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Valorization of Keting fish (*Mystus nigriceps*) viscera using papain for antioxidant protein hydrolysate production

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Abstract. Underutilized viscera from keting fish (*Mystus nigriceps*) processing in Kenjeran, Surabaya offer opportunities for resource recovery and pollution prevention. This study aimed to optimize the hydrolysis conditions of keting viscera using papain and to evaluate its potential as a source of antioxidant protein hydrolysate. The optimal hydrolysis conditions were determined based on the most effective scavenging activity of 2,2-diphenyl-1-picrylhydrazil (DPPH) radical. Viscera were hydrolyzed under various conditions, including four enzyme concentration levels (0, 1, 3, and 5%) and four hydrolysis durations (1, 2, 4, and 6 hours). Enzyme concentration, hydrolysis duration, and their interaction showed a significant influence on antioxidant activity ($p < 0.05$). Hydrolysate was produced under optimal conditions with a 5% papain solution applied for six hours. This resulted in a product with strong antioxidant properties, as measured by its IC_{50} value of 1.22 mg/mL against DPPH radicals. The proximate analysis of the hydrolysate showed the protein content was 30.04% and distributed its molecular weight in the range of 68 – 134 kDa and ≤ 10 kDa. This research demonstrates the feasibility of valorizing fish processing waste into a valuable product with prospective uses in the functional food, nutraceutical and pharmaceutical fields.

1. Introduction

Fish processing produces considerable quantity of by-products, including bones, viscera, skin, scale, and heads, which are mostly discarded [1, 2]. Improper disposal of such processing by-products might cause serious environmental issues due to their high organic load, creating favorable conditions for disease outbreaks. The organic matter present in fish waste contributes to the eutrophication of water bodies, thus depleting the oxygen levels in water and harming aquatic ecosystems [3].

The repurposing of underutilized fish by-products supports the principles of a circular economy, offering a sustainable pathway to high-value products and economic benefits. The valorization of fish by-products into bioactive peptides provides a sustainable route to high-value products [4]. Hydrolysis using enzyme is an green technology for producing transforming these by-products into value-added products, for example, fish protein hydrolysate (FPH), which possess valuable bioactivities, including antioxidant properties [5, 6]. FPH have attracted substantial interest recently given its prospective uses in food, pharmaceutical, and cosmetic industries. FPH is known to be rich in antioxidants, which can help in combating oxidative stress, a major cause of many chronic diseases [6, 7]. However, FPH production technology needs to focus on the development of the hydrolysis process to achieve higher yields, higher degree of hydrolysis and bioactivity. A number of investigations have focussed on the FPH production from diverse fish species using different enzymes. The hydrolysis of *Acipenser sinensis* using 3% papain for six hours and 3.5% alcalase for six hours [8], *Channa striata* using 3% papain for three hours [9], and Indian mackerel using 1% papain for six hours [10] resulted in FPH with antioxidant activity. Based on these studies, different fish species and enzymes have varying optimal hydrolysis durations and enzyme concentrations.

Keting fish (*Mystus nigriceps*) is a commonly consumed fish species in Surabaya, Indonesia. A popular processing method, smoking, generates significant amounts of fish processing by-products, including viscera. This study focused on optimizing the enzymatic hydrolysis of keting fish viscera with papain to produce high-quality FPH with high antioxidant activity. By investigating the impact of hydrolysis duration and papain concentration on FPH yield and antioxidant activity, this study contributes to the sustainable utilization of underutilized fish by-products and explores avenues for developing novel health-promoting food elements.

2. Materials and Methods

2.1 Viscera collection and processing
The viscera of *M. nigriceps* were obtained from a smoked fish producer in Kenjeran, Surabaya. The viscera were stored in an icebox to maintain freshness during transportation. The viscera underwent cleaning and homogenization in sterile distilled water (DW; 1:1, w/v). The homogenized viscera were centrifuged to obtain pellet from supernatant. The viscera pellet was stored in a freezer prior to further experiments.

2.2 Papain-assisted hydrolysis of viscera
The hydrolysis conditions were optimized by varying the papain concentration (0, 1, 3, and 5%) and hydrolysis duration (1, 2, 4, and 6 hours). Hydrolysis was carried out at 60 °C in an incubator with a stirring speed of 225 rpm. The hydrolysate was centrifuged, and the supernatant was collected. The enzymatic activity was terminated by inactivating the papain in a waterbath at 95 °C for 15 minutes.

2.3 Evaluation of antioxidant by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity
The hydrolysate's antioxidant activity was evaluated using the DPPH radical scavenging assay [11, 12]. Hydrolysate diluted to 5% in DW and mixed with DPPH reagent in a 1:1 ratio (v/v) in a microplate (96-well). The microplate covered with foil and maintained at room temperature for 30 min, followed by measurement of the absorbance at 517 nm (A) using an ELISA reader. The antioxidant activity was calculated according to the equation below:

$$\text{DPPH radical-scavenging activity (\%)} = \frac{(A_{control} - A_{blank\ 1}) - (A_{sample} - A_{blank\ 2})}{(A_{control} - A_{blank\ 1})} \times 100$$

$Blank_1$ is DW as a control blank; and $blank_2$ is sample without DPPH (replaced by methanol) as a sample blank. The hydrolysate concentration required to achieve 50% inhibition (IC_{50}) of the DPPH radical was determined by plotting the concentration (X-axis) against the percentage of inhibition (Y-axis). The hydrolysate used in IC_{50} measurements was oven-dried at 50°C for 48 hours, powdered, and dissolved in DW to obtain various concentrations (0.1-2.0 mg/mL).

2.4 The measurement of hydrolysate proximate composition

Moisture, ash, lipid, protein, and carbohydrate contents were measured as part of the proximate composition. Moisture content was measured gravimetrically after oven-dried at 100 °C. Ash content was measured gravimetrically after being processed at 550 °C in a furnace [13]. Lipid content was measured gravimetrically following extraction with a 2:1 ratio (v/v) of methanol and chloroform and subsequent solvent evaporation [14]. Protein content was analyzed using the Kjeldahl method after digestion with sulfuric acid at high temperature [15] Carbohydrate content was determined using the by-difference method by subtracting the moisture, ash, lipid, and protein analysis results from 100% [16].

2.5 Determination of Hydrolysate Molecular Weight using SDS-PAGE

The hydrolysate's molecular weight distribution was examined with SDS-PAGE [11]. Hydrolysate was dissolved in SDS-PAGE buffer and heated to denature the proteins. The hydrolysate sample was then loaded onto a gel and separated by electrophoresis. The protein bands were treated for an hour with Brilliant Blue R-250 and then underwent a destaining process for three hours. Protein bands were visualized with blue light transilluminator and compared with the protein molecular weight marker to determine their molecular weights.

2.6 Statistical analysis

Statistical evaluations were performed to analyze the data for DPPH radical-scavenging activity and hydrolysis yield. Normal data distribution was checked using the Shapiro-Wilk test, and homogeneity of variance was checked using Levene's test. Data conforming to these assumptions were analyzed using a two-way ANOVA with a significance level set at $\alpha = 0.05$ to evaluate the effects of enzyme concentration, hydrolysis duration, and their interaction on DPPH radical scavenging activity. Tukey's honestly significant difference test was used for post hoc comparisons. The proximate composition was analyzed using descriptive statistics to calculate the mean from three replicates.

3. Results and discussion

3.1 Antioxidant activity of papain hydrolyzed-fish viscera

This study aimed to evaluate the best conditions for producing fish viscera protein hydrolysates with enhanced DPPH radical scavenging activity. Hydrolysates generated under various hydrolysis conditions exhibited differing levels of antioxidant activity, as presented in Table 1. Antioxidant activity showed significant differences with increasing enzyme concentration and hydrolysis duration ($p < 0.05$). Tukey's post hoc test ($p < 0.05$) indicated that hydrolysis of viscera with 5% papain for 6 hours resulted in the highest antioxidant activity, reaching 69.57 ± 0.30%. The rise in antioxidant activity over the hydrolysis duration is attributed to the generation of antioxidant peptides. Enzymatic hydrolysis cleaved the proteins in keting viscera into smaller peptides, resulting in lower molecular weights. Peptides with lower molecular weights are recognized for their enhanced antioxidant effectiveness [17]. However, prolonged hydrolysis can reduce activity by breaking bioactive peptides into non-bioactive fragments [18], highlighting the importance of optimizing hydrolysis duration. The enhanced activity is also linked to the production of hydrophobic peptides during hydrolysis [19]. Ma et al. [20] reported that bioactive peptides from tilapia skin contain hydrophobic amino acids proline, alanine, and glycine that showed scavenging interactions with the free radicals. Higher enzyme concentrations increase the yield of bioactive peptides [21]. The hydrolysate produced under optimal conditions in this study showed a DPPH radical-scavenging activity of 73.80% at a concentration of 2 mg/mL. This value aligns to those reported by Jemil et al. [22] for stingray hydrolysate produced using *Bacillus subtilis* enzymes (75.00% at 6 mg/mL), and by Ktari et al. [23] for zebrafish hydrolysates produced using fish protease (76.56% at 6 mg/mL).

Table 1. The effect of various papain concentration (%) and hydrolysis duration (h) on the antioxidant activity (% DPPH radical scavenging) of the hydrolysate.

Papain concentration (%)	Hydrolysis duration (h)			
	1	2	4	6
0	47.13 ± 0.51[ab]	44.69 ± 0.13[a]	48.85 ± 2.18[ab]	50.94 ± 0.26[abc]
1	45.61 ± 1.79[a]	46.53 ± 1.81[ab]	51.64 ± 0.26[abc]	53.74 ± 0.39[bcd]
3	51.29 ± 3.39[abc]	53.31 ± 3.20[bcd]	60.19 ± 0.70[de]	60.88 ± 1.39[def]
5	57.68 ± 3.65[cde]	58.87 ± 2.53[cde]	65.93 ± 2.24[ef]	69.57 ± 0.30[f]

The DPPH radical scavenging activity values provided (mean ± standard deviation) were determined using a 5% diluted liquid hydrolysate. Tukey's HSD test reveals significant differences ($p < 0.05$) within the combinations of papain concentration and hydrolysis duration, as shown by different letters (a to f) in the columns.

The optimal hydrolysate, produced using 5% papain for 6 hours, exhibited a lower IC_{50} value (1.22 mg/mL; Figure 1) compared to scalloped hammerhead muscle hydrolysates (3.06 mg/mL) reported by Luo et al. [24], but higher than those smooth hound muscle (0.60 mg/mL) and stone fish tissue (0.49 mg/mL) reported by Bougatef et al. [25] and Bordbar et al. [26], respectively. The lower IC_{50} values indicating greater efficacy [27]. These findings suggest that *M. nigriceps* viscera hydrolysates possess promising antioxidant potential. Variations in fish species, tissue, enzyme type, concentration, and hydrolysis duration can influence antioxidant activity. Different fish species have varying protein and amino acid compositions, leading to diverse bioactive peptide profiles. The bioactivity of hydrolysates is partly determined by the specificity of the enzyme used [28].

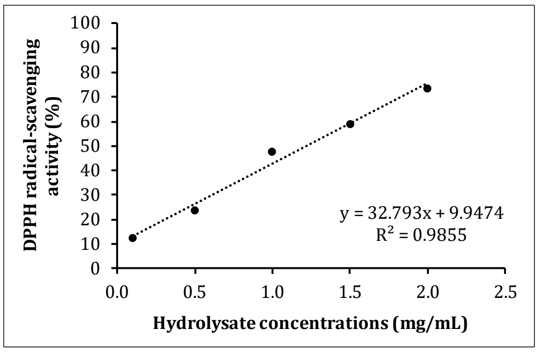

Figure 1. The DPPH radical-scavenging activity (%) of keting fish protein hydrolysate was evaluated at various concentrations (mg/mL). To determine the IC_{50} value, the concentration required to inhibit 50% of DPPH radicals, linear regression analysis was employed.

3.2 Yield of the papain hydrolyzed-fish viscera

Hydrolysate yield is the percentage of dry product relative to initial substrate, reflects hydrolysis efficiency. High yield values signify an efficient conversion of keting visceral protein into peptides by papain. However, the enzyme concentration significantly influenced yield ($p < 0.05$), while hydrolysis duration did not. Enzyme concentration affects hydrolysate yield because more enzyme means more active sites available to cleave proteins, thus increasing hydrolysis efficiency [29]. At the optimal conditions (5% papain, 6 hours), one kilogram of *M. nigriceps* viscera yielded approximately 97.1 grams of dried hydrolysate (9.71%; Table 2). This compares favorably to previous studies [30, 31], suggesting that hydrolysate yield varies with enzyme type, concentration, and fish part.

Table 2. The effect of various papain concentration (%) and hydrolysis duration (h) on the yield (%) of the hydrolysate.

Papain concentration (%)	Hydrolysis duration (h)			
	1	2	4	6
0	6.91 ± 0.66	7.02 ± 0.08	6.31 ± 0.14	5.22 ± 0.08
1	8.07 ± 0.54	7.58 ± 0.46	6.72 ± 0.19	5.28 ± 0.21
3	12.02 ± 0.12	11.16 ± 0.54	9.38 ± 0.74	7.68 ± 0.17
5	15.36 ± 0.21	14.09 ± 0.42	12.27 ± 0.69	9.71 ± 0.34

3.3 Proximate composition of the raw fish viscera and papain hydrolyzed-fish viscera

Proximate analysis of viscera hydrolysate from *Mystus nigriceps* revealed a protein content of 30.04%, lipid content of 0.78%, and moisture content of 22.14% (Table 3). The high protein content and antioxidant bioactivity of this hydrolysate suggest its potential applications in various fields, including human food, dietary supplements, and animal feed [32 – 34]. The relatively high moisture content indicates a need for further drying to enhance product stability [35]. Comparison with previous studies [33, 36 – 38] showed that the composition of hydrolysates can vary depending on the type of fish and processing methods. The FPH produced in this study exhibited a lower lipid content compared to the FPH from snapper fish waste meat hydrolysate (4.05%) reported by Prayudi et al. [39]. This difference could be explained by the higher lipid content in the raw material used in this study. The centrifugation process is also effectively reduced the lipid content, minimizing the risk of lipid oxidation in the hydrolysate [40].

Table 3. The proximate composition of the raw viscera, liquid and dried hydrolysates.

Proximate composition	Content (%)		
	Raw viscera	Liquid hydrolysate	Dried hydrolysate
Protein	7.60 ± 1.91	2.63 ± 0.16	30.04 ± 0.72
Lipid	0.19 ± 0.02	0.05 ± 0.03	0.78 ± 0.03
Ash	3.00 ± 0.01	0.18 ± 0.01	2.81 ± 0.07
Water	83.93 ± 0.01	93.75 ± 0.19	22.14 ± 0.53
Carbohydrate	5.27 ± 1.88	3.39 ± 0.00	44.24 ± 0.09

The proximate composition of the hydrolysate under optimal hydrolysis conditions (5% papain and hydrolysis for 6 hours). The dried hydrolysate is obtained by drying the liquid hydrolysate in an oven at 50°C for 48 hours.

3.4 The molecular weight distribution of the papain hydrolyzed-fish viscera

SDS-PAGE results revealed that papain successfully hydrolyzed *M. nigriceps* viscera proteins into smaller peptides over time (Figure 2). During the hydrolysis process, enzymatic cleavage of the protein can be observed. This is evidenced by the faint bands appearing on SDS-PAGE, which indicate the fragments resulting from enzymatic action [41]. Protein bands with high molecular weights (approximately 43 - 130 kDa) became thinner, while bands with low molecular weights (less than 10 kDa) became thicker. This indicates an increase in the amount of small peptides due to papain enzyme activity. These findings are consistent with previous studies [42 – 44] reporting that protein hydrolysates generally contain small peptides. Small peptides have the potential to exhibit high biological activities, such as antioxidant effects, due to their easy interaction with free radicals. Hydrolysis produces lower molecular weight peptides that are enriched in hydrophobic amino acids. These peptides can interact with free radicals via hydrogen bonding and hydrophobic interactions [20]. Subsequent study should focus on the purification and sequencing of antioxidant peptides to improve their efficacy and safety, and to gain a better understanding of their functional mechanisms.

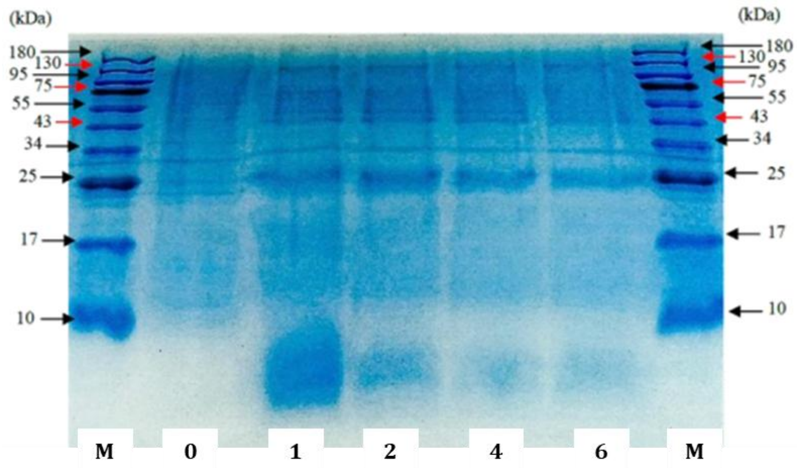

Figure 2. The effect of 5% papain hydrolysis on the molecular weight of *M. nigriceps* viscera. Molecular weights were observed using SDS-PAGE at 0, 1, 2, 4, and 6 hours of hydrolysis at 60°C. M represents the standard marker with a known molecular weight (kDa).

4. Conclusion

This study demonstrates that enzymatic hydrolysis of keting fish viscera using 5% papain for 6 hours is the optimal condition to produce hydrolysate with high antioxidant activity. The resulting hydrolysate is rich in low molecular weight proteins, which could be a valuable source of bioactive peptides. This indicates the great potential of hydrolysates as functional ingredients in various products, such as food, pharmaceuticals, and cosmetics. However, further investigation is necessary to define the active peptides and ensure the safety and stability of the hydrolysate before commercial application. These findings open new opportunities for the conversion of fishery waste into economically valuable products with health benefits.

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