Purification of Protease Enzymes from Wild Poinsettia (*Euphorbia Heterophylla*) Plant with Ammonium Sulphate

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Abstract. Protease enzymes are one of the essential enzyme groups that have been widely studied and have very high economic value. The study aimed to determine the optimum protease enzyme extraction method from the wild poinsettia (Euphorbia heterophylla) to produce the highest protease enzyme activity. It was a salting-out extraction method with adding ammonium sulphate salt at saturation levels of 50%, 60%, and 70%. The results showed that adding ammonium sulphate at a concentration of 50% is the most effective protease extraction from the latex of wild poinsettia. It showed the highest percentage yield (17.63 ± 4.74%) and the soluble protein content (4.76 ± 0.014%), while the protease enzyme activity was the lowest (2.9 x 103 ± 0.0142 unit/mg). The highest specific protease activity is achieved by extraction with 70% ammonium sulphate, i.e., 1.8 x 104 units/mg, at the saturation level of 50% and 60% ammonium sulphate; the specific activity of the protease wild poinsettia tended to be low. In conclusion, adding ammonium sulphate salt at saturation levels of 50%, 60%, and 70% showed the different characterization of protease enzymes from wild poinsettia plant latex.

Keywords: ammonium sulphate · plant latex · protease activity · protease enzyme

1 Introduction

Protease enzymes play a significant role and have great potential in developing biotechnology and its use in the food processing and pharmaceutical industries. Protease enzymes are one of the essential enzyme groups that have been widely studied and are highly economically valuable [1]. This enzyme functions as a catalyst in the hydrolysis of peptide bonds in proteins. Protease enzymes can be obtained from living organisms, i.e., in any part of the plant, i.e., organisms holding the highest percentage as protease producers (43.85%) compared to bacteria (18.09%), fungi (15.08%), animals (11.15%), algae (7.42%), and viruses (4.41%) [2]. The development of enzyme production technology has been widely carried out in the fields of food, pharmaceuticals, bioactive peptides, and various basic research [3]. Protein substrate is a substrate that is widely

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used in the field of enzymes, because of its simplicity, speed, and accuracy in determining the reaction constants that occur [4]. The potential economic impact of enzyme-based technology is the potential for efficient use of raw materials and reduced use of toxic chemicals so as to produce minimal waste. Another advantage is lower energy use with increased production yield and reduced keyword losses and by-products.

Several plants have been reported and developed as sources of protease enzymes in Indonesia. Include papain from papaya, bromelain from pineapples, *Calotropis gigantea* latex [5–7], moringa leaves [8], Agave Angustifolia leaves [9] and pineapple roots [10]. Using protease enzymes from plants is beneficial [11] in terms of the availability of raw materials and the simple extraction process. Alternatively, protease enzymes from the wild poinsettia (*Euphorbia heterophylla*) can increase the economic value and utilization of this plant itself, which has only been considered a wild plant and used as a traditional medicinal ingredient [12, 13] in Sumenep.

People use the wild poinsettia as a traditional medicine to maintain their diet. In addition to having therapeutic and health effects in the treatment or treatment of disease, the wild poinsettia is known to have the potential as a protease enzyme, namely in the sap. The wild poinsettia plant belongs to the Euphorbiaceae family. This plant is also known by the common names, namely patik mas in Java, kate mas in Ternate, or pargesi in Sumenep. This plant is considered a wild plant and weed and has a laxative effect [14, 15]. The first year of research aimed to determine the best method of extracting protease enzymes from the wild poinsettia (*Euphorbia heterophylla*) to produce the highest protease enzyme activity. This study employed the salting-out extraction method [16–19] as the initial step in the isolation and characterization of the protease enzyme from the latex of wild poinsettia.

The urgency of this research is that the demand for protease enzymes in the food industry is increasing and proportional to the abundant potential of wild plants in Indonesia. The results of this study provide information on the development of food biotechnology, support the sustainability of human life, food security, and the utilization of natural resources of traditional medicinal plants in East Java. In this modern era, several studies are developing new and appropriate methods to improve various production processes through green economy, increase added value for raw materials through processes that reduce energy and raw material consumption and produce less waste and toxic by-products.

2 Material and Methods

2.1 Sample

The raw material used in this research was the latex from the wild poinsettia (Euphorbia heterophylla) obtained from Sumenep, East Java. The latex was attained by injuring the stems and leaves of the wild poinsettia plant, then carrying them in cold conditions in a cool box. Chemicals used in the analysis were pure analytical purity (pro analyze). Meanwhile, the tools used included a cold centrifuge, centrifuge, freeze dryer, pH meter type 3320 (Germany), magnetic stirrer, vortex, Sanyo refrigerator, water bath, Ohaus analytical balance, and electric heater.

2.2 Preparation of Experiments

The first stage of the research was the extraction of protease enzymes from the latex of the wild poinsettia, carried out by salting-out [1] with the addition of ammonium sulphate salt at different levels of saturation. The treatments studied included adding ammonium sulphate salt at a saturation level of 50%, 60%, and 70%. The latex of wild poinsettia was diluted with 0.05 M phosphate buffer, pH 7, with a ratio of 1 g of latex: 5 ml of phosphate buffer. Degumming was carried out by centrifugation at 4000 rpm for 20 min. Furthermore, the latex supernatant was separated, and the enzyme activity and protein content were observed. The supernatant sample was added with ammonium sulphate salt at the saturation levels according to the treatment (50%, 60%, and 70%) [1], and the precipitate obtained was dialyzed using a dialysis bag having an MWCO (Molecular Weight Cut Off) 12–14 kDa [20].

2.3 Percentage Yield (Graphimetric Method) [21, 22]

The yield calculation was carried out by weighing the dry crude protease and then compared with the sample weight multiplied by 100%. The protein content of the protease enzyme was determined by the Lowry modified method [21] using bovine serum albumin (BSA) as the protein standard. The protein content is expressed as mg/mL.

2.4 The Soluble Protein Content (Lowry Method) [23]

The Lowry method carried out observation of protein levels of protease enzymes. Observations were made by taking a sample of 0.001 g dry protease sample. Then, protein hydrolysis was conducted to obtain dissolved protein using 0.1 ml of 2N NaOH at 100 °C for 10 min and cooled. The resulting dissolved protein was then reacted with 2 ml of Mix-Lowry reagent and allowed to stand for 10 min. Subsequently, the reacted result was added with 0.25 ml of Follin's reagent and left for 30 min. The reacted result was mixed with 5 ml of distilled water, then the absorbance was read with a spectrometer at a wavelength of 750 nm. The absorbance data were plotted on the BSA standard curve to calculate the protein content.

2.5 Protease Activity [23, 24]

The activity of the protease enzyme was tested using a soluble casein substrate at pH 7. Then, 0.01 g of soluble casein were weighed in a centrifuge tube and mixed with 3 ml phosphate buffer pH 7. Then, the mixture was pre-incubated at 37 °C for 4 min. A 0.250 ml sample for latex filtrate or 0.005 g for dry crude protease was added to the mixture, then incubated at 55 °C for 20 min. At the end of incubation, the hydrolysis reaction was stopped by adding 1 ml of 15% TCA solution. A control without incubation and hydrolysis reactions was carried out at 0 min. The addition of 15% TCA solution was performed before the addition of proteases and then centrifuged at 1000 rpm for 10 min. The supernatant was taken at 1 ml, then added with 2.5 ml of Lowry mix and left for 10 min. The mixture was then added with 0.250 ml of Follin's reagent and left for 30 min. The mixture was measured with 5 ml of distilled water, and the absorbance was

read using a spectrometer at a wavelength of 750 nm. The absorbance data were plotted on the tyrosine standard curve to calculate the hydrolysis activity.

Protease activity is expressed in activity units, where one unit means an increase in the concentration of dissolved protein by one mol per minute of incubation time. The specific activity is stated in units of activity per milligram of enzyme protein. The calculation of the specific activity of the enzyme used the following formula:

$$\textbf{\textit{Protein activity}} = \frac{[c]}{t} \times \frac{1000}{181.19}$$

Description:
[C] = dissolved protein concentration (μmol tyrosine/ml)
t = hydrolysis time (minutes)
189.19 = tyrosine molecular weight
1 unit = 1 mol tyrosine released from the substrate by each mg of enzyme at 37 °C/minute

3 Results and Discussion

Efforts to find sources of protease enzymes from plants need to be made to find potential sources of new enzymes that can be used in the food industry. The plant explored as a source of protease enzymes is *Calotropis gigantea* plant. Siduri is a type of wild tropical shrub that has not been used optimally in Indonesia, whose population is relatively abundant, growing on dry land and areas around the coast. In Witono's study [20], the most appropriate protease isolation technique from the *Calotropis gigantea* latex was the salting-out method using 65% ammonium sulphate with the highest specific activity (0.059 units/mg). The effect of the saturation level of ammonium sulphate on the percentage yield of wild poinsettia latex is shown in Table 1.

Table 1 demonstrates that the higher the saturation level of ammonium sulphate used for extraction from the precipitated preparations, the crude protease yield of latex decreases to 70% ammonium sulphate. The addition of ammonium sulphate at a concentration of 50% is the most effective protease extraction from wild poinsettia latex. Ammonium sulphate salt in solution will ionize and reduce protein interaction with the solvent so that the interaction between protein molecules increases and eventually precipitates. High salt ions will bind to water molecules, reducing ammonium sulphate [15, 16]. The proper extraction method to extract protease enzymes from melinjo fruit peel is the salting-out method (10–13) using ammonium salt with 50% saturation with enzyme activity of 62.15 x 10–2 mol tir.ml-1.min-1 and yield of 1.52% [7]. Fatimah and Wardani's research [8]. The protease extracted from Moringa leaves by homogenization using 100 mM potassium phosphate buffer pH 7.0 containing 10 ml 0.3% ascorbic acid and 10 ml 15 mM EDTA was the most effective extraction buffer and stabilizer. The protease enzyme from Moringa leaves with the highest activity was found in the addition of 0.3% ascorbic acid and 15 mM EDTA, which was 2.45 U/mg. The enzyme with the highest activity produced was precipitated with the best fraction of 60% ammonium sulphate salt.

Table 1. Characterization of crude protease extract from wild poinsettia latex

Ammonium sulphate saturation (%)	Percentage yiled (%)	Total soluble protein content (%)	Protease activity (unit/mg)
50	17.63 ± 4.74	4.76 ± 0.014	2.9 x 10^3 ± 0.0142
60	12.67 ± 2.13	4.56 ± 0.053	4.02 x 10^3 ± 0.0127
70	10.47 ± 2.41	4.25 ± 0.170	4.49 x 10^3 ± 0.0026

This is in line with the increase in the protease yield Table 1. The more proteases extracted were also followed by the increasing number of proteins within them. When the ammonium sulphate salt is added to the enzyme protein solution, most of the water molecules will bind to the salt ions, which will further reduce the amount of water available to bind to the protein so that the protein will precipitate. Crude protease results from precipitation with ammonium sulphate is still a mixed fraction consisting of enzyme protein and non-enzyme protein fractions.

The highest specific protease activity was achieved by extraction with 70% ammonium sulphate, i.e., 1.8 x 104 units/mg, at the saturation level of 50% and 60% ammonium sulphate. The specific activity of the protease wild poinsettia tended to be low. The extracted product is suspected of containing more protein enzymes with a lower protein content of protease. Specific activity is a unit of protease activity in each milligram of protein contained in the enzyme, which can also indicate an enzyme's purity level. If the specific activity is lower than expected, it is caused by denatured protein enzymes or much higher levels of non-enzyme proteins [19, 25, 26].

Evaluation of milk coagulation by crude protease enzymes produced from the sap of the *Euphorbiaceae* family plant in cheese production (Mahajan & Chaudhari, 2014) showed that the specific activity of the enzyme *Euphorbia circular* (78.4 ± 9.0 U/mg) > *Euphorbia nivulia* (60.5 ± 3.2 U/mg) > *Euphorbia nerifolia* (54.7 ± 3.9 U/mg) > *Euphorbia heterophylla* (37.3 ± 4.8 U/mg). Clumping milk is an early stage in cheese making; therefore, this crude enzyme is needed in cheese making. Purification of protease enzymes from *Calotropis gigantea* latex was carried out through column chromatography with Sephadex G-25 gel followed by CM Sephadex C-50 Cation Exchanger, and extraction of *Calotropis procera latex* by polyethylene glycol–salts biphasic system [27]. The protease enzyme production technique with the highest activity (0.080 units) was obtained by precipitation technique at pH 3.5, which was extracted using cold distilled water with 0.7% $Na_2S_2O_3$ content, followed by freeze-drying or vacuum drying at 40 °C [1].

4 Conclusion

Ammonium sulphate salt addition at saturation levels of 50%, 60%, and 70% showed different characterization of protease enzymes from wild poinsettia plant latex. Ammonium sulphate concentration of 50% is the most effective protease extraction from wild

poinsettia latex. It showed the highest percentage yield (17.63 ± 4.74%) and dissolved protein content (4.76 ± 0.014%), while the protease enzyme activity was the lowest (2.9 x 103 ± 0.0142 units/mg). The highest specific protease activity was achieved by extraction with 70% ammonium sulphate, ie 1.8 x 104 units/mg, at the saturation level of 50% and 60% ammonium sulphate; the specific activity of wild poinsettia protease tends to be low. The results of this study can provide new insights regarding the processing of wild plant that have the raw material potential as a green and sustainable approach to biotransform from latex to protease enzymes, a value-added product.

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