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Lactobacillus Plantarum Fermentation Effect on Tannin Reduction, Proximate Analysis, and Protein Profiles of Ganyong (*Canna edulis* Kerr) Flour

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ABSTRACT

One of the problems in using ganyong (*Canna edulis* Kerr) as food is the presence of tannin, an antinutritional substance, that can reduce the nutritional quality of ganyong. The purpose of this study was aimed to analyze the effect of fermenting ganyong using *Lactobacillus Plantarum* bacteria on the chemical compounds and the reduction of tannin level in its flour. The results showed that the optimum conditions of fermenting ganyong were at pH 6, the temperature of 40 ^{o}C, and at the fermentation time of 36 hours. At the optimum conditions, *Lactobacillus Plantarum* was able to decrease tannin content in ganyong from 2.53 mg/mL to 0.84 mg/mL. The reduction of tannin content is due to the activity of tannase produced by the microorganisms. Fermentation caused the enrichment protein content, from 1.87% to 2.01% and the reduction of starch, amylose, and amylopectin contents to 51%, 16.82%, and 38.08% from 60.19%, 18.27%, and 43.49% respectively. SDS-PAGE results showed that the protein profiles changed in ganyong sample after the fermentation process. Protein with a molecular weight of 72.49 kDa which showed in the non-fermented ganyong, did not appear in the fermented ganyong, and protein band intensities also showed changes.

Keywords: Ganyong; *Lactobacillus Plantarum*; tannin; protein profile

INTRODUCTION

Ganyong or edible canna (*Canna edulis* Kerr) is a type of tubers that are found in various regions of Indonesia. Ganyong tubers contain high carbohydrates, thus, they can be used as staple food [1]. Preparation of ganyong as food is usually done by boiling or steaming, and it can be prepared for extending the storage time as flour. Starch plays an important role in flour processing, especially in terms of providing energy for humans. Starch content is also one of the important criteria for improving the quality of the flour. Starch consists of two fractions that can be separated. The dissolved fraction is amylose and the unsolved fraction is amylopectin. Generally, starch contains more amylopectin than amylose. The comparison between amylose and amylopectin affects the solubility and degree of starch gelatinization [2]. Preparation of ganyong tubers to produce qualified products can be done through the fermentation phase. Fermentation has a variety of benefits, for example, to provide a texture of food products, preserving food products, and improving the taste of certain food products [3]. Fermentation can also reduce non-digestible carbohydrates and reduces the level of antinutritional compounds of the food.

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One of the obstacles to the utilization of ganyong as food is the existence of antinutritional compound which is tannin [4]. Tannin is a unique polyphenol because it has either a positive or negative effect on human health. Tannin can act as an antioxidant that binds free radicals, thus, the body can avoid cell damage and prevent diseases. However, tannin is an antinutritional compound because it can bind to proteins forming insoluble chemical complex causes a reduction of protein digestibility and can affect the activity of enzymes in the human digestive system [5]. It is necessary to make treatments for decreasing tannin compound in the ganyong tuber, thus, it is not harmful for consumption [6]. One of the treatments to decrease tannin content in ganyong tuber is to use enzymes produced by certain microorganisms through the fermentation process to hydrolyze tannin.

Lactobacillus plantarum is one of the microorganisms capable to hydrolyze tannin by producing an extracellular tannase that hydrolyzes ester bonds in tannin compound into glucose and gallic acid [7], [8]. Previous studies have shown that in addition to reducing anti-nutrition, *Lactobacillus Plantarum* fermentation can increase protein content in foods [9], [10]. The microorganism is not pathogenic that causes infection and intoxication, and it has been widely applied in the fermentation of food products so it can be used in this study. Moreover, the fermentation method to decrease tannin content in ganyong tuber using *Lactobacillus Plantarum* bacteria has never been done before. In this study, ganyong fermented flour was then analyzed by using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) technique to obtain the protein profile of ganyong fermented flour.

EXPERIMENT

Chemicals and Instrumentation

Ganyong tubers used in this study were obtained from Pujon-Malang. Microbial culture, chemicals, and reagents used include *Lactobacillus plantarum* bacteria obtained from Center for Food and Nutrition Studies Laboratory of Gadjah Mada University, deMan Ragosa Sharpe Agar (MRSA) (Oxoid), deMan Ragosa Sharpe Broth (MRSB) (Oxoid), bacterial peptone (Merck), ammonium iron(III) sulfate (Emsure), potassium ferricyanide (Merck), Nelson reagents (Merck), ammonium molybdate (Merck), sodium arcenate (Merck), sodium thiosulfate (Merck).

Main instrumentals used include incubator (Haraeus Type B 5042), oven (Memmert), pH meter (inoLab WTW), water bath (Memmer W 200), shaker (Edmund Buhler SM 25, 24B), autoclave (all American Model 20X), laminar air flow, UV-Vis Spectrophotometer (Shimadzu Model 160A double-beam), Visible spectrophotometer (Thermo Scientific Genesys 20), and electrophoresis equipment (SDS-PAGE).

Procedure reaction

Optimum Conditions of Fermentation

The determination of optimum conditions of fermentation included determination of optimum pH, optimum temperature, and optimum fermentation time which were done with 4 variations of treatment in triplicate, based on the lowest tannin content of each treatment. For the determination of optimum pH, the variations used are pH 4, pH 5, pH 6, and pH 7. The optimum pH obtained would be used to determine the optimum temperature of fermentation with temperature variations of 35 ^{0}C, 40 ^{0}C, 45 ^{0}C, and 50 ^{0}C. Furthermore, optimum pH and optimum temperature obtained would be used to determine the optimum fermentation time of the fermentation with a variation of time 12, 24, 36, and 48 hours.

Tannin Content Analysis

The content of tannin in ganyong flours was analyzed using Nishitani and Osawa's method [11] that modified by Setiarto and Widhyastuti [12] which was initiated by making a tannic acid calibration curve. Standard tannic acid solution with a concentration of 25 ppm was added to 5 of volumetric flasks 25 mL as much as 1, 2, 3, 4 and 5 mL respectively, then, each flask was added with 3 mL of ammonium iron(III) sulfate solution. The solution was stirred for 30 minutes and then added with 3 mL of potassium ferricyanide solution and stirred again for 30 minutes. The solution was diluted with distilled water to prepare a standard solution of 1, 2, 3, 4, and 5 ppm, and the absorbance was measured at 752.5 nm wavelength.

As much as 2.5 grams of the modified ganyong flour was dissolved into 50 mL of water and then taken as much as 5 mL and diluted in 10 mL volumetric flask. 1 mL of the solution was taken and diluted again by addition of ammonium iron(III) sulfate solution (shaken for 30 minutes), potassium ferricyanide solution (shaken for 30 minutes), and water, then the absorbance was measured at 752.5 nm wavelength.

Analysis of Starch Content

Two grams of the sample was suspended using 50 mL of water then centrifuged for 15 minutes at 5000 rpm. The suspension was filtered then the precipitate was washed and transferred into 500 mL Erlenmeyer flask containing 200 mL of water. A-25 mL of 25% HCl was added to the mixture. The solution was neutralized using 45% NaOH and filtered. Nelson-Somogyi method [13] was used to measure the starch content of the resulted filtrate.

Analysis of Amylose and Amylopectin Contents

1 mL of 95% ethanol solution and 9 mL of 1 N NaOH were added to 0.1 gram of ganyong flour and heated for 1 hour, then the solution was diluted to 100 mL. After that, 5 mL of the solution was placed into 100 mL volumetric flask and diluted again by adding 1 mL of 1 N CH_3COOH, 2 mL of 2% I_2, and water. The absorbance of the solution was measured using UV-Vis Spectrophotometer at 620 nm of wavelength and then calculated the amylose content. The content of amylose was used to estimate the content of amylopectin in the sample based on the total difference between amylose and the starch contents.

Protein Content Determination

Protein content determination was performed using the micro-Kjeldahl method [13]. A gram ganyong flour was diluted with water in a 10 mL volumetric flask, then transferred to the Kjeldahl's flask. Next, 10 mL of H_2SO_4 and 5 grams of Na_2SO_4-HgO mixture as catalyst was added to the solution and boiled for 30 minutes. The solution was then distilled with a quantity of NaOH-$Na_2S_2O_3$ to converted ammonium salt to ammonia. The end of the condenser was dipped into an Erlenmeyer flask containing a saturated boric acid solution, and then the distillation result was titrated with a hydrogen chloride solution by way of phenolphthalein indicator.

Protein Profile Analysis of Ganyong Flours Using Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS – Page) Method

Gel preparation

Gels used for the analysis were separation gel and stacking gel. The separation gel was prepared by mixing 1.9 mL of water with 1.3 mL of 1.5 M Tris-HCl buffer solution (pH 8.8), 1.7 mL of 30% acrylamide solution, 50 µL of 10% APS (ammonium persulfate) solution, and

2 µL of TEMED (N,N,N',N'-tetramethyl-ethylenediamine) solution. The mixture was stirred and formed in a gel plate. The stacking gel was made by mixing 1.4 mL of water, 250 µL of 1 M Tris-HCl buffer solution (pH 6.8), 330 µL of 30% acrylamide solution, 20 µL of 10% SDS solution, 20 µL of APS solution, and 2 µL of TEMED solution. The mixture was stirred and poured over the separating gel, then a well-forming comb was inserted and left for 30 minutes.

Preparation and Running Sample

Ganyong samples were suspended to the phosphate buffer solution and boiled for 5 minutes. The gel mold was placed into the electrophoresis box, the upper and lower reservoirs were filled with the separation buffer solution. As much as 10 µL prepared sample was loaded into the wells, and the protein marker was loaded into the first lane. Electrophoresis was run at 200 V voltage, and the running process was stopped when protein-dye markers reached the bottom of the gel.

Staining and Determination of Molecular Weight

The resultant gel was immersed in a dye solution and stirred for 6 hours. After the staining process, the gel was washed using water then soaked into the destaining solution. The destaining solution was changed until produced a clear gel. The determination of molecular weight was done by measuring the migration distance of protein bands.

Statistical Analysis

In this study, triplicate data of tannin content analysis were statistically analyzed using the Completely Randomized Design (CRD) model performed by the Statistical Package for Social Science (SPSS) 19.0 software program. The Least Significance Different (LSD) test at 5% level ($p < 0.05$) was then performed to determine the difference between the treatments.

RESULT AND DISCUSSION

Tannin Level of Ganyong Flours

The results of this study showed that the fermentation treatment of ganyong flours using *Lactobacillus Plantarum* bacteria could decrease tannin content (Figure 1). The tannin content in the non-fermented ganyong is 2.53 mg/mL. During the fermentation process, *Lactobacillus Plantarum* bacteria produce tannase (tannin acyl hydrolase) that can hydrolyze tannins contained in ganyong flour [14]. Tannin acyl hydrolase is an enzyme that catalyzes the hydrolysis reaction of ester bonds in the hydrolyzable tannin and gallic acid ester to produce gallic acid and pyrogallol which is antioxidants [15], [16].

In the determination of optimum pH, the largest decrease in tannin content was obtained at treatment with pH 6. pH 6 was then used as the optimum pH in the subsequent treatment to determine the optimum temperature. The result showed that in the fermentation at a temperature of 40 ^{0}C, the content of tannin was the lowest compared to the other temperatures. These results are in agreement with the previous study which reported that the activity of tannase is optimum to hydrolyze tannin at pH 6, and temperature of 40 ^{0}C [17].

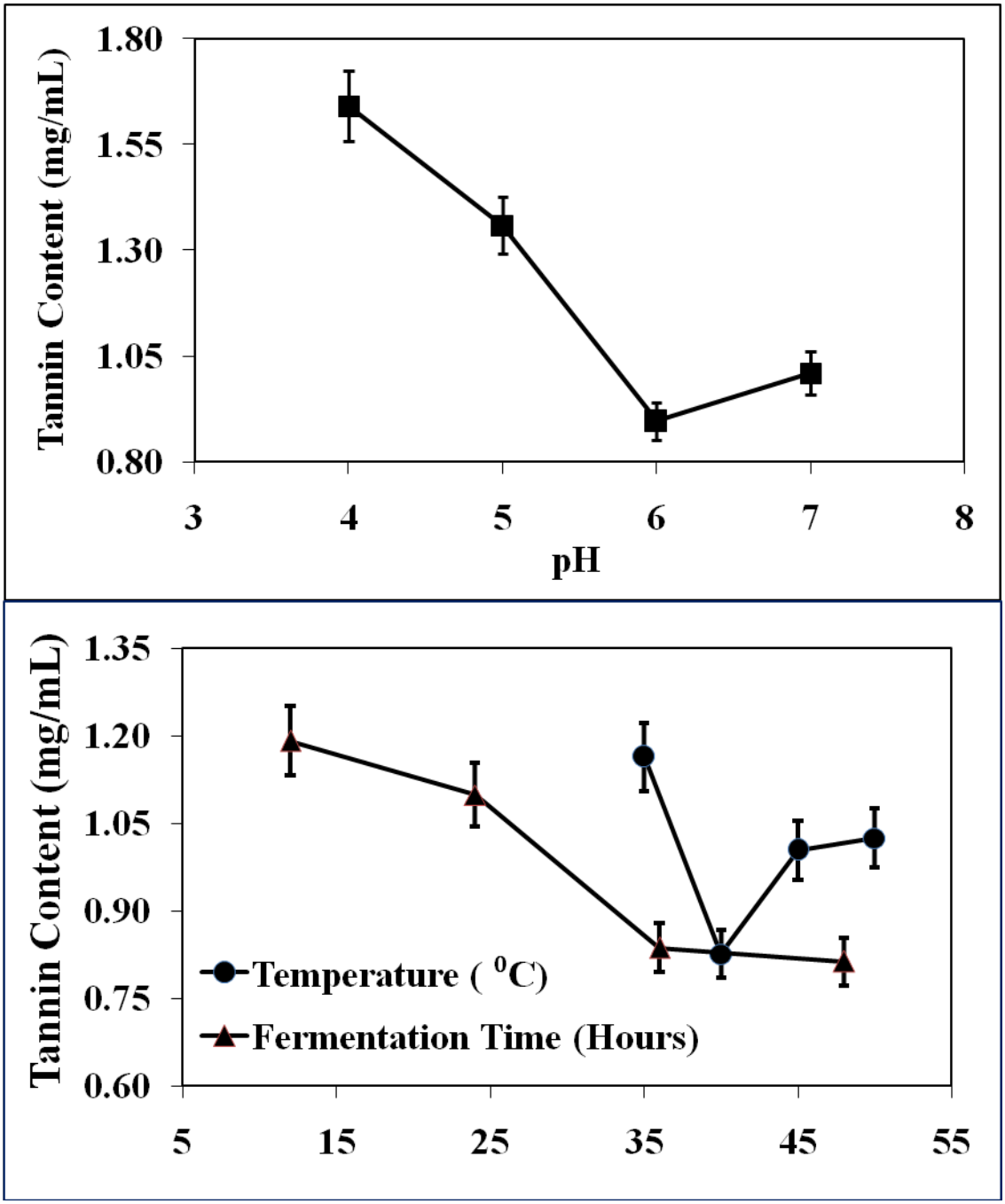

Figure 1. Tannin contents in the determination of optimum conditions of fermenting ganyong. (above) pH; (bottom) temperature and fermentation time.

It can be seen that in determining of optimum fermentation time, treatment with 48 hours produced the lowest tannin level, but treatment with 36 hours was statistically the best treatment, thus, it was used as the optimum fermentation time in this study.

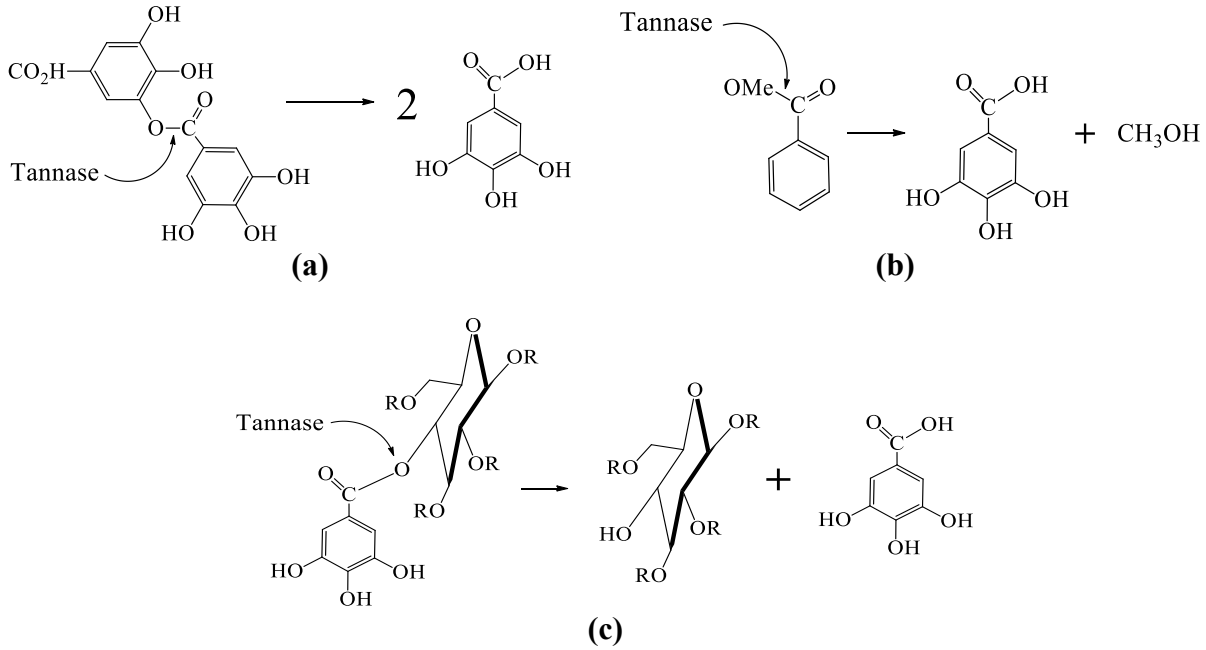

Figure 2. Mechanism of action of tannin acyl hydrolase (tannase) to catalyzes the breakdown of tannins. (a) Ethyl gallate; (b) Methyl gallate; and (c) Gallotannin [14].

Protein, Starch, Amylose, and Amylopectin Levels in Fermented and Non-Fermented Ganyong Flours

After obtaining the optimum condition of *Lactobacillus Plantarum* fermentation, measurements were made to compare contents of starch, amylose, amylopectin, and protein of the fermented and non-fermented ganyong flours (Table 2). In this study, carbohydrate levels in the form of starch, amylose, and amylopectin of ganyong flour decreased after 36 h fermentation treatment using *Lactobacillus plantarum*. The decrease in carbohydrates occurred because during the fermentation, the bacteria utilized carbohydrate components such as amylose and amylopectin as a carbon source for its growth [18].

Table 1. Data of protein, starch, amylose and amylopectin contents in ganyong flours

No	Ganyong Flour Contents	Fermented (%)	Non-fermented (%)
1	Starch	51.90 ± 2.12	60.19 ± 2.16
2	Amylose	16.82 ± 3.33	18.27 ± 3.39
3	Amylopectin	38.08 ± 1.61	43.49 ± 1.24
4	Protein	2.01 ± 0.04	1.87 ± 0.01

The results also showed that the fermentation of ganyong using *Lactobacillus Plantarum* bacterial increased protein concentration. A previous study confirmed that fermentation can convert substrates containing nitrogen and carbon into proteins [19]. It was seen in Table 2 that starch level decreased but protein level increased.

Protein Profile of Ganyong Flours

The protein profile of fermented and non-fermented ganyong flours can be determined by using electrophoresis technique with SDS-PAGE. The result shown in Figure 3 shows that there are differences in protein patterns in both fermented and non-fermented ganyong flours.

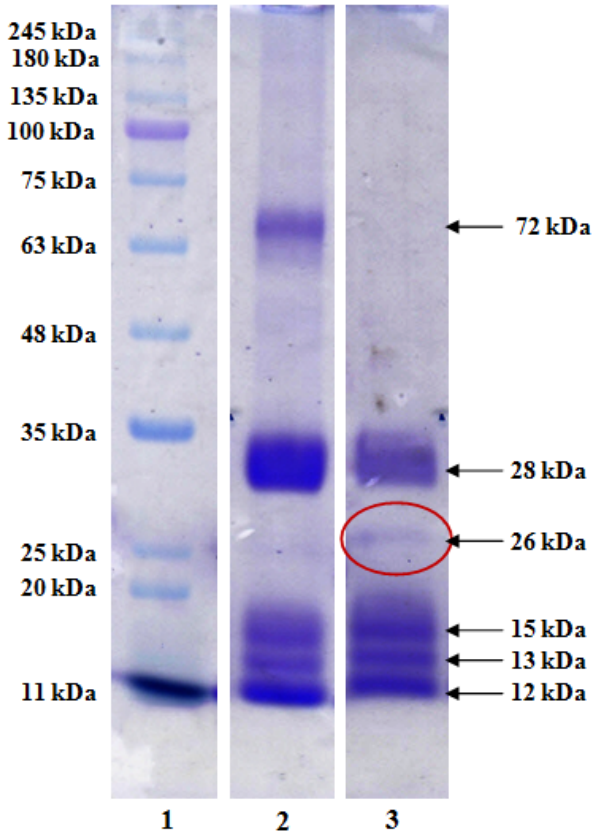

Figure 3. SDS-PAGE results of fermented and non-fermented ganyong flours. Lanes: (1) Marker; (2) Non-fermented ganyong flour; (3) Fermented ganyong flour.

Non-fermented ganyong flour (second lane) contained proteins with molecular weights of 72 kDa, 28 kDa, 15 kDa, 13 kDa, and 12 kDa. After fermentation in optimum conditions, the protein band with 72 kDa range disappeared, while the other protein bands re-appeared in the fermented ganyong flour (third lane) with different intensities. The disappeared 72 kDa protein is suspected to be a protein that binds with tannin causes antinutritional effects. The missing of protein is thought because the protein is converted to another substrate during the fermentation process. *Lactobacillus Plantarum* can produce proteinases that responsible for the degradation of protein [20].

It can be seen that there was a decrease in intensity in the 28 kDa protein band. Meanwhile, there was an increase in intensity in the molecular weights of 15 kDa, 13 kDa, and 12 kDa. This fermentation process may induce the synthesize of new proteins that have more functional properties. There was a new protein band in the fermented ganyong sample with a molecular weight of 26 kDa, but the intensity is very small.

The differences of protein band intensity shown in SDS-PAGE results of fermented and non-fermented ganyong flours indicated that the activity of *Lactobacillus Plantarum* bacteria in the fermentation process has an effect on protein profiles.

CONCLUSION

The results revealed that the optimum conditions of fermenting ganyong using *Lactobacillus plantarum* are at pH 6, the temperature of 40 ^{0}C, for 36 hours. Fermentation causes the enrichment of protein levels, also in the decrease of carbohydrate and tannin levels in ganyong flour. Fermentation treatment also caused a change in the protein component of ganyong flour shown by the SDS-PAGE results.

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