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EFFECT OF DIFFERENCES BROMELAIN ENZYME CONSENTRATION ON PROTEIN HYDROLYSATE FROM WASTE OF TILAPIA VISCERA (*Oreochromis sp.*) ON ANTIOXIDANT ACTIVITY

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Abstrak

Ikan nila merupakan salah satu komoditas yang banyak dikonsumsi oleh masyarakat, khususnya di Indonesia. Jeroan ikan nila segar memiliki komposisi kandungan protein sebesar 55,55%, sehingga untuk meningkatkan nilai jualnya sering dijadikan hidrolisat protein. Hidrolisis protein menggunakan enzim merupakan cara yang efektif, karena dapat membuat hidrolisat protein terhindar dari kerusakan asam amino tertentu. Enzim proteolitik memiliki banyak jenis dan salah satu yang dapat digunakan sebagai penghidrolisis, dalam menghasilkan hidrolisat protein adalah enzim bromelin. Tujuan dari penelitian ini adalah untuk mengetahui pengaruh jenis enzim proteolitik dan waktu hidrolisis terhadap aktivitas antioksidan hidrolisat protein dari limbah jeroan Oreochromis sp. Penelitian ini menggunakan RAL 2 faktor dan masing-masing diulang sebanyak 3 kali. Faktor pertama adalah konsentrasi enzim bromelin yang digunakan, yaitu konsentrasi kontrol, 1%, 2% dan 3%. Faktor kedua adalah waktu hidrolisis yaitu 4 jam, 6 jam dan 8 jam. Data yang diperoleh dianalisis dan dilanjutkan dengan uji aktivitas antioksidan, uji derajat hidrolisis dan uji kadar protein. Konsentrasi enzim bromelin terbaik sebagai antioksidan adalah konsentrasi 3% hidrolisis selama 6 jam dengan nilai IC50 adalah 82,53µg/mL, DH terbaik 82,25%±4,03 dan Kadar Protein tertinggi sebesar 54,55%.

Kata Kunci: Antioksidan, enzim bromelin, jeroan ikan nila, hidrolisat protein

Abstract

Tilapia is a commodity that is widely consumed by the public, especially in Indonesia. Fresh tilapia offal has a protein content composition of 55.55%, so to increase its selling value it is often made into protein hydrolysate. Protein hydrolysis using enzymes is an effective way, because it can make protein hydrolysates avoid damage to certain amino acids. Proteolytic enzymes have many types and one that can be used as a hydrolyzer, in producing protein hydrolysates is bromelain enzyme. The purpose of this study was to determine the effect of proteolytic enzyme type and hydrolysis time on antioxidant activity of protein hydrolysate from Oreochromis sp. offal waste. This study used RAL 2 factors and each was repeated 3 times. The first factor was the concentration of bromelain enzyme used, namely control concentration, 1%, 2% and 3%. The second factor was the hydrolysis time, which was 4 hours, 6 hours and 8 hours. The data obtained were analyzed and continued with antioxidant activity test, hydrolysis degree test and protein content test. The best concentration of bromelain enzyme as antioxidant was 3% concentration for 6 hours hydrolysis with IC50 value of 82.53µg/mL, the best DH of 82.25%±4.03 and the highest protein content of 54.55%.

Keywords: Antioxidant, bromelain enzyme, tilapia offal, protein hydrolysate

1. INTRODUCTION

Tilapia is a mainstay aquaculture commodity that is quite widely consumed by the wider community, especially in Indonesia. Apart from being in great demand, tilapia is Indonesia's leading export commodity and is included in the top 10 category among other fishery commodities. BPS 2021 data shows that tilapia exports for the last three years, namely 2018-2020 have increased by 17.13%. Tilapia export volume in 2020 was recorded at 12.29 tons per year with an export value of USD 78.44 million. The part of tilapia that is often used for processing is only fish meat, this causes fish parts such as scales, skin, bones and offal to be considered fishery waste. Raw materials from low-quality fish or waste if not processed optimally will cause environmental, health, and economic problems. Meilisa (2019) revealed that the majority of tilapia by-product waste is not utilized because it is considered to have no selling value compared to fish meat. In addition, tilapia waste is considered by the community to have no nutritional value and complicated handling in its processing.

Various efforts to overcome and minimize the amount of fishery waste are to utilize it optimally, one of which is by processing it as Tilapia offal protein hydrolysate (HPJN). Protein hydrolysates are generally processed through chemical and enzymatic hydrolysis processes. However, enzymatic hydrolysis is more efficient because according to Mawardani (2019) enzymatic hydrolysis is considered more efficient because it can make protein hydrolysates avoid damage to certain amino acids. Enzymatic hydrolysis is the most widely used technology to produce bioactive peptides, due to mild processing conditions, easy to control reactions, and little by-product formation (Cui et al., 2021). One of the protease enzymes that can hydrolyze proteins is bromelain enzyme.

Many studies on tilapia hydrolysate have been conducted on meat and skin using different enzymes and concentrations. However, there is still no research on the effect of different enzymes and hydrolysis time on the antioxidant activity of tilapia offal hydrolysate. Though the potential of offal is quite high as an antioxidant. In the research of Noman et al. (2022) Hybrid Sturgeon fish hydrolysate enzymatically using bromelain showed the highest reducing power in the DPPH test (IC50 3.14 mg/mL). While hydrolysis with bromelain makes it possible to obtain peptides with the highest ability to reduce free radicals. Research on the antioxidant activity of protein hydrolysates in fish and its offal has been widely done, but research on tilapia offal protein hydrolysates that have antioxidant activity does not yet exist so this research needs to be done. This study aims to examine the antioxidant activity and bioactive components of protein hydrolysates from tilapia offal.

2. RESEARCH METHOD

2.1 Research Time and Place

This research conducted in September 2022 - December 2022. The sampling process was carried out at Pabean Market Surabaya, East Java. The hydrolysis process, protein and antioxidant activity test were carried out at the Chemistry Laboratory, Faculty of Fisheries and Marine Sciences.

2.2 Tools and Materials

The main materials used in this study were tilapia offal (Oreochromis sp.) from Bawean Fish Market, Surabaya and bromelain enzyme with 50 USP/gram activity. Other materials used were distilled water, ethanol, trichloroacetic acid (TCA) (Merck), 1,1-diphenyl-1-picrylhydrazyl (DPPH) crystal (Sigma Aldrich), catalyst (K2SO4 anhydrous) (Pudak), H2SO4 (Merck 95%), H2O2 (Merck 30%), methyl red (Rofa), bromo kresol green (Rofa), NaOH (Merck 106498), KHP and HCl.

While the tools used in this study include tools for preparation, basin containers, digital scales, glass arlojo, spatula, aluminum foil, blender, waterbath shaker (Memmert), ice centrifuge (Hettich EBA 200), waterbath shaker (Memmert), filter paper, calico cloth, millipore paper No. 45, oven (Memmert). 45, oven (Memmert), electric stove, furnace, Kjeldahl flask, Soxhlet tube, spectrophotometer (Spectro UV Vis AMV11), incubator (Memmert),

desiccator, vortex, pipette, and other glassware such as test tubes, screw tubes, beaker glass, glass bottles, Erlenmeyer flasks, glass funnels, and measuring flasks.

2.3 Procedures and Data Analysis

The method used in this research is an experimental method to make tilapia offal protein hydrolysate by enzymatic hydrolysis and determine the antioxidant activity of the resulting protein hydrolysate. The results of the study were then tested for characteristics including the degree of hydrolysis, IC50 antioxidant activity and protein content.

Research Procedure

This study used a two-factor completely randomized design (CRD). The first factor was the variation of bromelain enzyme concentration, namely using conventional bromelain enzyme (P1/control), 1% bromelain enzyme concentration (P2), 2% bromelain enzyme concentration (P3) and 3% bromelain enzyme concentration (P4). The second factor is hydrolysis time, namely 4 hours, 6 hours and 8 hours.

Raw Material Preparation

Tilapia offal raw materials were obtained from Pabean Fish Market Surabaya, then washed using running tap water. The criteria for fish offal used as samples were stomach, intestines, liver, gall bladder, pancreas, gonads, spleen, and kidneys. After that, the offal was cleaned until all the dirt was removed and chopped with a blender for 2 minutes, packed in polyethylene bags, frozen, and stored at -18°C until use. The prepared samples were thawed overnight in a refrigerator at 4°C before undergoing the enzymatic hydrolysis procedure.

Preparation of Protein Hydrolysate

Protein hydrolysis was performed according to the method by Noman et al. (2022) with some modifications. A 100 gram sample of tilapia offal was dissolved in distilled water in a ratio of 1:1 (w/v). The dissolved offal was then added with different concentrations of bromelain enzyme: conventional bromelain enzyme (P1/control), 1% concentration of bromelain enzyme (P2) 2% concentration of bromelain enzyme (P3) and 3% concentration of bromelain enzyme (P4) and stirred until homogeneous. Then hydrolysis was carried out using an incubator for 4, 6 and 8 hours at 54oC and continued enzyme inactivation at 90℃ for 15 minutes using pH 6.5 using a waterbath or oven (Ovissipour et al., 2013). The hydrolysis was filtered with a filter and followed by centrifugation for 20 min at 10,000rpm at 4℃. The supernatant was freeze-dried for 48 hours at -55°C under 0.25 mbar vacuum lyophilized the protein hydrolysate obtained using bromelain and stored at -20°C in an airtight container until used for final product analysis.

Determination of Degree of Hydrolysis

The degree of hydrolysis was measured as the ratio of -amino acid nitrogen (AN) content to total nitrogen (TN) content in the tested sample. The formal titration procedure proposed by Taylor [1957] with modifications was applied to determine AN. The homogeneous hydrolysate mixture (1.5 g) obtained from the enzymolysis process was carefully taken, and its weight was increased to 50 g by adding distilled water. The pH of the hydrolysate solution was adjusted to 7.0 using 0.1 N NaOH solution, then 10 mL of 38% (v/v) formaldehyde was added, and the resulting mixture was left at 25±2°C for 5 minutes. Finally, the titration process was continued to pH 8.5 using the same NaOH solution. The equation used to calculate the AN content:

$$\mathrm{AN} = \frac{V \times C \times 14.007}{\mathrm{W} \times 1000} \times 100$$

where: V is the volume of NaOH (mL), C is the concentration of NaOH, and W is the weight of hydrolysate (g).

$$\mathrm{DH}\ (\%) = \frac{\mathrm{AN}}{\mathrm{TN}} \times 100$$

Degree of Hydrolysis was calculated after TN determination using the macro-Kjeldahl procedure [method 955.04; AOAC, 1998].

Protein Content

Determination of protein content using the Kjehdahl method with the process of deconstruction and distillation. A 5 ml sample of fish protein hydrolysate was put into the Kjehdahl flask, then 15 ml of H2SO4 and 3 ml of H2O2 were added using a pipette. The solution was deconstructed for approximately 60 minutes and then cooled for 10-20 minutes. After being allowed to cool transfer it into a 500ml distillation flask, rinse with H2O until the volume is 200ml. In the distillation process a distillator is used with a temperature of 100 0C. The distillate obtained was added with drops of indicator (0.1% methyl red and 0.1% bromo kresol green in ethanol). The distillate was then titrated with 0.2 M HCl until the color turned pink. Furthermore, the titrant volume was recorded and the calculation of nitrogen content was carried out. The calculation was done with the following formula:

Furthermore, protein content was calculated with the following formula:

% protein content = % Kjeldahl nitrogen x conversion factor (6.25) Description:

Antioxidant Activity Test

Antioxidant activity test of fish offal protein hydrolysate was measured based on the method of Wang et al. (2013). A 2 mL sample of tilapia (Oreochromis sp.) offal protein hydrolysate was mixed with 0.5 mL of 0.02% DPPH solution. The mixture was incubated for 30 minutes at room temperature, then the absorbance was measured using UV-VIS spectrophotometer with wavelength of 517 nm. Percent (%) of DPPH radical inhibition was calculated using the equation:

$$\text{DPPH radical scavenging activity \%} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Low absorbance value of sample solution indicates higher DPPH scavenging activity. The absorbance of the sample solution and the absorbance of the blank solution were calculated to determine the inhibition value. Then the inhibition value of each treatment was calculated using the regression equation to obtain the IC50 value. A lower IC50 indicates a higher DPPH radical scavenging activity of the sample (Noman et al., 2022).

3. RESULTS AND DISCUSSION

3.1 Degree of Hydrolysis

The test results of the degree of hydrolysis of Tilapia Offal Protein Hydrolysate produced through hydrolysis using bromelain enzyme with concentrations of 0%, 1%, 2%, and 3% with different observation times of 4 hours, 6 hours and 8 hours can be seen in Figure 1.

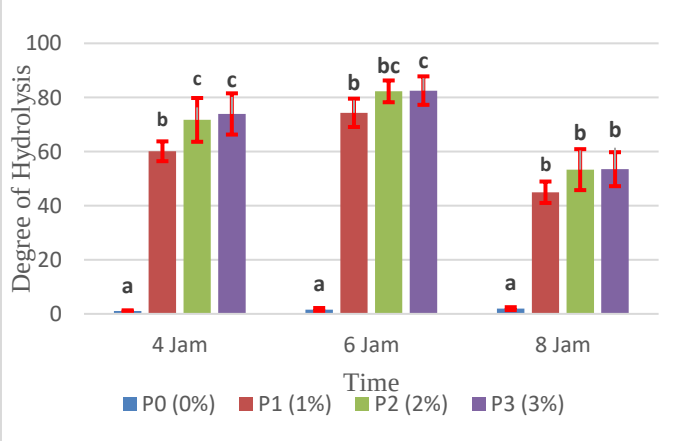

Figure 1. HPJN Hydrolysis Degree Test Results

Higher the concentration of bromelain enzyme affects the increase in the speed of product formation or protein hydrolysate. This is in accordance with the research of Prastyo *et al.* (2020) which states that the value of the degree of hydrolysis in fish protein hydrolysate is influenced by the length of time of hydrolysis and the concentration of enzymes given.

In this study, during the hydrolysis process, there was a phase of rapid increase in reaction when enzyme concentration was added (0%-1%) followed by a phase of steady increase until the end of the observation showing that the enzymatic reaction experienced a drastic decrease/risk phase. This is similar to the research of Ramakrishnan *et. al.* (2013) who observed mackerel waste protein hydrolysate. In the study, there was a

rapid reaction explosion phase followed by a stable phase until the end of the experiment, indicating that the enzymatic reaction followed a zipper mechanism. In the presence of sufficient substrate, the initial rate of reaction increased linearly when the enzyme concentration was increased to 2%, after which the reaction followed a zipper mechanism as dependent on reaction time during the extraction process.

Enzyme with 3% concentration hydrolyzed for 6 hours had the highest value with a degree of hydrolysis value of 82.25%. The DH value was maximum at 3% enzyme. The concentration was also reported for hydrolysis of cricket (*Gryllodes sigillatus*) protein using alcalase, with DH up to 52% (Hall et al., 2017). Likewise, protein hydrolysate from Chinese sturgeon (Acipenser sinensis) had the highest DH value when hydrolyzed using 3% enzyme concentration (Noman et al., 2018). This happens because the interaction of protein substrate using bromelain enzyme has reached its maximum saturation and the hydrolysis process will not run effectively and efficiently when the enzyme concentration is greater than 3% (Firmansyah and Abduh., 2019).

3.2 Protein Content

The test results of protein content of HPJN in each treatment of 0%, 1%, 2% and 3% observation time of 4 hours, 6 hours and 8 hours can be seen in Figure 2. The protein content of HPJN increased along with the addition of enzyme concentration. The relationship between different concentrations of bromelain enzyme and protein content can be seen in Figure 2.

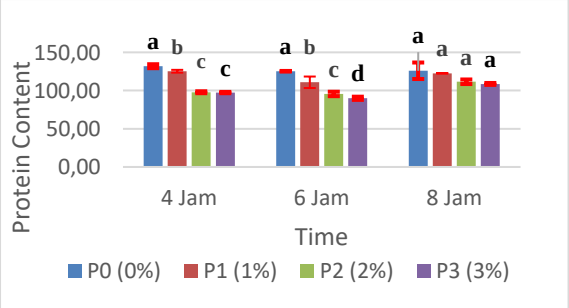

Figure 2 Protein Content Test

Protein levels in the 4-hour treatment at each concentration of bromelain enzyme addition increased (42.56%, 45.25%, 52.68 and 52.24%), while in the 6-hour and 8-hour observations decreased in treatment 2 (2%) to treatment 3 (3%). It is possible that the optimal hydrolysis process occurs at a concentration of 2% and protein denaturation at a concentration of 3%. While in treatment 8 also experienced protein levels because it was possible due to denaturation. This is almost the same as the research of Ramakrishnan et. al. (2013) who examined protein hydrolysates using enzymes from mackerel processing waste with the highest protein content obtained at an enzyme ratio of 2% after 4 hours of hydrolysis. This happens because the interaction of the protein substrate using the bromelain enzyme has reached its maximum saturation, so the protein is not formed completely at a concentration of 3% (Firmansyah and Abduh., 2019).

In this study, the enzyme used was bromelain enzyme to determine the optimal protein content during the hydrolysis process. The protein content of around 37.18-59.31% is lower than previous findings. Ovissipour et al. (2013) examined that protein hydrolysates of tuna fish range from 70-80%. Lyophilized protein hydrolysate from kingfish showed a protein content of about 85.57% (Abdulazeez et al., 2013). This may be due to differences in the type of enzyme used, incubation time and analytical method used for estimation.

The protein content of tilapia offal protein hydrolysate is still better than previous findings, namely the research of Witono et. al. (2014) with protein content of 34-41%. Many researchers have reported the protein content of fish protein hydrolysates ranging from 60% to 90% of the total composition contained therein. The high protein content in fish protein hydrolysate is due to the solubility of the protein during hydrolysis and the removal of insoluble solid materials by the centrifugation process (Chalamaiah et.al., 2012).

3.3 DPPH Test

The results of the antioxidant activity test using the DPPH method showed that HPJN was considered to have antioxidant activity. The percent

inhibition value in the protein hydrolysate of each treatment increased as the concentration of HPJN increased (Appendix 3). The concentration of hydrolysate samples used to determine the percent inhibition against DPPH free radicals were 62.5, 125, 250, 500 and 1000 mg/ml.

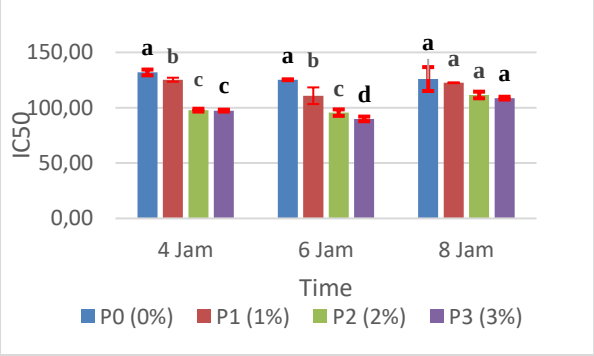

Figure 3 Antioxidant test results of DPPH test

Tilapia offal protein hydrolysate has an IC50 value of between 89.91- 131.88µg/mL while the IC50 value of vitamin C is 8.669 µg/mL. IC50 on vitamin C as a positive control in the study of 8.669 µg/mL is small. In the classification of Molyneux (2004), vitamin C is classified as a very strong antioxidant. While the IC50 of tilapia offal protein hydrolysate when matched in Molyneux's classification is classified as a moderate antioxidant category. A compound is said to be a very strong antioxidant if the IC50 value is less than 0.05 mg/mL (<50 ppm), strong if the IC50 value is 0.05-0.10 mg/mL (50-100 ppm), moderate if the IC50 value is 0.10-0.15 mg/mL (100-150 ppm), and weak if the IC50 value is 0.15-0.20 mg/mL (150-200 ppm) (Molyneux 2004 in Nurjanah et al., 2021).

Tilapia offal protein hydrolysate with different bromelain enzyme treatments and different time treatments showed a fairly good IC50 compared to previous studies. In the research of Luo et al. (2013) showed the IC50 value of shark protein hydrolysate (Sphyrna lewini) of 3,060 ppm. Research by Nurjanah et al. (2021) also states that protein hydrolysates in white snapper offal have very weak antioxidant activity, because they have a value greater than 200 ppm. However, it is still inferior to the research of Mutamimah et.al., (2018) who examined the HPI of Tuna fish eyes with a very good IC50 of 1.08-2.97 ppm.

The low IC50 value of tilapia offal protein hydrolysate is influenced by the bioactive peptide components contained therein. In general, all hydrolysates containing peptides or proteins can donate protons and can react with radical compounds to convert into more stable compounds. A compound is said to have antioxidant activity if when tested it is able to capture free radicals from DPPH. In this study, the DPPH solution which was initially purple then when reacted with tilapia offal protein hydrolysate as anticosidan, the color of the solution turned yellowish purple. This color change indicates that the unpaired electrons on the DPPH free radical have paired (Baehaki et. al., 2015).

The IC50 value obtained in tilapia offal protein hydrolysate using different bromelain enzymes carried out in this study is smaller than the research conducted previously. In this study, the IC50 value was 89.91-131.88µg/mL, while in the research of Mutamimah et.al., (2018) the IC50 obtained was 1.08-2.97 µg/mL. This is likely due to differences in the drying method used. This study used supernatant, while in the study the method used was the freeze-dry method, this method is considered the most effective for increasing antioxidant activity so that the components of active substances in tilapia offal protein hydrolysate are more stable. As a comparison, vitamin C is an exogenous antioxidant that can reduce free radicals so that it can inhibit lipid peroxidation and prevent cell damage (Yimcharoen et al., 2019), has an IC50 value of around 8.67 micrograms/mL, because the protein hydrolysate sample consists of many compounds that are not just antioxidants, while vitamin C consists of pure antioxidants (Witono et. al. (2018).

4 CONCLUSIONS AND SUGGESTION

Enzyme concentration and hydrolysis time have an influence on antioxidant activity. The higher the concentration of bromelain enzyme in the process of hydrolysis of tilapia offal protein, the better the hydrolysate product produced, so that the antioxidant activity is also higher. Meanwhile, the longer the hydrolysis time, the higher the degree of hydrolysis, IC50 and protein content produced. However, in this study, the constant condition was in the hydrolysis treatment of 6 hours because the antioxidant activity decreased in the treatment of 8 hours.

Tilapia offal protein hydrolysate using bromelain enzyme has antioxidant activity. In this study, the best concentration of bromelain enzyme as antioxidant was 3% concentration of hydrolysis for 6 hours with IC50 value of 82.53µg/mL. While the best hydrolysis degree

was found in the ratio of 2% bromelain enzyme with 6 hours hydrolysis time with a hydrolysis degree value of 82.25% ± 4.03. The highest protein content in the 6-hour treatment with 2% bromelain enzyme concentration was 54.55%.

The results of antioxidant activity analysis on tilapia offal protein hydrolysate with different concentrations showed that the best protein hydrolysate had strong antioxidant activity category with IC50 value of 89.91 µg/mL. Based on statistical analysis, the enzyme concentration that has high effectiveness as an antioxidant among 1%, 2% and 3% concentrations is at a concentration of 3% hydrolysis for 6 hours.

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