Extraction of PLA2 and antibacterial activity test of lionfish (Pterois volitans) spine venom

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Abstract. The population of Pterois volitans has caused significant damage to other fish populations and coral reef ecosystems. Population control of P. volitans consumes a considerable cost so that the utilization of these fish needs to be sought to be useful along with controlling the population. This fish is known to contain the enzyme Phospholipase A2 (PLA2), which used as an antibiotic against some bacteria. This study will examine the antibacterial activity of the phospholipase A2 enzyme extracted from P. volitans venom to Escherichia coli, Bacillus subtilis, and Staphylococcus aureus. The method used to isolate the enzyme PLA2 is by using precipitation of ammonium sulfate (AS) and precipitation with ethanol. The results of the precipitation tested with the Lowry protein concentration test, the Marinetti PLA2 activity test, and the identification of the SDS-PAGE protein. The agar diffusion disc method is used to test the antibacterial activity. The results obtained from this research are that 80% ammonium sulfate precipitation method has the highest protein and enzyme activity with a ratio of 1.32 times compared to toxic extract. For antibacterial activity test results, an 80% ammonium sulfate sample may inhibit the activity of S. aureus bacteria but does not affect B. subtilis and E. coli.

1. Introduction
Pterois volitans or red lionfish have become an invasive species in the Western Atlantic with few or no predators [1]. Lionfish populations continue to expand, threatening the well-being of coral reefs and other marine ecosystems [2]. Allegedly, there is Phospholipase A2 (PLA2) content, the protein that has antibacterial activity against several bacteria in lionfish venom. In its development, PLA2 can be used as an antibiotic or antibacterial treatment for some bacterial diseases. Since the bacterial resistance to various antibiotics had become a significant concern in terms of the extent of the clinical use of conventional antibiotics [3]. The antibacterial properties of PLA2 based on the ability of PLA2 to hydrolyze the phospholipid components of bacterial cell membranes so that bacteria undergo lysis and then died.

This research became a preliminary study of the antibacterial properties of P. volitans venom. The study started from the process of extracting P. volitans venom, purification, to testing the venom
activity as a standard basis for antibacterial testing afterward. Antibacterial testing uses a disc diffusion method to the extent of qualitative to see the presence or absence of clear zones in bacteria around the sample discs that dispensed with P. volitans venom extract. The use of antibacterial activity on P. volitans venom can help control the fish population also provide an alternative to antibiotics against certain bacteria.

2. Materials and methods

2.1. Pterois volitans venom extraction, Phospholipase A2 isolation, and protein concentration

The specimen was gathered from fisheries in Jakarta from March – April 2018, and the specimens stored in a saltwater tank before use. The method used to isolate Phospholipase A2 from crude venom is Savitri et al. (2011) method [4]. 50 gr spines of the collected sample immersed in 0.01M phosphate buffer pH7 of 100 mL and 10 mL CaCl2 0.1 M for 8 minutes at 40 °C. After the sample in the phosphate buffer has been prepared, it is then centrifuged 15,000 g for 30 minutes to separate the impurity deposit from the supernatant crude venom. The supernatant obtained from the initial extraction stage was heated in the water bath for 30 minutes at 60 °C, and every 10 minutes was stirred with a magnetic stirrer, then reconstituted with 15,000 g of centrifugation for 30 minutes. The supernatant added with 80% ammonium sulfate salt according to its degree of solubility. The precipitation is carried out using a magnetic stirrer, and the sample container remains immersed in ice to keep the temperature intact. Separation of precipitate was done by 15,000 g refrigeration centrifugation for 30 minutes. The centrifugation precipitate dissolved in 0.01 M phosphate buffer, pH 7.0, and CaCl2. The method used to determine protein concentration is the Lowry method, with Bovine Serum Albumin (BSA) as the standard.

2.2. Phospholipase A2 activity analysis

PLA2 activity assay was done using the Marinetti method [5]. The method analyzes the clearing or absorbance decrease of egg yolk suspension. A total of 0.1 ml of the sample from the first venom extract, heated venom extract, until the fractionation using ammonium sulfate, was inserted into a test tube with 1.5 ml of egg yolk substrate. The tube was then vortexed, and the absorbance reduction measured for 5 minutes. Substrate concentration of 2 mg/ml in Tris-HCl buffer 0.1 M, pH 8.0. The absorbance measured at a wavelength of 900 nm using a spectrophotometer. One unit of the enzyme determined as an activity that caused a decrease of 0.01 absorbance/min or equivalent to a substrate reduction of 1 μmol/min [5].

2.3. Antibacterial activity test

Antibacterial activity is tested by using Bauer et al. (1966) or Disc Diffusion Method. Bacteria tested in this experiment included gram-negative bacteria (E. coli) and bacterial Gram-positive (B. subtilis and S. aureus). The concentration of the sample varied according to the initial concentration of the toxic extract obtained, to concentrations based on testing of antibacterial properties of venom extract with the content of PLA2 (snake venom) previously tested at 100 μg/ml [6] for 20 l disc capacity. The positive control used antibiotic chloramphenicol with a dose of 30μg/disc, while the negative control is a disk that is not diluted by anything. The test is limited to a qualitative analysis of whether a clear zone is located around the disc paper that has spilled into the sample after 24 hours incubation.

3. Results and discussions

3.1. Phospholipase A2 isolation results

PLA2 isolated by using 2.064 grams ammonium sulfate 80% added for 4 mL crude venom. The Lowry protein concentration test shows that precipitation of ammonium sulfate 80% resulted in 361.92 μg/mL of protein concentration compared with crude venom extract 330.81 μg/mL. Lowry test results showed an increase in protein concentration along with the addition of ammonium sulfate salt to the
venom extract. The same results also are shown in the Marinetti test, which showed an increase in activity of the 80% ammonium sulfate precipitation sample compared with the specific activity of the crude venom samples. 80% ammonium sulfate sample has 0.020 unit/μg specific activity while the crude venom sample has 0.015 unit/μg specific activity.

**Table 1. Summary of isolation test result of Phospholipase A2**

| Sample      | Protein Concentration (μg/mL) | Vol (mL) | Enzyme Unit (unit) | Enzyme Activity (unit/mL) | Total Activity (unit) | Total Protein (μg) | Specific Activity (unit/μg) | Comparison to CV |
|-------------|-------------------------------|----------|-------------------|--------------------------|-----------------------|-------------------|---------------------------|------------------|
| Crude Venom | 330.81                        | 4        | 0.5               | 5                        | 20                    | 1323.22           | 0.015                    | 1                |
| Ethanol     | 310.61                        | 4        | 0.36              | 3.6                      | 14.4                  | 1242.44           | 0.012                    | 0.77             |
| AS20%       | 240.69                        | 4        | 0.18              | 1.8                      | 7.2                   | 962.78            | 0.007                    | 0.49             |
| AS40%       | 261.08                        | 4        | 0.2               | 2                        | 8                     | 1044.33           | 0.008                    | 0.51             |
| AS60%       | 351.19                        | 4        | 0.26              | 2.6                      | 10.4                  | 1404.78           | 0.007                    | 0.49             |
| AS80%       | 361.92                        | 4        | 0.72              | 7.2                      | 28.8                  | 1447.67           | 0.020                    | 1.32             |

3.2. **Antibacterial activity results**

Antibacterial activity result assay showed by measuring the inhibition zone of each tested sample. The positive control used antibiotic chloramphenicol with a dose of 30μg / disc, while the negative control is a disk that is not diluted by anything. The result showed that the AS 80% sample had shown the area of a narrow inhibition zone (14.35 mm) round disc against *Staphylococcus aureus*, whereas smaller compared to chloramphenicol (28 mm) as a positive control. This result indicates that PLA2 from *P. volitans* venom possess potent antibacterial activity against *S. aureus*. PLA2 from the venom of *P. volitans* possesses antibacterial activity against *S. aureus*, which is similar to snake venom PLA2 [7]. The PLA2 antibacterial activity can occur by permeabilizing the bacterial membrane by forming pores.

**Table 2. Antibacterial activity test results**

| Sample      | Time (hour) | Inhibition zone + (mm) |
|-------------|-------------|------------------------|
| Crude Venom |             |                        |
| *Staphylococcus aureus* | 48 | 0                      |
| *Bacillus subtilis* | 48 | 0                      |
| *Escherichia coli* | 48 | 0                      |
| *Staphylococcus aureus* | 48 | 14.35                  |
| AS 80%      |             |                        |
| *Bacillus subtilis* | 48 | 0                      |
| *Escherichia coli* | 48 | 0                      |
| *Staphylococcus aureus* | 48 | 26.46                  |
| Chloramphenicol |             |                        |
| *Bacillus subtilis* | 48 | 16.73                  |
| *Escherichia coli* | 48 | 14.69                  |

Although *B. subtilis* and *S. aureus* are both gram-positive bacteria, and antibacterial activity not shown in *B. subtilis* as in *S. aureus*, the result is similar to Ahmadi et al. (2010) research, which also does an antibacterial activity test against *B. subtilis* by using *Echis carinatus* venom extract [8]. Since venom consists of different substances of protein and enzyme that responsible for their biological activities, these compounds may interact with specific molecules of *S.aureus* but not affecting other strains like *B.subtilis*. Other studies in *Lachesis muta* venom had PLA2 activity, with no antibacterial effect. It can be concluded that the presence of PLA2 in the venom does not guarantee the antibacterial activity [9].

While antibacterial activity is also not found in *E. coli* bacteria. It because the *E. coli* bacteria are gram-negative bacteria. The distinctive feature of gram-negative bacteria is the presence of a double membrane surrounding each bacterial cell. Although all bacteria have an inner cell membrane, gram-
negative bacteria have a unique outer membrane. This outer membrane excludes certain drugs and antibiotics from penetrating the cell, partially accounting for why gram-negative bacteria are generally more resistant to antibiotics than are gram-positive bacteria [10].

![Figure 1. Antibacterial Activity Test on S. aureus, B. subtilis, dan E. coli.](image)

4. Conclusion
Lionfish P. volitans venom extract contains Phospholipase A2, which can be seen using the Marinetti method and SDS-PAGE result. Protein precipitation method using ammonium sulfate 80% effectively purifies PLA2 from lionfish P. volitans venom extract with specific activity 1.32 times the specific activity of crude venom and a relatively high degree of purity. Protein precipitation method using 80% ethanol is not sufficient to purify PLA2 from lionfish P. volitans venom extract with specific activity 0.77 times the specific activity of crude venom. PLA2 isolated from P. volitans venom with the salting-out method using ammonium sulfate on 80% saturation level has antibacterial activity against Staphylococcus aureus but does not affect Bacillus subtilis and Escherichia coli.

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