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Enzymatic Processing of Grouper Bone Waste as Fish Protein Hydrolysate Potential Bioactive Peptides

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ABSTRACT

Grouper fish is a type of reef fish that has high economic value and is widely used as fillets in the industry. It is estimated that 50% of the total fish catch is not used as food. Fish solid waste has been utilized as a source of protein and essential amino acids with high nutritional value over the last decade. Enzymatic hydrolysis is the most recommended method to produce fish protein hydrolysates. Enzymatic hydrolysis with various enzymes and hydrolysis time can determine the characteristics of the hydrolysate obtained. The results of hydrolysis of fish bone powder using variations of Papain, Bromelain, and alcalase enzymes, along with time variations of 30-300 minutes, showed the highest yield with papain enzymes at a hydrolysis time of 240 minutes. The degree of hydrolysis above 88% was achieved at an incubation time of 120 minutes with papain enzyme and alkalase and 3 hours with bromelain enzyme. The proximate content of protein hydrolysates from fish bones showed a moisture content of between 7% and 15%, an ash content below 0.5%, and a protein content of 0.46%. The results of protein hydrolysis molecular weight analysis using SDS-PAGE revealed that each enzyme yielded peptides at sizes of 5 and 3.4 kDa, which are expected to have potential as bioactive peptides.

HIGHLIGHTS

This study highlights the potential of grouper bone waste as a source of bioactive peptides through enzymatic hydrolysis.

1. INTRODUCTION

Indonesia is a major producer of reef fish that is traded as food, including grouper. According to FAO data, before 2016, Indonesia contributed 26.5% of the world's grouper catch. Groupers are caught for export and local consumption and are marketed alive and dead in fresh or frozen form (Amiin et al., 2023; Efendi et al., 2021; Khasanah et al., 2020; Petrova et al., 2018). The grouper fish is a significant export commodity to several countries, including Hong Kong, Japan, Singapore, and China (Mo et al., 2018; Nurhayati et al., 2014; Rumondang et al., 2023; Tirtadanu et al., 2023). In the industry, grouper fish is mainly utilized as fillets, taking only the meat, which results in the rest being discarded as fish waste, around 45-65%. The fish waste is not suitable for consumption or use as food and has not been widely utilized despite its high protein content. Fish waste produced includes muscle components (15-20%), skin and fins (1-3%), bones (9-15%), head (9-12%), viscera (12-18%), and scales around 5% (Coppola et al., 2021; Kumar et al., 2018; Martínez-Alvarez et al., 2015; Palla et al., 2022). Fish waste is typically not marketed due to low consumer acceptance or sanitary regulations that restrict its use, so it is primarily used for ensilage, fertilizer production, or disposal (Ishak

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and Sarbon, 2018). In China, fish and shrimp waste are currently used in conjunction with trash fish, anchovies, and lantern fish (*Benthosema pterotum*) to produce fishmeal (Mo et al., 2018; Raeesi et al., 2023). However, the utilization of fish waste for the production of fishmeal, fertilizer, and fish oil still yields a low level of profit. It can cause environmental pollution, which has a negative impact on society (Nguyen et al., 2022).

Over the last decade, solid waste from fish has gained attention from researchers by potentially utilizing fish by-products as a source of essential amino acids, collagen, gelatin, anti-inflammatory, blood pressure lowering, antidiabetic, antihypertensive, antimicrobial, and enzymes (Ahn et al., 2015; Gao et al., 2021; Henriques et al., 2021; Ishak and Sarbon, 2018; Korkmaz and Tokur, 2022; Zhang and Huang, 2019). Currently, research interest in fish waste is focused on the production of protein hydrolysates that are treated chemically, physically, enzymatically, or in combination. This is because it is more cost-effective and offers solutions that reduce environmental problems (Harnedy and FitzGerald, 2012; Korkmaz and Tokur, 2022). Enzymatic treatment of fish waste has been widely practiced to obtain fish protein hydrolysates (FPH) (Annisa et al., 2017; Araujo et al., 2021; Honrado et al., 2024; Nguyen et al., 2022). Enzymatic hydrolysis is the most recommended method for FPH production in the pharmaceutical and food industries because this method does not leave residual organic solvents or chemical compounds in the resulting product, is more efficient, inexpensive, produces fish protein hydrolysates without loss of essential amino acids, and avoids non-hydrolytic product changes or damage (Siddik et al., 2021; Zhang et al., 2020). Proteolytic enzymes that are endopeptidases break peptide bonds within protein molecules, while exopeptidases hydrolyze peptide bonds from the N or C terminus (Bernadeta et al., 2012; Clemente, 2000). Enzymatic hydrolysis with protease enzymes such as trypsin, chymotrypsin, flavourzyme, alcalase, ficin, papain, and bromelain on fish proteins can produce peptides and amino acids with different molecular weights depending on the degree of enzyme hydrolysis (Gao et al., 2021; Korkmaz and Tokur, 2022; Raksakulthai and Haard, 2003; Udenigwe and Aluko, 2012). Several studies have been conducted to obtain protein hydrolysate from tilapia (*Oreochromis niloticus*) using a combination of bromelain and pepsin enzymes, yielding 12.68% with a degree of hydrolysis of 61.46% (Nasution and Nasution, 2024). Chemical and enzymatic hydrolysis of tuna fish skin, scales, and bones yield 2.6-16.7% of dry matter (Ahmed et al., 2019). The results of research from Kaveh et al. 2024 showed that hydrolysis using alcalase and pancreatin on skipjack fish (*Katsuwonus pelamis*) with a hydrolysis time of 146.9 and 171.67 minutes and enzyme concentrations of 1.94 and 2.17% obtained a degree of hydrolysis of 25.12% and 20.35%, respectively.

2. METHODOLOGY

2.1 Grouper bone pretreatment

Grouper bones obtained from fish collectors in the Sidoarjo area of East Java, Indonesia, were stored at -20°C until needed. The steps taken to obtain fish bone powder involve separating the meat from the bones by boiling. The clean bones are then dried in an oven at 60°C for 24 h. After drying, the bones are ground into powder using a homogenizer.

2.2 Enzymatic production of fish protein hydrolysate (FPH)

Hydrolysis of fish bones was carried out enzymatically using three commercially available protease enzymes: papain, bromelain, and alcalase, according to the method modification by Pires et al. (2024). In an Erlenmeyer flask, 1 g of fish bone powder was added to the enzyme papain, bromelain, or alkaline phosphatase with an activity of 0.7 U. Then, phosphate buffer (pH 7) was added to a volume of 8 mL. The Erlenmeyer flask was closed using aluminum foil and incubated for 3 h at room temperature. Incubation was continued at 40°C with time variations of 30, 60, 120, 180, 240, and 300 min, followed by an additional incubation at 90°C for 5 min. The results of the incubation were filtered, and the filtrate was collected and then dried at 70°C for 24 h to obtain powder FPH.

2.3 Analysis of protein

Protein content was determined using the Bradford method (Walker, 2002). A total of 0.5 mL protein sample was added to 2.5 mL of biuret reagent and vortexed. The solution was incubated at room temperature (25°C) for 10 min. The solution was added 0.25 mL of Folin-Ciocalteu and vortexed again. The solution was incubated at room temperature for 20 min and then measured the absorbance with a UV-Vis Spectrophotometer (Shimadzu-1800) at a wavelength of 595 nm.

2.4 Degree of hydrolysis (DH)

Determination of the DH of FPH was carried out by measuring the soluble protein content of liquid FPH treated with the addition of trichloroacetic acid (TCA) and without the addition of TCA. This method is a modification of the total nitrogen method (Hoyle and Merritt, 1994). The TCA addition treatment was carried out by adding FPH dissolved in distilled water and 6.25% (v/v) TCA in a ratio of 3:2. The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 8,000 × g for 15 min. The supernatant formed was measured for soluble protein by the Bradford method and compared with the protein content without TCA addition treatment. DH value was measured with the equation:

$$\text{DH (\%)} = \frac{\text{TCA}-\text{Soluble N in sample}}{\text{total N in Sample}}$$

2.5 Determination of molecular weight

Molecular weight determination was performed using electrophoresis (Biorad mini protean II) with the Laemmli Method. The initial step of electrophoresis is the preparation of a separating gel with a concentration of 12% made from the following components: 3.35 mL of distilled water, 2.5 mL of Tris-Cl (pH 8.8), 100 µL of SDS (10%), 4 mL of polyacrylamide (30%), 100 µL of ammonium persulfate (10%), and 5 µL of TEMED. Furthermore, a 4% concentration stacking gel was made with a composition of 1.525 mL aquabidesh; 650 µL Tris-Cl pH 6.8; 25 µL SDS 10%; 333.5 µL polyacrylamide 30%; 25 µL ammonium persulfate 10% and 5 µL TEMED. Samples with a volume of 20 µL were added 10 µL of 5x sample buffer (Tris-Cl, 60 mM, pH 6.8; glycerol, 25%; SDS, 2%; 2-mercaptoethanol, 14.4 mM; and bromophenol blue, 0.1%). The denatured mixture was loaded into wells on the stacking gel and placed in a tank containing running buffer at pH 8.3 (Tris-base, glycine, and SDS). Electrophoresis was carried out at a voltage of 150 volts for 50 min. After electrophoresis is complete, the gel is stained with Coomassie blue or silver nitrate.

3. RESULTS AND DISCUSSION

3.1 Preparation of gouper bone powder

In the fish processing industry, demersal fish, such as grouper, are typically processed into fish fillets, which generate a considerable amount of by-products, including heads, scales, bones, skeletons, skin, fins, and viscera. These by-products can reach 45-65% of the total fish weight (Ishak and Sarbon, 2018; Palla et al., 2022). Bone and skeletal by-products, as well as other materials, have the potential to be utilized for FPH production. The FPH consists of relatively short peptides (2-20 amino acids) that can be produced by breaking proteins into peptides either by chemical or enzymatic hydrolysis (Patil et al., 2020). The dried bones were made into coarse powder, as shown in Figure 1(c), and produced as much as 25.3% of the initial grouper bone sample (Figure 1(b)). Bone generally has a lower moisture content compared to skin (Binsi et al., 2009).

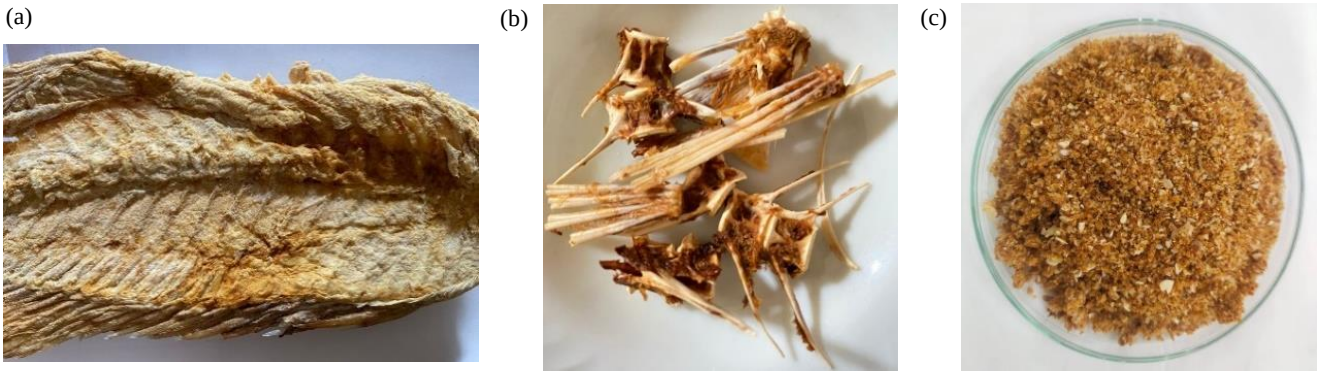

Figure 1. Grouper fish waste in the form of bones (a), boiled bones (b), and bones that have been made into powder (c)

Protein content ranges from 13 to 15 g/100 g based on wet weight, with grouper having higher protein than red snapper (Shakila et al., 2012). The high protein content is an advantage of fish waste that can be utilized in high-value products. With the rapid development of marine fisheries, grouper production is increasing year by year, producing by-products rich in protein, lipids, and minerals. It is a good source for the development of natural active ingredients (Liu et al., 2024). Fish bones, as a by-product, are considered waste and have low economic value. With special treatment, fish bone is a good source of protein and

bio-calcium, which can be leveraged to enhance its potential in food products (Dong et al., 2021). The utilization of fish bones as FPH is one of the alternatives that can be done as a promising solution to overcome the challenge of turning by-products into high-value products. This aligns with SDG 12, which aims to halve waste (Sapatinha et al., 2025). Grouper fish in addition to having a high selling value has been reported to have antiproliferative benefits from giant grouper (*Epinephelus lanceolatus*) egg hydrolysate against oral cancer cells (Yang et al., 2016); grouper bone hydrolysate (GBH) as a potential candidate for applications in health promotion and sports performance enhancement (Kao et al., 2024); enzymatic hydrolysis of fish waste can be used as a viable solution to obtain high value-added products such as collagen (Araujo et al., 2021).

3.2 Enzyme hydrolysis of fish bone grouper with different protease

Fishbone powder was hydrolyzed using three different types of protease enzymes, namely Bromelin, Papain, and Alcalase, with a variation of hydrolysis time of 30-300 min. Based on the results (Table 1), it is evident that the mass of hydrolyzate is proportional to the yield obtained, which increases with different hydrolysis times. In hydrolysis with papain and alcalase enzymes, the highest yield was obtained in 240 min, while the bromelain enzyme gave the highest yield in 30 min. The results of the one-way ANOVA test analysis showed a probability of $p<0.05$, indicating a significant difference in hydrolysis time and the resulting hydrolysate yield. The results of Duncan's further test showed that at an interval of 30 and 240 min, there was a significant difference in the yield of hydrolyzate produced.

Table 1. Effect of enzyme and time variation on the yield produced

Hydrolysis time (minutes)	Papain		Bromelain		Alcalase	
	Hydrolyzate mass (g)	Percent Yield (%)	Hydrolyzate mass (g)	Percent Yield (%)	Hydrolyzate mass (g)	Percent Yield (%)
30	0.914	18.28^{b}	1.978	39.56^{e}	1.675	33.50^{b}
60	0.980	19.60^{c}	0.886	17.72^{d}	1.713	34.26^{c}
120	0.867	17.34^{a}	0.846	16.92^{c}	1.480	29.60^{a}
180	1.061	21.22^{d}	0.842	16.84^{c}	1.488	29.76^{a}
240	2.007	40.14^{e}	0.646	12.92^{b}	1.794	35.88^{d}
300	0.908	18.16^{b}	0.587	11.74^{a}	1.710	34.20^{c}

Numbers followed by the same letter in the same column indicate no significant difference in the 0.05 Duncan test.

Protease enzymes have specific abilities on the cutting side of proteins, allowing for the production of different peptides with varying molecular weights, depending on the level of hydrolysis achieved with the enzyme used (Nguyen et al., 2022). Proteolytic enzymes are classified as endo and exopeptidases. Endopeptidases hydrolyze peptide bonds at specific residues and produce large peptides. The enzyme bromelain hydrolyzes proteins containing peptide bonds into simpler amino acids. In this case, cysteine endopeptidase specifically cuts peptide bonds on carbonyl groups, such as those found in arginine or aromatic amino acids, namely phenylalanine or tyrosine (Masri, 2013). Alcalase enzymes are endopeptidase proteases that belong to the serine protease group, which cleave the internal bonds of peptide chains that contain active sites with phenylalanine, tyrosine, and leucine (Jaziri et al., 2017). Meanwhile, the papain enzyme, which has a sulfhydryl functional group, is capable of hydrolyzing peptide bonds in lysine and glycine amino acids (Nilna et al., 2021).

The results of proximate analysis of protein hydrolysates from each hydrolysis of bromelain, papain, and alcalase enzymes (Table 2). Data on ash content and water content for each enzyme showed significant differences, whereas the protein content yielded nearly identical results. The protein yield in the hydrolysate obtained is minimal compared to several other studies, which is likely due to the suboptimal amount of enzyme used. Haslaniza et al. (2013) stated that the increasing concentration of proteolytic enzymes in the hydrolysis process will cause an increase in soluble nitrogen content in fish protein hydrolysate.

The results of research by Kristinsson and Rasco (2000) showed that FPH from Salmon fish muscle, using alcalase enzymes, obtained 88.39% protein, 0.92% water content, and 8.96% ash content. Another study reported that the results of chemical

hydrolysis of grouper skin waste aimed at obtaining gelatin showed an ash content of 1.32%, a protein content of 79.9691%, and a water content of 4.61% (Azara, 2017). The protein contained in this hydrolysate product is soluble, whereas the undissolved protein is wasted during the separation process. During hydrolysis, insoluble proteins are converted into soluble nitrogen compounds, which then break down into simpler compounds, such as peptides, amino acids, and ammonia. The protein content of catfish protein hydrolysate increased due to an increase in the concentration of enzymes used, resulting in a corresponding increase in soluble nitrogen content (Baehaki et al., 2015).

Table 2. Proximate analysis of fish bone protein hydrolysate

Hydrolysis results with enzyme	Ash content (%)	Moisture content (%)	Protein content (%)
Alcalase	0.295[a]	7.10[a]	0.425[a]
Papain	0.445[b]	15.20[c]	0.430[a]
Bromelain	0.285[a]	9.50[b]	0.460[a]

Numbers followed by the same letter in the same column indicate no significant difference in the 0.05 Duncan test

3.3 Degree of hydrolysis

The degree of hydrolysis (DH) is the level of protein breakdown into short-chain compounds, as measured by the ratio of α-amino nitrogen to total nitrogen (AN/TN), so the higher the level of protein breakdown into short-chain compounds, including α-amino nitrogen compounds, causes the higher the degree of hydrolysis. Conversely, the lower the rate of protein breakdown into short-chain compounds, the lower the degree of hydrolysis (Jaziri et al., 2017). Figure 2 shows that the best results with DH and papain enzyme were obtained at 180 min, while those with bromelain enzyme and alcalase were achieved at 120 min of hydrolysis time. The results of statistical test analysis, with $p<0.05$, showed a significant difference in hydrolysis time for DH with all enzymes. During the hydrolysis process with papain, bromelain, and alcalase enzymes, these enzymes cleave the peptide bonds in the initial protein into smaller protein molecules and peptides with increased solubility (Tacias-Pascacio et al., 2020). Hydrolytic enzymes break peptide bonds of protein substrates through their active sites, which can initiate catalysis through covalent interactions with protein substrates (Nothling et al., 2019).

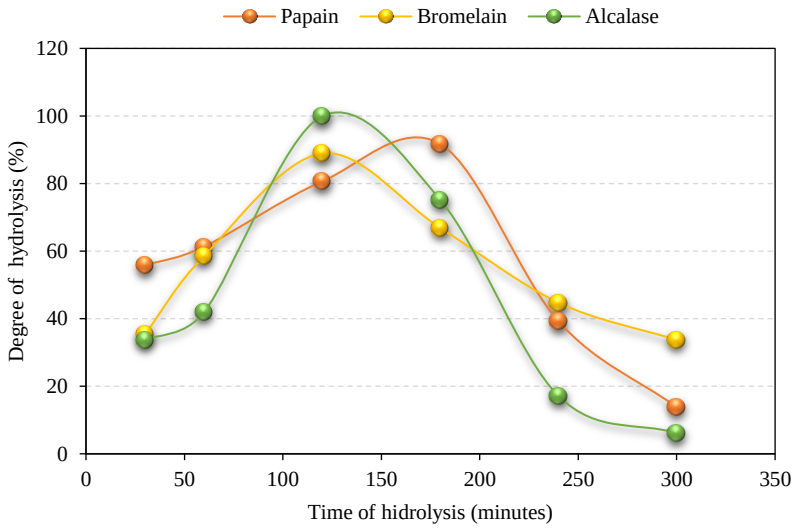

Figure 2. Effect of hydrolysis time with papain, bromelain, and alkalize enzymes on Hydrolysis Degree

The extent of proteolysis is quantified as DH, which refers to the percentage of peptide bonds broken down (Wang et al., 2007). The method used to evaluate the degree of hydrolysis of peptide bonds is based on the amount of nitrogen released by protein hydrolysis in the presence of precipitating agents, such as trichloroacetic acid (TCA) (Haslaniza et al., 2013). The degree of hydrolysis is the percentage (%) of free

amino groups released during the hydrolysis process to the total nitrogen contained in the substrate (Restiani, 2017). The degree of hydrolysis (DH) of protein is determined by several factors, including the type of protease used, enzyme concentration, temperature, pH, and hydrolysis time (Haslaniza et al., 2013; Liaset et al., 2000). Therefore, it is crucial to optimize certain factors to achieve the optimal DH. Enzymes exhibit different of DH when the same feedstock is used, which can be explained by the affinity of the enzyme for the substrate. Enzymes are highly efficient catalysts because they bind substrates (and cofactors) in the active site of a peptide bond that is stereospecifically oriented to the proximity of the group performing the catalytic reaction, forming an enzyme-substrate complex. A study conducted by Silva et al. (2014) using Alcalase showed a 15.5% of DH in fish bones. Hydrolysis of catfish protein with 0.04% bromelain enzyme and incubation time of 2.8 hours resulted in DH, pH, and antioxidant activity of 35.88, 7.07, and 29.86%, respectively, at optimum conditions (Nurdiani et al., 2024). Production of fish protein hydrolysate from chef carp (*Hypophthalmichthys nobilis*) using ficin enzyme at optimum conditions showed that hydrolysate yield increased with increasing DH, and the highest yield was obtained at DH 20.15% (Alahmad et al., 2022). Production of protein hydrolysate from Giant mudskippers using alkaline enzyme at optimum conditions, with a hydrolysis time of 2 hours, resulting in a hydrolysis degree of 67.44% (Edison et al., 2020). Zavareze et al. (2009) used chopped Bluewing searobin (*Prionotus punctatus*) to achieve a 25.41% of DH with Alcalase. Research by Gajanan et al. (2016) showed that the ability of papain to hydrolyze protein from curry fish bone waste was higher than that of bromelain, as indicated by the DH value (Gajanan et al., 2016). Restiani (2017) stated that the time of bromelain hydrolysis of protein hydrolysates influenced the degree of hydrolysis, where 240 minutes yielded the highest DH. The increase in hydrolysis speed at the beginning of the reaction is likely due to the rapid cleavage of peptide bonds, which reaches its maximum speed. The activity of cutting peptide bonds by the bromelain enzyme begins to decrease as the available substrate decreases. The soluble peptides or free amino acids produced from the hydrolysis process can cause inhibition of the active side of the enzyme so that the enzyme is unable to carry out the activity of cutting peptide bonds, causing the hydrolysis rate to reach a stationary phase (Zavareze et al., 2009; Dong et al., 2008; Gajanan et al., 2016; Restiani, 2017; Silva et al., 2014). Some studies report that increasing temperature can improve hydrolysis efficiency by resulting in greater release of amino acids or peptides. This phenomenon could be attributed to the higher ionization constant of water (Pires et al., 2024).

3.4 Protein pattern FPH based on molecular weight by SDS-PAGE

The results of the protein cutting pattern based on molecular weight using SDS-PAGE on each enzyme based on the best degree of hydrolysis, as shown in Figure 3. The results of hydrolysis with the three enzymes showed an almost identical pattern, where peptides were obtained at sizes ranging from around 30 to 10 kDa. Papain and alcalase enzymes were able to show hydrolyzed peptides at 5 and 3.4 kDa. The use of different enzymes can significantly impact the molecular weight (MW) of the peptides and the resulting amino acid sequence. In general, increasing the hydrolysis time can increase the degree of hydrolysis (DH), thereby reducing the molecular weight (MW) of the peptide, which can enhance its biopeptide activity. For example, biopeptides with high antioxidant activity are peptides of lower molecular weight (<3,000 Da), as it is easier for them to donate electrons or hydrogen atoms and react with free radicals to form more stable compounds (Honrado et al., 2024). Some studies have shown a relationship between the molecular weight (MW) size of peptides and their biological activity. The active peptide obtained has ACE (Angiotensin-I Converting Enzyme) activity with a size of less than 1,000 Da (Ngo et al., 2014). FPH from Blue Whiting (*Micromesistius poutassou*) fish waste with lower Mw resulted in outstanding production yield (12% w/b substrate), most excellent protein content (77% w/b), most excellent in vitro digestibility (>95%), highest presence of essential amino acids (43%), best antioxidant (DPPH=62%) and antihypertensive properties (IC_{50}-ACE=80 mg/L) (Vàzquez et al., 2024). Protein hydrolysates from fish waste, enzymatically broken down with alkaline-producing low-molecular-weight peptides (<13.7 kDa), can exhibit antioxidant and ACE inhibitor activities (Borges et al., 2023). Molecular weight is one of the important characteristics of FPH that needs to be considered, as it provides information on the expected physical and bioactive properties of the product. More than 50% of the molecules in protein hydrolysates

from sardine have a molecular weight of less than 10 kDa (Chiodza and Goosen, 2024). Another research attempted to improve the functional peptides of mackerel (*Scomber scombrus*) protein hydrolysates by reducing the particle size of the peptides via ultrasonication and obtained small size (<200 Da) and small peptides (500-2,000 Da) in FPH samples (Cropotova et al., 2024).

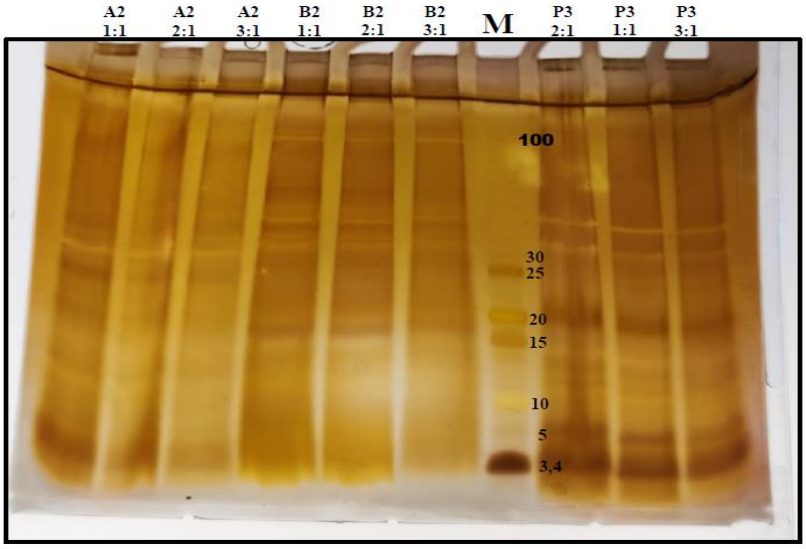

Figure 3. Electrophoresis results of protein hydrolysates at the best degree of hydrolysis using alcalase (A), bromelain (B) and papain (P) enzymes with various sample dilutions of 1:1 to 1:3; M is a protein marker of size 3.4-100 kDa.

4. CONCLUSION

Based on the results of hydrolysis using various types of enzymes in grouper bones, the best hydrolysis degree value was achieved at a 2-hour incubation time using papain and alcalase and at 3 hours with bromelain, with a hydrolysis degree above 88%. The protein pattern of electrophoresis results from papain enzymes that can produce sizes below 5.4 kDa and are expected to have activity as a biopeptide compound. The proximate content of protein hydrolysates from fish bones showed water content between 7-15%, ash content below 0.5%, and the yield of protein hydrolysate from each enzyme hydrolysis was 36-40%.

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AUTHOR CONTRIBUTIONS

Experimental run and Data Collection, Intan Fatma Listiandari, Browi Nugroho, Nur Indah Syamsiati, Mohammad Dhimas Adiputra, Ireniza Liano; Methodology, Validation, Supervision and Writing Original Draft Preparation, Nuniek Herdyastuti, Rudiana Agustini, Titik Taufikurohmah; Formal Analysis; Data Curation, Visualization, Writing-Review and Editing, Nuniek Herdyastuti, Tukiran, Tan Wen Nee.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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