ 100 มิลลิโมลาร์) ต่อการเปลี่ยนแปลงคุณภาพของกุ้งขาวขณะเก็บรักษาในน้ำแข็งเป็นระยะเวลา 12 วันพบว่ากุ้งขาวที่แช่ด้วยซิสเตอีนที่ผ่านการให้ความร้อนในสภาวะด่างที่ความเข้มข้นสูงมีคะแนนการเกิดเมลาโนซิส TBARS และปริมาณจุลินทรีย์น้อยกว่ากุ้งขาวที่แช่ด้วยซิสเตอีนที่ผ่านการให้ความร้อนในสภาวะด่างที่ความเข้มข้นต่ำและกุ้งขาวชุดควบคุม (ไม่แช่สารใดๆ)

ปัจจัยบางประการ เช่น พีเอชและความเข้มข้นของสารตั้งต้น มีผลต่อสมบัติของซิสเตอีนที่ผ่านการให้ความร้อน พบว่าซิสเตอีนที่ผ่านการให้ความร้อนในสภาวะที่เป็นด่างมีความสามารถในการยับยั้งกิจกรรมเอนไซม์พอลิฟีนอลออกซิเดส การจับกับทองแดง

ความสามารถในการรีดิวซ์ และความสามารถในการจับอนุมูลอิสระสูงกว่าซิสเตอีนที่ผ่านการให้ความร้อนในสภาวะที่เป็นกรด ทั้งนี้สมบัติต่างๆเพิ่มขึ้นเมื่อความเข้มข้นของซิสเตอีนเพิ่มขึ้น โดยเฉพาะอย่างยิ่งที่ระดับความเข้มข้นเท่ากับ 500 มิลลิโมลาร์ จากการศึกษาผลของซิสเตอีนที่ผ่านการให้ความร้อนในสภาวะที่เป็นด่างที่ระดับความเข้มข้นแตกต่างกัน (20, 100 และ 200 มิลลิโมลาร์) ต่อคุณภาพของกุ้งขาวที่เก็บรักษาในน้ำแข็งเป็นระยะเวลา 12 วัน พบว่ากุ้งขาวที่แช่ด้วยสารละลายซิสเตอีนที่ผ่านการให้ความร้อนในสภาวะที่เป็นด่างที่ระดับความเข้มข้น 200 มิลลิโมลาร์ มีการเปลี่ยนแปลงทางกายภาพ เคมี และจุลินทรีย์น้อยที่สุดเมื่อเปรียบเทียบกับกุ้งขาวชุดควบคุม ดังนั้นสารละลายซิสเตอีนที่ผ่านการให้ความร้อนในสภาวะที่เป็นด่าง ณ ระดับความเข้มข้นที่เหมาะสมสามารถใช้เป็นสารยับยั้งการเกิดเมลาโนซิส รวมทั้งยังมีสมบัติเป็นสารต้านออกซิเดชันและสารยับยั้งการเจริญเติบโตของจุลินทรีย์ เพื่อชะลอการเสื่อมเสียคุณภาพของกุ้งขาวระหว่างการเก็บรักษาในน้ำแข็ง

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ABSTRACT

Polyphenol oxidase (PPO) from the cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*) was characterized. The highest PPO activity towards DOPA (3, 4-dihydroxy phenylalanine) was observed at 45 °C and pH 6.0. PPO was stable in pH range of 5-7 and was unstable at the temperature greater than 50 °C. As determined by activity staining using DOPA as a substrate, the molecular weight of Pacific white shrimp PPO was 180 kDa. Sulfur-containing compounds, especially cysteine and glutathione, heated under alkaline condition exhibited PPO inhibitory activity in a concentration dependent manner.

Heated alkaline cysteine (HAC) (100 °C, pH 10) showed the greater PPO inhibitory activity than did heated alkaline glycine, methionine and glucose-cysteine Maillard reaction products ($P<0.05$). HAC also exhibited the higher copper-binding activity, reducing power and radical scavenging activity than did other heated alkaline sulfur-containing compounds. Those properties were dependent upon the concentration of HAC used. Effect of HAC at different concentrations (0, 20 and 100 mM) on the quality changes of Pacific white shrimp during 12 days of iced storage was investigated. Shrimps treated with HAC at higher concentrations had the lower melanosis score, thiobarbituric reactive substances and psychrophilic bacterial count, compared with shrimps treated with lower concentrations of HAC and without HAC treatment.

Some factors including pH and cysteine concentration affected the properties of resulting products. HAC exhibited the higher PPO inhibitory activity, copper-binding activity, reducing power and radical scavenging activity than heated

cysteine prepared at acidic condition. All properties increased when the concentration of cysteine increased, especially at 500 mM. Effect of diluted HAC with different concentrations (20, 100 and 200 mM) on the quality of Pacific white shrimp during 12 days of iced storage was investigated. Shrimps treated with 200 mM HAC had the retarded melanosis, lowered TBARS and psychrophilic bacterial count, compared with shrimps without HAC treatment. Therefore, HAC at an appropriate concentration could be used as a promising melanosis inhibitor, which possessed antioxidant and antimicrobial activities, in order to retard the loss in quality of white shrimp during the extended iced storage.

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Yoottana Phonpala

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CHAPTER 1

INTRODUCTION

Seafoods, especially shrimps, are one of the products which have become economically important for Thailand. However, shrimps are perishable, mainly associated with the spoilage microorganism. Additionally, shrimps undergo discoloration during iced or chilled storage. This phenomenon is caused by the action of polyphenol oxidase (PPO) (Kim *et al*., 2000). Melanosis or black spot in crustacean such as shrimp, prawn, lobster, and crab is rapid, even in iced or chilled storage (Montero *et al*., 2001b). The presence of black spot, although not necessarily an indicator of spoilage and seems to be harmless to consumers, detracts from the visual quality of crustacean and reduces the product's market value, leading to financial loss (Bartoro and Birk, 1998; Montero *et al*., 2001a).

Melanosis is triggered by a biochemical mechanism consisting of oxidation of phenols to quinones by enzymatic reaction of PPO. This is followed by non-enzymatic polymerization of the quinones, giving rise to pigments of high molecular weight and very dark or black coloring (Montero *et al*., 2001b). Several approaches have been used to eliminate from the reaction one or more of its essential components: oxygen, enzyme, copper, or substrate (Kim *et al*., 2000). PPO inhibition has been focused to prevent melanosis in crustacean. The inhibition of PPO can be achieved using physical methods, chemical methods, or a combination method. Melanosis in crustacean can be controlled by some compounds, especially sulfiting agents (Ferrer *et al*., 1989; Benjakul *et al*., 2006). However, sulfite has been prohibited, owing to its adverse health effect (Tan and Harris, 1995; Matmaroh *et al*., 2006). There are other alternative compounds to sulfiting agents. Most are of natural origin and their purpose is to inhibit, or slow down the onset of melanosis. These include ascorbic acid, benzoic acid, sorbic acid, kojic acid, phytic acid, proteases, and resorcinol derivatives. All of which are considered to be safe compounds (Taoukis *et al*., 1990). Additionally, cysteine, Maillard reaction products (MRPs), calamelization products (CPs) and honey have been reported as natural melanosis inhibitors.

MRPs have been found to exhibit PPO inhibitory activity due to metal chelating activity (Wijewickreme *et al*., 1997; Matmaroh *et al*., 2006) and reducing power (Tan and Harris, 1995; Matmaroh *et al*., 2006). In addition, MRPs also exhibited the antioxidant and antimicrobial activity. Thus MRPs may be applied to preserve many food products. Inhibitory activity of MRPs on PPO activity for many fruits, vegetables and seafood products has been widely studied, but a little information regarding the use of sulfur-containing compounds in inhibiting crustacean PPO has been reported. The knowledge and technology gained can be further applied to shrimp farmers or processors in order to retard the melanosis during handling and storage. As a consequence, the quality can be maintained.

Review of Literature

1. Polyphenol oxidase (PPO)

Polyphenol oxidase (PPO) is a copper-containing enzyme, which is also known as catechol oxidase, catecholase, diphenol oxidase, *o*-diphenolase, phenolase and tyrosinase (Martinez and Whitaker, 1995). It is responsible for catalyzing two basic reactions by using the substrates, phenols and O_2 (Kim *et al*., 2000). PPO can be classified as: (1) monooxygenase or monophenol oxidase in the *o*-hydroxylation of monophenols to *o*-dihydroxyphenols (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase EC 1.14.18.1) and (2) a two-electron oxidase or diphenol oxidase in the oxidation of *o*-diphenols to *o*-quinones (1,2-benzenediol : oxygen oxidoreductase EC 1.10.3.1) (Wong, 1989).

1.1 Molecular structures

PPO has been isolated from various sources. The primary structure of several PPOs from mammals, higher plants, insects, fungi, and bacteria have been characterized and their encoding genes have been cloned but the secondary and tertiary structures of a PPO have not been determined (Kim *et al*., 2000). PPO contains two Cu-binding sites, called CuA and CuB. The partial primary amino acid sequence and secondary and tertiary structures of spiny lobster (*Panulirus interruptus*) hemocyanin subunit *a* were determined. The subunit folds into three

domains. Domain 2 contains the binuclear copper site responsible for binding and transporting O_2 in the lobster (Gaykema *et al*., 1984). Each copper is liganded to three histidine residues (copper A, His 196, 200, and 226; copper B, His 346, 350, and 386). All of three histidine residues are located on four α helices that fold in proximity to each other (Whitaker, 1995). There are similarities between the amino acid sequence around the binuclear copper sites of the lobster hemocyanin and that of region B of the PPO. The spacing of the active site histidines along the PPO peptide chain is similar but not the same as that of the lobster hemocyanin (Whitaker, 1995). In *N. crassa* PPO, Huber *et al*. (1985) found that CuA is coordinated to His 187, 193, and 281, and CuB to His 95, 104, and 306.

In animals, PPO is referred to as tyrosinase because L-tyrosine is the major monophenolic substrate. The active site of tyrosinase consists of two copper atoms and three states: *met*tyrosinase, *deoxy*tyrosinase, and *oxy*tyrosinase (Garcia-Molina *et al*., 2005). In the oxygenated form, the binuclear cupric site is tetragonal with protein ligands (histidine residues) binding in the equatorial plane and one or two axial ligands such as H_2O. The two coppers are also bridged by an endogenous protein ligand (such as phenolate or carboxylate groups) and an exogenous oxygen molecule bound as peroxide (Figure 1) (Himmelwright *et al*., 1980). When the copper ions are bridged by an exogenous ligand other than peroxide, the enzyme is in the *met* form. The *deoxy* form contains a bicuprous structure without exo- or endogenous bridging (Wong, 1989).

The *met* form is converted, by a 2*e*-reduction, to the *deoxy* form which binds molecular oxygen reversibly to form *oxy*tyrosinase. Peroxide addition to the *met* form also yields the *oxy* enzyme (Wong, 1989).

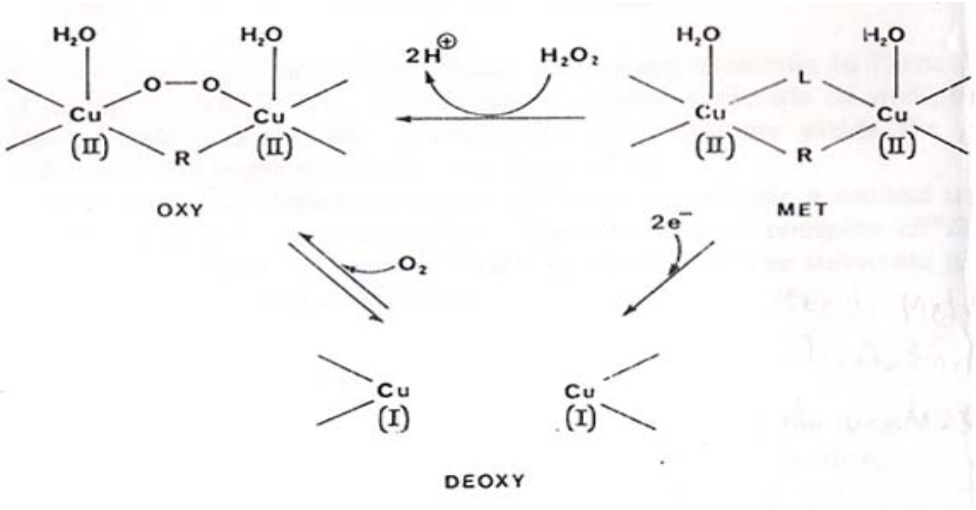

Figure 1. Three states of tyrosinase
Source: Himmelwright *et al*. (1980)

1.2 Molecular weights

Molecular weights of PPO vary with the origins. The generally accepted molecular weight of mushroom PPO is 128.0 kDa, while the molecular weights of higher plant PPOs range from 40.7 to 58.1 kDa (Whitaker, 1995). The molecular weights of crustacean PPO is summarized as shown in Table 1. The molecular weights of crustacean PPO range from 30 to 213 kDa. Florida spiny lobster PPO had three isoforms and the Western Australian lobster PPO had two isoforms. The molecular weights of the Florida spiny lobster PPO isoforms were determined to be 82, 88, and 97 kDa, while those of the Western Australian lobster were 87 and 92 kDa. The molecular weights of these isoforms were higher than those of white shrimp (30 kDa), pink shrimp (40 kDa), and Antarctic krill (75 and 83 kDa), but lower than that of kuruma prawn (160 kDa). Benjakul *et al*. (2005b) found that the molecular weight of PPO from the kuruma prawn cultured in Japan was estimated to be 160 kDa. Recently, Nirmal and Benjakul (2009) reported that the molecular weight of PPO from the cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*) was 210 kDa. Furthermore, two isoforms of PPO from deepwater pink shrimp (*Parapenaeus longirostris*) were estimated to have the molecular weight of 200 and 500 kDa (Zamorano *et al*., 2009)

1.3 Mode of action

PPO is responsible for a type of discoloration called melanosis in crustaceans. The enzyme catalyzes two basic reactions in melanosis: (a) hydroxylation of monophenols (monophenol oxidase activity) and (b) oxidation of diphenols (diphenol oxidase activity). The enzyme catalyzes the hydroxylation to the *o*-position adjacent to an existing hydroxyl group. The second reaction is the oxidation of the diphenol to *o*-quinones, which are then further oxidized to melanins (brown products), usually by non-enzymatic mechanisms (Figure 2). It is still not clear whether a single enzyme system is responsible for both reactions or if there are two distinct enzyme molecules (Kim *et al.*, 2000). When both monophenol- and diphenol oxidases are present, the ratio of monophenol to diphenol oxidase is usually 1:10 or as high as 1:40 (Nicolas *et al.*, 1994). PPO isolated from the insect cuticle appears to have only diphenol oxidase activity, while PPO from insect hemolymph has the monophenol oxidase activity. PPO from shrimp cuticle exhibits both activities (Kim *et al.*, 2000).

Table 1. Some characteristics of PPO from crustacean

Source	M.W. (Daltons)	Specific activity (units / mg)	K_m (mM)	Ref.
White shrimp (*Penaeus setiferus*)	30,000	59	2.83	Simpson *et al*. (1987)
Pacific white shrimp (*Litopenaeus vannamei*)	210,000			Nirmal and Benjakul (2009)
Pink shrimp (*Penaeus duorarum*)	40,000	82.8	1.63	Simpson *et al*. (1988)
Deepwater pink shrimp (*Parapenaeus longirostris*)	200,000 500,000	38.5	1.85	Zamorano *et al*. (2009)
Florida spiny lobster (*Panulirus argus*)	97,000 88,000 82,000	0.36	9.85	Chen *et al*. (1991)
Western Australian lobster (*Panulirus cygnus*)	92,000 87,000	0.03	3.57	Chen *et al*. (1991)
Lobster (*Homarus americanus*)	32,180 35,480 39,300			Opoku-Gyamfua and Simpson (1993)
Kuruma prawn (*Penaeus japonicus*)	160,000			Benjakul *et al*. (2005b)

Source: Adapted from Kim *et al*. (2000)

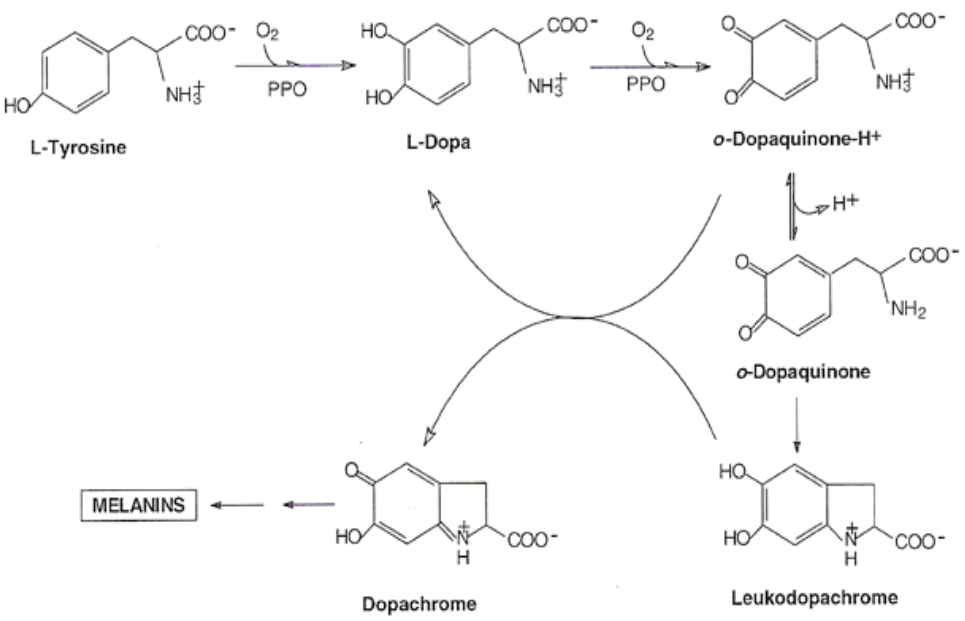

Figure 2. Melanosis pathway induced by polyphenol oxidase
Source: Garcia-Monila *et al*. (2005)

The proposed mechanisms of hydroxylation and dehydrogenation reactions are presented separately, but linked by a common *oxy*PPO intermediate (Solomon *et al*., 1992). O_2 is bound first to the two Cu(I) groups of *deoxy*PPO to give *oxy*PPO, in which the O_2 has a characteristic of a peroxide (Solomon *et al*., 1992). For monophenol oxidase activity, the *oxy* form then binds the monophenol via one of the Cu(II)s, displacing the water molecule. In the subsequent step, the steric orientation of the bound monophenol and O_2 are altered to place the *o*-position of the monophenol adjacent to the second Cu(II) in *oxy*PPO. Therefore, the *o*-position is hydroxylated by the bound –O-O– moiety. The initial product of the monophenol is diphenol bound to both Cu(II)s. For mechanisms of oxidation of *o*-diphenol, the two Cu(II)s groups of *oxy*PPO then bind the two hydroxyl groups of diphenol, replacing the two water molecules or OH groups, to form the O_2 · diphenol · enzyme complex. The diphenol is oxidized to quinone, leaving the enzyme as *met*PPO. The *met*PPO undergoes further reaction to form *deoxy* form, while Cu(II) is reduced to Cu(I). The *deoxy* form can then bind O_2 without cycling through the *met* form to give the oxygenated enzyme, allowing further turnover (Whitaker, 1995).

1.4 PPO activity in crustaceans

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). PPO is distributed in many parts of shrimps with different levels of activity (Montero *et al*., 2001b; Benjakul *et al*., 2005b). 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 jointed and where the cuticle is connected to the pleopods (Ogawa *et al*., 1984; Benjakul *et al*., 2005b). Montero *et al*. (2001a) reported that the highest PPO activity was found in the carapace of prawns (*Penaeus japonicus*), followed by the caudal zone (uropods and telson), the muscle and cuticle This could be the reason why melanosis starts in the head portions and spreads to the tails (Montero *et al*., 2001a). Nakagawa and Nagayama (1981) studied the distribution of catecholoxidase in the tissue and organs of several crustaceans and found that no activity was found in the muscle of tiger shrimp (*Penaeus japonicus*) and in other species. However, some activity was detected in some crab meats. In addition, Ferrer *et al*. (1989) reported that the crude PPO extracts from the cuticle of Florida spiny lobster (excluding pleopods and epidermis) had initially very low activity; but a gradual increase in PPO activity was observed during storage of extracts at 4 °C with the highest activity on day 3. Recently, Zamorano *et al*. (2009) reported the distribution of PPO in different tissues of deepwater pink shrimp (*Parapenaeus longirostris*). PPO activity was the highest in the carapace, followed by the abdomen exoskeleton, cephalotorax, pleopods and telson, respectively. No PPO activity was found in the abdomen muscle and in the pereopods and maxillipeds. Melanosis development in different anatomical parts of deepwater pink shrimp during 7 days of chilled storage (4 °C) was evaluated. Marked melanosis developed in the head (both as separated organ and attached to the shrimp) and cephalothorax was found on day 1, but the other individual organs showed no evidence of black spots even after 7 days of storage. On day 7, the melanosis markedly developed in the head without the carapace. Removal of the carapace resulted in a faster melanosis development in the excised heads and the whole shrimp, probably due to higher accessibility of oxygen to the enzyme and the substrates.

There are several factors affecting PPO activity. The control of those factors would provide a promising means to retard the melanosis induced by PPO.

1.4.1 pH

Factors influencing the stability of an enzyme are those affecting the secondary, tertiary, and/or quaternary structures of enzymes. Most enzymes undergo irreversible denaturation at very acidic and very alkaline pHs (Whitaker, 1995). In general, the conformational change at the active site of enzyme due to dramatic pH changes may cause the significant decline in enzyme activity. PPO activity markedly decreased in either acidic or alkaline pH ranges. Crustacean PPO is generally stable in the pH range of 5-9, while optimum pHs of crustacean PPO range from 5.0 to 8.0 (Table 2).

The shrimp and lobster PPO was most stable within a narrow pH range (5.0 – 9.0). Benjakul *et al*. (2005b) found that PPO from the cephalothorax of kuruma prawn (*Penaeus japonicus*) cultured in Japan was stable over a wide pH range (3.0 – 10.0) with the remaining activity above 90%. However, PPO from shrimp (*Penaeus japonicus*) cultured in Spain was not stable at acidic pH ranges (Montero *et al*., 2001a). In general, PPO is not stable at acidic pH. PPO of lobster (*Panulirus argus* ; Ferrer *et al*., 1989) and white shrimp (*Penaeus setiferus* ; Simpson *et al*., 1987) was unstable at pH below 5. Simpson *et al*. (1988) reported that pink shrimp PPO was stable over a broader pH range (6.5 - 9.0), while white shrimp was most stable at slightly acidic to alkaline pH and was least stable at acidic pH. The differences in pH stability indicated the different molecular properties including the bonding stabilizing the structure as well as the enzyme conformation among the various species and anatomical locations (Benjakul *et al*., 2005b).

The optimal pH of PPO depends to a large extent on the physiological pH, in which the enzyme activity occurs in nature or the locus of extraction. For example, the pH of the carapace of the cephalothorax was 7.16 ± 0.07, while the pH of abdominal cuticle was 8.76 ± 0.04. The optimal pH of crustacean PPO also varied with the species. Oshima and Nagayama (1980) reported that the optimal pH of catechol oxidase from Antarctic krill was 6.5. PPO from the carapace of shrimp (*Penaeus setiferus*) showed the maximal activity at pH 7.5 (Simpson *et al*., 1987), while the optimal pH of black tiger prawn PPO was 6.0 (Rolle *et al*., 1991).

Table 2**.** pH and temperature profile of PPO from crustacean

Source	pH optimum	pH stability	Temperature optimum (°C)	Temperature stability (°C)	Ref.
White shrimp (*Penaeus setiferus*)	7.5	6.0-8.0	45	25-50	Simpson *et al*. (1987)
Pacific white shrimp (*Litopenaeus vannamei*)	5.5	5.5-6.0	45	40-45	Thepnuan (2007)
Pink shrimp (*Penaeus duorarum*)	8.0	6.5-9.0	40	20-40	Simpson *et al*. (1988)
Deepwater pink shrimp (*Parapenaeus longirostris*)	4.5	4.5-9.0		30-35	Zamorano *et al*. (2009)
Florida spiny lobster (*Panulirus argus*)	6.5	6.5-8.0	35	30-40	Chen *et al*. (1991)

Source: Adapted from Kim *et al*. (2000)

Table 2**.** (Continued)

Source	pH optimum	pH stability	Temperature optimum (°C)	Temperature stability (°C)	Ref.
Western Australian lobster (*Panulirus cygnus*)	7.0	5.0-9.0	30	25-35	Chen *et al*. (1991)
Black tiger prawn (*Penaeus monodon*)	6.0	6.0-7.0	45	30-40	Matmaroh (2005)

In addition, PPO activity and pH stability differed according to enzyme state of activation (Ferrer *et al*., 1989). The differences in optimal pH might be because of the different nature of prototropic groups in the active site of enzymes (Whitaker, 1972).

1.4.2 Temperature

The temperature has a significant effect on the PPO activity. The effect of temperature on the stability of PPO from crustaceans indicates that the enzymes appeared to have similar thermal stability (20-50 °C) (Table 2). Most PPO is heat labile; a short exposure of the enzyme to temperatures at 70-90 °C causes a partial or total irreversible denaturation. In shrimps (*Penaeus duorarum* and *Penaeus monodon*), PPO has been found to be unstable at temperature over 30-35 °C (Rolle *et al*., 1991; Simpson *et al*., 1988). Nevertheless, PPO of shrimp (*Penaeus setiferus*) and Florida spiny lobster (*Panulirus argus*) was stable at temperature below 50 and 40 °C, respectively (Simpson *et al*., 1987; Ali *et al*., 1994). Crude PPO isolated from deep sea crab was shown to be inactivated at 70 °C (Mashall *et al*., 1984).

Vamos-Vigyazo (1981) pointed out that the thermo-tolerance of PPO was dependent on the enzyme source. Simpson *et al*. (1988) reported that pink shrimp PPO was more heat-labile than was white shrimp PPO. About 35% of the original activity of pink shrimp PPO was destroyed after 30 min at 50 °C, while the original activity of white shrimp PPO remained unchanged after the same treatment. Ali *et al*. (1994) found that the Florida spiny lobster PPO was stable up to 40 °C, but after incubation at 60 °C for 30 min the PPO was completely inactivated. The result was in accordance with Ferrer *et al*. (1989) who reported that the inert PPO from Florida spiny lobster was stable between 25-40 °C. Benjakul *et al*. (2005b) found that the PPO from kuruma prawn (*Penaeus japonicus*) cephalothorax was stable up to 40 °C and slightly lost its stability at 50°C, but no activity was remained when heated to 70 and 80 °C for 30 min. It could be the fact that enzyme undergoes denaturation and loses its activity at high temperature. Moreover, even within the same species, there are differences in thermal stability depending on the state of activation of the enzyme (Ferrer *et al*., 1989).

The optimal temperature of PPO has been reported to be varied, depending on species and habitat temperature. The optimal temperature of PPO from the kuruma prawn cephalothorax was 35 °C, whereas the optimal temperature of PPO from carapace of shrimp (*Penaeus japonicus*) cultured in Spain was 55 °C. Benjakul *et al*. (2005b) found that PPO from the cephalothorax of kuruma prawn showed a slightly lower optimal temperature than those of other species reported. It was suggested that a lower kinetic energy was required for the activity of PPO from kuruma prawn cephalothorax, compared with that needed for other species.

1.4.3 Metal ions

Some Metal ions such as Cu^{2+}, Zn^{2+}, and Mg^{2+} have a significant effect on PPO activity. The role of copper in catalysis of oxidation of monophenols and *o*-diphenols was elucidated (Kubowiz, 1938). The addition of $CuSO_4$ to plant extracts containing PPO increased the observed activity. The removal of copper by reducing compounds, including ascorbate, led to the complete inactivation of the enzyme. However, the activity can be restored by incubation of the inactive PPO with $CuSO_4$ (Dawson and Mallette, 1945). Simpson *et al*. (1987) reported that PPO activity

from shrimp (*Penaeus setiferus*) increased with the addition of the copper, whereas Benjakul *et al*. (2005b) 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.

However, Liu *et al*. (2006) reported that PPO activity from crab (*Charybdis japonica*) was strongly inhibited by Cu^{2+}, Zn^{2+}, and Mg^{2+}. In addition, they found that 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.

1.4.4 Protease and some chemicals

In crustaceans, the PPO system is considered a constituent of the immune system and forms an important part of an immuno-recognition process of the defense mechanism in invertebrates. It localizes in the hemolymph as a zymogen or proPPO form that has to be activated by protease (Brunet, 1980 ; Ferrer *et al*., 1989) , fatty acids and lipids (Heyneman and Vercauteren, 1968) , laminarin (β-1,3- glucan) (Soderhall and Unestam, 1979) , acetone and alcohol (Preston and Taylor, 1970) or sodium dodecyl sulfate (Funatsu and Inaba, 1962). However, Benjakul *et al*. (2005b) found that sodium dodecyl sulfate and methanol showed no influence on the activity of PPO from kuruma prawn cephalothorax.

The latent PPO from shrimp and lobster is activated by trypsin or an endogenous enzyme. The activation in these species was the result of proteolysis that produced the numerous isoenzymes. 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. Benjakul *et al*. (2005b) reported that trypsin had no effect on the activity of PPO from kuruma prawn cephalothorax and proposed that the activated PPO might be present in the extract. Simpson *et al*. (1987) reported that trypsin did not appear to alter the activity of shrimp (*Penaeus setiferus*) PPO. However, Norway lobster prophenolase 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 prophenolase from Norway lobster (Zotos and Taylor, 1996). Other proteases (chymotrypsin and pepsin) do not have the same activation toward PPO as trypsin, although chymotrypsin was shown to activate PPO in insects (Kim *et al*., 2000).

It has been reported that PPO is released from the pro-form by microbial products such as laminarin (β-1, 3- glucan). The activation causes the formation of melanins, which possess antimicrobial properties. The formation of secondary metabolites, such as glucans, glycoproteins, lipopolysaccharides, laminarins, and others by microorganisms may also induce the protease to activate the PPO. In addition, it is believed that the specific microorganisms can induce the protease that activates PPO (Kim *et al*., 2000).

2. Enzymatic browning: Melanosis or Blackening

The postmortem dark discoloration called "melanosis or blackening" is one of problems causing the loss of market value of crustaceans. This phenomenon is triggered by a biochemical mechanism involving the oxidation of phenols to quinones by polyphenol oxidase (PPO). This is followed by non-enzymatic polymerization of the quinones, giving rise to pigments of high molecular weight and very dark or black coloring (Montero *et al*., 2001b). The melanosis in chilled shrimps begins at the head and then spreads to the tails; the rate of spread of melanosis differs among the various species. The spread of melanosis in pink shrimp (*Penaeus duorarum*) is faster than in white shrimp (*Penaeus setiferus*). This could be related to the differences in levels of substrate or levels of enzyme concentration or enzymatic activity in each species (Simpson *et al*., 1987). Figure 3 illustrates the melanosis and provides a visual scale for the progression of melanosis in shrimp.

Although the pigmentation seems to be harmless to consumers, it drastically reduces the product' s market value and causes the financial loss. Its occurrence in seafood is considered a deteriorative process that must be controlled and/or eliminated. Controlling this reaction begins by understanding its mechanism(s), the properties of the enzyme(s), its substrates and inhibitors, and the chemical, biological, and physical factors affecting the reaction (Kim *et al*., 2000).

Figure 3. Melanosis progression scale of shrimp
Source: Kim *et al*. (2000)

2.1 Factor influencing melanosis or black spot development in crustaceans

2.1.1 Molting cycle

Crustacean must replace their exoskeleton with a larger one to increase in size. This phenomenon is called “molting”. Each molting cycle is clearly set off by the shedding of the old exoskeleton (ecdysis). The molting cycle is subdivided into four stages (postmolt, intermolt, premolt and molt) (Kim *et al*., 2000). At the postmolt, the shell is hard and PPO activity is very low. In the intermolt stage, crustaceans are beginning to develop the new cuticle under the old shell. During late premolt, the old shell is ready to be discarded and the new cuticle begins to harden. At molting stage, crustaceans are the most vulnerable because the new cuticle will not harden for a few days (Kim *et al*., 2000).

Melanosis appears to be related to the level of active PPO initially present in the cuticle, which is also related to the molting cycle (Ali *et al*., 1994). Lobster with high PPO activity (late premolt) was more prone to form black spot than those with low PPO activity (intermolt). Ogawa *et al*. (1983) reported the relationship between the appearance of black discoloration and molting cycle in the integumentary

tissue of the tails of *Panulirus laevicauda*. They suggested that the physiological factors in the live state may contribute to melanin formation. The preparation for molting is accompanied by an increase in oxygen consumption by the entire animal, resulting in increased metabolism in some or all tissues.

In addition, Brookhart and Kramer (1990) reported that the molting fluid contained more than 10 proteolytic enzymes, possibly a complex and unique mixture of endo- and exo- cleaving proteolytic enzymes, which related to the activation of proPPO to PPO. Ali *et al*. (1994) hypothesized that the molting fluid was the source of the natural activator(s) of proPPO. They also found that the proPPO forms were activated *in vitro* by molting fluid, which is secreted into the cuticle just before molt. Thus, crustaceans ready to molt should be avoided for marketing because of their increased susceptibility to melanosis.

2.1.2 Species

In chilled shrimp, the rate of spread of melanosis differs among the 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). PPO was much more active in some species. In pink shrimp (*Penaeus duorarum*; Simpson *et al*., 1987), PPO was more active than in the white shrimp (*Penaeus setiferus*; Madero and Finne, 1982; Simpson *et al*., 1987). However, the spread of melanosis was much slower in the black tiger shrimp (Rolle *et al*., 1991).

2.1.3 Storage time

Nonenzymatic melanin formation may also be important in black spot formation over prolonged iced storage (Ali *et al*., 1994). Bailey and Fieger (1954) suggested that the formation of PPO substrates from both bacteriological and enzymatic breakdown of protein during prolonged storage is important factor in black spot development. Additionally, Madero and Finne (1982) reported that the pH at the surface of shrimp may reach as high as 7.5 or 8 during iced storage due to production of nitrogen bases, which could accelerate non-enzymatic melanin formation.

Martinez-Alvarez *et al*. (2005) reported that the development of black spot in chilled tiger prawns (*Marsupenaeus japonicus*) was noticeable after 1 day and gradually increased in both cephalothorax and abdomen. However, the black spot

development did not correlate with PPO activity because decreasing in PPO activity (80% reduction) was found after 6 days while melanosis score reached 3 (scoring melanosis according to a scale of from 1 to 4, where 1 = complete absence of black spots; 2 = a few small spots on the carapace; 3 = considerable spotting on the carapace; 4 = substantial spotting over the entire shrimp). They suggested that this could be due to the double mechanism of melanosis. Melanosis starts by the action of PPO, which oxidizes naturally occurring phenols from amino acids into quinones. Subsequent polymerization of the quinones by non-enzymatic mechanism gives rise to the accumulation of black high-molecular-weight pigments, not necessarily correlated to the direct action of PPO at this point.

3. Inhibition of polyphenol oxidase (PPO) activity in shellfish

3.1 The use of some chemicals/inhibitors

3.1.1 Reducing agents

Melanosis in crustaceans can be controlled by some chemicals. The use of reducing agents is the most effective control method for melanosis (Martinez and Whitaker, 1995). The major role of reducing agents in the prevention of melanosis is their ability to reduce the *o*-quinones to the colorless diphenols, or react irreversibly with the *o*-quinones to form the stable colorless products (Kim *et al*., 2000). The most widespread treatment used by the food industry for control of malanosis is the addition of sulfiting agents. Sulfiting agents include sulfur dioxide ($SO_{2)}$ and several forms of inorganic sulfite that liberate SO_2 under the condition of use (Kim *et al*., 2000). 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 inhibiting irreversibly PPO causing complete inactivation (Ferrer *et al*., 1989). Martinez-Alvarez *et al*. (2005) reported that prawns (*Marsupenaeus japonicus*) treated with sulfite-based solution had the lowest melanosis score up to 8 days. Therefore, prawns treated with sulfite-based solution showed a slower melanosis development. However, prawns (*Penaeus japonicus*) treated with 1.25% sodium bisulfite did not display any appreciable

differences in the melanosis rating scores with respect to the untreated prawns during iced storage of 35 days (Lopez-Caballero *et al*., 2000).

Ascorbic acid and its various neutral salts and other derivatives have been the leading GRAS additive used in food to prevent browning and other oxidative reactions. The mechanism of ascorbic acid for melanosis inhibition has generally been attributed to the reduction of enzymatically formed *o*-quinones back to their precursor diphenols, thus preventing the formation of pigments (Walker, 1977). Montero *et al*. (2001b) found that ascorbic acid did not slow down the progress of melanosis in prawns (*Penaeus japonicus*) compared to the control treatment. The inhibitory activity of ascorbic acid, whereby the *o*-quinones are reduced to original phenols, was not effective under the experimental conditions. However, ascorbic acid is irreversibly oxidized to dehydroascorbic acid during the process and browning can occur after depletion. Therefore, ascorbic acid derivatives such as erythorbic acid, 2- and3-phosphate, phosphinate esters, and ascorbyl-6-fatty acid esters of ascorbic acid as more stable forms have been developed (Liao and Seib, 1990).

Sulfhydryl compounds, such as cysteine, tripeptide, reduced glutathione, *N*-acetylcysteine, and thiourea, have been found to be the potential inhibitors of browning in a variety of foods. Cysteine is an effective inhibitor of enzymatic browning. It is reported to be more effective than sodium bisulfite as an antibrowning agent (Kahn, 1985). Concentrations of cysteine and other thiols required for the achievement of acceptable levels of browning inhibition have however negative effects on taste. The inhibition of melanosis by cysteine is thought to be due to the formation of colorless thiol-conjugated *o*-quinones. Cysteine has also been shown to reduce *o*-quinones to their phenol precursors (Walker, 1977; Cilliers and Singleton, 1990). In addition, Cysteine-quinone adducts were proved to be competitive inhibitors of PPO (Kim *et al*., 2000).

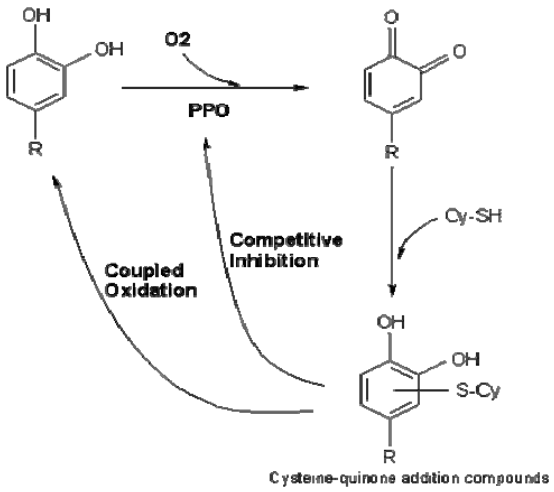

Figure 4. Effect of cysteine and cysteinyl addition products with o-quinones on the enzymatic oxidation of *o*-diphenols
Source: Kim *et al*. (2000)

The inhibitory effect of cysteine and glutathione on PPO from kuruma prawn (*Penaeus japonicus*) was investigated by Benjakul *et al*. (2006). Cysteine exhibited a slightly greater inhibition, toward kuruma prawn PPO, than did glutathione. For the inhibition kinetics, cysteine and glutathione showed competitive inhibition since K_m values increased with cysteine and glutathione concentrations, whereas V_{max} values remained unchanged. Competitive inhibition takes place when a molecule that is structurally similar to the substrate for a particular reaction competes for a position at the active site on the enzyme (Benjakul *et al*., 2006). In addition, cysteine and glutathione could also reduce *o*-quinone or interact with the intermediate, leading to colorless compounds. Thus, inhibition of PPO by both compounds might occur directly or indirectly (Benjakul *et al*., 2006).

3.1.2 Acidulants

The role of acidulants is to maintain the pH below what is needed for optimum catalytic activity of PPO. Acidulants such as citric acid and phosphoric acid, and malic acid lower the pH of the system to below 3 where PPO is inactive. The acidulant is often used in combination with other antibrowning agents. Benner *et al*. (1994) reported that brown shrimp (*Penaeus aztecus*) treated with L-lactic acid in combination with 4-hexylresorcinol (0.0025%) was effective as a melanosis inhibitor.

Citric acid is the one of the most widely used acid in the food industry with ascorbic acid (Kim *et al*., 2000). Citric acid exerts inhibition on PPO by reducing the pH as well as by chelating the copper at the enzyme-active site (Kim *et al*., 2000).

3.1.3 Chelators

The removal of metal ions by chelators would render the PPO inactivated, leading to the retarded melanosis (Kim *et al*., 2000). Chelating agents such as EDTA, citric acid, phosphates maltol, and kojic acid complex with prooxidative agents (copper and iron ions) through an unshared pair of electron in their molecular structure which provides the chelating action (Kim *et al*., 2000). Montero *et al*. (2001b) reported that prawns (*Penaeus japonicus*) treated with kojic acid showed no black spot until 5 days of storage. Apart from being antimicrobial agent, sodium benzoate has been reported to be a chelating agent (Kim *et al*., 2000). This compound showed a melanosis inhibitory effect in prawns (*Penaeus japonicus*) more effective than ascorbic acid and citric acid (Montero *et al*., 2001b).

3.1.4 Enzyme inhibitors

4-Hexylresorcinol (4-HR), which is also structurally related to phenolic substrates, is recognized as PPO inhibitors. 4-HR has several advantages over sulfites in food including its specific mode of inhibitory action, its lower use level required for effectiveness, its inability to bleach preformed pigments, and its chemical stability. 4-HR acts as an enzyme-competitive inhibitor due to structural resemblance to phenolic substrates (Kim *et al*., 2000). McEvily *et al*. (1990) reported that dipping 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. Montero *et al*. (2001b) reported that 4-HR was the most effective inhibitor for melanosis prevention in prawns (*Penaeus japonicus*) during chilled storage. 4-HR was used for controlling melanosis in crustaceans as an alternative to sulfites (Kim *et al*., 2000). Lambrecht (1995) reported that the headless shrimp dipped in 4-HR for 1 min controlled black spot formation for a longer period of time than fresh water (control) or 1.25% sodium metabisulfite. After 7 days of storage at 2 °C, raw head-off brown shrimp treated with water showed 54% black spot and the sulfite-treated shrimp had 11% black spot, whereas 4-HR-treated shrimp had only 3.6% black spot. At day 14, the black spots of control and sulfite-treated shrimp were increased to 75

and 25, respectively. However, the 4-HR-treated shrimp did not show an increased in black spot. Perez-Mateos *et al*. (2002) studied the effect of 4-HR on the PPO activity from oysters (*Ostraea edulis*) during 21 days of storage. PPO activity in the batch treated with 50 ppm of 4-hexylresorcinol was lower than the batch without inhibitors after day 7 of storage.

Recently, other anti-browning agents have been developed. Gokoglu and Yerlikaya (2007) reported that grape seed extracts with concentration of 15 g/l markedly exhibited the inhibitory activity on the melanosis formation of shrimp (*Parapenaeus longirostris*) stored at 4 °C. Additionally, Nirmal and Benjakul (2009) reported that ferulic acid (FA) (0.1%, 0.5%, 1% and 2% (w/v)) showed inhibitory activity towards PPO from Pacific white shrimp (*Litopenaeus vannamei*) in a dose dependent manner.

4.2 Physical methods

4.2.1 Heating

Heat treatment is the most widely utilized method for stabilizing foods because of its capacity to destroy microorganisms and to inactivate enzymes. Enzyme denaturation at high temperatures involves unfolding of the native structure to a random coiled structure that has lost catalytic activity (Kim *et al*., 2000). Thermal stability of an enzyme is related to its structure, the relative amounts of free and bound water, substrate binding, pH, and presence of salts (Adams, 1991). However, heat treatment is not a practical method for treatment of fresh foods. Heating is nutritionally disadvantageous since it results in losses in vitamins, flavors, colors, texture, carbohydrates and other water-soluble components. Requirements for large amounts of water and energy, coupled with waste disposal problems make heating technically disadvantageous (Kim *et al*., 2000).

4.2.2 High-pressure treatment

Enzyme denaturation is caused by rearrangement and/or destruction of noncovalent bonds such as hydrogen bonds, hydrophobic interactions, and ionic bonds of the tertiary protein structure. Pressure can influence biochemical reactions by reducing molecular spacing and increasing interchain reactions. High-pressure treatment is a potentially viable technique for preserving food quality through the

inactivation of endogenous food enzymes (Kim *et al*., 2000). Pressures exceeding 5 kbar generally cause irreversible denaturation of enzymes due to the weakening of hydrophobic interactions and the breaking of intramolecular salt bridges. High-pressure treatments can result in either reversible or irreversible changes in protein structure. Loss of catalytic activity under high-pressure conditions however varies in accordance with the enzymes, the nature of the substrates, the temperature and the duration of high pressure processing. Montero *et al*. (2001a) reported that the activity of PPO from prawns (*Panaeus japonicus*) was greater at atmospheric pressure (0.1 MPa) than in pressurized samples at basidic condition (pH 8.0). There was a decrease in activity as the pressure was gradually increased. They also reported that the slight decrease was observed from 100 to 200 MPa and more pronounced from 300 to 400 MPa. The PPO activity was therefore inactivated by pressure.

4.2.3 Exclusion of oxygen

PPO uses oxygen as a proton acceptor to convert the phenols into a variety of end products, including quinones that spontaneously rearrange into the end product melanin (Aspán *et al*., 1995). Tanner *et al*. (2006) reported that PPO activity from Atlantic blue crab (*Callinectes sapidus*) decreased by 33%, 49% and 70% of activity in air at 15%, 5% and 1% O_2, respectively. Perez-Mateos *et al*. (2006) reported that PPO activity from pressurized oysters (*Ostraea edulis*), stored in vacuum packaging was lower than the pressurized oysters, packaged aerobically. Thus the use of oxygen impermeable packages, modified aymosphere packaging or vacuum packaging may be useful in preventing the onset of browning induced by PPO (Martinez and Whitaker, 1995).

Objective

1. To characterize PPO from the cephalothorax of Pacific white shrimp.
2. To study PPO inhibitory activity and antioxidative activity of sulfur-containg compounds and their Maillard reaction products (MRPs).
3. To investigate some factors affecting PPO inhibitory activity of heated cysteine.
4. To study the uses of heated alkaline cysteine to retard the quality loss of Pacific white shrimp during iced storage.

CHAPTER 2

RESEARCH METHODOLOGY

1. Material

1.1 Pacific white shrimp samples

Pacific white shrimps (*Litopenaeus vannamei*) with a size of 55-60 shrimps/kg were purchased from a farm in Songkhla province, Thailand. The shrimps 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, shrimps were washed in cold water and the cephalothoraxes were removed and used as the source of PPO. The cephalothoraxes were powderized by grinding with liquid nitrogen in a Waring blender. The powder obtained was kept in polyethylene bags, sealed and stored at -20 °C until use. The storage time was not more than 1 month.

1.2 Chemicals

L-β-(3,4 dihydroxylphenyl) alanine (L-DOPA), Brij-35, tetramethylmurexide (TMM), 2,2-azino-bis(3-ethylbenzothaiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and methionine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ammonium sulfate, ferric chloride and sodium chloride were procured from Merck (Damstadt, Germany). Glycine and cysteine were purchased from Fluka (Messerchmittstr, Switzerland). Di-sodium hydrogen orthophosphate, sodium tetraborate (borax), hexamine and glucose were obtained from UNILAB (Auckland, New Zealand). Glutathione was purchased from Wako Pure Chemical Industries (Tokyo, Japan).

2. Method

2.1 Characterization of Pacific white shrimp PPO

2.1.1 Extraction and fractionation of Pacific white shrimp PPO

Extraction and fractionation were carried out according to the method of Benjakul *et al*. (2005b). Cephalothorax powder (50 g) was homogenized with 0.05 M sodium phosphate buffer containing 1.0 M NaCl and 0.2% brij 35 (pH 7.2) at a ratio of 1:3 (w/v) using a homogenizer (IKA Labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was then stirred for 30 min at 4 °C. After centrifugation at 8000 ×*g* for 30 min at 4 °C using a Sorvall Model RC-5B plus refrigerated centrifuge (Newtown, CT, USA), the supernatant was fractionated by the addition of solid ammonium sulfate to obtain 40% saturation. The mixture was allowed to stand at 4 °C for 30 min. The precipitate was collected by centrifugation at 12,500 × *g* at 4°C for 30 min. The pellet obtained was dissolved in a minimal volume of 0.05 M sodium phosphate buffer, pH 7.2 and dialyzed with 15 volumes of the same buffer with three changes at 4 °C. The dialysate was referred to as "PPO extract".

2.1.2 PPO activity assay

PPO activity was determined by monitoring the rate of dopachrome formation using L-DOPA as a substrate as described by Matmaroh *et al*. (2006) with some modifications. PPO extract (300 µl) was mixed with 300 µl of distilled water. Thereafter, 600 µl of 20 mM L-DOPA in McIlvaine buffer (0.2 M Na-phosphate and 0.1 M Na-citrate) (pH 6.0) were added to initiate the reaction. The reaction was run for 3 min at 45 °C and A_{475} was measured. One unit of PPO was defined as the change in 0.001 U of A_{475}/min/ml.

2.1.3 Characterization of Pacific white shrimp PPO

2.1.3.1 pH and temperature profiles

The activity of PPO was assayed at different pHs (3.0 to 9.0) using McIlvaine buffer for pHs 3-7 and 0.1 M phosphate-borate buffer for pHs 8-9. The assay system was prepared by mixing 300 µl of PPO extract with 300 µl of distilled water and the mixture was then added with 600 µl of 20 mM L-DOPA dissolved in buffers with various pHs to initiate the reaction. The reaction was run at 25 °C for 3 min and A_{475} was measured.

For temperature profiles study, 300 µl of PPO extract was mixed with 300 µl of distilled water before adding 600 µl of 20 mM L-DOPA dissolved in McIlvaine buffer (pH 6.0) preincubated at various temperatures (25, 30, 35, 40, 45, 50, 55, 60 and 70 °C). The reaction mixture was run at each temperature for 3 min and A_{475} was determined.

2.1.3.2 pH and thermal stability

The pH stability of PPO extract was determined by incubating PPO extract in various buffers with different pHs (4.0-9.0) for 30 min. The residual activity was determined under the optimum condition. For thermal stability, PPO extract was preincubated at different temperatures (25-70 °C) for 30 min. After rapid cooling in iced water, the residual activity was determined under the optimum condition.

2.1.3.3 Activity staining

PPO activity staining was carried out according to the method of Benjakul *et al*. (2005b). Enzyme extract was mixed with the sample buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, glycerol and 0.3% bromophenol blue) with and without 1.5M βME at a ratio of 1:1 (v/v). The mixture (20 µg protein) was loaded onto the gel made of 7.5% separating gel and 4% stacking gel. Electrophoresis was run at a constant voltage of 120 /gel. After separation, one of two identical gels was immersed in McIlvaine buffer, pH 6.0 containing 20 mM L-DOPA for 15 h at 25 °C. The activity zone appeared as the dark band. Another gel was stained with 0.125% Coomassie Brilliant Blue R-250 dissolved in 50% methanol and 7.5% acetic acid and destained in 25% methanol and 10% acetic acid. High-molecular-weight standards were used for estimation of the apparent molecular weight of PPO activity zone.

2.2Anti-browning and antioxidative activities of sulfur-containing compounds and their Maillard reaction products (MRPs) prepared under alkaline condition

2.2.1 Preparation of sulfur-containing compounds and Maillard reaction products (MRPs)

Different sulfur-containing compounds (cysteine, methionine and glutathione) were dissolved in 0.1 M borate buffer (pH 10) to obtain the concentrations of 2 and 20 mM. Those compounds were also used for preparation of

MRPs. To prepare MRPs, sulfur-containing compounds were mixed with glucose at equimolar in the same buffer. Glycine solutions (2 and 20 mM) were also prepared by dissolving glycine in the same buffer. All solutions were transferred to screw-sealed tubes, tightly capped and heated in an oil bath (Buchi labortechnik AG, Flawil, Switzerland) at 100 °C for 12 h. The heated samples were cooled immediately in iced water. Solutions were then neutralized using 6 N HCl. Additionally, all compounds dissolved in distilled water were also subjected to heating at 100 °C for 12 h, following by cooling and neutralization and were used as the controls. All solutions obtained were kept at 4 °C until analysis.

2.2.2 Assays

2.2.2.1 PPO inhibitory activity

PPO extract (300 µl) with activity of 380-400 units/ml was mixed with 300 µl of various solutions. The mixtures were allowed to stand for 10 min at room temperature. The mixture was then added with 600 µl of L-DOPA in McIlvaine buffer, pH 6, to initiate the reaction as described previously. The residual activity was determined and % inhibition was calculated.

2.2.2.2 Copper chelation

Copper binding capacity was determined according to the method of Wijewickreme *et al*. (1997). The samples (with appropriate dilution) (500 µl) were mixed with 1.5 ml of 10 mM hexamine buffer (pH 5.0) containing 10 mM KCl. The mixture was then added with 500 µl of 0.1 mM $CuSO_4$ and incubated for 10 min at room temperature. The reaction mixture was mixed with 100 µl of 1 mM TMM in hexamine buffer (10 mM, pH 5.0). The amount of free copper in the solutions was obtained from a standard curve, where the absorbance ratio A_{460}/A_{530} of solutions containing 500 µl of $CuSO_4$ (0.02-0.1 mM), 2.0 mL of hexamine buffer and 100 µl of TMM was plotted against the concentration of $CuSO_4$ (0.02-0.1 mM). The amount of copper bound to MRP mixtures was calculated as the difference between the amount of copper added and free copper present in the solution.

2.2.2.3 Reducing power

Reducing power was determined according to the method of Oyaizu (1986). One ml of samples (with appropriate dilution) was mixed with 1 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide

($K_3Fe(CN)_6$). The reaction mixture was incubated in a temperature-controlled water bath at 50 °C for 20 min, followed by addition of 1 ml of 10% trichloroacetic acid. The mixture was then added with 1 ml of distilled water and 200 µl of 0.1% $FeCl_3$. The absorbance at 700 nm was determined and used as the measurement of reducing power. Sample blank was prepared in the same manner, except distilled water was used instead of 1% potassium ferricyanide.

2.2.2.4 ABTS radical scavenging activity

ABTS radical scavenging activity was determined by ABTS assay as described by Arnao *et al*. (2001). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Fresh ABTS solution was prepared for each assay. Sample (150 µl) was mixed with 2850 µl of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance was then measured at 734 nm using the spectrophotometer. The blank was prepared in the same manner, but distilled water was used instead. For sample blank, ABTS solution was excluded and methanol was replaced. A standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was expressed as mmol Trolox equivalents (TE)/ml sample.

2.2.2.5 DPPH radical scavenging activity

DPPH radical scavenging activity was determined by DPPH assay according to the method of Wu *et al*. (2003). Sample (1.5 ml) was added with 1.5 ml of 0.15 mM 2,2-diphenyl-1-picryl hydrazyl (DPPH) in 95% ethanol. The mixture was mixed vigorously and allowed to stand at room temperature in dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same manner, except that distilled water was used instead of the sample. The sample blank was prepared by leaving out DPPH solution and using ethanol instead. A standard curve was prepared using Trolox in the range of 10-60 µM. The activity was expressed as µmol Trolox equivalents (TE)/ml sample.

Sulfur-containing compounds yielding the highest PPO inhibitory activity was chosen for further study.

2.2.3 Effect of heated alkaline cysteine on the quality changes of Pacific white shrimp during iced storage

2.2.3.1 Preparation of shrimps treated with heated alkaline cysteine

Heated alkaline cysteine (HAC) solution with the concentrations of 20 and 100 mM were prepared as previously described. Before used, the solutions were neutralized using 6 N HCl. Shrimps were soaked in the neutralized HAC with a shrimp/solution ratio of 1:10 (w/v) for 10 min. Treated shrimps were drained on the screen for 3 min. Shrimps without treatment were used as the control. All samples were stored in ice using a shrimp/ice ratio of 1:2 (w/w). The ice was changed everyday and shrimp/ice ratio was maintained constant. Twelve shrimps were taken for each treatment every 3 day up to 12 days for analyses.

2.2.3.2 Determination of melanosis

Melanosis or blackening of shrimp samples during storage was evaluated by visual inspection as described by Otwell and Marshall (1986) using a scale where 0 = absent; 2 = slight, noticeable on some shrimps; 4 = slight, noticeable on most shrimps; 6 = moderate, noticeable on most shrimps; 8 = heavy, noticeable on most shrimps; and 10 = heavy, totally unacceptable. Twelve trained panelists were used for the assessment.

2.2.3.3 Determination of thiobarbituric acid reactive substance (TBARS)

TBARS in the samples were determined according to Buege and Aust (1978). Shrimp meat (1 g) was mixed with 5 ml of a solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid 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 5500 × *g* for 25 min. The supernatant was collected and the absorbance was measured at 532 nm using a UV-160 spectrophotometer. TBARS value was calculated from the standard curve of molonaldehyde and expressed as mg malonaldehyde/kg sample.

2.2.3.4 Determination of psychrophilic bacterial count

Whole samples without peeling (10 g) were weighed into a stomacher bag containing 90 ml of 0.85% normal saline. Blending was conducted in a Stomacher (IUL Instrument, Spain) for 2 min. Normal saline (0.85%) was used for diluting the samples. Thereafter, the sample diluted in serial 10-fold dilution was spread on plate count agar (Merck, Damstadt, Germany). The plates were incubated at 4 °C for 1 week prior to psychrophilic bacterial counting as described by Speck (1976).

2.3 Effect of pH on anti-browning and antioxidative activities of heated cysteine

2.3.1 Preparation of heated cysteine

Cysteine (20 mM) was dissolved in different buffers with various pHs (0.1 M sodium citrate for pH 2-6; 0.1 M Tris-HCl buffer for pH 7; 0.1 M borate buffer for pHs 8, 9 and 10; 0.1 M sodium hydrogen carbonate buffer for pH 11). The solutions prepared were transferred to screw-sealed tubes, tightly capped and heated in an oil bath at 100 °C for 12 h. The heated samples were cooled immediately in iced water. Heated cysteine (HC) solutions were then neutralized and kept at 4 °C until analyzed.

2.3.2 Assays

All analyses were carried out as described in section 2.2.2

The HC with initial pHs rendering the highest PPO inhibitory activity was chosen for further study.

2.4 Effect of reactant concentration on anti-browning and antioxidative activities of heated alkaline cysteine (HAC)

2.4.1 Preparation of heated alkaline cysteine (HAC)

HAC were prepared by heating cysteine in 0.1 M borate buffer, pH 10 at 100 °C. Different reactant concentrations (20, 100 and 500 mM) were used. To prepare HAC, the mixtures were transferred to screw-sealed tubes, tightly capped and heated in an oil bath at 100 °C for 12 h. The heated samples were cooled immediately in iced water. HAC obtained were neutralized and kept at 4 °C until analyzed.

2.4.2 Assays

All analyses were carried out as described in section 2.2.2.

The HAC with reactant concentration rendering the highest PPO inhibitory activity was chosen for further study.

2.5 Effect of heated alkaline cysteine (HAC) on the quality changes of Pacific white shrimp (*Litopenaeus vannamei*) during iced storage.

2.5.1 Preparation of shrimp treated with heated alkaline cysteine

Heated alkaline cysteine (HAC) solution with the concentration of 500 mM was prepared by heating the solution (pH 10) at 100 °C for 12 h. Before used, the solutions were neutralized using 6 N HCl and then diluted into different concentrations (20, 100 and 200 mM). Shrimps were soaked in the neutralized HAC with a shrimp/solution ratio of 1:10 (w/v) for 10 min. Treated shrimps were drained on the screen for 3 min. Shrimps without treatment were used as the control. All samples were stored in ice using a shrimp/ice ratio of 1:2 (w/w). The molten ice was removed everyday and shrimp/ice ratio was maintained constant. Twelve shrimps were taken for each treatment every 3 day up to 12 days for analyses.

2.5.2 Chemical determination

2.5.2.1 Total volatile base (TVB) and trimethylamine contents (TMA)

TVB and TMA were determined using Conway's method according to the method of Conway and Byrne (1939).

2.5.2.2 Thiobarbituric acid reactive substance (TBARS)

TBARS was conducted according to the method of Buege and Aust (1978).

2.5.3 Determination of melanosis

Melanosis or blackening of shrimp samples during storage was evaluated by visual inspection as described by Otwell and Marshall (1986). Twelve trained panelists were used for the assessment.

2.5.4 Determination of psychrophilic bacterial count

Psychrophilic bacterial count was determined as described previously (section 2.2.3.4.)

3. Statistical analysis

Completely randomized design (CRD) was used throughout the study. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the SPSS static program (Version 11.0) for Window (SPSS Inc. Chicago, IL, USA).

CHAPTER 3

RESULTS AND DISCUSSION

1. Characterization of PPO from cephalothorax of Pacific white shrimp

1.1 pH and temperature profiles

The pH profile of PPO in the extract is shown in Figure 5. The maximal activity of PPO was observed at pH 6.0. The activity markedly decreased in a very acidic and alkaline pH ranges. Approximately 20% of activity was remained at pH 4.0 and the activity was completely lost at pH 3.0. A sharp decrease in activity was also noticeable at pH higher than 7.0. The optimal pH of PPO depends to a large extent on the physiological pH, in which the enzyme activity occurs in nature or the locus of extraction (Montero *et al*., 2001a). For prawns (*Panaeus japonicus*), the optimal pH of PPO from carapace of the cephalothorax was 7.16, while the optimal pH of PPO from abdominal cuticle was 8.76 (Montero *et al*., 2001b). The optimal pH of crustacean PPO also varied with the species (Rolle *et al*., 1991). PPO from the carapace of shrimp (*Penaeus setiferus*) showed the maximal activity at pH 7.5 (Simpson *et al*., 1987), while the optimal pH of black tiger prawn PPO was 6.0 (Rolle *et al*., 1991). In addition, PPO from *Penaeus duodorum* was active in the pH range of 6.5-9.0 (Simpson *et al*., 1988). Benjakul *et al*. (2005b) found that PPO from the cephalothorax of kuruma prawn (*Penaeus Japonicus*) cultured in Japan exhibited the maximal activity at pH 6.5. The differences in optimal pH might be owing to the different nature of prototropic groups in the active site of enzymes (Whitaker, 1972).

The optimal temperature of Pacific white shrimp PPO was 45 °C (Figure 6). The activity increased with increasing temperature up to 45 °C. However, the activity decreased when the temperature increased, mainly due to thermal denaturation of PPO. The optimal temperature of PPO has been reported to be varied, depending on species and habitat temperature. The optimal temperature of PPO from the kuruma prawn cephalothorax was 35 °C (Benjakul *et al*., 2005b), whereas the

optimal temperature of PPO from carapace of shrimp (*Penaeus japonicus*) cultured in Spain was 55 °C (Montero *et al*., 2001b). Maximal activity of PPO from *Penaeus setiferus* (Simpson *et al*., 1987; 1988) and from *Penaeus monodon* (Rolle *et al*., 1991) was observed at 40-45 °C.

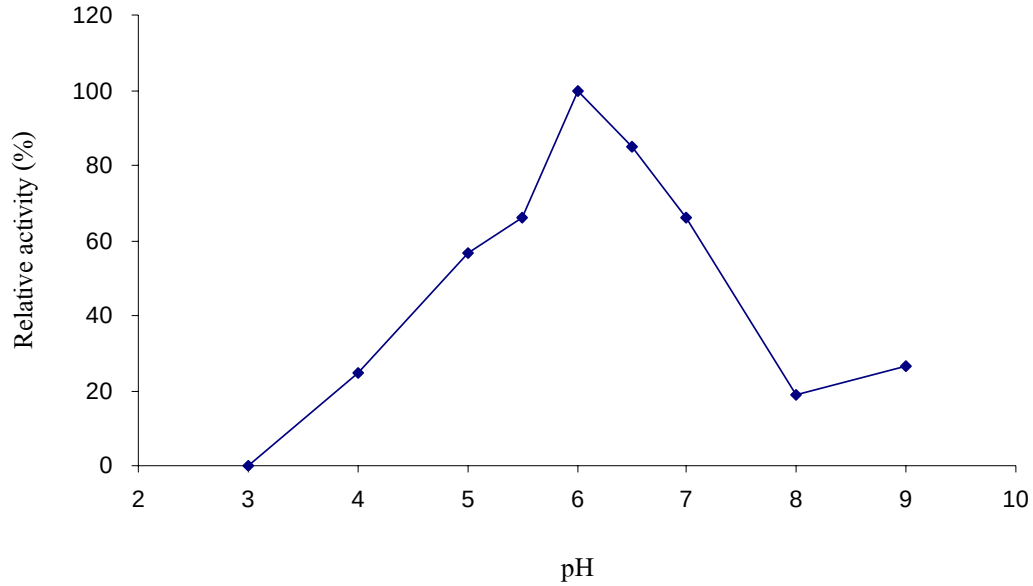

Figure 5. pH profile of PPO from the cephalothorax of Pacific white shrimp. The activity was assayed at 25 °C using 3,4 dihydroxy-L- phenylalanine as a substrate.

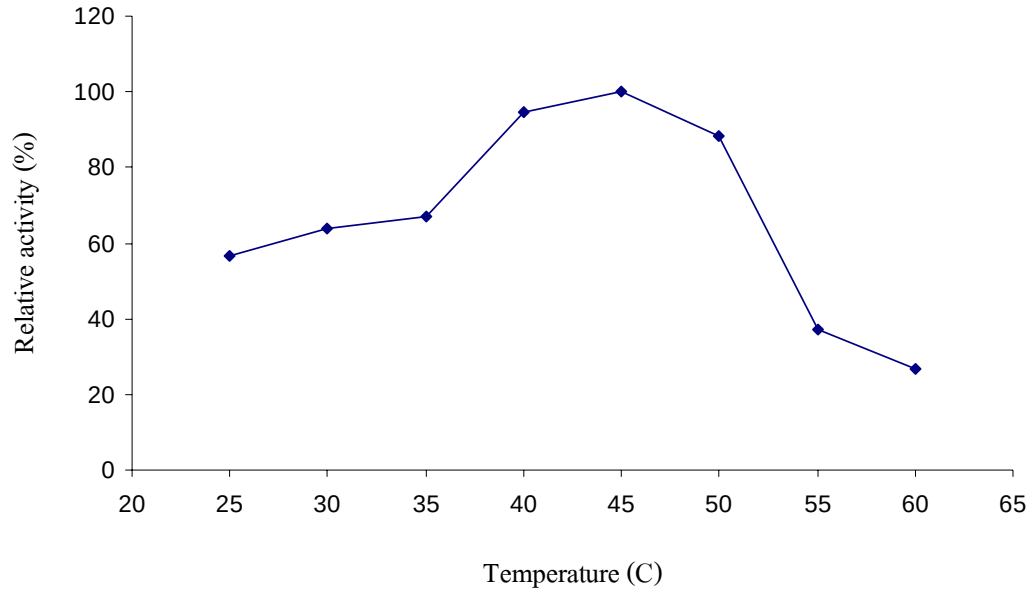

Figure 6. Temperature profile of PPO from the cephalothorax of Pacific white shrimp. The activity was assayed at pH 6.0 using 3,4 dihydroxy-L-phenylalanine as a substrate.

1.2 pH and thermal stability

PPO from Pacific white shrimp cephalothorax was most stable at pH 6.0 (Figure 7). At the lower pH, the sharp decrease in activity was obtained. Approximately 20% of activity was remained at pH 4.0. The result was in agreement with Rolle *et al*. (1991) who found that PPO from *Penaeus monodon* was unstable at pH below 5. PPO from white shrimp (*Penaeus setiferus*) and pink shrimp (*Penaeus duorarum*) was unstable at acidic pH (Simpson *et al*., 1988). Also, the activity also decreased at basic pHs. However, the degree of decrease was slightly lower than that found in the acidic pH range. Approximately 50% of activity was found when subjected to pH 9.0. However, Benjakul *et al*. (2005b) reported that PPO from the kuruma prawn cephalothorax was stable over a wide pH range (3.0-10.0). The differences in pH stability indicated the different molecular properties including the bonding stabilizing the structure as well as the enzyme conformation, which varied among the various species and anatomical locations (Benjakul *et al*., 2005b).

Thermal stability of Pacific white shrimp PPO is depicted in Figure 8. The activity was gradually lost when heated up to 50 °C. The marked decrease in

activity was noticeable when heated at temperature greater than 50 °C. Approximately 15% of activity was remained after heating at 70 °C. Most PPO are heat labile; a short exposure of the enzyme to temperature at 70-90 °C cause a partial or total irreversible denaturation. Vamos-vigyazo (1981) pointed out that the thermal stability of PPO was dependent on the enzyme source. PPO from white shrimp (*Penaeus setiferus*; Simpson *et al*., 1987) and kuruma prawn (*Penaeus japonicus*; Benjakul *et al*., 2005b) was unstable at the temperature greater than 50 °C, whereas pink shrimp PPO was not stable at temperatures higher than 40 °C (Simpson *et al*., 1988).

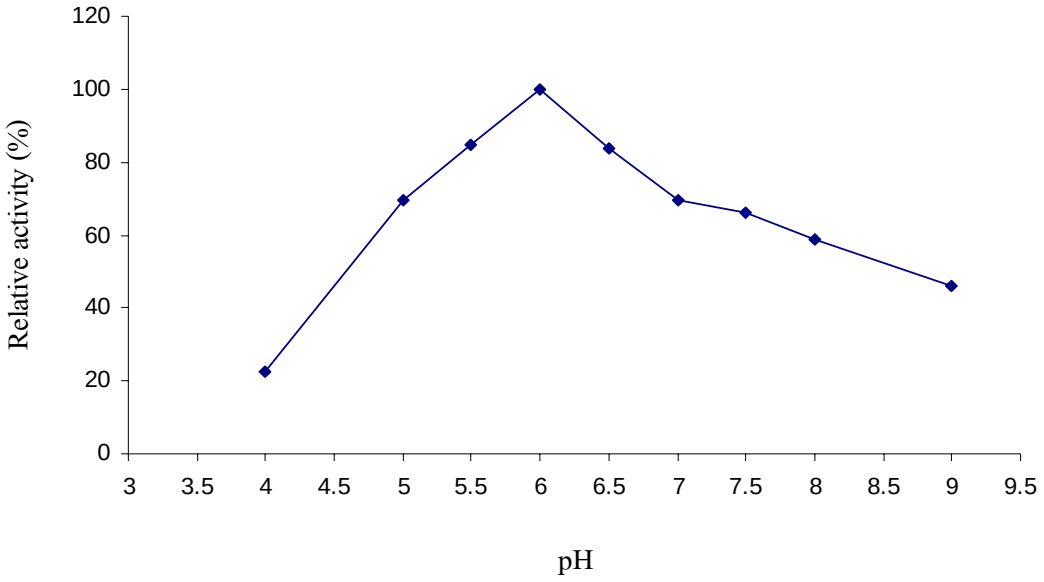

Figure 7. pH stability of PPO from the cephalothorax of Pacific white shrimp. PPO activity was assayed after incubating PPO extract for 30 min at various pHs. The residual activity was determined at 45 °C and pH 6.0 for 5 min using 3,4 dihydroxy-L-phenylalanine as a substrate.

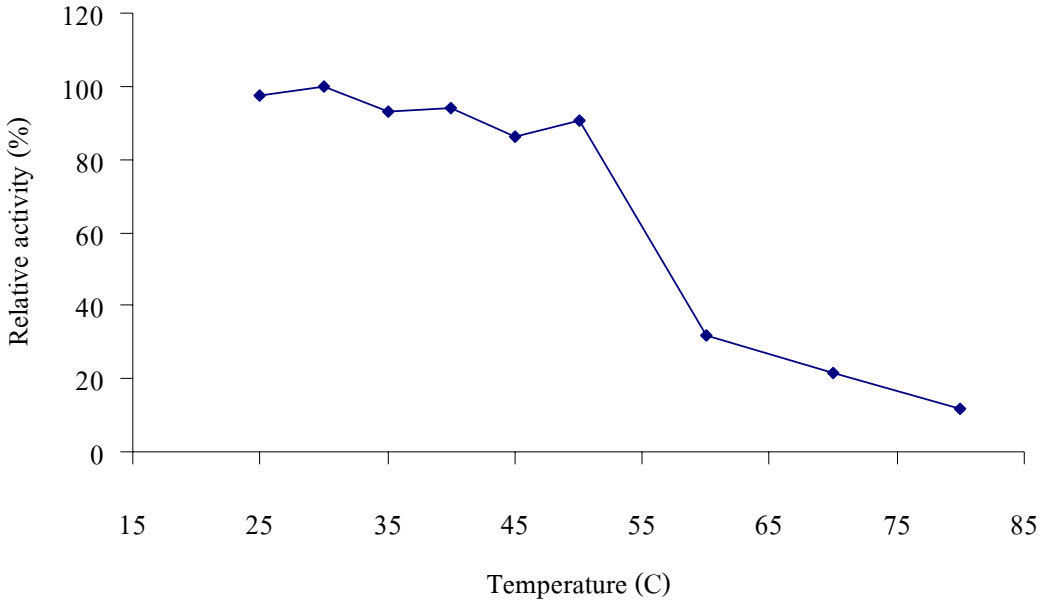

Figure 8. Thermal stability of PPO from the cephalothorax of Pacific white shrimp. PPO activity was assayed after incubating PPO extract for 30 min at various temperatures. The residual activity was determined at 45 °C and pH 6.0 for 5 min using 3,4 dihydroxy-L-phenylalanine as a substrate.

1.3 Activity staining of PPO

Activity staining of PPO from the cephalothorax of Pacific white shrimp is depicted in Figure 9. The activity band was observed as black color at the apparent molecular weight of 180 kDa. The result indicated that Pacific white shrimp PPO had no disulfide bond. No differences in activity band were observed between reducing and non-reducing condition. A wide range of molecular weight has been reported for PPO from different sources, especially in crustaceans. Benjakul *et al.* (2005b) reported that the apparent molecular weight of PPO from kuruma prawn was 160 kDa, while the molecular weights of PPO from pink shrimp were 30 and 35 kDa. PPO from different shrimps comprised the different isoforms with varying molecular weights (Chen *et al.*, 1991). Additionally, the molecular weight of PPO also varied with the molting stage (Ferrer *et al.*, 1989).

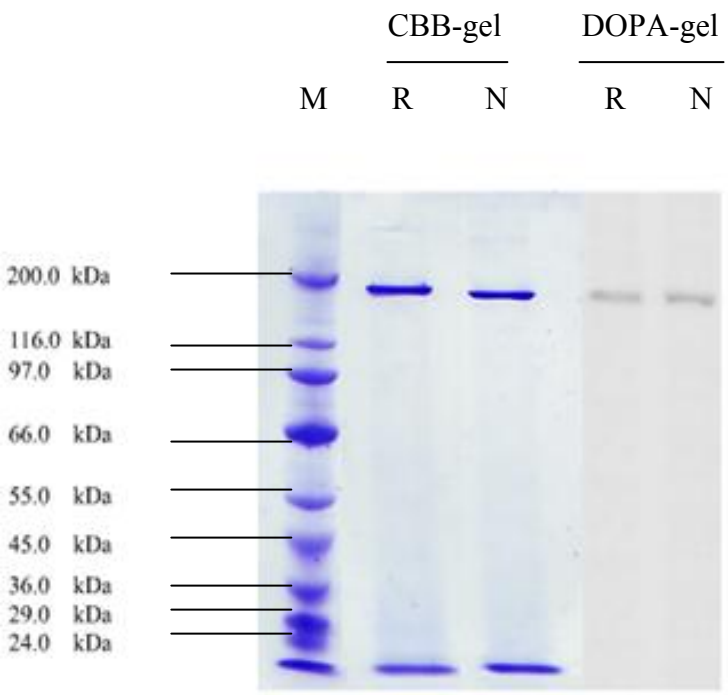

Figure 9. Activity staining of PPO from the cephalothorax of Pacific white shrimp. M: molecular weight marker; R: reducing condition; N: non-reducing condition

2. Some properties of sulfur-containing compounds heated under alkaline condition

2.1 PPO inhibitory activity and antioxidative activity

PPO inhibitory activity of sulfur-containing compounds heated under alkaline condition (pH 10) and their MRPs in comparison with that of glycine and glycine-glucose MRPs is shown in Table 3. Cysteine and glutathione had PPO inhibitory activity in a concentration dependent manner. The result was in agreement with Benjakul *et al*. (2006) who found the inhibitory effect of both compounds on PPO from kuruma prawn. After heating, cysteine and glutathione showed the higher inhibitory activity toward PPO ($P<0.05$), while no differences was found for glycine and methionine ($P>0.05$). In general, inhibitory activity increased as the concentration of reactant increased from 2 to 20 mM. At 20 mM, lower PPO inhibitory activity was observed in cysteine and glutathione when glucose was incorporated as evidenced by the lower inhibitory activity of MRPs of both compounds ($P<0.05$). However, no

differences were noticeable at a reactant concentration of 2 mM. MRPs derived from cysteine and glutathione exhibited the higher PPO inhibitory activity than those obtained from glycine and methionine. Billaud *et al*. (2005) reported that thiol-derived MRPs contained inhibitory compounds of PPO activity. Additionally, Tan and Harris (1995) reported that MRPs from cysteine-glucose heated for 1 h appeared to be the most effective inhibitor towards apple PPO. The result indicated that cysteine and glutathione with free sulfhydryl group at the side chain showed the remarkable PPO inhibitory activity after heating under alkaline pH, compared with the parent counterpart. However, methionine possessing sulfur residues inside molecule had no change in activity after heating under alkaline condition. Cysteine could be degraded under alkaline condition via β-elimination (Beeley and Levons, 1963). Degradation products of cysteine and glutathione might play a role in inhibiting PPO.

Table 3. PPO inhibitory activity of sulfur-containing compounds heated under alkaline condition and their Maillard reaction products.

Samples	% PPO inhibition					
	2mM			20mM		
	Original	Heated	MRPs	Original	Heated	MRPs
Glycine	$5.52^{*} \pm 0.51^{b,B**}$	$6.75 \pm 0.64^{b,D}$	$6.83 \pm 0.40^{b,C}$	$15.12 \pm 2.71^{a,C}$	$15.40 \pm 1.13^{a,C}$	$14.96 \pm 1.52^{a,B}$
Cysteine	$18.53 \pm 5.87^{d,A}$	$28.40 \pm 1.07^{c,A}$	$28.40 \pm 2.64^{c,A}$	$29.16 \pm 1.30^{c,A}$	$66.58 \pm 3.92^{a,A}$	$37.50 \pm 0.80^{b,A}$
Methionine	$16.02 \pm 0.99^{ab,A}$	$15.85 \pm 1.51^{ab,C}$	$11.26 \pm 1.63^{c,B}$	$19.04 \pm 1.68^{a,B}$	$18.45 \pm 1.40^{ab,C}$	$14.97 \pm 3.94^{bc,B}$
Glutathione	$17.65 \pm 0.46^{d,A}$	$24.48 \pm 0.85^{c,B}$	$28.55 \pm 1.49^{c,A}$	$27.47 \pm 1.15^{c,A}$	$56.05 \pm 5.46^{a,B}$	$40.28 \pm 0.47^{b,A}$

* Mean ± SD from triplicate determinations.

** The different letters in the same row indicated the significant differences ($P<0.05$).

The different capital letters within the same column indicate the significant differences ($P<0.05$).

2.2 Copper chelating ability

Copper chelating ability of sulfur-containing compounds without and with heating at alkaline pH in comparison with their MRPs is shown in Table 4. At low concentration of reactant (2 mM), cysteine exhibited the highest copper-chelating activity ($P<0.05$). Cysteine after heating at pH 10 and its MRPs had slightly lower activity than the parent counterpart. Much greater chelating activity was observed with all compounds having higher concentration (20 mM) ($P<0.05$). Among unheated compounds, cysteine also exhibited the greatest chelating activity. Proteins, peptides and α-amino acids are capable of forming stable complexes with Cu^{2+}. In addition, they are also capable of chelating copper at the active site of PPO (Kim *et al*., 2000). Histidine and cysteine have particularly high affinities for Cu^{2+}. Apart from having NH_2 and COOH group, histidine possesses an imidazole ring and cysteine contains a thiol group, both of which have metal binding capacity. Copper-chelating activity of amino acids at a concentration of 20 mM, except glycine, increased after heating at pH 10. MRPs also exhibited the chelating activity. Cysteine-glucose MRPs showed higher copper-chelating activity, followed by MRPs derived from glutathione and glycine, respectively. Generally, the lower activity was obtained when glucose was present, in which Maillard reaction took place. Matmaroh *et al*. (2006) reported that MRPs from the fructose-glycine model system showed copper chelating activity, especially when high concentrations of reactants (4.5-30 mM) were used. Melanoidin in MRPs behaves as anionic hydrophilic polymers, which can form stable complexes with metal cations. Ketone or hydroxyl groups of pyranone or pyridone residues act as donor groups in melanoidins and participate in the chelation with metals as melanoidins have net negative charge (Migo *et. al*., 1997). Different heavy metals (Cu^{2+}, Cr^{3+}, Fe^{3+}, Zn^{2+}, Pb^{2+}, etc.) form large complex molecules with melanoidins, amino acids, proteins and sugars in acidic medium and get precipitated (Migo *et al*., 1997). Inhibition of PPO by sulfur-containing compounds both cysteine and glutathione heated at pH 10 (Table 1) might result from their copper chelating ability. PPO from crustaceans has been known to contain Cu^{2+} in the active site (Kim *et al*., 2000).

Table 4. Copper chelation of sulfur-containing compounds heated under alkaline condition and their Maillard reaction products.

Samples	Bound copper (%)					
	2 mM			20 mM		
	Original	Heated	MRPs	Original	Heated	MRPs
Glycine	6.21* ± 0.23^{c,B**}	6.67 ± 0.29^{c,B}	6.47 ± 0.06 ^{c,A}	11.26 ± 0.23^{b,C}	12.17 ± 2.52^{b,C}	25.14 ± 0.29^{a,B}
Cysteine	8.13 ± 0.15^{c,A}	7.73 ± 0.06^{cd,A}	6.77 ± 0.32^{d,A}	29.91 ± 0.50^{b,A}	36.33 ± 0.76^{a,A}	34.83 ± 0.29^{a,A}
Methionine	5.25 ± 0.13^{c,B}	6.47 ± 0.06^{c,B}	2.43 ± 0.70^{d,C}	10.82 ± 0.73^{b,C}	20.50 ± 1.80^{a,B}	21.00 ± 0.43^{a,B}
Glutathione	5.95 ± 0.12^{d,B}	6.73 ± 0.15^{d,B}	5.87 ± 0.06^{d,A}	16.54 ± 0.07^{c,B}	33.50 ± 0.50^{a,A}	26.33 ± 1.26^{b,B}

* Mean ± SD from triplicate determinations.

** The different letters in the same row indicated the significant differences ($P<0.05$).

The different capital letters within the same column indicate the significant differences ($P<0.05$).

2.3 Reducing power

Reducing power of sulfur-containing compounds heated at pH 10 and their MRPs, as monitored by A_{700}, is shown in Table 5. At 2 mM, cysteine and glutathione heated at pH 10 exhibited the highest reducing power ($P<0.05$). However, a decrease in activity was observed for their MRPs ($P<0.05$). Glycine and methionine showed the low reducing power, regardless of heating at pH 10 or via Maillard reaction. When a concentration of 20 mM was used, higher reducing power was found, irrespective of treatment. Among all samples, cysteine heated at pH 10 had the highest reducing power ($P<0.05$). In the presence of glucose, where Maillard reaction took place, reducing power of MRPs derived from cysteine and methionine decreased ($P<0.05$), while those of MRPs from glycine and glutathione increased ($P<0.05$). Therefore, reducing power of all compounds used varied with treatment, in which different compounds with varying reducing power were formed. Hydroxyl group of MRPs plays an important role in reducing activity (Yoshimura *et al*., 1997). The intermediate compounds of MRPs were also reported to be capable of donating hydrogen atoms (Yen and Hsieh, 1995). Matmaroh *et al*. (2006) also reported that the reducing power of MRPs derived from fructose-glycine increased as the reactant concentration increased, however no differences in reducing power were observed when reactant concentrations ranged from 0.75 to 1.5 mM. Cysteine has been shown to reduce *o*-quinones to their phenol precursors (Walker, 1977; Cilliers and Singleton, 1990). The result suggested that sulfur-containing compounds including cysteine and glutathione heated at pH 10 possessed the ability of reducing quinone to reduced form, in which final brown products could not be developed.

Table 5. Reducing power of sulfur-containing compounds heated under alkaline condition and their Maillard reaction products.

Samples	Absorbance at 700 nm					
	2mM			20mM		
	Original	Heated	MRPs	Original	Heated	MRPs
Glycine	$0.10^{*} \pm 0.00^{d,C**}$	$0.06 \pm 0.00^{e,B}$	$0.03 \pm 0.02^{f,C}$	$0.12 \pm 0.00^{c,C}$	$0.22 \pm 0.01^{b,D}$	$0.72 \pm 0.02^{a,C}$
Cysteine	$0.05 \pm 0.02^{d,D}$	$0.29 \pm 0.01^{c,A}$	$0.10 \pm 0.01^{d,B}$	$0.13 \pm 0.01^{cd,B}$	$5.43 \pm 0.22^{a,A}$	$3.05 \pm 0.09^{b,A}$
Methionine	$0.12 \pm 0.00^{c,B}$	$0.09 \pm 0.02^{c,B}$	$0.08 \pm 0.00^{c,B}$	$0.14 \pm 0.00^{c,B}$	$0.95 \pm 0.09^{a,C}$	$0.33 \pm 0.03^{b,D}$
Glutathione	$0.25 \pm 0.01^{e,A}$	$0.31 \pm 0.04^{d,A}$	$0.22 \pm 0.00^{e,A}$	$2.40 \pm 0.00^{b,A}$	$1.74 \pm 0.05^{c,B}$	$2.82 \pm 0.02^{a,B}$

* Mean ± SD from triplicate determinations.

** The different letters in the same row indicated the significant differences ($P<0.05$).

The different capital letters within the same column indicate the significant differences ($P<0.05$).

2.4 Radical scavenging activity

ABTS radical scavenging activity of sulfur-containing compounds heated at pH 10 and their MRPs in comparison with glycine is shown in Table 6. ABTS assay is an excellent method for determining the capacity of hydrogen-donating (scavengers of aqueous phase radicals) and of chain breaking (scavengers of lipid peroxyl radicals) (Leong and Shui, 2002). All compounds had the increase in ABTS radical scavenging activity when heated at pH 10. However, their MRPs had the lower activity ($P<0.05$). With increasing concentration of reactant (20 mM), the increase in activity was noticeable. For cysteine and glutathione, the decrease in activity was found with their MRPs, in comparison with those heated at pH 10. Maillard reaction might contribute to lowering the ability of both compounds at pH 10 in scavenging the radicals. For glycine and methionine, MRPs possessed the higher activity than those heated at pH 10. Thus, ABTS radical scavenging activity of all compounds tested depended upon treatment. Maillard *et al.* (2007) reported that glucose-cysteine MRPs exhibited higher radical scavenging capacity than those obtained from glucose-glycine or glucose-proline MRPs, which could be attributed to the sulfhydryl group of cysteine. The result suggested that cysteine and glutathione heated at pH 10 could be used as antioxidant due to their ability to donate hydrogen or to scavenge the radicals.

No DPPH radical scavenging activity of all compounds tested at 2 mM was found (Table 7). The DPPH radical scavenging activity indicated the hydrogen donating abilities of antioxidant (Guerard and Sumaya-Martinez, 2003). PPH radical scavenging activity of cysteine and glutathione was noticeable after heating at pH 10. MRPs of those compounds showed the lower activity. DPPH radical was scavenged by MRPs through donating of hydrogen to form a stable DPPH-H molecule (Matthaus, 2002). The color changed from purple to yellow by acceptance of hydrogen radical from active compounds and it became a stable diamagnetic molecule. However, glycine and methionine had no activity regardless of treatment. Cysteine heated at pH 10 exhibited the highest DPPH radical scavenging activity ($P<0.05$). Thiol groups are well known as a free radical scavenger in its molecule. Benjakul *et al*. (2005a) found that MRPs derived from a porcine plasma protein

(PPP)-galactose showed the increase in DPPH radical scavenging activity in a concentration-dependent manner. Similar result was observed for all compounds at 20 mM, in comparison to that observed at 2 mM. The higher activity was obtained at higher concentration. At high concentration, glycine and methionine without and with heating at pH 10 or via Maillard reaction exhibited DPPH radical scavenging activity. However, they had the lower activity than glycine and glutathione. The result suggested that sulfhydryl group mainly involved in ability of donating hydrogen of compounds derived after heating. DPPH radical scavenging activity of cysteine and glutathione heated at pH 7 reconfirmed the antioxidant activity of these compounds, which can be used to prevent or retard lipid oxidation in food systems.

Table 6. ABTS radical scavenging activity of sulfur-containing compounds heated under alkaline condition and their Maillard reaction products.

Samples	ABTS radical scavenging activity (mmole TE/ml)					
	2mM			20mM		
	Original	Heated	MRPs	Original	Heated	MRPs
Glycine	3.39* ± 0.01^{d,C**}	4.04 ± 0.10^{c,C}	3.23 ± 0.01^{d,C}	3.80 ± 0.02^{c,D}	9.69 ± 0.10^{b,C}	10.57 ± 0.35^{a,C}
Cysteine	3.25 ± 0.01^{e,D}	11.43 ± 0.92^{c,B}	3.57 ± 0.03^{e,B}	9.13 ± 0.07^{d,B}	46.48 ± 0.81^{a,A}	18.05 ± 0.16^{b,B}
Methionine	3.71 ± 0.03^{d,B}	4.11 ± 0.03^{cd,C}	2.40 ± 0.02^{e,D}	4.55 ± 0.03^{c,C}	5.11 ± 0.83^{b,D}	9.00 ± 0.05^{a,D}
Glutathione	4.85 ± 0.01^{e,A}	16.67 ± 0.03^{c,A}	5.14 ± 0.00^{e,A}	12.97 ± 0.07^{d,A}	42.07 ± 0.54^{a,B}	25.08 ± 0.02^{b,A}

* Mean ± SD from triplicate determinations.

** The different letters in the same row indicated the significant differences ($P<0.05$).
The different capital letters within the same column indicate the significant differences ($P<0.05$).

Table 7. DPPH radical scavenging activity of sulfur-containing compounds heated under alkaline condition and their Maillard reaction products.

Samples	DPPH radical scavenging activity (μmole TE/ml)					
	2mM			20mM		
	Original	Heated	MRPs	Original	Heated	MRPs
Glycine	0.00* ± 0.00^{d,A}**	0.00 ± 0.00^{d,C}	0.00 ± 0.00^{d,B}	10.43 ± 0.68^{c,A}	29.53 ± 1.02^{a,C}	15.30 ± 0.58^{b,B}
Cysteine	0.00 ± 0.00^{e,A}	21.25 ± 0.12^{bc,A}	7.91 ± 1.68^{d,A}	15.93 ± 5.56^{c,A}	42.52 ± 0.00^{a,A}	26.36 ± 5.71^{b,A}
Methionine	0.00 ± 0.00^{c,A}	0.00 ± 0.00^{c,C}	0.00 ± 0.00^{c,B}	13.81 ± 1.32^{b,A}	34.01 ± 3.41^{a,B}	14.71 ± 1.00^{b,B}
Glutathione	0.00 ± 0.00^{e,A}	9.15 ± 1.53^{d,B}	8.03 ± 1.39^{d,A}	14.62 ± 0.63^{c,A}	39.67 ± 1.24^{a,A}	26.62 ± 0.61^{b,A}

* Mean ± SD from triplicate determinations.

** The different letters in the same row indicated the significant differences ($P<0.05$).
The different capital letters within the same column indicate the significant differences ($P<0.05$).

2.5 Effects of heated alkaline cysteine (HAC) on the quality changes of Pacific white shrimp during iced storage

2.5.1 Changes in melanosis

The development of melanosis of Pacific white shrimp was monitored during iced storage (Figure 10). Melanosis occurs in crustacean and shellfish during storage as a result of the action of polyphenol oxidase (PPO) on tyrosine or its derivatives, such as tyramine, to form melanin (Rolle *et al*., 1991). The control sample had the continuous increase in melanosis score during 12 days of iced storage. During the first 3 days of storage, melanosis was found in all samples at the low degree. Melanosis of the control was obvious at day 6 of storage, compared with those treated with HAC at both concentrations ($P<0.05$). Melanosis score of all treatments, except the shrimp treated with 100 mM HAC, increased as storage time increased ($P<0.05$). At day 12 of storage, HAC at a concentration of 100 mM exhibited the effectiveness on inhibition of melanosis as evidenced by the lowered score of shrimps in comparison with the control and those treated with 20 mM HAC. The retardation of black spot formation in Pacific white shrimp might be due to the reducing power and the copper-chelating activity of HAC. Additionally, quinones could be reduced to original phenols by HAC and the active site of PPO could be bound, resulting in the lower activity.

2.5.2 Changes in TBARS

TBARS value of Pacific white shrimp soaked without and with HAC at 20 and 100 mM during iced storage is shown in Figure 11. The increase in TBARS was observed in all samples when the storage time increased ($P<0.05$), suggesting that lipid oxidation occurred during extended storage. The initial values of TBARS of all samples were 0.55-0.66 mg/kg. It was suggested that lipid oxidation might occur during post-mortem handling to some extent. TBARS has been used to measure the concentration of relatively polar secondary reaction products, especially aldehydes (Nawar, 1996; Kolakoska *et al*., 1992). After 12 days of iced storage, the highest TBARS value was observed in the control, and lower TBARS values were found in samples soaked in HAC. The lower TBARS value in the shrimp treated with HAC, especially at 100 mM, indicated the decrease in lipid oxidation; this could be due to

antioxidative activity of HAC as shown by its ABTS and DPPH radical scavenging activity and reducing power (Tables 5, 6 and 7).

2.5.3 Changes in psychrophilic bacterial count

Changes in psychrophilic bacterial count of Pacific white shrimp during iced storage are shown in Figure 12. At day 0, no psychrophilic bacterial count was found in all samples, regardless of the treatment. Psychrophilic bacterial count in all samples increased as the storage time increased ($P<0.05$). No bacterial count was found in sample soaked with HAC at 100 mM up to 3 days of storage. At the end of storage (12 days), the control sample had the highest count (6.12 ± 0.09 log CFU/g), whereas the shrimp treated with 100 mM HAC showed the lowest count (5.01 ± 0.02 log CFU/g). HAC might chelate the metal ions required for microbial growth. As a consequence, the growth of psychrophilic bacteria could be retarded. It has been reported that micronutrients including various ions are important for microbial growth (Montville and Matthews, 2001). During iced storage, psychrophilic microorganisms most likely played a role in spoilage of shrimp. Thus, the use of HAC could retard the spoilage of Pacific white shrimp during iced storage.

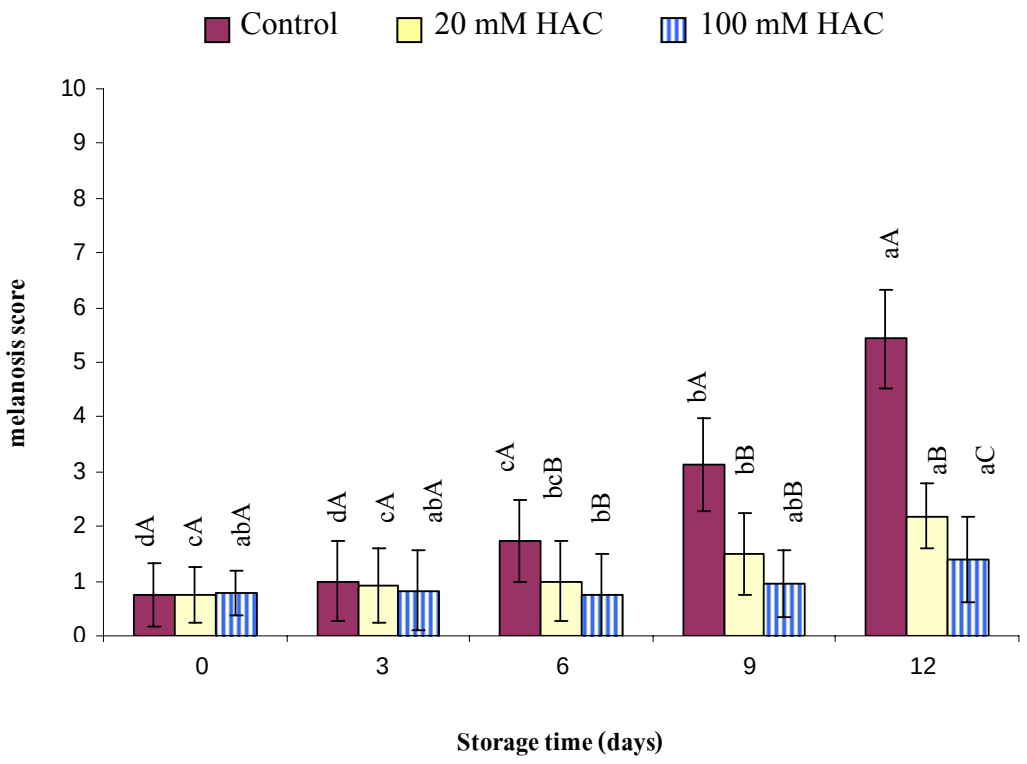

Figure 10. Changes in melanosis score of Pacific white shrimp soaked without and with HAC at different concentrations during iced storage. Different capital letters within the same storage time indicated significant differences ($P<0.05$). Different letters within the same treatment indicated significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

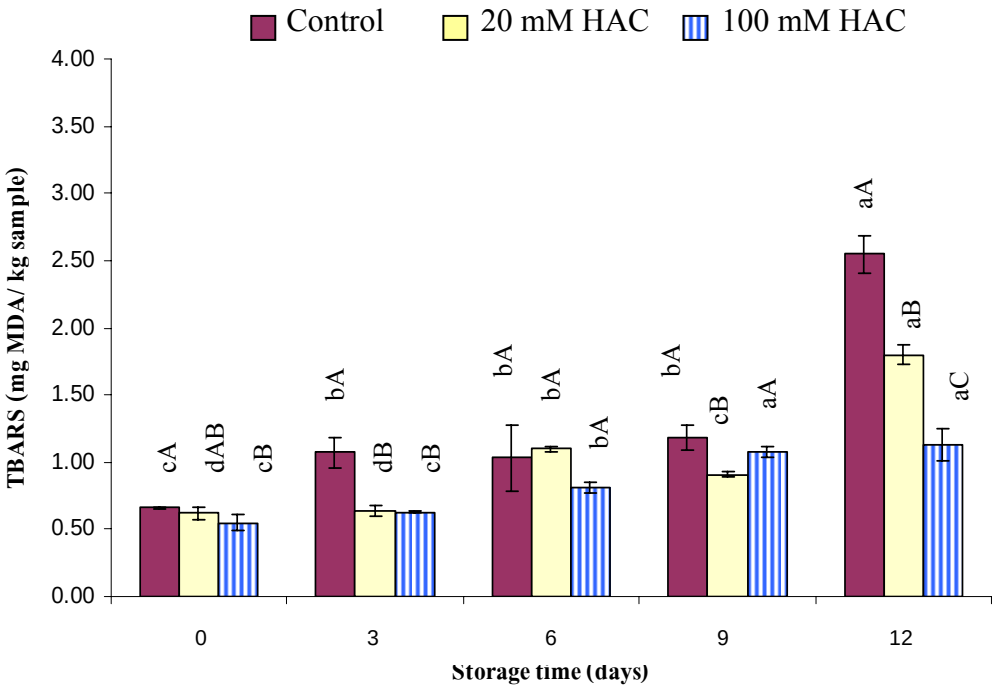

Figure 11. Changes in TBARS of Pacific white shrimp soaked without and with HAC at different concentrations during iced storage. Different capital letters within the same storage time indicated significant differences ($P<0.05$). Different letters within the same treatment indicated significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

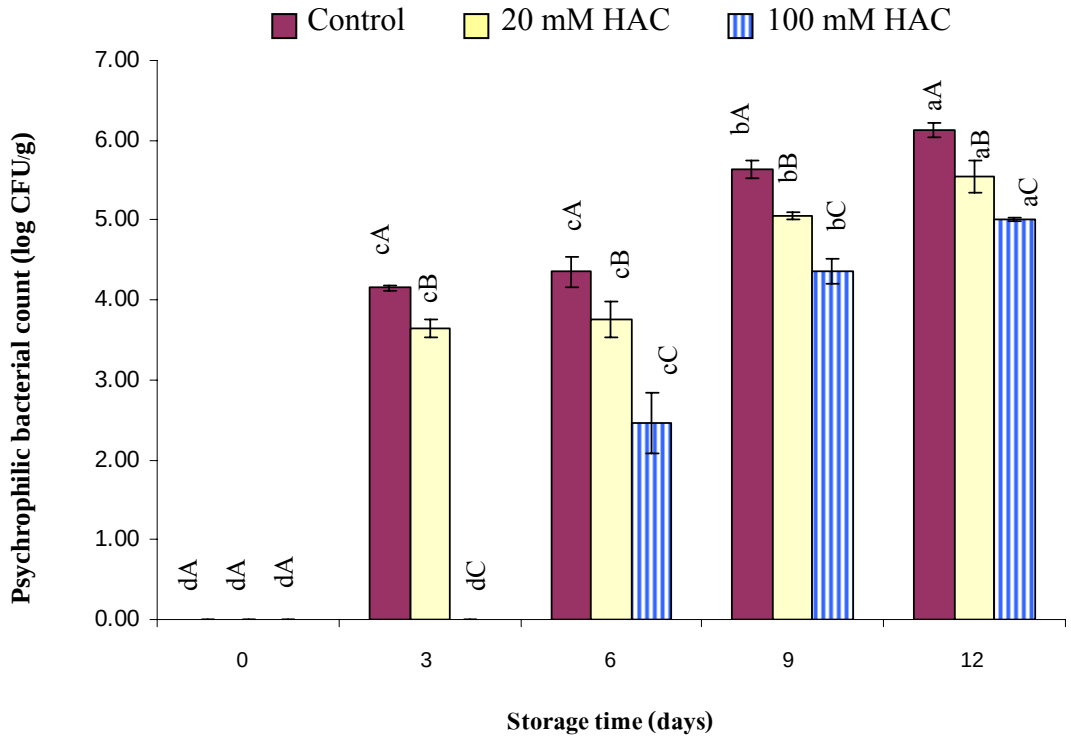

Figure 12. Changes in psychrophilic bacterial count (log CFU/g) of Pacific white shrimp soaked without and with HAC at different concentrations during iced storage. Different capital letters within the same storage time indicated significant differences ($P<0.05$). Different letters within the same treatment indicated significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

3. Effect of different pHs on some properties of heated cysteine

3.1 PPO inhibitory activity

PPO inhibitory activity of heated cysteine (HC) prepared at different pHs is shown in Figure 13. It appeared that an alkaline medium (pHs 8-11) was a more favorable reaction condition for the formation of potent inhibitory compounds against PPO ($P<0.05$). HC prepared at pH 8 showed the lower PPO inhibition than those prepared at pHs 9-10 ($P<0.05$). Conversely, HC prepared under acidic conditions had the decrease in PPO inhibitory activity when pHs decreased to 3.0 ($P<0.05$). Nevertheless, an increase in PPO inhibitory activity was found at pH 2. Matmaroh (2005) reported that PPO from black tiger prawn was inhibited by fructose/glycine MRPs prepared at alkaline pH. In general, cysteine is a competitive inhibitor of PPO (Kim *et al*., 2000). Richard-Forget *et al*. (1992) reported that cysteine exhibited competitive inhibition on apple PPO. Benjakul *et al*. (2006) also reported the competitive behaviour of cysteine and glutathione, when tested against PPO from kuruma prawn. HC prepared at alkaline pH might have anionic structure at higher pH, which can form complex with copper atom at the active site. Anionic hydrophilic polymers such as melanoidin have been reported to form stable complexes with metal cations (Chandra *et al*., 2007). Furthermore, cysteine could be degraded by heating under alkaline condition via β-elimination to form degradation products which might play a role in PPO inhibition (Beeley and Levons, 1963). At pH 3-5, the lower PPO inhibitory activity was found, probably due to the positive charge of cysteine. As a consequence, the repulsion between cysteine and Cu was presumed. At very acidic pH (pH 2), HC formed might contain the degradation products capable of inhibiting PPO by some mechanisms.

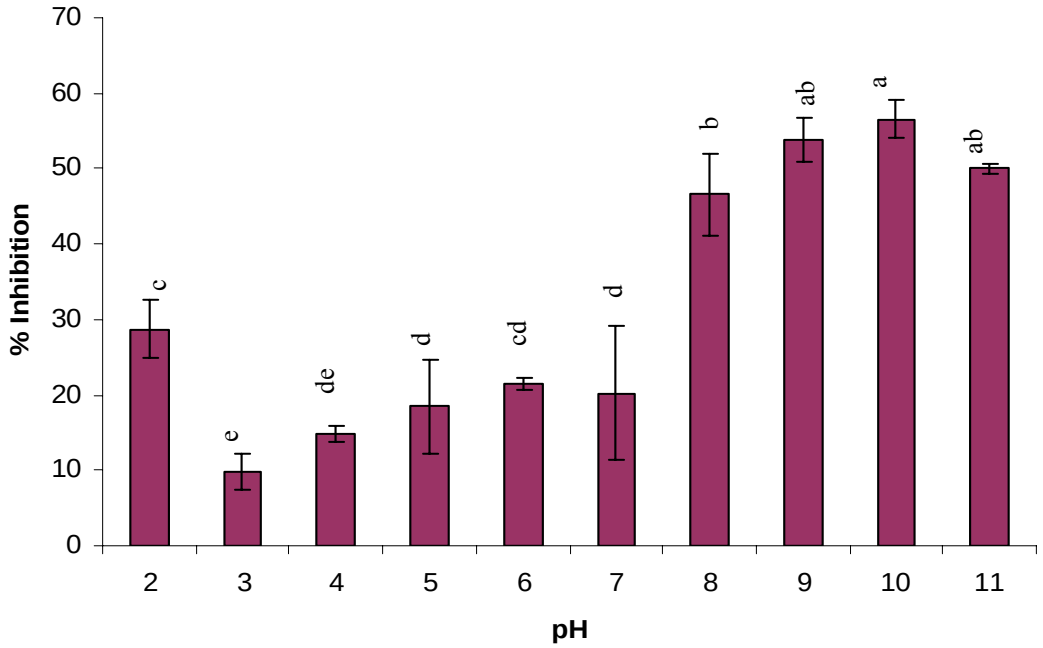

Figure 13. PPO inhibitory activity of heated cysteine (HC) prepared at different pHs. Different letters on the bars indicate significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

3.2 Copper chelation

PPO inhibitory activity of HC prepared at different pHs is shown in Figure 14. Generally, HC prepared at different pHs had the similar copper chelating activity, in which copper ions could be chelated completely under the assay condition used in the study. However, lower copper chelating activity was observed when pH of 3 was used ($P<0.05$). This result was in accordance with the lowest PPO inhibitory activity of HC prepared at pH 3 (Figure 13). PPO from crustacean has been known to contain Cu^{2+} in the active site (Kim *et al*., 2000; Jang *et al*., 2003). The compounds in HC possessing the copper chelating activity might form the complex with Cu^{2+} in the active site, leading to the loss in PPO activity. Matmaroh (2005) also reported that fructose/glycine MRPs prepared at very alkaline pH (pHs 10-12) had the greater copper chelating activity. Cysteine has particularly high affinities for Cu^{2+} since a thiol group has metal binding capacity (Kim *et al*., 2000). Thus, pH of HC was another important factor governing the PPO inhibitory activity, most likely associated with the ability in copper chelation.

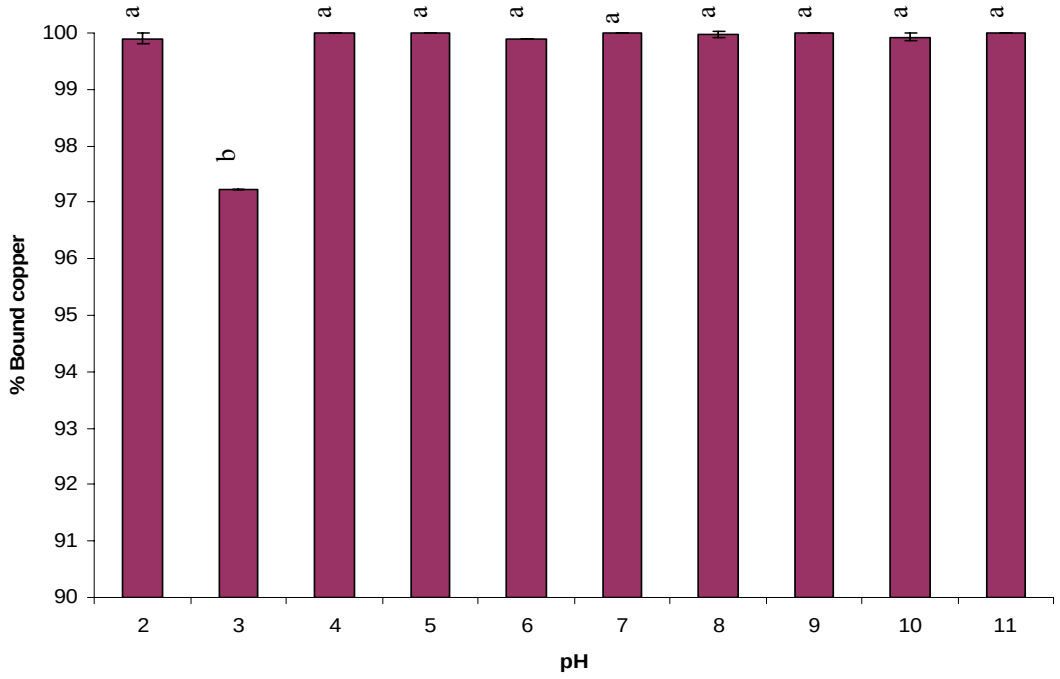

Figure 14. Copper chelating activity of heated cysteine (HC) prepared at different pHs. Different letters on the bars indicate significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

3.3 Reducing power

Reducing power, as the measurement of A_{700}, of HC prepared at different pHs is shown in Figure 15. HC prepared at alkaline pH (pH 8-11) showed higher reducing power when compared with those obtained at acidic and neutral pH ($P<0.05$). However, HC prepared at pH 2 had the reducing power comparable to that prepared at pH 9 but less than those obtain at pH 8, 10 and 11 ($P<0.05$). From the result, HC prepared at pH 10 showed the highest reducing power ($P<0.05$). The result indicated that reducing compounds were formed when the higher pHs were used and also implied that HC especially with high initial pH had hydrogen donating activity. The hydroxyl group (OH) of reducing compounds plays an important role in reducing activity (Yoshimura *et al*., 1997). Beeley and Jevons (1963) also reported the reducing power of cysteine and serine under alkaline condition. Reducing compounds were also found in fructose/glycine MRPs prepared at alkaline pH (Matmaroh, 2005).

Therefore, HC could reduce DOPA-quinone to DOPA in the browning pathway. As a result, the further reaction, in which melanin formation took place, could be retarded.

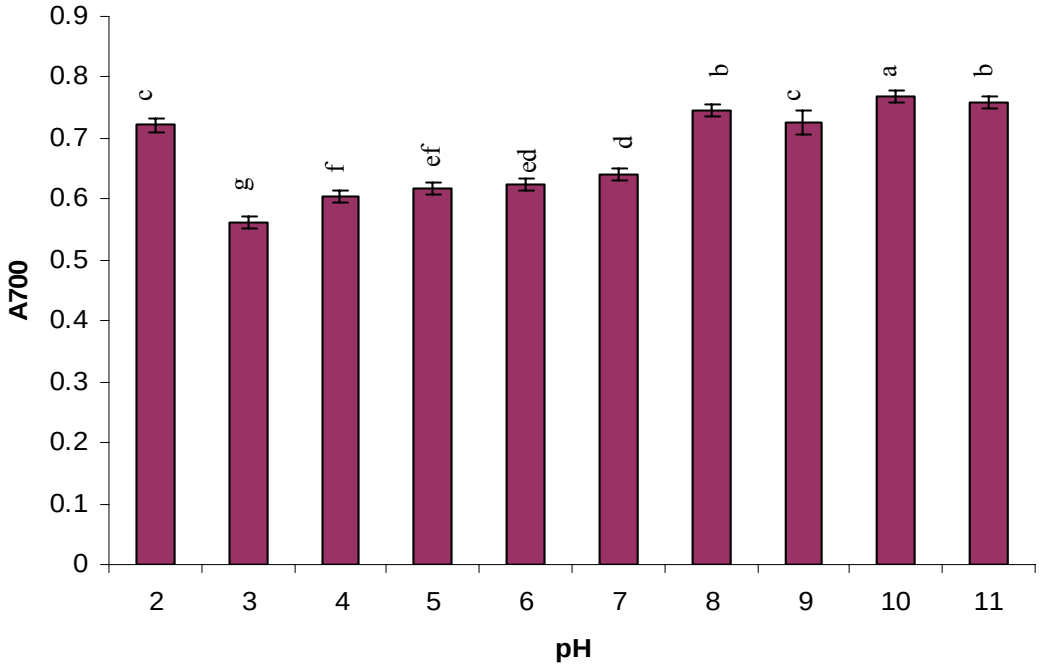

Figure 15. Reducing power of heated cysteine (HC) prepared at different pHs. Different letters on the bars indicate significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

3.4 Radical scavenging activity

ABTS scavenging activity of HC prepared at various pHs is depicted in Figure 16. Generally, approximately 1.5-2 fold increase in ABTS radical scavenging activity of HC was found when HC was prepared at alkaline pH, compared with at acidic and neutral pHs. In alkaline pH range, a slight decrease in ABTS radical scavenging activity was found at pH 11 and the highest ABTS radical scavenging activity was obtained at pH 10 and 11 ($P<0.05$). The results indicated that pH of the media significantly influenced the antioxidant activity of the HC. It is well known that ABTS assay is an excellent method for determining the capacity of hydrogen-donating (scavengers of aqueous phase radicals) and of chain breaking (scavengers of lipid peroxyl radicals) (Leong and Shui, 2002). Additionally, Lingnert

and Eriksson (1980) reported that basic medium (initial pH of 10.5 and 9.6 for Glucose–Arg and Glucose–Lys mixtures, respectively) was a more favorable reaction condition for the production of potent radical scavengers than pH 6.

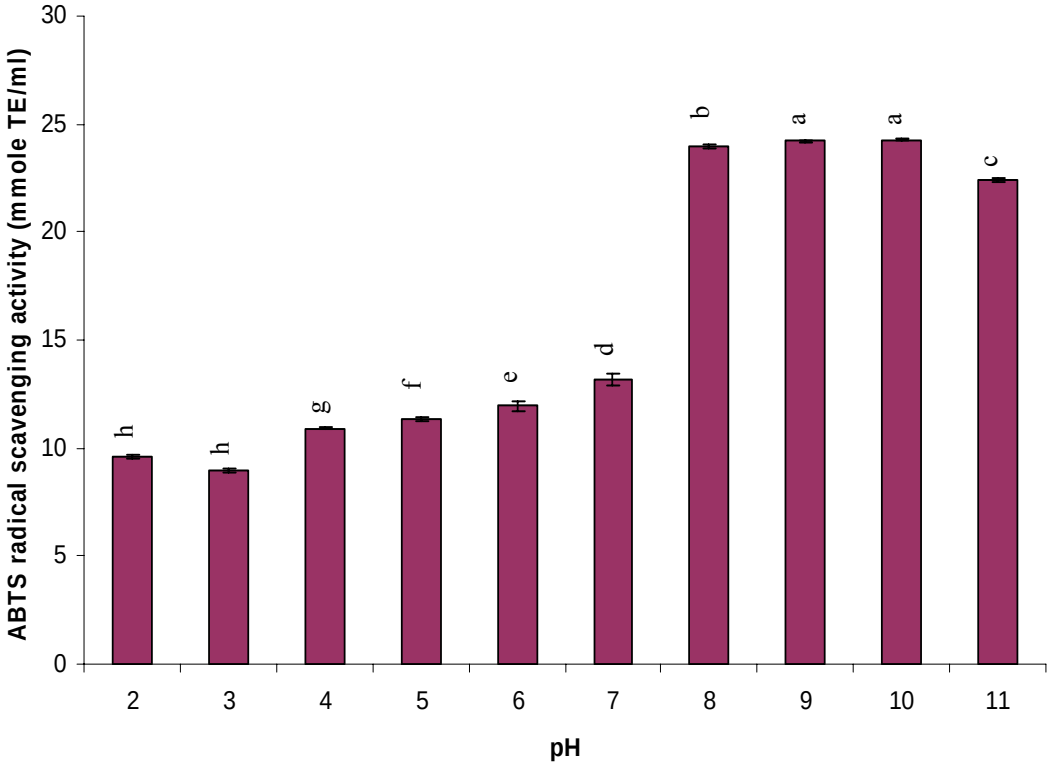

Figure 16. ABTS radical scavenging activity of heated cysteine (HC) prepared at different pHs. Different letters on the bars indicate significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

The scavenging activity of HC on DPPH radical, a molecule containing a stable free radical, is depicted in Figure 17. The DPPH radical scavenging activity indicated the hydrogen donating abilities of antioxidant (Brand-Williams *et al*., 1995). DPPH radical scavenging activity of HC prepared at alkaline pH was higher than those prepared at acidic pH. However, no differences in DPPH radical scavenging activity were observed between pH 10 and 11 ($P>0.05$). For acidic pH range, HC prepared at pH 2 exhibited the higher DPPH radical scavenging activity, compared with those prepared at other pHs. The result suggested that the

compounds generated at pH 2 during heating had the capacity of hydrogen donation to radicals. From the result, DPPH radical scavenging activity was coincidental with reducing power. Lertittikul (2005) reported that the higher pH of PPP-glucose model system resulted in the greater antioxidative activity of MRPs, compared with the lower pHs used. This result revealed that HC, especially at alkaline pH, could provide the hydrogen to radicals, in which the propagation could be terminated.

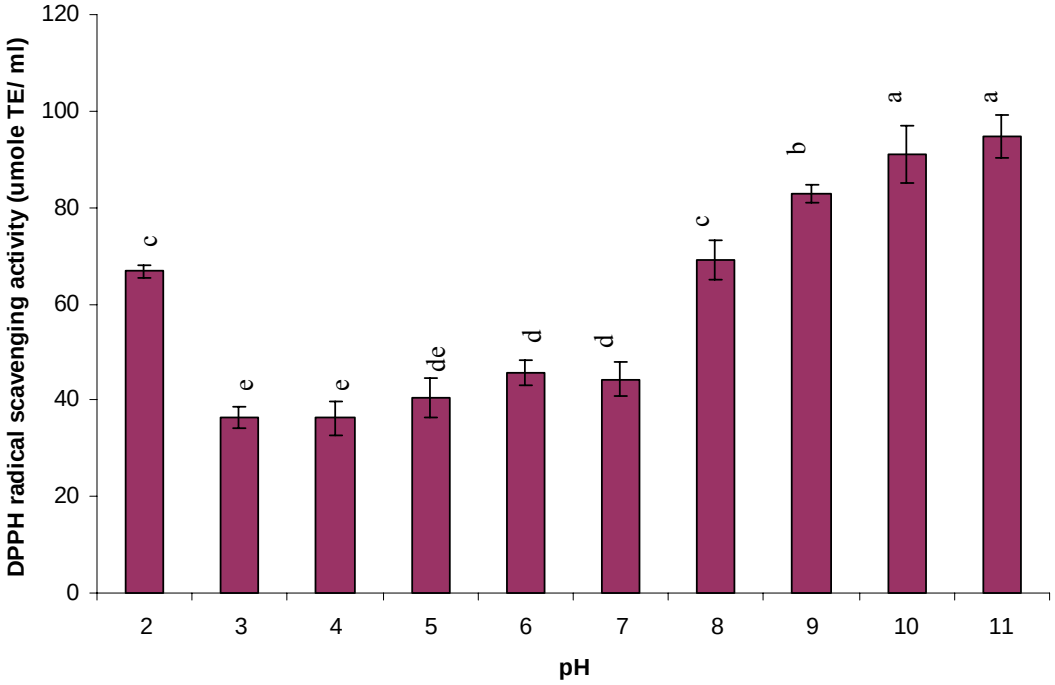

Figure 17. DPPH radical scavenging activity of heated cysteine (HC) prepared at different pHs. Different letters on the bars indicate significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

Therefore, pH of 10 was appropriate for HC preparation with the high PPO inhibitory activity.

4. Effect of reactant concentration on some properties of heated cysteine

4.1 PPO inhibitory activity

Heated cysteine prepared at pH 10 (HAC) with different concentrations showed different inhibitory activity towards white shrimp PPO (Figure

18). From the results, the inhibition of PPO increased as the cysteine concentration increased ($P<0.05$). HAC with the concentration of 500 mM exhibited the highest PPO inhibition, by which 71% of activity was inhibited. Matmaroh *et al*. (2006) also found that PPO inhibitory activity of MRPs prepared from fructose and glycine increased from 55% to 80% when the reactant concentration increased from 15 to 30 mM. Additionally, Lee and Park (2005) found that the inhibitory activity of MRPs from a glucose–glycine system, against PPO from potato, increased as the amount of glucose increased.

PPO from crustacean contains Cu^{2+} at its active site which is liganded to histidine residues (Kim *et al*., 2000). The compounds formed during heating cysteine at alkaline condition might form the complex with Cu^{2+} at the active site, leading to the loss in PPO activity. Dudley and Hotchkiss (1989) found that cysteine is an effective inhibitor of mushroom PPO and PPO inhibitory activity increased about 25% when the concentration of cysteine used in the reaction increased from 0 to 0.04 mM. Benjakul *et al*. (2006) also found that thiol-containing compounds such as cysteine and glutathione exhibited inhibitory activity towards PPO from kuruma prawn (*Penaeus japonicus*), via competitive inhibition mechanism. Thiol-containing compounds, such as cysteine and glutathione, have been reported to inhibit the enzymatic browning, especially in fruits and vegetables (Arslan *et al*., 2004). Thiol-containing compounds prevent the browning reaction by trapping the intermediates with the subsequent prevention of transformation to the brown pigment (Friedman, 1996). Furthermore, the compounds could inhibit PPO by changing the copper-containing active site of the enzyme (Friedman, 1996) or showed direct irreversible inhibition (Robert *et al*., 1996).

4.2 Copper chelation

Copper-chelating activity of HAC at different concentrations was shown in Figure 19. HAC with various cysteine concentrations had different copper-chelating activity. Copper-chelating activity increased with increasing cysteine concentration used ($P<0.05$). Thus, efficiency of copper-chelating activity was dependent on the reactant concentration. Matmaroh *et al*. (2006) also found that copper-chelating activity of MRPs prepared from fructose and glycine increased as

the reactant concentration increased. Sulfhydryl group of cysteine has been reported to chelate with metal ion (Kim *et al*., 2000). Moreover, some amino acids, especially histidine, have been reported to have copper-chelating activity. MRP obtained from heated histidine and glucose exhibit copper ion binding ability in oil/water mixtures (Bersuder *et al*., 2001). Therefore, the ability of HAC in chelating copper, which is localized in the active site of PPO, was dependent upon the initial cysteine concentration. During heating, some compounds possessing copper chelating property might be produced, particularly when cysteine at higher concentrations was used.

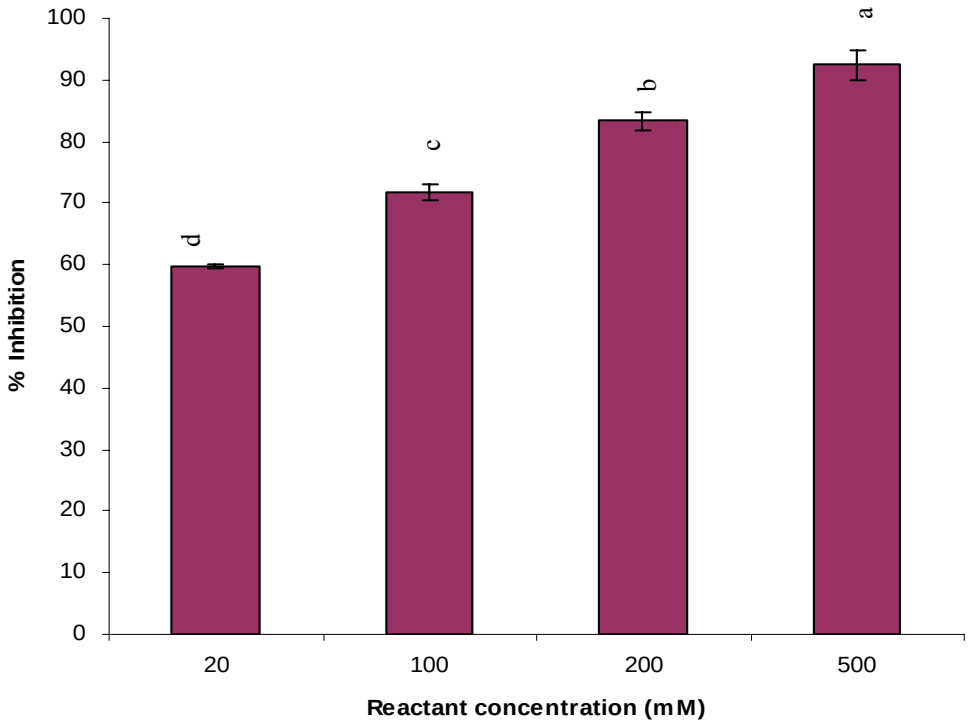

Figure 18. PPO inhibitory activity of heated alkaline cysteine (HAC) prepared with different cysteine concentrations. All samples were 50-fold diluted before assays. Different letters on the bars indicate significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

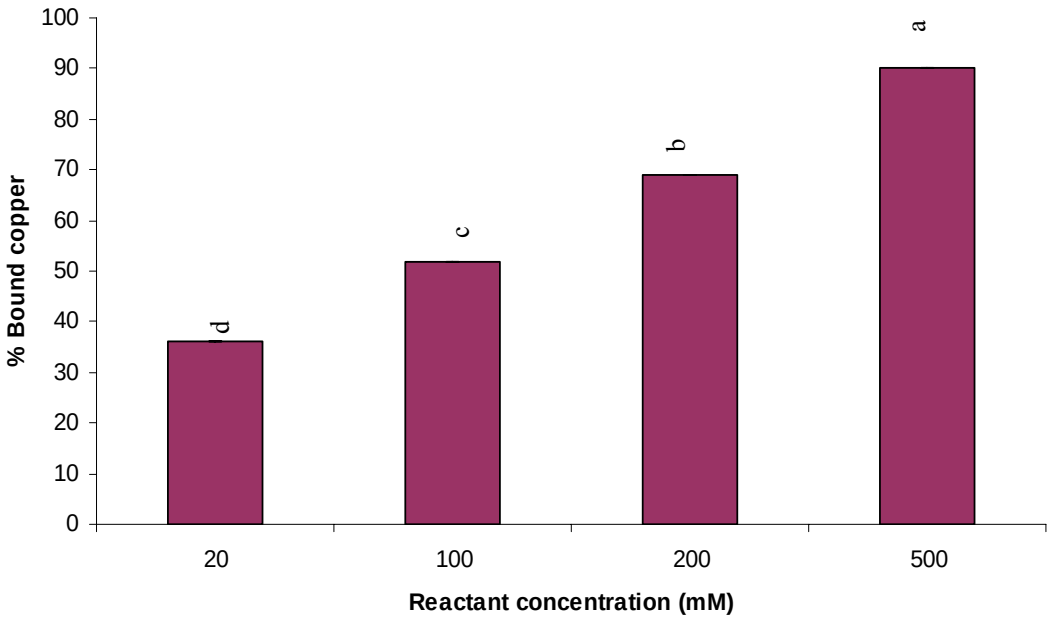

Figure 19. Copper chelating activity of heated alkaline cysteine (HAC) prepared with different cysteine concentrations. All samples were 50-fold diluted before assays. Different letters on the bars indicate significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

4.3 Reducing power

Reducing power of HAC, as monitored by A_{700}, with various cysteine concentrations, is shown in Figure 20. The reducing power of HAC increased with increasing concentrations of cysteine ($P<0.05$). From the result, reducing power was correlated with PPO inhibitory activity. HAC with the cysteine concentration of 500 mM exhibited the highest reducing power, which was in agreement with the highest PPO inhibitory activity. Conversely, HAC with the reactant concentration of 20 mM exhibited the lowest reducing power and PPO inhibition. Some amino acids have been reported to exhibit reducing power. Beeley and Jevons (1963) demonstrated that cysteine showed the highest reducing power when compared with other amino acids. A thiol group in its molecule was expected to play a key role in reducing property. Furthermore, Matmaroh *et al*. (2006) found that the reducing power of MRPs prepared from fructose–glycine system increased with increasing concentrations of reactant. With the high concentration of cysteine, the compounds with reducing power

could be produced to a higher extent during heating. This led to the increase in reducing power of resulting HAC.

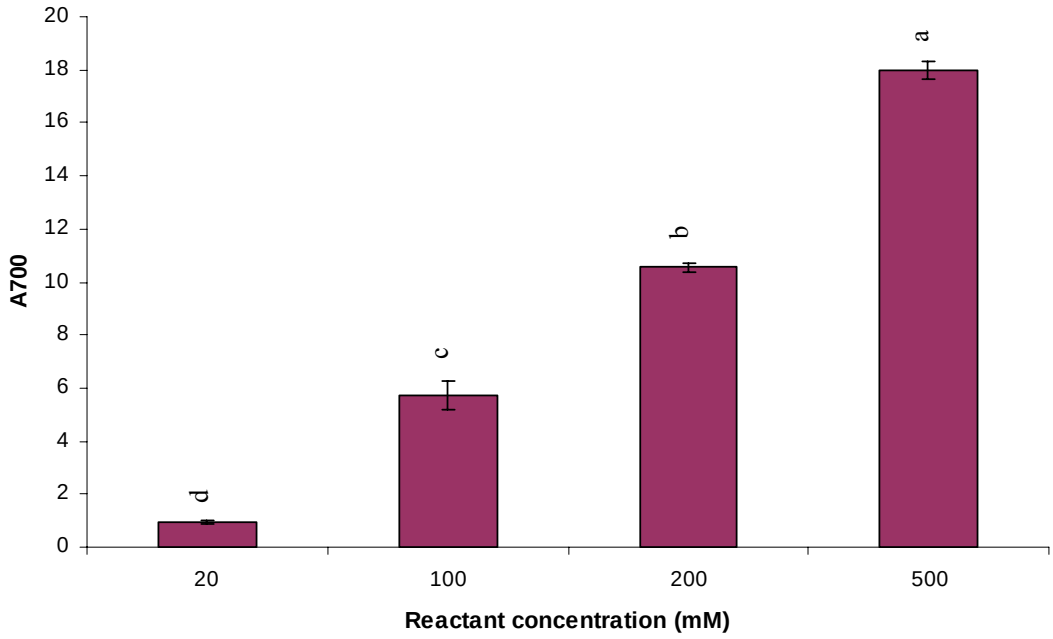

Figure 20. Reducing power of heated alkaline cysteine (HAC) prepared with different cysteine concentrations. All samples were 50-fold diluted before assay. Different letters on the bars indicate significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

4.4 Radical scavenging activity

The scavenging activity towards ABTS radicals of HAC prepared at different cysteine concentrations is depicted in Figure 21. HAC with different cysteine concentration exhibited varying ABTS radical scavenging activity. ABTS radical scavenging activity increased when the cysteine concentration used in the reaction increased. However, no differences in ABTS radical scavenging activity were observed in HAC when the cysteine at concentrations of 200 and 500 mM were used ($P>0.05$). Thiols are well known as free radical scavengers, and thus as antioxidants, in biological and other systems (Taylor and Richardson, 1980). It was more likely that HAC, especially prepared with the high cysteine concentration, could act as antioxidant in food systems.

DPPH radical scavenging activity of HAC markedly increased when cysteine concentration increased up to 500 mM ($P<0.05$) (Figure 22). The results indicated that the higher cysteine concentration induced the formation of HAC with a greater radical scavenging activity. Generally, DPPH radical scavenging activity of HAC correlated well with reducing power (Figure 20). The result confirmed that HAC was capable of donating hydrogen to the radicals, in which the oxidation could be retarded.

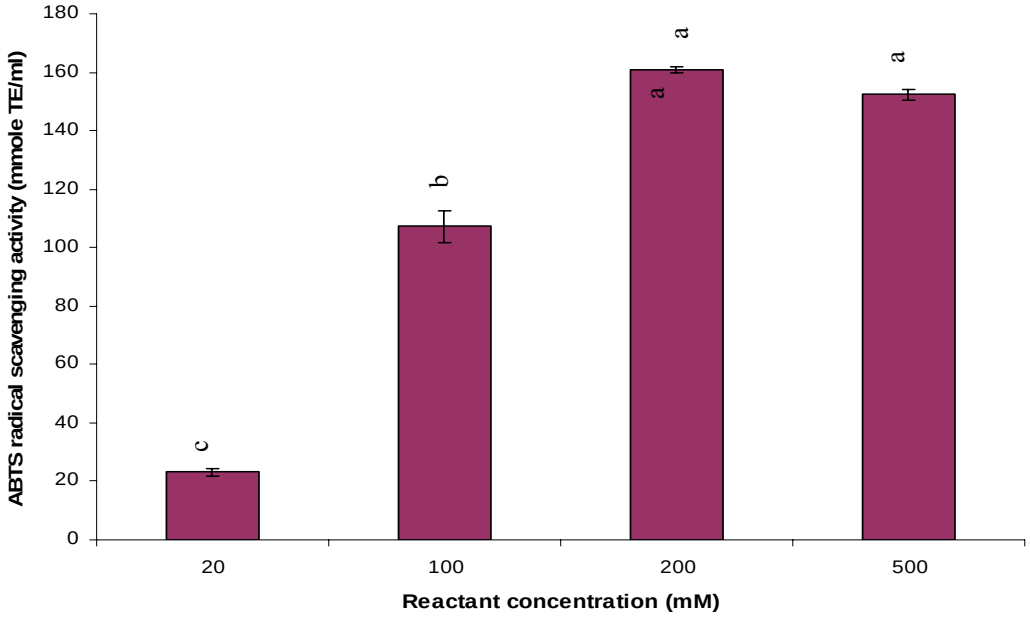

Figure 21. ABTS radical scavenging activity of heated alkaline cysteine (HAC) prepared with different cysteine concentrations. Different letters on the bars indicate significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

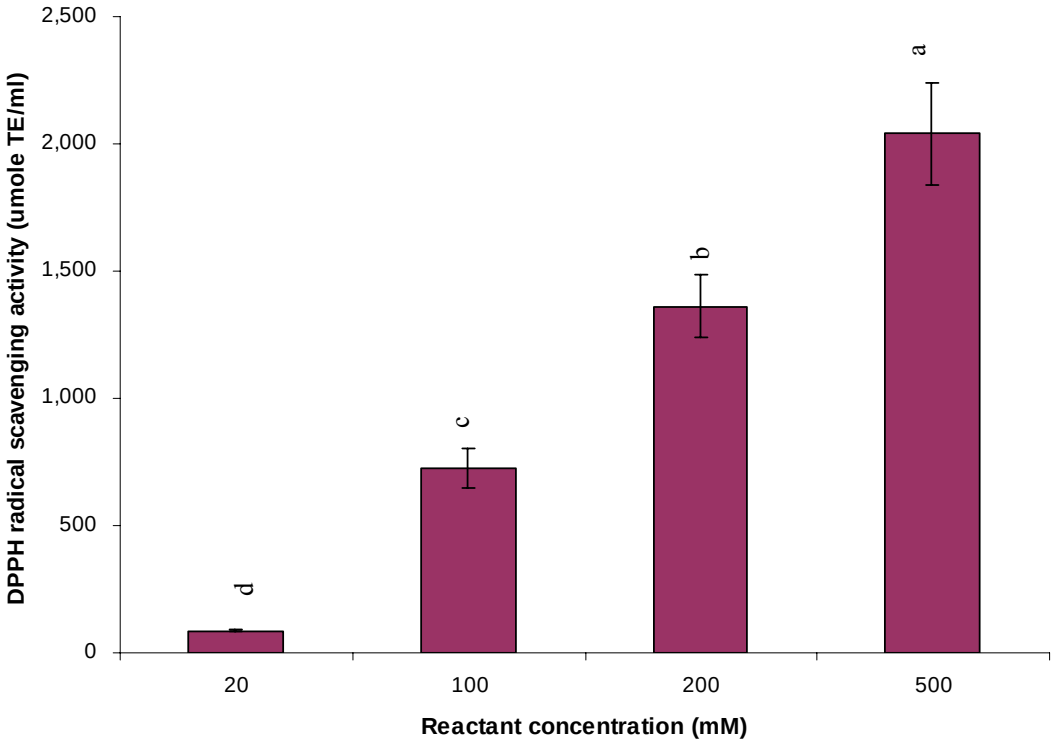

Figure 22. DPPH radical scavenging activity of heated alkaline cysteine (HAC) prepared with different cysteine concentrations. Different letters on the bars indicate significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

5. Effect of HAC on the quality changes of Pacific white shrimp (*Litopenaeus vannamei*)

5.1 Changes in Melanosis

Melanosis development in Pacific white shrimp without and with HAC treatment at different concentrations (20, 100 and 200 mM) is shown in Figures 23 and 24. Melanosis score of all treatments increased as storage time increased ($P<0.05$). The control sample (without treatment) had the continuous increase in melanosis score during 12 days of iced storage. The score of 6 was obtained in the control at the end of storage, whereas shrimps treated with HAC had the score lower than 4. Otwell and Marshall (1986) considered a score of 4 or more as indicative of product degradation. However, Otwell *et al*. (1992) did not consider the product to be

unacceptable until it scored 8. At day 12 of storage, shrimps treated with HAC at a concentration of 200 mM had the lower melanosis as evidenced by the lower score of shrimps in comparison with the control and those treated with 20 and 100 mM HAC. The retardation of Melanosis development in Pacific white shrimp might be due to the reducing power and the copper-chelating activity of HAC. When DOPA-quinone was reduced to DOPA by HAC along with the chelation of copper in active site of PPO, the development of melanosis was impeded. From the results, HAC could be used as a novel anti-browning agent to retard the melanosis development in shrimps during iced storage.

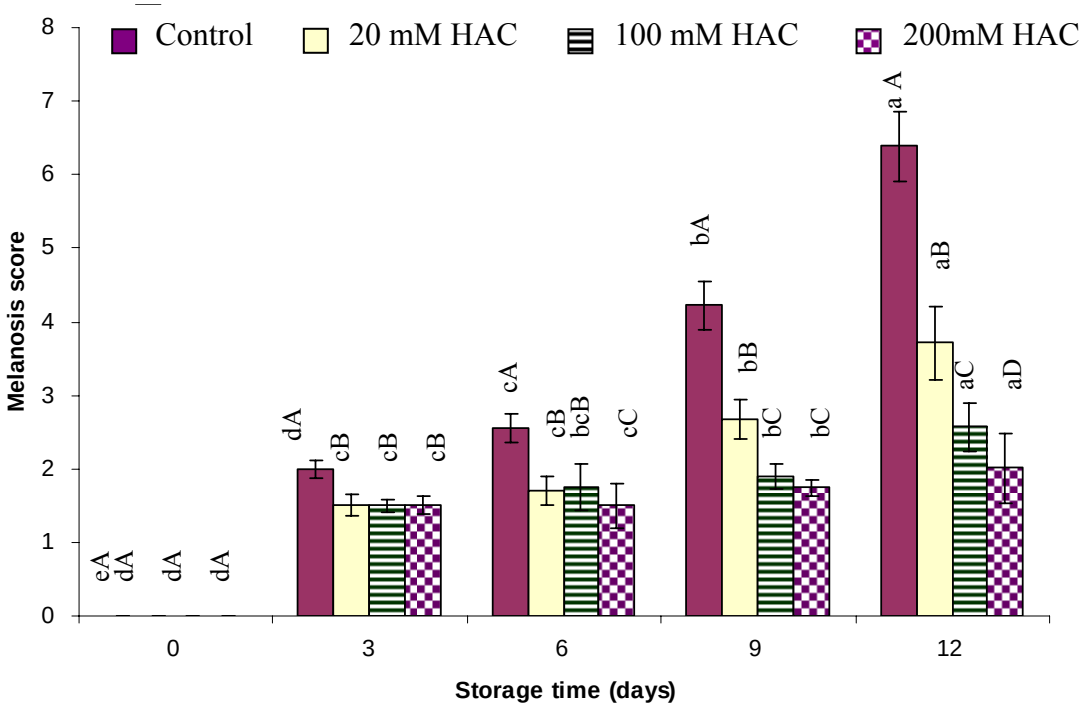

Figure 23. Changes in melanosis score of Pacific white shrimp soaked without and with HAC at different concentrations during iced storage. Different capital letters within the same storage time indicated significant differences ($P<0.05$). Different letters within the same treatment indicated significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

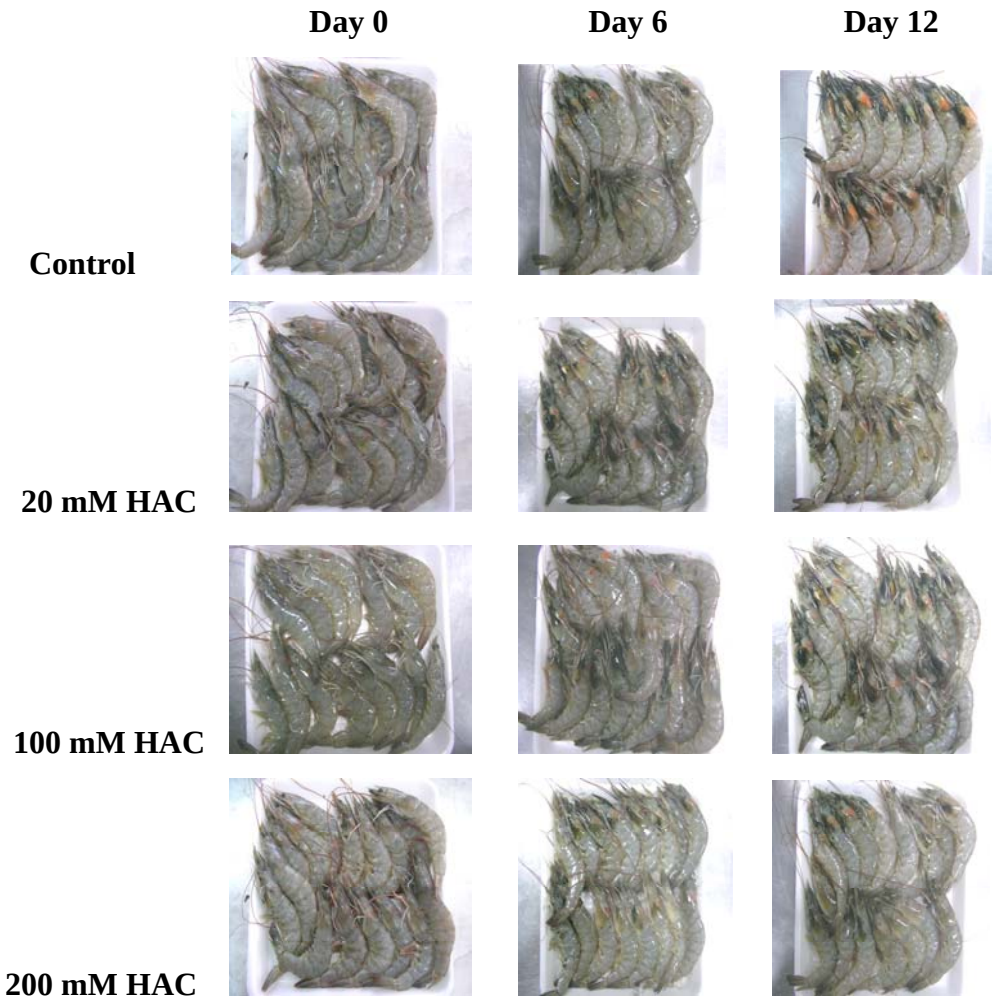

Figure 24. Shrimps treated without and with HAC at different concentrations during 12 days of iced storage.

5.2 Changes in TVB and TMA contents

TVB content of Pacific white shrimp treated with HAC at different concentrations is shown in Figure 24. No differences in TVB content were observed among all treatments at day 0. TVB content in shrimps without HAC treatment was higher than other treatments at the same storage time, except for day 0. In general, TVB contents increased with increasing storage time ($P<0.05$). However, shrimps soaked in 200 mM HAC had the slower increasing rate of TVB when compared with the control. Increasing rate of TVB content was lower when HAC concentration increased. At day 12 of storage, TVB content of control was the highest (6.98 mg N/100 g sample) whereas the lowest in TVB content was found in shrimps soaked in 200 mM HAC (4.25 mg N/100 g sample). The increase in TVB content indicated the breakdown of protein and the production of nitrogenous volatile compounds, which possibly gives rise to strong off-flavor (Ozogul and Ozogul, 2000). Generally, TVB content in shrimp stored in ice increased with increasing storage time (Baixas-Nogueras *et al*., 2002; Sadok *et al*., 2004; Campos *et al*., 2005; Matmaroh, 2005; Thepnuan, 2007). From the result, it was suggested that HAC at higher concentration could retard the spoilage, especially caused by microorganisms. HAC might inhibit the growth of microorganisms by chelating some ions required for microbial growth. Cysteine has thiol group which plays an important role in microbial inhibition. Additionally, Liu *et al*. (2007) reported the microbial inhibitory activity of cysteine-rich peptide, especially on gram-positive bacteria.

For TMA, the pattern of changes was similar to that TVB. TMA content was not detected in all treatments during the first 3 days of storage. At day 12 of storage, shrimps without HAC treatment showed the highest TMA content (1.03 mg N/100g sample), whereas the lowest TMA content was found in shrimps treated with 200 mM HAC (0.35 mg N/ 100g sample). Thepnuan (2007) reported that TVB content of white shrimp stored at 4 °C was 0.6 mg N/100g sample at the end of storage. Moreover, Matmaroh (2005) also found that TMA content could not be detected in black tiger prawn during 10 days of storage. TMA is produced by the reduction of trimethylamine oxide (TMAO) by TMAO reductase producing microorganisms. TMA formation is most likely depended on fish species. Rodriguez

et al. (2004) observed that TMA content of hake muscle stored in either flake ice or slurry ice hardly increased during 12 days of storage. Additionally, Yamagata and Low (1995) also found that TMA content of banana shrimp (*Penaeus merguiensis*) hardly increased during day 4 days of iced storage and increased to 1.51 mg N/100g sample on day 8 of storage.

TVB and TMA contents can be used for quality assessment of fish. TVB values of fresh and good quality fish are generally less than 12 mg N/100 g sample. Higher TVB values in the range of 25-35 mg N/100 g sample indicate that the fishes ranged from slightly decomposed/edible to decomposed/inedible (Ababouch *et al*., 1996). In case of shrimp, the limits of acceptability in some sectors of the Australian and Japanese markets are 5 mg TMA/100 g muscle and/or 30 mg TVB (Hebard *et al*., 1962). Based on TVB and TMA contents, all samples were still acceptable during 12 days of storage.

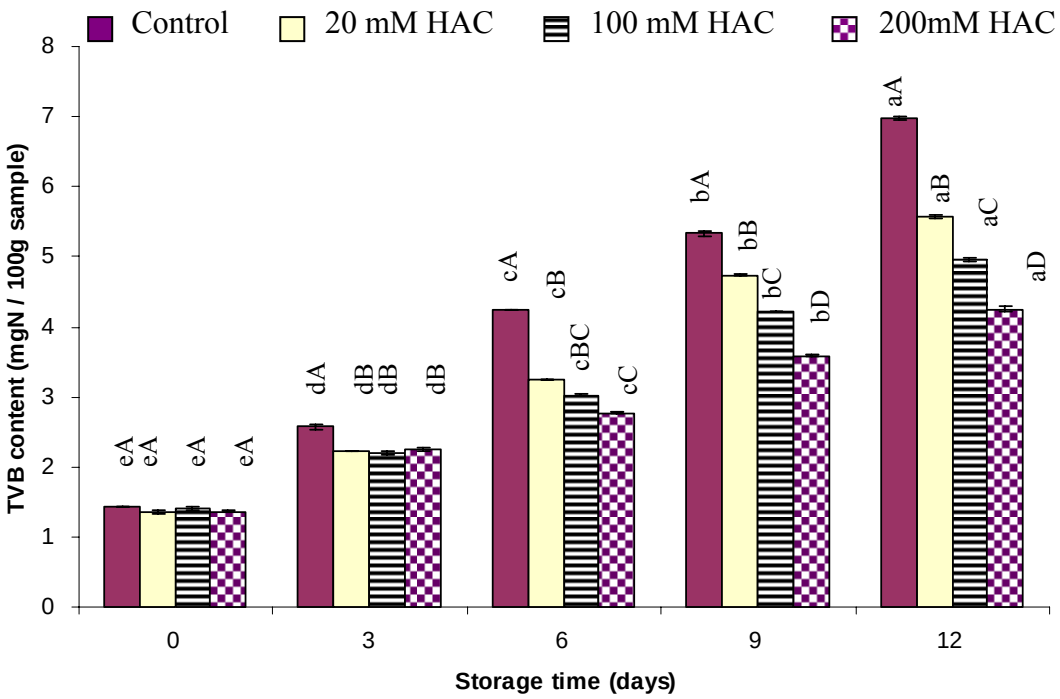

Figure 25. Changes in TVB content (mg N / 100g sample) of Pacific white shrimp soaked without and with HAC at different concentrations during iced storage. Different capital letters within the same storage time indicated significant differences ($P<0.05$). Different letters within the same treatment indicated significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

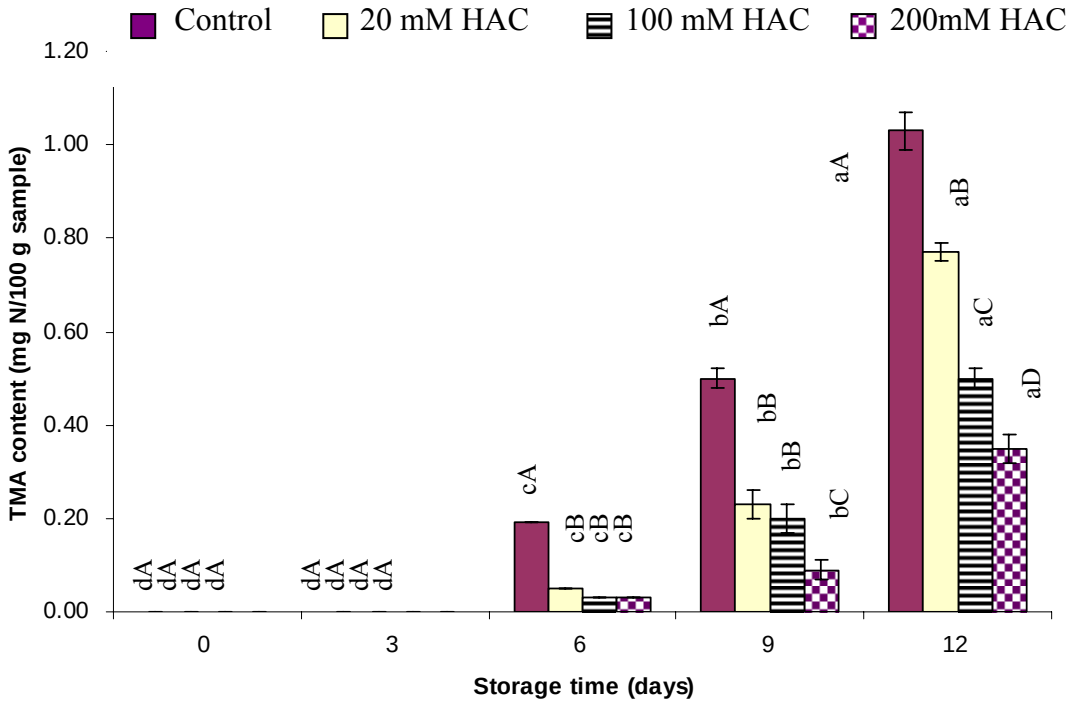

Figure 26. Changes in TMA content (mg N / 100g sample) of Pacific white shrimp soaked without and with HAC at different concentrations during iced storage. Different capital letters within the same storage time indicated significant differences ($P<0.05$). Different letters within the same treatment indicated significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

5.3 Changes in TBARS

TBARS value of Pacific white shrimp soaked without and with HAC at 20, 100 or 200 mM during iced storage is shown in Figure 26. The increase in TBARS was observed in all samples when the storage time increased ($P<0.05$), suggesting that lipid oxidation occurred during extended storage. A sharp increase in TBARS value during 12 days of iced storage was observed in the control shrimps. The initial values of TBARS of all samples were 0.52-0.60 mg/kg. After 12 days of iced storage, the highest TBARS value was observed in the control (3.95 mg/kg), and the lowest TBARS values was found in the sample soaked in 200 mM HAC (0.99 mg/kg), ($P<0.05$). Generally, the lower TBARS value was observed when the concentration of HAC used increased ($P<0.05$). However, no differences in TBARS

values of shrimps treated with all concentrations of HAC within the first 3 days of storage ($P>0.05$). The lowered TBARS value in the shrimp treated with HAC, especially at 200 mM, indicated the decrease in lipid oxidation; this could be due to antioxidative activity of HAC as shown by its ABTS and DPPH radical scavenging activities and reducing power (Figures 20, 21 and 22).

5.4 Changes in psychrophilic bacterial count

Changes in psychrophilic bacterial count of Pacific white shrimp during iced storage are shown in Figure 27. Psychrophilic bacterial count was negligible in all samples, regardless of the treatment, at day 0 of storage. No bacterial count was found in sample soaked with HAC at 200 mM within the first 3 days of storage. At the end of storage (12 days), the control sample had the highest count, whereas the shrimp treated with 200 mM HAC had the lowest count ($P<0.05$). At the same storage time, the lower psychrophilic bacterial counts of shrimps were observed when the concentration of HAC increased ($P<0.05$). Many chemicals were treated with shrimp during iced or chilled storage to inhibit microbial growth. Thepnuan (2007) reported that higher counts of psychrophilic bacteria were observed in shrimps kept in air at 4 °C during 12 days of storage, compared with those treated with 5 g/l ascorbic acid or 2% (w/v) pyrophosphate. Martinez-Alvarez *et al*. (2005) reported that 0.1% 4-hexylresorcinol mixed with citric acid or ascorbic acid could inhibit the microbial growth in tiger prawn (*Marsupenaeus japonicus*) during 12 days of chilled storage (4 °C). During iced storage, psychrophilic microorganisms most likely played a role in spoilage of shrimp. From the result, the use of HAC, especially at higher concentrations, could retard the spoilage of Pacific white shrimp during iced storage.

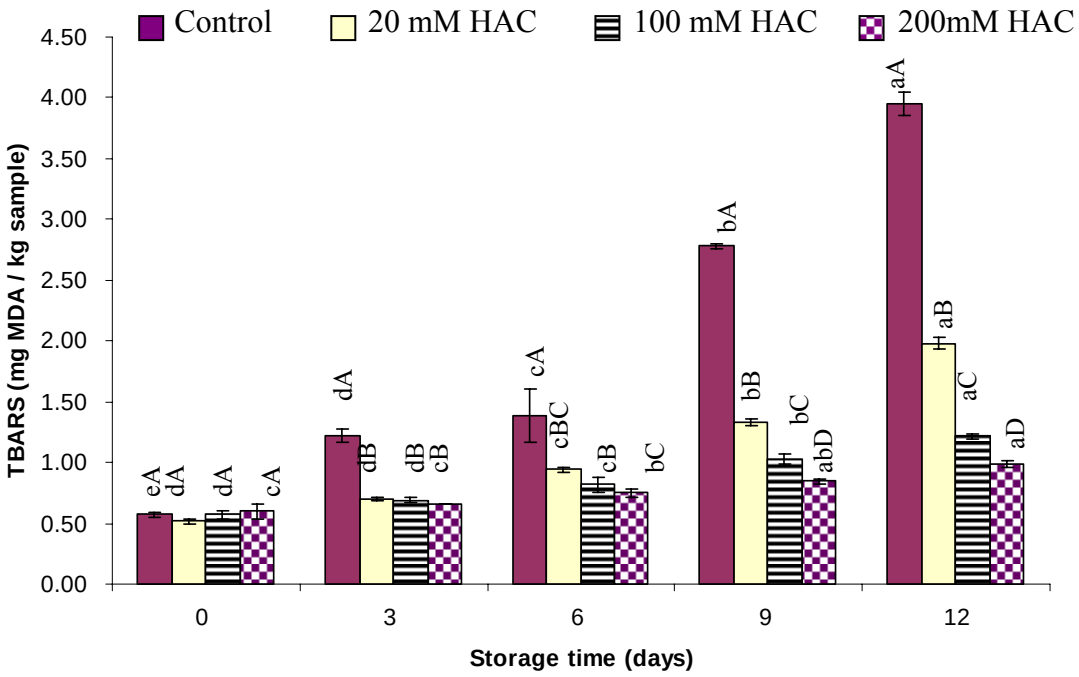

Figure 27. Changes in TBARS of Pacific white shrimp soaked without and with HAC at different concentrations during iced storage. Different capital letters within the same storage time indicated significant differences ($P<0.05$). Different letters within the same treatment indicated significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

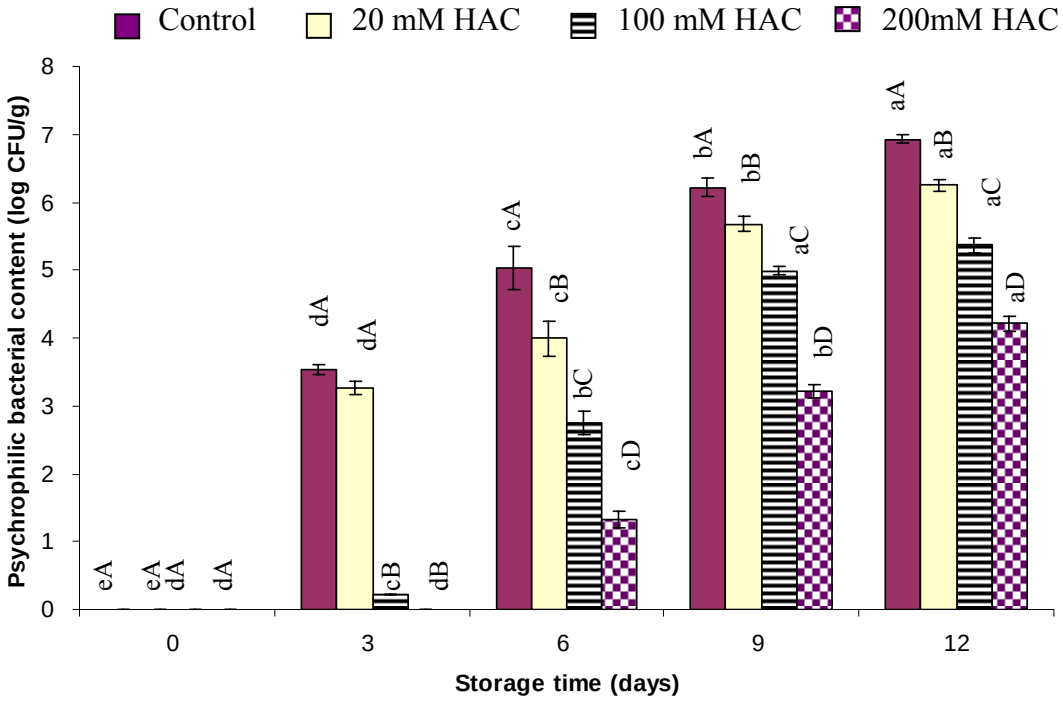

Figure 28. Changes in psychrophilic bacterial count (log CFU/g) of Pacific white shrimp soaked without and with HAC at different concentrations during iced storage. Different capital letters within the same storage time indicated significant differences ($P<0.05$). Different letters within the same treatment indicated significant differences ($P<0.05$). Bars represent the standard deviation from triplicate determinations.

CHAPTER 4

CONCLUSION

1. Polyphenol oxidase (PPO) from Pacific white shrimp (*Litopenaeus vannamei*) showed the highest activity at 45 °C and pH 6.0. PPO was stable in pH range of 5-7 and was unstable at the temperature greater than 50 °C. The molecular weight of Pacific white shrimp PPO was estimated to be 180 kDa.

2. Heated alkaline cysteine (HAC) exhibited the greater PPO inhibitory activity, copper-binding activity, reducing power and radical scavenging activity, compared with heated alkaline solution (pH 10) of glycine, methionine and glucose-cysteine Maillard reaction products.

3. Alkaline pH increased PPO inhibitory activity, copper-binding activity, reducing power and radical scavenging activity of heated cysteine, while acidic pH, except pH 2, lowered those activities. Cysteine concentrations determined these activities, which were increased with increasing concentrations.

4. Shrimps treated with HAC prepared under optimum condition had the retarded melanosis and lowered lipid oxidation. Microbiological growth of shrimp treated with HAC, especially at higher concentration, was also impeded.

SUGGESTIONS

1. Further study on toxicity of HAC should be carried out.

2. The effect of HAC treatment on the acceptance of Pacific white shrimp and other shrimps should be elucidated.

3. The use of HAC in combination with other preservation methods should be studied to maximize the shelf-life extension of shrimps.

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APPENDIX

ANALYTICAL METHODS

1. Determination of trimethylamine (TMA-N) and total volatile basic nitrogen (TVB-N) by Conway's method (Conway and Byrne, 1936)

Reagents

1. Inner ring solution (1% boric acid solution containing indicator): Dissolve 10 g of boric in 1 liter flask, add 200 ml of ethanol and make up volume to 1 liter with distilled water.

2. Mixed indicator solution: Dissolve bromocresol green (BCG) 0.01 g and methyl red (MR) 0.02 g in 10 ml of ethanol.

3. 0.02 N HCI

4. Saturated K_2 CO_3 solution: Weigh 60 g of potassium carbonate, and add 50 ml of distilled water. Boil gently for 10 min. After cooling down, filter the mixture through filter paper.

5. 4% trichloroacetic acid ($CC1_3$ COOH), TCA, solution: Dissolve 40 g of TCA in 960 ml of distilled water.

6. Sealing agent: Weigh 3 g of Trangacanth gum, add 30 ml of distilled water, 15 ml of glycerine and 15 ml of 50% saturated K_2CO_3 solution and mix well.

7. Neutralized 10% formaldehyde solution: Add 10 g of $MgCO_3$ to 100 ml of formaline (35% formaldehyde solution) and shake in order to neutralize the acidity of formalion. Filter and dilute the filtrate with 3 volume of distilled water.

Method

Sample extraction:

1. Weigh 2 g of fish meat and place in a mortar and grind well.
2. Add 8 ml of 4% TCA solution and grind well.
3. Stand for 30 min at ambient temperature with occasional grinding.
4. Filter through filter paper (Whatman No. 41) or centrifuge at 3,000 rpm, for 10 min.
5. Keep the filtrate in -20 °C freezing if necessary.

1.1 Determination of TVB-N

1. Apply sealing agent to Conway's unit.
2. Pipette 1 ml of inner ring solution into inner ring.
3. Pipette 1 ml of sample extract into outer ring.
4. Slant the Conway's unit with cover.
5. Pipette 1 ml of saturated K_2CO_3 solution into outer ring.
6. Close the unit.
7. Mix gently.
8. Stand for 60 min at 37 °C in incubator.
9. Titrate the inner ring solution with 0.02 N HCI using a micro-burette until green color turns pink.
10. Prepare the blank test using 1 ml of 4% TCA instead of sample extract.

1.2 Determination of TMA-N

1. Apply sealing agent to Conway's unit.
2. Pipette 1 ml of inner ring solution into inner ring.
3. Pipette 1 ml of sample extract into outer ring.
4. Pipette 1 ml of neutralized 10% formaldehyde into outer ring.
5. Slant the Conway's unit with cover.
6. Pipette 1 ml of saturated K_2CO_3 solution into outer ring.
7. Close the unit.
8. Mix gently.

9. Stand for 60 min at 37 °C in incubator.

10. Titrate the inner ring solution with 0.02 N HCI using a micro-burette until green color turns pink.

11. Prepare the blank using 1 ml of 4% TCA instead of sample extract.

Calculation

$$\text{TMA-N or TVB-N (mg N/ 100g)} = \frac{(V_S - V_B) \times (N_{HCl} \times A_N) \times V_E \times 100}{W_S}$$

Where V_S = Titration volume of 0.02 N HCI for sample extract (ml)

V_B = Titration volume

N_{HCI} = Normality of HCI (0.02 N×f, factor of HCI)

A_N = Atomic weight of nitrogen (× 14)

W_S = Weight of muscle sample (g)

V_E = Volume of 4 % TCA used in extraction

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