Food Research 7 (5) : 156 - 163 (October 2023)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Effects of peptide fractions and amino acids on antioxidant properties of autolyzed tuna viscera protein hydrolysate

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Article history:
Received: 24 May 2022
Received in revised form: 27 April 2022
Accepted: 8 October 2022
Available Online: 5 October 2023

Keywords:
ABTS,
By-product,
FRAP,
Protease,
Skipjack tuna,
Ultrafiltration

DOI:
https://doi.org/10.26656/fr.2017.7(5).270

Abstract

Tuna viscera as a common waste product from the tuna processing industry contributes to environmental pollution. The effects were determined of peptide fractions and amino acids on the antioxidant properties of protein hydrolysate from tuna viscera. We converted this waste into protein hydrolysate, a high added-value product, using autolysis. Tuna protein hydrolysate was fractionated by ultrafiltration into four fractions (>10 kDa, 5–10 kDa, 1–5 kDa and <1 kDa) and each was examined for its antioxidant properties (DPPH, ABTS, FRAP and metal chelating) and amino acids composition. The MW and amino acids of the tuna protein hydrolysate peptide fractions were not directly correlated with DPPH radical scavenging activity and metal chelating. The ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP) of the 1–5 kDa fraction were higher than for the other fractions. The tuna protein hydrolysate peptide fractions contributed to antioxidant activity and should be used to their full advantage by the nutritional and food industries.

1. Introduction

Thailand is the world's largest producer and exporter of tuna products, with most of the fresh and frozen raw materials being imported and used for canned tuna production. The imported tuna species consist of skipjack (67.3%), yellowfin (15.1%), albacore (9.3%), and others (5.9%). In 2020, 667,215 tonnes of tuna products were exported, with a value of THB 82,773 billion following an increasing trend (Thai Tuna Industry Association, 2020). However, the industry generates a large amount of waste during processing. Of the 757,644 tonnes of frozen whole tuna used as raw material in 2020 (Thai Tuna Industry Association, 2020), 7–8% was viscera and the total amount of waste was 60,611 tonnes (Hajihama, 2013). It is imperative to reduce these wastes to decrease environmental pollution, and instead use them for high added-value products (Saidi *et al*., 2014). Currently, most of the by-products are processed into animal feed, but with low value. To address this, efforts have been made to convert tuna viscera into alternative products, such as fish meal (Hernandez *et al*., 2004; Nguyen *et al*., 2011), tuna extract and protein hydrolysate (Ovissipour *et al*., 2012; Salwanee *et al*., 2013; Klomklao and Benjakul, 2017; Rutchanee *et al*., 2018; Taheri and Bakhshizadeh, 2020).

The production of protein hydrolysate uses endogenous enzymes for autolysis (Detkamhaeng *et al*., 2016; Ben Maiz *et al*., 2019) and selected protease (such as papain, alcalase, bromelain, pepsin, protamex, and flavourzyme) for the enzymatic modification of proteins (Barkia *et al*., 2010; Ben Maiz *et al*., 2019; Taheri and Bakhshizadeh, 2020). Tuna viscera is a good source of protease, which breaks down large proteins into smaller peptides and free amino acids with high solubility (Chalamaiah *et al*., 2012) Hydrolysis depends on the reaction conditions, including enzyme activity, temperature, and time (Barkia *et al*., 2010). The protease present in tuna viscera eliminates the need for expensive, synthetic enzymes (Nikoo *et al*., 2021). Protein hydrolysate is a natural source of many antioxidants. It has strong antioxidant activity against free radicals and reactive oxygen species (Qian *et al*., 2008).

The amino acid composition differs according to the source of raw materials, degree of hydrolysis, and type of protease (Sarmadi and Ismail, 2010; Li *et al*., 2013; Memarpoor-Yazdi *et al*., 2013). Bioactive peptides contain 2–20 amino acids (Saidi *et al*., 2014) and usually have a molecular weight <3 kDa (Le Gouic *et al*., 2018). Therefore, protein hydrolysate may need to be reduced to these smaller peptide fractions to take full advantage of their bioactive properties for nutritional, food, and pharmaceutical applications (Vandanjon *et al*., 2009).

Ultrafiltration (UF) is a useful technology for the

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eISSN: 2550-2166
Published by Rynnye Lyan Resources

fractionation of peptides based on molecular weight cut-off (MWCO) sizes, as well as an environmentally friendly and cost-effective process (Drioli *et al*., 2011). This research aimed to study the effects of peptide fractions and amino acids on the antioxidant properties of protein hydrolysate from tuna viscera.

2. Materials and methods

2.1 Raw materials

A sample of viscera from Skipjack tuna (*Katsuwonus pelamis*) was acquired from Thai Union Group PCL, Samut Sakhon, Thailand, packed in polyester bags, and transported on ice within 2 hrs to the Department of Fishery Products, Faculty of Fisheries, Kasetsart University, Bangkok. Then, the viscera tissue was blended and packed in polyester bags (2 kg per bag) and stored at -20°C until further experimentation.

2.2 Extraction of protein hydrolysate

Extraction of protein hydrolysate followed the method described by Detkamhaeng *et al.* (2016). A sample of the blended tuna viscera was autolyzed at 55° C for 24 hrs, and then temperature was increased to 100° C for 1 hr to terminate the reaction. The material was centrifuged at 10,000×*g* at 4°C for 30 mins. Samples of the supernatant were collected and sterilized in an autoclave at 121°C and 15 psi for 15 mins before storing until further analysis.

2.3 Determination of degree of hydrolysis

The total nitrogen content of the tuna protein hydrolysate was analyzed using the Kjeldahl method (Nielsen, 2010). The DH of the tuna protein hydrolysate was determined method TCA-solubility index as described by Rutherfurd (2010) according to the method of Tohmadlae *et al.* (2020) using the formula:

DH (%) = Total nitrogen in protein hydrolysate × 100 / Total nitrogen in material

2.4 Fractionation of peptides using ultrafiltration

Fractionation of peptides using ultrafiltration (UF) followed the method described by Charoenphun *et al.* (2013). Amicon® Stirred Cells and membranes with MWCO sizes of 10, 5, and 1 kDa were used, along with nitrogen gas and pressure of 50–75 psi. Retentate and permeate peptides were analyzed for soluble protein content**,** antioxidant properties, and amino acid composition.

2.5 Soluble protein content analysis

The soluble protein content was determined using the methods of Lowry *et al.* (1951) and Peterson and Johnson (1978), at a spectrophotometric wavelength of 750 nm. The amount of soluble protein (in mg/mL) was calculated from a standard curve of bovine serum albumin (BSA).

2.6 DPPH radical scavenging activity assay

DPPH radical scavenging activity assay followed the method described by Sukkwai *et al.* (2011), using a spectrophotometric wavelength of 517 nm. Trolox was used as the positive control at concentrations of 0–60 µM. The activity of each sample was compared to a standard curve and calculated by applying the absorbance to the standard equation.

2.7 ABTS radical scavenging activity assay

ABTS assay followed the methods of Alemán Pérez *et al.* (2011) and Ketnawa and Liceaga (2017), using a spectrophotometric wavelength of 734 nm. Trolox was used as the positive control at concentrations of 25–60 µM. The activity of each sample was compared to a standard curve and calculated by applying the absorbance to the standard equation.

2.8 Ferric reducing antioxidant power assay

FRAP assay followed the method described by Wu *et al.* (2003), using a spectrophotometric wavelength of 595 nm. Trolox was used as the positive control at concentrations of 0–850 µM. The activity of each sample was compared to a standard curve and calculated by applying the absorbance to the standard equation.

2.9 Metal chelating activity assay

Metal chelating assay followed the method of Boyer and McCleary (1987), using a spectrophotometric wavelength of 562 nm. EDTA was used as the positive control at concentrations of 0–150 µM. The activity of each sample was compared to a standard curve and calculated by applying the absorbance to the standard equation.

2.10 Amino acid composition

Samples were hydrolyzed with 6 M HCl at 110°C for 24 hrs and analyzed using high-performance liquid chromatography (Agilent 1260 Infinity, USA). The mobile phase A contained 10 mM of Na_2HPO_4, 10 mM of $Na_2B_4O_7$ (pH 8.2), and 5 mM of NaN_3; the mobile phase B contained acetonitrile:methanol:water (45:45:10 v/v/v). The flow rate was 1.5 mL/min. An injection volume of 1 µL was used, and the detector wavelengths were set at 230 nm and 450 nm. The derivatization reagents were borate buffer, o-phthalaldehyde (OPA), and 9-fluorenylmethyl chloroformate (FMOC). Determination of amino acids followed the method of the

https://doi.org/10.26656/fr.2017.7(5).270

Association of Official Analytical Chemists (AOAC, 1997).

2.11 Statistical analysis

A completely randomized design (CRD) was used for the experiments, with three replications. Data were presented as mean±standard deviation (SD) values. Means were compared using Duncan's multiple range test and statistical analysis of variance (ANOVA) at a significance level of 95%.

3. Results and discussion

3.1 Fractionation of peptides

The degree of hydrolysis of the tuna protein hydrolysate was 19.16±0.65%. The protein hydrolysate was fractionated using MWCO sizes of 10, 5, and 1 kDa. The resulting four peptide fractions (and relative proportions) were >10 kDa (56.40±1.20%), 5–10 kDa (14.28±1.03%), 1–5 kDa (10.17±0.74%) and <1 kDa (19.15±0.52%), as shown in Figure 1. The tuna protein hydrolysate had a soluble protein content of 1,031.66±14.26 mg protein, while the peptide fractions of >10 kDa, 5-10 kDa, 1-5 kDa and <1 kDa had protein contents of 586.67±19.82, 153.07±6.24, 106.42±0.49 and 184.98±12.25 mg protein, respectively. The soluble protein content of all peptide fractions was significantly lower than for tuna protein hydrolysate ($p<0.05$).

3.2 DPPH radical scavenging activity

DPPH radical scavenging capacity assay is a method for the analysis of the capacity of primary oxidation resistance in the reagent DPPH 2,2-diphenyl-1-picrylhydrazyl is radical in the methanol solvent that accepts an electron or hydrogen to become a stable free radical (Fatiha and Abdelkader, 2019). The DPPH radical scavenging activities of the tuna protein hydrolysate (60.34±1.34 μmol TE/mg protein) and the peptide fractions were not significantly different ($p\geq0.05$) as shown in Figure 2. The MW of the tuna protein hydrolysate showed some effect on the DPPH antioxidant activities as did the protein hydrolysates from mackerel (Wu *et al.,* 2003), salmon (Ahn *et al.,* 2014), cod (Sabeena Farvin *et al.,* 2016), and tuna (Wu *et al.,* 2003; Zhang *et al.,* 2019). DPPH free radicals can be dissolved only in inorganic media (especially in alcohol-based media) and are less sensitive in aqueous and organic media. Hydrophobic amino acids have a

Table 1. Amino acid composition of tuna protein hydrolysate and peptide fractions.

Amino acid (mg/100g sample)	Tuna protein hydrolysate	Fraction			
		>10 kDa	5–10 kDa	1–5 kDa	<1 kDa
Aspartic acid*	580.62	330.75	84.15	63.60	109.77
Alanine**	413.62	236.06	59.99	44.74	79.20
Arginine*	367.91	208.47	53.63	41.06	70.87
Cystine*	-	-	-	-	-
Glutamic acid*	893.98	512.98	131.09	97.74	169.04
Glycine*	464.94	266.07	67.63	50.95	87.30
Histidine*	136.99	79.88	20.05	15.82	24.88
Hydroxylysine	-	-	-	-	-
Hydroxyproline	-	-	-	-	-
Isoleucine**	295.78	169.61	43.19	32.39	57.13
Leucine**	460.01	263.31	66.39	49.86	88.13
Lysine*, ***	499.08	285.44	73.15	55.22	95.64
Methionine**	-	-	-	-	-
Phenylalanine**	250.73	-	-	27.14	-
Proline**	316.94	173.81	45.37	34.66	58.50
Serine*	304.20	177.70	44.81	33.86	60.40
Threonine*	309.83	177.86	44.69	34.00	58.01
Tryptophan**	-	-	-	-	-
Tyrosine*	-	-	-	-	-
Valine**	406.83	232.72	59.42	43.96	77.51
Hydrophilic amino acids	3557.55	2039.15	519.21	392.25	675.90
Hydrophobic amino acids	2143.91	1075.51	274.37	232.75	360.48
Essential amino acids	2359.25	1208.82	306.91	258.39	401.30
Total	5701.46	3114.66	793.58	625.00	1036.38

- = not detected

* Hydrophilic amino acids

** Hydrophobic amino acids

https://doi.org/10.26656/fr.2017.7(5).270

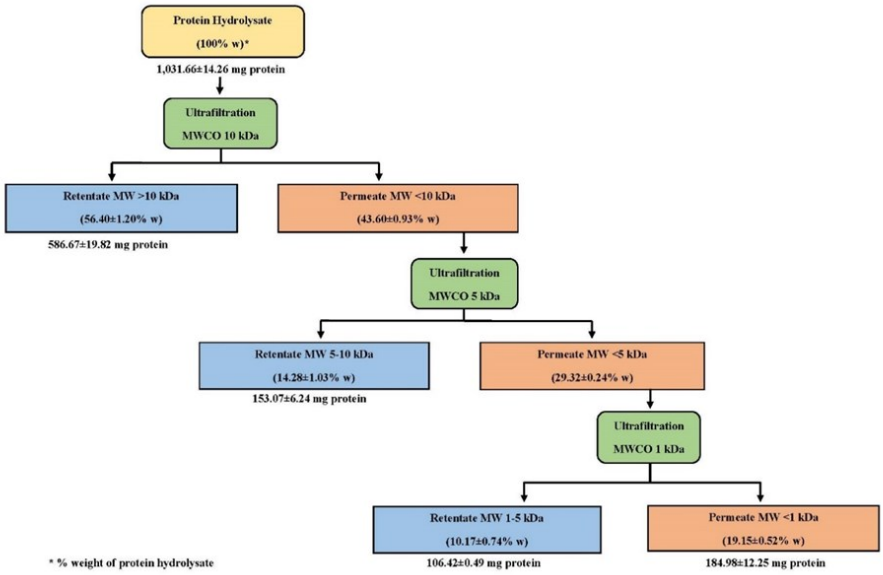

Figure 1. Diagram of fractionation of tuna protein hydrolysate using ultrafiltration (values shown as mean±SD).

strong effect on DPPH free radicals (Tang *et al.,* 2010). The tuna protein hydrolysate showed lower DPPH radical scavenging activity because it contained less hydrophobic amino acids (alanine, isoleucine, leucine, methionine, phenylalanine, proline, and valine), as shown in Table 1.

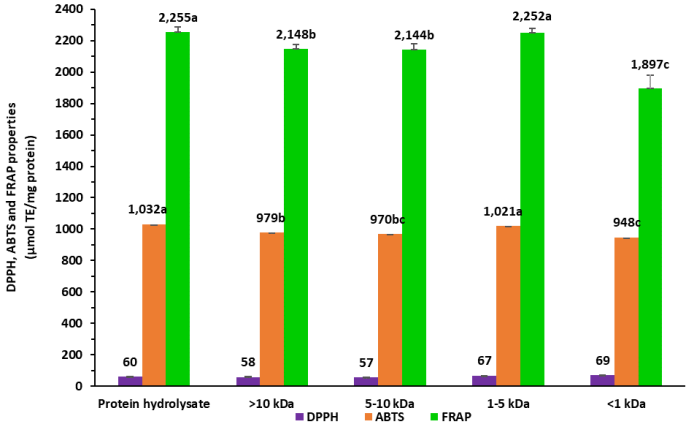

Figure 2. Antioxidant activities of tuna protein hydrolysate and peptide fractions. Results are presented as mean±standard deviation (n = 3). Values with different superscripts are statistically significantly different at p<0.05.

3.3 ABTS radical scavenging activity

ABTS assay radical scavenging capacity assay is a method for the analysis of the capacity of primary oxidation resistance in the reagent 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). The diammonium salt is a stable radical in an aqueous solution so the primary antioxidant acts to stop the reaction chain in the reaction as the oxidation acts as an electron donor (Tang *et al.,* 2010). The current results showed that the protein hydrolysates and peptide 1–5 kDa fraction had the significantly highest antioxidant activity (1031.16±69. 82 and 1021.8±13. 85µmol TE/mg protein, respectively) compared to the peptide fractions of >10 kDa, 5–10 kDa and <1 kDa (978.83±11.25, 970.05±28.34 and 947.96±22.43 µmol TE/mg protein, respectively). ABTS free radicals are more sensitive in aqueous and organic media (Tang *et al.,* 2010). The current results showed that the MW of the peptides had an effect on the ABTS radical scavenging activity. Factors affecting the level of ABTS antioxidant activity are the source of the raw material, the autolysis process, and the amounts of peptides and amino acids. In addition, high MW peptides are effective as antioxidants and increase and decrease hydrophilic and hydrophobic amino acids, respectively, improving the antioxidant effect of ABTS (Tang *et al.,* 2010). The tuna protein hydrolysate contained hydrophobic amino acids (alanine, isoleucine, leucine, methionine, phenylalanine, proline, and valine) and hydrophilic amino acids (aspartic acid, arginine, glycine, lysine, serine, and threonine), as shown in Table 1 that contributed to the high antioxidant effect of the ABTS. In the current research, the tuna protein hydrolysate and the 1–5 peptide fractions had the highest ABTS effect but they were not significantly different. Although the amount of the 1–5 peptide fraction was low (10%), the peptide fractions had a greater effect on ABTS than the hydrophilic and hydrophobic amino acids.

3.4 Ferric reducing antioxidant power

FRAP assay is used to evaluate the ability of an antioxidant to donate an electron to reduce the ferric iron)III) to ferrous iron (II). The reducing properties depend on the electron donor capacities of the peptides and amino acids (Bougatef *et al.,* 2010). The current results, shown in Figure 2, indicated that the tuna protein hydrolysate and the peptide fraction 1–5 kDa had high FRAP values of 2254.50±30.35 and 2251.85±28.03 µmol TE/mg protein, respectively, that were

https://doi.org/10.26656/fr.2017.7(5).270

significantly ($p<0.05$) higher than for all the other peptide fractions (>10 kDa, 2147.51±27.10; 5–10 kDa, 2143.71±35.35; and <1 kDa, 1896.84±70.28 µmol TE/ mg protein). Other research reported that in cod protein hydrolysate, the peptide fractions 3–5 kDa and <3 kDa had higher FRAP levels than the larger peptides (Sabeena Farvin *et al.,* 2016). In salmon by-product hydrolysate, peptides with MW <1 kDa had the highest FRAP (Ahn *et al.,* 2014). FRAP is related to the MW of peptides (Sabeena Farvin *et al.,* 2016). High amounts of low-MW peptides increase the donation of electrons to free radicals, thus the termination of the chain reaction (Nikoo *et al.,* 2019). Radical scavenging activity as measured using FRAP is also associated with hydrophobic amino acids (Pownall *et al.,* 2010). In the current study, the tuna protein hydrolysate contained hydrophobic amino acids (alanine, isoleucine, leucine, methionine, phenylalanine, proline, and valine), as shown in Table 1 and the protein hydrolysate also had a high FRAP. This was similar to reports on tuna dark muscle hydrolysate (Saidi *et al.,* 2014) and cod protein hydrolysates (Sabeena Farvin *et al.,* 2016).

3.5 Metal chelating activity

The metal chelating assay is used to determine the ability for oxidation because metal ions are the main reaction iron in the form of ferrous (Fe^{2+}) will oxidize with oxygen in the air to form a superoxide anion radical ($O_2^{-\bullet}$), which is a free radical initiator that produces other free radicals (Torres-Fuentes *et al.,* 2012; Da Rocha *et al.,* 2018). Long-chain peptides have higher numbers of amino acids than short-chain peptides; thus, short-chain peptides cannot form a complex with metals and long-chain peptides may have metal chelating activity compared to the shorter peptides (Taheri and Bakhshizadeh, 2020). Histidine, arginine, and lysine affect metal chelating via their carboxyl or amino side chains. Histidine has also been shown to have strong radical scavenging capacity as a result of metal chelating (Wu *et al.,* 2003; Samaranayaka and Li-Chan, 2011; Girgih *et al.,* 2013; Taheri and Bakhshizadeh, 2020). Tuna protein hydrolysate and peptide fractions contain less histidine and consequently may not show any metal chelating activity.

4. Conclusion

This research indicated that the peptide fractions had a greater effect on ABTS radical scavenging activity and FRAP than the hydrophilic and hydrophobic amino acids, while the MW and amino acids of the tuna protein hydrolysate did not have significant effects on DPPH and metal chelating. Tuna protein hydrolysate and the 1–5 kDa peptide fraction showed ABTS radical scavenging activity and FRAP levels that were higher than all the other peptide fractions. Choosing the most appropriate peptide fraction can be beneficial in the food industry.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was financially supported by the National Research Council of Thailand (NRCT) under the project "Production of food products for seniors from by-products of tuna processing".

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