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Biochemical properties of polyphenoloxidase from the cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*)

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Abstract

Melanosis is a major problem associated with the quality loss of Pacific white shrimp. This is mediated by polyphenoloxidase (PPO), in which biochemical and molecular properties can be varied with species, molting period, etc. The better understanding of PPO characteristics should pave a way for lowering melanosis, thereby extending the shelf life of Pacific white shrimp during handling and storage. PPO was extracted from the cephalothorax of Pacific white shrimp, partially purified by ammonium sulphate precipitation (0% to 40% saturation) and diethylaminoethyl-Sephacel anion exchange chromatography, and characterised. Partially purified PPO (83.8-fold purity) showed the maximal activity using L-β-(3,4-dihydroxylphenyl)alanine (L-DOPA) as a substrate at pH 6 and at 55°C. PPO was stable over a pH range of 5 to 10 but unstable at a temperature greater than 60°C. Based on the activity staining with L-DOPA, the apparent molecular weight of PPO was 210 kDa. The Michaelis constant (K_m) of PPO for the oxidation of L-DOPA was 2.43 mM. Trypsin, copper acetate and sodium dodecyl sulphate were unable to activate PPO, suggesting that the enzyme was in the active form. Cysteine, ethylenediaminetetraacetic acid and *p*-aminobenzoic acid showed PPO inhibitory activity in a dose-dependent manner. At the same concentration used (1 and 10 mM), cysteine exhibited a higher inhibitory effect towards PPO. Active PPO with higher stability could be responsible for the rapid melanosis formation in Pacific white shrimp, especially at the cephalothorax portion during postmortem storage. The use of an effective PPO inhibitor, especially cysteine, in combination with chilled storage could prevent melanosis in shrimp.

Keywords: Pacific white shrimp, Polyphenoloxidase, Enzyme characteristic, Activator, Inhibitor

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Background

Polyphenoloxidase (PPO) is known as phenolase, tyrosinase and catechol oxidase. PPO is a copper-containing metalloenzyme, which catalyses two basic reactions, in the presence of molecular oxygen, that include the *o*-hydroxylation of monophenols to give *o*-diphenols (monophenol oxidase, cresolase activity, EC 1.14.18.1) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenoloxidase, catecholase activity, EC 1.10.3.1) (Garcia-Molina et al. 2005). PPO in crustaceans is distributed mainly in the carapace, followed by the abdominal exoskeleton, cephalothorax, pleopods and telson (Zamorano et al. 2009). PPO is most commonly found in the cephalothorax of prawn and shrimp (Montero et al. 2001a). PPO is involved in the black spot formation in crustaceans during postmortem storage. Black spot formation (melanosis) is one of the problems that occur in crustaceans. It drastically reduces the consumer acceptability and the product's market value (Montero et al. 2001a). The intensity of melanosis formation in crustaceans varies with species, due to differences in the substrate and enzyme concentration (Benjakul et al. 2005).

Therefore, a better understanding of biochemical properties and kinetics of PPO is needed to control and inhibit its action. PPO from the cephalothoraxes of various crustaceans such as pink shrimp (*Parapenaeus longirostris; Penaeus duorarum*) (Zamorano et al. 2009; Simpson et al. 1988; Chen et al. 1997), white shrimp (*Penaeus striferus*) (Simpson et al. 1988; Chen et al. 1997), prawn (*Penaeus japonicus*) (Benjakul et al. 2005; Montero et al. 2001b) and lobster (*Nephrops norvegicus*; *Homarus Americanus*) (Yan et al. 1990; Opoku-Gyamfua and Simpson 1993) have been purified and characterised. PPO from different crustaceans showed different molecular weight, optimum pH, thermal stability and kinetic parameters.

Pacific white shrimp (*Litopenaeus vannamei*) is an important commercial species primarily cultured in Thailand and accounts for 90% of the global aquaculture shrimp production (Nirmal and Benjakul 2009a). This high-value shrimp is very perishable and susceptible to black spot formation during postmortem handling and storage. To impede melanosis in shrimp, the minimization of PPO by regulating pH or temperature as well as the use of appropriate PPO inhibitors should be implemented, thereby maintaining the quality of shrimp during handling, processing and storage. However, there is no information on biochemical properties of PPO from the cephalothorax of Pacific white shrimp cultured in Thailand. The aim of this study was to determine the biochemical properties of PPO from Pacific white shrimp and to elucidate the effects of some chemicals on PPO activity.

Methods

Chemicals

L-β-(3,4 dihydroxylphenyl)alanine (L-DOPA), Brij-35, phenylmethanesulfonyl fluoride (PMSF), bovine pancreatic trypsin (501.25 U/mg), *p*-aminobenzoic acid and cysteine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS), ammonium sulphate and ethylenediaminetetraacetic acid (EDTA) were obtained from Merck (Darmstadt, Germany). Coomassie Brilliant Blue R-250 and *N,N,N′,N′*-tetramethylethylenediamine were procured from Bio-Rad Laboratories (Hercules, CA, USA). High-molecular-weight markers and diethylaminoethyl (DEAE)-Sephacel were purchased from GE Healthcare UK Limited (Buckinghamshire, UK).

Shrimp collection and preparation

Pacific white shrimp (*L. vannamei*) with a size of 55 to 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 arrival, the shrimp were washed in cold water and stored in ice until used (not more than 3 h). The cephalothoraxes of the shrimp were separated, pooled and powdered by grinding with liquid nitrogen in a Waring blender (AY46, Moulinex, Foshan, Guangdong, China). The powder obtained was kept in a polyethylene bag and stored at −20°C for not more than 2 weeks.

Extraction and partial purification of PPO from the cephalothorax of Pacific' white shrimp

The extraction of PPO from the powdered cephalothoraxes of Pacific white shrimp was carried out according to the method of Nirmal and Benjakul (2009b) with a slight modification. The powder (25 g) was mixed with 75 mL of the extracting buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 1.0 M NaCl and 0.2% Brij-35). PMSF at 1 mg/mL was also added to the extracting buffer to prevent proteolysis by indigenous proteases. The mixture was stirred continuously at 4°C for 30 min, followed by centrifugation at 8,000 × *g* at 4°C for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). Solid ammonium sulphate was added into the supernatant to obtain 40% saturation and allowed to stand at 4°C for 30 min. The precipitates formed were collected by centrifugation at 12,000 × *g* for 30 min at 4°C. The pellets were dissolved in a minimum volume of extracting buffer and dialysed with 50 volumes of cold extracting buffer with three changes overnight. The insoluble materials were removed by centrifugation at 3,000 × *g* at 4°C for 30 min.

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

Measurement of PPO activity

The assay system consisted of 100 μL of PPO solution, 600 μL of 15 mM L-DOPA in deionised water, 400 μL of 0.05 M phosphate buffer, pH 6.0 and 100 μL of deionised water (Nirmal and Benjakul 2009a). The PPO activity was determined for 3 min at 45°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 using the deionised water instead.

pH and temperature profiles of PPO

The activity of PPO in the DEAE-Sephacel fraction was assayed at different pH (2.0 to 10.0) at 45°C using 15 mM L-DOPA as a substrate. Different assay buffers at pH 2.0 to 7.0 (McIlvaine buffer; 0.2 M sodium phosphate and 0.1 M sodium citrate) and pH 8.0 to 10.0 (0.1 M phosphate-borate buffer) were used. For temperature profile study, the assay was performed at different temperatures (25°C to 60°C) for 3 min at pH 6.0. The activity was measured as previously described.

pH and thermal stability of PPO

To study the pH stability of PPO in the DEAE-Sephacel fraction, the 100 μL of fraction was mixed with 100 μL of McIlvaine buffer or 0.1 M phosphate-borate buffer at various pH (2 to 10) and incubated at room temperature for 30 min (Benjakul et al. 2005). Residual activity was then determined using 15 mM L-DOPA as a substrate at pH 6.0 and at 55°C for 3 min. For thermal stability, 100 μL of DEAE-Sephacel fraction containing PPO was incubated at different temperatures (25°C to 60°C) for 30 min. The sample was cooled rapidly in the iced water (Benjakul et al. 2005). The residual activity was then assayed at pH 6.0 and at 55°C for 3 min as previously described.

Kinetics study of PPO

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

PPO activity staining

DEAE-Sephacel fraction containing PPO from the cephalothoraxes of Pacific white shrimp was subjected to activity staining as per the method of Nirmal and Benjakul (2009a). The fraction was mixed with the sample buffer containing SDS at a ratio of 1:1 (*v*/*v*). The sample (3-μg protein) was loaded onto the polyacrylamide gel made of 7.5% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, one of the two identical gels was immersed in a 0.05 M sodium phosphate buffer (pH 6.0) containing 15 mM L-DOPA for 25 min at 25°C. The activity zone appeared as a brown band. Another gel was stained with 0.125% Coomassie Brilliant Blue R-250 and destained in 25% methanol and 10% acetic acid. To estimate the molecular weight of PPO, the markers including myosin from rabbit muscle (200 kDa), β-galactosidase from *Escherichia coli* (116 kDa), phosphorylase b from rabbit muscle (97 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase from bovine liver (55 kDa), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (36 kDa) and carbonic anhydrase from bovine erythrocytes (29 kDa) were used.

Effect of trypsin and copper on PPO activity

Effects of trypsin and copper on PPO in the DEAE-Sephacel fraction were determined using various concentrations of trypsin and copper acetate as per the method of

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

Effect of SDS and inhibitors on PPO activity

The influence of SDS and inhibitors (cysteine, EDTA and *p*-aminobenzoic acid (PABA)) on the activity of PPO in the DEAE-Sephacel fraction was determined as described by Benjakul et al. (2005) with a slight modification. To obtain the various final concentrations (0.05%, 0.1% and 0.5%), 100 μL of SDS was added to 100 μL of fraction. The mixtures were incubated at room temperature for 30 min, and the residual activity of PPO was assayed using 15 mM L-DOPA as a substrate at 55°C. Absorbance at 475 nm was recorded up to 3 min against the blank, in which the deionised water was used instead of the L-DOPA solution.

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

Statistical analyses

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

Results and discussion

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

Table 1 presents the purification steps of PPO from the cephalothorax of Pacific white shrimp. PPO from the cephalothorax of Pacific white shrimp was extracted and fractionated using 40% ammonium sulphate (AS) saturation. After AS fractionation, 2.7-fold purity was achieved. Simpson et al. (1987) reported AS (40% saturation)-fractionated PPO from the cephalothorax of white shrimp (*Penaeus setiferus*) with 2.4-fold purity. A 1.6-fold increase in purity was reported for the PPO from the cephalothorax of kuruma prawn (*P. japonicus*) with 40% AS fractionation (Benjakul et al. 2005). AS fraction was further

Table 1 Summary of the purification of PPO on DEAE-Sephacel anion exchange chromatography

Purification steps	Total activity(U)	Total protein(mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	49,099.5	1,396.8	35.1	1	100
AS fractionation	40,537	423.5	95.7	2.7	82.5
DEAE-Sephacel column	36,062	12.3	2,943.8	83.8	73.4

AS, ammonium sulphate; DEAE, diethylaminoethyl.

purified by DEAE-Sephacel anion exchange chromatography, and the purity of 83.8-fold was obtained. During DEAE-Sephacel chromatography, most of the unbound positively charged proteins were removed. At the end of partial purification through AS fractionation followed by DEAE-Sephacel chromatography, PPO with a yield of 73.4% was recovered. PPO from the cephalothorax of white shrimp was purified to 65.6-fold with affinity chromatography (Simpson et al. 1987). PPO from the cephalothorax of Taiwanese black tiger shrimp (*Penaeus monodon*) with purity of 58-fold was prepared using a Phenyl Sepharose CL-4B column (Rolle et al. 1991). PPO from the cephalothorax of pink (*P. duorarum*) and white (*P. setiferus*) shrimps were purified to 64- and 45-fold by Phenyl Sepharose CL-4B chromatography, respectively (Chen et al. 1997). This result suggested that DEAE-Sephacel anion exchange chromatography of AS fraction was effective in the purification of PPO from the cephalothorax of Pacific white shrimp.

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

The maximal activity of PPO in DEAE-Sephacel fraction from the cephalothorax of Pacific white shrimp was observed at pH 6 (Figure 1A). The result was in accordance with that of Rolle et al. (1991) who reported that maximal activity of PPO from the cephalothorax of Taiwanese black tiger shrimp was found at pH 6. PPO from the carapace and the viscera of Norway lobster (*N. norvegicus*) had optimum pH at 7 and 8, respectively (Gimenez et al. 2010). The maximum activity of PPO from the cephalothorax of kuruma prawn (*P. japonicus*) was obtained at pH 6.5 (Benjakul et al. 2005). Nevertheless, partially purified carapace PPO from deepwater pink shrimp (*P. longirostris*) showed a maximum activity at pH 4.5 (Zamorano et al. 2009). PPO from the cephalothorax of white shrimp showed the maximum activity at pH 7.5 (Simpson et al. 1987). The optimal pH of PPO for different crustaceans varied with the species and anatomical location (Benjakul et al. 2005). PPO activity was markedly decreased in either acidic or alkaline pH range. At extreme acidic or alkaline pH conditions, unfolding of enzymes might occur due to disruption of electrostatic bonds stabilising enzyme molecules, thereby making PPO inactive.

DEAE-Sephacel fraction containing PPO from the cephalothorax of Pacific white shrimp had an optimal temperature of 55°C (Figure 1B). Montero et al. (2001b) reported that PPO from the carapace of imperial tiger prawn (*P. japonicus*) cultured in Spain showed a maximum activity at 55°C. However, PPO from the cephalothorax of kuruma prawn cultured in Japan showed a maximum activity at 35°C (Benjakul et al. 2005). PPO activity from Norway lobster (Gimenez et al. 2010) and deep water pink shrimp (Zamorano et al. 2009) continuously increased up to 60°C. The maximum activity was noticeable at 40°C to 45°C for PPO extracted from different shrimps including pink shrimp (Simpson et al. 1988), Taiwanese black tiger shrimp (Rolle et al. 1991) and white shrimp (Simpson et al. 1987). The variation in optimal temperature of different crustaceans was most probably related to their habitat temperature. Further increase in temperature above 55°C resulted in the decrease in PPO activity, more likely due to the thermal denaturation of PPO.

pH and thermal stability of PPO from the cephalothorax of Pacific white shrimp

The pH stability study of PPO in DEAE-Sephacel fraction revealed that PPO from the cephalothorax of Pacific white shrimp was stable over a pH range of 5 to 10 with a remaining activity of more than 80% (Figure 2A). PPO was found to be more stable in a neutral to alkaline pH range. However, PPO was unstable at a pH below 5. Remaining

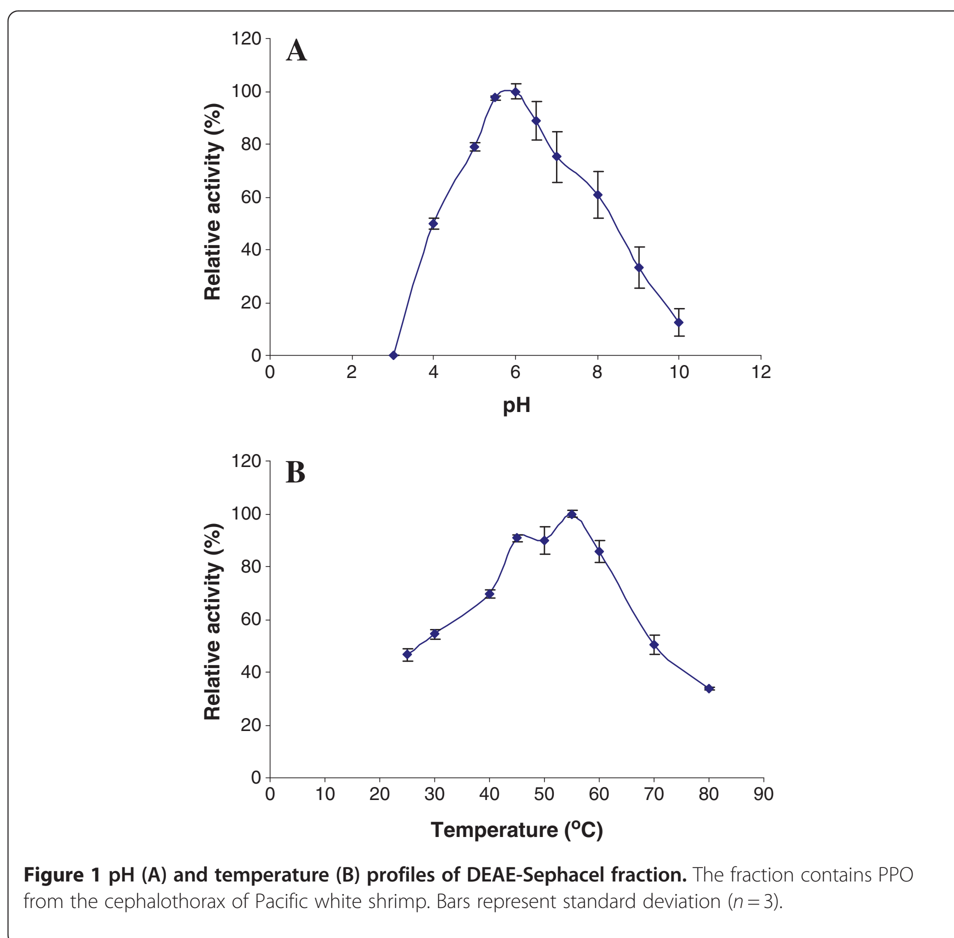

Figure 1 pH (A) and temperature (B) profiles of DEAE-Sephacel fraction. The fraction contains PPO from the cephalothorax of Pacific white shrimp. Bars represent standard deviation ($n = 3$).

activities of 43% and 25% were observed at pH 4 and 3, respectively. PPO from kuruma prawn was reported to be stable over a pH range of 3 to 10 (Benjakul et al. 2005). Nevertheless, PPO from pink shrimp (Simpson et al. 1988) and deep water pink shrimp (Zamorano et al. 2009) had high stability between pH 6 to 12 and pH 4.5 to 9.0, respectively. PPO from imperial tiger prawn (Montero et al. 2001a) and white shrimp (Simpson et al. 1987) were unstable below pH 5. PPO from the carapace and the viscera of Norway lobster was stable in a neutral to alkaline pH range (Gimenez et al. 2010). Stability of Pacific white shrimp PPO at alkaline pH suggested that the conformation of PPO active site was not affected.

Thermal stability of DEAE-Sephacel fraction containing PPO from the cephalothorax of Pacific white shrimp is presented in Figure 2B. PPO was stable up to 60°C for 30 min with more than 70% remaining activity. The result suggested that temperature below 60°C did not affect the three-dimensional structure of PPO. At temperature above 60°C, a sharp decrease in PPO activity was observed. PPO more likely underwent thermal denaturation at high temperatures. PPO of white shrimp (Simpson et al. 1987) and kuruma prawn (Benjakul et al. 2005) had the stability up to 50°C. PPO of pink shrimp (Simpson et al. 1988), Taiwanese black tiger shrimp (Rolle et al. 1991) and deepwater pink shrimp (Zamorano et al. 2009) were unstable at temperatures over 30°C to 35°C. PPO from Pacific white shrimp cultured in Thailand, a tropical country, was more stable than that from shrimp from temperate water.

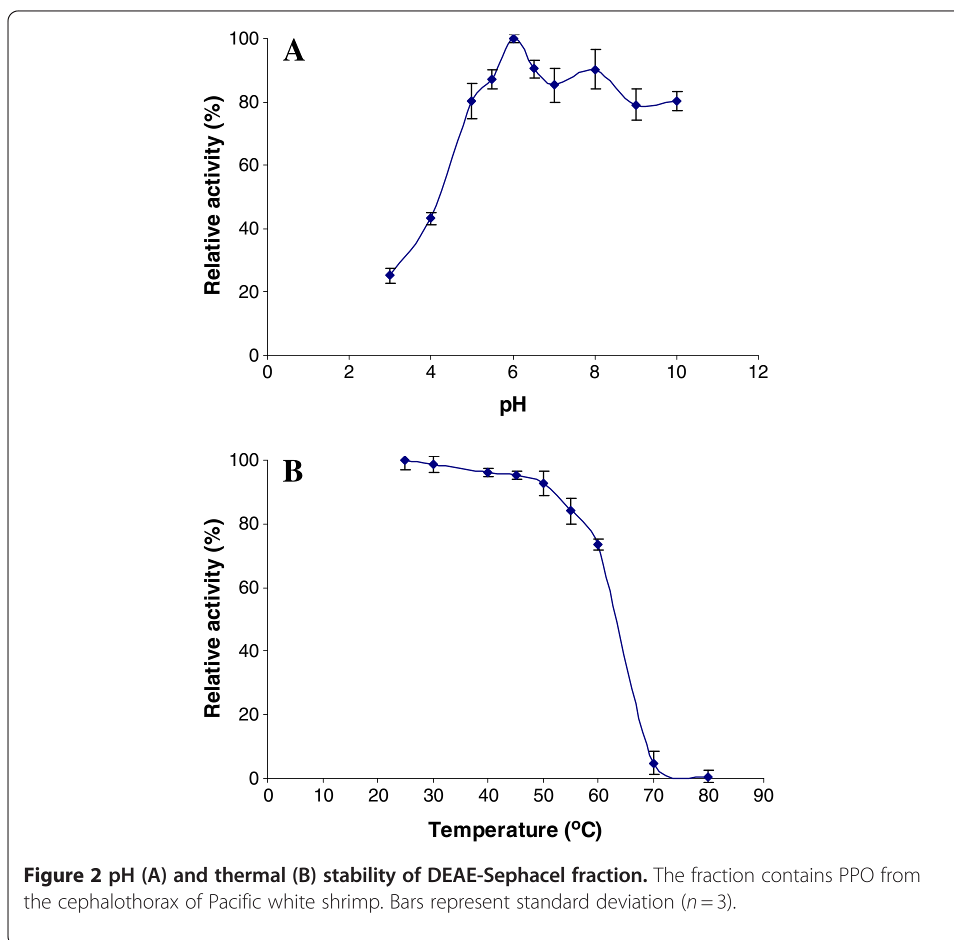

Figure 2 pH (A) and thermal (B) stability of DEAE-Sephacel fraction. The fraction contains PPO from the cephalothorax of Pacific white shrimp. Bars represent standard deviation ($n = 3$).

Kinetics and molecular weight of PPO from the cephalothorax of Pacific white shrimp

The K_m for the oxidation of L-DOPA by PPO in DEAE-Sephacel fraction was 2.43 mM, which was similar to the K_m for the oxidation of DL-DOPA by PPO from white shrimp (K_m 2.8 mM) (Simpson et al. 1988). K_m values reflect the affinity of enzymes to their substrates. K_m values of 1.6 and 1.85 mM were obtained using DL-DOPA as a substrate for PPO from pink shrimp (Simpson et al. 1988) and deep water pink shrimp (Zamorano et al. 2009), respectively. A lower K_m value indicates a higher catalytic efficiency of the enzyme towards its substrate. The K_m values for the oxidation of L-DOPA by white shrimp (Chen et al. 1991b) and kuruma prawn (Benjakul et al. 2006) PPO were 3.48 and 0.26 mM, respectively. PPO from the viscera of Norway lobster showed a higher catalytic affinity for catechol than PPO from the carapace with apparent K_m values of 5.97 and 19.40 mM, respectively (Gimenez et al. 2010). V_{max} for the oxidation of L-DOPA by PPO in DEAE-Sephacel fraction was 61×10^3 U/mg PPO. Simpson et al. (1988) reported that V_{max} for PPO from the cephalothorax of pink and white shrimp was 5.6×10^3 and 3.1×10^3 U/mg PPO, respectively. The result suggested that Pacific white shrimp PPO had higher reaction rate of L-DOPA oxidation than pink and white shrimp PPO. The differences in K_m and V_{max} of PPO from different species were plausibly owing to the differences in molting stage, method of capture, handling and storage conditions (Rolle et al. 1991).

The activity staining of DEAE-Sephacel fraction containing PPO from the cephalothorax of Pacific white shrimp is illustrated in Figure 3. Based on activity staining, the apparent

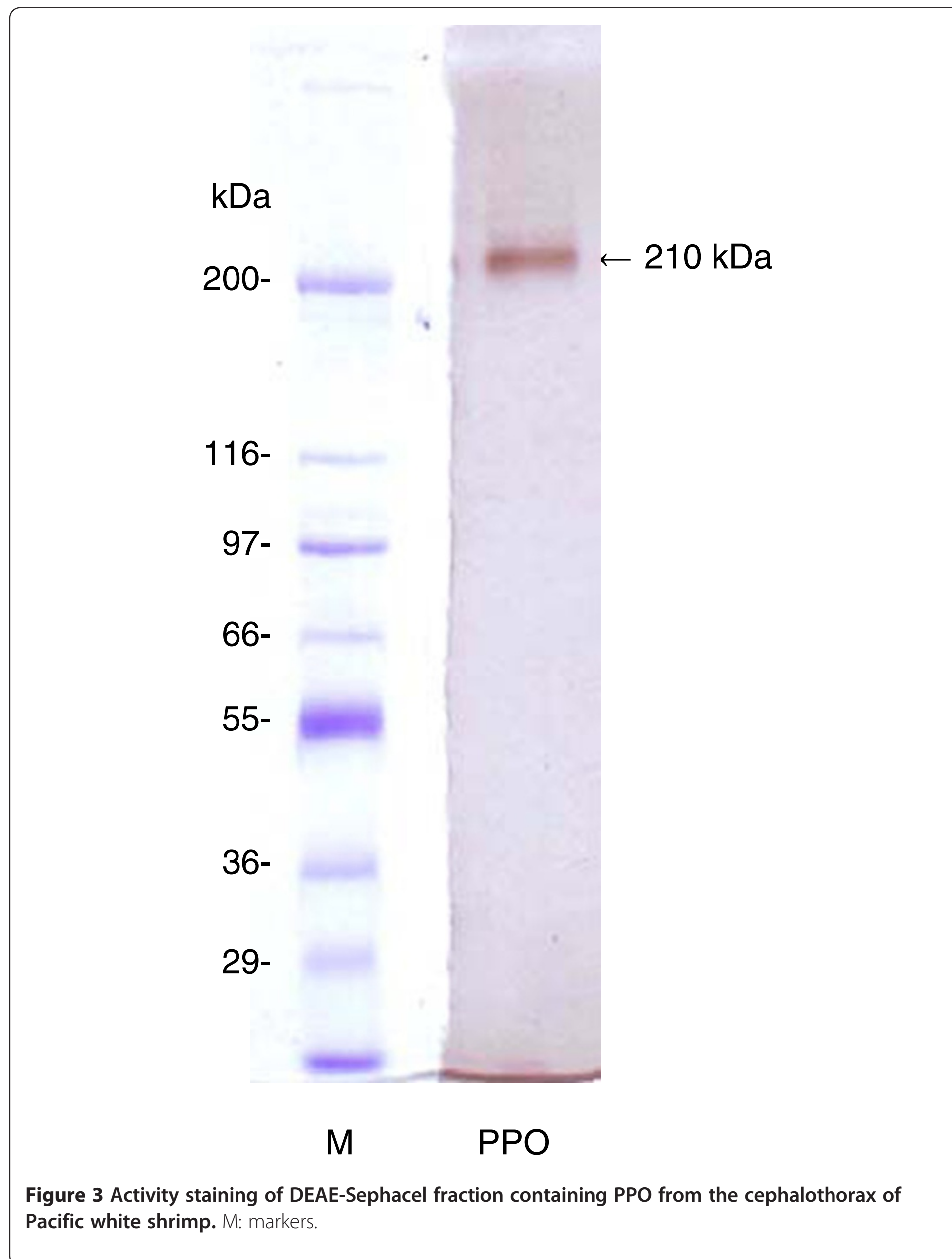

Figure 3 Activity staining of DEAE-Sephacel fraction containing PPO from the cephalothorax of Pacific white shrimp. M: markers.

molecular weight of PPO was 210 kDa. This result reconfirmed our previous report (Nirmal and Benjakul 2009a), where PPO from the crude extract of Pacific white shrimp cephalothorax showed a molecular weight of 210 kDa. PPO from the viscera and carapace extracts of the cephalothorax of Norway lobster had an apparent molecular weight of 200 to 220 kDa as determined by activity staining using L-tyrosine and 4-tert-butyl-catechol as substrates (Gimenez et al. 2010). Zamorano et al. (2009) studied the electrophoretic mobility of PPO from deepwater pink shrimp using nonreducing SDS-PAGE, followed by staining with DOPA, and found the activity band with a molecular weight of 200 kDa. From activity staining, PPO from the kuruma prawn cephalothorax showed an activity band with a molecular weight of 160 kDa (Benjakul et al. 2005). PPO from different species of crustaceans comprised the different isoforms with varying molecular weights (Chen et al. 1991a).

Table 2 Effect of trypsin and copper acetate at different concentrations on PPO relative activity in DEAE-Sephacel fraction

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

Values are means ± SD (n=3). Different letters in the same column indicate a significant difference ($P < 0.05$). PPO, polyphenoloxidase.

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

The effect of trypsin and copper acetate at different concentrations on PPO in DEAE-Sephacel fraction is shown in Table 2. Increasing concentration of trypsin up to 80 μg/mL had no impact on PPO activity ($P > 0.05$). This result was in agreement with that of Simpson et al. (1987), Benjakul et al. (2005) and Zamorano et al. (2009) who found that trypsin had no effect on PPO from white shrimp, kuruma prawn and deepwater pink shrimp, respectively. However, a slight increase in PPO activity was observed in the presence of trypsin at a concentration of 100 μg/mL. Trypsin had a slight effect on the conversion of hemocyanin (Hc) of white leg shrimp (*Penaeus vannamei*) to HcPPO (Garcia-Carreno et al. 2008). Opoku-Gyamfua et al. (1992) reported that PPO activity from lobster increased with increasing concentration of trypsin of up to 20 μg/mL. Trypsin could activate proPPO to PPO by cleaving propeptide from proPPO, thereby enhancing its activity (Benjakul et al. 2005). However, PPO in DEAE-Sephacel fraction more likely existed in an active form.

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

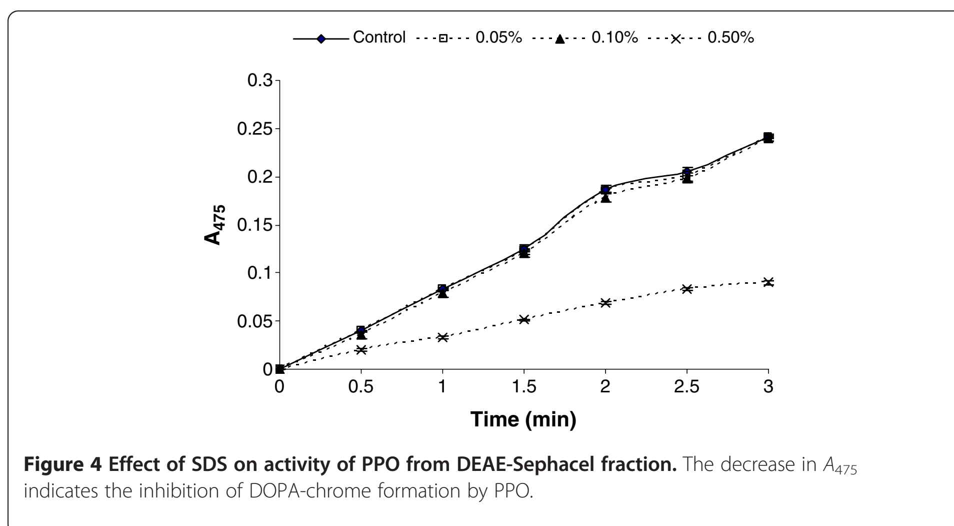

Figure 4 Effect of SDS on activity of PPO from DEAE-Sephacel fraction. The decrease in A_{475} indicates the inhibition of DOPA-chrome formation by PPO.

The effect of SDS at different concentrations on PPO in DEAE-Sephacel fraction is presented in Figure 4. SDS at 0.05% and 0.1% had no influence on PPO activity. However, SDS at a concentration of 0.5% showed an inhibitory effect on PPO from Pacific white shrimp. The results are supported by Adachi et al. (1999) who observed the inactivation of PPO from the hemocyte of kuruma prawn (*P. japonicus*) by SDS. Nevertheless, Garcia-Carreno et al. (2008) reported that Hc from white leg shrimp was converted to HcPPO by SDS treatment. PPO from the cephalothorax of kuruma prawn was not affected by 0.05% SDS (Benjakul et al. 2005). These results reconfirm that PPO in DEAE-Sephacel fraction more likely existed in an active state, which was not affected by SDS of up to 0.1%. However, a concentration of 0.5% SDS might disturb the hydrophobic interaction in PPO, thereby lowering PPO activity.

Table 3 shows the effect of cysteine, EDTA and PABA on PPO in DEAE-Sephacel fraction. In general, increasing concentration of cysteine, EDTA and PABA resulted in the increase in PPO inhibition ($P < 0.05$). Cysteine at the concentration used showed a higher inhibitory effect towards PPO, compared with EDTA and PABA. At the concentration of 10 mM, cysteine totally inhibited PPO activity, whereas EDTA and PABA showed 22.41% and 58.60% inhibition towards PPO, respectively. The results were in accordance with Opoku-Gyamfua et al. (1992) who reported that cysteine, PABA and EDTA inhibited PPO from lobster. However, oxidation of DOPA by pink and white shrimp PPO was not inhibited by cysteine (Simpson et al. 1988). The inhibition of PPO activity by EDTA might relate to its metal-chelating capability, thus making Cu^{2+} unavailable at the active site of the enzyme. PABA could compete with DOPA in binding

Table 3 Effect of cysteine, EDTA and PABA at different concentrations on PPO relative activity (%) in DEAE-Sephacel fraction

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

Values are means ± SD (n=3). Different letters in the same row indicate a significant difference ($P < 0.05$). EDTA, ethylenediaminetetraacetic acid; PABA, *p*-aminobenzoic acid.

to the active site of PPO. Cysteine containing sulphur group might be involved in the PPO inhibition. Cysteine exhibited a competitive type of inhibition on PPO from kuruma prawn (Benjakul et al. 2006) and mulberry (Arslan et al. 2004). Thiol reagents might interact with copper at the active site of PPO, leading to the loss of the activity (Benjakul et al. 2006). Cysteine can react with *o*-quinones by forming cysteinyl adduct, a colourless compound (Richard-Forget et al. 1992). Therefore, oxidation of L-DOPA by Pacific white shrimp PPO was inhibited by cysteine due to the various inhibitory mechanisms of cysteine towards PPO. Recently, catechin and ferulic acid were shown to inhibit PPO from Pacific white shrimp via different inhibition kinetics (Nirmal and Benjakul 2012).

Conclusions

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

Abbreviations

AS: Ammonium sulphate; DEAE: Diethylaminoethyl; EDTA: Ethylenediaminetetraacetic acid; Hc: Hemocyanin; Km: Michaelis constant; L-DOPA: L-β-(3, 4 dihydroxylphenyl) alanine; PABA: p-aminobenzoic acid; PAGE: Polyacrylamide gel electrophoresis; PMSF: Phenilmethanesulfonyl fluoride; PPO: Polyphenoloxidase; SDS: Sodium dodecyl sulphate; Vmax: Maximum reaction velocity.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NPN participated in the design of the experiment, carried out all the experiments mentioned in the manuscript, performed the statistical analysis and drafted the manuscript. SB conceived the study, participated in its design and coordination and helped draft the manuscript. Both authors read and approved the final manuscript.

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