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ORIGINAL RESEARCH

Analysis of protease activity of melinjo *(Gnetum gnemon L.)* skin on the tenderness of buffalo meat

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Abstract. To enhance the tenderness of meat, it can be accomplished physically or chemically. In chemical way, the process can be accomplished using protease enzymes that can be obtained from fruit, the higher the protease enzyme activity, the softer the meat. In this experiment, the extract and juice of melinjo skin (*Gnetum gnemon* L) was used to examine the protease enzyme activity. Papaya (*Carica papaya*) and pineapple (*Ananas comosus*) were used as a comparison. The research was conducted to understand the influence of incubation time (t) and incubation temperature (To) towards the optimum activity characteristic of protease enzyme on the juice and the extract of melinjo skin, papaya and pineapple. The incubation time of juice and extract was from 0 to 90 minutes with incubation temperature of 37°C and incubation time at 20 minutes with incubation temperature range from 20 to 80°C. The protease enzyme activity of juice and extract expand significantly ($p<0.05$) in conjunction with the increase of incubation time and temperature. The research results revealed that the maximum activity of protease enzyme detected at incubation time of 90 minutes for melinjo juice and pineapple skin, and 10 minutes for papaya. The optimum incubation temperature of melinjo skin at 70°C, papaya at 60°C and pineapple juice at 60°C. Meanwhile, the maximum activity of protease enzyme from melinjo extract identified in the incubation time at 80 minutes, papaya at 90 minutes and pineapple 60 minutes, whereas the incubation temperature of melinjo skin juice at 60°C, papaya at 80°C and pineapple at 30°C.

Keywords: Buffalo Meat, Juice, Protease,

INTRODUCTION

Meat is a food rich of nutrients like protein, fat, minerals, vitamins and other important substances needed by the body. The quality of meat is strongly influenced by factors of slaughter processes [1]. The factors before slaughter are examples such as genetics, species, gender, age of livestock and others, while the factors that affect the quality of meat after cutting are withering, pH of carcasses and meat, cooking methods, electrical stimulation, hormones, anti-biotics, texture, doneness, fat bonds, color and additives of meat enzymes [2]. For food connoisseurs, the tenderness of the meat is the most important factor apart from the already mentioned criteria.

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In the process of meat softening, generally it can be done by physical and chemical processes [3]. Physically, it can be done by cooking, while chemically it can be done by adding proteolytic enzymes to the meat. Meat softening using proteolytic enzymes is the most used method. This enzyme can be found in fruits and vegetables, for example papain and bromelain enzymes [4]

Papain enzyme is a protease enzyme produced by papaya plants *(Carica papaya* L.*),* especially young papaya, while bromelain is produced by pineapple and its skin *(Ananas comosus).* Bromelain in pineapple can strike the connective tissue of proteins and degrade them, so the meat can be more tender. Meanwhile, the papain and chymopapain enzymes found in papaya could attack proteins in muscle fibers and then control them into smaller peptide parts [4],[5].

Beside of papaya and pineapple, melinjo skin *(Gnetum gnemon* L.*)* also has the characteristics of the enzyme protease. It can be known by

rubbing its skin on human skin that can cause softer [6], but there has been no scientific research to prove it. Therefore, this research was conducted to prove that melinjo skin also contains enzymes which is able to soften the flesh with higher tenderness compared to papain and bromelain enzymes and will not cause allergies. This study is expected to provide a new formulation result in the food industry development, especially the meat industry.

Generally, this research aimed to know the influence of protease enzyme from melinjo (*Gnetum gnemon* L.) skin towards meat tenderness. It has been done deeply regarding comparison of protease enzyme from melinjo skin and papain from papaya as well as bromelain from pineapple.

METHODOLOGY

Research Samples

The samples used for this research were old buffalo meat, young melinjo skin, papaya and pineapple. The meats were taken from frozen food market "CCK" in Kajang imported from India named as "Allana". Young melinjo aged of 45 days obtained from Bandar Baru Bangi Area, the samples were extracted from the skin with the comparison of 100 grams sample/100 ml water to achieve liquid extract. Papaya was taken from garden of University Kebangsaan Malaysia (UKM) and young pineapple was obtained from Bandar Baru Bangi market.

Sample Preparation Juice and Extract Preparation

Preparation was done by separating seeds from melinjo skin. Melinjo skin, young papaya and pineapple were weighed. Every sample had been weighed as much as 100 grams, then 100 ml aquadest was added. The samples were then blended for 1 minute in slow speed and for 2 minutes in high speed. The resulting liquid was filtered using a masline fabric, screening was continued using Whatman no.1 paper. In the process of extract, the result of the filter was put in a petri dish and was feezed in a refrigerator with a temperature of -2ºC for 2 hours, so that an ice slab was formed. These frozen samples were then dried using an ice dryer from 48 to 72 hours, so that they formed into powder.

Chemicals Determination

As much as 4 grams Sodium Hydroxide (NaOH) 1mol/l was dissolved into 100 ml of distilled water, stirred until reaching homogeneous solution form. Lead Phosphate (1mol/l pH 75), as much as 34 grams of KH_2PO_4(a) was dissolved into 250 ml of distilled water and 57 grams of $K_2HPO_4.3H_2O$(b) was dissolved into 250 ml of distilled water. Then the pH of the solution (b) was adjusted to pH 7.5 using solution (a). Furthermore, a concentrated 1 mol/l of Hydrochloric Acid (HCl) solution of 9.8 ml was added to distilled water 72 ml stirred until a homogeneous solution is formed. Casein Lead Solution (2% w/v), phosphate 0.1 mol / pH 7.5), 1 gram of casein was added with 5 ml of distilled water and dissolved in a measuring flask of 50 ml. It had been added 2.5 ml of Sodium Hydroxide (NaOH) solution (3.2.4.1) and 30 ml of distilled water to the measuring flask. The solution was stirred until it was completely dissolved and formed a homogeneous solution. A total of 5 ml of lead phosphate (3.2.4.2 solution) was added to the clear solution and the pH was changed at 7.5 using an HCl solution (3.2.4.3 solution) and dissolved again with distilled water so that it reached a volume of 50 ml. Trichloroacetic acid (0.3 mol/l) was prepared by dissolving as much as 4.9 grams of trichloroacetic acid solution into 100 ml of distilled water and stirring until a homogeneous solution. Sodium Hydroxide (NaOH) solution was prepared by dissolving 50 ml of NaOH solution with 50 ml of distilled water and stirring until a homogeneous solution is formed.

TCA Deposition (Trichloroacetat)

TCA deposition was carried out according to Bergmeyer (1984) method after modified. Three sets of test tubes were provided, each set consisted of 10 test tubes. 2.5 ml of casein buffer (3.2.4.1 solution) was added to each test tube. Then each test tube was added as much as 0.5 ml of cider and melinjo extract, papaya, and pineapple peels with a concentration of 1%. The mixture of all sets of tubes was incubated at a temperature of 37°C with a time of 0,10, 20, 30, 40, 50, 60, 70, 80 and 90 minutes, respectively. As much as 2.5 ml of TCA (3.2.4.5 solution) was added to each test tube. Each test tube was allowed to stand for 30 minutes before being filtered using Whatman No.1 filter paper. The absorption of the clear filter was measured using the Thermo Spectronic Helios instrument at 280 nm by using absorption at 280 nm.

Optimum temperature determination kinetic characteristics of protease

It had been used seven strata of incubation temperature 20ºC, 30ºC, 40ºC, 50ºC, 60ºC, 70º C and 80ºC. The method ujsed is the same as TCA deposition, only the incubation period is set for 20 minutes.

Protease enzyme activity testing

The protease enzyme activity testing and meat ripening was carried out with following to the [7] with several modifications. Frozen meat was

thawed in the refrigerator at 4°C for 24 hours until water comes out of the meat. Meat was cut with a weight of 200 grams, measured for 10 cm x 20 mm, and put in a plastic container. Every 100 ml of melinjo skin juice, papaya and pineapple were mixed with the meat and soaked at room temperature with a soaking time of 20, 40 and 60 minutes, every 10 minutes the meat was turned over until all parts were completely submerged. Then, the meat was rinsed and put in an aluminum pan, 400 ml of water is added to the sample and then boiled on the stove at an outside temperature of 100°C, with the inner temperature of the meat was 75°C for 30 minutes. The meat was cooled at room temperature for 30 minutes and completely ready for analysis.

Statistics Analysis

ANOVA test was conducted by SAS application, then continued by DUNCAN test, if there are significant differences of $p<0.05$.

RESULTS AND DISCUSSION

Incubation time and the behavior of protease enzyme on juice and extract samples

The initial experiment step was conducted to find out the influence of incubation time towards protease enzyme activity, all samples were incubated in the temperature of 37ºC, this was done commonly due to the optimum activation of enzyme at temperature between 37ºC and 40ºC. Generally, if the enzyme having optimum temperature from 37ºC to 40ºC were heated on higher temperature, hence the enzyme will be degraded, then, the enzyme activation will be reduced (Kusumaningrum dkk, 2019). The results of the experiment can be seen in the Table 1.

Table 1 reveals that incubation time influences significantly on ($p<0.05$) protease enzyme sample behaviors examined (juice and extract). In general, the protease enzyme in the fruit extract has higher activity compared to protease enzyme activity in juice sample. This is probably due to the juice contains lower proteins compare to its extract, therefore the activity of protease enzyme in juice shows slightly low. In this melinjo juice sample, papaya and pineapple, the differences of protease enzyme activity significantly showed in time range between 0, 70, 80 and 90 minutes ($p<0.005$), whereas in the sample extract, the differences of protease enzyme activity in all experiment samples significantly occurred during 0 to 90 minutes ($p<0.005$). The differences of melinjo skin sample and pineapple were remarkably appeared in the incubation time of 20, 40, 60 and 70 minutes ($p<0.05$).

These dissimilarities of protease enzyme on juice sample were exceptionally shown in minutes ranges of 0, 70, 0, and 90, where protease enzyme activity found melinjo was higher that the activity of protease enzyme in papaya and pineapple. However, in the extract sample, the protease enzyme activity differences in all samples happened during first treatments until minutes of 90th. The papaya

Table 1. The behavior of protease enzyme juice and melinjo skin extract, papaya and pineapple during incubation time from 0 to 90 minutes.

Incubation Time (minutes)	Juice			Extract		
	Melinjo	Papaya	Pineapple	Melinjo	Papaya	Pineapple
0	0.865^{a}	0.703^{b}	0.762^{b}	8.378^{a}	1.719^{b}	3.905^{a}
10	1.073^{a}	0.832^{a}	0.838^{a}	7.354^{a}	1.768^{b}	7.149^{a}
20	1.278^{a}	0.854^{a}	1.092^{a}	7.930^{a}	1.897^{c}	7.203^{b}
30	1.465^{a}	0.854^{a}	1.086^{a}	8.046^{a}	1.889^{b}	7.913^{a}
40	1.665^{a}	0.914^{a}	1.149^{a}	8.168^{b}	1.973^{c}	8.351^{a}
50	1.946^{a}	0.870^{a}	1.316^{a}	8.246^{a}	2.014^{b}	8.786^{a}
60	2.651^{a}	0.843^{a}	1.468^{a}	8.268^{b}	2.168^{c}	9.384^{a}
70	2.778^{a}	0.938^{b}	1.524^{b}	8.273^{b}	2.249^{c}	8.792^{a}
80	3.224^{a}	0.970^{b}	1.642^{b}	8.705^{a}	2.273^{b}	8.703^{a}
90	3.178^{a}	0.986^{b}	1.749^{b}	8.703^{a}	2.276^{b}	8.649^{a}

a, b, c: the difference letter on the same line indicates a notable difference in $p<0.05$

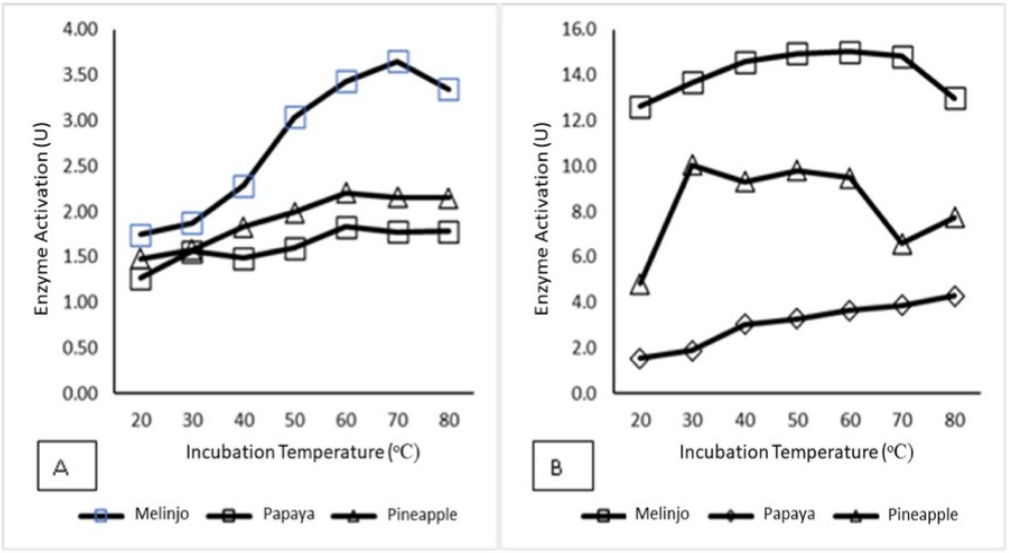

Figure 1. The relationship between protease enzyme activity and temperature of samples: (A) Juice and (B) Extract.

extract had lower protease enzyme activity rather than protease extract in melinjo and pineapple.

The protease enzyme activity was influenced by its substrate, the higher the feeding substrate the bigger its enzyme activation. The research conducted by [8] utilized substrate as a measure tool to understand the activity of certain enzyme. Protease enzyme activity are considered by its ability in hydrolyzing casein substrate which could break down the polypeptide chains on casein. Casein substrate will be decomposed into peptide strands and amino acids. To determine the magnitude of protease activity, it was carried out by adding up the tyrosine produced by hydrolyzing casein [8]. Beside the substrate factors, it was likely there were other factors that caused protease enzyme activity in fruit extract to be higher that its juice, for instance the pH condition, and the presence of cofactors or inhibitor in each of these samples [9].

Protease enzyme activity is determined by the length of incubation time, each protease enzyme has a different time to achieve its optimum activity. However, this is influence by many factors [10]. Melinjo seed extracts take shorter time to be able to work optimally compared to papaya which takes up to 80 minutes to achieve optimal activity. Pineapple has optimum activity of protease enzyme at 40th and 60th minutes. The enzyme takes different time to reach it optimum activity, it is caused by enzyme production that are primary metabolites occurred in logarithmic phase of initial phase of stationer (Oktavia et al., 2018; Sonia & Kusnadi, 2015). This is in accordance with the reasearch experiented by (Kurniawati et al, 2021) they found that optimum enzyme activity occured at the 12th hour had a niticeable difference compared to the 4th and the 8th hour.

Incubation temperature and protease enzymes behavior in juice and extract samples

The treatment results of different temperature (20°C, 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C) on protease enzyme activity from each sample in the experiments were provided in the Figure 1.

Figure 1 above express an idea of enzyme activity rose as the temperature increase. However, the enzyme activity had optimum limits on their work. In melinjo juice sample, the optimum working temperature of protease enzyme was 70°C with an enzyme activation of 3,655. Meanwhile, the optimum temperature of enzyme in papaya and pineapple was 60°C with enzyme activation of 1,830 and 2,205 respectively. As for the extract sample, the optimum temperature for enzyme behavior in the melinjo sample was 60°C with enzyme activation of 15,025, while in the papaya extract sample, the optimum temperature was 80°C with enzyme activation of 4,285. Pineapple has low optimum enzyme activation of 30°C with enzyme activation of 10,005.

The analysis results revealed that temperature had very paramount role on protease enzyme activity in the mixed sample with melinjo seed extract and juice, papaya and pineapple. Each enzyme had certain temperature to work optimally [11].

In the optimal utilization of enzymes, it is necessary to comprehend better the factors affecting the stability and enzyme activity. Enzymes have biological component and contain high percentage of proteins which have an impact to the environment. Several factors influencing are the concentration of enzymes and their reaction, temperature and their effects, pH, radiation (light), enzyme inhibitor and activator. In general, temperature affects exponentially to the enzyme activity in which the temperature increases of 10°C will escalate doubled enzyme activity [8],[11]. However, if the optimum temperature has been reached, then the temperature enlargement will decrease enzyme activity because it will be degraded [10].

These results aligned with the research conducted by [4], depicted that protease enzyme (papain, bromelain, ficin) were still active at the temperature of 60-70°C, whereas the protease enzyme of papaya juice had optimum incubation temperature between 60°C and 80°C. This was caused by papain enzyme and chymopapain found in papaya were protease protein classified in the sulfidrin protease which had higher heat resistance compared to other protease enzyme, She mentioned that papain enzyme can be stable at the temperature of 80 to 120°C [12]. The increase activity of protease enzyme proved that protein breakdown into amino acid increased especially tyrosine, tryptophan and phenylalanine [10].

CONCLUSION

Protease enzyme activity was determined by difference time length to reach it optimum activity. Generally, protease enzymes in the fruit juice need more incubation time to achieve its optimum activation compared to the extract. Therefore, it is suggested to utilize protease enzyme in the extract form on its applications. The temperature had important role on the protease enzyme activity in the juice and extract of melinjo, papaya and pineapple, this is due to of each enzyme has certain temperature to work optimally.

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