

Production of protein hydrolysate from overripe tempeh catalyzed by bromelain

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Article history:

Received: 18 January 2023

Received in revised form: 18 April 2024

Accepted: 9 May 2024

Available Online: 27 December 2024

Keywords:

Bromelain,

Enzymatic hydrolysis,

Overripe tempeh,

Protein hydrolysate

DOI:

[https://doi.org/10.26656/fr.2017.8\(6\).032](https://doi.org/10.26656/fr.2017.8(6).032)

Abstract

Solid-state fermentation is an important food processing technology, often utilized in the food industry because of its production of secondary metabolites, amino acids, organic acids, and enzymes. Prolonged solid-state fermentation of tempeh can increase the protein and free amino acid content in it. By using bromelain, the production of overripe tempeh protein hydrolysate (OTPH) as a food ingredient may be possible. Bromelain was extracted from pineapple core and its optimum temperature and pH for its activity were determined. OTPH is made from soybeans that were fermented for 2, 3, 4, and 5 days, added with crude bromelain enzyme of 0.10, 0.15, 0.20, 0.25% (v/w), and incubated at the optimum condition obtained from the preliminary stage. A longer fermentation time and higher concentration of enzyme added resulted in significant increases in the protein content (ranging from 45.96% to 50.87%), water-soluble amino acids content (ranging from 14.55% to 39.86%), and total amino acid content with 0.25% crude bromelain produced the highest total amino acid content amounting to 36682.002 mg/100 g. These results showed the potential for the application of bromelain to produce protein hydrolysate from overripe tempeh.

1. Introduction

Solid-state fermentation is one of the excellent food processing technologies. During the fermentation process, a series of biochemical processes occur which produces secondary metabolite, amino acids, organic acids, and enzymes. Solid-state fermentation is often utilized in various research to produce enzymes, flavor compounds, food coloring, and other substances that can contribute to the food industry (Couto and Sanroman, 2006). This shows that solid-state fermentation continues to develop and still has a variety of applications that can be further investigated, including in the popular native Indonesian fermented food, tempeh.

Tempeh is a fermented food product made from yellow soybeans. Generally, tempeh is fermented for 24 to 48 hrs (Utami *et al.*, 2016). During the fermentation process, a series of biochemical processes occur in the presence of *Rhizopus* sp. One of them is protein hydrolysis which breaks down protein into amino acids and peptides. According to Handoyo and Morita (2006), *Rhizopus* can hydrolyze 65% soy protein during fermentation. Therefore, it can be said that tempeh is an excellent source of plant-based protein.

Overripe tempeh is defined as yellow soybeans that are being fermented for a longer time than ordinary tempeh. In Indonesia, it is currently often used as a flavor enhancer in various traditional food. Overripe tempeh has a pungent aroma, a darker appearance, and a softer texture when compared to ordinary tempeh (Utami *et al.*, 2016). In a study conducted by Gunawan-Puteri *et al.* (2015), prolonged fermentation of tempeh (up to 5 days) can result in different amino acid profiles from tempeh that is fermented in a shorter time. Nout and Kiers (2005) also observed that overripe tempeh has a higher protein content (53%) compared to ordinary tempeh (40-50%). The increase in protein content is due to continual protein hydrolysis that occurs during fermentation.

In general, protein hydrolysates are made from animal-based foods, such as fish and cow's milk. Research on the production of protein hydrolysate derived from plant-based food sources is still scarce. Protein hydrolysate has a very broad application in the food industry, including as a supplement in energy drinks and food products, the production of hypoallergenic foods, or the treatment of certain diseases (Clemente, 2000). Generally, in the production of

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hydrolysate, the protein in the raw material will be hydrolyzed into short-chain peptides and free amino acids. Soybean is an alternative that can be utilized as a source of plant-based protein hydrolysate. In the study of Wijayanti *et al.* (2016), bromelain can be used to produce hydrolysate from milkfish. Bromelain was also seen to successfully hydrolyze meat and soybean protein with a degree of over 80% (Utami *et al.*, 2019). Purwaningsih (2017) observed that bromelain can be used to increase the protein content in tofu. Therefore, bromelain isolated from pineapple can be utilized to hydrolyze protein-containing food to produce protein hydrolysate. The objective of this study was to determine the chemical characteristics of the protein hydrolysate from overripe tempeh in variations of fermentation time and concentration of enzyme.

2. Materials and methods

2.1 Materials and equipment

Cayenne pineapple cores were obtained from plantations in Bali, Indonesia. Yellow soybeans were obtained from Pasar Modern Paramount Serpong, Tangerang, Indonesia; commercial tempeh starter Raprima® was obtained from Rumah Tempe Indonesia, Bogor. Casein, L-tyrosine, and other chemical reagents were purchased from Sigma (Sigma-Aldrich). The equipment used such as blender (Panasonic), centrifuge (Boeco M-240R), cabinet dryer (Wangdi W), Ultra Performance Liquid Chromatography, AccQtag column 18 (2.1 mm × 100 mm), and various standard glassware.

2.2 Extraction of bromelain enzyme

Bromelain was extracted from the pineapple cores. Extraction was carried out according to Al-Sa'ady *et al.* (2016) method. Pineapple cores were crushed in a blender for 2 mins with the addition of cold sodium phosphate buffer (0.1 M; pH 7) in a ratio of 2:1. The blended slurry was filtered through a filter cloth, separating the filtrate and fibrous parts. The filtrate was centrifuged at 5000 rpm for 15 mins at 4°C to obtain supernatant and precipitate. The precipitate obtained was removed and the supernatant was crude bromelain extract. The crude enzyme was stored at -20°C.

2.3 Determination of protein content

The protein content of crude bromelain was determined using the Bradford method (Nielsen, 2010). Coomassie brilliant blue G-250 was dissolved in 95% ethanol and acidified with phosphoric acid (85%). The crude enzyme was dissolved in phosphate buffer and centrifuged (6000 rpm, 15 mins). The supernatant was then added with Bradford reagent and the absorbance was measured using a UV-Vis spectrophotometer at 595

nm wavelength. Bovine serum albumin solution was used as standard.

2.4 Determination of the optimum temperature

Determination of the optimum temperature of crude bromelain enzyme was done by incubating the crude enzyme and substrate at different temperatures. In this assay, casein was used as the substrate. 5 mL of 0.65% casein was added with 1 mL of crude enzyme and incubated at 40, 45, 50, 55, and 60°C for 10 mins. To stop the reaction, 5 mL of trichloroacetic acid solution (TCA) (100 mM) was added. Enzymatic activity was determined for each incubation temperature.

2.5 Determination of the optimum pH

The optimum pH of crude bromelain enzyme was determined by incubating the enzyme and substrate at different pHs. A 0.65% casein solution was prepared by dissolving casein in phosphate buffer with different pHs ranging from 5.5, 6.0, 6.5, 7.0 and 7.5. 5 mL of the substrate was added with 1 mL of crude bromelain and incubated at the optimum temperature for 10 mins. To stop the reaction, 5 mL of TCA (100 mM) was added. Enzymatic activity was determined for each pH variation.

2.6 Activity of crude bromelain

The proteolytic activity assay of crude bromelain was done by colorimetry method at 37°C, pH of 7.5, and using casein as substrate (Cupp-Enyard, 2008). 5 mL of 0.65% (w/v) casein in potassium phosphate buffer (0.05 M, pH 7.5) was incubated in a water bath (37°C) for 5 mins, then added with 1 mL of the enzyme. The mixture was then incubated for 10 mins. To stop the reaction, 5 mL of 110 mM TCA was added to the mixture and incubated for 30 mins. The mixture was then centrifuged (6000 rpm, 10 mins). The supernatant obtained was called the test filtrate. Blank filtrate was obtained by doing the same procedure without any enzyme added. Such 2 mL of each test filtrate and the blank filtrate were added with 5 mL of 0.5 M Na₂CO₃ and 1 mL of Folin-Ciocalteu's phenol and incubated for 30 mins in a 37°C water bath. The absorbance at 660 nm was measured. A standard graph was generated using a varying concentration of L-Tyrosine (0.055; 0.11; 0.22; 0.44; 0.88 μmol).

2.7 Fermentation of tempeh

Tempeh and overripe tempeh fermentation were carried out according to a method developed by Handoyo and Morita (2006) with some modifications. A total of 300 g of yellow soybeans were soaked with clean water (1:2) for 12 hrs, rinsed, and then dehulled. The soybeans

were steamed for 45 mins, cooled to room temperature, and inoculated with a tempeh starter (0.5% w/w). Soybeans (100 g) wrapped in banana leaves and incubated at room temperature for 2, 3, 4, and 5 days. After the fermentation, all tempeh was stored at refrigeration temperature until required for use.

2.8 Enzymatic hydrolysis

The enzymatic hydrolysis procedure was done according to Gunawan-Puteri *et al.* (2015) and Machin (2012) with some modifications. Fresh tempeh and overripe tempeh were sliced and steamed for 10 mins and crushed using a blender with the addition of 100 mL of water. The pH of tempeh was adjusted using NaOH and/or HCl to reach the optimum pH of the bromelain enzyme. Crude bromelain extract was added ranging from 0.10, 0.15, 0.20 and 0.25% (v/w). Tempeh slurry was incubated in a water bath at the optimum temperature for 1 hr. Then, it is heated to 100°C for 10 mins. The slurries were then dried in a cabinet dryer at 50°C for 24 hrs, ground using a dry blender, and sifted with a 40-mesh sieve.

2.9 Determination of crude protein content

Crude protein content was assayed using the Kjeldahl method (Association of Official Analytical Collaboration (AOAC) International, 2005). A mixture of the sample (2 g), K₂SO₄ (7 g), selenium (5 mg), concentrated H₂SO₄ (10 mL), and 30% H₂O₂ (10 mL) was added to the digestion tube. The mixture was digested until clear and cooled down to room temperature. The sample was then added with 50 mL of 35% NaOH, connected to a distillation unit and was distilled for 5 mins. An Erlenmeyer flask containing 25 mL of 4% H₃BO₃ added with a mixed indicator (0.2% methyl red and bromocresol green in alcohol) was prepared and used to capture the distillate of the sample. The distillate was then titrated using HCl until the endpoint (color changes from green to faint pink). A blank sample was prepared by exchanging the sample with distilled water.

2.10 Determination of water-soluble amino acid content

Water-soluble amino acid content was assayed according to Djunaidi *et al.* (2017). The sample was diluted in a ratio of 1:5 with distilled water. Extraction was done at room temperature with constant stirring for 6 hrs. The mixture was then filtered with a filter cloth. The filtrate obtained was centrifuged (8000 rpm, 15 mins) to obtain the supernatant. The supernatant was stored in the freezer (-20°C). The sample was thawed before the assays.

The supernatant was added with 500 µL distilled

water and phosphate buffer (pH 8.0), incubated in a water bath (37°C) for 5 mins, and added with 750 µL of 10% (w/v) TCA. The mixture was then centrifuged (10000 rpm, 10 mins). Approximately 300 µL of the supernatant obtained was added with 1000 µL of 0.5 M Na₂CO₃ and 200 µL of Folin-Ciocalteu's phenol. The absorbance at 578 nm was measured and calculated according to a standard graph that was obtained using varying concentrations of L-Tyrosine (0.005, 0.01, 0.02, 0.04, 0.08 mg/mL).

2.11 Amino acid profile analysis

The amino acid profile was assayed using ultra-performance liquid chromatography. The assay was done in the Saraswanti Indo Genetech Laboratory (SIG) located in Bogor, Indonesia. The procedure was done according to the SIG internal module. The assay was done in three main steps, which are sample preparation, preparation of the standard solution, and derivatization procedure.

In the sample preparation step, a sample of 0.1-1.0 g was put into a 20 mL headspace vial and added with 5 mL of 6 N HCL and closed. The vial was then heated in a 110°C oven for 22 hrs, was cooled down to room temperature, and transferred into a 50 mL volumetric flask. The headspace vial was rinsed into the volumetric flask and was diluted down to the volume using distilled water, and then it was homogenized. The sample was then filtered using grade 42 ashless filter paper and syringe filter GHP/RC 0.2 µm. 500 µL of the filtrate was then pipetted into a 2 mL tube, added with 40 µL of internal standard AABA 2.5 mM and 460 µL of distilled water, and was vortexed. The sample was then derivatized.

Derivatization of the sample and standard was done by transferring 10 µL of the sample into an insert vial, topped up with 70 µL of Accq. Tag fluor borate buffer and it was then vortexed. The mixture was then heated on a heating block at 60°C for 10 mins, cooled down to room temperature, and injected into the UPLC system. The standard solution was prepared by mixing 40 µL of AABA solution with 40 µL of 2.5 µmol/mL amino acid standard solution and 920 µL of distilled water in a 2 mL tube. The mixture was then vortexed and derivatized.

The specification for the chromatography condition used is as follows:

Column: AccQ. Tag Ultra C18 1.7 µm (2.1 × 100 mm), Mobile phase: A: Accq. Tag Ultra Eluent A 100%, B: Accq. Tag Ultra Eluent B:aquadest (90:10), C: Aquadest, D: Accq. Tag Ultra Eluent B 100%, Flow rate: 0,5 mL/min, Pump system: Gradient, Injection Volume: 1 µL, Column temperature: 49°C, Detector: PDA 260 nm

2.12 Statistical analysis

Data were analyzed in a Completely Randomized Design using the analysis of variance procedure. The mean difference was determined using the Duncan multiple range test (SPSS 23.0 Version). The significance of the difference was determined at $p < 0.05$.

3. Results and discussion

3.1 Protein content of crude bromelain

The Bradford method that was used to quantify the protein content of the crude bromelain measures it through colorimetry and compares it to a BSA standard curve. The crude bromelain extracted was measured to have a protein content of 0.4703 ± 0.0291 mg/mL. This does not correspond well with research done by Sarkar *et al.* (2017), where it was found that the protein content of crude bromelain was 0.21 ± 0.005 mg/mL. The difference in the protein content might be because of the difference in buffer and pH that was used in the extraction. The higher protein content that was seen in this research might also show that there might still be some other protein compounds or other enzymes that were extracted together.

3.2 Activity of crude bromelain

Cupp-Enyard's (2008) method of testing proteolytic activity works by hydrolyzing the substrate (casein) with the enzyme, which results in the production of several derivative products, amino acids and short-chain peptides, one of which is L-Tyrosine. L-Tyrosine reacts with the Folin-Ciocalteu's phenol reagent, resulting in a blue color, which can be quantified using a spectrophotometer, the higher the absorbance implies a higher concentration of L-Tyrosine which is produced from the hydrolyzation of casein, thus signalling a higher proteolytic activity. The absorbance value of the enzyme is also compared to a standard L-Tyrosine, thus it can be quantified using Unit. The unit reflects the amount of tyrosine (μmol) which is released per min by the casein hydrolyzation.

The extracted crude bromelain enzyme in this research was measured to have a proteolytic activity of 0.6309 ± 0.2973 U/mg. A low enzymatic activity might be because of the bromelain being crude, which might be contaminated with other compounds. According to Mohan *et al.* (2016), these other compounds include phosphates, glucosidases, peroxidases, cellulases, glycoproteins, and carbohydrates.

The proteolytic activity is also affected by the method of extraction. Sarkar *et al.* (2017) observed that

the proteolytic activity from pineapple cores that were extracted using phosphate citrate buffer at a pH of 6.5 was 0.0025 U/mL. Ketnawa *et al.* (2012) observed that the protease enzymatic activity from pineapple cores that were extracted using distilled water was 0.7954 U/mg. According to Muntari *et al.* (2012), there are some chemical methods to modify the activity and stability of an enzyme, including the immobilization using various media, the addition of ammonium sulphate salt and ethanol as a precipitant, and cross-linking the enzyme with chemical compounds.

3.3 Determination of optimum temperature

In Figure 1, specific protease activity was observed the lowest at 40°C , which was 0.6671 ± 0.0862 U/mg and steadily increased until the temperature of 55°C (1.5481 ± 0.1643 U/mg) after which it started to decrease, as at 60°C , the specific protease activity was observed to be at 1.2037 ± 0.0205 U/mg. This result showed that the optimum temperature for the enzyme to catalyze the breaking down of substrate into products is 55°C . Whitaker *et al.* (2018) explained that enzymes are proteins that have tertiary structures, they denature in unsuitable conditions, i.e. high temperature. Denaturation breaks the hydrogen bonds, ionic bonds, and hydrophobic interaction in proteins, thus changing the conformation of the tertiary structure from folded to unfolded (Noviyanti *et al.*, 2013). This phenomenon causes a change in the active side of the enzyme thus the substrate cannot bond to this active side, which lowers the enzymatic activity.

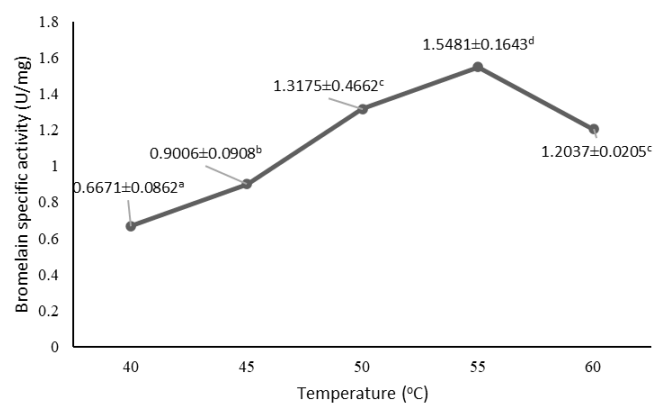


Figure 1. Effect of temperature towards specific activity of bromelain. Values are presented as mean \pm SD. Values with different superscripts are statistically significantly different ($p < 0.05$).

At temperatures lower than 55°C , the specific protease activity was also observed to be lower. This was because of the low activation energy that was available. The increase in temperature increases the kinetic energy of the system by accelerating the vibrational, rotational, and translational motion from the enzyme and substrate, this increases the intensity of the collisions between the substrate and enzyme, which results in a higher number

of E-S (enzyme-substrate) complexes produced, thus more end products were produced.

3.4 Determination of optimum pH

The effect of pH towards the specific proteolytic activity can be seen in Figure 2. The lowest activity observed was at the pH of 5.5, which was 0.3929 ± 0.0727 U/mg. The activity continues to increase until the optimum pH (6.5), with a specific activity of 0.9677 ± 0.0390 U/mg.

The tertiary structure of an enzyme is stabilized by electrostatic non-covalent bonds or ionic bonds, hydrogen bonds, hydrophobic interactions, and disulfide covalent bonds. pH affects the formation of electrostatic bonds which are formed between the groups inside of an enzyme which are negatively charged with a positively charged group. At the optimum pH, the enzyme side chains are ionized into $-\text{COO}^-$ and H_3N^+ which can form a strong ionic bond (Whitaker et al., 2018).

The result was also supported by research by Masri (2014) which observed that at the pH of 6.5–7.0, bromelain has the optimal conformation for the formation of bromelain-casein E-S complex, thus forming more end products.

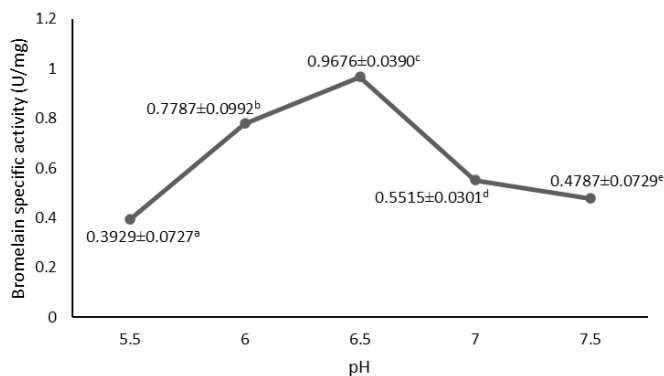


Figure 2. Effect of pH towards specific proteolytic activity of bromelain. Values are presented as mean \pm SD. Values with different superscripts are statistically significantly different ($p < 0.05$).

3.5 Effect of fermentation time and enzyme concentration on crude protein content

The effect of fermentation time and enzyme concentration on the crude protein content of overripe tempeh protein hydrolysate is seen in Figure 3. According to the statistical analysis, both fermentation time and enzyme concentration had a significant effect towards the parameter ($p < 0.05$) and interaction is seen between the 2 factors. Three treatments which were seen to have the highest crude protein content were at 5 days of fermentation, with 0.25% enzyme concentration added; 5 days of fermentation, with 0.2% enzyme concentration added; and 4 days of fermentation, with

0.25% enzyme concentration added.

Generally, longer fermentation time results in a higher crude protein content in the protein hydrolysate. This is also supported by research by Utami et al. (2016), where tempeh which was fermented for 96 hrs had an increase in the crude protein content when compared to tempeh which was fermented for 24 hrs. Solid-state fermentation occurs in tempeh because of the presence of *Rhizopus* sp. which is a mold that produces proteolytic enzymes. It uses protein from the yellow soybeans as a nitrogen source for its growth (Muthmainna et al., 2016). The protease which is produced will then hydrolyze the yellow soybean's protein into short-chain peptides and amino acids thus increasing the crude protein content contained in the tempeh. Handoyo and Morita (2006) also support the observation of fermentation causing complex proteins to break down into smaller short-chain peptides and amino acids. In their research, a significant decrease in the albumin and globulin content in tempeh can be seen, the longer the fermentation time applied.

A higher enzyme concentration used also increased the crude protein content of the overripe tempeh protein hydrolysate, as the enzyme hydrolyzes protein into short-chain peptides and increases the content of the NH_3^+ group (Witono et al., 2016). A higher concentration of enzyme used leads to a higher reaction rate, increasing the production of end products in a shorter amount of time (Wijayanti et al., 2016).

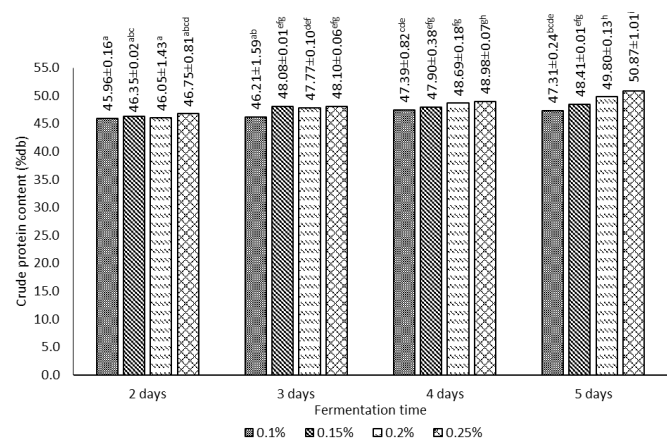


Figure 3. Effect of fermentation time and enzyme concentration on the crude protein content of overripe tempeh. Values are presented as mean \pm SD. Values with different superscripts are statistically significantly different ($p < 0.05$).

The higher value of crude protein content might also be attributed to the production of ammonia at prolonged fermentation times as the deamination of amino acids during fermentation produces ammonia (Stodolak et al., 2015). According to Muthmainna et al. (2016), peptides and amino acids in tempeh will be converted into NH_3 and NH_2 as the fermentation is prolonged. This accumulation of ammonia and nitrogen from peptides

and amino acids contributes to the high value of crude protein content observed, as it was measured using the Kjeldahl method.

3.6 Effect of fermentation time and enzyme concentration on water-soluble amino acid content

The effect of fermentation time and enzyme concentration on the water-soluble amino acid content of the overripe tempeh protein hydrolysate can be seen in Figure 4. Based on the statistical analysis, both the fermentation time and enzyme concentration added had a significant effect towards the water-soluble amino acid content ($p < 0.05$). In accordance with the crude protein content results, the longer the fermentation time results in a higher content of water-soluble amino acid in the overripe tempeh protein hydrolysate. Fermentation causes the hydrolyzation of the proteins into smaller and simpler compounds, which have a lower molecular weight and thus are more soluble in water (Utami et al., 2016).

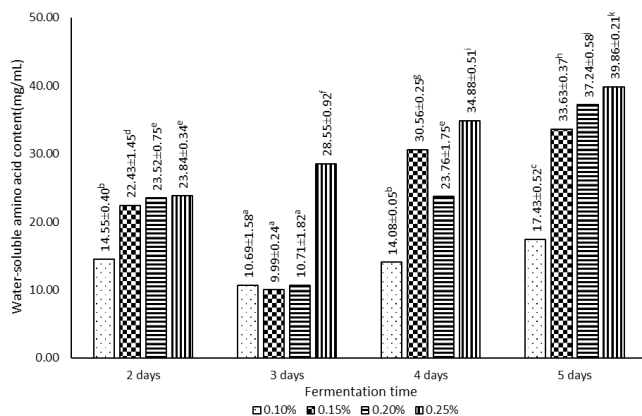


Figure 4. Effect of fermentation time and enzyme concentration on water-soluble amino acid content. Values are presented as mean±SD. Values with different superscripts are statistically significantly different ($p < 0.05$).

As seen in Figure 4, the increase of enzyme concentration added causes fluctuation of the water-soluble amino acid content in overripe tempeh protein hydrolysate. Meanwhile, Wijayanti et al. (2016) observed that the higher enzyme concentration that is added should produce a higher water-soluble amino acid content. Bromelain is an endopeptidase enzyme which has a specificity towards various substrates (Raftani et al., 2016), thus should hydrolyze the various proteins contained in the tempeh. The discrepancy between the observed results in this research and previous research might be caused by the decrease in enzyme activity along its storage period. Truc et al. (2011) observed that bromelain enzymes stored at 4°C and -30°C for 14 days will have a significantly lower enzyme activity. Differences in the enzyme activity might explain why fluctuations are seen in this research.

3.7 Amino acid profile of overripe tempeh protein hydrolysates

The treatments with the highest crude protein and water-soluble amino acid content were then analyzed to identify its' amino acid profile. Witono et al. (2015) observed that amino acid is one of the parameters that are usually used in the research of overripe tempeh, especially in determining the nutritional value of overripe tempeh. Other than that, the amino acid profile also affects the functional properties of the overripe tempeh protein hydrolysate. The overripe tempeh produced in this research was seen to have an amino acid profile which is quite complete.

Table 1 shows that between the selected treatments, the treatment with the highest total amino acid content was the overripe tempeh protein hydrolysate which was fermented for 5 days with 0.25% enzyme concentration added, with a total content of 36682.002 mg/100g. The results also show that the longer the fermentation time and the higher enzyme concentration added resulted in an increase in the total amino acid content, this result shows that crude bromelain activity affected the total amino acid of the overripe tempeh protein hydrolysate. This is in accordance with research by Witono et al. (2015) which observed that there was an increase in total amino acid content of 2 to 3 times its' original value when comparing overripe tempeh with the addition of exoprotease from *Calotropis gigantea* and overripe tempeh without enzyme addition.

Bromelain structurally is in the sulfhydryl protease functional group, which contains a cysteine group as a side branch. Cysteine acts as a nucleophile which breakdown peptide bonds, however, as a weak nucleophile, cysteine needs to be activated by cysteine residues located near the cysteine groups (Laskar and Chatterjee, 2009). The higher the concentration of enzyme added, the higher the reaction rate of the breakdown, which results in a higher total amino acid content produced (Witono et al., 2007).

Leucine was seen to be the highest percentage of essential amino acid observed in the overripe tempeh protein hydrolysate, with histidine being the lowest. This is supported by the research of Witono et al. (2015), which observed that the high leucine content is expected, as the protein source used in the production of the tempeh was soybeans. Norton et al. (2012) also observed that the essential amino acid with the highest concentration in soybean is leucine. For non-essential amino acids, it is seen that glutamic acid was the highest. This might be because of transamination that occurs during fermentation (Chasanah et al., 2019).

Table 1. Free amino acid content (mg/100 g) of overripe tempeh protein hydrolysates.

Amino acid	Treatment				
	4 days, 0%	4 days, 0.25%	5 days, 0%	5 days, 0.20%	5 days, 0.25%
Essential amino acid					
Isoleucine	1846.250	1819.619	1826.372	1847.520	1923.774
Leucine	2882.775	2905.531	2906.210	2929.765	3065.506
Lysine	1975.763	1931.889	1900.629	2074.145	1919.130
Phenylalanine	2437.188	2651.691	2371.915	2517.815	2755.193
Threonine	1630.842	1639.374	1612.951	1643.460	1753.728
Valine	1890.861	1851.845	1901.696	1917.298	1971.373
Histidine	1354.953	1401.306	1233.783	1299.425	1662.167
Non-essential amino acid					
Arginine	2539.316	2677.537	2405.941	2710.257	2770.936
Glycine	1686.019	1692.649	1738.685	1700.604	1852.908
Tyrosine	1496.150	1659.800	1443.952	1594.274	1723.575
Alanine	1736.572	1655.263	1763.532	1767.389	1803.444
Aspartic acid	3615.747	3435.858	3643.900	3743.393	3529.691
Glutamic acid	5830.027	5818.186	6476.399	6133.801	5979.055
Proline	1741.026	1843.776	1666.751	1844.258	1877.117
Serine	1885.771	1893.991	1829.687	1891.016	2094.405
Total	34549.260	34878.315	34722.403	35614.420	36682.002

4. Conclusion

Fermentation time and the concentration of bromelain added significantly affect the chemical characteristics of the overripe tempeh protein hydrolysate. The longer the fermentation time and the higher concentration of bromelain enzyme added causes an increase in the crude protein content, water-soluble amino acid content, and the total amino acid content of the overripe tempeh protein hydrolysate. The highest crude protein ($49.10 \pm 1.01\%$), water-soluble amino acid (39.86 ± 0.21 mg/mL), and total amino acid content (36682.002 mg/100 g) was found in the overripe tempeh protein hydrolysate produced from 5 days of fermentation and the addition of 0.25% bromelain enzyme concentration.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors would like to acknowledge the Head of the Food Technology Study Program of Universitas Pelita Harapan (Ir. Wilbur Donald Raymond Pokatong, MSc., PhD), the Dean of Faculty of Science and Technology Universitas Pelita Harapan (Eric Jobiliong, PhD.) and the Center of Research and Community Development of Universitas Pelita Harapan for the continual support of this research, and Mario Deny Pereira for providing assistance during the research.

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