Collagen peptides from *Pangasius* fish skin as antioxidants

N Azizah¹, Y Ochiai² and M Nurilmala*¹

¹ Department of Aquatic Product Technology, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University (IPB University), Indonesia
² Graduate School of Agricultural Science, Tohoku University, Japan

*E-mail: mnurilmala@ipb.ac.id

**Abstract.** Fish skin can be extracted and hydrolyzed into collagen peptide to enhance the antioxidant activity. The research objectives were to determine the characteristics of collagen peptides from pangasius fish skin as antioxidants. The fish skin was extracted and hydrolyzed using 2% Alcalase and fractionated using a molecular sieve membrane. The gelatin yield was 15.07±1.45% against the starting material. The pH value of gelatin was 4.76±0.02. SDS-PAGE showed β band 221.3 kDa, α1 band 137.1 kDa and α2 band 117.7 kDa. The hydrolysis of gelatin resulted in the degree of hydrolysis of 48.06±1.97% with Alcalase at 55°C for 180 min. The total antioxidant activity (TEAC) was calculated with ABTS assays. The TEAC values were 24.53±0.78 μmol TE/g for the gelatin, 44.40±0.13 μmol TE/g for the peptides, 45.07±0.12 μmol TE/g for the >30 kDa fraction, 45.85±0.04 μmol TE/g for the 10-30 kDa fraction, 45.93±0.04 μmol TE/g for the 3-10 kDa fraction, and 45.98±0.04 μmol TE/g for the <3 kDa fraction.

**Keywords:** antioxidant, collagen peptide, extraction, fractionation, hydrolysis

1. Introduction

*Pangasius* (*Pangasius hypophthalmus*) is one of the most well-known freshwater fish in Indonesia. Indonesia is the second-largest country as a producer of pangasius products. According to the Ministry of Marine Affairs and Fisheries Republic of Indonesia, pangasius production in 2017 was 578,344 tonnes (KKP 2018). Pangasius skin is about 6% of the whole fish body weight and becomes a substantial raw material for production of collagen (Mahmoodani et al 2014).

Collagen is a biomaterial of animal protein which is a major component of connective tissue. Pretreatment of collagen with acid or alkali and heating at high temperatures will cause the structure of the triple helix polypeptide chain to be denatured to form gelatin. Most commercial gelatin is presently from pig and bovine sources, thus gelatin from fish skin is an alternative source of halal gelatin (Karim and Bhat 2009). Gelatin can be increased its biological activity by hydrolysis to collagen peptides (Song et al 2017).

Collagen peptides have several biological activities including anticancer, antihypertensive, and antioxidants (Aleman et al 2011). The antioxidant activity of collagen peptides can be influenced by...
the molecular weight of peptides. Previous studies have shown that peptides from squid skin with small molecular weights produce high antioxidant activity (Nam et al 2008). Therefore, the research objectives were to determine characteristics of collagen peptides from pangasius fish skin as antioxidants.

2. Materials and methods

2.1. Materials
Pangasius (P. hypophthalmus) skins were kindly obtained from a pangasius fillet company in Karawang, Jawa Barat, Indonesia, and chilled on ice while transporting to the laboratory. Other materials used are NaOH (Merck, New Jersey, USA), sulfuric acid (Merck, New Jersey, USA), citric acid (Cap Gajah), deionized water, and Alcalase 2.4 units/g (Sigma-Aldrich, St. Louis, USA).

2.2. Extraction of gelatin
The method was modified from Zhang et al (2016). The pangasius skin was cut in a square shape (1×1 cm) and washed with tap water. The skin was soaked in 0.25% citric acid with the skin:solution ratio of 1:4 (w/v) for 12 h. The skin washed by using deionized water (DI), then extracted using deionized water with the skin:solution ratio of 1:1 (w/v) at 65°C for 7 h. The resulting of the extraction process was filtered through double-layered cheesecloth so it becomes gelatin solution. The gelatin solution was dried in the oven at 50°C for 15 h so it becomes gelatin powder.

2.3. Hydrolysis of gelatin
The method was modified from Chalamaiah et al (2014). The gelatin solution (6.67%, w/v) was hydrolyzed by Alcalase (pH 8, 55°C) with a ratio for the enzyme: substrate was 2% (w/v) for 3 h. The collagen peptide was frozen for 15 min to inactivate the enzyme. Finally, the collagen peptide was centrifuged at 6,000×g for 30 min at 4°C. The collagen peptide was freeze-dried and stored at -20°C.

2.4. Fractionation of collagen peptides
The method was modified from Kusumaningtyas et al (2015). The collagen peptides fractionated through a 30 kDa, 10 kDa, and 3 kDa molecular weight cut off (MWCO) membrane and were centrifuged at 7,500×g for 10, 15 and 40 min, respectively. These four collagen peptide fractions (>30, 10-30, 3-10, <3 kDa) were freeze-dried and stored at -20°C.

2.5. Proximate composition
The proximate composition such as moisture, ash, fat, and protein in the pangasius skin was determined according the methods described by AOAC (2005).

2.6. Yield
The gelatin yield was determined by the equation:

$$\text{Gelatin yield (\%) = \frac{\text{Weight of dried gelatin (g)}}{\text{Weight of wet skin (g)}} \times 100}$$

(1)

2.7. pH measurement
The pH measurement of gelatin solution 6.67% (w/v) (GMIA 2012) was determined by using a pH meter (T9000, WalkLab, UK). The pH meter electrode was dipped into the sample for a while until a stable number is obtained on the pH meter monitor.

2.8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
The method was modified from Laemmli (1970). The SDS-PAGE analysis used a Mini Protean TGX Precast Gels (BioRad Laboratories, CA, USA) for the gelatin sample. About 2 mg of gelatin sample
was dissolved in 1 mL SDS 5%, heated at 85°C for 1 h, then centrifuged for 5 minutes at a speed of 12,400×g, then taken 20 μL and added a sample buffer in a ratio of 1:1 (v/v), then heated at 85°C for 10 min before loaded onto the well. About 15 μL gelatin sample was put into a polyacrylamide gel. Electrophoresis was carried out at a constant voltage of 75 V for 15 min and 150 V for 1 h. Gel staining was done by immersing the gel in a silver staining kit solution (BioRad Laboratories, CA, USA). Protein bands showed the molecular weight of gelatin and were analyzed using Software Photocapt.

2.9. Degree of hydrolysis
The method was modified from Adler-Nissen (1986). About 20 mL of collagen peptide was added to 20 mL of 20% trichloroacetic acid (TCA) (w/v) to produce TCA soluble material. The mixture was allowed to stand for 30 minutes so that precipitation occurs, then centrifuged at a speed of 6,000×g for 30 minutes. The resulting supernatant was analyzed its nitrogen content by the Kjeldahl method (AOAC 2005). The degree of hydrolysis was determined by the equation:

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DH (%) = \frac{\text{Soluble N in TCA 20% (w/v)}}{\text{Total N in the sample}} \times 100
\]  

(2)

2.10. Antioxidant activity
The method was modified from Hazra et al (2008). The ABTS solution was made by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulphate. The oxidation reaction was carried out under dark conditions for 12-16 h. The solution was further diluted with deionized water to obtain an absorbance of 0.70±0.02 units at a wavelength of 734 nm. About 50 μL sample and 150 μL ABTS solution were put into a microplate well then incubated for 6 min at 37°C in a microplate. Absorbance measurements were carried out at a wavelength of 734 nm with an ELISA plate reader. Standard curves for measuring total antioxidant activity using trolox with concentrations of 10, 20, 30, 40, 50, 60 μg/mL. The percentage inhibition of absorbance was calculated to determine the trolox equivalent antioxidant concentration (TEAC).

3. Results and discussion

3.1. Proximate composition
The moisture content of pangasius fish skin was 39.08±0.6%. The ash content was 0.26±0.02%, while the lipid content was 12.33±0.23%. The crude protein content was 39.75±0.12%, higher than those of Pangasius sutchi skin 30.91±0.28% (Mahmoodani et al 2014) and Chanos chanos 23.74% (Perceka et al 2015). The crude protein content of the collagenous material represented the maximum possible yield of gelatin.

3.2. Yield
The yield of extracted pangasius skin gelatin was 15.07±1.45% against the starting material, a value is comparable with that (6-19%) reported by Karim and Bhat (2009). See et al (2010) reported that the yield was 10.78% when P. sutchi skin was extracted at 45°C for 18 h. The yield value can be influenced by several factors including the type of raw material, the pretreatment process, and the extraction temperature.

3.3. pH value
The pH value of pangasius skin gelatin was 4.76±0.02. See et al (2010) reported that the pH value of snakehead gelatin was 5.39±0.02 and red tilapia gelatin was 5.50±0.02. The pH value of gelatin was influenced by the type of immersion solution and the concentration used to extract the gelatin. The pH value of this pangasius skin gelatin was corresponding with the Gelatin Manufacturers Institute of America (2012) for edible gelatin, which is around 3.8-5.5 (GMIA 2012). It could be concluded that the gelatin from pangasius skin could be applied for food industry.
3.4. **SDS-PAGE**

The molecular weight measurements of gelatin using SDS-PAGE namely the presence of β, α₁ and α₂ bands. As shown in figure 1, the molecular weight of the β band is 210 kDa, the α₁ band is 137.057 kDa and the α₂ band is 104.470 kDa. Nurilmala *et al* (2017) reported the results of SDS-PAGE gelatin yellowfin tuna skin extracted at 65°C has a molecular weight of β component was 250 kDa, component α₁ was 129.670 kDa and component α₂ was 116.364 kDa. The β and α bands are the dominant components in gelatin whose chains of the two components are increasingly degraded with increasing temperature and extraction time.

![Figure 1](image)

**Figure 1.** Results of SDS-PAGE. (M) molecular weight standard and (G) gelatin.

3.5. **Degree of hydrolysis**

The hydrolysis of gelatin with Alcalase resulted in a degree of hydrolysis of 48.06±1.97% at 55°C for 180 min. It was higher than degree hydrolysis of *Oreochromis* spp 8.13% (Aziz *et al* 2014). The higher DH indicates that more protein can be broken down into small molecules. The DH of a peptide is influenced by the type of enzyme used in hydrolysis, hydrolysis time, hydrolysis temperature and pH of hydrolysis. Alcalase is a protease enzyme from *Bacillus licheniformis* which has a high DH with a relatively short time under moderate pH conditions compared to neutral enzymes or other acids. Protease enzymes are used to obtain low molecular weight hydrolysates and high antioxidant activity.

3.6. **Antioxidant activity**

The antioxidant activity of gelatin, collagen peptide, and fractions (>30, 10-30, 3-10, <3 kDa) with ABTS assay are shown in figure 2. 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) is produced by reacting strong oxidizing agents (potassium persulphate) with ABTS. The principle of the ABTS method is the removal of cation colors because antioxidant compounds donate hydrogen to ABTS radicals (Hazra *et al* 2008).
Figure 2. Results of total antioxidant activity with ABTS assays. The letters indicate significant differences (p <0.05).

The TEAC values demonstrated that gelatin, collagen peptide, and fractions (>30, 10-30, 3-10, <3 kDa) are potent antioxidants. The <3 kDa fraction was the highest in the total antioxidant activity. A previous study by Hizbullah (2018) showed that the <3 kDa fraction was the highest antioxidant activity (IC\textsubscript{50} 30 µg/L) and Nurilmala et al (2019) showed that collagen peptide fraction was the highest antioxidant activity (IC\textsubscript{50} 113.66 µg/L). The present data also shows a significant difference (p <0.05) for all the samples. The molecular weight of peptide is responsible for antioxidant activity, thus lower molecular weight fractions possess higher antioxidant activity.

4. Conclusion

Fish skin can be extracted and hydrolyzed into collagen peptide. The collagen peptide from pangasius skin has the potential to expand its health beneficial effect in the food industry. The molecular weight of collagen peptide fractions is closely related to antioxidant activity, thus lower molecular weight fractions possess higher antioxidant activity.

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