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In Vitro Antioxidant Activity Dpph Assay of Protein Hydrolysate Mackerel (Scomber Scombrus) Viscera

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ABSTRACT

Based on exports data mackerel (*Scomber sombrus*) in 2023 approximately 2,702 tons, while by-products had produced around 60%. This creates serious problems with its disposal. Fish by-products can be processed into value-added products, one of which is fish protein hydrolysates. The purpose of the study was to determine the molecular weight of the SDS-PAGE (*Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis*) method and the antioxidant activity DPPH assay (2,2-diphenyl-1-picrylhydrazil) hydrolysate of mackerel viscera protein. The results showed a molecular weight in the range of 12 – 16 kDa. Antioxidant activity was shown by an inhibition percentage value of 50.117 ± 0.4793 % with an antioxidant capacity of 0.9141 ± 0.0606 mmol TE/g of Trolox equivalent samples. Based on antioxidant activity, mackerel viscera protein hydrolysate has the potential to be a natural source of antioxidants.

INTRODUCTION

Atlantic mackerel (*Scomber scombrus*), a pelagic and migratory fish, is abundant in the north Atlantic and in the Mediteranean seas (Ennaas *et al*., 2015). Marine capture production of Atlantic mackerel (*Scomber scombrus)* in 2020 1,049,000 tons, live weight (FAO, 2022). According to statistical data from the Ministry of Maritime Affairs and Fisheries 2023, mackerel imports in Indonesia are 122,971 tons, with an export volume of 2,702 tons.

The fish processing industry produces more than 60% of the by-products as waste such as heads, skins, trimmings, fins, frames, visceras and roes, only about 40% fish products for human consumption. These large quantities of fish by-product waste fisheries would create serious problems on disposal and pollution in both

developed and developing countries. By-product waste contains protein-rich ingredients that are generally marketed in low-cost markets, such as animal feed, fishmeal and fertilizer (Chalamaiah *et al*, 2012).

In view of protein-rich fish processing waste, biotechnology was developed to obtain nutritional and physiological peptides by enzymatic hydrolysis of fish proteins that produce protein hydrolysate production from processed fish waste and become a solution to the problem of disposal and pollution (Chalamaiah *et al*., 2012). Several proteolytic enzymes are commonly used to hydrolyze fish protein to produce fish protein hydrolysate products including alcalase, papain, pepsin, trypsin, α-chymotrypsin, pancreatin, flavorzyme, pronase, neutrase, protamex, bromelain, cryotin F, protease N, protease A, orientase, thermolysin and validase (Halim *et al*., 2016).

According to Halim *et al*. (2016), the source of fish protein hydrolysate comes from three main components of fish, namely muscle (meat); skin; and waste (head, *trimming*s, fins, frames, viscera and roe). Villamil *et al.* (2017), reviewing the potential of fish waste, especially viscera as a source to obtain protein and hydrolysate. The ability of hydrolysates from several sources of fish protein as antioxidants, antihypertensives, and antimicrobials has been reported (Ryan *et al*., 2011), such as antioxidant peptides from the black pomfret (*Parastromateus niger*) viscera (Ganesh *et al*, 2011), anti-hypertensive peptides from Sardines and Tuna heads and viscera (Martinez-Alvarez *et al*., 2016), antibacterial hydrolysate from Smooth Hound viscera (*Mustelus canis*) (Abdelhedi *et al*., 2016).

Oxidation is a vital process in all living organisms even though it has the side effect of producing free radicals (Di Bernardini *et al*., 2011). The resulting radicals are highly unstable and can cause to cell or tissue injury (Cheung *et al*., 2015). Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates, and many studies focus on finding natural antioxidants from marine fish by-products (Wang *et al*., 2018). Souissi *et al.* (2007) evaluated hydrolysates obtained from heads and viscera of sardines (*Sardinella aurita*) in terms of scavenging effect on DPPH free radicals and inhibition of linoleic acid autoxidation, more than 50% inhibition of linoleic acid peroxidation and an antioxidant activity of about 40%. Nurdiani *et al.* (2022), has carried out the characteristics of fish protein hydrolysate from the *Scombridae family*, mackerel (*Scomber japonicus*).

Based on the above background, this study aims to determine molecular weight and antioxidant activity of DPPH assay hydrolysate protein from mackerel (*Scomber scombrus*) viscera.

METHOD

Sample Preparation

By-products (viscera) sample were obtained from fish processing company that produces mackerel fillets in Gresik district, East Java. Viscera were freezing with Air Blast Freezer at -35˚C, further stored in Cold Storage temperature -20±2° C.

Hydrolysis Process

The hydrolysis followed procedure by Ramakrishnan *et al*. (2013) with modification, as many as 500 grams of minced mackerel viscera mixed with aqueous in a ratio of 1:1 (w/v), then added Alcalase® enzyme (E.C 3.4.21.14; Millipore, Germany) concentration 0.5 % (by weight of raw material) and pH 7.0. The hydrolysis time were 240 minutes with a stirring speed of 140 rpm and incubated at 55° C. Inactivation of enzymes at 90° C for 10 minutes using a water bath. The hydrolysis results are allowed to cool and centrifuge (Rotanta 460 R; Hettich; Germany) at 4,100 rpm for 40 minutes at 4° C to separate the insoluble suspended material and fat from the hydrolysate.

Molecular Weight Analysis (SDS-PAGE)

Fish protein hydrolysate molecular weight was determined by sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE), based on the Laemmli (1970) method. SDS-PAGE analysis uses 15% separating gel and 5% stacking gel. Premix 20 µL protein hydrolysate sample with 5 L loading buffer was inserted in a microcentrifuge tube (tube) and heated at 95°C for 5 minutes then centrifuged at 13,000 rpm for 1 minute. 10 µL of protein sample is put in SDS gel and electrophoresis is run in two steps; (1) 30 minutes at 60V; (2) 90 minutes at 140 V. Incubate the gel by whisking slowly for 1 hour in the fume hood. The dye solution drained and the gel rinsed with distilled water. The destaining solution poured to soak the gel and incubated for 30 minutes with slow shaking.

Antioxidant Activity of DPPH

Testing of DPPH antioxidant activity using a microplate reader. Preparations are carried out in accordance with Donkor *et al.* (2012) with modifications. A sample of 0.1 mL was added to 0.2 mL of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) solution with a concentration of 0.02 mM at 95% methanol, followed by dark incubation at room temperature for 60 minutes. The solution was measured using an ultraviolet-visible spectrophotometer at λ 517 nm. The absorbance data obtained was calculated as the percentage (%) of antioxidant activity or percentage (%) inhibition. The value is obtained by the following equation:

$$\%inhibition = \frac{(blank\ absorbance) - (sample\ absorbance)}{blank\ absorbance} x100\%$$

Trolox standard curves starting from 0.78125, 1.5625, 3.125, 6.25 and 12.5 µM are used as standards and antioxidant capacities are expressed in Trolox Equivalent (TE) mmol per gram sample.

Antioxidant capacity

The calculation of antioxidant capacity followed Apak *et al*., (2006) method, the weight of material used 0.5 grams and prepared in a volume of 5 ml as an initial volume. The volume of the solution sample was 0.1 ml and the total final volume of the solution (color change after the addition of reagents) in the DPPH method was 0.2 ml. Trolox Equivalent Capacity Equation:

Antioxidant Capacity (mmol TR/g) = $(A_f/\varepsilon_{TR}) \times (V_f/V_s) \times r\ (V_{cup}/m)$

Where :

m : mass of the sample in grams

V_s : volume of sample added for analysis in mL

r : dilution ratio

Abs : absorbance

V_f : colour development (after the addition of reagents) was made in a final volume

V_{cup} : initial volume

Data analysis

The data analysis in the testing of antioxidant activity and antioxidant capacity was presented in an average ± standard deviation with 3 tests and 2 replicated (duplo). Rigor control of antioxidant capacity analysis using *Relative Percent Difference* (RPD) 5%. According to Habibi (2019), the difference in relative percent (*Relative Percent Difference*/RPD) is used in duplo analysis with a frequency of 5% - 10% per batch or at least 1 time for the number of test samples less than 10 as a control for the rigor of the analysis. The percentage of RPD:

$$\%RPD = \frac{sample\ result - duplicate\ result}{[sample\ result + duplicate\ result]/2} x100\%$$

RESULT AND DISCUSSION

Molecular Weight Analysis (SDS-PAGE)

Results of molecular weight hydrolysate protein mackerel offal (*Scomber scombrus*) presented on **Figure 1.** The results of the measurement of 2 wells showed a molecular weight in the range of 12 kDa to 16 kDa.

This shows that protein hydrolysis using the enzyme Alcalase® can break the bonds of protein peptides into peptides and amino acids with low molecular weight. The molecular weight of protein hydrolysate mackerel (*Scomber scombrus*) viscera is higher than mackerel head protein hydrolysate (*Scomber japonicus*) which is in the range of 10.85 kDa to 90.24 kDa (Nurdiani *et al*., 2022), while the molecular weight of parrotfish head (*Chlorurus sordidus*) protein hydrolysate in the range of 18.05 kDa to 75.89 kDa (Priharto *et al*.. 2019). Low molecular weight indicates that the hydrolysis process works effectively because it breaks peptide bonds (myosin, actin, troponin and tropomyosin) by proteases and enzymes (Nurdiani *et al*., 2022).

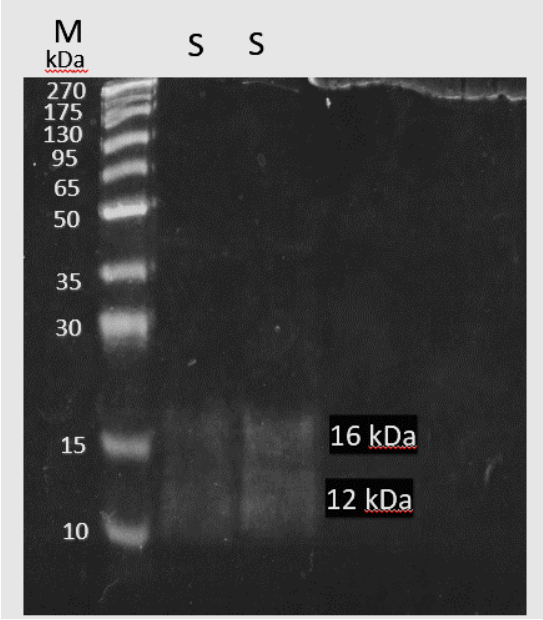

Figure 1. Results of SDS-PAGE protein hydrolysate mackerel viscera; (M) Marker; (S) Wells

Antioxidant Activity DPPH Assay

Trolox Antioxidant Standard Curve

Trolox is one of the antioxidant standards. Trolox is a water-soluble analogue of vitamin E. Trolox as an antioxidant can be applied in biological and biochemical systems to reduce oxidative stress or damage. Trolox can be used for the determination of the antioxidant activity of both Single compounds and their mixtures. In addition, these compounds have little impact on environmental conditions on the number of electrons exchanged in the observed reactions. The antioxidant value corresponding to Trolox is expressed in units introduced as Trolox Equivalents Antioxidant Capacity (TEAC), calculated from the ratio of the reaction of the test compound (e.g. measured as an inhibition) to the Trolox reaction. It is stated that the measurements for Trolox and its sample must be taken under the same conditions and the tested solution must have the same concentration. Antioxidant capacity tests that apply Trolox as a standard include DPPH, ORAC and FRAP (Olszowy-Tomczyk, 2021).

The standard curve antioxidant (Trolox) testing in this study is presented in **Table 1.** and standard curve of antioxidants in **Figure 2.,** the value of the regression equation obtained, y = 5.841x – 0.1295.

Table 1. Trolox standard curve

Final Concentration (µM)	% inhibition 1	% inhibition 2	% inhibition 3	Average % inhibition	Std. dev
12.5	73.2464	72.6206	73.0117	72.9596	0.3162
6.25	36.5059	35.5671	36.7666	36.2799	0.6309

Final Concentration (µM)	% inhibition 1	% inhibition 2	% inhibition 3	Average % inhibition	Std. dev
3.125	17.9922	17.7575	18.1486	17.9661	0.1969
1.5625	8.9179	9.1525	8.9961	9.0222	0.1195
0.78125	4.5372	4.3807	4.8501	4.5893	0.2390

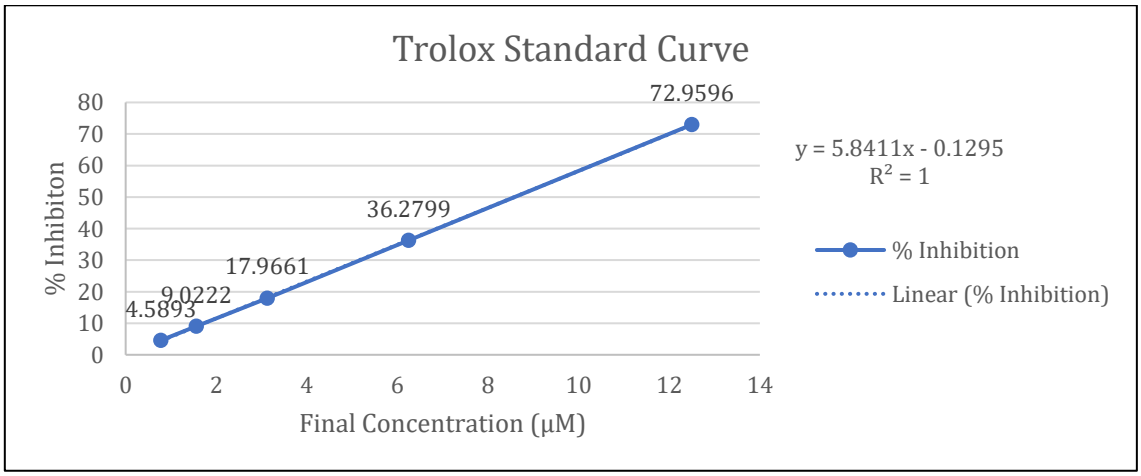

Figure 2. Standard curve antioxidant (Trolox).

Percentage (%) Inhibition and Antioxidant Capacity

The antioxidant activity of DPPH assay indicated by percentage value (%) of inhibition in **Table 1.**

Table 1. Protein hydrolysate mackerel (*Scomber scombrus*) viscera in percentage (%) inhibition value

R	% inhibition 1	% inhibition 2	% inhibition 3	Average % inhibition	Std. dev
1 (Simplo)	49.7784	49.8566	49.7001	49.7784	0.0782
2 (Duplo)	50.3781	50.5085	50.5085	50.4563	0.0689
Average	50.0782	50.1825	50.0912	50.1173	
Std. dev	0.4240	0.4609	0.5531	0.4793	

Note : R : Replicate

The results of antioxidant activity test obtained a percentage value (%) inhibition with an average value 50.117 ± 0.4793%. The antioxidant activity of mackerel viscera protein hydrolysate (*Scomber scombrus*) obtained was higher than mackerel head protein hydrolysate antioxidant activity (*Scomber japonicus*) as much 36.95 ± 0.82% (Nurdiani *et al*., 2022). However, the antioxidant activity of mackerel (*Scomber scombrus*) viscera protein hydrolysate was lower than that of parrotfish

head protein hydrolysate (*Chlorurus sordidus*) at 58.20 ± 0.55% (Priharto *et al.* 2019).

According to Baehaki *et al.* (2015), the DPPH (2,2-diphenyl-1-picrylhydrazyl) method widely used to experiment with the ability to capture free radical compounds or hydrogen donors, and determine the antioxidant activity of food. The DPPH method is a simple, fast and inexpensive method for filtering the activity of catching radicals of several compounds. The DPPH method can be used for solids or liquid samples and is not specific to specific antioxidant components. Therefore, the DPPH method is most often used compared to other methods. DPPH can be shown at a maximum absorbance of 517 nm in both ethanol and methanol.

Wirayuda *et al.* (2022) added, high antioxidant activity is generally found in products with low molecular weight with broken oligopeptide chains. In addition, other factors such as specificity of protease enzyme used in hydrolysis process can also affect the size, number and sequence of amino acids. This can then affect the antioxidant activity of the hydrolysate products produced.

The antioxidant capacity of mackerel viscera protein hydrolysate (*Scomber sombrus*) obtained a value of 0.9141 ± 0.0606 mmol TE/g of Trolox equivalent samples. The antioxidant capacity obtained is higher compared to the head protein hydrolysate of snakehead fish (*Channa striata*) 13 mmol TR/mg (Agustin *et al.*, 2023). The results obtained on **Table 2.**

Table 2. Antioxidant (AC) capacity in mmol TR/g protein hydrolysate sample mackerel (*Scomber scombrus*) viscera

R	Abs 1	Abs 2	Abs 3	AC 1	AC 2	AC 3	Average AC	AC Final	% RPD AC
R1	0.1926	0.1923	0.1929	0.9076	0.9090	0.9061	0.9076	0.0106	2.2514
R2	0.1903	0.1898	0.1899	0.9192	0.9215	0.9211	0.9206		

Note : R (Replicate); Abs (Absorbance); AC (Antioxidant Capacity)

According to Koleva *et al.* (2001), the measurement of antioxidant capacity was carried out to determine the ability of a compound as an antioxidant. More DPPH radicals are reduced, the greater the value of the sample's antioxidant capacity. According to Theafelicia and Wulan (2023), the DPPH method has the principles of reducing free radicals, reducing redox active compounds, and applying appropriate standards to measure antioxidant capacity using spectrophotometry tools.

CONCLUSION

Hydrolysate protein mackerel (Scomber scombrus) viscera hydrolyzed with the enzyme Alcalase® has a molecular weight of 12 kDa to 16 kDa. The antioxidant activity of DPPH was obtained with an inhibition percentage value of 50.117 ±

0.4793 % with Trolox Equivalent Antioxidant Capacity (TEAC) as much 0.9141 ± 0.0606 mmol TE/g sample. However, futher research is required to determine their application into preservative food self-life.

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