Protein isolation and identification of *Pterois volitans* spine venom coagulant activity

A N Sommeng¹, A K Eka¹, M Y A Ramadhan¹, M J Ginting², 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*, or commonly referred to lionfish, are fish species originating from Indo-Pacific waters but are becoming invasive in other regions such as the Caribbean and Atlantis. Various efforts have been made to reduce the number of lionfish, and one of them is by utilizing the venom on the spine. The venom extraction of *P. volitans* spines is done mechanically using sonication and centrifugation, and then protein isolation is carried out using salt. Coagulant activity from extract (crude venom) and lionfish venom protein isolate was done by counting PT (prothrombin time) and aPTT (activated partial thromboplastin time) which resulted that the crude venom and protein isolate of lionfish venom can accelerate blood clot (procoagulant) respectively up to 8.5 seconds and 6 seconds. Protein identification was made using LC-MS/MS device. The LC-MS/MS analysis showed that the protein isolate of lionfish venom contains Nomega-nitro-L-arginine methyl ester (L-NAME) compounds known to have procoagulant effects. From a series of tests mentioned, it concluded that *P. volitans* venom have procoagulant activity and one of the compounds responsible for it is L-NAME.

1. Introduction

Lionfish (*Pterois volitans*) are fish species that originate from Indo-Pacific waters[1]. Lionfish has white and red or brown stripes. Adult lionfish can grow up to 47 cm in length and live for approximately ten years[2]. The venom owned by lionfish makes it have little or almost no predators[3]. A large-scale invasion of the shallow waters of Atlantis and the Caribbean by two species of lionfish (*P. miles* and *P. volitans*) is known to have been a problem for the local ecosystem[4]. These problems arise because lionfish are predators that eat invertebrates and smaller fish up to 4% of its body weight per day that could increase competition for food[5-7]. Types of fish that lionfish prey is also the catch for fishers so that their revenue decreased by 30– 45%[8]. Also, if the prey is fish that consume the algae, the algae growth can be remarkably rapid[9].

Lionfish has venom found in its spines. If punctured, it will cause burning for 15 to 20 minutes. The venom that this fish has is very dangerous and can cause death, even though the chances are minimal. Symptoms that arise after being exposed to toxins from lionfish are hypertension,
tachycardia (increase in heart rate), and numbness. Within three hours of being exposed to venom, it can cause limb paralysis[10].

In previous lionfish venom studies [11], pro-coagulant activity found in P. russelli. The study tested its whole venom (without extraction or purification) by calculating the Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT). By varying the amount of venom tested, the highest coagulant activity found at 30 and 60 μg in 100 μl of blood plasma for both tests. The discovery of the presence of pro-coagulant activity in lionfish venom opens the possibility of finding active coagulation factors and new treatments for thrombotic disorders and anti-hemorrhagic agents that will be needed if there are severe injuries due to accidents. One type of disease that requires such substances with pro-coagulant activity is hemophilia. Coagulant activity in lionfish venom will not be separated from the role of the proteins contained in it. If the type of protein that plays a role in the activity is known, then the use of lionfish venom as an alternative treatment for blood clotting disorders, such as hemophilia, can be maximized. Therefore, isolation and identification of proteins in lionfish toxins need to be done.

2. Materials and methods

2.1. Pterois volitans venom extraction

The study began by taking spines from lionfish. The spines then rinsed with seawater to remove the sticking dirt and salt. Then, the sample soaked in 100 ml of 0.01 M phosphate buffer solution pH 7.0 containing 0.001 M calcium dichloride, for every 50 grams of spine/sting. After that, the mixture authenticated for two times 8 minutes with an 80% pulse and an output of 10 at 20 kHz. The extracted toxin then centrifuged at 15,000 × g for 30 minutes to separate impurities such as spines particles or denatured proteins. Crude venom obtained through the extraction process still contains many impurities, so the protein is isolated by using ammonium sulfate. Isolation is carried out by adding the required ammonium sulfate solids into a solution of crude venom then incubated for 30 minutes to ensure that ammonium sulfate dissolved. After that, the solution centrifuged at 30,000 × g for 30 minutes. The proteins that have been isolated were formed solids.

2.2. Lowry test and freeze-drying

Venom sample and protein isolates were mixed (vortex) with 2 ml pH 7.0 phosphate buffer until homogeneous. Then 0.04 ml of the sample was put into a test tube. In the tube that has contained the sample, 4 ml of the biuret / Lowry reagent was added then incubated at room temperature for 10 minutes. After incubation, six drops of Folin–Ciocalteu reagent added to each tube, and then it was mixed and re-incubated at room temperature for 30 minutes. Venom samples from the extraction and isolation results then dried using the freeze-drying method. The results of this drying powder form that can facilitate researchers in testing.

2.3. Calculation of Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT)

Blood plasma was taken as much as 100 μL of the four blood types (A, B, AB, and O). 20 μL of a crude venom solution with a concentration of 750 ppm was added to the citrated plasma and incubated for 5 minutes at 37 °C. 200 μL of PT reagent solution added. The mixture incubated for 60 seconds. After 60 seconds, the freezing (coagulation) time calculated until fibrin threads seen. Citrated plasma and PT control reagents used as normal controls.

Crude venom with different doses of 20 μL was added to 100 μL of citrated plasma (which had the most considerable activity of type variation) and incubated for 5 minutes at 37 °C then added 200 μL of PT reagent solution. The mixture is incubated first for 60 seconds. After 60 seconds, the freezing (coagulation) time calculated until fibrin threads seen. Citrated plasma and PT control reagents used as normal controls.

Different amounts of protein isolate were added as much as 20 μL into 100 μL of citrated plasma (which had the most considerable activity of type variation test) and incubated for 5 minutes at 37 °C
then added 200 μL of PT reagent solution. The mixture silenced for 60 seconds. After 60 seconds, the freezing time calculated until fibrin threads seen. Citrated plasma and PT control reagents used as normal controls.

Crude venom with different doses, each added as much as 20 μL into 100 μL of citrated plasma and incubated for 5 minutes at 37 ℃. 100 μL of aPTT reagent was added and stirred. The mixture incubated first for 180 seconds then added 100 μL of calcium dichloride 0.025 M (preheated to 37 ℃) and the freezing time calculated. Citrated plasma and aPTT reagent control used as normal controls. Different amounts of protein isolate, each of 20 μL added to 100 μL of citrated plasma and incubated for 5 minutes at 37 ℃. 100 μL of aPTT reagent control solution is added and stirred. The mixture incubated first for 180 seconds then added 100 μL of calcium dichloride 0.025 M (preheated to 37 ℃) and calculated the freezing time. Citrated plasma and aPTT reagent control used as normal controls.

2.4. LC-MS/MS analysis
This analysis starts with sample preparation using a thin layer chromatography method. The instrument system used is LC-ESI-QTOF and MS used is the Xevo G2-S TOF system with positive ionization mode. The results data is then analyzed using the MassLynx V4.1 SCN884 2012 program.

3. Results and discussions

3.1. The protein concentration of samples (Lowry test)
The samples calculated for concentration are crude venom and protein isolates with a saturation of 20%, 40%, 60%, and 80%. Figure 1 shows the absorbance and concentration of each sample obtained using the calculation of the standard curve equation.

![Figure 1. The protein concentration of venom and protein samples.](image)

Based on research journals on animal venoms, the venom majority samples tested were crude venom and protein isolates. Figure 1 shows that the resulting protein concentration increases with increasing saturation of ammonium sulfate used. It shows that ammonium sulfate proved to be able to separate the protein venom lionfish from other components or content.

In this study, isolated protein using 80% ammonium sulfate saturation produced the highest protein concentration compared to other samples. However, the protein concentrate produced at 80% saturation was not so far or significant, with 60% saturation when compared with 40% and 20% saturation. It shows that the use of ammonium sulfate to separate protein from other content almost at the stationary stage, so the sample of protein isolates that used in this study was the result of isolation with 80% ammonium sulfate saturation.
3.2. Coagulant activity of lionfish venom protein in the variation of blood type

Figure 2 shows that the venom has procoagulant activity and is most significant in donor with blood type A. This shown from the most considerable prothrombin time difference between control (no treatment) and the sample gave the crude venom, which is 4.5 seconds. In humans, many factors that influence blood clotting. These factors include antigen concentration, thromboplastin presence[12], the range of time between blood taking and testing, the presence of other biochemical compounds in the blood[13] and others that are very dependent on the condition of each donor[14].

3.3. Calculation of Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT) in lionfish crude venom (CV)

As discussed earlier, PT and aPTT are parameters used in calculating the coagulant activity of a compound against human blood plasma. From the previous test, the blood type which had the most significant coagulant activity was A. Therefore, blood plasma from a donor with type A was used in the calculation of PT and the aPTT in this study to reduce the factors that could differentiate results. From the calculation of PT and aPTT in crude venom lionfish against blood plasma, it obtained that crude venom from lionfish was able to accelerate the process of blood clotting (procoagulant). In figure 3, shows that the time needed by blood plasma to form fibrin fibers in samples given by crude venom is less than the control.
Figure 3 shows that crude venom from lionfish has procoagulant activity in both coagulation pathways, which are extrinsic (PT) and intrinsic (aPTT). It was consistent with the results of research conducted by Memar et al. in 2015, where it stated that the crude venom from *P. russelli* had procoagulant activity on both coagulation pathways. Figure 4 shows the pattern of crude venom coagulant activity from lionfish. It is known that the more samples added to the blood plasma, the higher the activity. In the addition of a sample of 7.5 to 30 μg, coagulant activity from crude venom lionfish experienced a significant increase — similarly, the addition of a sample of 60 to 120 μg. However, the blood clotting time at the addition of a sample (CV) of 30 μg had an activity equal to 60 μg which was twice the number.

If allosteric properties of thrombin are associated with this study, it assumed that there is a similarity of activity between the addition of a sample of 30 μg with 60 μg because the blood clotting reaction reaches the equilibrium stage in that amount. When compared again with the research conducted by Memar *et al.*, we found similarities in the pattern of coagulant activity of crude venom *P. volitans* and *P. russelli*. Coagulant activity in *P. russelli* crude venom reached the equilibrium stage in the addition of a sample of 60 μg, whereas in *P. volitans* (this study) the equilibrium stage was achieved in the addition of a sample of 30 μg. However, there is a difference between the research conducted by Memar *et al.* and this study is that there is a much more significant increase in return activity after achieving the equilibrium stage.

3.4. Calculation of Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT) in protein lionfish (IP) isolates

In addition to the activity of crude venom from lionfish, the isolated proteins also tested for their coagulant activity in this study. From the calculation of PT and aPTT on lionfish protein isolates against blood plasma, the results showed that protein isolates from lionfish were able to accelerate the process of blood clotting (procoagulant). In figure 5, shows where the time taken by the blood plasma to form fibrin threads in the sample given protein isolates was less than the control.

![Figure 5. Blood clotting time in addition to protein isolates. Line A is the coagulant time of protein isolate PT and line B is coagulant time of protein isolate aPTT.](image1)

![Figure 6. Coagulant activity of *P. volitans* protein isolates. Line A is the coagulant activity of protein isolate PT, and line B is the coagulant activity of protein isolate aPTT](image2)

Just like the activities of crude venom, protein isolates accelerate the blood clotting process so that it has procoagulant activity. However, in contrast to its crude venom, protein isolates from lionfish venom can only accelerate the blood clotting process for up to 6 seconds, which is the addition of a sample of 7.5 μg. From the graph in figure 6, it knows that there is a decrease in coagulant activity in addition to protein isolates as much as 7.5 to 30 μg. It caused by the discovery of L-Arginine compounds in protein isolates during protein identification.
As mentioned before, one of many coagulation factors, namely thrombin, is allosteric, which allows it to have two different coagulation properties. The most significant possibility that occurred was, in addition to protein isolates between 7.5 to 30 μg, the procoagulant activity of the L-NAME compound (which also found during protein identification) was disrupted by the presence of L-arginine. Whereas in addition to protein isolates between 30 to 120 μg, the activity of L-NAME increases again, which causes the reaction to move towards the formation of fibrin threads.

3.5. Protein identification (LC-MS/MS analysis)

From the results of LC-MS / MS analysis, as shown in figure 7, it was found that the lionfish venom protein isolate contained the Nomega-Nitro-L-arginine methyl ester (L-NAME) compound with the molecular formula C$_7$H$_{15}$N$_5$O$_4$ and weight m/z 188.1035. The identification of the L-NAME compound is following the database made by the National Center for Biotechnology Information. According to research conducted by Dambisya (1996), L-NAME has a procoagulant effect on the blood. So, it concluded that L-NAME is a compound, one of the compounds (does not rule out the possibility of other procoagulant compounds) which are responsible for procoagulant activity in lionfish venom.

![C$_7$H$_{15}$N$_5$O$_4$](image)

**Figure 7.** LC-MS/MS analysis using the MassLynx program.

Besides L-NAME, we also found L-arginine compound with molecular formula C$_6$H$_{14}$N$_4$O$_2$ and weight m/z 175.1195. Based on research conducted by Dambisya (1996), L-arginine has an anticoagulant effect on the blood. This compound has properties that are contrary to L-NAME. With the discovery of L-arginine, the pattern of coagulant activity analyzed shown in the activity test. The highest possibility that occurred was, at a concentration of protein isolates between 375 to 1500 ppm. The presence of L-arginine disrupted L-NAME compound procoagulant activity. Whereas in the concentration of protein isolates between 3000 and 6000 ppm, L-NAME can again reach equilibrium which causes the reaction to move towards the formation of fibrin threads.

4. Conclusion

*P. volitans* spine toxins have procoagulant activity, both in intrinsic or extrinsic blood coagulation pathways. Crude venom from *P. volitans* can accelerate blood clotting to 8.5 seconds in addition to a 120 μg sample, whereas protein isolates from *P. volitans* with 80% saturation can accelerate blood clots up to 6 seconds in addition to a 7.5 μg sample. *P. volitans* spines venom has a protein with a molecular weight range of 6-210 kD. *P. volitans* spine venom contains the Nomega-Nitro-L-arginine
methyl ester (L-NAME) compound, which is known to have a procoagulant effect on human blood plasma.

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