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Isolation and Antibacterial Activity of Honey Bee Venom Bioactive from *Apis cerana*

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Abstract. Honey bee venom from Apis species has garnered considerable attention in medicine due to its bioactive, which have antimicrobial activity. This research aims to isolate bioactive melittin and phospholipase A2 (PLA2) of honey bee venom from Indonesian *Apis cerana* and examine the antibacterial activity of these bioactive. Honey bee venoms were isolated from four locations using an electric shock method and purified using Fast Protein Liquid Chromatography. Purified protein was analyzed by SDS PAGE and measured by the Lowry method. Further, the antibacterial activity of melittin and PLA2 against Gram-positive (*Staphylococcus aureus* and *Salmonella typhi*) and Gram-negative (*Escherichia coli*) bacteria was defined by inhibition zone. Our results show that the average of all bee venoms obtained from four locations with eight sampling times was 17.1 mg proteins. Melittin was purified using gradient steps with 55%, 90%, and 100% elution buffer, while PLA2 was purified using 55% elution buffer. Melittin and PLA2 showed molecular weights of less than 7.5 kDa and 19 kDa, respectively. Protein concentrations in melittin and PLA2 were 95 µg/mL and 142 µg/mL, respectively. Among the two bioactive, only PLA2 showed antibacterial activity. The diameter of the inhibition zone was 7.76 mm when the concentration of PLA2 was 45 µg/mL against *E.coli*. This result showed that PLA2 from Indonesian *Apis cerana* has antibacterial activity and may be a promising candidate for the antibacterial agent on Gram-negative bacteria. These findings may contribute to developing new antibacterial substances derived from Indonesian *Apis cerana*.

Keywords: Antibacterial; *Apis cerana*; Honey bee venom; Melittin; PLA2

1. Introduction

Bacteria are microorganisms that are useful in a variety of areas, including food (Ahangari et al., 2021), agriculture (Afzal et al., 2019), and health (Douillard & de Vos, 2019). However, bacteria are frequently recognized as the cause of infectious diseases (Vouga & Greub, 2016). Medicines called antibiotics are used to treat bacterial infections. Antibiotic treatment may become ineffective due to antibiotic resistance (Maglangit et al., 2021). Antibiotic resistance is one of the public health issues that could threaten everyone.

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The impact of this issue increases the morbidity and mortality rate (Frieri et al., 2017). Failure to develop new antibiotics and inappropriate use of antibiotics are factors that accelerate the occurrence of antibiotic resistance (Nwobodo et al., 2022).

Various studies have been conducted to discover new antibacterial compounds (Belete, 2019; León-Buitimea et al., 2020), including antibacterial substances derived from honey bee venom (Frangieh et al., 2019). The honey bee is an economically beneficial insect that is extensively employed as a pollinator in agriculture and generates various natural products (Pucca et al., 2019). These products can be used in medicine, such as honey (Bouacha et al., 2018), royal jelly (Mehrnoush & Darsareh, 2021), pollen (Sevin et al., 2022), propolis (Sahlan et al., 2020), and honey bee venom (Sarhan et al., 2020). Honey bee venom is produced by the venom glands, which are found at the back of the abdomen and are kept in the venom reservoir (Pucca et al., 2019).

The water content of honey bee venom is approximately 88%. The final 12% is made up of peptides (melittin, apamin, and adolapin), enzymes (e.g., Phospholipase A2 or PLA2), biologically active amines, and nonpeptide components (lipid, carbohydrates, and free amino acids). Melittin is the constituent component in bee venom consisting of 26 amino acids. Melittin has a conformation capable of causing damage to the double layer of lipids when binding to lipid cell membranes (Bellik, 2015). Melittin is also known to activate the PLA2 enzyme and work synergistically through enzyme-peptide complexes (Soltan-Alinejad et al., 2022). The PLA2 from bee venom *Apis mellifera* is known to have antimicrobial activity in some Gram-negative bacteria (Boutrin et al., 2008).

Apis cerana is one of the honey bees found in Indonesia (Hadisoesilo, 2001). Harvesting and utilization of honey bee venom still need to be developed in Indonesia. Extraction of venom content from honey bees must be done because of active substances that act as antibacterial. Therefore, this study aims to isolate bioactive melittin and PLA2 of honey bee venom from Indonesian *Apis cerana* and examine the antibacterial activity of these bioactive. This study may contribute to discovering new antibacterial substances obtained from Indonesian *Apis cerana*.

2. Methods

2.1. Honey Bee Venom Collection

Honey bee venom was collected from *Apis cerana*, cultivated in Ciburial Village, Bandung Regency, Indonesia. Electric shocks were used during the harvesting process. The microcomputer bee venom collector was connected with a set of tools consisting of copper, a glass plate, and a pedestal. A microcomputer was used to set the voltage and time interval of the circuit. The bee was given an electric shock and the sting pierced the glass plate. Venom was secreted and dried on a glass plate (Figure 1). Then, in the freezer, venom was kept at a cool temperature.

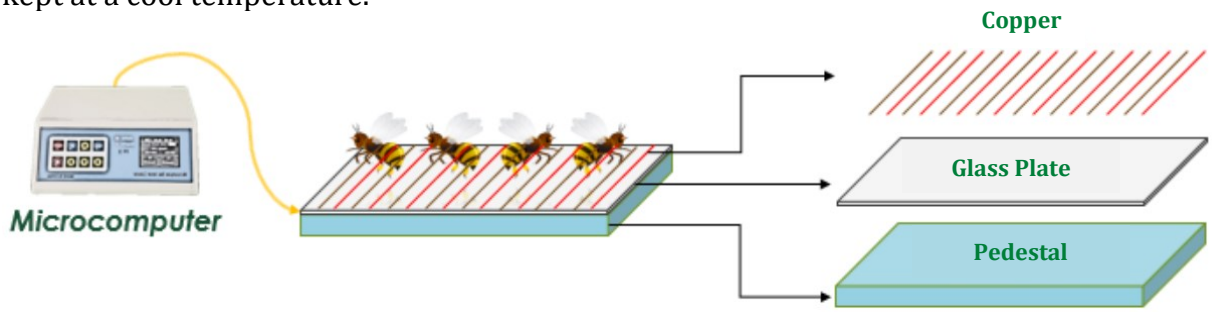

Figure 1 Honey bee venom collector

2.2. Protein Purification from Honey Bee Venom

Crude bee venom was purified based on the literature. Samples were dissolved in deionized water and centrifuged at 3000 rpm for 20 minutes. Centrifugation aims to remove impurity components that are insoluble in water. The venom was then filtered using a 0.2 µm microfilter to remove small debris, pollen, and bee glue in the venom sample. Melittin and PLA2 were then purified using a strong cation exchange column Fast Protein Liquid Chromatography (FPLC) (Teoh et al., 2017). The principle of protein purification by FPLC [Amersham Pharmacia Biotech] is the difference in affinity of the components distributed in the mobile phase and stationary phase and also uses a small diameter stationary phase to achieve high resolution (Walls & Walker, 2017). Melittin has an isoelectric point (pI) of 10 (Habermehl, 2012). The selection of the column and buffer aims to bind the target protein to the column. pH conditions below pI cause positively charged proteins and bind to cation exchangers (Jungbauer & Hahn, 2009). In this study, 50 mM pH 6.0 sodium phosphate buffer was used as the binding buffer and 1 M NaCl in sodium phosphate buffer was used as the elution buffer. The elution process was carried out in stages, starting from 55%, 90%, and 100%.

2.3. Protein Analysis

Qualitatively protein purity was tested using the Sodium Dodecyl Sulphate Polyacrylamide Gel electrophoresis (SDS-PAGE) with Coomassie blue staining method. Protein concentrations were tested quantitatively using the Lowry assay (Redmile-Gordon et al., 2013), and bovine serum albumin was used as standard. The absorbance was read at 650 nm using a spectrophotometer [NanoDrop].

2.4. Antibacterial Activity Assay

Staphylococcus aureus (Gram-positive), *Salmonella typhi* (Gram-positive), and *Escherichia coli* (Gram-negative) were used to study the antibacterial activity as these bacteria were used in previous studies (Omar et al., 2020). Before testing, all microorganisms were freshly produced. The disc diffusion method assessed the antibacterial activity of crude bee venom, melittin, and PLA2. Tetracycline, amoxicillin, and chloramphenicol were used as positive controls for *S. aureus*, *S. typhi*, and *E. coli*. All bacteria were incubated at 30^{o} -35^{o} C for 18-24 hours. The inhibition of microbial growth was measured edge to edge across the zone of inhibition over the centre of the disk.

3. Results and Discussion

3.1. Honey Bee Venom Collection

Honey bee venom was harvested four times at different locations. In the first location, honey bee venom was harvested at the original location of the bee colony, Ciburial Village. Variation of electrical impulse shock was applied (3.2 V, 4.8 V, and 5.2 V) and set for 5, 10, and 30 minutes. The optimum duration for honey bee venom collection was 3.2 V for 10 minutes because not many bees fainted or died after an electric shock. Honey bee venom was harvested at 28.2 mg.

The second and third locations were at the Universitas Indonesia (UI). The venom obtained was 15.6 mg and 5.8 mg. There was a significant decrease in the amount of venom obtained in the third harvest due to the need for more time for the colony's adaptation from Ciburial Village to the UI.

Ciburial Village represented the fourth location, where 87.3 mg of poison was collected. The average of all acquired poisons is 17.1 mg. A previous study obtained 24-164 mg of poison from *Apis mellifera* from the harvest during the day and summer (Sanad et al., 2013). The results obtained in this study were less than the previous study (Table 1) because the

type and size of colonies used were different, as well as the harvest time between noon and evening.

3.2. Protein Purification from Honey Bee Venom

Crude bee venom was purified based on the literature (Teoh et al., 2017). Figure 2 shows several peaks of absorbance values when the concentration gradients of buffer B are 0%, 55%, 90%, and 100%. The absorbance value is measured at a wavelength of 280 nm because the protein absorbs a wavelength of 280 nm due to the presence of amino acids, such as tryptophan, tyrosine, and cysteine (Reinmuth-Selzle et al., 2022). Therefore, the high absorbance value indicates the presence of proteins through the column, then measured by a UV monitor.

Table 1 Parameters for collection and results of honey bee venom

Location	Level / Voltage	Interval ON (s)	Interval OFF (s)	Time (min)	Colony	Sampling	Results (mg)
(Sanad et al., 2013)	-	-	-	-	-	1x	24-164
Bandung	10-75 / 3.2-5.2 V	4	4-8	5-30	5	1x	28.2
UI (EngPark)	10 / 3.2 V	4	8	10	1	3x	15.6
UI (ILRC)	10 / 3.2 V	4	8	10	1	3x	5.8
Bandung	10 / 3.2 V	4	8	10	6	1x	87.3

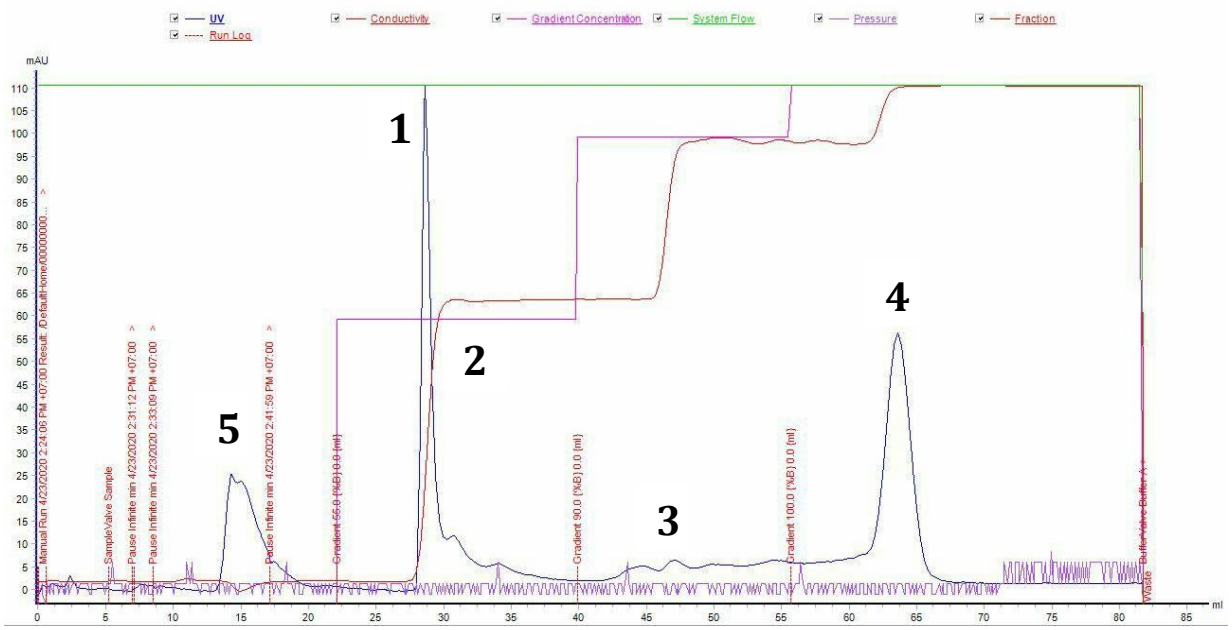

Figure 2 FPLC chromatogram of crude venom protein separation

Five samples (numbers 1 to 5 in Figure 2) were taken from the FPLC fractions for SDS-PAGE analysis (Figure 3). FPLC was used in this study because it was designed for proteins and the availability of stationary phases in the most common chromatography modes. Furthermore, an FPLC column costs approximately ten times cheaper than an HPLC column (Walls & Walker, 2017).

There are various molecular size proteins in the lane CV because bee venom has a lot of protein content and size varies from small to large. Two bands were observed at approximately 19 kDa in lane 1, indicating the PLA2. It was purified using 55% elution buffer. Bellik's research showed that 40-50% of the dry weight constituents of *Apis cerana* honey

bee venom is melittin at 2.8 kDa and was observed in this study (lane 4 in Figure 3, less than 7.5 kDa). It shows that melittin was purified using elusion gradient steps ranging from 55%, 90%, and 100%. The protein bands in lanes 1 and 4 demonstrate that PLA2 and melittin were successfully purified from honey bee venom in this study.

The total protein concentration of the purified sample was obtained by measuring protein absorbance at 650 nm. The results of measuring protein concentrations in crude venom, melittin, and PLA2 were 1373.656; 95.432; and 142.034 µg/ml, respectively. These results show that the largest protein concentration is crude venom because it was not purified. Protein samples were then used for antibacterial tests with various concentrations.

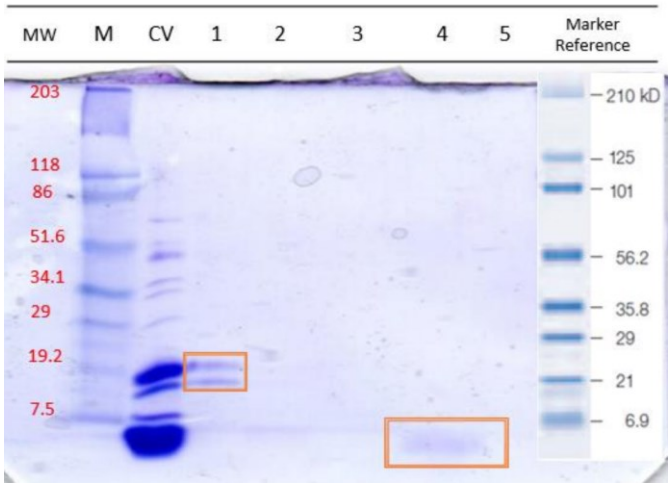

Figure 3 SDS PAGE gel of samples from the protein purification. Lane MW: size of molecular weight markers in kDa; Lane M: protein markers; Lane CV: crude venom; Lane 1 to 5: purified protein from FPLC method

3.3. Antibacterial Activity Assay

An assay of antibacterial activity was performed in triplicate. The bacteria used are *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli*. These bacterial strains were selected to represent bioactive testing of bacteria that are distinguished by their cell wall constituents. *S. aureus* and *S. typhi* are Gram-positive, while *E. coli* is a Gram-negative. In addition, these bacteria are widely found to be the cause of human disease infections. Tetracycline, amoxicillin, and chloramphenicol were used as antibiotics on positive control assays in *S. aureus*, *S. typhi*, and *E. coli*, respectively.

The bioactive tests were crude venom, melittin, and PLA2. Sample concentrations were varied, ranging from low to high concentrations. Crude venom samples were used at 0.1; 0.5; 1; 5; 10; and 150 µg/mL, melittin samples were tested at 0.1; 0.5; 1; 5; 10; and 40 µg/ml, and PLA2 sample was used only at 45 µg/mL. The antibacterial activity assay against Gram-positive bacteria showed that the inhibition zones produced by tetracycline and amoxicillin were 20.1 and 33.95 mm, respectively. This study showed that there was no inhibition against Gram-positive bacteria. The antibacterial activity test against Gram-negative bacteria showed that the inhibition zone by chloramphenicol and PLA2 was 18.8 mm and 7.76 mm, respectively (Table 2). This study showed that antibacterial activity is only on the bioactives of PLA2 against Gram-negative bacteria (Figure 4). The previous study showed that PLA2 from *Acanthaster plancii* and snakes showed antibacterial activity. This enzyme was known to react with phospholipid and change it into phosphodiester and fatty acid. This reaction yielded conformation loss on the cell membrane. Therefore, the bacteria or cells with cell membranes become broken and inactive. Meanwhile, for other compounds,

it will use different activities. It tried to attach to the membrane and make the hole. There is the possibility that this compound needs to specifically bind to a specific protein absent in bacteria (Ibrahim et al., 2013). However, further investigation is needed. The PLA2 may be applied using various extracts from these studies (Gunawan-Puteri et al., 2021; Haryuni et al., 2019).

The bioactive honey bee venom from *Apis mellifera* has antibacterial activity against Gram-negative and Gram-positive bacteria. Activity against Gram-negative bacteria has a more significant inhibition zone than Gram-positive bacteria because Gram-negative bacteria have less peptidoglycan (Zolfagharian et al., 2016). In other microorganisms, 6.29 µg of PLA2 from *Acanthaster plancii* can inhibit *S.aureus* (Gram-positive) by 8.5 mm (Ibrahim et al., 2013) and PLA2 from *Pterois volitans* has antibacterial activity against *S. aureus* with an inhibition zone of 14.35 mm (Sommeng et al., 2020).

Table 2 Results of antibacterial activity assay

Substance Type	Concentration	Inhibition Zone Diameter (mm)			Note
		S. aureus	*S. typhi*	*E. coli*	
Crude Venom	0.1 - 150 µg/mL	0	0	0	-
Melittin	0.1 - 40 µg/mL	0	0	0	-
PLA2	45 µg/mL	0	0	7.76	-
Amoxicillin	1000 ppm	not assayed	33.95	not assayed	Positive Control
Tetracycline	1000 ppm	20.1	not assayed	not assayed	Positive Control
Chloramphenicol	30 µg	not assayed	not assayed	18.8	Positive Control
Negative Control	-	0	0	0	-

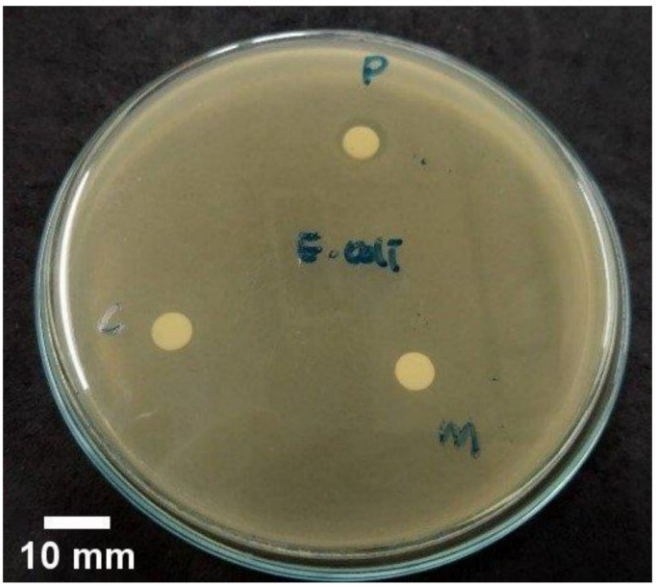

Figure 4 Inhibition zone from antibacterial activity test on *E. coli*; P: PLA2 45 µg/mL; M: Melittin 40 µg/mL; C: crude venom 150 µg/mL

4. Conclusions

In conclusion, bioactive melittin and PLA2 were isolated from Indonesian *Apis cerana*. The FPLC approach, utilizing varying concentrations of elution buffer, is an excellent way to purify melittin and PLA2 from bee venom. Melittin was purified using elusion gradient steps ranging from 55%; 90%; and 100%, while PLA2 was purified using 55% elution

buffer. Melittin and PLA2 were examined for antibacterial activity against Gram-positive (*S. aureus* and *S. typhi*) and Gram-negative (*E. coli*) bacteria. The result suggests that antibacterial activity is only on the PLA2 against *E.coli* by producing an average value of inhibition zone of 7.76 mm.

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