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Enzymatization of Nila Fish (*Oreochromis Niloticus*) Protein Hydolysate by Combination of Bromelin and Pepsin Enzymes

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ABSTRACT

Nile Tilapia (*Oreochromis niloticus*) is a freshwater fish with better nutritional content compared to other freshwater fish, making it suitable for use as a source for protein hydrolysate production. Protein hydrolysate is the result of the breakdown of protein into short-chain compounds through hydrolysis. This research aims to hydrolyze the minced Nile tilapia meat using a combination of bromelain and pepsin enzymes at a concentration of 5% and pH 7 conditions for 5-6 hours. The hydrolysis results using the combination of bromelain and pepsin enzymes (B100: P0) have a yield value of 12.68%, ash content of 2.94%, protein of 57.25%, water 2.65%, and fat 10.25% with a degree of hydrolysis of 61.46%. The hydrolysate has antibacterial properties against *Staphylococcus aureus* and *Escherichia coli* bacteria with inhibition zones of approximately 8.3 mm and 8.5 mm, respectively, at a concentration of 1 (mg/µL), contains 15 types of amino acids with the highest composition being lysine 5.12% and the highest non-essential amino acid being aspartic acid 5.75%.

Keywords: Amino Acids, Antibacterial, Enzymatic, Nila Tilapia, Protein Hydrolysate

ABSTRAK

Ikan Nila (*Oreochromis niloticus*) merupakan ikan air tawar dengan kandungan gizi lebih baik dibandingkan ikan air tawar lainnya, menjadikannya dapat dimanfaatkan sebagai sumber pembuatan hidrolisat protein. Hidrolisat protein merupakan hasil dari penguraian protein menjadi senyawa berantai pendek melalui hidrolisis. Penelitian ini bertujuan menghidrolisis daging ikan nila yang telah dihaluskan menggunakan kombinasi enzim bromelin dan enzim pepsin pada konsentrasi 5% dan kondisi pH 7 selama 5- 6 jam. Hasil hidrolisis menggunakan kombinasi enzim bromelin dan enzim pepsin (B100 : P0) memiliki nilai rendemen sebesar 12,68%, kadar abu 2,94%; protein 57,25%; air 2,65%; dan lemak 10,25% dengan derajat hidrolisis sebesar 61,46%. Hidrolisat tersebut memiliki sifat antibakteri terhadap bakteri *Staphylococcus aureus* dan *Escherichia coli* dengan dengan zona hambat masing-masing sekitar 8,3 mm dan 8,5 mm pada konsentrasi 1(mg/µL), mengandung 15 jenis asam amino dengan komposisi tertinggi adalah lisin 5,12% dan asam amino non-esensial tertinggi adalah asparticacid 5,75%.

Kata kunci : Antibacterial, Asam Amino, Enzimatis, Hidrolisat Protein, Ikan Nila

1 Introduction

Nila Tilapia (*Oreochromis niloticus*) is one of the most widely cultivated fish in Indonesia because it can proliferate. Tilapia fish also contains high-quality nutrients, namely essential amino acids, which add value as protein hydrolysate [1]

Fish protein hydrolysate is a product resulting from the decomposition of fish protein into simpler peptides in the form of amino acids through hydrolysis by enzymes, acids, bases, and fermentation [2]. The high degree of hydrolysis makes protein hydrolysates more soluble in water due to the formation of small-sized peptides and amino acids [3]. The process of protein hydrolysis can be carried out chemically or biochemically. Additionally, the biochemical process using enzymes is much easier to control, more specific in breaking peptide bonds, and there is no reduction in the nutritional value of the resulting HPI [4].

Protein hydrolysates are generally used as food additives because they contain a high nutritional value in the form of amino acids; they are also commonly used as flavour enhancers and emulsifying agents [5]. Dinakarkumar et al. (2022) also conducted a study on the functional characteristics of enzymatic hydrolysis of fish protein from tilapia (*Oreochromis niloticus*) using bromelain and papain enzymes with different hydrolysis lengths, followed by testing parameters such as yield, proximate, and degree of hydrolysis [6]. In addition, bromelain enzyme is more active in hydrolyzing animal proteins compared to papain enzyme, which is more active in hydrolyzing plant proteins [7]. The combination of calotropin enzyme and papain enzyme in producing fish protein hydrolysate and its antioxidant capacity also was studied by [8].

Antibacterial activity is beneficial in the fields of health and food. Antibacterial agents are used as preservatives to prevent oxidation and help inhibit the growth of bacteria that cause food spoilage [9]. A research experiment on the antibacterial and antioxidant activities of goat milk protein hydrolysate hydrolyzed with crude bromelain extract was also reported by [10] . The research results showed that peptides from goat milk hydrolyzed with bromelain enzyme could inhibit the growth of *E. coli, S. Typhimurium, and L. monocytogenes* bacteria.

Wijayanti et al. (2016) conducted research on the characterization of protein hydrolysate from milkfish with different concentrations of bromelain enzyme. With the concentration results, the bromelain enzyme has a significant effect on the proximate levels of protein, fat, water, and ash [11]. However, it does not have a significant effect on the carbohydrate level.

Based on the explanation above, the researcher is interested in utilizing Nila tilapia fish (*Oreochromis niloticus*) as a protein source in the production of protein hydrolysate enzymatically with a combination of bromelain and pepsin enzymes".

2 Materials and Methods

2.1 Equipment

The tools used in this study include: beaker glass, blender, analytical balance, measuring cup, measuring flask, desiccator, oven, centrifugator, burret, Kjedahl flask, pedestal flask, soklet apparatus, electromantel, autoclave, freeze dryer, HPLC, and thanur.

2.2 Materials

The materials used in this study include tilapia, distilled water, bromelin enzyme, pepsin enzyme, N-hexanes, NaOH 30%, Selenium mix, pp indicator, Tashiro indicator, HCL (p), H_3BO_3 3%, H_2SO_4 (p), TCA 20%, orthophthalaldehyde reagent, NaOH 6 N, *Staphylococcus aureus* and *Escherichia coli* bacteria, MHA, NA, and NaCl 0.9%.

2.3 Preparation of Sample

The cleaned-scales tilapia meat is separated from fish bones and cut into small pieces. Furthermore, the fish meat is mashed with a blender until smooth.

2.4 Preparation of 5% Bromelin Enzyme and 5% Pepsin Enzyme Solution

As much as 5 grams of bromelain enzyme powder were added to a measuring cup containing a small amount of distilled water and stirred until homogeneous. The mixture was then diluted using a 100 ml volumetric flask. the same treatment is also given to 5% Pepsin Enzyme Solution.

2.5 Preparation of Nila Tilapia Protein Hydrolyzate

In this manufacture, the ratio of bromelain enzyme and pepsin enzyme is carried out with the ratio of 5% pepsin enzyme concentration. The preparation of protein hydrolysate was carried out with 5 variations of bromelain enzyme and pepsin enzyme combinations as shown in Table 1.

Table 1 Ratio of bromelain enzyme and pepsin enzyme

Bromelin Enzyme	Pepsin Enzyme
100	0
75	25
50	50

The crushed fish meat is placed into a measuring cup, then distilled water in a 1:4 (w/v) ratio of the sample weight and enzymes with various concentrations of 5%. It is then hydrolyzed at 55ºC for 5-6 hours with a pH of 7, followed by enzyme activation at 90ºC for 20 minutes, and then centrifuged at 3500 rpm to obtain the soluble fraction. Twenty minutes, then centrifugation at 3500 rpm to obtain the soluble fraction. This fraction is then dried using a freeze dryer to obtain a powder from the protein hydrolysate.

2.6 Drying Process with Freeze Dryer

The sample was first prepared for freeze-drying by freezing it in a deep freezer. While the sample was freezing, the freeze dryer was activated, and its chamber was cleaned. The chamber temperature was then set to -50°C. Once frozen, the sample was transferred to the freeze-dryer chamber, which was subsequently sealed and filled with nitrogen gas. The drying process was then monitored at 30-minute intervals.

2.7 Characterization and Testing

2.7.1Yield Analysis

The yield of Tilapia protein hydrolysate was calculated according to Hadiwiyoto (1993) with the following formula:

$$Yield\ (\%) = \frac{weight\ of\ protein\ hydrolyzate\ (g)}{weight\ of\ chopped\ tilapia\ meat\ (g)} \times 100\%$$

2.7.2Proximate Test

Total Ash Content Test:

The protein hydrolyzate powder was weighed as much as 1 gram in a porcelain cup of known weight. Dried in the oven. Ashed in an ashing kiln at 600ºC for 3 hours. Cooled in a desiccator. Then weighed until a fixed weight is obtained.

$$Ash\ content\ (\%) = \frac{weight\ of\ ash\ (g)}{weight\ of\ sample\ (g)} \times 100\%$$

Total Water Content Test:

The protein hydrolyzate powder was weighed as much as 2 grams into a porcelain cup of known weight. It was dried in the oven at 105oC for 3 hours, cooled in a desiccator, and then weighed until a fixed weight was obtained.

$$Water\ content\ (\%) = \frac{weight\ of\ water\ (g)}{weight\ of\ sample\ (g)} \times 100\%$$

Test Total Fat Content:

As much as 2 grams of protein hydrolysate powder is dissolved in a mixture of 25 mL of 25% HCl and 45 mL of distilled water. The solution was refluxed for 15 minutes. After being filtered hot and washed, the precipitate is dried at a temperature of 105°C. The dry precipitate is then extracted with n-hexane for 2-3 hours. The n-hexane extract is evaporated until dry and weighed.

$$Fat\ content\ (\%) = \frac{weight\ of\ fat\ (g)}{weight\ of\ sample\ (g)} \times 100\%$$

Total Proteint Content Test:

The total protein content of the sample was determined using the Kjeldahl method. A protein sample (1 gram) is digested with concentrated sulfuric acid and a selenium catalyst to convert organic nitrogen into ammonia. The ammonia formed is then released by adding a strong base and distilled into a boric acid solution. The amount of ammonia is determined volumetrically by acid-base titration using standard hydrochloric acid.

$$Nitrogen\ content\ (\%) = \frac{\big(HCl\ (mL) - Blanco(mL)\big) \times N\ of\ HCl \times 14.008}{weight\ of\ sample\ (g)} \times 100\%$$

$$Proteint\ content\ (\%) = Nitrogent\ content\ (\%) \times 6.25$$

2.7.3Antibacterial Activity Test

Preparation of MHA (Muller Hilton Agar) Media:

As much as 9.5 grams of MHA media was dissolved in 250 ml of distilled water and then heated while stirring until homogeneous and boiling. Next, the media was sterilized in an autoclave at 121°C for 15 minutes.

Preparation of Slanted NA (Nutrient Agar) Media and Bacterial Inoculation:

As much as 7 grams of NA were dissolved in 250 mL of distilled water. The mixture is stirred and heated until dissolved, then sterilized in an autoclave at 121°C for 15 minutes. Next, 3 ml of the NA medium is poured into test tubes and allowed to be set at room temperature in an inclined position until solidified. After that, Staphylococcus aureus and Escherichia coli bacteria are suspended in the medium and incubated for 1-2 hours at 35ºC. Next, the turbidity of the solution was measured at a wavelength of 560-600 nm, resulting in a transmittance of 25-28.

Protein Hydrolyzate Antibacterial Activity Test:

Amount of 0.1 millilitres of bacterial suspense *Escherichia coli* and *Staphylococcus aureus* are all placed in a sterilized Petri dish. 15 ml of MHA media is added, and the mixture is homogenized on a flat surface to ensure that the bacterial suspense and media are uniformly homogeneous. The mixture is then allowed to solidify. Next, a well is used to create a hole in the solidified media, and 50 µ/L of each sample concentration—0.50 µ/ml, 0.75 µ/ml, and 1 µ/ml—is added to each cup. During about a day of incubation at 37 C, the diameter of the inhibitory area (DDH) that appeared was measured using a calliper.

2.7.4 Analysis of Degree of Hydrolysis of Nila Tilapia Protein Hydrolyzate

The degree of hydrolysis is calculated based on the percentage ratio of trichloroacetic acid (TCA). 20 ml of fish protein hydrolysate was added with 20 ml of 20% TCA solution (w/v). The mixture was allowed to stand for 30 minutes to enable sedimentation to occur, then centrifuged at a speed of 7800 rpm for 15 minutes. After that, the formed supernatant was analyzed for nitrogen content using the Kjeldahl method. The degree of hydrolysis can be calculated using the following equation.

$$Hydrolysis\ (\%) = \frac{dissolved\ Nitroten\ in\ 20\%\ of\ TCA}{Nitrogent\ Total\ (\%)} \times 100\%$$

2.7.5 Amino Acid Analysis by HPLC

As much as 60 grams of protein hydrolysate powder were added with 4 mL of 6N HCl. Then, the mixture was heated for 24 hours at a temperature of 110°C. After heating, the mixture was cooled to room temperature and then neutralized to pH 7 using a 6N NaOH solution. Next, aquabides were added until the total volume reached 10 ml, and the mixture was filtered using Whatman filter paper sized 0.2 µm. A sample of 50 µl was taken, then mixed with 300 µl of orthophthalaldehyde (OPA) solution and stirred for 5 minutes. Finally, 10 µl of this mixture is injected into the HPLC for analysis.

3 Results and Discussion

3.1 Results of Nila Tilapia Protein Hydrolysate Preparation

In this study, Tilapia protein hydrolysate was produced through an enzymatic hydrolysis process using a combination of bromelain and pepsin enzymes. The tilapia meat was hydrolyzed at a temperature of 55°C for 5-6 hours under pH 7 conditions, with variations in the ratio between bromelain and pepsin enzymes (100:0, 75:25, 50:50, 25:75, and 0:100) at a total enzyme concentration of 5%. After the hydrolysis process, enzyme activation was carried out at a temperature of 90°C for 20 minutes. The hydrolysis results are then filtered and centrifuged to separate the soluble fraction. The obtained soluble fraction is then dried using a freeze dryer until a protein hydrolysate powder is obtained [12]. The yield from this drying process can be seen in Table 2.

Table 2. Yield of drying process

Variation of sample	Results (%)
B100:P0	12.68
B75:P25	12.15

B50:P50	12.52
B25:P75	12.48
B0:P100	12.39

Analysis of the results of tilapia protein hydrolysis shows significant variation. The combination of 100% bromelain enzyme (B100:P0) produced the highest degree of hydrolysis at 12.68%, while the combination of 75% bromelain and 25% pepsin enzyme (B75:P25) yielded the lowest result at 12.15%. These results indicate that the increase in the proportion of bromelain enzyme in the hydrolysis process is directly correlated with the rise in the degree of hydrolysis of tilapia protein as reported [13]. This shows that the bromelain enzyme is more effective in breaking down tilapia protein compared to the pepsin enzyme under these experimental conditions.

3.2 Proximate Analysis Results

3.2.1 Test Results of Ash Content of Tilapia Fish Protein Hydrolysate

Table 3 Ash content test results

Sample variation	**Results (%)**
B100 : P0	2.94
B25 : P75	2.72
B50 : P50	3.92
B25 : P75	3.00
B0 : P100	3.96

The analysis of ash content in tilapia protein hydrolysate shows significant variation, with the highest percentage (3.96%) found in the enzyme combination B0:P100 and the lowest (2.72%) in B75:P25. These results indicate that the increase in pepsin enzyme concentration positively correlates with the increase in ash content in the hydrolysate. However, the ash content values in this study were overall lower compared to the findings of Riyadi et al. (2019) [14]. The addition of acid or base compounds during hydrolysis aims to achieve the optimal pH for the enzyme, which in turn can form salts and increase the ash content of the final product [15].

3.2.2 Test Results of Tilapia Hydrolysate Protein Content

Table 4. Protein content test results

Sample variation	Results (%)
Nila Tilapia	24.93
B100 : P0	57.25
B75 : P25	55.25
B50 : P50	54.93
B25 : P75	54.43
B0 : P100	52.87

Analysis of tilapia protein hydrolysate shows that treatment variations result in a wide range of protein content, with the B100:P0 variation being the highest (57.25%) and B0:P100 being the lowest (50.37%). This value is significantly higher than the protein content of fresh tilapia. The increase in protein content can be explained through the enzymatic hydrolysis mechanism, which breaks down proteins into smaller-sized peptides. The higher the enzyme activity and incubation time, the more peptide bonds are hydrolyzed, thereby increasing the concentration of dissolved protein in the solution. In addition, the dehydration process of the raw materials also contributes to the increase in protein content in the hydrolysate, as it reduces the water content that can dilute the protein solution.

3.2.3 Test Results of Tilapia Protein Hydrolysate Water Content

Table 5 shows a relatively wide range of variation. The highest moisture content was observed in the B0:P100 treatment, with a value of 5.66%, while the lowest was found in the B100:P0 treatment, at 2.65%. These fluctuations in moisture content indicate that the water content in tilapia protein hydrolysate can be

manipulated by adjusting the composition of materials and the parameters of the hydrolysis process. This has important implications in determining the physicochemical properties and stability of the final product, as well as its potential use in various applications.

Table 5. Tilapia Protein Hydrolysate Water Content

Sample variation	Results (%)
B100 : P0	2.65
B75 : P25	3.28
B50 : P50	4.08
B25 : P75	5.53
B0 : P100	5.66

Although the increase in the amount of pepsin enzyme in various enzyme combinations was expected to enhance the water content in tilapia protein hydrolysate, the results of this study showed otherwise. The moisture content obtained was actually lower compared to the previous study by Riyadi et al. (2019) [14]. The B50:P50 variation approaches the SNI 2886:2015 standard, but the B100:P0 and B75:P25 variations are still below the standard. This difference is likely due to the hygroscopic nature of tilapia protein hydrolysate, which tends to absorb water during the drying process but then rerelease it. The results of this study indicate that optimization of the hydrolysis and drying processes needs to be carried out to achieve moisture content that meets the standards.

3.2.4 Test Results of Fat Content of Tilapia Fish Protein Hydrolysate

Table 6. Test results of fat content

Sample variation	Results (%)
B100 : P0	10.25
B75 : P25	8.08
B50 : P50	6.86
B25 : P75	6.02
B0 : P100	5.52

The fat content of the Nile tilapia protein hydrolysate shows significant variation, with the highest percentage (10.25%) recorded in the B100:P0 treatment and the lowest (5.52%) in the B0:P100 treatment. These results indicate a correlation between fat content and the raw material ratio. The lower the water content in the hydrolysis process, the higher the fat content produced. Nevertheless, the fat content of the Nile tilapia protein hydrolysate overall still meets the standards set in SNI 2886:2015. It should be noted that an increase in fat content can affect the shelf life of the product. Therefore, further research is needed to optimize the hydrolysis process to reduce fat content without compromising product quality. These findings are consistent with previous research that shows the hydrolysis process can increase the fat content in products compared to fresh raw materials.

3.3 Hydrolysis Degree Results of Tilapia Protein Hydrolysate

Table 7. Hydrolysis degree results

Sample variation	Results (%)
B100 : P0	61.46
B75 : P25	64.47
B50 : P50	67.00
B25 : P75	68.42
B0 : P100	71.86

The highest degree of hydrolysis (71.86%) was obtained in the B0:P100 variation, while the lowest value (61.46%) was found in the B100:P0 variation. These results indicate that the composition of materials and

the conditions of the hydrolysis process significantly affect the degree of protein breakdown. If compared to previous studies, the degree of hydrolysis obtained in this study is generally higher. The increase in the degree of hydrolysis indicates that more peptide bonds are being hydrolyzed, resulting in a higher amount of free peptides and amino acids. According to Kurniawan's findings (2012), the degree of hydrolysis is influenced by the amount of peptides and free amino acids dissolved in the solution, which is a direct result of protein breakdown by enzymes [16]. Hasnaliza et al. (2010) also support this finding, where the increase in the degree of hydrolysis indicates an increase in the number of free amino groups produced from the cleavage of peptide bonds [17].

3.4 Antibacterial Activity Test of Protein Hydrolysate

Table 8. Antibacterial activity test results

Bacteria	**Sample concentration µg/ml**	**Clear zone (mm)**				
		B100:P0	**B75:P25**	**B50:P50**	**B25:P75**	**B0:P100**
Staphylococcus aureus	0.50	7.4	6.7	6.9	6.5	6.5
	0.75	8.0	7.2	7.2	7.6	7.1
	1	8.3	7.6	7.5	8.1	7.7
Escherichia coli	0.50	7.1	6.8	7.3	6.9	6.3
	0.75	7.8	7.6	7.7	7.9	7.0
	1	8.5	8.3	8.0	8.1	7.6

Protein hydrolysates have proven to have great potential as a source of bioactive peptides with various activities, including antioxidant, antibacterial, and antihypertensive. Research shows that the length of the amino acid chain in antibacterial peptides plays a crucial role in their effectiveness. Peptides with amino acid chains of up to 50 residues generally have a higher killing power against both Gram-positive and Gram-negative bacteria. This antimicrobial mechanism involves the interaction between the peptide and the bacterial cell membrane. Antibacterial peptides often contain specific amino acid residues, such as essential amino acids (lysine, arginine), hydrophobic (alanine, leucine, phenylalanine, isoleucine, valine), and aromatic. (triptofan, tirosin). The side chains and peptide bonds of these amino acids play a synergistic role in disrupting the integrity of the bacterial cell membrane. In addition, the concentration of protein hydrolysate also affects its antibacterial activity. The higher the concentration, the stronger its ability to inhibit the growth or even kill microorganisms. This phenomenon aligns with the basic principle of microbiology, which states that the effectiveness of an antimicrobial agent depends on the dosage.

3.5 Analysis of Amino Acid Composition of Nila Tilapia Protein Hydrolysate Powder

The analysis of the amino acid composition in tilapia protein hydrolysate powder shows the presence of 15 different types of amino acids. Among them, 9 essential amino acids are very important for the human body. Still, they cannot be produced by the body itself, namely histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Additionally, 6 non-essential amino acids were also found such as alanine, arginine, aspartic acid, glutamic acid, glycine, and serine. The complete results of the amino acid composition analysis can be seen in detail in Table 9.

Table 9. Amino Acid composition

Amino acid	Nila Tilapia (%)	B100:P0 (%)
Asparticacid	1.82	5.75
Glutamicacid	2.89	4.04
Serin	0.63	1.25
Histidin	1.67	1.72
Glisin	0.91	1.33
Threonin	0.66	3.75
Arginin	0.76	4.20
Alanin	0.96	2.47
Tyrosin	0.99	2.87

Methionin	0.25	0.86
Valin	2.80	3.10
Phenylalanin	0.93	3.25
Ileusin	0.80	3.11
Leusin	1.35	3.39
Lysin	1.64	5.12

Hydrolysis of tilapia protein using bromelain enzyme produces smaller-sized peptides and free amino acids. This process increases the availability of protein nutrients, especially essential amino acids such as lysine and arginine, which are necessary for growth and tissue repair. The increase in free amino acid levels, along with the extended hydrolysis time, indicates the efficiency of the protein breakdown process. The increase in the polarity of the hydrolyzed protein also implies an increase in its solubility and bioavailability. The amino acid profile produced by hydrolysis is greatly influenced by various factors, including the type of fish, the enzymes used, and the process conditions.

4 Conclusion

This research successfully optimized the production of tilapia protein hydrolysate using a combination of bromelain and pepsin B100 enzymes. The resulting hydrolysate has high nutritional value, characterized by significant protein content and a complete amino acid profile. Additionally, this hydrolysate also has interesting biological activities, such as antibacterial activity. The results of this study show the great potential of tilapia protein hydrolysate as a functional raw material in the development of food and nutraceutical products.

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6. Conflict of Interest

Authors declare no conflicts of interest.

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