Research Article

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POLYPHENOL OXIDASE: CHARACTERIZATION, ENZYME ACTIVITY AND ANTIBACTERIAL ACTIVITY AGAINST BACTERIAL PATHOGENS

Shimna. AK [1]* and Thangavel [2]

[1]Research Scholar, PG and Research Department of Microbiology, Sree Narayana Guru College, Coimbatore, India

[2]Head, Associate Professor, PG and Research Department of Microbiology, Sree Narayana Guru College, Coimbatore, India

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***Corresponding author**
E-mail: ayshashimna@gmail.com

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ABSTRACT

The antibacterial action of shrimp extracts against the pathogenic microorganisms was selected as the primary objective of the present research. In this research the protein profiles of polyphenol oxidase (PPO) and its enzyme activity was investigated. Enzyme activity of PPO was reported in the present research under optimized parameters. To determine the optimized condition for maximum enzyme activity of PPO, a design experiment illustrating Box-Behnken method was used in the research work. SDS-PAGE electrophoretic patterns crude and partly purified PPO extracts of carapace, cephalothorax and muscle were presented. Two separate bands were obtained for crude and purified PPOs. Crude showed 64KDa and 52KDa protein bands; and purified PPOs showed 64KDa and 48KDa. The Specific enzyme activity of PPO enzyme was measured at different temperature (33, 35 and 37°C), pH (5-7) and Time (5, 10 and 15 min) by using DL-DOPA as a substrate. Maximum enzyme activity of 28.98 U/mg of protein was obtained for the reaction mixture analysed at pH 6 at 35ºC in 10 mins. The antibacterial activity of shrimp PPO extracts against the test organism was presented. Maximum inhibitory zone of 23mm were obtained against a significant pyogenic organism (MRSA). Another important Gram-Negative pathogen *Escherichia coli* exhibited 21mm of inhibitory zones for the enzyme extracts. *Pseudomonas aeruginosa* (MDR) and *Enterococcus fecalis* (ESBL) exhibited 19mm of inhibitory zones and *Klebsiella pneumoniae* showed 18mm of inhibitory zones. The mode of action of antibacterial peptide on the cell membrane of organisms has displayed the promising antibacterial potential of marine metabolites.

Keywords: Polyphenol oxidase, Pacific white shrimp, enzyme activity, antimicrobial peptides, inhibitory zones

INTRODUCTION

Polyphenoloxidase (PPO) is most commonly found in the cephalothorax of prawn and shrimp[1]. Polyphenoloxidase (PPO) is a copper-containing metalloenzyme catalyses reaction in the presence of molecular oxygen[2]. PPO in Pacific White shrimps is present in the cephalothorax, carapace, pleopods and telson[3]. PPO is also known as zymogen (pro PPO) activated by a proteinase cascade induced by the compounds like carbohydrates and lipopolysaccharides and other proteins[4]. Zymogen in the cascade system plays a significant role in the primary immune response. It involves in the cuticle sclerotization and healing injuries in the crustaceans. During the post-mortem storage of crustaceans, zymogen will be converted and further activated into PPO by proteolytic actions. The enzyme activation was regulated after leaching from the digestive tract[5]. In this study different tissue of Pacific White shrimp was selected as the source to extract the enzyme PPO. It was reported that minimizing the action of PPOs reduces the melanosis in shrimp and other crustaceans. Melanosis is commonly known "black spot" formation in the carapace and cephalothorax of crustaceans during post-harvest storage[6]. It was earlier reported that melanosis causes no direct harm to consumers, but reduces the quality, shelf life, and commercial value of crustaceans[7]. Hence, different preservation methods were reported to prevent melanosis. Different methods like slurry ice treatment with an anti-melanostic agents[8], precooking[9] and modified atmosphere packaging[10] was used. Minimization of PPO was regulated by optimizing the pH, temperature and by using inhibitors to maintain the quality of shrimp during handling, processing and storage. During this condition, the action of PPO against the indigenous microbial flora and other spoilage microbes was evaluated. The antibacterial action of PPO against some of the selected pathogenic microorganisms (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterococcus fecalis*) was selected as the primary objective of the present research. In this present research the protein profiles of PPO and its enzyme activity was also investigated. Enzyme activity of PPO was also reported in the present research under optimized parameters. To determine the optimized condition for maximum enzyme activity of PPO, a design experiment illustrating Box-Behnken method was used in the research work

MATERIALS AND METHODS

Collection of Pacific White shrimps

Normal sized Pacific White shrimps weighing about 2kg were procured from a commercial fish market, at Coimbatore, Tamil Nadu, India. Procured shrimps were washed once, packed in a transport polystyrene box covered with ice packs and brought to Microbiology Laboratory, Sree Narayana Guru College, Coimbatore, Tamil Nadu, India for further processing. Collected shrimps were stored at 0° C. Shrimps were then thawed and washed under cold tap water, and the carapace (exoskeleton of the cephalothorax), cephalothorax including the first three thoracic segments, exoskeleton of the abdomen, the remaining thorax segments and the abdomen (here in after named as muscle), pereopods and maxillipeds, pleopods and telson (including the uropods) were individually segmented using a sterile cutter and stored at 0°C until further analysis (Fig-1). During analysis, the well frozen segmented shrimp parts were

mixed with dry ice, ground to a fine powder in a mixer and again stored at 0°C before enzyme purification process.

Extraction of Poly phenol oxidase (PPO)[3]

Fine powdered shrimp tissue materials were processed in the laboratory for the extraction of Poly phenol oxidase (PPO) enzyme. The extraction of PPO was carried out by the method described by Zamorano et al[3]. About 10g of fine powdered shrimp tissue material was added to 0.1M sodium phosphate buffer (pH 7.2) containing 1M NaCl, 0.2% Brij-35 and 2% poly vinyl poly pyrrolidone (PVPP). The whole suspension was prepared for the total volume of 10ml at the concentration of 1mg/ml. The prepared shrimp tissue concentrate containing the enzyme was stirred for 6h at 4°C. The content was centrifuged at 8,000 to 10,000 rpm for 20min at 4°C. Pellet containing the tissue debris was discarded; the supernatant containing enzyme extracts were filtered through a membrane filtration apparatus (2µ). The crude extracts containing enzyme concentrate was collected and stored at 0°C for further analysis.

Partial Purification of PPO using Dialysis membrane[11]

The crude enzyme extracts stored at 0°C processed further to obtain partially purified components after saturating with ammonium sulfate precipitates. The crude enzyme extract was added with ammonium sulfate salts until it reaches the saturation point. The entire saturated solution was centrifuged at 10,000 rpm for 30min. The supernatant containing the enzyme content was suspended in sterile vials and stored at 4°C to obtain the protein precipitates. The precipitate in needle crystal structure was observed after overnight incubation. These precipitates were resuspended in 0.1M sodium phosphate buffer (pH 7.0) and stirred for 30min at 10,000 rpm to remove other lipid contents and debris. The collected fractions were then subjected to desalting procedure using a dialysis tube method against 0.1M sodium phosphate buffer (pH 7.0). Specific molecular cut off (12 to 14kDa) dialysis tubes were procured commercially (Hi Media) and pretreated using 2% sodium carbonate and 1% EDTA. The solution was heated to boiling temperature and the dialysis tubes were immersed for 30min to activate the pores. After activating the pores, the tubes were filled with the enzyme fractions and tied tightly at both the ends. The tubes with filled in contents were desalted by immersing into the buffer solution. The beaker was placed on the magnetic stirrer and the dialysis was started with constant stirring condition (120rpm and 30°C). For every 30 min the buffer solution was changed without disturbing the dialysis tube. Finally the partially purified enzyme contents from the tubes were resuspended in cell free vials and stored at 0°C.

SDS-PAGE analysis of PPO[3]

Determination of molecular weight and activity of PPO using electrophoresis

Molecular weight of the crude extract and dialysis protein fractions containing PPO was identified. 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 (Genei Electrophoresis, Bangalore, India). After separation, the 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 from rabbit muscle (36 kDa) and carbonic anhydrase from bovine erythrocytes (29 kDa) (Fermentas) were used.

Optimization of enzyme activity using Design Experiment - Box-Behnken Statistical tools

Enzyme activity of purified and crude enzyme fractions was determined using the method described by José-Pablo Zamorano et al., (2008). To determine the optimized condition for maximum enzyme activity of PPO, a design experiment illustrating Box-Behnken method was used in the research work. Using this statistical tool, maximum enzyme activity of PPO at a specific pH and temperature was determined. In Table-1, the statistical run with varying pH and temperature as two major factors were presented. Using a standard substrate, DL-3,4-dihydroxyphenylalanine (DL-DOPA) the enzyme activity was measured at three varying incubation time periods. PPO activity was determined using a UV-Vis spectrophotometer equipped with a CPS-240 thermostatic controller. The enzyme activity procedure is briefly described below. The reaction mixture was prepared and exposed as per statistical run presented in Table-1. All the crude and purified enzyme samples were incubated with the standard substrate, 10mM DL-3,4-dihydroxyphenylalanine (DL-DOPA). After incubating the samples at their respective optimized conditions, the absorbance value of each mixture was measured for enzyme activity at 475nm using a UV-Vis spectrophotometer equipped with a CPS-240 thermostatic controller. One unit of enzymatic activity was defined as an increase in absorbance of 0.001 per minute. Experiments were performed in triplicate, and the results expressed as units of enzymatic activity per mg of protein. The protein content was measured according to the method of Bradford (1976) using bovine serum albumin as standard.

Evaluating the antibacterial activity of shrimp PPO against the challenge bacteria

The antibacterial activity of extracted shrimp PPO crude and purified fractions was evaluated against the predominant isolates by well diffusion method. Sterile Nutrient Agar (Composition g/L: Peptone: 5g; Yeast extract: 5g, Beef extract: 3g, Sodium chloride: 5g, Agar 15 g; Final pH (7.0 ± 0.2) plates were prepared and allowed to solidify. About 0.1% inoculum suspensions of five bacterial cultures (ESBL *Escherichia coli*, ESBL *Klebsiella pneumoniae*, MDR *Pseudomonas aeruginosa*, Methicillin resistant *Staphylococcus aureus* and Vancomycin resistant Enterococci) were swabbed uniformly over the agar surface. Under sterile conditions, 6mm wells were cut on the agar surface of each NA plates. About 50µl each of crude and dialysate PPO fractions in 5% dimethyl sulfoxide (DMSO) were loaded into the well and the plates were incubated at 37ºC for 24 - 48h. The antibacterial activity was evaluated in terms of zone of inhibition around the wells of each extract in all the inoculated NA plates. The inhibition clear zones were measured and recorded in millimeter.

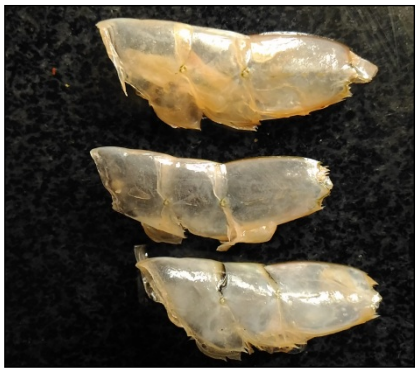
Table-1: Design Experiment – Box-Behnken statistical method to optimize conditions for maximum PPO activity

Run	pH	Temperature (°C)	Time (mins)	Specific activity (U/mg)
1	6	37	15	27.99
2	5	35	15	27.20
3	**5**	**35**	**5**	**27.03**
4	7	35	15	27.40
5	6	33	5	28.14
6	7	37	10	28.86
7	**6**	**35**	**10**	**28.98**
8	5	33	10	27.56
9	7	35	5	28.46
10	6	37	5	28.23
11+	7	33	10	28.77
12	6	33	15	27.88
13	5	37	10	27.66

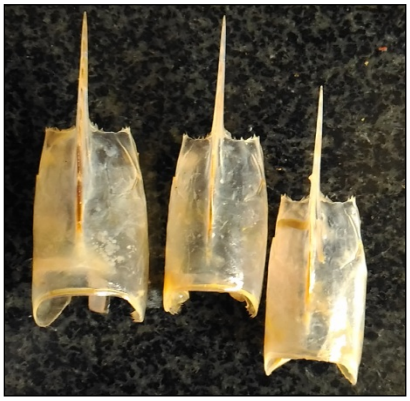
Table-2: Antibacterial activity of PPO extracts against the test organism

S. No	Test organisms	Character	Zone of inhibition (mm)
1	*Escherichia coli*	ESBL	21
2	*Klebsiella pneumoniae*	ESBL	18
3	*Pseudomonas aeruginosa*	MDR	19
4	*Staphylococcus aureus*	Methicillin resistant	23
5	*Enterococcus fecalis*	Vancomycin resistant	19

Head with the carapace



Carapace



Cephalothorax

Figure 1: Different parts and tissues of Pacific White shrimps for PPO extractions

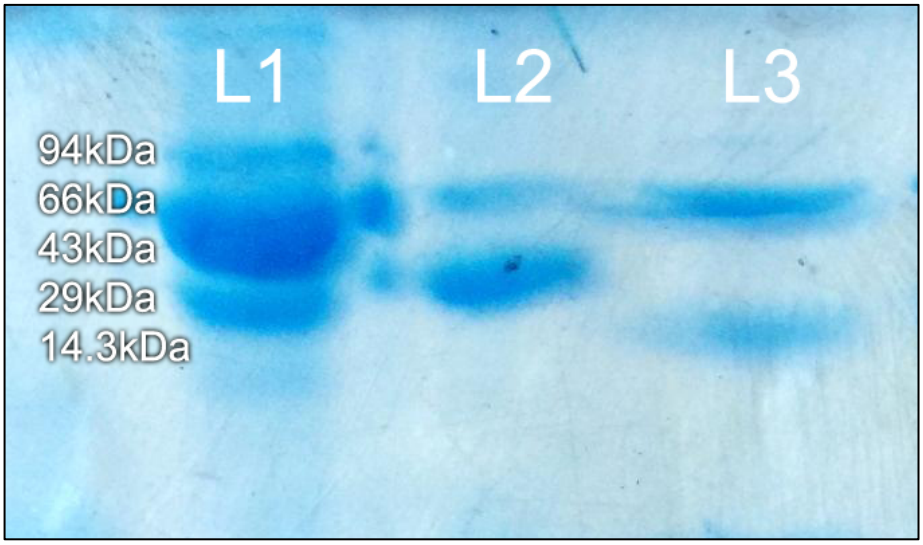
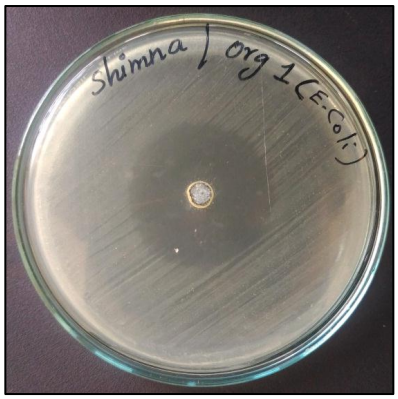
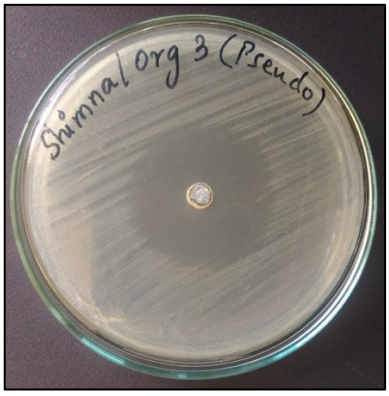


Figure 2: SDS PAGE: Protein profile of polyphenol oxidase (PPO)
Lane 1: Marker (94, 66, 43 29 and 14.3kDa)
Lane 2: Crude PPO (64 and 52kDa)
Lane 3: Purified PPO (64 and 48kDa)

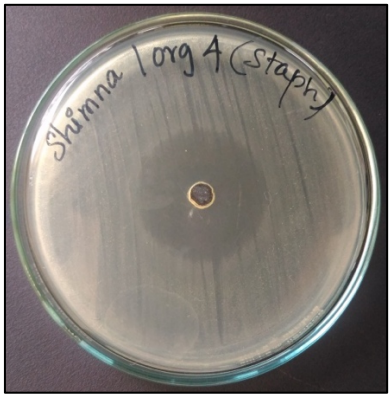


3a. ***Escherichia coli***

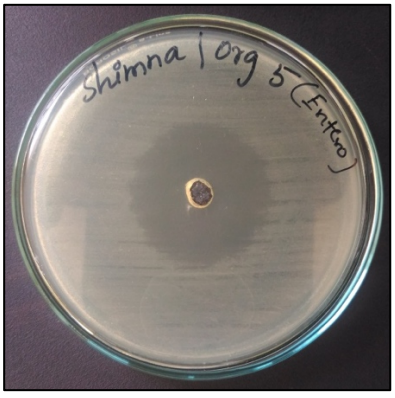
3b. ***Klebsiella pneumonia***



3c. ***Pseudomonas aeruginosa***



3d. ***Staphylococcus aureus***



3e. ***Enterococcus fecalis***

Figure 3: Antibacterial activity of shrimp extracts against bacterial pathogens

RESULTS AND DISCUSSION

SDS-PAGE analysis of PPO

SDS-PAGE electrophoretic patterns crude and partly purified PPO extracts of carapace, cephalothorax and muscle were presented in Fig-2. Two separate bands were obtained for crude and purified PPOs. Crude showed 64KDa and 52KDa protein bands; and purified PPOs showed 64KDa and 48KDa. The obtained results were found supportive with molecular weight of PPOs during the literature survey. PPO enzyme from black Pacific White shrimp (*Penaeus monodon*) obtained the molecular weight of 63kDa and 80kDa[12]. PPO obtained from *Charybdis japonica* obtained three protein bands of 80, 75, and 70kDa. The enzyme activity from cephalothorax kuruma prawn was had a molecular weight of 160 kDa[13]. Zamorano et al[3] reported that the extracts from carapace and cephalothorax showed a band of approximately 500kDa. Martínez-Alvarez et al[14] also found that protein band corresponded to the presumptive PPO oligomer of about 526 kDa in the same species. Nurhayati et al[11] obtained lot of bands with high (>200 kDa) and low (<60 kDa) molecular weight indicating that the enzyme was not pure.

Enzyme activity of purified and crude enzyme fractions

The Specific enzyme activity of PPO enzyme was measured at different temperature (33, 35 and 37°C), pH (5-7) and Time (5, 10 and 15 min) by using DL-DOPA as a substrate (Table-1). Maximum enzyme activity of 28.98 U/mg of protein was obtained for the reaction mixture analyzed at pH 6 at 35ºC in 10 mins. Minimum enzyme activity of 27.03 U/mg of protein was obtained for the reaction mixture analysed at pH 5 at 35ºC in 5 mins. Nirmal and Benjakul[6] reported similar amount of enzymatic activity for crude and ammonium sulphate (AS) fractions of PPOs. Crude PPOs showed 35.1U/mg and AS fractions showed 95.7U/mg of specific activity. Nurhayati et al[11] in another study aimed to purify PPO partially from black Pacific White shrimp (*Penaeus monodon*). Extraction, precipitation with ammonium sulphates, partial purification, and determination of molecular weight was analyzed. Precipitation showed best concentration to obtain the highest specific activity for ammonium sulfate fractions. Zamorano et al[3] studied PPO activity of deepwater pink shrimp (*Parapenaeus longirostris*) from different tissues. Maximum enzyme activity was found in the carapace, followed by that in the abdomen exoskeleton, cephalotorax, pleopods and telson. No PPO activity was found in the abdomen muscle and in the pereopods and maxillipeds. The authors also found that PPO had the highest activity at pH 4.5 and was most stable at pH 4.5 and 9.0. Apparent kinetic constants in the partly purified PPO were KM = 1.85 mM and Vmax = 38.5U/mg of protein.

Antibacterial activity of shrimp PPO extracts against the test organism

In Table-2, the antibacterial activity of shrimp PPO extracts against the test organism was presented. All the five test organisms were found susceptible to the PPO extracts. Maximum inhibitory zone of 23mm were obtained against a significant pyogenic organism (MRSA). Another important Gram-Negative pathogen *Escherichia coli* exhibited 21mm of inhibitory zones for the enzyme extracts. *Pseudomonas aeruginosa* (MDR) and *Enterococcus fecalis* (ESBL) exhibited 19mm of inhibitory zones and *Klebsiella pneumoniae* showed 18mm of inhibitory zones (Fig-3a to 3e). The reason for the antibacterial action of Crustacean extracts on the bacterial species may be due to the presence of significant antimicrobial peptides. Many marine sources have shown promising antibacterial potential against the bacterial pathogens when tested under standard *in vitro* conditions. Tincu and Taylor[15] reviewed different types of antimicrobial peptides from marine invertebrates. Antimicrobial peptide from Crustacea was highlighted against different bacterial and fungal pathogens. Penaeidins are prominent among crustacean antimicrobial peptides isolated from the hemolymph of the shrimp *Penaeus vannamei*. Antibacterial activity of penaedins was reported as specific against Gram-Positive bacteria[16]; and also against Gram-Negative *Vibrio* spp.[17]. Penaeidins are reported as strongly cationic composed of N-terminal proline-rich region and a C-terminal domain stabilized by intramolecular disulphide cross-links[16]. The cation was found to be attributed for its defensin class of antimicrobial peptides. The antibacterial mode of action of the marine peptides on the bacterial cell membrane was described. Clavanins are the antimicrobial peptide produced by ascidian *Styela clava*. The clavanins are amphipathic histidine-rich antimicrobial peptides contain 23 amino acids and exhibit C-terminal amidation[18]. Antimicrobial activity of clavanin A against *Escherichia coli*, *Listeria monocytogenes*, and *Candida albicans* was found maximum at pH 5.5. Clavanin A has the ability to permeabilize the outer and inner membranes of *Escherichia coli* very effectively at pH 5.5. In addition, clavanins were broadly effective against gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*. This is likely a function of the high net positive charge of clavanins at pH 5.5 due to the histidine component, which has a pKa of ca. 6.5[19]. Clavanin A efficiently inserts into different phospholipid monolayers via hydrophobic interactions, suggesting that the membrane is the target for this molecule[20], probably through interactions with membrane proteins that generate transmembrane ion gradients[21].

CONCLUSION

In this present research the protein profiles of polyphenol oxidase (PPO) extracted from and its enzyme activity was also investigated. Two separate bands were obtained for crude and purified PPOs. Crude showed 64KDa and 52KDa protein bands; and purified PPOs showed 64KDa and 48KDa. The Specific enzyme activity of PPO enzyme was measured at different temperature (33, 35 and 37°C), pH (5-7) and Time (5, 10 and 15 min) by using DL-DOPA as a substrate. Maximum enzyme activity of 28.98 U/mg of protein was obtained for the reaction mixture analysed at pH 6 at 35ºC in 10 mins. The antibacterial activity of shrimp PPO extracts against the test organism was presented. All the five test organisms were found susceptible to the PPO extracts. Maximum inhibitory zone of 23mm were obtained against a significant pyogenic organism (MRSA). Another important Gram-Negative pathogen *Escherichia coli* exhibited 21mm of inhibitory zones for the enzyme extracts. *Pseudomonas aeruginosa* (MDR) and *Enterococcus fecalis* (ESBL) exhibited 19mm of inhibitory zones and *Klebsiella pneumoniae* showed 18mm of inhibitory zones. The present research work concluded that the antibacterial activity of the shrimp extracts was mainly due to the presence of selective antimicrobial peptide. The mode of action of peptide on the cell membrane of organisms has displayed the promising antibacterial potential of marine metabolites.

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