



## Development of Enzyme Production Technology with Double Protease Immobilization Method (Calotropin and Papain)

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**Abstract**—Enzymes are biocatalysts widely applied in agriculture, food processing, chemical industries, and medical fields. Enzymes can be used singly or immobilized within a matrix. However, free enzymes often exhibit lower stability compared to immobilized enzymes. Therefore, this research aimed to evaluate the combined two proteolytic enzymes for extracting protein hydrolysates. The objective was to analyze enzymes' immobilization characteristics using carrageenan and determine the optimal concentration combination. Calotropin and papain were applied at concentrations of 2.5 mL, 3.5 mL, and 4.0 mL, respectively. The analysis parameters included yield, enzyme activity, soluble protein content, specific enzyme activity, stability, and kinetic parameters ( $K_m$  and  $V_{max}$ ). The results showed that the combined proteases exhibited yields ranging from 44.43% to 48.63%; enzyme activities of 9.23–53.63 U/mL; soluble protein contents of 2.40–2.89 mg/mL; and specific enzyme activities of 3.54–18.62 U/mg protein. The enzymes were maintained stability at temperatures ranging from 30 to 60 °C. The kinetic parameters were determined as  $K_m = 0.0034$  g/mL and  $V_{max} = 17.094$  U/min. The best immobilization is calotropin 4 ml and papain 4 ml with stability at temperatures of 30–50°C.

**Keywords**— Calotropin, papain, immobilization, carrageenan, enzyme.

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### I. INTRODUCTION

Enzymes are biocatalysts widely utilized across various sectors, including agriculture, food, chemicals, and medicine. Enzymes are environmentally friendly and have beneficial properties, namely efficient, selective, and predictable reaction processes without by-products. According to [1], the global use of enzymes has been increasing at 10–15% annually. [2] stated that approximately 59% of industrial applications involve enzymes, particularly proteases. According to [3], enzyme utilization is projected to grow by USD 5 billion, with an estimated annual demand increase of around 4%. Indonesia has plants that produce protease enzymes and must optimize its potential and production to minimize imports. Tropical plants that have great potential to be developed as protease producers include calotropin (*Calotropis gigantea*) and papaya (*Carica papaya*).

Enzyme applications in industry can involve free enzymes or enzymes immobilized within a matrix. The use of free enzymes that are dispersed or suspended in a substrate medium is often less stable than immobilized enzymes. [4] Immobilized papain exhibited relatively constant activity over repeated use compared to the free enzyme. Immobilization technology using carrageenan has been carried out by [5], which showed that the higher the concentration of carrageenan, the more stable the enzyme. [6] reported that the activity of immobilized calotropin was 7.39 U/ml and that of immobilized papain was 6.37 U/ml.

Given the growing demand for calotropin and papain, innovative approaches are required to ensure sustainable availability. One-way strategy combines a single protease into a double protease using an entrapping immobilization technique with carrageenan. Therefore, this research aimed to analyze the characteristics of calotropin and papain immobilization and determine the best treatment.

## II. MATERIALS AND METHODS

### A. Materials

The main materials consisted of calotropin obtained from Watu Ulo, Jember, East Java, Indonesia, and papain from Bedadung Pakusari, Jember, East Java, Indonesia. The materials for analysis consisted of: Na<sub>2</sub>CO<sub>3</sub>, CuSO<sub>4</sub>, Na-tartrate, mix Lowry reagent, distilled water, ammonium sulfate, Follin, trichloroacetic acid (TCA), NaOH, phosphate buffer pH 7, tyrosine, and casein.

### B. Methods

#### Production of enzyme [7]

The crude enzyme was mixed with 0.05 M phosphate buffer, pH 7, in a 1:1 ratio, and continued with the degumming process using a cold centrifuge (40°C, 8000 rpm, for 10 minutes). The supernatant was separated from sediment, which mostly contained gum.

#### Double protease production [8]

The concentration of calotropin used was 2.5 ml, 3.5 ml, and 4 ml, while papain was 2.5 ml, 3.5 ml, and 4 ml. The combination of papain and calotropin enzymes was then added with carrageenan in a ratio of 1:1. The mixture was stirred and put into a petri dish and then heated for 4 hours in a vacuum oven at 50°C. The last step was crushing the dry ingredients using a mortar.

### C. Parameters

#### Yield [9]

The yield calculation was conducted by weighing the enzyme before immobilization (enzyme + wet carrageenan) and comparing it with the weight of the enzyme after immobilization (enzyme + dry carrageenan), then multiplying by 100%. The yield calculation uses the following formula:

$$\% \text{ Yield} = \frac{A}{B} \times 100\% \quad \text{eq (1)}$$

Description:

A : enzyme after immobilization (grams)

B : enzyme before immobilization (grams)

#### Enzyme activity [10]

There are three treatments in enzyme activity, namely blank, standard, and sample. In the sample, 0.1 mL of enzyme solution was put into a test tube and, added 0.5 mL of 2% w/v casein and 0.5 mL of buffer phosphate pH 7. In the blank and standard, the enzyme was replaced with distilled water and 0.1 mM tyrosine. The solution was incubated at 37°C for 10 minutes. The hydrolysis reaction was stopped by adding 1 mL of 0.1 M TCA (trichloroacetic acid). In the blank and standard, 0.1 mL of enzyme solution was added, while in the sample, 0.1 mL of distilled water was added. The solution mixture was re-incubated at 37°C for 10 minutes, followed by centrifugation at 1000 rpm for 10 minutes. A total of 0.75 mL of supernatant was added to a test tube containing 2.5 mL of 0.4 M Na<sub>2</sub>CO<sub>3</sub>, and 0.5 mL of Follin Ciocalteu reagent was added and incubated

at 37°C for 20 minutes. The incubation results were measured using a spectrophotometer at  $\lambda = 670$  nm. Protease enzyme activity is calculated using the formula:

$$UA = \frac{(A_{sp} - A_{bl})}{(A_{st} - A_{bl})} \times p \times \frac{1}{T} \quad \text{eq (2)}$$

Description:

Asp : Sample absorbance

Abl : Blank absorbance

Ast : Standard absorbance

P : Dilution factor

T : Incubation time

#### Soluble protein [11]

A 0.05 g sample was dissolved in 10 ml of distilled water. The sample was centrifuged for 5 minutes; 0.125 ml was taken and reacted with 2.5 ml of Mix-Lowry reagent for 10 minutes in the dark. 0.25 ml of Follin was added for 30 minutes, and then distilled water was added up to 5 ml. The absorbance was measured at a wavelength of 750 nm. The absorbance data were plotted on a standard curve of BSA (*Bovine Serum Albumin*) to calculate the soluble protein content.

#### Specific activity of enzymes [12]

The specific activity of enzymes is obtained by dividing the enzymes activity with the protein content. The specific activity of the enzyme indicates that the protein produced is the desired target protein. The specific activity of the enzyme is calculated based on the formula:

$$\text{Specific activity of enzymes} = \frac{\text{enzyme activity}}{\text{protein content}} \quad \text{eq (3)}$$

#### Temperature stability of enzyme [10]

Enzyme stability was carried out on the measurement of the optimal temperature required for enzymatic reactions (30, 40, 50, 60, and 70 °C). The test steps used were as in determining enzyme activity. The stability of the protease enzyme to temperature was measured by incubating the enzyme solution for 80 minutes at varying temperatures (30, 40, 50, 60, and 70°C).

#### Km/Vmax [13]

Enzyme kinetic parameters (Km and Vmax) are based on the relationship between substrate concentration (S) and enzyme activity. The substrate solutions were prepared with concentrations of 0.02, 0.01, 0.0075, 0.005, and 0.0025 grams, and enzyme activity was determined using a buffer phosphate pH 7. After that, a table (V) and (S) was made and changed to 1/V and 1/S to calculate the Km and Vmax values. The Vmax and Km values are obtained from the 1/Vmax and 1/Km values.

### D. Data analysis

The data obtained were processed using Microsoft Excel 2016 and analyzed using the Analysis of Variance Test (ANOVA) at a significance level of 5%. If significantly different, the results are then continued with the DMRT (Duncan Multiple Range Test). The data obtained are presented in the form of tables and bar charts to make it easier to analyze the data.

### III. RESULT AND DISCUSSION

#### E. Yield

The yield of the double protease enzyme ranges from 44.43% to 48.64%. The results of the yield test are in **Figure. 1**.

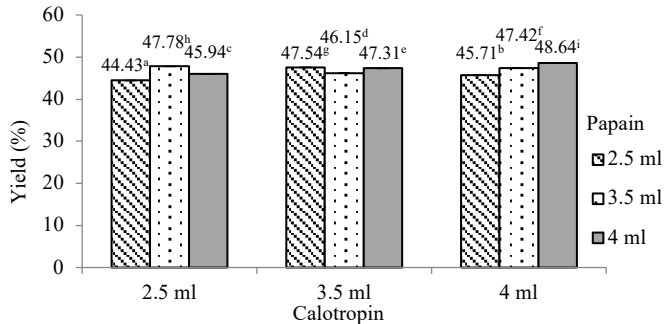


Fig. 1 Yield of double protease enzymes

Based on ANOVA analysis, the calotropin and papain concentrations significantly affected the yield. The highest yield (48.64%) was obtained at a concentration of 4.0 mL calotropin and 4.0 mL papain, while the lowest yield (44.43%) was observed at 2.5 mL calotropin and 2.5 mL papain. The proportion of enzyme concentrations strongly influences the yield value. The use of papain: calotropin enzyme (60:40), produced the highest yield (75.18%) in smart flavor catfish [14]. [16] stated that using calotropin and papain (without combination) resulted in yields ranging from 37.23% to 41.30%. The results of this research indicate that combining calotropin and papain through double protease immobilization leads to an increase in yield. The use of higher enzyme concentrations enhances the role of carrageenan as an immobilization matrix. According to [15], carrageenan can encapsulate enzymes effectively, reduce free water content and protein precipitation during drying. Enzymes that are optimally entrapped within the carrageenan matrix tend to exhibit higher yields [15].

#### F. Enzyme activity

Enzyme activity was carried out to determine the ability of double protease enzyme combination to hydrolyze proteins into peptide bonds and simple amino acids [8]. The value of double protease enzyme activity is shown in **Figure. 2**.

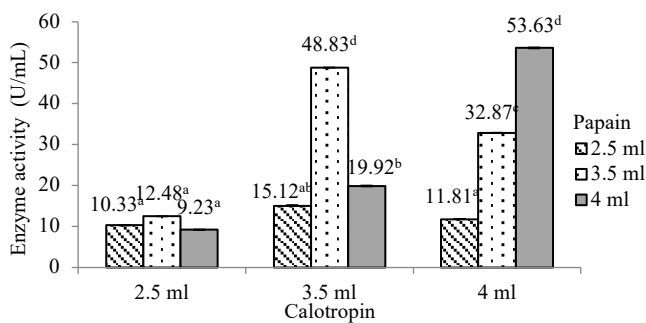


Fig. 2 Enzyme activity of double protease

Based on ANOVA analysis, the calotropin and papain concentrations significantly affected the enzyme activity. The highest activity (53.63 U/mL) was observed at 4.0 mL calotropin and 4.0 mL papain, while the lowest (9.23 U/mL) occurred at 2.5 mL calotropin and 4.0 mL papain. Enzyme concentration plays a critical role in determining the activity level. In general, increasing enzyme concentration proportionally enhances enzyme activity up to a certain optimal point. According to [16], the ratio of calotropin and papain (50:50) is the optimum concentration that can produce the highest degree of hydrolysis (82.32%). Low enzyme concentration will also low enzyme activity. Because the enzyme is unable to survive at ambient temperatures during the immobilization process, carrageenan may be unable to entrap the enzyme effectively, and partial denaturation may occur at 50°C. Furthermore, combinations with lower concentrations of calotropin than papain tend to yield lower enzyme activity. [8] stated that calotropin activity is higher than the papain enzyme.

Another factor that influences is the enzymatic mechanism, because calotropin is exopeptidase and papain is endopeptidase. The exopeptidase-type enzyme breaks down protein structures from the outside, producing smaller fragments than the endopeptidase-type enzyme, which breaks down proteins from the inside [8]. These functional differences may influence the efficiency and pattern of protein hydrolysis in the combined enzyme.

#### G. Soluble protein

The soluble protein of immobilized double protease can be seen in **Figure. 3**.

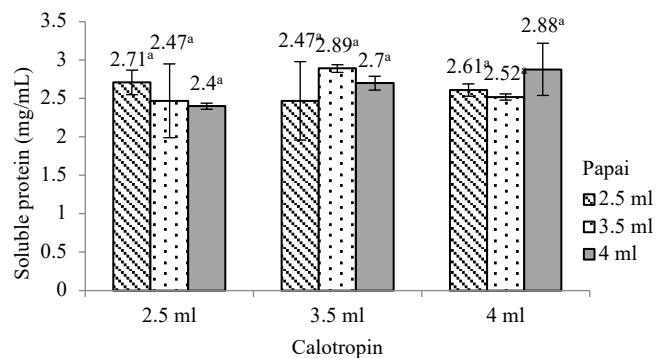


Fig. 3 Soluble protein of double enzyme

The highest soluble protein was in the treatment of 3.5 mL calotropin and 3.5 mL papain (2.89 mg/mL) and 4 mL calotropin and 4 mL papain (2.88 mg/mL), while the lowest was in the treatment of 2.5 mL calotropin and 4 mL papain (2.4 mg/mL). However based on ANOVA, the difference calotropin and papain concentrations did not significantly affect the levels of soluble protein. The combination of enzymes can increase the soluble protein. This enhancement is likely due to the synergistic of calotropin (exopeptidase) with papain (endopeptidase) which together accelerate peptide bond hydrolysis, leading to increased production of soluble proteins

[17]. In a previous study, a 50:50 ratio of calotropin and papain in tempeh hydrolysate the highest soluble protein content (58.85%) [16]. Similarly, [17] reported that the combination of calotropin and papain can increase soluble protein levels in flavor enhancer of sword bean protein.

#### H. Double enzyme specific activity

According to [18], the higher specific activity, the greater amount of enzyme that is successfully precipitated compared to other proteins at a certain saturation level. The specific activity of double protease enzyme is shown in **Figure. 4**.

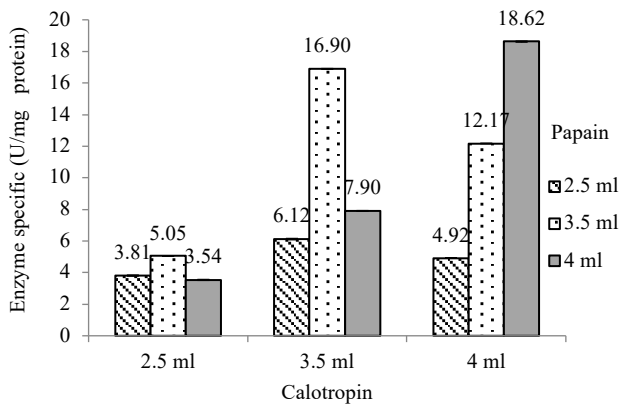


Fig. 4. Enzyme specific of double protease

The highest specific enzyme activity was observed in the treatment with 4.0 mL calotropin and 4.0 mL papain, reaching 18.62 U/mg protein. The lowest specific activity (3.54 U/mg protein) occurred in the combination of 4.0 mL calotropin and 2.5 mL papain. Based on **Figure. 4**, the second highest is 3.5 ml calotropin and 3.5 ml papain at 16.90 U/mg protein. These results are consistent with enzyme activity, where both treatments showed high overall activity. Since specific activity is calculated by dividing enzyme activity with soluble protein, higher enzyme activity with relatively lower protein content leads to higher specific activity values. According to [19], specific enzyme activity is strongly correlated with enzyme activity; greater enzyme activity typically results in higher specific activity. Moreover, higher specific activity is indicative of greater enzyme purity. As reported by [6], the specific activities of immobilized protease enzymes without combination were 15.46 U/mg protein for papain and 13.53 U/mg protein for calotropin when immobilized using maltodextrin. These findings suggest that the combination of calotropin and papain, particularly at higher concentrations, can enhance the specific activity beyond the levels achieved with individual enzymes.

#### I. Temperature stability of enzyme

Enzyme stability was conducted to determine the stability of enzyme activity at its optimum temperature. Based on enzyme stability analyzed at five temperature levels, a curve is obtained as in **Figure. 5**.

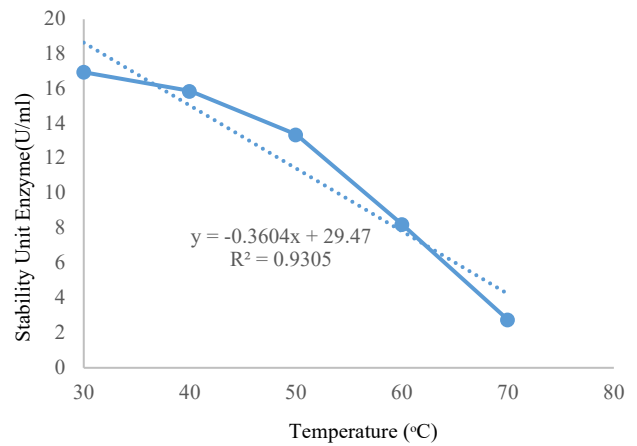


Fig. 5 Temperature stability of enzyme

Enzyme stability against temperature was evaluated using the treatment of 4 ml calotropin and 4 ml papain. Based on **Figure. 5**, the stability of the double protease enzyme decreased with increasing temperature. The decreased enzyme stability is caused by active group starting damage. According to [20], enzymes possess various chemical bonds including hydrogen bonds, ionic and hydrophobic interactions, that help maintain their structural integrity under normal conditions. However, at elevated temperatures, these bonds and active groups may break, leading to enzyme denaturation. Denaturation alters the conformation of the active site, which in turn reduces the enzyme's catalytic activity.

The double protease enzyme is relatively stable at temperature of 30-50 °C. However, at 60-70°C enzyme stability began to decline. The reduced activity observed at higher temperatures is likely due to enzyme denaturation. According to [6], the stability of calotropin with 50% corn starch immobilization and 22.50% maltodextrin is relatively stable at a temperature of 50-60°C with an enzyme activity ranging from 5.10 to 4.89 U/mL, and at a temperature of 70°C there is decrease in enzyme activity, namely 1.56 U/mL. Carrageenan as an immobilization matrix plays an important role in enzyme thermal stability. According to [15], enzymes immobilized with carrageenan are more resistant to elevated temperatures because they are trapped within the gel pores of carrageenan, which provides strong structural support and can withstand high thermal conditions.

#### J. Km/Vmax

The Km and Vmax of the double protease combination were determined based on enzyme activity at various substrate concentrations. The Lineweaver-Burk transformation results are shown in **Figure. 6**.

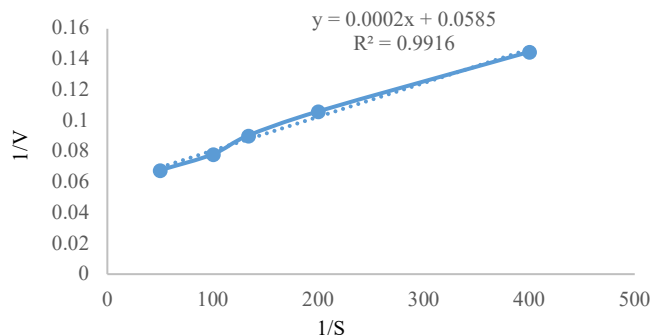


Fig. 6. Double protease value Km/Vmax

The Km and Vmax values were determined using the 4 mL calotropin and 4 mL papain treatment, as this condition exhibited the highest enzyme purity. Based on **Figure 6**,  $y = 0.0002x + 0.0585$  and  $R^2 = 0.9916$ , so the Vmax calculation is 17.094 U/minute/mL, and the Km is 0.0034 g/mL. This value shows that the Vmax of 17.094 U/minute/mL is the maximum speed with a substrate (Km) of 0.0034 g/mL. This value indicates the maximum amount of substrate to increase the enzyme speed, and if substrate is added again, there is no increase in the enzymatic reaction. This is because an enzymatic reaction will increase with increasing substrate concentration [S] until the speed is constant. The speed of enzymatic reaction cannot increase anymore with increasing substrate [S] called (Vmax) maximum speed [21].

#### IV. CONCLUSION

Double protease has a yield ranging from 44.43-48.63%; enzyme activity 9.23-53.63 U/mL; soluble protein content 2.40-2.89 mg/mL; specific activity 3.54-18.62 U/mg protein; enzyme stability at temperatures of 30-60°C; and Km 0.0034 g/mL Vmax 17.094 U/minute/mL. The best immobilization is calotropin 4 ml and papain 4 ml with stability at temperatures of 30-50°C with Km 0.0034 g/mL Vmax 17.094 U/minute/mL.

#### ACKNOWLEDGMENT

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#### CONFLICT OF INTEREST

The Authors declare no conflict of interest to disclose.

#### USE OF ARTIFICIAL INTELLIGENCE (AI) TOOLS STATEMENT

We used Grammarly (Grammarly Inc., 2025) to improve the clarity and grammar of the manuscript. The authors reviewed and approved all changes.

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