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Protease from Paddy Oats (*Gnetum gnemon* L.) Seed Peel and Its Potential for Extracting Virgin Coconut Oil

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***Abstract*—** *This study investigates the possibility of agricultural waste, paddy oats fruit peel, as a source of protease especially for extracting virgin coconut oil from coconut milk. Efficacy of protease from paddy oats was compared to efficacy of various treatments. Paddy oats protease showed the high performance for extracting virgin coconut oil since it resulted in high yield (25.7%) and good quality oil with free fatty acids of 0.092%, density of 0.924 g/mL, and also consistently ranked high for all evaluated sensory parameters. Virgin coconut oil produced from protease treatment resulted in aroma score of 7.6, taste score of 7.3 and color score of 9.2. In overall, the result of this study revealed that paddy oats fruit peel, especially the ripe peel, has great potential as a source of protease for extracting virgin coconut oil.*

***Keywords*—** *Agricultural waste; coconut oil; plant protease.*

I. INTRODUCTION

Protease is an important biocatalyst, a unique catalytic activity of protease that makes it an inexpensive choice for hydrolysing peptide bonds for industrial use [1]. As a commercial enzymes it is widely used in various industrial fields, it is not surprising that protease occupy 60% of the total market in the world [2]. The total sales of enzymes in the world which are estimated at 600 million per year and three-fourths of them are hydrolytic enzymes including protease [3]. Protease has been used for a long time as an active compound that plays an important role in various industries to increase the efficiency of industrial processes. Protease Protease is degradative enzymes which accelerate the process of protein hydrolysis. They break down the protein chain by a process and convert protein into smaller chains called peptides or even into amino acids [4,5]. Protease was applied in cheese making, detergent and leather industry several hundred years ago. Recently the use of protease become more important both in food industry as well as non-food industry.

Protease from plants get the attention of researchers because of several advantages they have. New proteases from plants do not need to go through rigorous tests of safety as well as new proteases from microorganisms. So that many researchers conducted research to explore new proteases from plants. Most proteases extracted from latex include proteases from *Calotropis procera* latex for meat tenderizers [6], 2013), milk clotting activity from *Euphorbia nivulia* latex [7]. However, proteases from plant tissues have also been widely extracted and applied to various fields. Protease from ginger was used in cheese making [8], protease from *Cynara* L. was successfully applied as milk coagulant [9]. Many studies also reported that protease extracted from various plant tissue such as protease from rice, *Cucumis trigonus, Pleioblastus hindsii, and Calotropis procera* [10-13], There is a need to find new sources of protease, with the goal being to produce it at an inexpensive cost.

Agricultural residues such as grasses, tree wastes, fruit peel and many other part of plant whose disposal is considered as an environmental problem as they have been accumulating and need high cost for their utilization processes. Paddy oats (*Gnetum gnemon* L.) is grown commercially in Indonesia, India, Malaysia, Philippines, and Fiji. The fruit-like strobilus consists of a small amount of skin or peel and a large nut-like seed 2-4 cm long inside [14], with both the fruits and leaves being very popular in Indonesian cuisines. Approximately 38% of the whole fruit is in paddy oats fruit peel. As agricultural waste, it has only been disposed of or used as organic fertilizer. The abundant waste that might cause environmental problems, then 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, as far as we know there has been no previous research of protease from paddy oats fruit peel. Hence, this research aims to investigate the potential of paddy oats fruit peel as a new source of protease especially for extracting virgin coconut oil from coconut milk. The superiority of virgin coconut oil quality compared to other vegetable oils causes this kind of oil to be called healthy vegetable oil. The main component of virgin coconut oil is saturated fatty acids up to 90% and the rest are unsaturated acids. Saturated fatty acids are dominated by lauric acid 84 from 46% to 49% [15] which has a C12 chain and about 7% caprylic acid. Both are medium chain saturated fatty acids, commonly called medium chain fatty acids or MCFA [16]. The presence of medium-chain saturated fatty acids in coconut oil gives some advantages. The most of lauric acid is converted instantly to be energy rather than stored as fat. Then, although coconut oil commonly used for cooking, it has many everyday uses. It is often used as an active compound in medicine and skin moisturizing products.

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Protease has the ability to break down protein include protein emulsifier coconut milk, the resulting oil will be released from the coconut milk emulsion system. After this process coconut milk separated into three components, namely: oil in the upper layer, curd or broken emulsifier in the middle layer, and water at the bottom layer. There have been many studies on the use of protease for extracting virgin coconut oil. However, not all of proteases are suitable as an enzyme for extracting virgin coconut oil. Researches are still needed especially to explore proteases that have the best performance, which have characteristics that are in accordance with the conditions of the virgin coconut oil extracting process.

II. Materials and Method

A. *Paddy Oats Seed Peel and Chemicals*

The main material in this study, fresh paddy oats seed 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 red colored (ripe peel) were selected from paddy oats fruits peel. 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.

B. Extraction of Protease

Red colored (ripe) and fresh paddy oats peel was selected and then cleaned with distilled water. After chopping the peel 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) was collected and stored at 4^{o}C before using for next purification.

C. (NH4)2SO4 Precipitation

Protease extract was concentrated by using the precipitation method with the gradual addition of (NH4)2SO4 to final saturation of 60%. The precipitate was separated by 10,000g centrifugation for 30 min and temperature maintained at 4^{o}C. Then, it was dissolved in a phosphate 127 buffer, and transferred into a tube (OrDial D80-6000-8000, Cellulose Dialysis Tubing) for overnight dialysis at 4°C. The dialysate was used for the next experiment and analysis.

D. Virgin Coconut Oil (VC)

The coconut milk was obtained from an old coconut kernel, which was extracted using water with ratio coconut and water 1: 3. The resulting coconut milk was left for 1 hour until two layers were formed, the top layer was cream, and the bottom layer was water. Water was then removed and the cream was used for further research. The process of producing virgin coconut oil was carried out with four different treatments: 1) Boiling the coconut cream with a temperature of 100^{o}C until oil resulted; 2) Acidification treatment with the addition of 3% acetate acid into coconut cream and incubated for 15 hours; 3) Centrifuging the coconut cream at 10000 rpm for 1 hour; 4) Addition of paddy oats protease with a concentration equal to 100 mg protein/100mL coconut milk and incubated for 15 hours. The obtaining virgin coconut oil was analyzed of yield (%), specific gravity (gr/mL); Physico-chemical attributes; and sensory evaluation.

E. Yield

Yield is calculated based on the weight of virgin coconut oil produced with the weight of the sample (cream coconut milk). All conditions for extracting virgin coconut oil are controlled both the maturity of the coconut fruit and the process of separating virgin coconut milk from curd (broken emulsifier). Virgin coconut oil is weighed (g) and divided by the weight of coconut milk cream. Yield is expressed in% as the following formula:

$$\text{Yield}\ (\%) = \frac{\text{Sample}\ (\text{g})}{\text{Virgin coconut oil}\ (\text{g})}$$

F. Physico-Chemical and Sensory Attributes

Free fatty acid and specific gravity of virgin coconut oil were analyzed according to standard procedure. Briefly, 5 g of sample (virgin coconut oil) was put into 250 ml erlenmeyer and then the sample is added 50 ml of 96% ethanol, and 2 ml of pp indicator. The sample was titrated with a standardized 0.05 N NaOH solution until the pink color was reached and did not disappear for 30 minutes. Free fatty acids expressed as % FFA.

G. Sensory Attributes

The sensory evaluation of virgin coconut oil sample was assessed by a trained panel of seven members. Discussion sessions were held to orient panel members before evaluating for preferred virgin coconut oil. Panelists evaluated the samples using a 10-point hedonic scale (Thompson et al. 2005) as follows: Aroma (1= least preferred and 10 = most preferred); Taste (1 = least preferred and 10 = most preferred) and Color (1= least preferred and 10 = most preferred).

H. Statistical Analysis

SPSS version 11.5 was used for data analysis. Data were collected from triplicate measurement. Analysis of varian was employed 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. Yield

Yield of virgin coconut oil from various treatments are presented in the following figure. The highest yield is virgin coconut oil produced by the boiling method of 27.4%. The yield of virgin coconut oil extracted using protease was 25.7% while the lowest yield of virgin coconut oil was 20.2% resulted from centrifugation treatment. Statistical analysis showed that the yield of virgin coconut oil from the centrifugation treatment was not significantly different ($p < 0.05$) with yield of virgin coconut oil from acid treatment.

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The boiling method produces the highest yield of virgin coconut oil because the high temperature from boiling process is able to damage the structure of the protein and cause the coconut milk emulsifier to be damaged and virgin coconut oil separated from the emulsion. Extracting virgin coconut oil by the method of adding proteases also gave a high yield, because the protease has ability to hydrolyze the protein of coconut milk emulsifier. The protease could hydrolyze protein molecule into small fragments, and resulted in the structure of protein being damaged [4,5,17], as well as the emulsifier in the coconut milk which was damaged and finally virgin coconut oil separated from the coconut milk emulsion system.

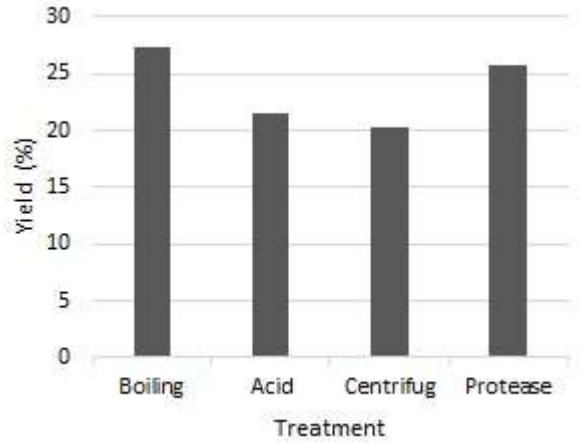

Fig. 1. Yield of virgin coconut oil from different treatment of extraction.

B. Free Fatty Acid and Density

The amount of free fatty acids could be related to the quality of oil, the higher free fatty acids content, the quality of oil considered lower.

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

Treatment	Free fatty acid (%)	Density (g/mL)
Boiling	0.207[a]	0.910[c]
Acid	0.085[b]	0.938[a]
Centrifugation	0.112[b]	0.919[b]
Protease	0.092[b]	0.924[b]
		Aquadest: 1.008

The same letter superscripts in the same column indicate not significant difference at $p<0.05$.

Table above shows free fatty acids of virgin coconut oil extracted with various treatments. Among the oil samples produced with different treatments, the virgin coconut oil resulted from acid treatment achieved the lowest free fatty acids (0.085%). It was not different ($p<0.05$) from those oil samples extracted with centrifugation and addition of paddy oats protease. Therefore, free fatty acids of all treated samples were significantly lower than that of boiling treatment. All treatments in this study produced oil that met the standards for free fatty acids of VCO, where the maximum allowable free fatty acids of 0.5% [18]; as well as Asian and Pacific Coconut Community Standard [19]. Free fatty acids and also glycerol are produced from hydrolysis of fat in the VCO. The hydrolysis process, one of vegetable oil deterioration factors, is generally accelerated by the presence of water and high temperatures beside metals, light, and air [19], therefore VCO oil produced by the boiling treatment had a higher level of free fatty acids compared to those of other treatments. Density could be used as a measure of the VCO purity, high-density oil shows an imperfect extraction process due to VCO contains high 370 moisture. The density of VCO that is required by Malaysian Standard (2007) is between 0.908-0.926 g/mL. In this study, boiling treatment yielded VCO with the highest purity of oil since it resulted in a density of 0.905 g/mL lower than those of other treatments. Oil treated with centrifugation had a slight increase in density (0.919 g/mL) and it did not differ from the oil sample treated with paddy oats protease. There was a marked sharply increase in the density of oil sample treated with acid and cause it to have a significant difference from other treatments. The boiling process allows for the complete evaporation of water in the process of extracting coconut oil, resulting in water-free coconut oil. The slightly higher water content in oil extracted by the addition of acid, protease and centrifugation due to oil collection formed after the extraction process is imperfect, allowing water to be included in the oil.

C. Sensory Attributes

Sensory attributes Means for sensory attributes are shown in Table II.

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

Treatment	Sensory attributes		
	Aroma	Taste	Color
Boiling	8.7[a]	8.5[a]	7.4[b]
Acid	7.2[c]	6.7[c]	9.3[a]
Centrifugation	7.9[b]	7.5[b]	8.9[a]
Protease	7.6[bc]	7.3[b]	9.2[a]

The same letter superscripts in the same column indicate not significant difference at $p<0.05$.

All treatments to produce VCO resulted in a high score of all evaluated parameters. As a coconut oil, VCO has a very strong coconut fragrance. The high average score of preference for all attributes of aroma, taste, and color of VCO is probably due to panelists comparing with common vegetable oils in the market they normally consume. The oil samples treated with boiling scored highest for aroma (8.7) and taste (8.5), while the score of color was the lowest compared to those of other treatments. The heating of boiling treatment allowed curd (broken emulsifier) cooked and contribute fragrance aroma and taste of resulted oil. Whereas in other treatments, fresh curd was immediately separated from the resulted oil without the heating process. The oil sample treated with centrifugation and addition of paddy oats protease received a lower score in aroma and taste, and it was no difference between them ($p<0.05$). The lowest score of aroma and taste (7.2 and 6.7) respectively subjected to the oil sample treated with the addition of acid because the oil formed slightly smells sour. Boiling treatment resulted in VCO with the lowest color score compared to those of other

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treatments. The heating process provides an increase in a chemical reaction including the formation of browning in the oil, besides that the heating process causes curd cooked and contributes to the emergence of brown yellow in the oil. While the oil treated with the addition of acid, centrifugation, and the addition of paddy oats protease received the highest score of color and no significantly different among them ($p<0.05$), since the oil obtained was very clear (colorless). Overall, the result of this study showed that VCO produced with the addition of paddy oats protease had a similar characterization to samples treated with acid and centrifuge. Hence, paddy oats protease extract could be used for extracting VCO, because consistently ranked high for all evaluated parameters.

IV. Conclusion

This study shows that protease extracted from paddy oats seed peel have excellent proteolytic activity, especially the activity for hydrolyzing proteins in emulsifier coconut milk. Extraction process by using protease produces high yields and also produces good quality virgin coconut oil. Paddy oats seed peel protease able to breakdown protein of coconut milk emulsifier properly and finally coconut oil easy to be extracted. Some research are needed to develop the use of protease to extract virgin coconut oil, so that it can improve the quality of virgin coconut oil, especially the sensory attributes.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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C. Indarto and Pomin Li, "Protease from Paddy Oats (*Gnetum gnemon* L.) Seed Peel and Its Potential for Extracting Virgin Coconut Oil," *International Research Journal of Advanced Engineering and Science*, Volume 4, Issue 1, pp. 303-306, 2019.