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Inhibitory Effect of Mimosine on Polyphenoloxidase from Cephalothoraxes of Pacific White Shrimp (*Litopenaeus vannamei*)

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

KEYWORDS: mimosine, inhibition kinetic, polyphenoloxidase, Pacific white shrimp, melanosis

1. INTRODUCTION

Black spot formation (melanosis) is one of the serious problems occurring in crustaceans during post-mortem handling and storage. Melanosis in shrimp drastically reduces the consumer acceptability and the product's market value, leading to considerable financial loss.[1] Melanosis is triggered by a biochemical mechanism which oxidizes phenols to quinones by polyphenoloxidase. Polyphenoloxidase (PPO) is also known as phenoloxidase, tyrosinase, and catechol oxidase and is involved in vertebrate pigmentation and browning of fruits and vegetables.[2] PPO is a bifunctional, copper-containing enzyme, which catalyzes two basic reactions in the presence of molecular oxygen. Those include *o*-hydroxylation of monophenols to give *o*-diphenols (Monophenol oxidase, EC 1.14.18.1) and the subsequent oxidation of *o*-diphenols to *o*-quinones (Diphenoloxidase, EC 1.10.3.1).[3] PPO exists in three different types of isoforms, namely, oxy-PPO [Cu (II) Cu (II) O_2], met-PPO [Cu (II) Cu (II)], and deoxy-PPO [Cu (I) Cu (I)].[4]

The intensity of melanosis formation in the crustacean varies with species, most likely due to the differences in substrate and enzyme concentration.[5] Many studies have focused on either inhibiting or preventing PPO activity by eliminating one or more of the essential components, e.g., enzyme, oxygen, and copper from the reaction.[6] To control the undesirable browning in crustaceans, sulfite derivatives and 4-hexylresorcinol have been intensively used. Owing to the strict regulation for the use of sulfiting agents and the high price of the commercial PPO inhibitor, the interest in natural additives for the retardation of melanosis in shrimp has increased.[7] Recently, plant phenolic compounds including grape seed extract,[6] ergothioneine from mushroom extract,[8] and green tea extract[1] were found as the effective additives to retard melanosis in shrimp. Benjakul et al.[9] reported that amino acids such as cysteine or glutathione had an inhibitory effect on PPO from kuruma prawn (*Penaeus japonicus*).

Pacific white shrimp (*Litopenaeus vannamei*) accounts for 90% of the global aquaculture shrimp production.[10] Thailand is the world's leading shrimp farming country and has become the top supplier of farmed shrimp to the USA.[11] Shrimp is a very perishable product and generally has a limited shelf life due to the formation of black spots.[12] Recently, lead (*Leucaena leucocephala*) seed extract has been reported to prevent melanosis formation in Pacific white shrimp during iced storage.[13] *L. leucocephala* contains a nonprotein amino acid called mimosine, (β-(3-hydroxy-4-pyridon-1-yl)-L-alanine).[14] Mimosine causes alopecia and other toxic signs in various species of animals.[15] Teratogenic effects have been demonstrated in pigs and rats, but no species of *Mimosa* has been shown to have any such effect in humans.[16] Therefore, better understanding in kinetics and mode of inhibition toward PPO of Pacific white shrimp should pave the way for melanosis control in shrimp and its products. However, there is no information on the inhibitory mechanism of mimosine toward PPO from Pacific white shrimp cultured in Thailand. The objective of this study was to elucidate the inhibition mechanism of mimosine toward PPO from Pacific white shrimp.

2. MATERIALS AND METHODS

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

2.2. Shrimp Collection and Preparation. Pacific white shrimp (*Litopenaeus vannamei*) with the size of 55−60 shrimps/kg were purchased from a supplier in Songkhla, Thailand. The shrimp, freshly caught and completely free of additives, were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 1 h. Upon

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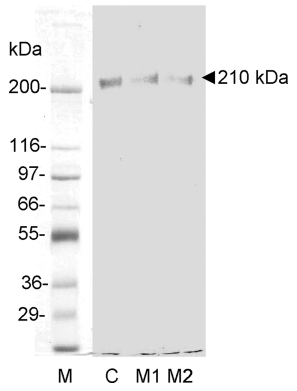

Figure 1. Activity staining of the DEAE-Sephacel fraction containing polyphenoloxidase from the cephalothorax of Pacific white shrimp in the absence and presence of mimosine at different concentrations. M, molecular weight marker; C, DEAE-Sephacel fraction; M1 and M2, DEAE-Sephacel fraction with 5 and 10 mM mimosine, respectively.

arrival, shrimp were washed in cold water and stored in ice until used (not more than 3 h). The cephalothoraxes of shrimps were separated, pooled, and powdered by grinding with liquid nitrogen in a Waring blender (AY46, Moulinex, Guangdong, China). The powder obtained was kept in a polyethylene bag and stored at −20 °C for not more than 2 weeks.

2.3. Extraction of PPO. Cephalothorax powder (50 g) was mixed with 150 mL of 0.05 M sodium phosphate buffer (pH 7.2) containing 1.0 M NaCl, 0.2% Brij 35, and 1 mg/mL of phenilmethanesulfonyl fluoride (PMSF). The mixture was stirred continuously at 4 °C for 30 min, followed by centrifugation at 8000*g* at 4 °C for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). Solid ammonium sulfate was added into the supernatant to obtain 40% saturation.[10] The precipitate was collected by centrifugation at 12,500*g* at 4 °C for 30 min using a refrigerated centrifuge. The pellet obtained was dissolved in a minimum volume of 0.05 M sodium phosphate buffer, pH 7.2, and dialyzed against 50 volumes of the same buffer at 4 °C with three changes of dialysis buffer. The insoluble materials were removed by centrifugation at 3000*g* at 4 °C for 30 min.

2.4. DEAE-Sephacel Column Chromatography of PPO. Ammonium sulfate fraction was applied onto the DEAE-Sephacel column (1.6 × 16 cm), previously equilibrated with 0.05 M phosphate buffer, pH 7.2.[17] The column was then washed with the same phosphate buffer until A_{280} was lower than 0.05. PPO was eluted with a linear gradient of 0 to 1.2 M NaCl in 0.05 M phosphate buffer (pH 7.2) at a flow rate of 0.5 mL/min. Fractions of 1.5 mL were collected, and those with PPO activity were pooled. The pooled fractions were dialyzed with 50 volumes of 0.05 M phosphate buffer (pH 7.2) with two changes within 12 h. PPO from the cephalothorax of Pacific white shrimp was purified to 83.7-fold after being chromatographed using the DEAE-Sephacel column.

2.5. PPO Activity Assay. PPO activity was assayed using L-DOPA as a substrate according to the method of Nirmal et al.[10] with a slight modification. The assay system consisted of 100 μL of PPO solution, 600 μL of 15 mM L-DOPA in deionized water, 400 μL of 0.05 M phosphate buffer (pH 6.0), and 100 μL of deionized water. The PPO activity was determined for 3 min at 55 °C by monitoring the formation of dopachrome at 475 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of PPO activity was defined as an increase in the absorbance at 475 nm by 0.001/min/mL. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture, and the deionized water was used instead.

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

2.7. Inhibition Kinetics of Mimosine on PPO. The DEAE-Sephacel fraction (100 μL) was mixed with mimosine solution (100 μL) to obtain final concentrations of 0.5, 2.5, and 5.0 mM. The mixtures were incubated for 5 min at room temperature (25 °C). To initiate the reaction, 1000 μL of L-DOPA in 0.05 M sodium phosphate buffer (pH 6.0) was added. At each concentration of mimosine, L-DOPA with seven different concentrations (0.5− 5 mM) was used as the substrate. The reaction mixtures were incubated for 3 min at 55 °C, and the absorbance at 475 nm was measured using a UV-1800 spectrophotometer. The Michaelis constant (K_m) for PPO was determined by Lineweaver−Burk plots,[18] and the K_i value was obtained from a Dixon plot.[19]

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

2.9. Determination of Copper Chelating Capacity of Mimosine. The copper chelating capacity of mimosine (0.5, 2.5, and 5.0 mM) was determined according to the method of Wettasinghe et al.[21] as modified by Nirmal et al.[1]

2.10. Effect of Mimosine on the Intermediate Browning Product. The reaction mixture containing 100 μL of DEAE-Sephacel fraction, 400 μL of assay buffer, and 600 μL of L-DOPA was incubated at 25 °C for 3 min. To the reaction mixtures, 100 μL of mimosine (10 and 20 mM) was added immediately and mixed thoroughly. The red-brown color that developed was monitored by measuring the absorbance at 475 nm up to 5 min at 25 °C. For the control, deionized water (100 μL) was added instead of mimosine solution. Decrease in absorbance at 475 nm indicates the reduction of *o*-quinone to phenols or the formation of the quinone−mimosine complex.[9]

2.11. Statistical Analyses. All analyses were performed in triplicate, and a completely randomized design (CRD) was used. Analysis of variance (ANOVA) was performed, and mean comparisons were done by Duncan's multiple range tests.[22] Analysis was performed using an SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1. Effect of Mimosine on PPO from the Cephalothorax of Pacific White Shrimp. Activity staining of the DEAE-Sephacel fraction containing PPO from the cephalothorax of Pacific white shrimp in the absence and presence of mimosine at the concentrations of 5 and 10 mM is illustrated in Figure 1. Mimosine showed the inhibitory effect on PPO from the cephalothorax of Pacific white shrimp as indicated by the lowered band intensity, as compared to that of control (without mimosine). Inhibitory activity of mimosine toward PPO was in a dose dependent manner. Nirmal et al.[13] reported that lead (*Leucaena leucocephala*)

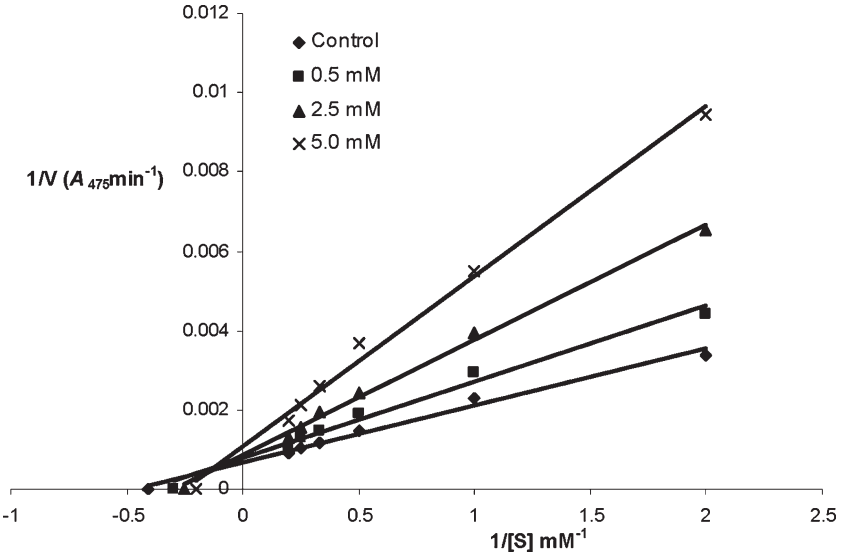

Figure 2. Lineweaver− Burk plot of polyphenoloxidase in the DEAE-Sephacel fraction from the cephalothorax of Pacific white shrimp in the absence and presence of mimosine at different concentrations. L-DOPA at levels of 0.5−5 mM was used as the substrate.

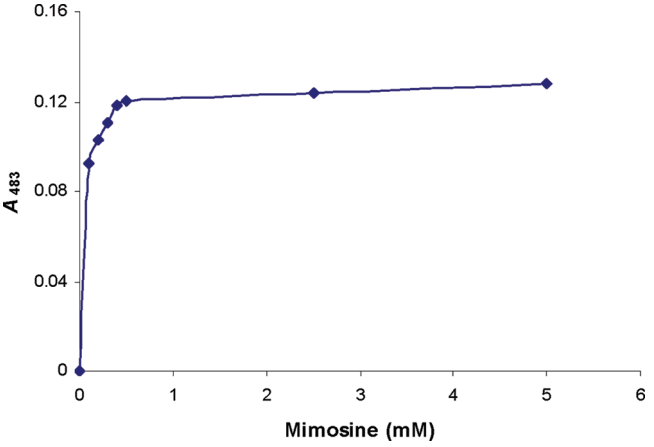

Figure 3. Copper reduction capacity of mimosine at different concentrations. The reaction mixture was incubated at 25 °C for 20 min. Absorbance was measured at 483 nm.

seed extract powder containing mimosine had an inhibitory effect on PPO from Pacific white shrimp. Mimosine is chemically similar to dihydroxyphenylalanine with a 3-hydroxy-4-pyridone ring instead of a 3,4-dihydroxyphenyl ring.[23] Mimosine most probably inhibited PPO by competing with the substrate in binding with the active site of PPO. On the basis of activity staining, PPO had a molecular weight of 210 kDa. The DEAE-Sephacel fraction containing PPO from the cephalothorax of Pacific white shrimp was in the active state and did not require activators including trypsin, sodium dodecyl sulfate, and isopropanol (data not shown). This result reconfirmed our previous report[10] where PPO from thecephalothorax of Pacific white shrimp showed a molecular weight of 210 kDa. Zamorano et al.[24] studied the electrophoretic mobility of PPO from deep-water pink shrimp (*Parapenaeus longirostris*) using nonreducing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), followed by staining with DOPA, and found the activity band with a molecular weight of 200 kDa. The viscera and carapace extracts from the cephalothorax of the Norway lobster (*Nephrops norvegicus*) showed both mono and diphenol-oxidase activity when activity staining was carried out using L-tyrosine and 4-*tert*-butyl-catechol as substrates. Apparent molecular weight of PPO from the cephalothorax of the Norway lobster was 200−220 kDa.[25] PPO from the cephalothorax of Pacific white shrimp could not use L-tyrosine as the substrate (data not shown), suggesting the lack of monophenoloxidase activity of PPO. *Leucaena leucocephala* seed extract powder containing mimosine and phenolic compounds could chelate a copper ion at the active site of PPO, thus inactivating PPO.[13]

3.2. Mode of PPO Inhibition of Mimosine. From the Lineweaver−Burk plots, the Michaelis constant (K_m) for the oxidation of L-DOPA by PPO in the DEAE-Sephacel fraction was 2.43 mM. K_m values reflect the affinity of enzymes for their substrates. The K_m observed for Pacific white shrimp PPO was approximately equal to that reported for the oxidation of DL-DOPA (K_m 2.8 mM) by *Panaeus setiferus* PPO.[26] The K_m value of 0.26 mM was found for the oxidation of L-DOPA by PPO from the kuruma prawn (*Penaeus japonicus*).[9] The K_m value for the oxidation of L-DOPA by *Charybdis japonica* PPO was 3.41 mM.[27] The higher K_m value indicates the lower catalytic efficiency of the enzyme toward the substrate.[27] Variations in enzyme preparation and assay methods can be associated with differences in K_m values.[17]

Inhibition kinetics of mimosine toward PPO from the cephalothorax of the Pacific white shrimp was studied from a Lineweaver−Burk plot as shown in Figure 2. Mimosine at different concentrations affected both the K_m and V_{max} values of PPO. Since the K_m value increased and the V_{max} value decreased with increasing mimosine concentrations, the inhibitory mode of mimosine was found to be mixed type. Results indicated that mimosine could bind with both the enzyme and enzyme−substrate complex, but with different affinities. Mimosine and kojic acid was reported to be a standard inhibitor for mushroom tyrosinase with competitive type inhibition.[28,29] Kojic acid showed a mixed type inhibition toward white shrimp, grass prawn, and lobster PPO.[17] The K_i value of mimosine obtained from Dixon

Table 1. Copper Chelating Activity of Mimosine at Different Concentrations[a]

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

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

plots was 3.7 mM. The K_i value of dodecyl gallate on mushroom tyrosinase was 0.636 mM.[30] Benjakul et al.[9] reported that cysteine and glutathione showed competitive inhibition toward kuruma prawn PPO with K_i values of 0.45−0.46 mM. The plots of residual enzyme activity vs the concentrations of enzyme in the presence of different concentrations of mimosine showed straight lines, which passed through the origin (data not shown). Mimosine at higher concentrations resulted in the decrease in the slope of the line, suggesting its reversible inhibition toward PPO. The decrease in the slope of the lines with increasing concentration of dodecyl gallate indicated the reversible inhibition toward mushroom tyrosinase.[30] Therefore, mimosine showed mixed type reversible inhibition on PPO from the cephalothorax of Pacific white shrimp.

3.3. Copper Reduction Capacity of Mimosine. Copper reduction capacity of mimosine at different concentrations is shown in Figure 3. Increases in the absorbance at 483 nm indicate the formation of Cu^+. Increased absorbance was observed as the concentration of mimosine increased up to 0.5 mM. Thereafter, no changes in absorbance were found, indicating that all Cu^{2+} ions were reduced to Cu^+. With increasing kojic acid concentration up to 0.28 mM, the copper reduction capacity of kojic acid increased.[20] PPO at the met-[Cu^{2+} Cu^{2+}] form is reduced by a reductant to deoxy-PPO [Cu^+ Cu^+], which then interacts with oxygen to form oxy-PPO [Cu^{2+} Cu^{2+} O_2]. Oxy-PPO is a highly active isoform, capable of catalyzing mono and diphenols.[20] Copper in the active site of PPO is primarily involved in the browning reaction.[9] Reduction of Cu^{2+} to Cu^+ at the active site of PPO by mimosine could convert PPO into the deoxy form. Ascorbic acid can reduce met-tyrosinase into deoxy-tyrosinase, which modifies the enzymatic turnover.[31] This result suggested that mimosine could lower or slow down the dopachrome formation by reducing met-[Cu^{2+} Cu^{2+}] to deoxy-PPO [Cu^+ Cu^+].

3.4. Copper Chelating Capacity of Mimosine. The copper chelating activity of mimosine at different concentrations is shown in Table 1. Mimosine showed copper chelating activity at all concentrations tested. The copper chelating activity of mimosine increased as the concentrations increased ($P < 0.05$). The copper chelating activity of 97.2% was observed in the presence of 5 mM mimosine. The copper chelating activity of mimosine was in accordance with their PPO inhibitory activity (Figure 1). The inhibition of metal-dependent enzymes by L-mimosine was related to its chelating ability.[32] The *N*-nitroso and *N*-hydroxy groups of hydroxylamine were suggested to be essential for the tyrosinase inhibitory activity, probably due to the copper chelating ability.[33] Cysteine and glutathione might interact with copper at the active site of kuruma prawn PPO, leading to a loss of activity.[9] Therefore, the copper chelating capacity of mimosine is one of the important inhibitory mechanisms involved in the inhibition of PPO from Pacific white shrimp.

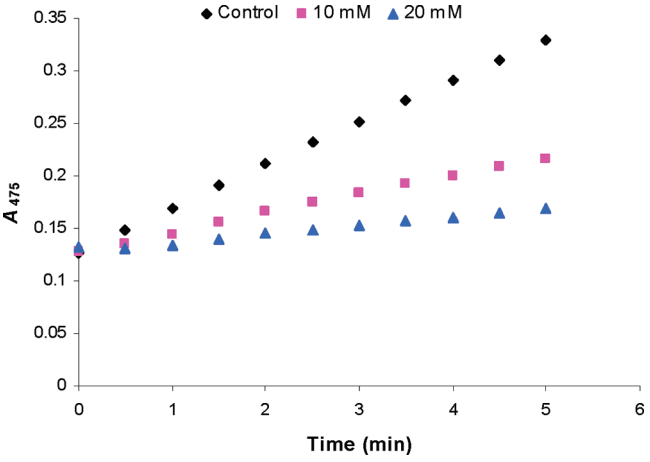

Figure 4. Absorbance of dopachrome after the addition of mimosine at different reaction times. The assay mixture was incubated at 25 °C for 3 min prior to the addition of mimosine. The absorbance at 475 nm was monitored for another 5 min at 25 °C.

3.5. Effect of Mimosine on the Intermediate Browning Product. A_{475} representing dopachrome formation without and with the addition of mimosine into the prior incubated assay mixture is illustrated in Figure 4. In general, continuous increase in dopachorme formation was observed in the control when reaction time increased. The formation of dopachrome was retarded in the presence of mimosine. Lower rate of increase in A_{475} indicated that mimosine might chelate copper at the active site of PPO, rendering PPO inactive. Additionally mimosine could form a complex with dopaquinone, in which a yellow complex with the maximum absorbance at 440 nm was observed. This result was concomitant with the inhibition kinetics, where mimosine showed mixed type inhibition (Figure 2). Cysteine or glutathione could lower the dopachrome formation by the reduction of quinone or the formation of the cysteinyl adducts.[9] The result indicated that the formation of the red-brown color compound in the reaction mixture through the action of PPO and L-DOPA was lowered by mimosine. Therefore, mimosine could inhibit dopachrome formation by reacting with PPO and the PPO−DOPA complex. Furthermore, mimosine might chelate the copper ion at the active site of PPO or react with dopachrome to form the yellow colored complex.

4. CONCLUSIONS

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

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