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Radical-Scavenging Activity of Fish Gelatin Hydrolysates from Bone of Pangasius catfish (*Pangasius sutchi*) by Microbial Proteases Hydrolysis

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Abstract: The gelatin extracted from the bone of Indonesian *Pangasius sutchi* was hydrolyzed using two proteases, namely protease *Lactobacillus plantarum* S31, which isolating from bekasam (an Indonesian fermented fish product) and flavourzyme (a commercial enzyme from *Aspergillus oryzase*). The concentrations of enzymes for hydrolysis were 1% and 6% based on enzyme/substrate [E/S] ratio. The bioactivity of the hydrolysates as an antioxidant was measured based on their capacity to inhibit 1,1-*diphenyl*-2-*picylhydrazyl* (DPPH). The ascorbic acid or vitamin C in the range 50–150 ppm was used as positive controls. The result showed that the bioactivities of all hydrolysates were increased compared to gelatin before hydrolysis. The hydrolysates have a percent of inhibition range that higher than the ascorbic acid. The hydrolysates of gelatin derived from the bone of *Pangasius sutchi* would be a potential source as raw material for bioactive peptides production with antioxidant activity.

Keywords: antioxidant; bioactive peptide; fish gelatin; gelatin hydrolysate; protease.

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1. Introduction

Fish based gelatin has been developed in producing bioactive peptides regarding their bioactivities as antioxidants [1], antihypertensive [2], antimicrobial [3], anti-diabetic (type 2) [4] and anticancer [5]. As antioxidants, fish gelatin is taking great attention concerning safety aspects [6], abundance amount, and promising for industrial application [7], as well as their simply hydrolyzed enzymatically in order to obtain higher activity [8, 9]. In addition, some of the antioxidant activity of bioactive peptides influenced by the presence of Glycine (Gly) and Proline (Pro) in which both these amino acids dominantly contain in fish gelatin [10]. A work conducted by Zheng *et al*. (2018) also concluded that the antioxidant activity of fish gelatin higher than porcine and bovine gelatin [11]. Studies investigating of antioxidant properties attaching to hydrolysates and bioactive peptides from fish gelatin was covering from skins and scales of Nile tilapia [8, 12, 13, 14], Sole fish [15], the skin of Amur sturgeon [16], Cobia [17], Unicorn leatherjacket, Thornback ray [18], Black-barred halfbeak [19], Carp [20], Tuna, Halibut [21], and bone of Skipjack Tuna [22].

Actually, as a source of fish gelatin, the bone of pangasius catfish has higher in yield compared to other fish-based origins [23]. So, this research provides a work using the most promising alternative source of gelatin in terms of considering the quantity of gelatin yield.

Moreover, this research was conducted to attempt using an enzyme from lactic acid bacteria which convenient for humans, i.e., protease from *Lactobacillus plantarum* S31. *L. plantarum* S31 is a bacterium that originated from bekasam (an Indonesian fermented fish product). A study by Budiarto *et al*. (2016) found that protease from *L. plantarum* S31 is thermostable [24]. There are several benefits by using thermostable enzymes, including preventing the contaminations, increasing catalysis rates, and reducing viscosity in order to simplify hydrolysates production [25]. The protease from *L. plan*tarum S31 is also proven hydrolyzed protein to become hydrolysates with antioxidant activity [26]. Furthermore, this research was also using commercial protease such as flavourzyme to obtain comparative results. Flavourzyme is a protease expressed by *Aspergillus oryzae* with endopeptidase and exopeptidase hydrolysis sites. Through more sites that can be hydrolyzed, may implicate to the great possibility of hydrolysates against oxidant agents.

The capacity of fish gelatin hydrolysates against oxidants agents measured by some methods. For instance, radical scavenging, metal chelating or reducing, and lipid peroxidation inhibition activities. It is commonly believed that the radical scavenging activity is the most and represents the parameter measuring the antioxidant activity. The majority of previous researches were using DPPH (1,1 *diphenyl* 2 *picylhydrazyl*) radical to determine the radical scavenging activity of fish-based gelatin [18, 19, 22, 27]. Cai *et al*. (2015) support that opinion by stating the DPPH is generally used as a substrate to measuring the antioxidant activity [28]. The method is fast, convenient, and efficient in predicting the antioxidant activity, so it has been broadly adopted to test the ability of compounds to act as free radical scavengers. Yang *et al*. (2019) mentioned that DPPH is a stable and cell-permeable radical that is commonly applied for analysis antioxidant activity [29]. Thus, for the reasons of deal with efficiency, in this research, we have analyzed the DPPH inhibition activity of pangasius gelatin hydrolysates and terminated the study on hydrolysate activity without purified further because number prior publications were showing the activity of gelatin hydrolysates are better than smaller purified gelatin peptides [11, 28, 30].

2. Materials and Methods

2.1. Enzyme preparation.

Lactobacillus plantarum S31 from Research Center for Biotechnology, Indonesian Institute of Science, was used as a source of the first enzyme. The production of extracellular protease from this lactic acid bacteria (LAB) and determination of the protease activity was done by using a method described by Budiarto *et al*. [24]. Whilst, the second enzyme is flavourzyme (from *Aspergillus oryzae*), which manufactured by Novozyme Corp. (Bagsvaerd, Denmark).

2.2. Fish bone gelatin hydrolysates preparation.

The gelatin from the bone of Pangasius catfish (*Pangasius sutchi*) extracted in our previous work [31] was used for hydrolysates preparation. The gelatin from the refrigerator placed at room temperature, then mixed using vortex until it became a liquid. Then, the gelatin solution added with each protease in different concentration (E/S ratio) i.e., 1% and 6% (v/v). After that, the gelatin-enzyme solution was incubated for 3 hours. The gelatin with protease from *L. plantarum* S31 incubated at pH 5, 37 ^{o}C, while the gelatin mixed with flavourzyme

from *A. oryzae* incubated at pH 7, 50 °C. The enzymatic hydrolysis was stopped by heating the mixture solutions at 100 °C for 10 minutes. The hydrolysates were obtained by separation technique using centrifugation at 10,000 rpm, 15 min, and 4 °C. Finally, the supernatant was collected as fish gelatin hydrolysates and stored at -20 °C before further analysis. The protein concentration of gelatin and their hydrolysates quantified using bicinchoninic acid (BCA) assay kit (Thermo Fisher, Rockford, IL, USA) by the method described in our previous work [31].

2.3. Determination of molecular weight.

Molecular weight (MW) identification was performed through vertical electrophoresis by using *sodium dodecyl sulfate polyacrylamide gel electrophoresis* (SDS-PAGE) as explained in our previous study carried out by discontinues Tris/HCl/glycine buffer system [23, 31]. Then, the appearance of bands protein was quantified for their *retardation factor* (Rf) value by using the formula of Rf = [the distance of bands of protein movement from starting point] / [the distance of marker movement from starting point]. The molecular weight of each hydrolysate quantified using linear equation (y=ax+b) made from a log of MW (y-axis) and Rf (x-axis).

2.4. Antioxidant activity.

Antioxidant activity was determined by measuring the radical scavenging activity of fish gelatin hydrolysates against DPPH (1,1 *diphenyl* -2- *picylhydrazyl*). The capacity of gelatin hydrolysates to inhibit free radicals of DPPH was conducted according to Clarke *et al*. (2013) procedure with some modification through ethanol 96% addition [32]. Firstly, 20 µL samples added with 180 µL DPPH solution (a distilled water used as control). After mixing vigorously for 10 s, then the absorbance measured using enzyme-linked immunosorbent assay (ELISA) Microplate Reader (Multiskan Ex, Champaign, IL, USA). The reaction/mixed solution was read at 540 nm against a reagent blank. The radical scavenging activity was calculated by the following formula: antioxidant activity (%) = [(As - An)/(Ab - An)] x 100 where As, An, and Ab were the absorbance values determined at 540 nm of the samples, the negative control, and the blank after a reaction, respectively [16, 32]. The IC_{50} was defined as a concentration of fish gelatin hydrolysates to inhibit free radical as many as fifty percent. The scavenging activity of ascorbic acid (vitamin C) as control positive with different concentrations (50-150 ppm) was also measured as the same as the procedure for quantifying the antioxidant activity of samples.

2.5. Statistical analysis.

All value determinations were performed in average values. Numbers were expressed as the means followed by their standard deviations. Analysis of variance was conducted, and differences between variables were analyzed for their significance by Tukey's range test ($p < 0.05$).

3. Results and Discussion

3.1. Fishbone gelatin hydrolysis.

Gelatin after hydrolysis was determined their molecular weight (figure 1 and table 1) and protein content (Table 2). It confirmed that gelatin hydrolysates have lower MW compared to crude extract gelatin. The MW of fishbone gelatin hydrolysates is various depend on enzyme

concentration for hydrolysis. In this study, the MW of gelatin hydrolysates were in the range of 41 – 63 kDa (figure 1). Fish gelatin that hydrolyzed with extracellular protease from *L. plantarum* S31 has MW slightly higher (51 and 63 kDa) than which hydrolyzed with flavourzyme (41 and 43 kDa).

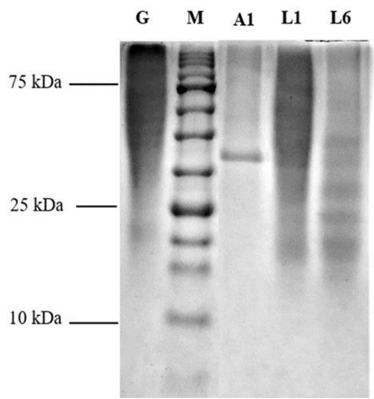

Figure 1. Electroforegram fish gelatin (G) and their hydrolysates. A1=hydrolysate derived from 6% [E/S] protease from *A. oryzae*, L1=1% [E/S] protease from *L. plantarum* S31 and L6= 6% [E/S} protease from *L. plantarum* S31, M=marker protein.

Table 1. Molecular weight (MW) of fish gelatin and their hydrolysates.

Samples	Rf samples	MW (kDa)
Fish gelatin (before hydrolysis)	0.059	**163**
	0.072	137
Hydrolysate (1% E/S ratio *L. plantarum* S31)	0.147	**63**
	0.188	44
	0.407	26
Hydrolysate (6% E/S ratio *L. plantarum* S31*)*	0.166	**51**
	0.194	43
	0.269	30
	0.327	27
	0.544	21
Hydrolysate (1% E/S ratio *A. oryzae)*	0.189	**43**
	0.309	28
	0.458	25
Hydrolysate (6% E/S ratio *A. oryzae)*	0.204	**41**
	0.316	28

Table 2. Protein content of fish gelatin and their hydrolysates.

Samples	**Protein content**
Fish gelatin	$1.83_{cd} \pm 0.100$
Hydrolysate (1% E/S ratio *L. plantarum* S31)	$0.80_{d} \pm 0.155$
Hydrolysate (6% E/S ratio *L. plantarum* S31*)*	$1.18_{d} \pm 0.005$
Hydrolysate (1% E/S ratio *A. oryzae*)	$3.44_{bc} \pm 0.109$
Hydrolysate (6% E/S ratio *A. oryzae)*	$12.46_{a} \pm 1.574$

[1] Values in the same row, followed by different letters are significant differents ($P < 0.05$).

A study conducted by Baehaki *et al*. (2015) found that fish protein hydrolysates derived from fish meat protein in which hydrolyzed using papain were producing four hydrolysates in MW range of 11.90-42.62 kDa [33]. While the protein content of fish gelatin and their hydrolysates were measured by using 96-well ELISA reader with bovine serum albumin (BSA) as standard in range 0-2 mg/mL. The result has shown that the protein concentration of hydrolysates is increased gradually by increasing enzyme for hydrolysis (Table 2). Hydrolysate derived from flavourzyme hydrolysis has the highest protein content.

3.2. Free radical scavenging activity of gelatin hydrolysates.

The bioactivities of fish gelatin hydrolysates as antioxidant measured by their capability to inhibit free radical 2,2-*diphenyl*-1-*picrylhydrazyl* (DPPH). This method is mention as a general method in order to determine the antioxidant capacity of bioactive compounds. The DPPH radical scavenging activity of an antioxidant is based on its ability to donate a hydrogen atom or an electron to stabilizing radicals, by converting it to the non-radical species [34]. In this work, the test was adopted from Clarke *et al*. (2013) in which the sample is mixed with DPPH solution, and 96% ethanol was added, followed by incubation for 30 minutes [32]. Theoretically, the DPPH solution reacts with the bioactive compound to create the *diphenylpycrylhydrazine*. The absorbance of reaction then measured in wavelength of 540 nm. The measurement was done using a 96-well microplate coupled with ELISA reader. The antioxidant activity determined by the ratio of sample absorbance with absorbance without bioactive compound then multiply 100%.

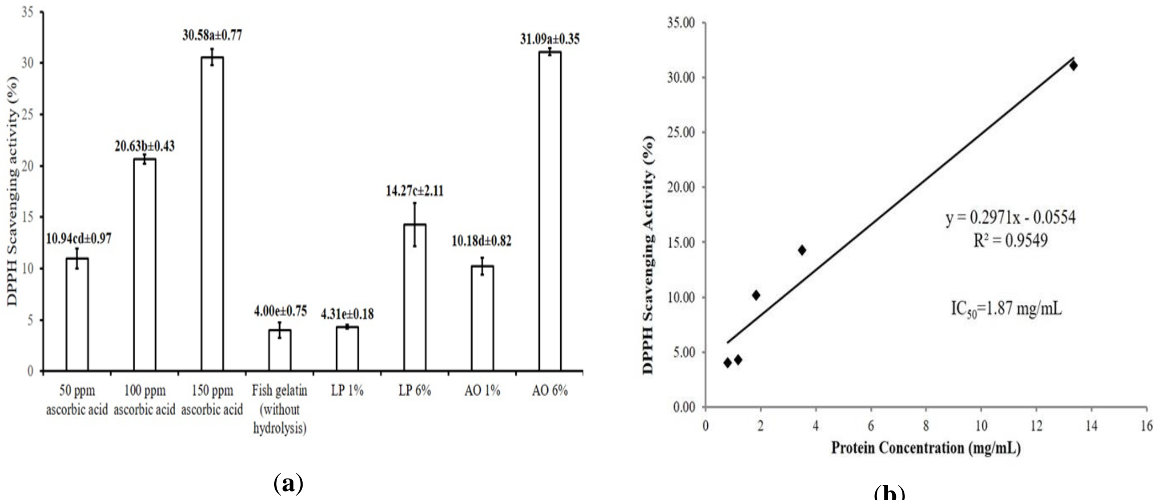

Figure 2. (**a**) Scavenging activity of fish gelatin and their hydrolysates compared to ascorbic acid. LP1% means 1% [E/S] protease from *L. plantarum* S31 and LP 6% is 6% [E/S] protease from *L. plantarum* S31. AO 1%= 1% [E/S] ratio from protease of *A. oryzae* and AO 6%= 6% [E/S] ratio from protease of *A. oryzae*. Values in each bar followed by different letters are significantly different ($P < 0.05$); (**b**) Standard curve for IC50 value quantification of DPPH•-scavenging activity of fish gelatin hydrolysate from the bone of Pangasius catfish.

In this study, ascorbic acids (vitamin C) with concentration 50-150 ppm used as a positive control of scavenging activity. Figure 2a shows that radical scavenging of gelatin hydrolysates higher than gelatin before hydrolysis. Gelatin hydrolysate from a 6% E/S ratio of flavourzyme is higher than other hydrolysates. The highest one seems comparable to the free radical scavenging capacity of 150 ppm ascorbic acid. Based on figure 2 also indicates that the inhibition of fish gelatin hydrolysates from 6% E/S ratios toward DPPH radical is outnumbered hydrolysates from 1% E/S ratios. A previous study has shown that gelatin hydrolysate from the skin of Amur sturgeon has antioxidant activity around 40% [16] in which they are also using the ability of hydrolysate to neutralize the free radical of DPPH to measure the antioxidant activity, however, they use Alcalase for hydrolysis.

The IC_{50} value of gelatin hydrolysate from the bone of Indonesian Pangasius catfish also determined (figure 2b). It is because IC_{50} generally accepted as an indicator of the effectiveness of bioactive compounds wherein this term as an antioxidant through free radical DPPH inhibition. The IC_{50} fish gelatin hydrolysate from Indonesian *Pangasius sutchi* is 1.87 mg/mL. The IC_{50} value of Tilapia skin gelatin hydrolysate (TSGH) was 3.66 mg/mL [13]. You

et al. (2010) reported the IC_{50} value of the DPPH·-scavenging activity of Loach protein hydrolysate was 2.64 mg/mL [35].

The successful hydrolysis process was analyzed by *sodium dodecyl sulfate polyacrilamide gel electrophoresis* (SDS-PAGE). Figure 1 provides the electroforegram of fishbone gelatin and their hydrolysates. The figure shown that the fish gelatin before hydrolysis has thick band in higher MW, where the vague band depicted by the hydrolysates particularly at higher enzyme concentrations. Interestingly, the hydrolysates have a thicker band in lower MW that, in fact, the intact fish bone gelatin has too vague in that MW. It is indicated that the protease was hydrolyzed the gelatin. Accordingly, to support the result that not display of MW of hydrolysate derived from 1% E/S ratio of flavourzyme in electroforegram (for the reason of inappropriate SDS-PAGE picture), the quantification of MW through Rf values was done. Based on this quantification, the intact fish bone gelatin has MW 163 kDa, where their hydrolysates three-fold lower. Our previous work found that fishbone gelatin hydrolysates from similar species have MW ~ 50 kDa after hydrolyzed with flavourzyme [36]. Although the MW of the unhydrolyzed gelatin in this study slightly below our previous identification [31], the range is still in MW range of fish gelatin from the bone of Pangasius catfish. The intact gelatin from this species has MW of ~100–116 kDa, ~150–200 kDa, and >225 kDa [23]. In addition, the dominant factor that affects the MW of gelatin is protein hydrolysis during storage, concerning in this case, the gelatin was stored for few months before running to electrophoresis, while in our previous analysis, the gelatin had injected to a well of gel electrophoresis directly after extraction.

The protein content of fish gelatin hydrolysate derived from flavourzyme hydrolysis is larger than hydrolysates from protease *L. plantarum* S31 hydrolysis. The method for analyzing protein content in this research was by using *bicinchoninic acid* (BCA) assay kit. Besides affected by amino acid residues, the quantification of protein using BCA is also influenced by the peptide bonds or peptide backbones of protein [37], which means that more peptide bonds in protein solution imply to greater protein concentration. Hence, it is announced that the flavourzyme was actively hydrolyze the gelatin compared to protease *L. plantarum* S31, which also confirmed by their MW ranges. Flavourzyme is an endopeptidase and exopeptidase enzyme. They have wider and greater hydrolytic sites, thus their hydrolytic possibility also larger. Moreover, the purify of an enzyme in like manner contributes to their hydrolytic activity. The flavourzyme in this research was purified before commercialized, otherwise, the protease *L. plantarum* S31 was a crude extract even the specific activity (2,000 U/g) [26] over the flavourzyme (500 U/g).

The antioxidant activity through DPPH radical scavenging ability of fish gelatin increased subsequent hydrolysis. The radical scavenging activity of gelatin hydrolysate reach almost eight times higher than fish gelatin before hydrolysis. The activity of hydrolysates from flavourzyme hydrolysis is two-fold higher than hydrolysates from their counterpart even though their MW lower. These results contradict the Abuine *et al*. (2019) statement remarking that high molecular peptides have higher DPPH inhibition activity than low molecular peptides [10]. In fact, radical scavenging activity of hydrolysates depends on many factors, including peptide size, sequence, amino acid composition enzyme used, and degree of hydrolysis, among which amino acid composition and peptide sequence play a crucial role in determining the antioxidant power [15]. Lassoued *et al*. (2015) reporting that the presence of certain amino

acids in the hydrolysate enhance scavenging activity of peptide, among of them like tryptophan, tyrosine, methionine, cysteine, histidine, and phenylalanine [18].

Some previous studies have observed the percentage of radical scavenging activity of fish gelatin and protein hydrolysates against DPPH in which using ascorbic acid as a positive control. As results, the DPPH scavenging activity of gelatin hydrolysates derived from skin gelatin of sole fish, thornback ray, black-barred halfbeak, and rainbow trout were ~68% [15], ~70% [18], 43.39% [19] and ~40% [27] respectively. While radical scavenging activity of gelatin hydrolysates from bone of sole fish was ~ 62% [15]. Furthermore, protein hydrolysates from the skin of grass carp and collagen milkfish having DPPH scavenging activity as much as 40% [28] and 35% [38], respectively. These data have shown us that gelatin hydrolysates from the bone of pangasius catfish have comparable DPPH scavenging activity than other hydrolysates. Indeed, the percent of inhibition toward DPPH still below, however comparing to the IC_{50} of other gelatin and protein hydrolysates, the gelatin from the bone of pangasius catfish, is still better. A study conducted by Chi *et al*. (2014) found that their hydrolysates derived from alcalase hydrolysis was lower than other enzymes hydrolysis [30]. They had found that the IC_{50} from that hydrolysate was 5.23 mg/mL. Other researchers also mentioned the IC_{50} from their leading hydrolysates, surprisingly all of their hydrolysates have higher IC_{50} than our work. For example, the IC_{50} from skin gelatin hydrolysates of tilapia, amur sturgeon, and thornback ray was 3.66 [13], 5.38 [16], and 1.98 mg/mL [18] respectively. Although the IC_{50} antioxidant from protein hydrolysates is still higher than antioxidant compounds from a medical plant (such as IC_{50} *Veronica biloba* fraction extracts which around 1.70±0.05μg/mL) [39], however, the protein has their own superiority regarding well-known as macronutrients so that generally safe and digestible. Thus, it is a signal that gelatin from the bone of pangasius catfish would be potential as a source of a bioactive peptide with antioxidant activity.

4. Conclusions

Fishbone gelatin hydrolysates from Pangasius catfish have a capacity as an antioxidant through inhibition free radical 2,2-*diphenyl*-1-*picrylhydrazyl* (DPPH). The activity of hydrolysate derived from enzymatic hydrolysis of 6% protease of *A. oryzae* was higher than gelatin before hydrolysis and other hydrolysates, even compared to 150 ppm ascorbic acid. In terms of DPPH scavenging activity, the gelatin hydrolysate from the bone of Pangasius catfish has lower IC_{50} than previous fish-based hydrolysates.

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Sai-ut, S.; Benjakul, S.; Sumpavapol, P.; Kishimura, H. Antioxidant Activity of Gelatin Hydrolysate Produced from Fish Skin Gelatin using Extracellular Protease from Bacillus amyloliquefaciens H11. *J Food Process Preserv* **2014**, *39,* 394–403, https://doi.org/10.1111/jfpp.12244.
2. Mahmoodani, F.; Ghassem, M.; Babji, A.S.; Yusop, S.M.; Khosrokhavar, R. ACE Inhibitory Activity of Pangasius catfish (Pangasius sutchi) Skin and Bone Gelatin Hydrolysate. *J Food Sci Technol* **2014**, *51,* 1847–1856, https://doi.org/10.1007/s13197-012-0742-8.
3. Gómez-Guillén, M.; López-Caballero, M.; Aleman, A.; López de Lacey, A.; Giménez, B.; Montero, P. *Antioxidant and Antimicrobial Peptide Fractions from Squid and Tuna Skin Gelatin.* Le-Buhan E., Eds.; Transworld Research Network: Karela, India, Volume 661, **2010;** pp. 89-115.
4. Wang, T.Y.; Hsieh, C.H.; Hung, C.C.; Jao, C.L.; Chen, M.C.; Hsu, K.C. Fish Skin Gelatin Hydrolysates as Dipeptidyl Peptidase IV Inhibitors and Glucagon-Like Peptide-1 Stimulators Improve Glycaemic Control in Diabetic Rats: A Comparison Between Warm- and Cold-water Fish. *J Funct Foods* **2015**, *19,* 330–340, https://doi.org/10.1016/j.jff.2015.09.037.
5. Fu, Y.; Zhao, X. In Vitro Responses of hFOB1.19 Cells Towards Chum Salmon (Oncorhynchus keta) Skin Gelatin Hydrolysates in Cell Proliferation, Cycle Progression and Apoptosis. *J Funct Foods* **2013,** 5, 1–10, https://doi.org/10.1016/j.jff.2012.10.017.
6. Amiza, M.A.; Wan Maizatul Shima, W.M.; Nor Hayati, I.; Nizaha Juhaida, M. Optimization of Gelatin Extraction Conditions from Cobia (Rachycentron canadum) Skin and Its Physicochemical Characteristics as Compared to Bovine Gelatin. *Int Food Res J* **2015**, *22,* 213–224.
7. Karim, A.A.; Bhat, R. Fish Gelatin: Properties, Challenges, and Prospects as An Alternative of Mammalian Gelatins. *Food Hydrocoll* **2009**, *23,* 563–576, https://doi.org/10.1016/j.foodhyd.2008.07.002.
8. Choonpicharn, S.; Jaturasitha, S.; Rakariyatham, N.; Suree, N.; Niamsup, H. Antioxidant and Antihypertensive Activity of Gelatin Hydrolysate from Nile Tilapia Skin. *J Food Sci Technol* **2015**, *52,* 3134–3139, https://doi.org/10.1007/s13197-014-1581-6.
9. Karayannakidis, P. D.; Zotos, A. Fish Processing By-Products as A Potential Source of Gelatin: A Review. *J Aquat Food Prod Technol* **2016**, 25, 65–92, https://doi.org/10.1080/10498850.2013.827767.
10. Abuine, R.; Rathnayake, A. U.; Byun, H. Biological Activity of Peptides Purified from Fish Skin Hydrolysates. *Fish Aquat Sci* **2019**, *22,* 1–14, https://doi.org/10.1186/s41240-019-0125-4.
11. Zheng, L.; Yu, H.; Wei, H.; Xing, Q.; Zou, Y.; Zhou, Y.; Peng, J. Antioxidative Peptides of Hydrolysate Prepared from Fish Skin Gelatin using Ginger Protease Activate Antioxidant Response Element-Mediated Gene Transcription in IPEC-J2 Cells. *J Funct Foods* **2018**, *51,* 104–112, https://doi.org/10.1016/j.jff.2018.08.033.
12. Sun, L.; Zhang, Y.; Zhuang, Y. Antiphotoaging Effect and Purification of An Antioxidant Peptide from Tilapia (Oreochromis niloticus) Gelatin Peptides. *J Funct Foods* **2013**, *5,* 154–162, https://doi.org/10.1016/j.jff.2012.09.006.
13. Zhang, Y.; Duan, X.; Zhuang, Y. Purification and Characterization of Novel Antioxidant Peptides from Enzymatic Hydrolysates of Tilapia (Oreochromis niloticus) Skin Gelatin. *Peptides* **2012,** *38*, 13–21, https://doi.org/10.1016/j.peptides.2012.08.014.
14. Ngo, D.; Qian, Z.; Ryu, B.; Park, J. W.; Kim, S. In Vitro Antioxidant Activity of A Peptide Isolated from Nile Tilapia (Oreochromis niloticus) Scale Gelatin in Free Radical-Mediated Oxidative Systems. *J Funct Foods* **2010**, *2*, 107–117, https://doi.org/10.1016/j.jff.2010.02.001.
15. Viji, P.; Phannendra, T.S.; Jesmi, D.; Rao, B.M.; Das, P.H.D.; George, N. Functional and Antioxidant Properties of Gelatin Hydrolysates Prepared from Skin and Scale of Sole Fish. *J Aquat Food Prod Technol* **2019**, *28,* 976–986, https://doi.org/10.1080/10498850.2019.1672845.
16. Nikoo, M.; Benjakul S.; Ehsani, A.; Li, J.; Wu, F.; Yang, N.; Xu, B.; Jin, Z.; Xu, X. Antioxidant and Cryoprotective Effects of A Tetrapeptide Isolated from Amur Sturgeon Skin Gelatin. *J Funct Foods* **2014**, *7*, 609–620, https://doi.org/10.1016/j.jff.2013.12.024.
17. Razali, A.N.; Amin, A.M.; Sarbon, N.M. Antioxidant Activity and Functional Properties of Fractionated Cobia Skin Gelatin Hydrolysate at Different Molecular Weight. *Int Food Res J* **2015**, *22,* 651–660.
18. Lassoued, I.; Mora, L.; Nasri, R.; Jridi, M.; Toldra, F.; Aristoy, M.C.; Barkia, A.; Nasri, M. Characterization and Comparative Assessment of Antioxidant and ACE Inhibitory Activities of Thornback Ray Gelatin Hydrolysates. *J Funct Foods* **2015**, *13,* 225–238, https://doi.org/10.1016/j.jff.2014.12.042.
19. Abdelhedi, O.; Nasri, R.; Mora, L.; Toldrá, F.; Nasri, M.; Jridi, M. Collagenous Proteins from Black-Barred Halfbeak Skin as A Source of Gelatin and Bioactive Peptides. *Food Hydrocoll* **2017**, *70,* 123–133, https://doi.org/10.1016/j.foodhyd.2017.03.030.
20. Tkaczewska, J.; Bukowski, M.; Mak, P. Identification of Antioxidant Peptides In Enzymatic Hydrolysates of Carp (Cyprinus carpio) Skin Gelatin. *Molecules* **2018**, *24,* 1–15, https://doi.org/10.3390/molecules24010097.
21. Alemán, A.; Giménez, B.; Montero, P.; Gómez-Guillén, M. C. Antioxidant Activity of Several Marine Skin Gelatins. *LWT - Food Sci Technol* **2011**, *44*, 407–413, https://doi.org/10.1016/j.lwt.2010.09.003.
22. Qiu, Y.; Wang, Y.; Yang, X.; Zhao, Y.; Chi, C.; Wang, B. Gelatin and Antioxidant Peptides from Gelatin

Hydrolysate of Skipjack Tuna (Katsuwonus pelamis) Scales: Preparation, Identification and Activity Evaluation. *Mar Drugs* **2019,** *17,* 1–17, https://doi.org/10.3390/md17100565.

23. Mahmoodani, F.; Ardekani, V.S.; See, S.F.; Yusop, S.M.; Babji, A.S. Optimization and Physical Properties of Gelatin Extracted from Pangasius Catfish (Pangasius sutchi) bone. *J Food Sci Technol* **2014**, *51,* 3104–3113, https://doi.org/10.1007/s13197-012-0816-7.
24. Budiarto, B.R.; Mustopa, A.Z.; Idarmawan, T. Characterization of Partially Extracellular Proteases from Bekasam-Isolated Lactobacillus plantarum S31 and Its Application to Hydrolyze Skimmed-Milk with Antibacterial Property. *Int Food Res J* **2016**, *23*, 340–349.
25. Cheng, L.; Mu, W.; Jiang, B. Thermostable L-Arabinose Isomerase from Bacillus stearothermophilus IAM 11001 for D-Tagatose Production: Gene Cloning, Purification and Characterisation. *J Sci Food Agric* **2010**, *90,* 1327–1333, https://doi.org/10.1002/jsfa.3938.
26. Herlina, N.; Mustopa, A.Z.; Surachma, R.S.; Triratna, L.; Kartina, G.; Alfisyahrin, W.N. Aktivitas Antibakteri dan Antioksidan Peptida Susu Kambing Hasil Hidrolisis dengan Protease Lactobacillus plantarum S31 (Antibacterial and Antioxidant Activity of Goat Milk Peptide Hydrolyzed with Protease of Lactobacillus plantarum S31). *J Biol Indones* **2019**, *15*, 23–31.
27. Ramezanzade, L.; Hosseini, S.F.; Nikkhah, M.; Arab-Tehrany, E. Recovery of Bioactive Peptide Fractions from Rainbow Trout (Oncorhynchus mykiss) Processing Waste Hydrolysate. *Ecopersia* **2018**, *6,* 31–40.
28. Cai, L.; Wu, X.; Zhang, Y.; Li, X.; Ma, S. Purification and Characterization of Three Antioxidant Peptides from Protein Hydrolysate of Grass Carp (Ctenopharyngodon idella) Skin. *J Funct Foods* **2015**, *16,* 234–242, https://doi.org/10.1016/j.jff.2015.04.042.
29. Yang, X.; Zhao, Y.; Qiu, Y.; Chi, C.; Wang, B. Preparation and Characterization of Gelatin and Antioxidant Peptides from Gelatin Hydrolysate of Skipjack Tuna (Katsuwonus pelamis) Bone Stimulated by In Vitro Gastrointestinal Digestion. *Mar Drugs* **2019**, *17*, 1–18, https://doi.org/10.3390/md17020078.
30. Chi, C.; Wang, B.; Hu, F.; Wang, Y.; Zhang, B. Purification and Identification of Three Novel Antioxidant Peptides from Protein Hydrolysate of Bluefin Leatherjacket (Navodon septentrionalis) Skin. *Food Res Int* **2014**, *73,* 124–129, https://doi.org/10.1016/j.foodres.2014.08.038.
31. Atma, Y.; Lioe, H.N.; Prangdimurti, E.; Seftiono, H.; Taufik, M.; Fitriani, D.; Mustopa, A.Z. The Proportion-Ratio on Dipeptidyl Aminopeptidase-4 (DP-4) Inhibition by Gelatin Compared to Synthetic Sitagliptin. *J Immunoass Immunochem* **2019**, *40*, 386–395, https://doi.org/10.1080/15321819.2019.1613243.
32. Clarke, G.; Ting, K.N.; Wiart, C.; Fry, J. High Correlation of 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging, Ferric Reducing Activity Potential and Total Phenolics Content Indicates Redundancy in Use of All Three Assays to Screen for Antioxidant Activity of Extracts of Plants from The Malaysian Rainforest. *Antioxidants* **2013**, 2, 1–10, https://doi.org/10.3390/antiox2010001.
33. Baehaki, A.; Lestari, S.D.; Romadhoni, A.R. Hidrolisis Protein Ikan Patin Menggunakan Enzim Papain dan Aktivitas Antioksidan Hidrolisatnya. *J Pengolah Has Pertan Indones* **2015**, *18,* 230–239..
34. Chandrasekara, A.; Shahidi, F. Inhibitory Activities of Soluble and Bound Millet Seed Phenolics on Free Radicals and Reactive Oxygen Species. *J Agric Food Chem* **2011**, *59,* 428–436, https://doi.org/10.1021/jf103896z.
35. You, L.; Zhao, M.; Regenstein, J.M.; Ren, J. Purification and Identification of Antioxidative Peptides from Loach (Misgurnus anguillicaudatus) Protein Hydrolysate by Consecutive Chromatography and Electrospray Ionization-Mass Spectrometry. *Food Res Int* **2010**, *43,* 1167–1173, https://doi.org/10.1016/j.foodres.2010.02.009.
36. Atma, Y.; Lioe, H. N.; Prangdimurti, E.; Seftiono, H.; Taufik, M.; Mustopa, A. Z. Dipeptidyl Peptidase IV (DPP-IV) Inhibitory Activity of Ultrafiltration and Gel Filtration Fractions of Gelatin Hydrolysates Derived from Bone of Fish for Antidiabetes. *Int J Adv Sci Eng Inf Technol* **2019**, *9*, 2096–2103, https://doi.org/10.18517/ijaseit.9.6.10658.
37. He, F. BCA (Bicinchoninic Acid) Protein Assay Fanglian. Available online: https://bio-protocol.org/pdf/bio-protocol44.pdf (accessed on 18 Februari 2020)
38. Kusumaningtyas, E.; Nurilmala, M.; Sibarani, D. Antioxidant and Antifungal Activities of Collagen Hydrolysates from Skin of Milkfish (Chanos chanos) Hydrolyzed Using Various Bacillus Proteases. *IOP Conf Ser Earth Environ Sci* **2019**, *278,* 1–7, https://doi.org/10.1088/1755-1315/278/1/012040.
39. Hassan, A.; Ullah, H.; Israr M. The Antioxidant Activity and Phytochemical Analysis of Medicinal Plant Veronica biloba. *Lett. Appl. NanoBioScience* **2019**, 8,732–738, 2019, doi: 10.33263/lianbs84.732738.