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The Beginning Study of Protease from Paddy Oats (*Gnetum gnemon* L.) Seed Peel and Its Potential as a New Source of Protease

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***Abstract*—** *This study investigates the possibility of agricultural waste, paddy oats fruit peel, as a source of protease extract. The ripe peel yielded the extract with the highest proteolytic activity of 343.5 U/mg. The effect of temperature and pH on protease activity of the extracts were evaluated. Profile of optimum pH and optimum temperature of three stages of peel maturity indicated the similar tendency. Paddy oat seed peel extracts showed high activity over a broad temperature range, with maximum activity at 45-55^{o}C. The optimum pH of crude extracts were about 6.0-8.5. In order to identify the classes of protease, the effect of different protease inhibitors has been examined. Thiol reagent, dithio-bis-nitrobenzoic acid (DTNB) inhibited protease activities, while EDTA showed stimulatory effect. This inhibition profile suggesting that the crude extracts contain cysteine protease. In overall, the results revealed that paddy oats fruit peel, especially the ripe and the mature peel, have great potential as a source of protease.*

***Keywords*—** *Agricultural waste; plant protease; crude extract.*

I. INTRODUCTION

Proteases are the most important classes of commercial enzymes and account for at least 60% of the total industrial enzyme in the market [1]. Among the world sales of commercial enzymes of around US$ 300-600 million per annum, 75% of those are hydrolytic enzymes, of which two-third are proteolityc enzymes [2]. Protease has been used for a long time as alternatives to chemicals to improve the efficiency of a wide range of industrial system and processes.

Proteases are degradative enzymes which accelerate the process of hydrolysis of proteins. They break down the chains by a process and convert protein into smaller chains called peptides or even into amino acids [3]. Because of this ability, proteases have an important position with respect to their application. Proteases have been developed for the use in industrial production, for instance it is used in the food industry, for meat tenderizing, brewing, baking, cheese manufacturing. Other applications are in animal feed, textiles industries, skin softening, medical use and detergent formulations [3-7].

Protease could be obtained from microorganisms, animals, and plants. Proteases from plant sources have received great attention from the industrial enzymes because of their functions and properties. Papain, bromelin, and ficin are the most popular proteases from plant tissue origin. Increasing demand of protease and obtaining a variety of proteases has lead researchers to explore newer sources of proteases. Serine protease extracted from the sprouts of *Pleioblastus hindsi* and protese from melon fruit [8,9]; Protease isolated from *Calotropis procera* latex [10]; Cheese making with vegetable coagulant [11]; Tenderization of buffalo meat using protease from *Cucumis trigonus* Roxb [12]. There is a need to find new sources of protease, with the goal being to produce it at an inexpensive cost.

Paddy oats (*Gnetum gnemon L.*) are grown commercially in Indonesia, India, Malaysia, Philippines, and Fiji. It is a medium size tree, growing to 15-20 m tall. The leaves are evergreen, opposite, 8-20 cm long and 3-10 cm wide, entire, emerging bronze-coloured, maturing glossy dark green. The fruit-like strobilus consists of a small amount of skin or peel and a large nut-like seed 2-4 cm long inside [13], with both the fruits and leaves being very popular in Indonesian cuisines. Approximately 38% of the whole fruit is in paddy oats fruit peel, which is only discarded during seed processing, as waste or used as organic fertilizer. This discarded fruit peel may cause environmental problems, and the treatment of such waste has been very costly for industries. The utilization of agricultural waste as a source of protease is an alternative mean.

Although many proteases have been extracted from plant latexes, fruits, leaves, and seeds, to the best of our knowledge, there is no previous study on purification and characterization of protease extracted from paddy oats fruit peel. Hence, the objective of this study was to evaluate the potential of paddy oats fruit peel as a new source of protease and to provide basic information about its main characteristics.

II. MATERIALS AND METHOD

A. Paddy Oats Seed Peel and Chemicals

Fresh paddy oats fruit peel was bought from a local market (Malang, East Java, Indonesia). Paddy oats fruits peel was selected based on size uniformity at the same stage of maturity and lack of visual defect. The green colored (immature peel), yellow colored peel (mature), and red colored (ripe peel) were selected from paddy oats fruits respectively. The peel was kept in the laboratory at 4^{o}C until used for the experiment. All the chemicals used in the experiment were analytical grade unless otherwise stated.

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B. Extraction of Protease

Fresh paddy oats peel was cleaned with distilled water and drained. The peel was chopped into small pieces, and then they were blended (Philips HR-2011 Blender) with 0.1 M sodium phosphate buffer at pH 7.2 for 4 minutes. The resulting blend was filtered using a cheese cloth and then centrifuged for 10 minutes at 10000g and temperature was kept at 4^{o}C. The pellet was discarded and the supernatant (crude extracts) from the 3 types of peel (immature, mature, and ripe) were collected and stored at 4^{o}C before purification.

C. Protease Activity Assay

Protease activity of the crude extract was measured according to the method described by Kunitz [14], using casein as a substrate. Briefly, the crude extracts solution was added to substrate casein 1% (w/v) in 100 mM sodium phosphate, pH 7.2. The reaction was carried out at 37^{o}C for 10 minutes and was stopped by 3 ml of 5% (w/v) TCA; the mixture was centrifuged at 5000g for 20 minutes. The absorbance of the supernatants was measured at 280 nm using a UV/visible spectrophotometer (UVmini-1240, Shimadzu). A standard curve was created using tyrosine solution. One unit of proteolytic activity (U) described the amount of protease that produced 1 microgram of tyrosine per minute under specified conditions.

D. Effect of Temperature on Proteolytic Activity

To determine the optimum temperature, the proteolytic activity of the crude extracts was evaluated at various temperatures (25-70^{o}C) under standard assay conditions, with casein as a substrate. The caseinolytic activity was expressed as the relative proteolytic activity, and the highest activity was taken as 100% relative activity.

E. Effect of pH on Proteolytic Activity

To investigate the effect of pH, the protease activity of the crude extracts were tested at various pH ranging from 3 to 10. The effect of pH on protease activity was studied by measuring protease activity of extracts at the optimum temperature with the following buffers: Glycine (pH 3), sodium acetate (pH 4-5), sodium phosphate (pH 6-7), tris-HCl (pH 8-10). The activities of the 3 different extracts were expressed as the relative proteolytic activity.

F. Effect of Inhibitors and Metal Ions on Proteolytic Activity

The effects of inhibitors on protease activities of the crude extracts were studied using phenyl methyl sulfonyl fluoride (PMSF) 2 mM, ethylene diamine tetra acetic acid (EDTA) 2 mM and dithio-bis-nitrobenzoic acid (DTNB) 1 mM. The effects of various metal ions (10 mM) were examined by adding metal ions Zn^{2+} and $Mg2^{+}$. The activity of protease extracts assayed in the absence of inhibitors and metal ions was taken as 100%.

G. Statistical Analysis

ANOVA was conducted for statistical analysis. Differences between means were assayed by Least Significance Different (LSD) test with a significance level of 5%.

III. Results and Discussions

A. Proteolytic Activity

Figures of total protein and proteolytic activity showed similar tendency to increase with the increasing maturity stage of paddy oats seed peel. The highest proteolytic activity (343.5 U/mg) was found in the ripe peel crude extract, while proteolytic activity was lower in mature peel extract, and the lowest proteolytic activity was identified in immature peel extract, there was a significant difference among the three.

TABLE I. The activities of paddy oats seed peel protease extracted from different stages of maturity.

Maturity	Protease activity (U/mg)
Immature	343.5^{a}
Mature	319.7^{b}
Ripe	308.3^{c}

Different superscript letters in the same column indicate the significant differences ($p \leq 0.05$).

The ripening process is a unique phase which involves several stages such as fruit development, maturation, ripening and finally senescence. It is widely known that the physiological and biochemical characteristic changes throughout the ripening period of fruit. Generally, ripening is believed to be a modification of fruit compounds which occurs as a result of an increase in synthesis of several enzymes, organic acid, amino acid accumulation, and solubilization of the pectin substances that result in progressive loss of tissue firmness. This mechanism may explain, the main reason for the low pH, high total protein, as well as a high protease activity in ripe peel extract. Nevertheless, the decrease of extract volume by increasing stage of maturity might be due to the peel's texture being too soft causing blockage in the filtration process.

B. Thermal Profile

The effects of temperature on the proteolytic activity of crude protease extracts were measured and reported as relative activities. The highest proteolytic activity of each crude extract was considered as 100%. The proteolytic activity was tested at a temperature range of 25-75^{o}C in each crude extract (Figure 1). The crude extracts from the three stages of fruit peel maturity performed a high proteolytic activity within a wide temperature range. Proteolytic activity increased rapidly with increasing temperature, with maximum activity being reached at the optimum temperature. The maximum activities from all of the crude extracts (immature, mature, and ripe) were between temperature 45-55^{o}C, relative activities ranging from 96% to 100%, with no significant different among activities at temperature 45, 50, and 55^{o}C. As the incubation temperature was increased, the relative proteolytic activity constantly decreased. At a temperature of 65^{o}C the proteolytic activity of mature and ripe peel extract was still considered high (more than 80% activity), however, the proteolytic activity of immature peel extract decreased sharply to 61%.

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The proteolytic activities of all crude extracts dropped to below 55% at a temperature of 70°C.

At the optimum temperature, the activity of the enzyme reaches the highest performance, because the optimum temperature is the most appropriate temperature for the active side of the enzymes to breakdown substrate. Mostly, protease enzyme from plants are resistant to high temperatures and remain active in a broad temperature range, hence the optimum temperature of plant protease is relatively high.

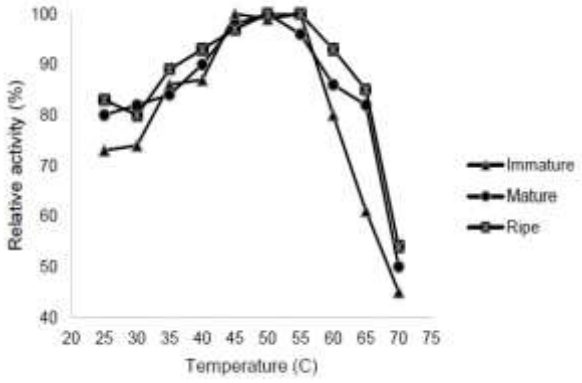

Fig. 1. The optimum temperature of paddy oats seed peel protease extracted from different stages of maturity.

Previous studies reported that the optimum temperature of bromelin from pineapple was 60°C [15,16]. The optimum temperature of serine protease extracted from *Cucumis trigonus* R. was 70°C [17], and the protease activity rapidly decreased as the reaction temperature increased higher than 80°C due to thermal denaturation of the enzyme's protein.

B. pH Profile

The effect of pH on the proteolytic activity of the crude extract from paddy oats fruit peel was determined over a pH range from 3 – 10. The highest proteolytic activity of each crude extract was considered as 100%. All crude extracts from the three types of peel maturity exhibited a wide pH activity profile (Figure 2).

The crude extracts from both mature and ripe peel showed high proteolytic activities within a pH range 6.0- 8.5 with no significant difference from the maximum activity. This result indicated that optimum pH of proteolytic activities from both mature and ripe peel extracts was in a wide temperature range (6.0-8.5), while the optimum pH of proteolytic activity from immature peel extract was narrower at a pH range 6.5-7.5. Proteolytic activity decreased dramatically out of pH range 6.0-8.5. All of the protease activities from 3 stages peel maturity fall down below 40%, at pH 9 because of protein denaturation.

Protease activity is affected by the conformation of the active site of the enzyme, whereas the conformation of the active site of the enzyme is affected by the concentration of H+ ions. At the optimal pH, protease showed the highest activity, because the correspondence between the active site of the protease with substrate had better conformation. Several previous studies reported that protease extracted from plants, in general, is a kind of neutral protease: protease from *chaya* leave, protease of ginger, *Euphorbia nivulia* protease [18-19]. However, protease extracted from rice seeds was reported such as an acid enzyme [20]. Our study demonstrates that protease enzyme extracted from paddy oats fruit peel was stable over a wide range of pH (6.0 – 8.5) and the stability profile highlighted the suitability of this protease for possible application in industrial process, particularly in the neutral condition.

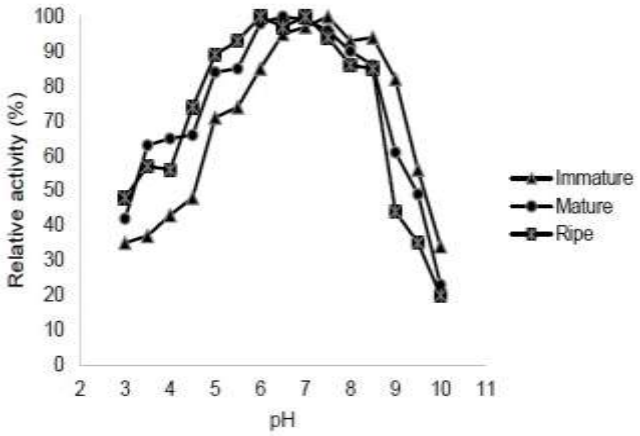

Fig. 2. The optimum pH of paddy oats seed peel protease extracted from different stages of maturity.

C. Active site of Protease

Proteases can be classified base on their sensitivity to various inhibitors. In order to confirm the type of protease, the effects of various inhibitors and metal ions on proteolytic activities of the crude extracts were studied and reported in Figure 3.

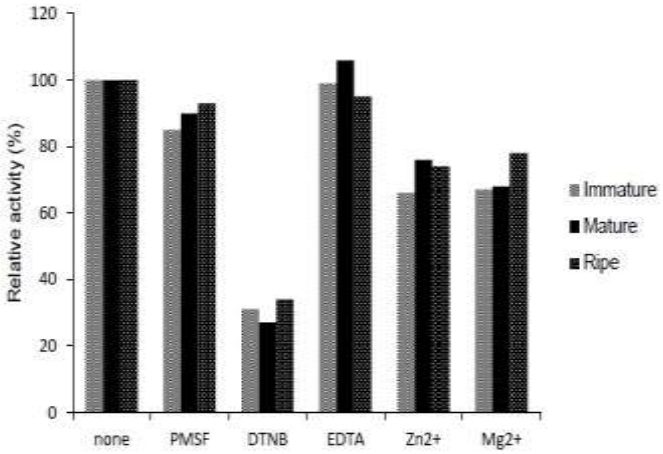

Fig. 3. Effect of inhibitors on proteolytic activity of paddy oats seed peel protease extracted from different stages of maturity.

As shown in the Figure, All of the crude extracts from different maturities of paddy oats fruit peel showed a similar

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tendency in proteolytic activity regarding the presence of various inhibitors and metal ions. Protease activities of crude extracts from all stages of fruit peel were not significantly inhibited by PMSF, a serine inhibitor; indicated protease is not a serine. EDTA showed the stimulatory effect on the protease activity suggesting that protease is not a metalloprotease. Finally, metal ions divalent (Zn^{2+} and Mg^{2+}) showed proteolytic activity inhibition; and DTNB strongly inhibited the proteolytic activity of crude extracts with remaining activities of 31% from the immature peel, 27% from the mature peel and 34% from the ripe peel. These evidences suggested that proteases of paddy oats fruit peel contain cysteine protease. Likewise, previous study reported that papain, a typical cysteine protease, was inhibited by some divalent metal ions [21]. The most plant proteases have been classified as cysteine protease or, rarely, as aspartic proteases [5].

IV. Conclusion

In general, these findings suggested that the protease of paddy oats fruit peels, especially from the mature and the ripe peel would be a potential candidate for various industrial applications mainly in the food industry. The investigation of proteolytic activity reported here is the beginning steps, further studies are needed to explore its molecular structure, the potential application of this protease, as well as to produce commercial protease from paddy oats fruit peel.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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