Protein isolation of *Pterois volitans* venomous with a heating process for antibacterial activity assay

A N Sommeng¹, F Riswandha¹, M J Ginting², S Pebriani¹, M Sahlan¹,³, H Hermansyah¹ and A Wijanarko ¹*

¹Department of Chemical Engineering, Faculty of Engineering, Universitas Indonesia, Depok 16424, Indonesia
²Marine Science Postgraduate Program, Faculty of Mathematics and Natural Science, Universitas Indonesia, Depok 16424, Indonesia
³Research Centre for Biomedical Engineering, Faculty of Engineering, Universitas Indonesia, Depok 16424, Indonesia

*Corresponding author's email: anondho.wijanarko@yahoo.com

**Abstract.** *Pterois volitans* is an invasive native predatory fish species in the Indo-Pacific ocean that disrupt the food chain and damage coral reefs which cause ecosystem imbalances. These fish have venomous spines that made them inedible and avoided by a predator. Due to its rapid growth, utilization of this venom can be useful along with controlling the population. Recent studies show the benefits of phospholipase A2, which is a protein compound that contained in the venom has antibacterial activity and expected to be an antibacterial agent. In this study, we used heating and gradual purification to obtain the optimum concentration of Phospholipase A2. The protein isolates were analyzed using the Marinetti method, determination of concentration by Lowry test, identification of protein with SDS-Page, and the antibacterial activity test using agar diffusion. The results of phospholipase A2 obtained from the extract of *P. volitans* venom by purification method ammonium sulfate at 80% saturation with a heating time of 35 minutes had a specific enzyme activity of 0.0206 units / µg and can inhibit *E. coli* bacteria 98.81% and *Salmonella sp.* inhibit 89.28% with a concentration of 3.77 µg / ml.

1. Introduction

*Pterois volitans* is an invasive predator species with stings that have a profound impact on living things because the venoms found in spines have cardiovascular, neuromuscular, and cytolytic effects that cause cell lysis that obtain food in the form of crustaceans, small fish and crabs have [1]. *Pterois volitans* invasion attacks can disrupt the food chain and damage coral reefs that cause ecosystem imbalances resulting in population changes [2]. Uncontrolled *P. volitans* population is caused by the lack of natural predators, due to its venomous spines. This incident can be a threat to Indonesia's marine biodiversity, given that the native habitat of these animals is the Indo-Pacific Ocean adjacent to the Indian Ocean.

Venom in *P. volitans* has the potential to be utilized as an antibiotic or antibacterial treatment for bacterial diseases where bacterial resistance to various antibiotics had become a significant concern in terms of the extent of clinical use of conventional antibiotics. *Pterois volitans* has a relationship with *Pterois ruselli*, which have a venomous spine that containing protein compounds such as
phospholipase A2 [3]. Phospholipase A2 (PLA2) has antibacterial activity and expected to be an antibacterial agent [4]. One of the conventional protein isolation methods is precipitating protein using ammonium sulfate. This method has been done before by Savitri, et al. to observe the phospholipase A2 (PLA2) activity on *Acanthaster planci*. Protein isolation extraction method using sonicator to extract venoms with the help of phosphate buffer solution [5] combined with heating process and protein precipitation using ammonium sulfate for the purification process to obtain the optimum concentration of Phospholipase A2 [6].

This study explains the effects of the heating process in protein isolation, purification, and evaluates the antibacterial activity as an alternative antibacterial agent. The protein extract activity and concentration identified and its antibacterial activity further studied by testing the sample onto *E. coli* and *Salmonella sp.* both gram-negative bacterium.

2. Materials and methods

2.1. *Pterois volitans* venomous spines

Lionfish samples used in this experiment obtained from Java island, Indonesia. The collected samples then prepared in a basin containing blocks of ice cubes that are already in medium-size cracks. The fish left for 5 – 10 minutes until then the fish spines in the dorsal fin, side fins, and anal fins are cut using scissors to the base of the fins.

2.2. Crude venom extraction

The pre-treatment process was performed based on the standardized method reported by Ibrahim et al. on *Acanthaster planci* [7]. The fish spines separated from the body and rinsed with 0.01M phosphate buffer pH 7 containing 0.001 M CaCl₂ to obtain crude venom.

2.3. Protein heating purification and ammonium sulfate fractionation

Crude venom was heated to obtain a purer phospholipase A2 enzyme with a hot plate at 60° with variations of time 25, 30, 35, and 45 minutes. Then the crude venom centrifuged to separate phospholipase A2 from other proteins that have been denatured at a speed of 4500 r/min for 30 minutes [8].

The method of purification carried out using ammonium sulfate fractionation. Ammonium sulfate fractionation carried out by adding ammonium sulfate to crude venom with a saturation level of 20%, 40%, 60%, and 80% little by little for approximately 20 min with a magnetic stirrer. Each enzyme protein sediment centrifugated at 4500 r/min for 30 min to separate from the filtrate. The *P. volitans* PLA2 fraction formed a precipitate which then dissolved in a 0.01 M phosphate buffer solution of pH 7.0 containing 0.001 M of CaCl₂.

2.4. Phospholipase A2 activity analysis

*Pterois volitans* phospholipase A2 enzymatic activity assessed using Marinetti methods in a solution of 0.1 m Tris-HCl buffer pH 8.0 prepared with 2 mg/ml egg yolk concentration in the solvent. [9]. 0.1 ml of enzyme sample solution then reacted with 1.5 ml of egg yolk suspension, and then the absorbance decrease value for 5 min observed without enzyme samples addition using Shimadzu UV – 2450 ultraviolet-visible (UV-VIS) spectrophotometer at a wavelength of 900 nm. Any decrease in absorbance of 0.01 / minute was defined as one unit of the enzyme.

Protein concentration determination was assessed with the Lowry method [10] using Folin–Ciocalteu Phenol 1N reagent and a biuret solution (1 ml of 1% NaK-tartrate solution and 1 ml of 1% CuSO₄ solution in 100 ml of 2% Na₂CO₃ solution). Bovine serum albumin 200 μg/ml used to make standard curves. Biuret solution added to the sample then incubated for 10 minutes. Then Folin reagent added to the sample then incubated for 30 minutes. The sample absorbance values tested using a UV–VIS spectrophotometer at a wavelength of 750 nm [12].
Protein purity assessed using the SDS-PAGE method. The sample dissolved with a ratio of 1:2 in a buffer and heated at 95°C for 1 min. Then, the gel loaded into the gel mold tube and homogenized until solidifies while the stacking gel prepared in the same way. The gel inserted into the electrophoresis chamber after the gel and hole entering the sample formed. The separation performed at 20 mA until the dye reached 0.5 cm of the gel bottom and removed the gel from the plate then immersed in a fixing buffer solution for 30 minutes, followed by staining with staining buffer solution. The gel then rinsed with a destaining solution to make the protein visible[6].

2.5. Antibacterial activity test
The antibacterial activity based on minimum inhibitory concentration using the agar dilution method reported by Wiegand et al. [11]. The test bacteria used were *E. coli* and *Salmonella sp.* Preparation of bacterial inoculum was started by making *E. coli* and *Salmonella sp.* inoculums with a concentration of 25% in one day and then dilution to a bacterial concentration of $10^3$ cfu / mL each. The sample was prepared by inserting 100μl of the sample into each sterile dependent on both bacteria. The controls were prepared, namely, positive control (C+) and negative control (C-). Positive control preparation begins weighing 10mg of chloramphenicol added to 10 mL so that a concentration of 1000ppm obtained, the same for both bacteria. Negative controls for each bacteria sample were prepared by entering sterilized 100μl aquadest into Eppendorf in both bacteria. Samples, C+ and C- with a volume of 100μl in the Eppendorf were added with bacterial inoculum ($10^3$ Cfu / mL) as much as 100μl into each sample, C+, C- then homogenized with the vortex. A liquid of 100μl was taken from each Eppendorf into sterilized petri dishes, and 20–25 mL TSA was added, homogenized, and incubated at 30–35°C for 24 hours. Bacterial growth was observed, which is formed with minimum inhibitory concentrations expressed when the cup is not entirely overgrown with microbes. The precise area of the chromatogram showed the inhibition of bacterial growth.

3. Results and discussions

3.1. Protein concentration and specific activity
The results of data processing from six samples of Marinetti Test and Lowry Test selected based on the most considerable specific activity of 30 samples tested on the precipitation of 20%, 40%, 60%, and 80% ammonium sulfate with variations in heating time for 25, 30, 35 and 40 minutes at 60°C. The samples obtained positively have an enzymatic activity that can be measured as shown at table 1 below.

| Sample  | Protein Concentration ($\mu$/mL) | Vol (mL) | Enzyme Unit (unit) | Enzyme Activity (unit/mL) | Total Activity (unit) | Total Protein (μg) | Specific Activity (unit/μg) |
|---------|---------------------------------|----------|-------------------|--------------------------|----------------------|------------------|--------------------------|
| AS 20%, 35 m | 6.93 | 2.2 | 0.00992 | 0.992 | 2.1824 | 15.26 | 0.1429 |
| AS 40%, 40 m | 6.38 | 2.2 | 0.0024 | 0.24 | 0.528 | 14.05 | 0.0375 |
| AS 60%, 40 m | 4.15 | 2.2 | 0.00088 | 0.088 | 0.1936 | 9.14 | 0.0211 |
| AS 80%, 30 m | 3.35 | 2.2 | 0.00106 | 0.106 | 0.2332 | 7.37 | 0.0316 |
| AS 80%, 35 m | 3.77 | 2.2 | 0.00078 | 0.078 | 0.1716 | 8.3 | 0.0206 |
| AS 80%, 40 m | 4.51 | 2.2 | 0.0012 | 0.12 | 0.264 | 9.94 | 0.0265 |

There was an increase in activity from the sample as the heating time increases. Table 1 showed that there is an effect of heating time with the enzymatic activity of the protein phospholipase A2, which was isolated from the venom of *Pterois volitans*. The specific activity obtained was even more significant when compared to the previous study by Sommeng [12] which obtained the most significant specific activity of 0.027 units / μg while this test obtained the highest specific activity of
0.1429 units / μg from result of ammonium sulfate saturation of 20% saturation level with heating time 35 minutes (AS20%, 35m). It showed that there is an effect of the precipitation using Ammonium Sulfate with heating to the phospholipase A2 enzymatic activity from Lionfish venomous spines

3.2. Protein molecular weight
From results of the SDS-PAGE test in figure 1 can be seen that the isolation of phospholipase A2 protein using a phased fractionation method of ammonium sulfate of AS40%, 40m; AS80%, 35m; and AS80%, 40m is the sample with the highest purity level with protein bands at the molecular weight level of about 85kDa when compared to the marker. The increase in purity of protein per salting saturation, where the reduced number of protein bands seen in the SDS-PAGE gel from a saturation level of 20% to a saturation level of 80%. Phospholipase A2 with a high molecular mass dependent on Ca²⁺ ions [13], so that by using a buffer containing CaCl₂, Pterois volitans can be extracted adequately.

![Figure 1. SDS-PAGE Test Result](image)

3.3. Antibacterial activity test
The results of testing for antibacterial activity showed a reduction in the concentration of *E. coli* bacteria in AS60%, 40m; AS80%, 30m; AS80%, 35m; AS80%, 40m and *Salmonella sp.* at AS80%, 30m; AS80%, 35m; AS80%, 40m as shown at Figure 2 below.
Figure 2. Phospholipase A2 sample before (A) and after (B) being incubated

| Sample           | Number of colonies E. coli (CFU/100µl) | Number of colonies Salmonella sp. (CFU/100µl) |
|------------------|----------------------------------------|---------------------------------------------|
|                  | I           | II           | I           | II           |
| AS 20%, 35m      | >300        | >300         | >300        | >300         |
| AS 40%, 40m      | >300        | >300         | >300        | >300         |
| AS 60%, 40m      | >300        | >300         | >300        | >300         |
| AS 80%, 30m      | 116         | 96           | 317         | 300          |
| AS 80%, 35m      | 23          | 48           | 338         | 305          |
| AS 80%, 40m      | 142         | 101          | 321         | 298          |
| K + (Chloramphenicol 1000ppm) | 0           | 0            | 0           | 0            |
| K – (Sterile Aq.) | 2.4 x 10²   | 2.1 x 10²   | 3.8 x 10²   | 4.2 x 10²   |

From table 2, samples with a saturation level of 80% can inhibit bacteria shows that there is an influence of saturation level with the ability to reduce the concentration of bacteria. Reduction of concentration in E. coli bacteria was more significant than that of Salmonella sp. because the largest component of phospholipid in the E. coli cell membrane is phosphatidylethanolamine, where phospholipase A2 prefers phosphatidylethanolamine as a substrate even though phospholipase A2 is not a substrate-specific enzyme [14].

The level of antibacterial activity based on the most significant bacterial concentration reduction were obtained in the US sample of 80%, 35m at a concentration of 3.77 µg / ml by inhibiting the bacteria E. coli 98.81%, which is very close to LD 99%, and in Salmonella sp. inhibits 89.28%, which is very close to LD 90%. For comparison, the venom Naja naja (Indian cobra) has a minimum inhibitory concentration for E. coli at 19.3 µg / ml and Salmonella sp. at 22.1 µg / ml [15]. Thus phospholipase A2 has the potential to be developed into an antibacterial material.

4. Conclusion
Phospholipase A2 isolated from the extract of Pterois volitans venom more effective at the higher saturation level of ammonium sulfate. Phospholipase A2 showed antibacterial activity against Salmonella sp. and E. coli, both gram-negative bacteria, with 89.28% and 98.81% inhibition rate respectively for AS80% sample with a heating time of 35 minutes. High bacterial inhibition for both bacterial cultures suggests that phospholipase A2 from the extract of Pterois volitans venom has the potential to become an antibacterial agent. For further development, the evaluation of the antibacterial activity of phospholipase A2 would be needed analysis in vivo studies. Phospholipase A2 potentially provide as an antibacterial agent with the promising source of environmentally friendly antibacterial agent.
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