The hydroxyproline content of fish bone gelatin from Indonesian *Pangasius catfish* by enzymatic hydrolysis for producing the bioactive peptide

YONI ATMA1,*, HANIFAH NURYANI LIOE2**, ENDANG PRANGDIMURTI2, HERMAWAN SEFITIONO3, MOH. TAUFIK1, DITA FITRIANI1, APON ZAENAL MUSTOPA3

1Department of Food Science and Technology, Faculty of Bioindustry, Universitas Trilogi. Jl. Taman Makam Pahlawan Kalibata, Jakarta 12760, Indonesia. Tel./fax.: +62-21-7980011, *email: yoniatma@trilogi.ac.id
2Department of Food Science and Technology, Faculty of Agricultural Engineering, Institut Pertanian Bogor. Jl. Raya Dramaga, Bogor 16680, West Java, Indonesia. **email: hanifahloie@agps.ipb.ac.id
3Research Center for Biotechnology, Indonesian Institute of Sciences. Jl. Raya Jakarta-Bogor Km 46, Cibinong, Bogor 16912, West Java, Indonesia

Abstract. Atma Y, Lioe HN, Prangdimurti E, Seftiono H, Tahufik M, Fitriani D, Mustopa AZ. 2018. The hydroxyproline content on fish bone gelatin from Indonesian Pangasius catfish by enzymatic hydrolysis for producing of bioactive peptide. Biofarmasi J Nat Prod Biochem 16: 64-68. Gelatins has been widely used in food, medicines, cosmetics, photography industries. In the food industry, gelatin used as food additives and functional foods. The applications of gelatin as a functional food due to their bioactivity in a form of peptides. Bioactive peptides from gelatin are mostly obtained through enzymatic hydrolysis processes. This study was conducted to measure the hydroxyproline content of gelatin bone of Indonesian Pangasius catfish (*Pangasius sutchi*) before and after enzymatic hydrolysis. The hydroxyproline is one of the dominant amino acids in gelatin. Gelatin hydrolysis was carried out using a flavourzyme at concentration of 6% and the incubation series of 0, 4, 6 dan 8 hours. The standard concentration of hydroxyproline was used in range of 0-1 µg. The results showed that the linear curve of the hydroxyproline standard solution was y=0.0554x+0.0406, with the coefficient of determination (R²) = 0.9435. The incubation time of enzymatic hydrolysis (6% enzyme concentration) affected the hydroxyproline content. The hydroxyproline from fish bone gelatin was 18.91±2.87 mg/mL, 63.81±1.28 mg/mL, 46.21±1.28 mg/mL and 37.64 ± 0.64 mg/mL respectively during 0, 4, 6 dan 8 h incubation time. This hydroxyproline content was significantly different at 95% confidence level from each treatment time.

Keywords: Bioactive peptide, enzymatic hydrolysis, fishbone gelatin, hydroxyproline

INTRODUCTION

Gelatin is a collagen hydrolysate with molecular weight ranging from 97 kDa until >250 kDa in which it is produced in acid or alkaline condition. It has been used in various industries including food, medicines, cosmetic and photographs (Mariod and Fadul 2013). In food industries, gelatin was used as food additives and functional food. As food additive, gelatin was used as emulsifier, stabilizer, gelling former, thickener, adhesive agent and biofilm. While as functional food, gelatin has been developed in form of peptide for antidiabetic, antimicrobial, antioxidant and antihypertensive (Gómez-Guillén et al. 2011).

The research regarding on gelatin as a functional food has been growing rapidly especially about fish-based gelatin utilization (Aleman et al. 2011; Koli et al. 2014; Nikoo et al. 2014). It is because the fish based gelatin could be potential to replace the mammalian gelatin, which is the most source of gelatin however unacceptable due to religion, sociocultural and health aspect consideration (Nurul and Sarbon 2015). Many previous studies have been reported that the fish gelatins have their bioactivities, for example, the gelatin from salmon, hake, halibut, nila tilapia, pangasius catfish and etc (Li-Chan et al. 2012; Mahmoodani et al. 2014a; Wang et al. 2015). Fortunately, most of fish based gelatin comes from by-product or waste of fish processing (Karayannakidis and Zotos 2016). Therefore, the utilization of this source could be promising in the future especially in Indonesia.

Indonesia is a country with wide area of water so that it provides the biodiversity in fisheries. There are two types of fish based on their habitat including warm-water fish and cold-water fish. In cases for gelatin source, it has been known that the gelatin from warm-water fish having superiority characteristic compared to the other one (Gómez-Guillén et al. 2009). One of the warm-water fish, which have the high production yield in Indonesia, is Pangasius catfish (*Pangasius sutchi*). In Indonesia, Pangasius catfish spread out in Sumatera and Kalimantan. In addition, the consumption and production rate of this fish has been increased every year. The Ministry of Marine Affairs and Fisheries targets the Pangasius catfish production in 2018 reaching 604.587 ton. It will inflict to the waste especially bone of fish, which contribute about 12.44% of the total fish weight. Based on previous studies, it concluded that gelatin from bone of Pangasius catfish was better compared to another fish based on gelatin in physicochemical characteristic. The gelatin from this...
source was also comparable with commercial bovine gelatin (Mahmoodani et al. 2014b)

The aim of this research was to analyze the hydroxyproline of gelatin from Indonesian Pangasius catfish (Pangasius sutchi) before and after hydrolysis enzymatic. This because most of bioactive peptide extraction was from gelatin resulted from gelatin hydrolyzation followed by hydroxyproline content measurement (Li-Chan et al. 2012; Mahmoodani et al. 2014a; Nikoo et al. 2014). The hydrolysis of gelatin is mostly and more efficient using the protease. The determination of this hydroxyproline content could become a basic knowledge to know and characterize the influence of hydrolysis toward hydroxyproline of fish bone gelatin from Pangasius catfish. It could also become a comparable study in hydroxyproline content of fish based gelatin in accordance with the bioactive peptide production.

**MATERIALS AND METHODS**

**Study area**

This experimental research was conducted in four stages including the gelatin extraction of Pangasius catfish, the process of fish bone gelatin hydrolysis, the measurement of the hydroxyproline content and data analysis. The fish bones used for gelatin extraction was Pangasius sutchi from Indonesian rivers in Riau province.

**Procedures**

**Gelatin extraction**

The gelatin extraction was done in two steps, i.e., pre-treatment and main extraction. The pre-treatment of fish bones was done by soaking them with a mild acid (citric acid) for 48 h. After the pre-treatment steps, the leached bone (ossein) was separated with pre-treatment solvent using centrifuge (Hettich, USA) at 4000 rpm for 15 minutes followed by main extraction steps using hot water (75°C) for 5 h. Afterward, the main extraction solvent was separated with liquid extract using filter paper (Whatman Grade 4, USA). Finally, this liquid extract namely liquid extract of fish bone gelatin was collected in a tube and stored at 4°C until further hydrolysis and analysis.

**Gelatin hydrolysis**

Fishbone gelatin obtained from gelatin extraction stages was firstly incubated at 50°C for 10 minutes. Subsequently, the flavourzyme (Sigma, Germany) was added with enzyme/substrate (E/S) with ratio of 6%. Afterward, this solution contained gelatin and flavourzyme (6%) was incubated for 4, 6 and 8 h. The hydrolysis enzymatic process was then stopped using hot water (100°C) for 10 min followed by soaking in cool water for 20 min. The last step, the hydrolysis gelatin was obtained by separation using centrifuge (Hitachi, Japan) at 1000 rpm for 15 min at temperature of 4°C. The supernatant called as fishbone gelatin was stored at 18°C for measurement analysis of their hydroxyproline content.

**Hydroxyproline analysis**

Hydroxyproline analysis of fish bone gelatin carried out as follows: preparation sample, reagent addition, and absorbance measurement as described by Koli et al. (2014). The preparation sample of gelatin was done by adding 12 N hydrogen chloride into sample followed by incubation at 100°C for 3 h. Afterward, the gelatin was filtered through filter paper Whatman no. 4. The filtrate gelatin was then added chloramine reagent containing 1.4% chloramine T (Biovision, USA) and oxidant buffer (Biovision, USA) in ratio of 1: 10. After the incubation for 5 min at room temperature, the sample of gelatin was added *p*-dimethylaminobenzaldehyde (DMAB) reagent containing 10% of DMAB concentration (Biovision, USA) and 60% of perchloric acid (Biovision, USA) in ratio 1: 1 (v/v). This solution was then incubated at 60°C for 90 min and continued with cooling for 2-3 min. At last, the solution was measured its absorbance using spectrophotometer (Thermo Multiskan, USA) at wavelength 540 nm. Hydroxyproline e kit (Biovision, USA) was used as standard for measuring the hydroxyproline content in fishbone gelatin before and after enzymatic hydrolysis. The concentration of hydroxyproline used in this analysis was 0-1 μg/well. The hydroxyproline quantification was done after the linear curve of hydroxyproline standard solution was obtained.

**Data analysis**

Data analysis was performed using one-way Analysis of Variance (ANOVA) at level 5% and continued with Tukey’s HSD (Honestly Significant Different) test or Tukey’s range test to determine the statistical analysis at the level of significant differences between data.

**RESULTS AND DISCUSSION**

**Fishbone gelatin hydrolysis**

There are some stages for production bioactive peptide from the gelatin including (i) gelatin extraction, (ii) gelatin hydrolysis, (iii) the hydrolysate filtration by ultrafiltration, (iv) gelatin purification and (v) analysis of the sequence after purification (Zhang et al. 2012; Li-Chan et al. 2012). The bioactivities of gelatin in each stage must be measured. In this research, a part of gelatin extraction and gelatin hydrolysis using bone of Pangasius catfish (Pangasius sutchi) as source of gelatin (without bioactivities analysis) was obtained. The most alternative source of gelatin came from fish processing products especially skin and bone. Based on previous studies, it showed that the gelatin from bone of Pangasius catfish had a better gelatin yield, ash composition compared to the commercial gelatin (Mahmoodani et al. 2014b). In addition, this source was also abundance in Indonesia. Figure 1 presents three series of stages to obtain the fishbone gelatin after hydrolysis.
The hydrolysis is a one of key part in producing bioactive peptide. The successful process of the hydrolysis would determine activities of peptide. Most of gelatin from fish processing products was hydrolyzed by enzymatic method. There are some enzymes has been used for hydrolysis of gelatin such as alcalase, flavourzyme, pepsin, trypsin, chymotrypsin, bromelain and papain (Choonpicharn et al. 2014; Himaya et al. 2012; Zhang et al. 2012; Li-Chan et al. 2012; Li-Chan et al. 2012; Wang et al. 2015). The research of Li-Chan et al. (2012) concluded that the gelatin from fish processing products was the best in their bioactivities after they were hydrolyzed using flavourzyme. Some fish-based gelatin hydrolyzed using flavourzyme in order to obtain the bioactive peptides was gelatin from skin of tun, nila tilapia and salmon (Atma 2016).

**Hydroxyproline content**

The hydroxyproline is one of dominant amino acid in gelatin besides glycine, proline, alanine and glutamic acid (Atma 2017). The hydroxyproline was analyzed to determine the extraction yield, representing the successful process of the extraction in gelatin production (Mahmoodani et al. 2014b; Sanei et al. 2013). Recently, most of research regarding with gelatin extraction from fish skin, bone, head or others part usually quantified their hydroxyproline content. It is because this amino acid has been unique and become differentiator between gelatin and others protein (Taheri et al. 2009; Sanei et al. 2013). In this study, the hydroxyproline content of fish bone gelatin from Pangasius catfish before and after enzymatically hydrolysis was analyzed. The quantification of hydroxyproline was done to know the correlation of the influence enzymatic hydrolysis of fish bone gelatin toward hydroxyproline concentration.

In the quantification of hydroxyproline, the series of hydroxyproline standard solution was set up. It is to quantify the linear curve for determining the relation between hydroxyproline absorbance and hydroxyproline concentration. In this research, the standard concentration of hydroxyproline was in range of 0-1 µg and the linear curve of the hydroxyproline standard solution was $y=0.0554x+0.0406$ with the coefficient of determination $(R^2) = 0.9435$. Figure 2 presents the standard curve of hydroxyproline in concentration ranging from 0-1 µg.

Based on linear equation of hydroxyproline standard curve, the content of hydroxyproline in sample gelatin was quantified. The y value is the absorbance at 560 nm wavelengths, while the x is the quantified concentration of hydroxyproline. Table 1 presents the hydroxyproline content of gelatin before hydrolysis process was lower compared to that after hydrolysis process. In this research, the fishbone gelatin was hydrolyzed using 6% flavourzyme and incubated for 4 h had a higher hydroxyproline content compared to another fishbone gelatin.

![Figure 2. The standard curve for absorbance at wavelength 560 nm of hydroxyproline in concentration range 0-1 µg](image)

Table 1. The hydroxyproline content on fish bone gelatin from Pangasius catfish (Pangasius suirchi) by different time of incubation during enzymatic hydrolysis

<table>
<thead>
<tr>
<th>Hydrolysis incubation time</th>
<th>Hydroxyproline content (mg/mL)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h (before hydrolysis)</td>
<td>16.88</td>
<td>20.94</td>
</tr>
<tr>
<td>4 h</td>
<td>62.91</td>
<td>64.71</td>
</tr>
<tr>
<td>6 h</td>
<td>45.31</td>
<td>47.11</td>
</tr>
<tr>
<td>8 h</td>
<td>37.18</td>
<td>38.09</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Average</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>37.65±0.64 d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>47.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>64.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.94</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Average are mean ± standard deviation from twice analysis. Value of average in row bearing different letter are significantly different at p < 0.05

Discussion

The hydroxyproline is an amino acid contained in fishbone gelatin around 5.3-9.6% (Atma 2017). The study conducted by Mahmoodani et al. (2014b) stated that fishbone gelatin from Pangasius catfish contained hydroxyproline 5.97 g/100 g. It means around 5970 mg/mL hydroxyproline was obtained from the fishbone gelatin of Pangasius catfish. In this study, the gelatin from Pangasius catfish contained hydroxyproline about 18.91 mg/mL. However, the extraction method in the research conducted by Mahmoodani et al. (2014b) was using hydrochloric acid (strong acid) in the pre-treatment, while in this study was using citric acid in the pre-treatment. The extraction method of gelatin affected the yield of hydroxyproline. Therefore, the hydroxyproline content in the previous study was higher. Furthermore, Mahmoodani et al. (2014b) were optimizing the gelatin extraction by response surface methodology, where are in this studies no optimization is done. The optimize condition for gelatin extraction from Pangasius catfish bone obtained by pre-treatment using hydrochloric acid, however, this chemical utilization for extraction have been limited because of safety and environmental issues consideration.

In this study, the hydroxyproline content increased after the hydrolysis process. The higher concentration of hydroxyproline was on fish bone gelatin incubated for 4 h (63.81 mg/mL). The increase of hydroxyproline content probably caused by the hydrolysis of polypeptide chain in gelatin, so that it increased the detectable amount of hydroxyproline. If the incubation time was longer (6 and 8 h), the hydroxyproline content decreased. It might cause by the denaturation or another factor that influence the hydroxyproline in the gelatin solution for being detected. The hydroxyproline content of fish bone gelatin during various incubation times was significantly different (p < 0.05) in each treatment (Figure 3).

Overall, the hydroxyproline concentration in this research is different with other studies. Li-Chan et al. (2012) reported that the hydrolysis of gelatin derived from Atlantic Salmon skin using flavourzyme found that the gelatin contained the hydroxyproline with the concentration around 88.24 mg/mL. Furthermore, Li Chan et al. (2012) also used enzyme with the concentration of 6% enzyme-substrate [E/S] in order to obtain the gelatin hydrolysate with the inhibitory activity toward dipeptidyl peptidase IV (DPP-IV) as anti-diabetic treatment approach. Previously, Benjakul et al. (2009) also measured the hydroxyproline of gelatin from two species bigeye snapper, i.e., Priacanthus tayenus and Priacanthus macracanthus with the result of the hydroxyproline content in each species were 87.75 mg/mL. and 90.86 mg/mL, respectively. This has been done to characterize the gelatin from fish skin of bigeye snapper. Another study conducted by Sun et al. (2012) was conducted to analyze the antiphotoaging and antioxidant activity of gelatin from Tilapia (Oreochromis niloticus) using in vivo method and the hydroxyproline was quantified after the gelatin was feed to mice. Furthermore the hydroxyproline in mice after feeding was 50 mg/kg, 100 mg/kg and 200 mg/kg fish gelatin was 0.018 mg/mL, 0.020 mg/mL and 0.022 mg/mL respectively (Sun et al. 2012). The hydrolysis of fish skin gelatin in intestine of animal occurs due to the protease that contains in digestive tract (Tabata et al. 2017).

The hydroxyproline content of fish bone gelatin was affected by the enzymatic hydrolysis. The enzymatic hydrolysis increased the hydroxyproline content. The hydrolysis with flavourzyme (6% enzyme/substrate concentration) was better during 4 h incubation compared 6 and 8 h. Nevertheless, the most important in hydrolysis gelatin for producing the bioactive peptide is their bioactivities.

ACKNOWLEDGEMENTS

This study is part of Research Grant of Penelitian Kerjasama Antra Perguruan Tinggi (PKPT). We would like to acknowledge the Ministry of Research, Technology and Higher Education of the Republic of Indonesia (RISTEKDIKTI) for providing the research fund.

REFERENCES


Himaya SWA, Ngo D, Ryu B, Kim S. 2012. An active peptide purified from gastrointestinal enzyme hydrolysate of Pacific cod skin gelatin


