

Original Paper

Characteristics of Flavor Enhancer Based on Moringa Leaves and Tempeh Using Protease Enzymes From Sap of Papaya and Crown Flower

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Abstract— Flavor enhancer is a food additive that's designed to enhance flavor. This research aims to determine the effect type (papaya, crown flower, and mixed protease) and percentage addition (1%, 2%, and 3%) of enzyme on the characteristics of flavor enhancer based on moringa leaves and tempeh. The design used in this study was a completely randomized design in factorial pattern. The variables observed included the degree of hydrolysis, moisture content, soluble protein content, maillard product, water solubility index, oil absorption, and glutamic acid content. Data were analyzed by Analysis of Variance (ANOVA) and followed by Duncan's test. The results showed that the interaction between the type and percentage addition of enzyme had a very significant effect on the degree of hydrolysis, maillard product, and glutamic acid content, significantly affected the soluble protein content and water solubility index, and had no significant effect on water content and oil absorption. The treatment type and percentage addition of enzyme had a very significant effect on the degree of hydrolysis, soluble protein content, maillard product, water solubility index, oil absorption and glutamic acid content, and had no significant effect on water content. Hydrolysis using mixed protease enzymes with an addition percentage of 3% produced flavor enhancer that had the best characteristics based on the highest glutamic acid content of 30.583 mg/g glutamic acid equivalent with a degree of hydrolysis of 65.781%, moisture content of 5.096%, soluble protein content of 1.529 mg/g tyrosine equivalent, maillard product of 0.221 AU, water solubility index of 0.0980 g/mL, and oil absorption of 1.55 mL/g.

Keywords— hydrolysis, protease, flavor enhancer, moringa leaves, tempeh

I. INTRODUCTION

Flavor enhancer is one of the products added to food that designed to enhance flavor. Flavor enhancer compounds generally have no nutritional value and are only used to enhance and improve the taste and aroma of food ingredients [1]. During this time, flavor enhancer ingredients used in almost all products sold in Indonesia are synthetic compounds, one of that is Monosodium glutamate (MSG) [2]. The MSG is beneficial in food processing, but highly consumption causes oxidative stress to the body [3]. One organ that is vulnerable to oxidative stress due to excessive MSG induction is the liver

[4]. To reduce the amount of MSG consumption, people can utilize natural high-protein ingredients as alternatives to flavor enhancer ingredients, including moringa leaves and tempeh.

Moringa (*Moringa oleifera*) is one type of plant which contains rich nutrients. Due to high levels protein in Moringa leaves, Moringa leaves contain various kinds of amino acids, one of them is glutamic acid [5]. Glutamic acid levels in moringa leaves amounted to 75.06 mg/g protein, the highest among other amino acids [6]. Meanwhile, research conducted by the Ministry Health of the Republic Indonesia on the nutritional content of tempeh, in 100 grams of tempeh there is protein (20.8 g), fat (8.8 g), carbohydrates (13.5 g), fiber (1.4 g), Ca (155 mg), P (326 mg), Fe (4 mg), and Water (55.3 g) (BSN, 2012). In 100 grams of steamed tempeh contains 1.74% w/w glutamic acid [7].

Proteins in foods can act as flavor precursors, especially when it is hydrolyzed into amino acids and peptides with flavor enhancer properties [8]. Short-chain peptides and free amino acids, especially glutamic acid, hydrolyzed by protease enzymes in proteins can provide savory taste [9]. Therefore, it is necessary to develop the utilize of protease enzymes from local Indonesian materials because most protease enzymes for the food industry are still imported and relatively expensive, then protease enzymes in papaya sap (papain) and protease enzymes in crown flower sap (calotropin) are used for the hydrolysis process.

The hydrolysis process in making flavor enhancer can be influenced by enzymes, characteristics of substrate, and hydrolysis process conditions such as pH, temperature, and enzyme concentration used [10]. Tondais et al. [11] obtained results with the best characteristics using the calotropin enzyme at the addition of 1.5% for 1 hour in flavor enhancer from mackerel scad. Meanwhile, in the hydrolyzed milkfish, the best characteristics were obtained when using 5% papain enzyme [12]. According to Witono [2], using a mixture of proteolytic enzymes is more efficient than using a single enzyme in the processing of koro pedang hydrolysate, with the same amount of enzyme, the mixture of papain and calotropin enzymes can hydrolyze more koro pedang substrates. In Putri, Winarti, and Djajati's [13] research, the proportion of crown

flower and papain enzymes (50%: 50%) produced the best tempeh flavor enhancer powder. Meanwhile, natural flavor enhancer from white mussel and sunflower seeds obtained the best characteristics from the hydrolysis of papain and calotropin enzymes with the addition of 3% for 2 hours [14]. Fajriyah and Winarti [15] used papain and calotropin enzymes with the addition of 3% and a hydrolysis time of 1 hour to produce the best characteristics in mulberry leaf and shrimp head flavor enhancers. The purpose of this research was to determine the effect of enzyme type and percentage of enzyme addition on the characteristics of natural flavor enhancer based on moringa leaves and tempeh.

II. MATERIAL AND METHODS

A. Time and Place

This research was conducted at the Food Processing Laboratory, Biochemistry and Nutrition Laboratory, and Food Analysis Laboratory, Faculty of Agricultural Technology, Udayana University, Bali, Indonesia. The research was conducted from February 2023 until June 2023.

B. Materials

The materials used in the processing and physicochemical analysis were the main ingredients consisting of moringa leaves from the 2nd to 7th stalk after the shoots obtained in Tegal Tugu Village, Gianyar Regency and 2-day fermented tempeh obtained from a tempeh producer with Putra Malang stamp in Peteluan Sidan, Gianyar Regency. Meanwhile, the enzyme preparation includes sap of young local red papaya fruit aged 2.5-3 months obtained in Tegal Tugu Village, Gianyar Regency, sap of purple flowering crown flower plant obtained in Purnama beach area, Gianyar Regency. Materials for analysis included distilled water, Na-metabisulfite (Sigma), soluble casein, phosphate buffer, Trichloroacetic Acid (TCA), follin ciocalteu reagent, tyrosine (Sigma), sodium carbonate, local glucose, technical salt, technical maltodextrin, local vegetable oil, glutamic acid (Sigma), and ninhydrin (Sigma).

C. Research Design and Data Analysis

This research used a Completely Randomized Design (CRD) factorial pattern with the factors of enzyme type and percentage of enzyme usage used at the hydrolysis stage with 2 replicates so that 18 experimental units were obtained. The enzyme type and percentage of enzyme usage were E1K1 (1% papaya protease), E1K2 (2% papaya protease), E1K3 (3% papaya protease), E2K1 (1% crown flower protease), E2K2 (2% crown flower protease), E2K3 (3% crown flower protease), E3K1 (1% mixed protease), E3K2 (2% mixed protease), and E3K3 (3% mixed protease). The data obtained were analyzed by ANOVA and if the treatment influenced the observed variables, it was continued with the Duncan test.

D. Protease activity test

Protease enzyme activity testing refers to the research of Fajriyah and Winarti [15] and Rahayu and Susanti [16]. Measurements were made using 0.01 g of soluble casein substrate mixed with 3 mL of phosphate buffer at pH 7 and pre-incubation at 37°C for 4 minutes. Subsequently, 0.250 mL of sap filtrate was added to the substrate, then incubated at 55°C for 20 minutes. After incubation, 1 mL of 15% TCA

solution was added to stop the hydrolysis reaction (the control was made without incubation and the reaction and addition of 15% TCA solution were done before the addition of protease). Then centrifuge at 1000 rpm for 10 minutes. The supernatant obtained was taken 1 mL and mixed with 2.5 mL of 0.5 M Na₂CO₃ and added 1 mL of Folin Cioceltau reagent. The solution was homogenized with a vortex and incubated at 37°C for 30 minutes. After that, the resultant solution was measured for absorbance at 660 nm using a UV-Vis spectrometer (Shimadzu, Japan). The absorbance data was plotted on a standard curve of tyrosine (Sigma, USA) to calculate the hydrolysis activity.

Calculation of enzyme activity can use the following formula:

$$1 \text{ unit activity} = [C]/t \times 1000/181,19$$

Description: [C] = concentration of soluble protein (μmol tyrosine/mL), t = hydrolysis time (minutes), 181.19 = molecular mass of tyrosine.

E. Making Flavor Enhancer

The main raw materials used in making flavor enhancer are moringa leaves and tempeh. Before steaming, moringa leaves are washed well. Steaming is conducted using hot steam temperature for 10 minutes. Steaming aims to soften the tissue and inactivate the lipoxidase enzyme. Tempeh that had been cut ± 1 cm thick was steamed for 10 minutes. Next, moringa leaves and tempeh were crushed using a crusher with a water ratio of (1:2) (b/v). After obtaining the mixed slurry, the pH was adjusted to 7 and enzymes were added based on the type and proportion according to the treatment. Furthermore, it was hydrolyzed at 55°C for 1 hour on a waterbath. Then heated at 100°C for 15 minutes for enzyme inactivation. Centrifugation was carried out to separate the filtrate and sludge, using speed of 3000 rpm for 20 minutes. The supernatant obtained is a protein hydrolysate added with other ingredients, such as 12.5% technical maltodextrin from the weight of the starting material. Dried using an oven at 60°C to a water content of ± 5%, then added 6% local glucose, 13.3% technical salt, and ground to obtain flavor enhancer powder [14]. Flowchart of flavor enhancer production can be seen in Fig. 1.

F. Degree of hydrolysis by soluble protein content in TCA

The degree of hydrolysis (DH) was determined according to Silvestre et al. [17] as follows: 1 mL of sample was added to 1 mL of 10% TCA solution (Sigma, USA), then incubated for 30 minutes, next centrifuged at 3000 rpm for 15 minutes. The resulting supernatant was analyzed for soluble protein content using the Anson method.

The percentage degree of hydrolysis (DH) was determined by the following equation:

$$DH (\%) = \frac{\text{Soluble protein content in TCA}}{\text{Total Protein}} \times 100\%.$$

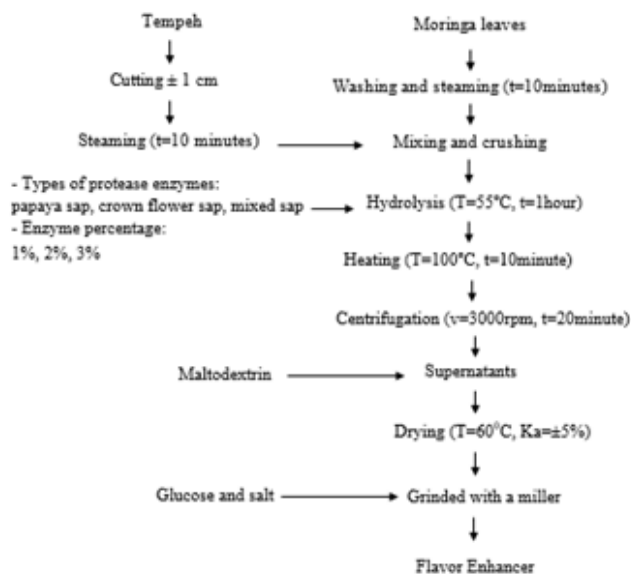


Fig. 1. Flowchart of flavor enhancer production

G. Water Content

Water content was determined by the drying method [18]. The aluminum cup was preheated at 105°C for 1 hour, then cooled for ± 15 minutes in a desiccator and the empty cup was weighed. Samples weighed ± 2 g in an aluminum cup whose mass is already known, then dried in an oven at 100 - 105°C for 4 hours, cooled in a desiccator and weighed. Warmed in the oven again at a temperature of 100-105°C for ± 1 hour, cooled in a desiccator ± 15 minutes and weighed. This treatment was repeated until constant mass (mass difference ± 0.002 g).

$$\text{Water Content (\%)} = \frac{\text{first mass (g)} - \text{final mass (g)}}{\text{first mass (g)}} \times 100\%.$$

H. Soluble protein content by anson method

Soluble protein content was determined by Anson's method, as follows: sample weighing 0.01 g was dissolved into 10 mL. The solution was taken 1 mL and then added 2.5 mL Na₂CO₃ 0.5 M and 1 mL Folin Cioceltau reagent. The solution was homogenized and then incubated at 37°C for 30 minutes. The absorbance of the sample was measured at a wavelength of 660 nm using a UV-Vis spectrophotometer. Blank was prepared in the same way but without sample. Soluble protein was calculated by the formula obtained from the L-tyrosine standard curve using the formula $y = ax + b$. Then based on the concentration results obtained from the calibration standard and expressed as tyrosine equivalents in mg/g equivalent.

Calculation of protein content was calculated by the formula:

$$\text{Soluble protein content} = \frac{\text{Concentration} \times \text{volume} \times \text{dilution factor}}{\text{sample mass}}$$

I. Maillard product

Maillard product was determined based on the absorbance value of the sample as follows: 0.1 gram sample was weighed then dissolved with distilled water and vortexed for 3 minutes. The sample solution was then measured for absorbance at a

wavelength of 420 nm. The absorbance amount was recorded in AU (absorbance unit).

J. Solubility index on water

The solubility index on water was determined as follows: 1 g of sample was added 10 mL distilled water into a centrifuge tube and shaken using a vortex. The tube was then centrifuged at 2000 rpm for 15 minutes. The supernatant obtained was taken as much as 2 mL and placed on a weighing cup that has known mass. The cup was then put into the oven and dried at 110°C until all the water evaporated. The cup is then cooled and weighed to determine the mass of dry matter contained in the supernatant.

The solubility index was determined by the following equation:

$$\text{Solubility index (g/mL)} = \frac{\text{mass of cup after oven} - \text{mass of cup}}{2 \text{ mL}}$$

K. Oil absorption

Oil absorption was determined as follows: 1 g of sample was placed into a tube and then added with 10 mL of local vegetable oil. Then stirred with a spatula and allowed to stand for 30 minutes at room temperature. Then centrifuged at 3000 rpm for 30 minutes. The volume of oil that is not absorbed by the sample is measured with a measuring cup.

Calculation of oil absorption is as follows:

$$\text{Oil absorption capacity (mL/g)} = \frac{\text{first volume} - \text{final volume}}{\text{sample mass}}$$

L. Analysis of glutamic acid content

Glutamic acid content was determined as follows: 0.05 g of solid glutamic acid (BM = 147.13 g/mol) was measured and dissolved in 100 mL of distilled water to have a concentration of 500 ppm. The 500 ppm glutamate standard solution was then diluted with distilled water to obtain concentrations of 100, 150, 175, 200, and 250 ppm.

The glutamate standard curve was determined by reacting 1 ml of various concentrations of glutamate solution with 1 ml of 0.5% ninhydrin. After that, put it in boiling water for 15 minutes. Furthermore, the solution was measured to determine the optimum wavelength and make a standard curve to determine glutamate levels. Determination of glutamate content was done by reacting 1 mL of hydrolyzed filtrate with 1 mL of 0.5% ninhydrin. Furthermore, it was heated for 15 minutes in boiling water. Then allowed to stand 10 minutes at room temperature. The absorbance value of the solution was then measured at a wavelength of 566 nm using UV-Vis spectrophotometry. Glutamate levels were calculated using the glutamate standard curve made and the y value and regression on the standard curve were calculated. The y value will be used to determine glutamate levels based on filtrate absorbance data with the resulting unit is ppm. Then based on the concentration results obtained from the calibration standard and expressed as glutamic acid equivalents in mg/g equivalent.

$$\text{Glutamic Acid Content} = \frac{\text{Concentration} \times \text{volume} \times \text{dilution factor}}{\text{sample mass}}$$

III. RESULTS AND DISCUSSION

A. Protease enzyme activity

The crude protease enzyme extracted from young papaya sap (papain enzyme) and crown flower plant sap (calotropin enzyme) was tested for its activity with casein substrate. The protease enzyme activity values of crude papaya, crude crown flower and their mixture can be seen in Table I.

TABLE I. ENZYME ACTIVITY VALUES BY TYPE

Enzyme Type	Enzyme Activity (Unit/mL)
Crude Papaya Protease	9,560 ± 0,145
Crude Crown Flower Protease	6,312 ± 0,077
Mixed protease	8,063 ± 0,192

Crude papaya protease enzyme has the highest enzyme activity value compared to the others, which is 9.560 U/mL. While the value of crude crown flower enzyme activity was the lowest at 6.312 U/mL and for the mixed enzyme activity value of papaya protease and crown flower at 8.063 U/mL. The value of enzyme activity in this study is greater than the research of Fajriyah and Winarti [15] with the value of crude papain enzyme activity of 1.65 U/mL and crude calotropin enzyme of 1.40 U/mL. This was influenced by the mixture ratio between tapped sap and metabisulfite solution, this study used a ratio of 1:2 while Fajriyah and Winarti [15] used a ratio of 1:4 because of that the crude protease enzyme obtained in this study was more concentrated than reported by Fajriyah and Winarti [15].

This research can also illustrate that each type of enzyme has a different enzyme activity. According to Onsaard, Ngam, and Onsaard [19], the value of enzyme activity can be related to the catalytic site and reaction mechanism. The proteolytic activity of several protease enzymes has different characteristics, so that in hydrolyzing peptide bonds protein molecules have different specifications [20]. The structure around the protease active site decides how the substrate can bind to the protease site [21]. Papaya protease enzyme contains papain which is a class of endopeptidase, breaking protein bonds in the middle of the peptide chain to produce peptide fragments. In breaking down proteins, the activity of papain enzymes hydrolyzes proteins starting with the process of breaking substrates into products by histidine and cysteine groups on the active side of the enzyme [22]. Meanwhile, calotropin contained in the crown flower protease enzyme is a class of exopeptidase that breaks peptide bonds located at the ends of protein molecules. The use of a combination of endopeptidase and exopeptidase enzymes in the hydrolysis process has a mechanism of breaking proteins into peptides by endopeptidase enzymes, followed by the breakdown of peptides into amino acids by exopeptidase [23].

B. Degree of hydrolysis of flavor enhancer based on moringa leaves and tempeh

The results of ANOVA showed that the type and percentage addition of enzymes in the hydrolysis process and their interaction had a very significant effect ($P < 0.01$) on the value of the degree of hydrolysis of flavor enhancer based on moringa leaves and tempeh. Hydrolysis degree in hydrolysis

process for flavor enhancer based on moringa leaves and tempeh can be seen in Fig. 2.

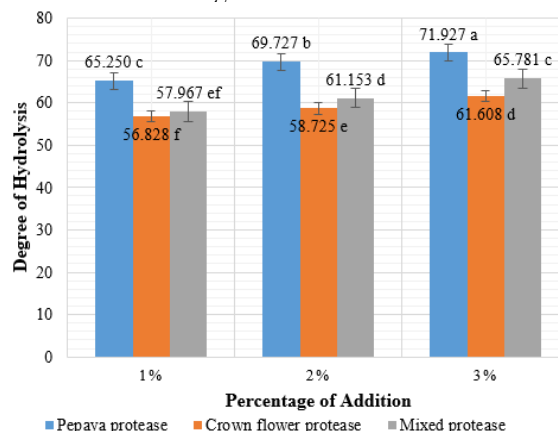


Fig. 2. Degree of hydrolysis in hydrolysis process for flavor enhancer based on moringa leaves and tempeh (%).

The average value of the degree of hydrolysis was 56.828%-71.927%. The increasing percentage of enzyme addition showed an improvement in the degree of hydrolysis in each treatment. The treatment of 3% enzyme addition percentage and enzyme type of papaya protease had the highest value of 71.927% compared to the treatment of crown flower protease (61.608%) and mixed protease (65.781%). The value of the degree of hydrolysis obtained in this research is almost close to that reported by Baehaki, Shanti, and Achmad [24] which obtained a hydrolysis degree value of 71.98% from the treatment of adding 6% concentration of papain enzyme in the catfish hydrolyzed. So it is indicated that papaya protease enzyme breakdown more peptide bonds than crown flower protease enzyme or protease enzyme mixture. Papaya protease enzyme contains papain, which is a sulfhydryl protease with an S-H group on its active side that influences its activity [22]. According to Verma et al. [25] papain is classified in cysteine proteases that process proteins more extensively than pancreatic protease compounds. While the calotropin enzyme in crown flower protease is included in the sulfhydryl exopeptidase group with a sulfhydryl group on the active side that can break down proteins in their terminal groups [2].

The different degrees of hydrolysis are caused by protein structure and/or composition from different proteolytic enzyme that cause different DH [26]. Research by O'Keeffe and FitzGerald [27] also showed that the DH values of whey protein hydrolysates produced by Alcalase, Neutrase and Flavorzyme were different at 20.5%, 12.5%, and 11.4%, respectively. In addition, the degree of hydrolysis is also influenced by the specificity of protease in hydrolyzing the substrate. According to Fox in Witono [2], the specificity of each protease enzyme is different in hydrolyzing peptide chains in proteins. In line with Whitaker in Witono [2], some protease enzymes have special requirements in their proteolytic activity. In addition, the increase in enzyme concentration is directly proportional to the increase in the degree of hydrolysis. According to Fajriyah and Winarti [15], the higher the enzyme concentration can increase the speed of hydrolysis so that it improves the results of protein breakdown in the form of short chain peptides and amino acids which will affect the value of the degree of hydrolysis. Puspitasari et al. [46] also found that

DH increased when the enzyme concentration increased in the hydrolysis process with bromelain enzyme.

C. Water content of flavor enhancer based on moringa leaves and tempeh

The results of ANOVA showed that the type and percentage addition of enzymes in the hydrolysis process and their interaction had no significant effect ($P > 0.05$) on the water content value of flavor enhancer based on moringa leaves and tempeh. Water content of flavor enhancer based on moringa leaves and tempeh can be seen in Fig. 3.

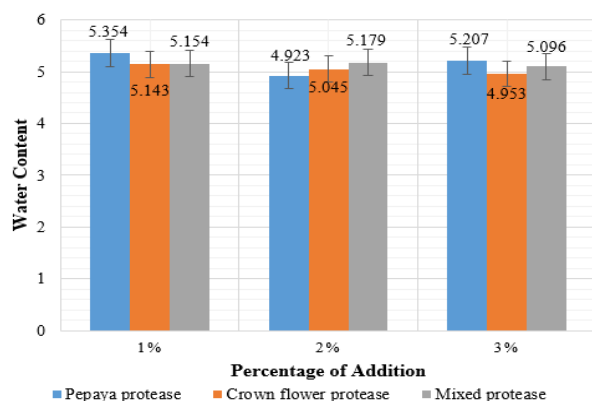


Fig. 3. Water content of flavor enhancer based on moringa leaves and tempeh.

The average value of water content of flavor enhancer powder based on moringa leaves and tempeh is 5.354%-4.923%. The treatment of hydrolysis process with papaya protease enzyme at 2% concentration has the lowest water content value but there is no difference with other treatments. Water content is one of the proximate characteristics that are important for food products because water contained in food can affect organoleptic quality ranging from texture, appearance and taste [28]. The value of water content in this study is lower than the value of water content reported by Wicaksono and Winarti [14] which is 6.28%-7.77% in mulberry leaf and shrimp head flavor enhancer powder. While the results of research by Elfian, Mappiratu, and Razak [29] on the production of anchovy flavor enhancer with crown flower sap crude protease enzyme obtained a water content in the product of 4.4%-6.22%. According to Whittaker in Witono [30], the hydrolysis process on protein can damage its globular structure so that the water binding capacity will be reduced. However, the value of water content in this study is higher than the quality requirements in SNI 01-4273-1996, which is a maximum of 4% so it needs to be dried again until it complies with National Indonesian Standard (SNI).

D. Soluble protein content of flavor enhancer based on moringa leaves and tempeh

The results of ANOVA showed that the type of enzyme and percentage of enzyme addition in the hydrolysis process of flavor enhancer based on moringa leaves and tempeh had a very significant effect ($P < 0.01$) and the interaction of both had a significant effect ($P < 0.05$) on the value of soluble protein content. Soluble protein content of flavor enhancer based on moringa leaves and tempeh can be seen in Fig. 4.

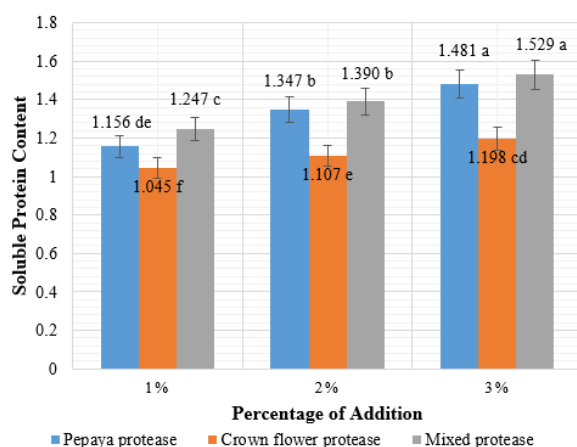


Fig. 4. Soluble protein content of flavor enhancer based on moringa leaves and tempeh (mg/g tyrosine equivalent).

The average value of soluble protein content in flavor enhancer powder based on moringa leaves and tempeh is 1.045-1.529 mg/g tyrosine equivalent. It is known that hydrolysis with mixed protease enzymes at a percentage of 3% addition produces the highest soluble protein content value of 1.529 mg/g tyrosine equivalent, but is not significantly different from using papaya protease enzymes at a percentage of 3% addition. Meanwhile, the lowest protein content value was obtained in hydrolysis using crown flower protease enzyme at 1% addition percentage. The higher percentage of enzyme addition in the hydrolysis process can increase the soluble protein content in the product. Similar to the opinion of Tondais et al. [11] that the higher addition of protease will increase the soluble protein content because enzymes can act as catalysts in denaturing proteins. The greater enzyme concentration can accelerate enzyme reactions in breaking peptide bond chains [2]. It is due to the hydrolysis of the protein causes the breakdown of the protein chain into proteose, peptone, short peptides, and a series of soluble amino acids [31].

The results of this research show that the hydrolysis process using mixed enzymes at a percentage of 3% addition can produce more soluble protein compared to the use of a single enzyme. Research by Witono et al. [40] also showed that the ratio of 50:50 crown flower and papain enzyme was the optimal ratio in producing soluble protein. Papaya protease enzymes from the endopeptidase group work to break protein chain bonds from within and away from the end of the polypeptide chain [32]. So that the hydrolysis products produced are still mostly insoluble peptide chains because they have a large molecular weight. Meanwhile, according to Witono [2], crown flower protease enzymes can break down proteins at their terminal groups, but the rate of hydrolysis can be reduced due to limited peptide bonds. Azmi et al. [33] stated that different types and properties of proteases can be combined to obtain the preferred results. Endopeptidase and exopeptidase can be combined to create proteolytic synergism that can accelerate the breaking of protein bonds to create simple proteins that can increase soluble protein levels [2].

E. Maillard product of flavor enhancer based on moringa leaves and tempeh

The results of ANOVA showed that the type and percentage addition of enzymes in the hydrolysis process and their interaction had a very significant effect ($P < 0.01$) on the Maillard product value of flavor enhancer based on moringa leaves and tempeh. Maillard product of flavor enhancer based on moringa leaves and tempeh can be seen in Fig. 5.

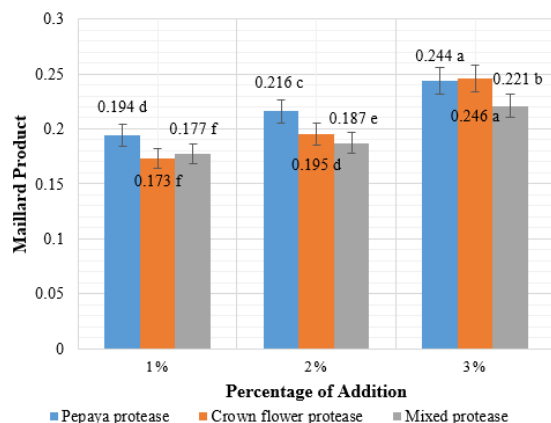


Fig. 5. Maillard product of flavor enhancer based on moringa leaves and tempeh.

The average value of Maillard products in flavor enhancer powder based on moringa leaves and tempeh was 0.173-0.246 absorbance units (AU). Hydrolysis with crown flower protease enzyme at 3% addition percentage produced the highest Maillard product value of 0.246 AU, but it was not significantly different from using papaya protease enzyme at the same addition percentage. Meanwhile, the lowest Maillard product value was produced in the hydrolysis process using crown flower protease enzyme at 1% addition percentage. The average Maillard product of flavor enhancer from hydrolysate of sunflower seeds and white mussels in Wicaksono and Winarti's [14] research was lower, ranging from 0.051-0.189 AU. This indicates that there are many primary amine groups from hydrolysis that react with reducing sugars.

Melanoidin compounds become one of the Maillard products, which is a brown pigment resulting from the interaction of hydrolysis products as primary amines with reducing sugars, which can be analyzed by absorbance method [34]. Amino acids and short-chain peptides from the hydrolysis act as precursors for the Maillard reaction by providing primary amine groups [35]. The higher concentration of enzyme added can accelerate the enzyme reaction with the substrate so that the primary amine group produced increases [2]. The larger peptides produced cause more primary amine groups to react with glucose so that the more intensive Maillard reaction, the more dark-colored Maillard products are produced [36]. Along with the report of Witono [2] that the more enzyme concentration added to the soybean substrate causes more Maillard products. Crown flower protease enzyme breaks protein bonds at the end of the peptide chain [2] so that it can accelerate the formation of primary amines that will react with reducing sugars to produce Maillard products. Meanwhile, papaya protease enzyme as an endopeptidase is responsible for

providing short chain peptides so as to increase the amino end [14].

F. Solubility index of flavor enhancer based on moringa leaves and tempeh on water

The results of ANOVA showed that the type of enzyme and the percentage of enzyme addition in the hydrolysis process of flavor enhancer based on moringa leaves and tempeh had a very significant effect ($P < 0.01$) and the interaction of both had a significant effect ($P < 0.05$) on the value of solubility index on water. Solubility index of flavor enhancer based on moringa leaves and tempeh on water is shown in Fig. 6.

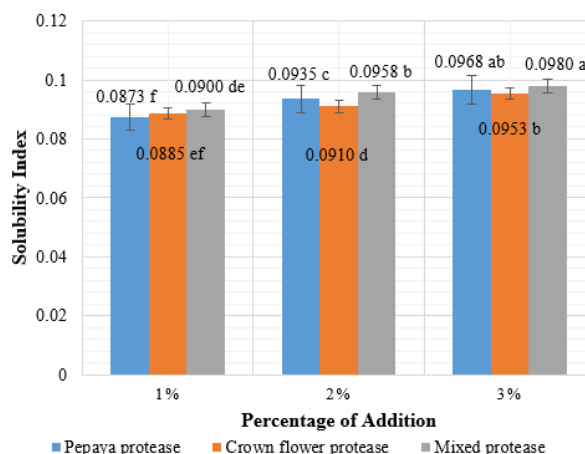


Fig. 6. Solubility index of flavor enhancer based on moringa leaves and tempeh on water (g/ml).

The average value of water solubility index in flavor enhancer powder based on moringa leaves and tempeh was 0.0873-0.0980 g/mL. Hydrolysis with mixed protease enzymes at 3% addition percentage produced the highest water solubility index value of 0.0980 g/mL. While the lowest water solubility index was produced in the hydrolysis process with papaya protease enzyme at 1% addition percentage. The water solubility index value in this study is higher than the water solubility index value reported by Wicaksono and Winarti [14] on flavor enhancers from sunflower seeds and white kupang which ranged from 0.012-0.037 g/mL, but close to the water solubility index value reported by Fajriyah and Winarti [15] which is 0.071-0.088 g/mL on flavor enhancers from mulberry leaves and shrimp heads.

The increasing percentage of enzyme addition will improve the water solubility index. According to Wilson and Walker in Witono [2] several changes in proteins occur during the hydrolysis of peptide bonds causing increased solubility due to increased NH_3^+ and COO^- content and reduced protein molecular weight. The water solubility of the product is related to its soluble protein content. Based on the research of Nalinanon et al. [37], different values of the degree of hydrolysis affect the peptide size, hydrophobic balance, and the number of peptides produced in the hydrolysis process. The hydrophobic balance of peptides can influence the solubility value. In line with the test results, the highest soluble protein content using mixed protease enzymes led to high results in the water solubility index. Short-chain peptides from hydrolysis

have a low molecular weight, so that it increases their solubility [14].

G. Oil absorption capacity of flavor enhancer based on moringa leaves and tempeh

The results of ANOVA showed that the type of enzyme and the percentage of enzyme addition in the hydrolysis process of flavor enhancer based on moringa leaves and tempeh had a very significant effect ($P < 0.01$) and the interaction of both had no significant effect ($P > 0.05$) on the value of the oil absorption capacity. Oil absorption capacity of flavor enhancer based on moringa leaves and tempeh is shown in Table II.

TABLE II. OIL ABSORPTION CAPACITY OF FLAVOR ENHANCER BASED ON MORINGA LEAVES AND TEMPEH (mL/G).

Treatment	Percentage			Average
	1%	2%	3%	
Papaya protease	0,90 ± 0,00	1,05 ± 0,07	1,20 ± 0,00	1,05 ± 0,15 b
Crown flower protease	0,85 ± 0,07	1,10 ± 0,14	1,25 ± 0,07	1,07 ± 0,20 b
Mixed protease	1,20 ± 0,00	1,35 ± 0,07	1,55 ± 0,07	1,37 ± 0,18 a
Average	0,98 ± 0,19 c	1,17 ± 0,16 b	1,33 ± 0,19 a	

Description: - The average value followed by the same notation in the same row and/or column indicates that the value is not significantly different ($P > 0,05$).

The average value of oil absorption in flavor enhancer powder based on moringa leaves and tempeh was 0.85-1.55 mL/g. Hydrolysis with mixed protease enzymes at 3% addition percentage produced the highest oil absorption value of 1.55 mL/g. The oil absorption value in this research is lower than the oil absorption value reported by Wicaksono and Winarti [14] on flavor enhancers from sunflower seeds and white kupang which ranged from 1.82-3.99 mL/g, but close to the oil absorption value reported by Fajriyah and Winarti [15] which is 1.147-1.786 mL/g on flavor enhancers from mulberry leaves and shrimp heads.

Oil absorption testing is indicated as the physical binding properties of lipids by proteins to absorb, hold and react in an emulsion system. Large oil absorption can improve the flavor and mouth feel of food [14]. During hydrolysis, protein molecules break down into short peptide pieces or amino acids that can have different groups, namely hydrophobic and hydrophilic, hydrophobic groups are involved in oil absorption capacity [14]. According to Kristinsson and Rasco [38], oil absorption by proteins is related to the hydrophobic surface. Other protein hydrolysates have been studied in relation to fat absorption. Casein hydrolysate prepared using papain enzyme has good oil absorption, suggesting that hydrophobic interaction is the main cause. The substrate specificity of the enzyme also seems to play a major role in this [38]. Kristinsson and Rasco's [38] research on salmon muscle protein hydrolysis showed that the use of different enzymes had different oil-absorbing abilities.

H. Glutamic acid content of flavor enhancer based on moringa leaves and tempeh

The results of ANOVA showed that the type of enzyme and the percentage of enzyme addition in the hydrolysis process and their interaction had a very significant effect ($P < 0.01$) on the value of glutamic acid content of Moringa leaf-based flavor enhancer and tempeh. Glutamic acid content of flavor enhancer based on moringa leaves and tempeh can be seen in Fig. 7.

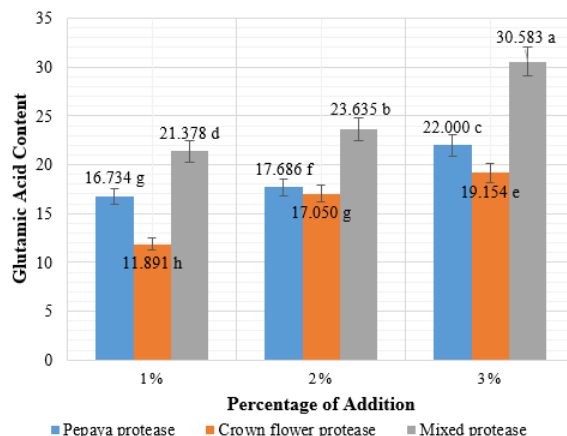


Fig. 7. Glutamic acid content of flavor enhancer based on moringa leaves and tempeh (mg/g glutamic acid equivalent).

The average value of glutamic acid content in flavor enhancer powder based on moringa leaves and tempeh was 11.891-30.583 mg/g glutamic acid equivalent. Hydrolysis with mixed protease enzymes at 3% addition percentage produced the highest glutamic acid content of 30.583 mg/g glutamic acid equivalent. The value of glutamic acid content in this study is lower than the value of glutamic acid content reported by Wicaksono and Winarti [14] in flavor enhancers from sunflower seeds and white kupang, which is 78.678 mg/g glutamic acid equivalent. This is due to differences in hydrolysis time, where in the preparation of flavor enhancers from mulberry leaves and shrimp heads using hydrolysis time for 2 hours. Increasing the percentage of protease enzyme addition will increase the glutamic acid content of the product. A high percentage of enzyme can hydrolyze more substrate. Rosida, Priyanto, and Putra [39] stated that high concentrations increase glutamic acid levels in protein hydrolysates. Ernawati and Dedin [45] said that the high protein content in the raw materials produces higher glutamic acid in the flavor enhancer. Meanwhile, different types of enzymes can produce different glutamic acid levels because of their different mechanisms in hydrolysis. Similar to the research of Tezano et al. [44], which analyzed *C. sorokiniana* protein isolates hydrolyzed with pepsin, bromelain and thermolysin, obtained different glutamic acid levels.

The results of this study showed that the hydrolysis process using mixed enzymes at a percentage of 3% addition can produce more glutamic acid compared to the use of a single enzyme. Similarly, Nam et al. [41] explored the potential for using purified pear protease as a meat tenderizer and noted that the combination of pear protease and kiwi fruit protease has a synergistic effect and can produce good quality meat products. Research by Linberg et al. [42] also showed that the

combination of proteases resulted in an increase in soluble protein yield of about 15% compared to a single protease. Hydrolysis efficiency can be improved through the combined use of synergistic proteases from different biological sources used sequentially or in parallel [43]. The calotropin enzyme in crown flower protease in the form of exopeptidase to break the terminal peptide bond of intact globular protein requires pretreatment or combination with endopeptidase, which is papaya protease to break the middle chain peptide bond [14].

IV. CONCLUSION

Based on the results of the study, it was concluded that the interaction between the type of protease enzyme and the percentage of enzyme addition had a very significant effect on the degree of hydrolysis, maillard product, and glutamic acid content, had a significant effect on soluble protein content and water solubility index, and had no significant effect on water content and oil absorption. The treatment of protease enzyme type and percentage of enzyme addition had a very significant effect on the degree of hydrolysis, soluble protein content, maillard product, water solubility index, oil absorption and glutamic acid content, and had no significant effect on water content. The results showed that hydrolysis using mixed protease enzymes with an addition percentage of 3% produced flavor enhancer based on moringa leaves and tempeh which had the highest glutamic acid content of 30.583 mg/g glutamic acid equivalent with a degree of hydrolysis of 65.781%, water content of 5.096%, soluble protein content of 1.529 mg/g tyrosine equivalent, maillard product of 0.221 AU, water solubility index of 0.0980 g/mL, and oil absorption capacity of 1.55 mL/g.

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