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The Protein Content and Protease Activity of Local Green Fly, *Chloroprocta* sp., Maggot Crude Extracts

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Abstract. Maggot extracts of local green flies, *Chloroprocta* sp., is a potential anti-biofilm candidate who can inhibit biofilm formation, break down the biofilm matrix, and reduce the viability of embedded *Staphylococcus epidermidis*. One component of *S. epidermidis* biomatrix is protein thus protease activity is presumably needed for biofilm detachment. Hence, this study aimed to analyze the protease activity and the protein content of maggot crude extracts from green flies, *Chloroprocta* sp. A quantitative enzymatic assay of protease activity with casein as a substrate was performed with some modifications. The protein content was measured with the Lowry method at a wavelength of 750 nm with tyrosine as a standard. The results revealed that the crude extracts from 0.425 grams maggots /mL PBS has protease activity of 4.875 U/mg and protein content of 29.252 ppm. These results indicated that the crude extract of maggot from local green flies, *Chloroprocta* sp. has protease as the active compound which could potentially be an anti-biofilm.

INTRODUCTION

Biofilm producing bacteria have a significant role as a cause of Healthcare-associated infections (HAIs). The mortality rate caused by HAIs in adult patients from 2003 to 2008 in Asia, Latin America, and Africa was 23.6%, 18.5%, and 29.3%, respectively [1]. The selection of antibiotics that are sensitive and have high penetration ability is very limited due to the increased incidence of antibiotic resistance to biofilm producing bacteria. One ingredient that has been widely investigated for its effect on biofilms and virulence factors is the maggot (larva) of green fly from the family *Calliphoridae* [2]. Maggot that has been studied in Europe comes from green flies *Lucilia sericata* [3], while in Singapore *Lucilia cuprina* is used [4]. Maggot requires a relatively short time to develop which are four days at 20ºC and three days at 27ºC. That condition causes obstacles in purchasing maggot from overseas countries' producers. Thus, the obtainability of indigenous green flies from Indonesia is important for organism alternative.

Recently, Anjarwati et al. [5] have reported that maggot from *Chloroprocta sp*. has anti-biofilm activity towards biofilm produced by *Staphylococcus epidermidis*. *Chloroprocta sp*. is a green fly species found predominantly in Semarang, the capital of Central Java Province, Indonesia. Maggot filtrate from *Chloroprocta sp*. was able to reduce the extracellular biofilm matrix and reduce the viability of biofilms produced by *S. epidermidis* [5,6]. However, the mechanism of biofilm degradation by *Chloroprocta sp.* is still unclear.

Generally, the composition of biofilm matrix is complex and varied among microorganism. The biomatrix composition is also varied even within the same species under different conditions. The essential component of biofilm matrix is exopolysaccharides such as cellulose and the poly-β-1,6-N-acetylglucosamine as the typical

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components [7]. Surface proteins and proteinaceous components are also crucial in biofilm formations that provide biofilm structure in which the bacterial cells are initially attached [8]. Various approaches to treat biofilm have been developed [9]. The treatment of biofilm with broad specificity proteases leads to biofilm disintegration [10]. Another approach to biofilm treatment is using anti-biofilm peptide which inhibits developed biofilm and also destroys multiple species in biofilms [11]. Indeed, maggot extract from *L. sericata* was reported having an active component to overcome the causes of resistance in biofilms which were proteolysis enzymes or proteases, and peptides [12]. Hence, this research aims to identify protease activity and protein content of crude extract of *Chloroprocta sp*. The findings will give an insight on how biofilm disruption by indigenous *Chloroprocta sp*.

MATERIALS AND METHODS

Materials

The green flies *Chloroprocta sp*. was reared in the Laboratory of Nutrition in the Faculty of Agriculture and Animal Husbandry, Diponegoro University, Semarang. Phosphate buffered saline was from Oxoid (Thermo Fisher Scientific, USA). Casein, *Folin Ciocalteau* and L-tyrosine were from Sigma-Aldrich (Germany). Trichloroacetic acid and Na_2CO_3 were from Merck (Germany). Ethanol technical grade and deionized water were from local chemical supplier.

Rearing and Collecting Maggots

The maggots was reared following the methods described by Arora et al. [4] and Anjarwati et al. [6]. The green flies were trapped by placing raw fish in containers. Then, the eggs were collected from the containers and washed three times with sterile deionized water and ethanol to sterilize them. After that, the eggs were allowed to hatch into maggots and collected when they entered the late of second phase or the early third phase of the *Chloroprocta sp.* maggot growth. The maggots were washed three times by using ethanol and sterile deionized water in different containers.

Maggot Extraction

Maggot extraction was performed following the methods described by Honda et al. [13] and Anjarwati et al. [6]. The maggots were extracted by soaking the maggots in the sterile phosphate buffered saline (PBS) pH 7.3 for 1 hour at room temperature (25°C) in the dark. After that, the maggots were incubated in PBS for 48 hours at 37°C. The mixture was centrifuged at 25°C, 10.000 rpm for 15 minutes. The supernatant was collected and sterilized using the syringe filters (0.2 µm, Corning NY 14831). Finally, the maggots' extracts were stored in the freezer at -20°C before further examination.

Determination of Protease Activity

Protease activity was measured following protease activity assay by Sigma [14] in which the casein was used as substrate. 1 mL of maggot crude extract was added to 5 mL of 0.65% casein substrate. 1 mL of distilled water was added to 5 mL of 0.65% casein as blank. The mixture was incubated at 37°C for 10 minutes. Termination of the reaction was carried out through the addition of 5 mL of 110 mM Trichloroacetic acid reagent. After termination, 1 mL of maggot crude extract was added to the blank. Next, the mixture was re-incubated at 37°C for 30 minutes. 2 mL of filtrate was separated by centrifugation at 10,000 rpm for 10 minutes. 5 mL of Na_2CO_3 and 1 mL of *Folin Ciocalteau* reagent were added to the filtrate and the mixture was incubated at 37°C for 30 minutes. The absorbance of the mixture was measured at 660 nm.

Tyrosine standard curve was prepared by reacting 2 mL of standard tyrosine and distilled water (as blank) with 5 mL of Na_2CO_3 and 1 mL of *Folin Ciocalteau* reagent. The mixtures were incubated at 37°C for 30 minutes, then the absorbance was measured at 660 nm. One unit of protease activity is designated as the amount of protease needed to release 1 µmol tyrosine from the casein substrate per minute.

Determination of Protein Content

The protein content was measured following the Lowry method [15]. 0.5 mL of maggot crude extract was added 0.7 mL of Lowry solution and incubated for 20 minutes at room temperature in the dark. Then, 0.1 mL of diluted *Folin Ciocalteau* was added followed by incubation for 30 minutes at room temperature in the dark. After incubation, the samples were measured the absorbance at 750 nm.

RESULTS AND DISCUSSION

Recently, several countries have developed research on greenfly maggots as a therapy in the health sectors. Green fly maggot that has been studied in Indonesia was the genus *Chloroprocta sp.* which is found in the area of Central Java. The Green fly species, *Chloroprocta sp.*, is a subfamily of *Chrysomyinae* from the family *Calliphoridae*, kingdom *Diptera*. The study of Anjarwati et al. [5] suggested that the maggot extract of *Chloroprocta sp*. has a protease component judged by a qualitative protease test using the gelatin hydrolysis method. Maggot extract with protease components in that study could damage extracellular biofilm matrix and reduce embedded *S. epidermidis* viability. This present study further analyzed the protease activity and the protein content of maggot crude extracts from greenflies, *Chloroprocta* sp.

The protein content of maggot crude extract was measured using Lowry method [15]. The Lowry method is a development of the Biuret method. In this method, two reactions are involved. Initially, the Cu (II)-protein complex will be formed as the biuret method, which in the alkaline atmosphere Cu (II) will be reduced to Cu (I). The Cu^{+} ion will then reduce the *Folin-Ciocalteu reagent*, the phosphomolybdate-phosphotungstate complex, resulting in the blue heteropoly-molybdenum due to Cu catalyst aromatic oxidation (amino acid side chain) reaction, which gives intense blue color and can be detected colorimetrically. The strength of blue depends mainly on the content of tryptophan and tyrosine residues. The advantage of the Lowry method is more sensitive (100 times) than the Biuret method so that it requires fewer protein samples. The detection limit ranges from a concentration of 0.01 mg/mL. However, Lowry's method had more interference due to its sensitivity.

The protease activity of maggot crude extract was determined following Sigma's method [14]. The casein was used as a substrate. The proteases hydrolyze casein and release tyrosine as well as other amino acids. *Folin Ciocalteus* reacts mainly with free tyrosine and produce blue colored compounds, which can be quantified by spectrophotometric as absorbance. The more tyrosine released from casein, the more is absorbance value which indicates the higher protease activity. The absorbance values generated by the samples are compared to a tyrosine standard curve to determine the activity of protease samples which is the equivalent amount of released tyrosine in micromoles from casein per minute.

The results of this research showed that 0.425 grams/mL maggot crude extract in PBS has protease activity of 4.875 U/mg and protein content of 29.252 ppm (Table 1).

TABLE 1. Analysis of protein content and protease activity of maggot crude extract only.

	Vol. Enzyme (µL)	Total Vol. (mL)	A 750 nm	A Tyrosine (As - Ab)*	Tyrosine (ppm)	Protease Activity (U/mg Enzyme)
Crude extract	0.1	238	0.888	0.839	29.252	4.875

*Absorbance for Blank (Ab) at 750 nm was 0.049.

The proteases are class of enzymes that hydrolyze peptide bonds. They can act as anti-biofilm due to their ability to disrupt surface proteins in biofilm matrix. Commercial protease enzymes have been reported as a good anti-biofilm agent which target the protein produced by *S. epidermidis* and *Staphylococcus aureus* during early biofilm formation [10]. The anti-biofilm activity was proportional to enzyme quantity. The commercial Flavourzyme which is a combination of exoprotease and endoprotease has anti-biofilm activity specific against *S. epidermidis* at a concentration of 3 U/mL and over [10]. Extracellular protease from actinomycetes strains AN090250 and AN091562 have been found to inhibit *S. aureus* biofilm development and degrade pre-existing *S. aureus* biofilm [16]. The extracellular proteases are known to have a broad substrate specificity hence the enzymes can degrade any protein in biofilm matrix that maintains the biofilm integrity.

In this research, maggot extract of *Chloroprocta* sp. affirmatively contains protein and protease activity. These findings may explain why the extract can reduce biofilms produced by *S. epidermidis* [6, 7]. Proteases in maggot

extract can accelerate the natural release of biofilms by endogenous enzymes. In addition, the biofilm structural damage reduces the ability of biofilms to provide nutrients for embedded bacteria [17]. Further research that can be done includes identification of types of protease in *Chloroprocta* sp., protease effects on biofilm regulator genes, increased protease production in maggot, in vivo tests and clinical trials of maggot extract as anti-biofilm on biofilms produced by monospecies and multispecies bacteria.

CONCLUSION

The maggots extract from indigenous green flies *Chloroprocta sp*. contained protein and protease activity. The ability of protease enzyme to disintegrate peptide bond makes the indigenous green flies *Chloroprocta sp*. as anti-biofilm producing organism to reduce surface protein in biofilm matrix from *S. epidermidis*.

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