Michaelis-Menten Parameters Characterization of Commercial Papain Enzyme “Paya”

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Abstract. The use of enzymes is increasing nowadays, both for research and for the industry. This happens due to the advances in various fields such as fermentation technology, genetic engineering, and enzyme applications technology. One kind of enzymes that is very widely used and plays an important role in biotechnology and industrial applications is a protease enzyme, especially papain enzyme. Papain is a proteolytic enzyme which is isolated from papaya fruit sap tapping, or it could be derived from the leaves of papaya (Carica papaya L.). Actually, in the market, there is already a commercial papain enzyme called "Paya" which serves as a meat tenderizer with a relatively low price, but the enzyme papain was not pure and has unknown activity. Therefore, this study aims to examine the concentration of dissolved papain enzyme and casein as the substrate. By using the Lowry protein assay method, the initial concentration of papain enzyme and casein respectively 20,000 ppm. The result of protein assay we found that the concentration of dissolved papain enzyme and dissolved casein are respectively 71.069 ppm and 764.8276 ppm. The results from the first experiment are used for the second experiment which is to measure and determine the enzyme kinetics parameters Km and Vmax of that commercial papain enzyme with casein as the substrate. From this research, we found that the Km and Vmax of this commercial papain enzyme respectively 248.68 ppm dan 1.514 ppm casein/minute.

Keywords: casein, papain enzyme; enzyme kinetics; Lowry protein assay method

1. Introduction
The use of enzymes is increasing nowadays, both for research and for the industry. This happens due to advances in various fields such as fermentation technology, genetic engineering, and technology applications of enzymes. One type of enzymes which are very widely used and play an important role in biotechnology and industrial applications is a protease enzyme. Protease enzymes, also called proteolytic enzymes are enzymes that are specific to unravel or break down proteins. Protease enzyme can be produced by many sources either microorganisms or from plants, fruits, etc. The uses of protease enzymes are very effective and profitable. For example in the food industry, the protease enzyme used for the processing of milk, bread, biscuits, cheese ripening process, meat tenderizer, and manufacture of products from soybeans. In addition to the food industry, protease enzymes are also used in some industrial applications such as detergents, pharmaceuticals, leather products, and industrial waste treatment processes.

One type of enzyme that is widely used in the industry and also the field of research is the papain enzyme. Papain enzyme is a proteolytic enzyme isolated from papaya fruit sap tapping, or it could be derived from the leaves of papaya (Carica papaya L.). Besides containing as much as 10% papain, papaya fruit sap is also composed of chymopapain and lysozyme enzyme by 45% and 20% [1]. Papain enzyme’s activity is quite specific because papain can only catalyze the hydrolysis process well under conditions of pH and temperature within a specified time range. Papain is usually active at pH values between 5.0 to 7.0 with an isoelectric point of 8.75, and a temperature of 50 to 60 °C. The activity of papain was reduced by 20% when heated at 75 °C for 30 min and 50% on heating using a temperature of 76 to 85 °C for 56 min at pH 7.
The papain enzyme used to tenderizes meat, protein hydrolyzate manufacture, purification material in the beer industry, textile industry, tanning industry, pharmaceutical and cosmetic industries, and others. Papain is usually traded in the form of yellowish white powder and should be stored below 4°C [2]. Traded pure papain enzyme is relatively expensive, whereas usually for research to save it using the enzyme papain which is extracted directly from the sap of the leaves of papaya or papaya.

Actually in the market has sold commercial enzyme brand "Paya" (Figure 1) which serves as a meat tenderizer with a relatively low price, but the enzyme papain was not pure but is mixed with other ingredients such as sugar and salt. Disadvantages of commercial papain enzyme Paya brand are besides being already mixed is due to this papain enzyme’s activity is unknown, but this commercial papain enzyme has been used in several studies. This study aims to measure and determine the kinetic parameters Km and Vmax of this papain enzyme with substrates such as casein. In addition, to measuring and determine the parameters of enzyme kinetics, this study also aims to examine the levels of protein in the enzyme papain this commercial and casein as well as its substrate by using the method of Lowry protein assay [3].

Research on the characterization of the enzyme is already conducted a long time ago; many methods are also being used which have been published. For the study of a few years ago until today in Indonesia, for example, in 2006 there is research conducted on the isolation and characterization of crude extract enzyme bromelain from pineapple stem by Herdyastuti using fractionation method [4]. In 2007 research was conducted on the production and characterization of the enzyme β-endoxilanase of termite’s intestinal system bacteria by Ratnadewi by using electrophoresis [5]. In 2013 then also research conducted on polyphenol oxidase enzyme characterization of crude extract of black tiger shrimp by Made Suhandana et al. by using a buffer solution in increments [6].

Research on the purification of the enzyme papain was conducted in 2000 by Monti et al. from Brazil, then research on the characteristics of the enzyme papain in 2007 by Oktaviani RA [7,8]. In 2013 research was conducted on the characteristics of the activity of the rough papain enzyme by Khamim Nugroho et al., and also in 2014 were characterized enzyme papain from papaya by Zusfahair et al. [9,10].

Almost all of the research on the use of enzymes and enzyme characteristics that have been done over the extraction process is usually preceded by the enzyme itself from its source be it plants, bacteria, etc.

2. Experimental Methods

2.1. Materials

The main ingredients of this study are a commercial enzyme papain "Paya" and casein. Commercial enzyme papain "Paya" obtained by purchasing it in Giant supermarket, whereas casein which is used as a substrate is obtained from the thesis supervisor. To test the protein content by using the method of Lowry, we require a standard solution, and the standard solution which we use is Bovine Serum Albumin (BSA). BSA was made with a weight of 5 mg that was dissolved in distilled water to 25 mL. Other materials needed for this research are biuret solution (mixture of 1 mL of CuSO4 solution 1% (w/v), 1 mL of NaK-Tartrate solution 1% (w/v), in 100 mL of Na2CO3 solution 2% (w/v)). A solution of 1% CuSO4 and 1% NaK-Tartrate respectively were made in a volume of 25 mL), 1N Folin Ciocalteau reagent, 0.05M pH seven phosphate buffer solution, one mL solution of trichloroacetic acid (TCA) 0.4M, and 0.5M sodium carbonate solution.

![Figure 1. Commercial Papain Enzyme "Paya" and Casein Which are Used](image)
2.2. Lowry protein assay method.

The dissolved concentration of papain enzyme and casein are obtained by making a standard solution of BSA. Standard protein solution (Bovine Serum Albumin 200 µg/mL) and distilled water are mixed with a certain amount by the Table 1 below in a test tube to obtain a wide range of concentrations between 20-120 mg in 1 mL of the standard solution.

| Table 1. Determination of Proteins by Using the Method of Lowry |
|------------------|--------|------|------|------|------|------|
|                   | Blank  | Standard | Sample |
| Concentration (mg/L) | 20     | 40     | 60     | 80     | 100   | 120   |
| BSA Standard (mL)   | 0,1    | 0,2    | 0,3    | 0,4    | 0,5   | 0,6   |
| Sample (mL)         | -      | -      | 0,1    | -      | -     | -     |
| Aquadest (mL)       | 1      | 0,9    | 0,8    | 0,7    | 0,6   | 0,5   |
| Biuret Solution (mL)| 3      | 3      | 3      | 3      | 3     | 3     |
| Folin Reagent (mL)  | 0,3    | 0,3    | 0,3    | 0,3    | 0,3   | 0,3   |

In the other tubes were also mixed two kind of protein samples (the first protein sample is a commercial papain enzyme "Paya," and the second is casein) and distilled water so that the total volume of the sample solution was one mL. Add 3 mL of biuret solution (mixture of 1 mL of CuSO4 solution 1% (w/v), 1 mL of NaK-Tartrate solution 1% (w/v), in 100 mL of Na2CO3 solution 2% (w/v)) into each test tube. Immediately shake it by using a vortex mixer. The solution mixture was incubated at room temperature for exactly 10 minutes using a stopwatch and time is calculated at the time of adding biuret. After exactly 10 minutes of incubation, add 0.3 mL of the 1N Folin-Ciocalteau reagent into each test tube. Immediately shake it again by using a vortex mixer. The solution was then incubated again at room temperature for 30 minutes after the addition of Folin reagent. The absorbance of each solution is measured precisely in the 30th minute at a wavelength of 750 nm.

2.3. The Determination of Km and Vmax.

This study was conducted to calculate the enzyme kinetics parameters of commercial papain enzyme "Paya" by calculating the Michaelis-Menten constant and a maximum velocity of papain enzyme to hydrolyzes casein as the substrate. 0.5 mL of enzyme solution added with 0.5 mL of 0.05 M phosphate buffer pH 7, then preincubated at 37°C for 5 minutes. Then also add 0.5 mL of substrate (made in 5 variations of concentrations, ranging from 191.2075 ppm, 95.604 ppm, 47.802 ppm, 23.901 ppm and 11.95 ppm which were obtained by diluting the substrate solution similar to that used in the first experiment), then incubated at 37°C for 10 minutes. To stop the reaction, then add one mL of 0.4 M trichloroacetic acid (TCA). After that, centrifuge it with a speed of 4000 rpm for 30 minutes. After centrifugation, a total of 0.5 mL of the filtrate was added with 2.5 mL of 0.5 M sodium carbonate, and then preincubated for 10 minutes. The next step is to add to 0.5 mL of Folin Ciocalteau reagent, incubated for 30 minutes. The final step is to read the optical density at 660nm. (Blank which is used is enzyme solution with the same treatment, except for the addition of TCA conducted before addition of substrate).

3. Results

3.1 Lowry protein assay method.

To test the levels of papain enzyme and casein dissolved required standard curve equation of the line which will be used to calculate the concentration. Figure 2 shows the absorbance measurement data for a standard solution of BSA, papain enzyme sample, and casein sample.
Figure 2. Calibration curve of BSA Standard Solution

From Figure 2, the equation line which will then be used to calculate the concentration of dissolved papain and casein. The trick is the variable \( y \) in the equation is equal to the value of the absorbance of the papain and casein sample which were also measured at a wavelength of 750 nm. Absorbance measurement is done as much as each of the three repetitions.

Of the three repetitions, the average value obtained for the sample absorbance Papain is at 0.2123 and for casein samples diluted 10 times is 0.228. Based on the average absorbance values obtained here then dissolved papain concentration is equal to:

\[
y = 0.0029x + 0.0062
\]

\[
x = 71.069 \text{ ppm}
\]

The concentration of dissolved casein also calculated based on the average absorbance values are, but it should be multiplied by the dilution factor because it has been diluted ten times. Here are the results:

\[
y = 0.0029x + 0.0062
\]

\[
x = 76.4827 \text{ ppm}
\]

\[
x = 76.4827 \text{ ppm} \times 10 = 764,8276 \text{ ppm}
\]

3.2. The Determination of \( K_m \) dan \( V_{max} \).

From the papain and casein dissolved concentration data which is got from the first experiment, we made five variations of casein concentration obtained by diluting the initial solution with a concentration of 764.8276 ppm to a solution with a concentration of casein 191.2075 ppm, 95.604 ppm, 47.802 ppm, 23.901 ppm, and 11.95 ppm. For a solution of papain enzyme, we do not need to dilute it but using a solution of papain enzyme the same as the solution at the study test of Lowry protein content.

Figure 3. Graph of Absorbance Average Value vs Substrate Concentration
In Figure 3, we obtained the equation of the line that will be used to calculate the value of Km and Vmax of the commercial papain enzyme "Paya" by using Michaelis-Menten equation which is transformed into its opposite or Lineweaver-Burk into the equation as below:

\[
y = 164.25x + 0.6606 \\
\frac{1}{V} = \frac{K_M}{V_{max} [S]} + \frac{1}{V_{max}} \\
\frac{1}{V_{max}} = 0.6606; V_{max} = 1.514 \text{ ppm casein/minute} \\
\frac{K_M}{V_{max}} = 164.25; K_M = 248.68 \text{ ppm}
\]

So, Km and Vmax of the commercial papain enzyme respectively are 248.68 ppm and 1.514 ppm casein/min.

4. Discussion.
Protein content in the enzyme affects the catalytic power of the enzyme. In general, with the increasing levels of protein in the enzyme, the catalytic power will also increase. One method can be used to determine the protein content is the method of Lowry. In the first experiment was obtained from the protein content test using the Lowry method that the concentration of dissolved enzyme papain is equal to 71.069 ppm of the initial concentration of 20000 ppm which was prepared by dissolving 2 g of the enzyme papain in distilled water until reaching a volume of 100 mL. The concentration of dissolved enzyme papain is very small when compared by initial concentration. This indicates that the maximum concentration of the enzyme papain in the enzyme solution which can hydrolyze casein as substrates they are at the 71.069 ppm.

To the concentration of dissolved casein showed a more concentrated so great absorbance value. Therefore it is necessary to dilute it as much as ten times to make the absorbance values are within the range of normal absorbance value. The result is the concentration of total dissolved casein 764.8276 ppm. These results indicate that the maximum concentration of casein which can be hydrolyzed by the papain enzyme. By knowing the concentration of the papain enzyme and casein dissolved can be seen how long the casein hydrolysis reaction by papain enzyme will take place.

The next phase of this research is to determine the kinetic parameters Km and Vmax of commercial papain enzyme “Paya.” In the enzyme reaction we know about the rate of the hydrolysis reaction, decomposition or other catalyze reactions called by velocity (V). The value of V of an enzymatic reaction will increase with increasing substrate concentration, but after further increasing the substrate concentration will arrive at a fixed velocity. In particular enzyme concentration value, V is almost linear with the increasing substrate concentration. In conditions where V cannot be increased further with increasing substrate concentration is called the maximum velocity (Vmax). Vmax is one enzyme kinetic parameters [11].

Another enzyme kinetic parameters are Michaelis-Menten constant, which is better known as Km. Km is the substrate concentration that is half of the active sites have been filled, i.e. when the enzyme reaction rate has reached ½ Vmax [11].

From the results of the calculation of Km and Vmax values obtained from commercial enzyme papain "Paya" respectively amounted to 248.68 ppm and 1.514 ppm casein/min. If the value of Km and Vmax unit is converted into µM will be respectively 3.316 µM and 0.020187 µM casein/min.

For comparison, in 1998, Sowmya Ganapathi-Desai et al. from the University of Kentucky, USA examined immobilized papain enzyme, which is the papain enzyme has values of Km and Vmax are respectively 500 µM and 7.6 µM/min [12]. Ruben Monti et al. in 2000 researching papain enzyme extracted from fresh papaya latex which results in Km and Vmax values on average respectively 1336.5 µM and 19.825 µM/min [7]. The result of the conversion of Km and Vmax values above can be compared to the value of Km and Vmax of commercial enzyme papain "Paya" which is found that much smaller than the immobilized enzyme papain and papain enzyme derived from fresh papaya latex. According to Fox (1991), Km is a measure of the affinity between the enzyme and its substrate, but it is also associated with the dissociation equilibrium constant of the enzyme-substrate complex into the enzyme and the substrate. Fox also added that this small Km value means that the enzyme has a high affinity for the substrate, therefore complex ES will be strong and will run equilibrium toward the ES complex, and vice versa [13].
The differences of Km and Vmax values of each enzyme associated with purity levels of the enzyme. As we know that commercial papain enzyme "Paya" is not pure, but a mixture of the enzyme papain, sugar, and salt produced by Enzyme Development Enterprise and sold as meat tenderizer products.

5. Conclusion
Commercial papain enzyme "Paya" and the substrate casein dissolved concentration from the average yield is respectively 71.069 and 764.8276 ppm ppm. Dissolved papain enzyme concentration value then used for the second phase of the study.

Km and Vmax values of commercial papain enzyme "Paya" of the average yield is respectively at 248.68 ppm and 1.514 ppm casein/min, it means that this small Km value means the enzyme has a high affinity for the substrate, the maximum velocity of commercial papain enzyme "Paya" to hydrolyze casein as its substrate casein is equal to 1.514 ppm casein/min, Km value indicates the concentration of the substrate in which will be achieved half Vmax, the value obtained from this experiment was 248.68 ppm. When compared by some previous studies on the enzyme Papain, the Km and Vmax values of commercial Papain enzyme "Paya" is much lower.

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