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Partial purification of polyphenoloxidase of black tiger shrimp *(Penaeus monodon)* from Indonesian waters

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Abstract

Shrimps are a very important resource all over the world because of their high market value. Their post-mortem discoloration (blackspot) is one of the serious problems occuring in crustaceans and become an issue for the industry. Biochemical properties analysis of PPO from black tiger shrimp had been conducted, but information of partial purification techniques of the PPO of the shrimp from Indonesian waters had not yet been reported. The study aimed is to partial purifying the enzyme polyphenoloxidase (PPO) from black tiger shrimp *(Penaeus monodon)* responsible for blackspots discoloration. Extraction, precipitation with ammonium sulphates, partial purification, and determination of molecular weight were conducted. Precipitation showed best concentration to obtain the highest specific activity was using ammonium sulfate at 70% saturation. Four hours was the best dialysis periode. Polyphenoloxidase from carapace of black tiger shrimp was purified to 1.64-fold with Sephadex G-150 gel filtration chromatography. Activity staining of the PPO showed that the enzyme consisted of two isoform with the apparent molecular weight of 258.29 kDa and 222.05 kDa.

Introduction

Shrimp is a very important fisheries resource all over the world because of high market value and nutrition value. However, shrimp is a highly perishable product with limited shelf life due to melanosis and biological composition. Blackspot formation (melanosis) is one of the serious problems occuring in crustaceans during pot-mortem handling and storage. Melanosis in shrimp drastically reduces the consumer acceptability and the product's market value leading to considerable financial loss (Kim *et al.*, 2000). PPO easily catalyzes phenolic substances and polymerize to form insoluble melanin that are not attractive to consumers (Begona *et al.*, 2010).

Blackspot is a natural post-mortem process originated by the polymerization of phenols into insoluble black pigments, the melanins. Melanosis formation correlates with post mortem tissue degradation caused by protease enzymes associated with activation of the enzyme PPO (Wang *et al.*, 2011). 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, EC1.10.3.1) (Garcia-Molina *et al.*, 2005).

Melanosis is a process that is triggered by a biochemical mechanism oxidation of phenol into quinone by a complex enzyme called PPO. This process is followed by polymerase non-enzymatic quinone compounds, producing a high molecular weight and very dark pigment (Montero *et al.*, 2001). The phenoloxidase (PO) have an important role in the process of melanosis and have an inactive form called ProPO (Hellio *et al.*, 2007). ProPO activated by trypsin and zymozan A (Cardenas and Dankert, 1997) and rPPO1-GFP is cleaved and activated by endogenous serine protease (Yang *et al.*, 2013).

PPO is an essential enzyme for shrimp, especially for the formation of a new shell. PPO plays a role in self-defense mechanism and also important in healing. Post mortem PPO becomes radical, thus handling becomes very important to reduce PPO activity (Perdomo-Moraleset *et al.*, 2007). PPO are abundant in shrimp shells. PPO is most commonly found in the cephalothorax of prawn and shrimp (Montero *et al.*, 2001). PPO in crustaceans is distributed mainly in the carapace, followed by the abdominal exoskeleton, cephalothorax, pleopods and telson (Zamorano *et al.*, 2009). High PPO activities in deep water pink

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shrimp were monitored in the extracts of carapace, cephalothorax, abdomen exoskeleton and pleopods, and lower activity was shown by the extract from the uropods to telson (Çaklı *et al*., 2013).

PPO from the cephalothoraxes of various crustaceans and clam such as *Peneaus japonicus* (Benjakul *et al*., 2005), *Ruditapes philippinarum* (Cong *et al.*, 2005), *Artemia sinica* (Fan *et al*., 2011), *Nephrops norvegicus* (Gimenez *et al*., 2010), and Pacific white shrimp (Nirmal and Benjakul, 2012) had been purified and characterized.

Black tiger shrimp is a major commercial commodity in Indonesia. Black spot is also a quality issues that related directly to commercial value of the shrimp. Suhandana *et al.* (2013) managed to isolate and characterize PPO enzyme of black tiger shrimp originated from Indonesian water. PPO were multi-stage extracted using buffer ratio of 1:3. with the following characters: optimum pH and temperature were 7 and 35°C respectively. $K_{m\ app}$ values were 1.24 mM and Vapp 23.26 U. Na^{+}, Ca^{2+}, Zn^{2+}, and EDTA (5 and 10 mM) inhibits PPO activity. Purification techniques of PPO enzyme isolated from tiger shrimp from Indonesian waters has not been reported. Therefore, the aimed of study is to partial purify the PPO enzyme from tiger shrimp *(Penaeus monodon)*.

Materials and Methods

Sampling of shrimp tissue

Black tiger shrimp *(Penaeus monodon)* with a size of 55 to 60 shrimps/kg were purchased from supplier in Bogor, Indonesia. The shrimp were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Aquatic Product Technology, Bogor Agricultural University within 1 h. Upon arrival, the shrimp were washed in cold water. The muscle, carapace, and abdomen exoskeleton of the shrimp were separated, pooled, and powdered by grinding with liquid nitrogen in a warring blender (Phillips).

Extraction of PPO from muscle, carapace, and abdomen exoskeleton of black tiger shrimp

The extraction of PPO from the powdered muscle, carapace, and abdomen exoskeleton of black tiger shrimp was carried out according to the method of Simpson *et al.* (1987) with a modification. Samples (50 g) then mixed with 150 mL of buffer (0.05 M sodium phosphate buffer, pH 7.2; containing 1.0 M NaCl and 0.2% Brij 35 mixture) (Merck) was stirred continuously at 4°C for 30 minutes, followed by centrifugation (8000 x g) at 4°C for 30 minutes using a refrigerated centrifuge (Sorvall). Supernatant obtained as a crude extract.

Partial purification of PPO from carapace of black tiger shrimp

Solid ammonium sulphates (technical) was added to the supernatant to obtain 30-80% saturation and allowed to stand at 4°C for 30 minutes. The precipitates were collected by centrifugation (12500 x g) at 4°C for 30 minutes. The pellets were dissolved in minimum volume of extracting buffer (0.05 M sodium phosphate buffer at pH 7.2) (Merck) and was dialyzed (MWCO 12 kDa, Sigma) with 50 times the volume of the buffer 0.01 M for 4 and 8 hours. Ammonium sulphates fraction was applied onto Sephadex G-150 (Sigma) column (1×100 cm), previously equilibrated with 0.05 M sodium phosphate buffer at pH 7.2 (Sigma). PPO was eluted with in 0.05 M phosphate buffer (pH 7.2) (Sigma) at a flow rate of 0.3 mL/min. Fraction of 3.0 mL was collected. The gel filtration was monitored at 280 nm with UV spektofotometer (CE 292 Series 2) and the eluted fractions with the PPO activity were pooled. The protein concentration fraction was determined according to the method of Bradford (1976).

Determination of molecular weight and activity of PPO from carapace of black tiger shrimp using electrophoresis

Crude extract, dialysis results, and Sephadex-G150 fraction protein containing PPO from the carapace of black tiger shrimp was to estimated of molecular weight as the method of Laemmli (1970). Crude extract and dialysate were determined activity staining as the method of Benjakul *et al*. (2005). The fraction was mixed with the sample buffer containing SDS (Merck) at a ratio of 1:1 (v/v). The sample (20 µg protein) was loaded onto the polyacrylamide gel made of 8% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 50 mA/gel and 120 V 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 phosphate buffer pH 6,5 (Merck) containing 15 mM L-DOPA (Sigma) for 15 hours at 25°C. The activity zone appeared as a brown band. Another gel was stained with 0.1% Coomassie Brilliant Blue R-250 and destained in 25% methanol and 12% 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

Table 1. Summary of the purification of PPO on Sephadex G-150 gel filtration colomn chromatography

Purification step	Volume (mL)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	150	47.73	1125.00	23.57	100.00	1.00
Precipitation	20	8.34	250.00	29.97	22.22	1.27
Dialysis	10	3.14	91.00	28.99	8.09	1.23
Gel Filtration	3	0.77	30.00	38.76	2.67	1.64

from rabbit muscle (36 kDa) and carbonic anhydrase from bovine erythrocytes (29 kDa) (Fermentas) were used.

Measurement of PPO activity

PPO activity was measured according to the method of Bono *et al.* (2010). A 0.2 mL aliquot of supernatant was added to 2.8 ml of 0.01 M L- DOPA solutions in 0.05 M phosphate buffer (pH 6.5). The PPO activity was determined for 5 min at 35°C by monitoring the formation of dopachrome at 475 nm using a spectro UV-VIS RS Spectrophotometer UV-2500. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture and using the deionised water instead. One unit of PPO activity was defined as an increase in the absorbance at 475 nm by 0.001/min at pH 6.5 at 35°C in a 3 mL reaction mix containing L-DOPA. Specific activity (U/mg) was U per mg protein the extract.

Results

Enzim PPO extracted using sodium phosphate buffer containing NaCl and Brij 35. Sample were homogenated, added extraction buffer and centrifugated. PPO sources were meat, shells and abdominal exoskeletons. Carapace showed highest activity (1.2 U), followed by abdominal exoskeleton (0.933 U), and meat (0.533 U). Partial purification extractions by iterative methods were conducted to increase the yield of crude extract (triplicates, sample and extraction buffer ratio 1:1) (Suhandana *et al.*, 2013).

Enzyme could be purified by separating from other non-enzyme proteins. Precipitation was carried out using ammonium sulphates salt. Precipitation saturation levels were 30-80%. Enzyme activity showed in relative activity by absorbance at a certain saturation level compared to the maximum enzyme activity at a particular saturation (Figure 1). Precipitation with ammonium sulfate result showed three separate parts which is pellet, supernatant and the clotting part that coagulates called as other fraction. The pellet and the other fraction that were precipitated using 70% saturation have high activity.

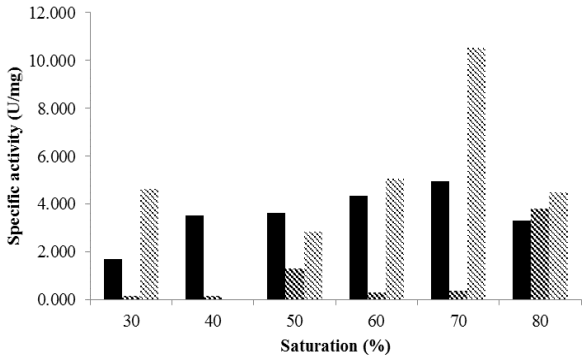

Figure 1. Precipitation of the crude extract PPO from carapace of black tiger shrimp using ammonium sulfate in various saturation; ■ pelet ▧ supernatant ▨ other fraction

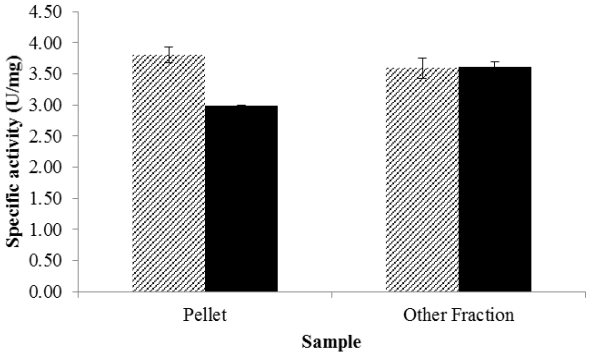

Figure 2. Specific activity of PPO from carapace of black tiger shrimp after dialysis for ▨ 4 hour ■ 8 hour

After ammonium sulphates fractionation, 1.27-fold purity was achieved (Table 1).

The ammonium sulphates fraction was future dialyzed (MWCO 12 kDa) to remove the salt on pellets. Dialysis was performed using dialysis bag and immersed in sodium phosphate buffer. Dialysis performed for 4 and 8 hours. The results showed that a decrease in dialysis-specific activity in the pellet after dialyzed for 8 hours, while the other faction specific activity increased after dialyzed for 8 hours (Figure 2). After dialysis process, 1.23-fold purity was achieved (Table 1). Dialysate from pellet and other fraction were mixed and then was purified by Sephadex G-150. The results showed that fraction 6 had the highest activity and purity of 1.64 fold (Table 1). Fraction 5-9 was subsequently used to analysis estimation of the molecular weight of the PPO (Figure 3).

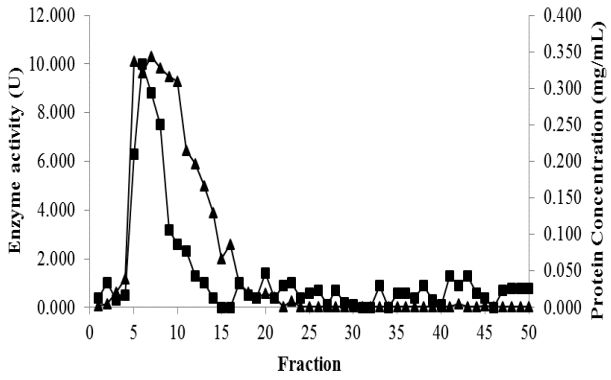

Figure 3. Fractions activity and protein concentration of PPO from carapace of black tiger shrimp after Sephadex G-150 gel filtration chromatography

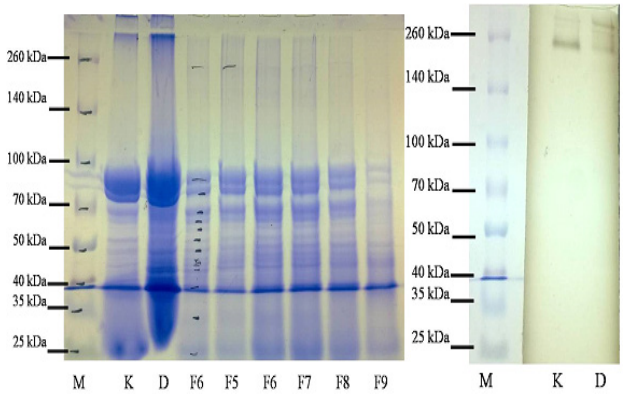

Figure 4. Protein pattern (A) and activity staining (B) of PPO from carapace of black tiger shrimp (M : marker, K : crude extract, D : dialysate, F5-F9 : fraction 5-9 of Sephadex G-150 gel filtration chromatography

Crude extract, ammonium sulphates fraction, and dialysate had several molecular weight. Purified by gel filtration chromatography also had several fractions with high molecular weight (> 200 kDa) to low molecular weight (<60 kDa) (Figure 4). The results showed that purification had not been optimum. The activity staining of crude extract and dialysate containing PPO from the carapace black tiger shrimp is illustrated in Figure 4. Based on activity staining, the apparent molecular weight of PPO were 258.29 and 222.05 kDa, while that purified fraction 5-9 of PPO show no apparent band with high molecular weight in SDS PAGE, thus no activity enzyme in zymogram (data not shown). Presumably because the PPO enzyme had been damaged due to the instability of the enzyme.

Discussion

Polyphenoloxidase (PPO) isolated from black shrimp tiger *(Penaeus monodon)* had the highest activity on carapace. PPO were also found on other shrimp *(Penaeus japonicus)* carapace and cuticle located at pleuron ventral next to pleopods, but with a lower activity (Montero *et al.*, 2001). Highest PPO activity found in the carapace of deepwater pink shrimp *(Parapenaeus longirostris)*, followed by abdominal exoskeleton, cephalothorax, pleopods, and telson (Zamorano *et al*., 2009). The spread of the melanosis (browning) at deep water pink shrimp was observed between the parts head and pereopods in male samples and activity of PPO different based on fishing season (Çaklı *et al*., 2013), sex, and age (Bono *et al.*, 2010). Meat had a lower PPO activity than the carapace and abdominal exoskeleton. PPO activity contained in meat suspected to be caused by turbidity in the sample. Zamorano *et al*. (2009) stated that PPO activity in the meat due to the turbidity of the sample. Partial purification lowered PPO activity in meat. The method to extract PPO is iterative methods (3 times) with the ratio sample and buffer is 1: 1 (Suhandana *et al.*, 2013). Stirring of pink and white shrimp powder in extraction buffer from 30 min to 3 h significantly ($P<0.05$) enhanced protein extraction. However, the extract showed significant decreases in total PPO activity and specific activity with increased extraction time. Therefore, a 30-min stirring time was chosen to extract PPO (Chen *et al*., 1997).

Ammonium sulphates precipitation had their advantages, namely high solubility and generally doesn't affect the structure of the protein, and dispose of non-enzyme proteins. Precipitation with ammonium sulphates saturation levels were species dependent because PPO biochemical characteristics of each species were different. Deepwater pink shrimp PPO best activity showed when precipitated using ammonium sulphates with 40-70% saturation (Zamorano *et al.,* 2009). The highest PPO activity was the use of ammonium sulphates with 0-40% saturation. Precipitated PPO relative activity increase up to seven times larger than crude extract. PPO precipitated with 40-70% saturation showed decreased relative activity by 5-10% compared to the 0-40% saturation levels (Montero *et al*., 2001). The specific activity of purified AsPO increased almost 53.87-folds compared with the crude AsPO, with an overall recovery of 46.34% of the initial activity (Fan *et al*., 2011).

PPO precipitation with ammonium sulphates activities were not yet optimal. Pigments that were bound to protein were still present in the sediment. The specific activity of the enzyme that was precipitated using ammonium sulphates was lower compared to the ones using butanol. Astaxanthine present in the mixture caused elution blockade from PPO. The pigment hinders elution of PPO. Butanol could separate pigments with proteins and

lipids. Protein precipitation with butanol results will be dissolved in the aqueous fraction (Chen *et al*., 1997). Therefore, pigments and lipids must be removed from the extract to obtain a high activity. Repeated washing and centrifugation at high speed could increase specific activity and remove lipids, pigments, and unwanted proteins (Rolle *et al.*, 1991).

Precipitation with ammonium sulphates followed by dialysis. Dialysis aims to eliminate existing salt due to amonium sulphate precipitation. Unfortunately the dialysis process could decrease the activity of PPO. Decrease in enzyme activity after dialysis occurs because the enzyme PPO was unstable. PO and its precursor (Propo) was not stable and suffered loss of activity when stored for a week. However, unlike the case with hemocyanin which had similarities with the PO, this pigment was more stable than the PO (Adachi *et al.*, 2004). Decline in PPO activity could also caused by improper dialysis time. The process of dialysis was usually done for a few hours until one night. Dialysis causes loss of activity of PPO because it was done for at least 8 hours. Therefore desalting column is the right solution because the process was faster (5-10 min) (Montero *et al*., 2001).

Purification using Sephadex G-150 increases purification by 1.64. The purification multiplications are lower than in other studies. It was caused by a single-stage purification performed, namely gel filtration which only based on the principle of separation by molecular weight. PPO from cephalothorax of Pacific white shrimp was purified to 83.8-fold with DEAE Sephacel anion exchange chromatography with a yield of 73.4% was recovered (Nirmal and Benjakul, 2012). PPO from *Ruditapes philippinarum* was purified to 62.5 fold with Q Sepharose FF which was followed by Sephacryl S-100 (Cong *et al*., 2005). PPO from *Artemia sinica* was purified to 18.73 times with Superdex 200 which was followed by Q Sepharose FF and to 53.87 fold when purified again with Superdex 200 (Fan *et al.*, 2011). PPO from *Branchiostoma belcheri* was purified to 60 fold with Sephadex G-200 which was followed by DEAE FF (Pang *et al*., 2005). PPO from the hemocytes of *Eurygaster integriceps* was purified to 7.31 fold with Sephadex G-100 which was followed by CM-Sepharose (Zibae*e et al*., 2011). This result suggested that purification with gel filtration (Sephadex G-150) could not be done one in step, but should be followed by other type of chromatography colomn.

PPO molecular weight determined by SDS PAGE electrophoresis. The results showed that there was still a lot of bands with high (>200 kDa) and low (<60 kDa) molecular weight. indicating that the enzyme was not pure. Activity staining of PPO from the carapace black tiger shrimp showed that the PPO consisted of two isoform with the apparent molecular weight of 258.29 and 222.05 kDa. PPO from carapace of Pacific white shrimp consisted of two isoform (210 and 220 kDa) (Manheem *et al.*, 2012), while that PPO from chepalothorax of Pacific white shrimp was only one activity band with molecular weight of 210 kDa (Nirmal and Benjakul, 2011; Manheem *et al.*, 2012). PPO was able to induce the oxidation of DOPA to DOPA-quinone and the intermediate products, which subsequently underwent polymerization to melanin. Molecular weight of PPO from the Pacific white was possibly due to the enhanced PPO activity by proteases, which were active at that temperature (Nirmal and Benjakul, 2010).

Several studies had been conducted to determine the molecular weight of the enzyme PPO. The enzyme activity from cephalothorax kuruma prawn was had a molecular weight of 160 kDa (Benjakul *et al*., 2005). Molecular weight PO clam *(Ruditapes philippinarum)* of 76.9 kDa (Cong *et al*., 2005). PO molecular weight of 125.5 kDa *Artemia sinica* (Fan *et al*., 2011). PPO enzyme from black tiger shrimp taiwanese *(Penaeus monodon)* had a molecular weight of 63 and 80 kDa (Rolle *et al*., 1991). PO obtained from hemocyanin *Charybdis japonica* had a molecular weight of 80, 75, and 70 kDa (Fan *et al.*, 2009). Deepwater pink shrimp *(Parapenaeus longirostris)* had the PPO enzyme with a molecular weight of 500 and 200 kDa (Zamorano *et al*., 2009). Zang *et al.* (2011) was found PPO from Pacific white shrimp had a molecular weight of 170 kDa.

Conclusions

Highest specific activity was obtained by precipitation using 70% ammonium sulphates and dialysis time of 4 hours. Purification was performed using gel filtration chromatography with Sephadex G-150. Determination of molecular weights get two bands with a molecular weight of 258.29 and 222.05 kDa kDa. The spesific activity of PPO after partial purification using Sephadex G-150 was 1.64 times higher than crude extract.

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