Producing armyworm (spodoptera sp.) Bioinsecticide based on cysteine protease of red ginger (zingiber officinale var. Rubrum)

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Abstract. Armyworm (Spodoptera sp.) is highly polyphagous defoliator on various horticulture and grain plants. Various chemical insecticides have been created to control it. There is a need to create an eco-friendly and specific insecticide which only affect armyworm’s nervous system. This research investigates cysteine-protease’s enzyme activity of red ginger (Zingiber officinale var. Rubrum) which is called zingibain. Its catalytic site matches with residue site in armyworm’s body so it can be used as bioinsecticide raw material which meets the criterias above. Fresh red ginger rhizomes were washed and extracted. The juice was then deposited in low temperature and centrifuged to get rid of its starch content. It was filtrated to remove large contaminants and poured into Potassium Phospate buffer. The liquid was then centrifuged again for 30 minutes before collecting the supernatant. Fresh leaves were then dipped into crude ginger protease extract and fed to fourth instar-armyworms. Leaves dipped into non-diluted extract were barely eaten by armyworm while the 50% and 25% dilution was half eaten and most eaten. The crude red ginger extract was not strong enough to kill them although the research showed its enzymatic activity reaches up to 169 PU. It still needs improvement to be produced as commercial bioinsecticide.

Keywords: Acetylcholinesterase (AChE) Inhibitor; Armyworm (Spodoptera sp.); Bioinsecticide; Cysteine Protease; Red Ginger (Zingiber officinale var. Rubrum)

1. INTRODUCTION

Indonesia is an agricultural country that has 8 million hectares agricultural land and will be expanded 500 thousand hectares due to national food self-sufficiency plan [1]. Beside allocating some space for agricultural land, food self-sufficiency needs supporting factor such as insecticide. In the other hand, chemical insecticide usage can polute environment, mutates insects become resistant or tolerant [2], degrades the quality of surface water [3], accumulated in mammals body, and exposes human fetus through pregnancy period [4]. Those side effects can be avoided by replacing chemical insecticide with bioinsecticide which specifically affects certain insect. So that, it less harm to environment, under water-living organisms, mammals, and human.

Armyworms become the object of this research because its polyphagous characteristic [5]. It destroy plants by eating every part of leaves included its tissue, bone (midrib), and vein. Armyworm’s life cycle starts from egg which laid down in groups which consist of up to 300 eggs each group and only
needs at least 30 days to repeat its cycle. Armyworms active at night while hiding during the day. So that, its activity barely detected by farmer. A plant that attacked by armyworms can lose most of its leaves in a night and gradually wilt and die.

Red ginger (Zingiber officinale var. Rubrum) is known for its powerful proteolytic enzyme called zingibain. Its enzyme is included as cysteine proteases and match perfectly with armyworm’s (Spodoptera sp.) catalytic site in nervous system [4]. In addition, red ginger can be found relatively easy in Indonesia. That makes red ginger’s enzyme is potential to be used as the main material of bioinsecticide [6] to attack armyworm.

The test result shows that dipped-leaves were less favored than the negative control (leaves which wasn’t dipped into red ginger extract). The leaves was dipped into red ginger extract with three various concentration such as 25%, 50%, and 100%. Leaves which was dipped into 25% concentration remained the least leaves, the 50% concentration remained half leaves, and the 100% concentration barely eaten by armyworms.

2. MATERIAL AND METHODS
This research was conducted at Bioprocess Laboratory, Chemical Engineering Department, Engineering Faculty, University of Indonesia, Depok. Fresh red ginger rhizomes were purchased from local market in Depok, armyworms were purchased from Bogor Agricultural University, tyrosin, caseine, and trichloroacetic acid (TCA) (technical grade) were purchased from PT. Pasifik Kimia Indonesia.

2.1. Extraction of crude red ginger cystein protease (zingibain)
800 g fresh red ginger rhizomes were cleaned and wind dried then be shredded and filtered to obtain its 100% concentration [6]. Then Potassium phospate buffer 0.1M, pH 7, was added to crude extract with 1:1 ratio to keep the pH neutral. Crude extract was centrifuged at 4600 rpm for 30 minutes to get rid of its starch [7]. The concentration was then diluted with demineral water to a concentration of 50% and 25%.

2.2. Ginger protease activity determination
The definition of protease activity is under a certain temperature the concentration of protease which is used to produce 1µg tyrosine by hydrolyze casein. The steps of determining the activity of ginger protease is as following:

Mix 1 ml of crude extract and 1 ml of 0.5% casein then the solution is incubated for 10 min at 37°C. The Assay is stopped by adding 1 ml of 0.4 M TCA [7]. The color reaction was observed by pouring 2 ml of extract-casein-TCA solution with 6 ml of biuret and 0.3 ml Folin-Ciocalteau reagent. Then using the solution of blank group to adjust the extinction, and measured the absorbance at 750 nm. The absorbances were compared with free tyrosin standard curve which was made by checking the absorbance of free tyrosin in 20, 40, 60, 80, and 100 ppm. The formula of protease activity calculation is:

\[ C_t = \frac{A_t}{A_{std}} \times C_{std} \]  

Where  
\( C_t \): Concentration of free tyrosin in crude extract (ppm)  
\( C_{std} \): Concentration of free tyrosin in standard curve (ppm)  
\( A_t \): Crude enzyme absorbance  
\( A_{std} \): Tyrosin standard absorbance
2.2.1. Incubation time determination
Incubation time was considered to obtain the highest enzyme activity of crude red ginger extract. The mixed solution of extract-casein was incubated in 37°C for 10, 20, and 30 minutes. These solutions were measured the absorbance with UV-Vis spectrofotometer.

2.3. Organoleptic and lifetime test
Organoleptic and lifetime test was conducted to observe if there’s any physical changes during the storage time and condition. The organoleptic test was done everyday for two weeks while lifetime test was done everyday for 4 days.

Crude extracts without dilution was placed in different temperature storage (10°C, 27°C, and 37°C) and observed for its smell and colour changes. To make sure that the crude extracts still had protease activity, the solutions (25%, 50%, and 100% concentration) were tested by colour reaction and absorbance test which had been mentioned above.

2.4. Efficacy test
Efficacy test was the major test of this research which observed the correlation between dipped-leaves with remaining leaves uneaten. The test conducted by dipping 5 leaves into each concentration of crude extract (25%, 50%, and 100%) plus negative control. Each day for 3 days, the leaves were observed and replaced by the new one while armyworm’s movement was observed.

3. RESULTS AND DISCUSSION
3.1. Protease activity assay
3.1.1. Incubation Time Determination
Incubation time determination was done before doing protease activity assay. The determination of optimum incubation time was the least time with the highest activity. The result shown on the Table 1 below:

| No. | Incubation Time (minutes) | Protease Activity (ppm) |
|-----|--------------------------|-------------------------|
| 1.  | 10                       | 82.7159                 |
| 2.  | 20                       | 81.233                  |
| 3.  | 30                       | 81.5897                 |

The result shows that there’s only slight different of protease activity among the variables (10, 20, and 30 minutes). It seems that incubation time didn’t really affect protease activity as long as it was incubated in enzyme’s optimum temperature (37°C). Various amount of protease activity among the variables might be caused by the solutions (crude extract-casein) didn’t homogenize the exact same way. But since 10 minutes incubation showed the highest protease activity with the least time, there’s no need to incubate the extract for longer time.

3.1.2 Protease Activity
Protease activity for each concentration and each storage condition is provided in Table 2 below:

| No. | Storage time | Protease Activity (ppm) |
|-----|--------------|-------------------------|
|     |              | 37°C | 27°C | 10°C |
| 1.  | Day 1        | 25%  | 50%  | 100% | 25%  | 50%  | 100% | 25%  | 50%  | 100% |
|     |              | 32.19| 57.88| 168.4| 12.82| 58.36| 151.9| 21.61| 33.47| 67.35|
Those data are visualized in graphics for better comparison. These are the graphics:

**Figure 1.** Protease Activity that Stored in 37°C

**Figure 2.** Protease Activity that Stored in 27°C

**Figure 3.** Protease Activity that Stored in 10°C

From data above, there were inconsistency of protease activity measured. The data should be steady or decreasing since there must be denaturation within the crude extract. But since each sample was analyzed by its absorbance through UV-Vis spectroftometer the denaturation was not detected.
Meanwhile, colour reaction test by Lowry method gave better understanding of what happened with those samples. Theoretically, peptide bonds of protein react with copper under alkaline conditions to produce Cu$^+$ which reacts with Folin-Ciocalteu reagent [8]. The reactions result in a blue colour. In first day of measurement, every sample turned into blue due to colour reaction test. But since the third day, samples which stored in 27$^0$C and 37$^0$C turned into greenish. Proved that there’s chemical changes of enzyme within the crude extract. The changes of colour reaction test in first day and third day is shown in Figure 4 below:

![Figure 4](image.png)

**Figure 4** Colour reaction test, colour changes in: (a) first day (b) third day

The colour reaction test result doesn’t neglect protease activity assay through UV-Vis spectrofotometer but completes the understanding that the samples still had protease activity. But the activity was not as good as it was first which strongly indicated denaturation process.

3.2 Organoleptic test

Organoleptic test was a qualitative test which measured subjectively by author’s part of body such as nose and eyes. Parameters were made so that the qualitative data can be quantified. The parameters are shown in Table 3 below:

| No. | Value | Smell Parameters       | Colour Parameters          |
|-----|-------|------------------------|----------------------------|
| 1.  | 5     | Fresh and Strong       | Brown and Homogen          |
| 2.  | 4     | Fresh, less strong     | Brown, little sediment     |
| 3.  | 3     | Fresh                  | Brown, much sediment       |
| 4.  | 2     | Acidic                 | Blackish brown             |
| 5.  | 1     | Highly acidic           | Blackish brown, much sediment |

Result of organoleptic test is shown on the Figure 5 below:

![Figure 5](image.png)

**Figure 5** Organoleptic Test: Smell & Colour changes
Organoleptic test was conducted to know the lifetime of crude red ginger extract. The result shows that the extract which stored in 10°C had the longest lifetime (longer than 15 days). Crude extract that stored in 27°C and 37°C only last for 3 days before its smell and colour got worse. Smell and colour changes in this test shows the declining quality of protein content in each extract. These data strengthen the understanding of denaturated enzyme which estimated in the discussion of protease activity assay.

Extract which stored in 10°C had the longest lifetime because denaturation process was slowed down. In the other hand, denaturation process gets faster in room temperature (27°C) and enzyme’s optimum temperature (37°C). From the organoleptic test, lifetime of crude red ginger extract in various temperature can be assumed and the storage condition can be determined.

3.3 Efficacy test
Result of efficacy test is shown on the Figure 6 below:

![Figure 6 Remaining leaves dipped into 100%, 50%, 25% crude extract and negative control](image)

**Table 4** Amount of armyworms alive during the experiment

| No. | Crude extract concentration | Amount of died-larvae | Mortality |
|-----|-----------------------------|-----------------------|-----------|
| 1   | Negative control (-)        | 0                     | 0%        |
| 2   | 25%                         | 1                     | 20%       |
| 3   | 50%                         | 1                     | 20%       |
| 4   | 100%                        | 0                     | 0%        |

Based on the observation done, leaves that dipped into various concentration of crude red ginger extract are eaten and affect the armyworm. It shows that leaves which were dipped into 25%, 50%, and 100% concentration gradually eaten out. From the third day, most of armyworms entered the fourth instar so that there were significant area of eaten-leaves. Only 25% and 50% concentration can kill the worm with 20% mortality. The efficacy test needs to be done to third instar-armyworm or below which armyworm’s immune system still develop and not complete yet.

4. Conclusion
Crude red ginger extract’s protease activity assay was best conducted with 10 minutes incubation time. Spectrofotometer absorbance shows that extract stored in 27ºC-37ºC had more protease activity than the
one stored in 10⁰C. Protease activity of crude red ginger extract reaches up to 169 ppm (in 27⁰C). In the other hand, organoleptic test shows that crude red ginger extract which stored in 10⁰C hadn’t spoiled for 14 days or more. So, crude red ginger extract is best to be kept in 10⁰C and pretreated 10 minutes in 27⁰ or 37⁰C before used. The efficacy test shows that the dipped-leaves affected the armyworms with 20% mortality. Crude red ginger’s low purity level can be the cause of the low mortality.

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