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Utilization of Partially Purified Papain Enzyme in Mallika Black Soybean Tempeh Hydrolysate as Umami Seasoning

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Abstract

Tempeh made from Mallika black soybean (*Glycine max* (L.) Merr. *var*. Mallika) can be fermented for up to 4 days and can be further optimized by adding partially purified papain enzyme obtained from California variety papaya leaves (*Carica papaya* (L.) *var*. California). Enzyme can be added to the hydrolysates to degrade protein into short-chain peptides and free amino acids, contributing to umami taste sensory attributes. The study aimed to determine the best ammonium sulfate fractionation of crude papain enzyme and the best physicochemical characteristics of black soybean tempeh protein hydrolysate. The addition of ammonium sulfate fractionation used was 0% to 80%; fermentation time was 2 to 4 days; and the concentration of enzyme added was 0%(w/v) to 1.5%(w/v). The results showed that the 40% fractioned papain enzyme gave the highest protease activity value (0.98±0.04 U ml^{-1}) and most of the papain enzyme was precipitated in this fraction leaving impurities. The black soybean tempeh hydrolysates with 4 × 1% showed the best physicochemical characteristic because it produced the highest umami substance. The best characteristics were moisture content (17.97±0.46%), glutamic acid content (171.58±5.72 mg g^{-1}) that was caused by a transamination reaction, dissolved protein content (470.66±19.50 mg g^{-1}), degree of hydrolysis (43.64±1.99%) and lightness (46.02±0.97). The umami substance's amino acids are high in content, such as glutamic and aspartic acids (59.89±0.31 mg g^{-1} and 26.47±0.09 mg g^{-1}). Sensory evaluation showed that treatment 4 × 1% demonstrated no significant difference in umami intensity with MSG (monosodium glutamate).

Keywords: enzyme purification; flavor enhancer; Mallika; "*semangit*"; tempeh hydrolysate

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INTRODUCTION

Tempeh has long been a traditional and popular Indonesian food. It has now spread to international markets, and many people are more familiar with it. Tempeh is made from soybeans and produced by solid-state fermentation using *Rhizopus oligosporus* and *Rhizopus oryzae* molds. The stages in making tempeh are washing and soaking the soybeans, heating/boiling the soybeans, peeling the soybeans' epidermis, inoculating tempeh molds, packaging and fermentation. During the fermentation process of tempeh, aeration is needed because of semi aerobic properties of molds to help their growth and development (Laksono et al., 2019). Tempeh molds can produce several enzymes, like phytases, proteases and amylases, which degrade macromolecules into micromolecules, such as amino acids and peptides (Wahyudi, 2018). Protease enzymes produced by tempeh molds have optimum pH of 3 to 5.5 (Sine and Soetarto, 2018). Tempeh is generally consumed by frying, steaming or being served as a food condiment.

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Processing method diversification of tempeh and its derivative products can be carried out particularly to utilize the amino acid content as an umami seasoning and to create a more sustainable and independent Indonesian agriculture using local raw materials. One common diversification is to lengthen the tempeh fermentation time for up to four days, known as "*tempe semangit*" which produces glutamic acid and aspartic acid as an umami flavor. Utami et al. (2016) and Djunaidi et al. (2017) found that four-day fermentation can produce the highest umami: bitter intensity ratio of up to 1.6:1 and potential use as umami seasoning. Furthermore, the fermentation time needs to be investigated to find the best physicochemical characteristics of tempeh protein hydrolysates.

The protein hydrolysates are generally produced using animal sources and underutilized from a vegetable source, such as Mallika black soybean (Yazid and Nuha, 2017). Mallika black soybean is a local Indonesian raw material, whose utilization is limited to the soy sauce manufacturing industry and is still inferior to the tempeh industry compared to imported yellow soybeans, whereas it has protein content (39.09%) higher than import yellow soybean (37.84%) and highest-level glutamic acid by 98.75 mg g^{-1} (Nurrahman, 2015). Utilization of Mallika black soybeans in making tempeh can be a concrete form of support for agricultural sustainability.

The way to increase the value of tempeh is by increasing the micromolecular components through fermentation and hydrolysis processes. Protein hydrolysis can be performed by using acid, base and protease enzymes. Enzymatic hydrolysis has several advantages, such as being readily obtained, working specifically on protein substrates, and having high catalytic power. Hydrolysis of black soybean tempeh protein can produce hydrolysate, which contains small molecules, such as peptides and amino acids, and can be used as seasoning powder (Yazid and Nuha, 2017). One type of protease is the papain enzyme. The papain enzyme has a higher level of protease activity on protein substrates derived from vegetable sources than other proteases, such as the bromelain enzyme, which has a higher level of protease activity on animal protein substrates. Papain enzyme also has high-temperature resistance, does not cause side reactions, is easy to obtain, is not toxic and has high activity (Taqwdasbriliani et al., 2013; Yazid and Nuha, 2017). Crude papain can be extracted from several sources from papayas, such as the leaf, fruit or sap. Crude papain extracted from California papaya leaf has an enzyme activity of 1.88 U ml^{-1} higher than crude papain extracted from young papaya sap (1.05 U ml^{-1}) or commercial papain (1.32 U ml^{-1}) (Zusfahair et al., 2014). Kai et al. (2015) found that papain enzymes at 0.2% and 2% can significantly hydrolyze protein in soybean to less than 32 kDa within one hour. Papain enzyme has high stability at pH 8 for at least three freeze-thaw cycles and temperature maintained below 4 ^{o}C for 24 hours (Marković et al., 2021). The enzyme can be partially purified by using ammonium sulfate, which separates enzymes and their impurities by the salting out principle. Partial purification can be done at a 40% to 80% fraction and increase the level of protease activity and purity level up to 6.27 times higher than the unpurified sample (Rohmah et al., 2019). Therefore, the ammonium sulfate concentration variation from papain enzyme extracted from California papaya leaf needs to be studied to obtain the best fractionation level based on the enzyme activity. Moreover, the concentration added of the partial purification of the papain enzyme on the black soybean tempeh hydrolysates needs to be investigated to obtain the best partial concentration of the addition of the papain enzyme purification based on its physicochemical characteristics.

The commonly used flavorings are MSG (monosodium glutamate) and HVP (hydrolyzed vegetable protein). Research conducted by Jeon et al. (2020) found that HVP flavorings hydrolyzed by proteases enzyme had higher overall preferences, umami taste intensity, and higher acceptability than MSG flavorings. Therefore, black soybean tempeh protein hydrolysate should be investigated to determine whether it can be used as a substitute for MSG commercial flavorings by its physicochemical characteristics. Furthermore, this research will find added value through a more diverse amino acid content from black soybean protein tempeh hydrolysate, utilizing local Indonesian products, especially black soybeans as a substitute for imported yellow soybeans, utilizing natural enzymes that can be obtained from local sources, and find the best protease enzyme separation fractionation method. Therefore, the study aimed to determine the best ammonium sulfate fractionation of crude papain enzyme extracted from the papaya California variety leaf and the best physicochemical characteristics of black soybean tempeh protein hydrolysate.

MATERIALS AND METHOD

Study design and participants

The research was conducted in the Department of Food Technology, Faculty of Science and Technology, Universitas Pelita Harapan, Tangerang. The raw materials, such as the leaves of California papaya, with minimum age of six-month planting, were obtained from Klampok Village (Bojonegoro, East Java Province), and Mallika black soybean with 85 to 90 days after planting was obtained from UD. Agro Aji Perkasa (Pacitan, East Java Province).

The research was divided into two stages. The first stage was to determine the best activity of the partially purified papain enzyme extracted from California papaya leaves for later use in the following step based on variations of ammonium sulfate concentration fractionation of 0%, 20%, 40%, 60% and 80%. The experimental design used was one factor completely randomized design with two replications. The second stage was to determine the physicochemical characteristics of black soybean tempeh protein hydrolysates based on the effect of fermentation time (2, 3 and 4 days) and the concentration of the addiction of the partially purified papain enzyme (0%, 0.5%, 1% and 1.5% w/v). The two-factor completely randomized design was applied with three replications. The post hoc test was conducted using Duncan Multiple Range Test (DMRT). The data was processed using IBM SPSS Statistics 25 version and Microsoft Excel.

Partial purification of papain enzyme extracted from California papaya leaves

The crude papain enzyme was extracted from the leaves of California papaya as stated in Zusfahair et al. (2014) with modification using phosphate buffer 0.1 M at pH 8. The juice temperature during the process was maintained at 4 ^{o}C using a chiller and ice cubes. The juice was filtered with a filter cloth and centrifuged for 15 minutes at 4,000 rpm "Hermle tipe Z 206A" and "Frilab", and the supernatant was taken as crude papain enzyme extract.

With modification, partial purification of crude papain enzyme was conducted based on previous research by Duong-Ly and Gabelli (2014) and Malle et al. (2015). Ammonium sulfate with treatment variations (0%, 20%, 40%, 60%, 80%) was added according to the fractionation table following Amer (2019). Ammonium sulfate was diluted to crude papain enzyme solution using a magnetic stirrer at 4 ^{o}C and centrifuge at 4,000 rpm for 15 minutes. The residue was taken, cut by phosphate buffer at pH 8, and placed in the pre-activated dialysis bag. The dialysis process was conducted by soaking the bag in phosphate buffer pH 8 for 21 hours at 4 ^{o}C. The buffer solution was replaced at the 3rd, 18th and 21st hours. The solution in the dialysis bag was taken as a partially purified papain enzyme. Purified papain enzyme was stored in freezer (0 ^{o}C) prior to use.

Protease activity analysis was conducted using a method developed by Cupp-Enyard and Aldrich (2008) with modification. The standard curve was made by reacting standard L-tyrosine with sodium carbonate and Folin-Ciocalteu Phenol reagent. Casein, 0.65% solution, was used as the enzyme-substrate throughout the incubation for 10 minutes. The reaction was stopped by adding trichloroacetic acid, and the solution was then centrifuged to obtain the supernatant. The supernatant was collected and measured using a spectrophotometer UV-Vis at 660 nm wavelength. Absorbance obtained was compared with a standard curve. The difference between the amount of L-tyrosine in the sample and the blank was referred to as the number of amino acids released during the hydrolysis process by the enzyme. Enzyme activity was measured as the amount of enzyme used to release 1 μmol (181 μg L-tyrosine) from substrate per minute at optimum conditions (pH 7 to 8 at 60 ^{o}C) (Wicaksono and Winarti, 2021).

Black soybean protein tempeh hydrolysates production

The Mallika black soybean was cleaned and soaked in acidified pH 4 water for 24 hours. After immersing, the soybeans were peeled from the epidermis and boiled for 30 minutes (100 ^{o}C). The soybeans were cooled to 30 ^{o}C and dried before the inoculation of 1% (w/w) tempeh yeasts (Raprima). Packaging was conducted using polypropylene (PP) plastic with holes given every 1 cm. The fermentation process was carried out based on the treatment (2, 3 and 4 days) at 25 ^{o}C to transform into black soybean tempeh, as presented in Figure 1 (Machin, 2012; Zusfahair et al., 2014; Nurrahman, 2015; Yazid and Nuha, 2017; Sine and Soetarto, 2018; Suparno et al., 2018; Wahyudi, 2018; Perdani and Utama, 2020 with modification).

Black soybean tempeh was cut, steamed for 10 minutes at 100 ^{o}C, ground with a blender with water 2:1, and adjusted to pH 8 by adding acetic acid or sodium bicarbonate. Purified papain enzyme with the highest fractionation activity was

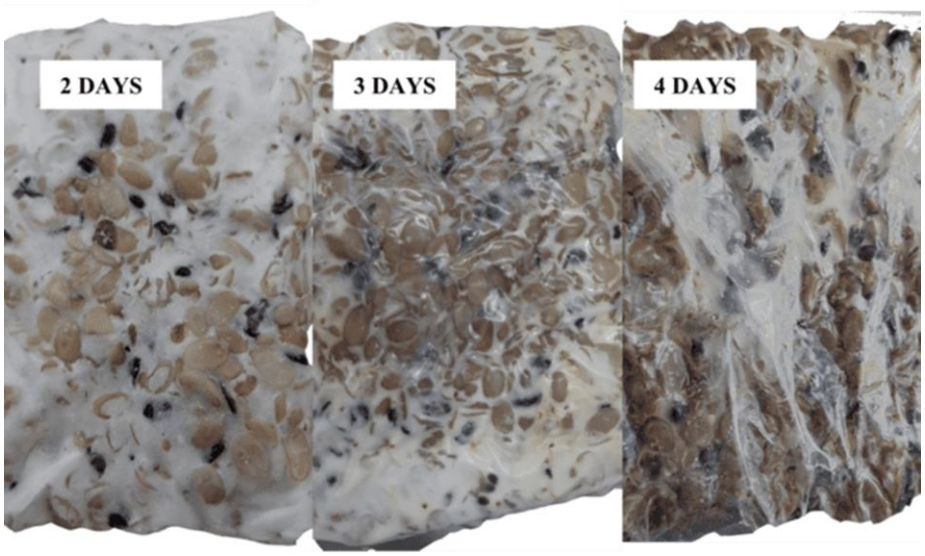

Figure 1. Black soybean tempeh with 2-, 3-, and 4-days fermentation time

added to the slurry based on the treatment variations (0%, 0.5%, 1% and 1.5% w/v). Incubation was carried out for two hours at 60 ^{o}C and stopped by increasing the temperature to 80 ^{o}C for 15 minutes. Glucose (13.3%), salt (13.3%) and maltodextrin DE 10-12 (0.4%) were added and homogenized with slurry. Glucose and maltodextrin were used as fillers whereas salt was utilized to produce a synergistic effect and enhance the perception of umami taste. The slurry was then dried at 50 ^{o}C for 24 hours, and flakes were obtained. Flakes were reduced in size using a dry blender and sieved with a size of 60 mesh. Black soybean tempeh protein hydrolysates were displayed in Figure 2 (Machin, 2012; Wicaksono and Winarti, 2021 with modification).

The black soybean tempeh protein hydrolysates were analyzed based on important parameters following previous research conducted by Djunaidi et al. (2017); Putri et al. (2020); Sitanggang et al. (2020); and Wicaksono and Winarti (2021), such as moisture content (AOAC, 2005), glutamic acid content (Khokhani et al., 2012; Mayasari et al., 2018; Muliadi and Anugrahati, 2022), soluble protein content (Muyassaroh et al., 2020; Wicaksono and Winarti, 2021), degree of hydrolysis (Alahmad et al., 2022), lightness (Djunaidi et al., 2017); and total amino acid profile (Saraswanti Indo Genetech, 2022).

The sensory evaluation was conducted on the best treatment sample by 12 selected panelists. The steps taken included basic taste test panelist

Figure 2. Black soybean tempeh protein hydrolysates with various treatment combinations

selection using sequential-triangle test (da Silva et al., 2014 with modification), umami taste threshold using BET 3-AFC test (Giguere et al., 2016; Adawiyah and Setiawan, 2017; Maulidiah and Fibrianto, 2019 with modification), and umami taste intensity (scoring test).

RESULTS AND DISCUSSION

Effect of ammonium sulfate fractionation on purified papain enzyme activity

Ammonium sulfate fractionation showed significant results on the activity of purified papain enzyme (< 0.05), which can be seen in Figure 3. Papain enzyme had a globular structure, three sulfide bridges, and a sulfhydryl group from cysteine residue on its active site. The sulfhydryl group was a weak nucleophile and needed to be activated by histidine, which was also presented in the enzyme's active site. The synergism between histidine and cysteine could cleavage the peptide bonds by attacking the carbonyl group in the peptide bond (Laskar and Chatterjee, 2009).

Figure 3 shows that 40% ammonium sulfate fractionation gave the highest enzyme activity of 0.98±0.04 U ml^{-1} significantly compared to 0%, 20%, 60% and 80%. The high value of proteases activity at 40% fractionation showed that the most precipitated fraction was papain enzyme, which had proteases activity. Previous research conducted by Malle et al. (2015) and Rohmah et al. (2019) disclosed that 40% to 80% fractionation of papain enzyme extracted from papaya sap gave the highest protease activity. Most of the papain enzyme was extracted at a concentration of 40% because the protein would experience salting out. Salting out happened because there were solubility differences between the protein and impurities. Proteins would tend to precipitate at salting out conditions because the salt would tend to bind water rather than protein. In contrast, the protein would tend to bond with each other and trigger the formation of hydrophobic bonds between proteins and precipitated aggregates. This mechanism would easily separate proteins and impurities and increase the enzyme activity value because the enzyme's active site would be free from impurities.

Otherwise, lower enzyme activity on 0% (0.34±0.05 U ml^{-1}) and 20% fraction (0.25±0.04 U ml^{-1}), which can be seen in Figure 3, indicated that the protein was still experiencing salting in. The 20% fractionation gave lower proteases activity than 0% or crude enzyme because it experienced salting in and most of papain enzyme precipitated, otherwise at 0% fractionation, the protein did not experience salting in or salting out, which allowed the presence of protease activity. According to Duong-Ly and Gabelli (2014), salting in was the condition in which a small part of the protein was soluble in pure water and needed a small amount of salt to remain stable in its folded conformation. Some proteins without the existence of salt would tend to aggregate. A small amount of salt triggered the neutralization of positive and negative charges of protein, prevented aggregation, and increased solubility.

The lower protease activity at 80% fractionation concentration compared to 40% fractionation, as seen in Figure 3, demonstrated that only a tiny portion of the precipitated protein

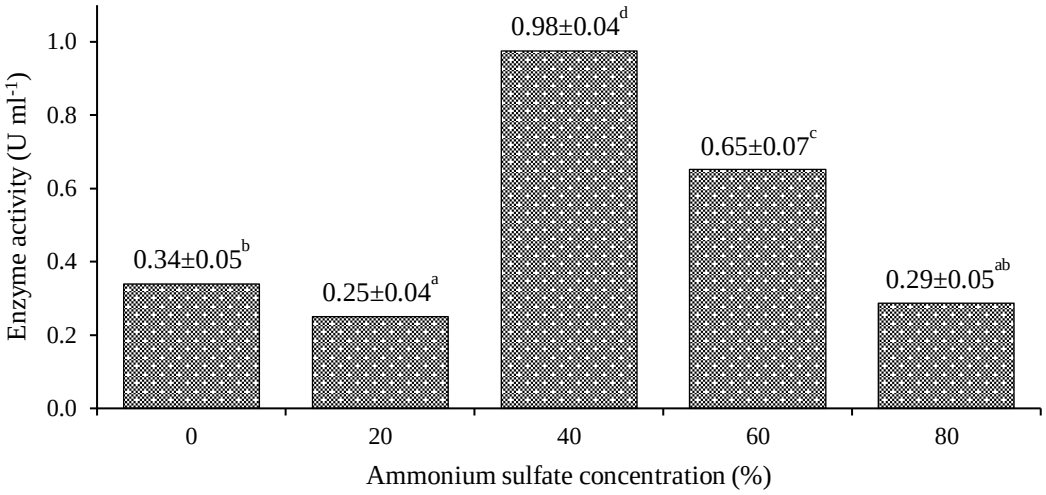

Figure 3. Partial purified papain enzyme activity with various concentrations of ammonium sulfate

Note: The notation of different superscript letters in the figures shows a significant difference (< 0.05)

was the papain enzyme, which had protease activity. Ginting et al. (2020) reported the presence and high activity of amylases in the leaf stalks of papaya plants produced by endophytic bacteria. Enzymes that were not included in the protease group could undergo aggregation and precipitate at 80% fractionation, but they did not affect the activity of the protease with casein as substrate.

A recent study showed that 40% fractionation gave the highest protease activity at 0.98±0.04 U ml^{-1}, as seen in Figure 3, and would be used for the following research stage as the best treatment. This value was higher than that reported in the previous studies on the protease extracted from papaya sap of 0.92 U ml^{-1} (Ratnayani et al., 2015) but lower than that of protease extracted from papaya sap (Wicaksono and Winarti, 2021), commercial papain (Yazid and Nuha, 2017), and Bangkok and California papaya leaves (Zusfahair et al., 2014). These differences were assumed because other factors, such as temperature, pH, activator, inhibitor, fractionation and dialysis method, were influenced.

Physicochemical characteristics

This stage was conducted to determine the effect of two research factors, fermentation time and concentration of the addition of partial purification papain enzyme, on the physicochemical characteristics of black soybean tempeh protein hydrolysates. The physical characteristics analyzed were lightness and molecular weight. The chemical characteristics analyzed included water content, glutamic acid content, soluble protein content, degree of hydrolysis, molecular weight and amino acid profile of black soybean tempeh protein.

Moisture content

Moisture content was an important quality parameter in food products because high moisture would make food easy to spoil. The value of moisture content in food products could be reduced by the drying process so that evaporation occurs (Hassanein et al., 2015). Indonesian Standard 01-3751-2006 has stated that maximum moisture content of flour is 14.5% (wet basis) or 17% (dry basis) and as presented in Table 1, almost all the treatments met this requirement.

As summarized in Table 1, the moisture content varied between 14.25±0.86% and 17.97±0.46%. There was a significant difference at 4 × 0% and 4 × 1%, but the rest did not show a significant difference (< 0.05). The treatments did not have a significant effect because all samples underwent the same drying process to become hydrolyzed powder. Water bound to the protein surface was categorized as type II, which had hydrogen bonding and still could be removed and cleaved by conventional drying, which reduced water activity and prolonged shelf life. The highest water content value was in the 4 × 1% treatment of 17.97±0.46%, indicating an increase of the levels of free amino acids and hydrophilic peptides, which could bind the water during extended fermentation time and increased amount of enzyme. Some amino acids bound with water were assumed of lysine and arginine, which had positive charges on side groups; otherwise, glutamic acid and aspartic acid had negative charges. Another

Table 1. Physicochemical characteristics of black soybean protein tempeh hydrolysates with various fermentation times and papain enzyme concentrations

Fermentation time	Papain enzyme (%)	Moisture content (%)	Free glutamic acid content (mg g^{-1})	Soluble protein content (mg g^{-1})	Degree of hydrolysis (%)	Lightness
2	0.0	17.04±3.35^{ab}	71.98±1.94^{b}	58.21±6.27^{a}	20.97±1.36^{a}	53.13±0.89^{de}
	0.5	15.46±1.44^{ab}	93.35±1.96^{c}	82.37±8.66^{b}	21.27±1.60^{a}	52.34±1.52^{d}
	1.0	15.32±0.70^{ab}	107.41±5.30^{e}	186.78±17.04^{e}	21.37±3.09^{a}	54.94±1.27^{f}
	1.5	17.51±1.70^{ab}	122.44±4.24^{f}	101.18±6.57^{c}	25.94±1.06^{b}	54.14±0.42^{ef}
3	0.0	16.22±2.02^{ab}	64.45±2.44^{a}	170.68±9.93^{d}	30.45±3.32^{c}	47.55±1.82^{abc}
	0.5	14.99±2.92^{ab}	94.06±2.43^{cd}	169.22±15.39^{d}	37.89±1.97^{e}	48.72±1.72^{bc}
	1.0	14.73±2.50^{ab}	97.78±2.81^{ad}	252.18±13.72^{f}	36.33±1.86^{de}	49.00±1.75^{c}
	1.5	15.15±2.41^{ab}	93.74±2.53^{cd}	276.80±15.22^{g}	34.32±1.31^{d}	48.60±1.40^{bc}
4	0.0	14.25±0.86^{a}	104.23±2.15^{e}	430.50±15.69^{i}	37.29±1.86^{e}	47.00±0.69^{ab}
	0.5	16.53±3.11^{ab}	91.11±3.00^{e}	363.48±11.60^{h}	38.56±1.36^{e}	46.32±0.74^{a}
	1.0	17.97±0.46^{b}	171.58±5.72^{h}	470.66±19.50^{j}	43.64±1.99^{f}	46.02±0.97^{a}
	1.5	14.35±0.38^{a}	145.85±3.26^{g}	357.53±9.35^{h}	43.03±1.03^{f}	48.40±2.43^{bc}

Note: The notation of different superscript letters in each column shows a significant difference (< 0.05)

amino acid that could bind water was amino acids with hydroxyl groups such as serine and threonine, as well as amide groups of asparagine and glutamine (Putri et al., 2020).

Free glutamic acid content

Free glutamic acid was the most critical parameter, which gave the characteristics of umami flavorings. Commonly, umami flavorings are produced by an enzymatic hydrolyzing process (Airaodion, 2019). Fermentation time and concentration of partially purified papain enzyme added showed significant differences (< 0.05), as seen in Table 1.

Table 1 presented that treatment with four days fermentation time and 1% partially purified papain enzyme concentration showed the highest glutamic acid content of 171.58±5.72 mg g^{-1}. Glutamic acid content increased simultaneously prolonged fermentation time and decreased at 1.5% concentration of partially purified papain enzyme added, especially on the three and fourth day of fermentation. The black soybean variety was reported to have higher glutamic acid by 98.75 mg g^{-1} than other varieties of soybeans (Nurrahman, 2015). An increase in fermentation time could provide a longer time for enzymes produced by tempeh molds to hydrolyze proteins and release glutamic acid. *R. oryzae* produced an alpha-amylases enzyme, which would be used by *R. oligosporus* as a substrate in producing proteases (Wahyudi, 2018). Extraction of papain enzyme from California papaya leaves yielded some proteases, such as papain, chymopapain, glutamine transferase, lysozyme, and other peptidases (Ratnayani et al., 2015). Purification increased hydrolysis activity due to the loss of impurities that could interfere with the performances of enzymes. The constant state of the reaction rate indicated the enzyme had reached its saturation point, the condition in which all the substrates had been bound to the active sites of the enzyme so that the rate of enzyme activity remained constant and did not affect the reaction rate (Permanasari et al., 2018). This phenomenon could also be seen on the third day of fermentation, showing a lower value at high enzyme concentrations than two days fermentation because the enzymes almost reached their saturation point. An increase in glutamic acid content could also occur due to a transamination reaction that accumulated glutamic acid production from other amino acid precursors. Transamination occurred in mold's mitochondria, which required oxygen in their metabolism; excess amino acid would be converted into alpha-keto acid and entered the citric acid cycle. Transamination occurs by releasing an amino group to one of the keto compounds, such as pyruvic acid, alpha-ketoglutarate or oxaloacetate. The amino group was accepted by the keto compound to form glutamic acid (Nelson and Cox, 2017). The amount of glutamic acid in a recent study was higher than the amount in previous study by Gunawan-Puteri et al. (2015) that was 150 mg g^{-1} at yellow soybean hydrolysate without adding an enzyme and oven drying method.

An increase of glutamic acid in black soybean tempeh protein hydrolysate powder, especially on the fourth day of fermentation time with the addition of 1% partially purified papain enzyme, as summarized in Table 1, could be utilized as umami seasoning powder because the high level of glutamic acid influenced the umami perception once added in a food product. Glutamic acid also modulates the desire to enjoy a dish, modulates the work of channel digestion, and increases the protein metabolism in the body (Gunawan-Puteri et al., 2015).

Soluble protein content

Soluble protein content indicates the number of protein peptide bonds that could be dissolved in water. The measurement of soluble protein content was performed to protein, short-chain peptides that had high solubility in water, and a small portion of amino acid, which could be detected by Lowry methods such as tyrosine, tryptophan and cysteine (Djunaidi et al., 2017; Shen, 2019; Arham et al., 2021).

Table 1 presented that fermentation time and concentration of the addition of partially purified papain enzyme showed a significant difference (< 0.05) in soluble protein content. Total soluble protein content increased simultaneously and prolonged fermentation time, while variations in enzyme concentration showed a decreasing trend, especially on the second and fourth-day fermentation. On the third day of fermentation, the protein content increased, indicating that the enzymatic degradation process remained taking place at high enzyme concentrations. The increased dissolved protein content in hydrolysates occurred because of the breakdown of protein into peptone, small chain peptides, and several soluble amino acids detected by the Lowry reagent (Putri et al., 2020). The presence of peptides was caused by the papain enzyme's mechanism in breaking the peptide bonds. Papain enzyme was included in the endopeptidases class, which cut peptide

bonds from the middle. This mechanism led to more peptide yield than exopeptidases, which tend to produce more amino acids. This phenomenon occurred due to protein degradation to soluble amino acids. Zhang et al. (2017) reported that some peptides had umami sensory attributes, such as dipeptides, tripeptides, tetrapeptides, pentapeptides, hexapeptides, octapeptides and undecapeptides. Black soybean contains more L-tyrosine, L-serine and L-histidine than other soybean varieties (Perdani and Utama, 2020). L-tyrosine, which had a high level in black soybean, was released through the hydrolysis process by proteases and partially purified papain enzyme, as well as the length of fermentation process. The free L-tyrosine could be detected by Lowry reagent to increase the absorbance, which indicated an escalation of soluble protein (Shen, 2019).

The increase in soluble protein content in a recent study, as seen in Table 1, showed a different result from previous studies. Muthmainna et al. (2017); Afifah et al. (2019); Beaubier et al. (2021) reported a decrease in soluble protein content with increasing fermentation time and a higher rate of enzymatic reaction. Black soybean globular proteins, such as glycinin and beta-conglycinin, degraded to amino acids because of high solubility in water and were easily denaturized by pH and temperature in the pre-treatment process. The denaturation caused proteins to be easier to digest by an enzyme produced by molds and partially purified papain enzyme.

Degree of hydrolysis

The degree of hydrolysis indicates the percentage of released peptides during the hydrolysis reaction (Putri et al., 2020). Fermentation time and concentration of partially purified papain enzyme added showed significant differences (< 0.05), as displayed in Table 1 degree of hydrolysis increased until it reached its peak on the fourth day of fermentation, and 1% addition of partially purified papain enzyme by 43.64±1.99%. This value was lower than reported in the previous study by Sitanggang et al. (2020) by 38.55±0.01% for two days of tempeh fermentation and 63.17±1.05% for four days of tempeh fermentation. Factors, including raw material and type of enzyme, influenced this difference.

The increase of the degree of hydrolysis by extending fermentation time and adding partially purified papain enzyme, as detailed in Table 1, occurred because the proteases produced by molds degraded protein into smaller peptides and amino acids. The addition of partially purified papain enzyme had undergone a purification process to have a more optimal active side to bind the substrate. Accumulation of small peptides and amino acids occurred on the last day of fermentation and was optimized by adding papain enzyme as endopeptidase (Sitanggang et al., 2020).

The initial protein content of pre-treated Mallika black soybean showed a value of 25.24%, which gave enough substrate for the enzyme to be hydrolyzed. On the third day, the degree of hydrolysis tended to decrease. On the fourth day, the addition of partially purified papain enzyme from 1% to 1.5% did not show any significant difference (< 0.05), as shared in Table 1. A previous study by Permanasari et al. (2018) stated that the enzyme saturation point was when the addition of enzymes increased the rate of reaction because mostly all the substrates had been bounded by the enzyme.

Lightness

The fermentation process and Maillard reaction could influence the lightness of black soybean tempeh protein hydrolysate. Color development from the fermentation process and Maillard reaction was an essential parameter in the acceptance and appearance of food products (Pathare et al., 2013). Therefore, fermentation time and the addition of partially purified papain enzyme had significant lightness differences especially those that could be seen on the two and three-four days fermentation time, as seen in Table 1.

The lightness value in Table 1 showed a significant decrease, especially in the third- and fourth-day fermentation compared to that of in the second-day fermentation. The lightness value in the second-day fermentation varied from 52.34±1.52 to 54.94±1.27, whereas the value in the third- and fourth-day fermentation ranged from 46.02±0.97 to 49.00±1.75. The phenomena could occur because in the third day and fourth day, the Maillard reaction and the stationary process and the death of the mold reached their maximum rate. A previous study by Muzdalifah et al. (2017) using yellow soybean as raw material showed lightness value varied from 71.52±0.53 to 78.91±0.32, which indicated the use of black soybean raw material affected the lower lightness value. The decrease of lightness was in line with the report by the previous studies conducted by Muzdalifah et al. (2017) and Lo et al. (2022), that *R. oryzae* and *R. oligosporus* experienced

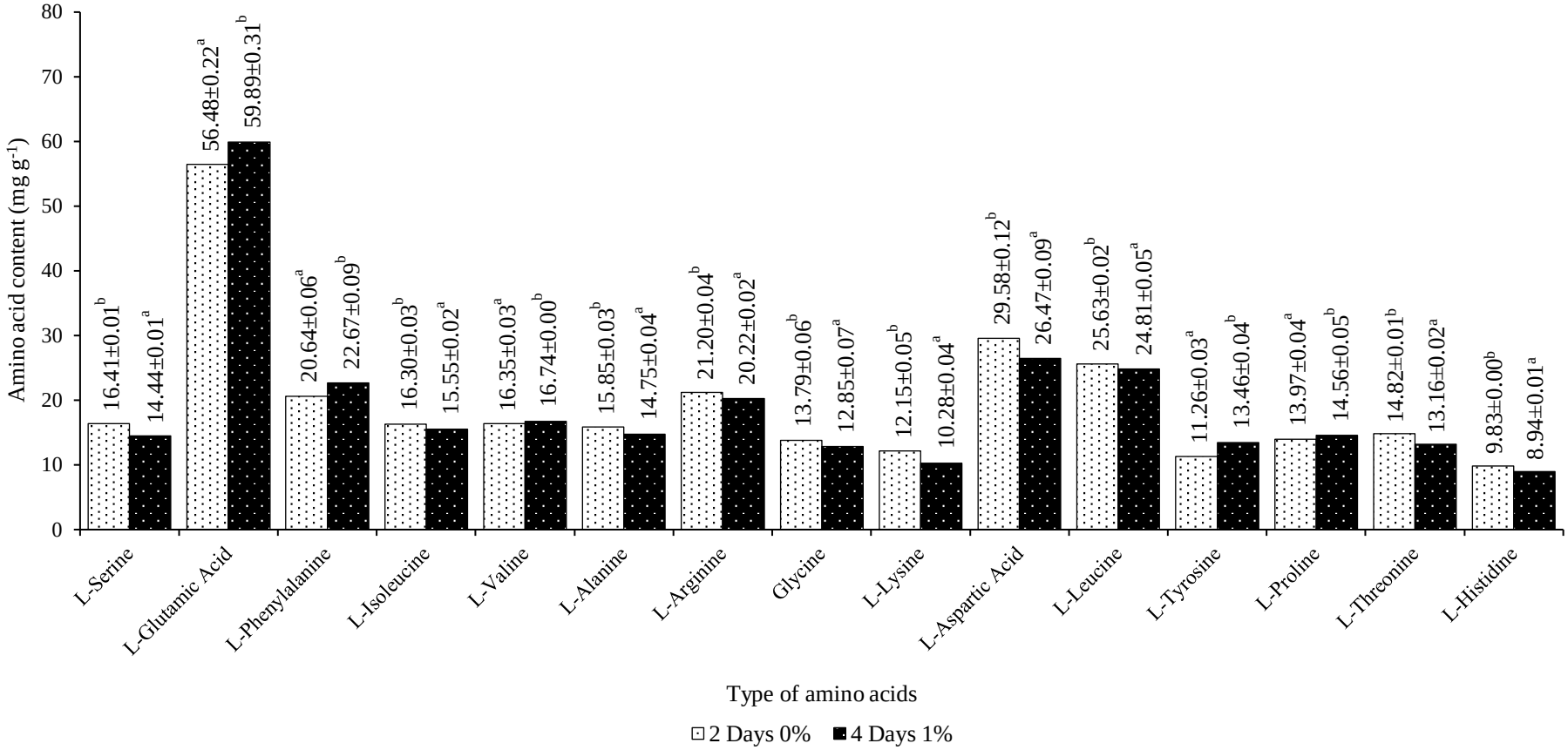

Figure 4. Amino acid profile in black soybean tempeh protein hydrolysate

Note: The notation of different superscript letters in the figures shows a significant difference (< 0.05)

some growth states. The longer fermentation process, the molds would enter a stationary phase where the substrate supplies were limited, and its growth would stagnate. At this stage, the mycelia of molds would become darker and blacker due to the production of spores.

The decrease of lightness value, as seen in Table 1, which was influenced by the addition of partially purified papain enzyme, occurred due to the Maillard reaction. Carbonyl groups of reduction sugar reacted with the components produced by the hydrolysis process, such as amino acids, which released melanoidin, especially in the drying process. The value of amino acids and short-chain peptides increased with the addition of enzymes. The most reactive amino acid in the Maillard reaction was L-lysine, which contained two amide groups (Sitanggang et al., 2021).

Amino acids profile

The fermentation and enzymatic hydrolysis produced micro components, such as amino acids and peptides, which influenced flavor. Amino acid profile analysis was conducted on two selected samples, which were 2 × 0% and 4 × 1%. The selection of this sample was based on the consideration of the highest and lowest results that could describe how the fermentation time and the concentration of the enzyme addition affected free glutamic acid, as can be seen in Figure 4.

The most type of amino acid detected was glutamic acid; at 4 × 1% treatment, the value was 59.89±0.31 mg g^{-1} or 20.74% of total amino acids, which was higher than that of previous study conducted by Gunawan-Puteri et al. (2015) by 15.9% at oven-dried "*semangit*" tempeh powder. The 2 × 0% treatment showed a lower glutamic acid value, indicating the effect of extended fermentation time and 1% enzyme addition. The second-highest amino acid content was aspartic acid, as seen in Figure 4. The two highest amino acid components were grouped into "MSG-like" amino acids, producing umami sensory attributes. Transamination reaction also influenced the high level of glutamic and aspartic acid because, by the time, those two amino acids were produced from amino acid precursors (Nelson and Cox, 2017).

Gunawan-Puteri et al. (2015) grouped several amino acids by their sensory attributes. Glutamic and aspartic acid had umami sensory; alanine, glycine, serine and threonine were responsible for giving sweet sensory characteristics, while arginine, histidine, isoleucine, leucine, methionine, phenylalanine, tryptophan, trypsin and valine gave bitter sensory attribute. As can be seen in Figure 4, amino acids with bitter and sweet characteristics had a lower value than glutamic and aspartic acids, which gave umami sensory attributes. At two days of fermentation, amino acids, which provided bitter sensory attributes, had more quantities than those in the four days of fermentation, thus giving them a more bitter taste. These results were relevant to those of the previous studies by Puteri et al. (2018) that in the second-day fermentation, the bitter taste intensity was scored 20.6 to 25, whereas, in the fourth-day fermentation, the umami taste intensity was scored highest by 23. Puteri et al. (2018) also stated that functional nutritional value increased with the addition of amino acids because it contained several increases of essential amino acids such as lysine, leucine, isoleucine, phenylalanine, valine, threonine and methionine. Leucine was the third highest value of essential amino acids after glutamic and aspartic acid.

Sensory evaluation

Sensory testing was carried out by selecting 12 people out of 14 people who took part in the selection with a basic taste test using the sequential test method. The second stage of the sensory test was to find the threshold value, namely the minimum concentration of the panelists to detect and recognize the presence of umami taste in hydrolysate. The selected hydrolysate sample had the best physicochemical characteristics based on the previous main parameters, such as glutamic acid content and amino acid profile, which was the four-day fermentation time with addition of 1% enzyme concentration. Tests were carried out on spinach vegetable soup with salt concentration of 0.3% and variations of flavorings concentration to obtain a threshold value. The recognition threshold value of this protein hydrolysate is 0.19%. The threshold value for the comparison sample (MSG) was obtained from previous research by Adawiyah and Setiawan (2017), which was 0.18% at a salt concentration of 0.3%. At the threshold concentration, the two samples were tested for the intensity of the umami taste and showed a value of 3.2±1.31 for hydrolysate sample and 4.25±1.76, which was not statistically significantly different. The value of 4 for umami intensity showed a rather strong taste of umami taste. The results of this sensory test showed the black soybean tempeh protein

hydrolysate to match the intensity of the umami taste of MSG at its threshold value.

CONCLUSIONS

Utilization of local Mallika black soybean in the manufacture of umami seasoning can be added with 40% partially purified papain enzyme. Fermentation treatment for four days and addition of 1% enzyme gave the best hydrolysate physicochemical results. Increasing the fermentation time up to four days and adding enzymes up to a maximum of 1% increased the value of glutamic acid content, which is the main parameter, dissolved protein content, and degree of hydrolysis, as well as decreased the value of lightness. Sensory testing also showed a threshold value that matched MSG and the intensity of umami taste was the same as MSG so that hydrolysate could be used as an alternative to MSG. Further research on other locally based enzyme sources, specific activity of enzymes on Mallika black soybean tempeh, commercialization of tempeh-based flavorings, and extension of the fermentation time can be carried out to find the maximum degree of hydrolysis.

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