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Short Communication

Effects of Different Acetic Acid Immersion Time on the Properties of Collagen from Pangasius Skin

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Abstract

Pangasius sp. production in Indonesia has reached 384,310 tons in 2019. One of the main waste in pangasius fillet industry is the skin, which reached to 6% of body mass. Fish skins are alternative for making collagen because it has no restrictions for certain religions and ethnicities. Collagen is a protein biomaterial which acts as the main component of connective tissue. Extracting collagen using organic acids such as acetic acid is the most common extraction method. Herein, we report the effects of different immersion time of acetic acid to collagen properties from pangasius skin. In this study, pangasius skin was treated using 10% alcohol with the ratio 1:10 (w/v) for defatting and 0.1M NaOH with the ratio 1:10 (w/v) for eliminating non-collagenous protein. The immersion of 0.5M acetic acid was carried out on pangasius skin with the ratio of 1:20 (w/v), for 24, 48, and 72h at temperature less than 15°C. The sample was salted-out for 24 hours with NaCl until the concentration reached 2.5M, then the sample was freeze-dried at -40°C. This study investigated that longer immersion time affected the properties of pangasius skin collagen. Different immersion time significantly affected the yield of collagen from pangasius skin ($p<0.05$). Pangasius skin has a potential to be used as collagen raw material, and 72h of immersion time (P3) is suggested to produce pangasius skin collagen with the highest yield and protein content (6.15 % and 9.26 %). Production of collagen from the fish skin will contribute to increase pangasius waste valorization in pharmaceutical industry.

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1. Introduction

Pangasius production in Indonesia continues to increase and reached 384,310 tons in 2019 (KKP, 2021). The yield of pangasius fillet processing was about 45%, resulting in a large amount of waste according to Sathivel *et al.* (2012). Moreover, pangasius skin weighs up to 6% of the total body weight (Mahmoodani *et al*., 2014). Utilization of fish skin by-product as raw material to produce collagen has a potential to reduce waste while increasing the added value of the waste. The untapped value of pangasius skin waste products causes this material to have the potential for further exploration. This is in line with United Nation Sustainable Development Goals, SDGs12: Responsible Consumption and Production which aims to reduce global food waste at retail and consumer ends. In addition, fish skin and bones are sources of collagen that can be accepted by all consumers, be it Muslim, Hindu, or Jewish (Choi *et al*., 2013).

Collagen is an animal protein biomaterial which acts as the main component of connective tissue (Azizah *et al*., 2020; Yu *et al*., 2014; Fabella *et al*., 2018). Collagen plays an important role in various industries because it is easily absorbed by the body, non-toxic, biocompatible, relatively stable, can be produced in various forms as needed, and easily dissolved in water or acid (Ahmad *et al*., 2019; Lee *et al*., 2001; Singh *et al*., 2011). Fish skin and bones are acceptable sources of collagen for all consumers (Choi *et al*., 2013), because it has no restriction for certain religion and ethnicity unlike cow and pig. The advantages of fish collagen include having a lower melting point and higher viscosity, thus providing excellent affinity for the skin (Kim *et al*., 2013; Draelos and Thaman, 2006). Studies on collagen production using fish and its by-products has been conducted by researchers, on various fish types and extraction techniques (Normah and Afiqah, 2018; Tangkaa *et al*., 2020; Devita *et al*., 2021; Sahubawa and Putra, 2011).

Before the fish skin is processed into collagen, a degreasing process is needed or the removal of fat from the raw materials that can interfere with the collagen extraction process. However, this pre-treatment was rarely explained by previous studies. The degreasing process is carried out using organic solvent such as ethanol. Research from Sasmal and Begam (2014) showed that the degreasing process on sea-fish skins carried out using 15% butyl alcohol immersion takes up to 48 hours. One method to increase the efficiency of the degreasing process is using ultrasonic waves. Ultrasonic waves are able to break cell membranes in solids or materials so that the content within them can easily loosen up, come out, and dissolve in the solvent (Falleh *et al*., 2012).

Collagen extraction using organic acids is the most common type of extraction, one of which uses acetic acid. The use of acid facilitates the solubility of collagen due to the increase in H^+ ions (Wulandari *et al*., 2015). Extraction with acetic acid usually produces acid soluble collagen (ASC). Producing collagen from fish skin can result 5-30% of yield (Nurhayati and Astiana, 2018), depending on the extraction parameter, one of which is the extraction time. Therefore, the aim of this study is to investigate the effect of different acetic acid immersion time on the collagen composition of pre-treated pangasius skin.

2. Materials and Methods

2.1 Materials

Pangasius skin was obtained from a fish processing company in East Java, Indonesia. The pangasius skin samples were cleaned from the remains of dirt and meat. Samples were washed with running water 5 times, then washed with distilled water 3 times. After washing, the samples were cut to a size of 1x1 cm^2. Skins were stored in freezer (<-18°C) until further use.

2.2 Method

2.2.1 Extraction of collagen

Chemical reagents used to produce collagen from pangasius skin was glacial acetic acid (Fulltime, China), absolute ethanol (SmartLab, Indonesia), sodium hydroxide (Merck, Germany), sodium chloride (Merck, Germany), and tris (hydroxymethyl) aminomethane (Vivantis, Malaysia). Pre-treatment of samples were carried out to remove fat content using ultrasonic cleaner (Elmasonic S 10 H) and 25% ethanol for 10 minutes at temperature less than 15°C. The samples were then evaporated by air-drying to remove the solvent. After reducing the fat content on the pangasius skin, decolouring and removal of non-collagen proteins were carried out by using immersion method with NaOH solution. The concentration of NaOH for the immersion was 0.1M NaOH for 24 hours and then replaced once after 8 hours of incubation.

Extraction of acid soluble collagen (ASC) was carried out using a combination of different concentrations of acetic acid and immersion time. The pangasius skin samples from the previous process were immersed in 0.5M acetic acid with ratio of 1:20 for 24h (P1), 48h (P2), and 72h (P3) at chilling temperature in refrigerator. The sample was then filtered and the liquid precipitated with the addition of NaCl until the solution reaches a

concentration of 2.5M, and 0.05M TRIS buffer is added to neutralize the pH. The resulting precipitate was centrifuged (Hettich Zentrifugen EBA 20) for 15 minutes at a speed of 6000 rpm. The pellets were dried using a freeze dryer. The samples were then stored at 4°C temperature for further analysis. All treatments were conducted in three replicates.

2.2.2 Yield analysis

The yield of collagen were calculated based on weight of wet and freeze-dried sample (Normah and Afiqah, 2018) by the following equation:

$$\text{Collagen yield(wb)} = \frac{\text{weight of wet collagen(g)}}{\text{wet of weight skin(g)}} \text{ x } 100\%$$

$$\text{Collagen yield(db)} = \frac{\text{weight of freeze-dried collagen(g)}}{\text{wet of weight skin(g)}} \text{ x } 100\%$$

2.2.3 Moisture, ash, and crude protein content

The moisture, ash, and crude protein content of the collagen were determined through standard procedures. Moisture was determined by moisture analyzer (BEL i-Thermo 163L, India), to determine ash content, sample was placed in furnace at 550°C until the sample turns grey ash, and crude protein content was analyzed using Kjeldahl method including destruction, distillation, and titration. Analyses were calculated on wet basis of collagen.

2.2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-Page)

Collagen samples were prepared by dissolving in 1g/L SDS solution to a concentration of 10mg/ml. Then, the mixture was heated at 85°C for 1 hour in a water bath, and centrifuged at 6000 rpm for 5 minutes. The dissolved samples were then analyzed by SDS-PAGE using 5% stacking gel and 7.5% gel solvent, with a ratio of 20µl of sample in 5µl of loading buffer. The preparation of measurement molecular weight of protein was carried out with Coomassie Blue R250 and then staining by using ethanol and acetic acid for overnight (Laemmli, 1970)

2.3 Data Analysis

The research layout to determine the impact of extraction time and concentration of acetic acid on acid soluble collagen (ASC) was analysed with 2 element ANOVA using Microsoft Excel program version 16.59.

3. Results and Discussion

3.1 Yield

The yield show the contribution of economic value in the process of manufacture of a material or product. Collagen was produced using pangasius skin that has been pre-treated using 25% ethanol in ultrasonic waves for 10 min, as it was the best result of previous experiment (unpublished). Collagen produced using pre-treated skin yielded 4.83 – 6.15% (dry basis). Results showed that longer time of immersion in acetic acid significantly increased the yield of wet collagen made from pangasius skin ($p<0.05$) (Table 1).

3.2 Moisture, Ash, and Crude Protein Content

Different immersion time has no significant effect on ash content of collagen ($P>0.05$), while the 24h treatment significantly reducing the moisture content (Table 2). The highest protein content was obtained by immersing in acetic acid for 72h (P3).

3.3 SDS-Page Profile

Result of SDS-Page showed that protein bands of collagen from pangasius skin ranged from 130 to >180 kDa from 72h treatment (Figure 1).

3.4 Discussion

The acid-soluble collagen (ASC) from fish skin has yield of 5-30%, depending on the type of fish, solvent, and extraction technique (Nurhayati and Astiana, 2018). Data showed that longer immersion time increased the yield of collagen produced. This result was in line with results from Normah and Afiqah (2018) that extracted collagen from sin croaker waste using 0.5M acetic acid, resulting in higher collagen yield in 5 days extraction (3.35%) compared with 3 days extraction (2.84%). Suparno and Prasetyo (2019) also stated that different soaking time of acetic acid significantly affected the solubility of chicken feet collagen. Acid soluble collagen (ASC) yield obtained from pre-treated pangasius skin was higher than collagen from Makaira indica skin (1.12 – 4.77%), bigeye tuna skin (3.05%), and black tilapia skin (2.24 – 5.97%) (Tangkaa *et al*., 2020; Devita *et al*., 2021; Sahubawa and Putra, 2011). The low ASC yield resulted using acetic acid also can be caused by the difficulty of acetic acid to break the cross-linked collagen telopetide between aldehyde, lysine and hydroxylysine which causes difficulty of collagen to dissolve (Nurhayati and Astiana, 2018).

Table 1. Wet and dry basis of collagen from pangasius skin

Characteristics	24h	48h	72h
Yields (% wb)	59.74 ± 3.71[a]	74.41 ± 2.82[b]	89.93 ± 0.61[c]
Yields (% db)	4.83	5.45	6.15

Description: Means ± standard deviation of triplicate analysis. Same letter indicates no statistical difference among means ($p < 0.05$)

Table 2. Protein, ash, and moisture content of collagen from pangasius skin

Characteristics	24h	48h	72 h
Protein content (%)	8.31	8.35	9.26
Ash content (%)	0.22 ± 0.01[a]	0.23 ± 0.006[a]	0.22 ± 0.003[a]
Moisture content (%)	74.19 ± 2.06[a]	78.16 ± 1.21[b]	79.91±1.07[b]

Description: Means ± standard deviation of triplicate analysis. Same letter indicates no statistical difference among means ($p < 0.05$)

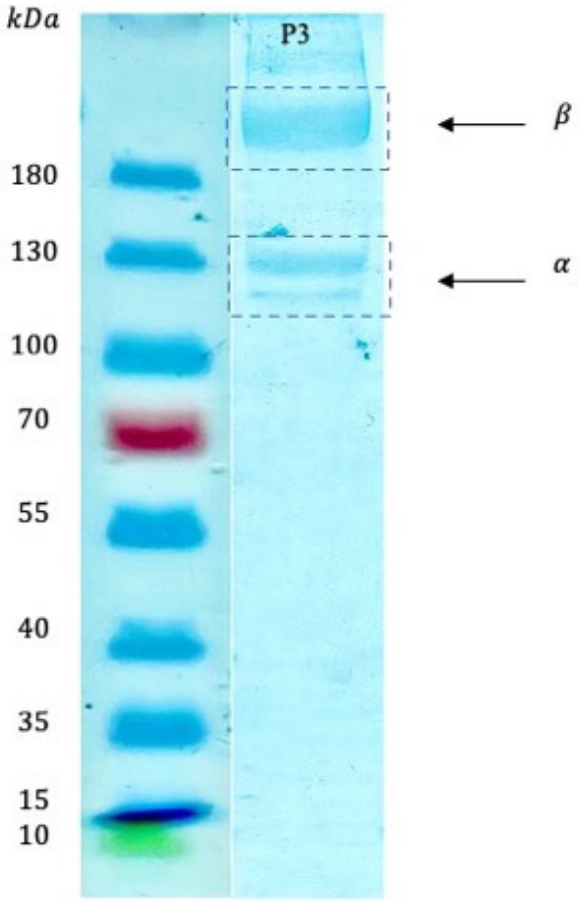

Figure 1. SDS-Page profile of pangasius skin collagen (72h of acetic acid immersion)

Protein content of pre-treated pangasius skin collagen in this research was slightly lower than the protein content of rainbow trout skin collagen (96.2%), mackerel skin collagen (87.74%) and sin croaker waste collagen (23.17%) (Tabarestani *et al*., 2012; Sonavane *et al*., 2018; Normah and Afiqah, 2018). The protein content of collagen with maceration for 24, 48 and 72 h were 8.31, 8.35 and 9.26%, respectively. The low protein content of collagen in this research was due to the sample used for proximate analysis, which is wet basis collagen that has high water content on the sample. Water content was higher due to swelling of pangasius skin, as the result of soaking in acetic acid. Soaking pangasius skin in acetic acid will cause the entry of water in collagen fiber as the result of the formation of hydrogen bonds between H^+ of acetic acid and non-polar group of the collagen (Jaswir *et al*., 2011). The formation of hydrogen bonds facilitates the extraction of collagen by disrupting the non-covalent bonds of collagen fiber (Suptijah *et al*., 2018).

The immersion time treatments were not affecting the molecular weight, as all samples (24h, 48h, and 72h) showed a similar range of molecular weight. Collagen is an extracellular matrix protein which consist of three helical chains. Type I collagen usually consist of identical alpha chains (α1 and α2) of around 100 kDa and a beta component of about 200 kDa (Kurien and Scofield, 2012). The result showed collagen from this research can be considered as type I collagen, as it con-

tain α and β bands of protein that is considered as main component of collagen (Figure 1). However, the clearest bands emerged within 72h treatment among other treatments. Poor, distorted, or fainted bands could be due to inadequate sample buffer-to-protein ratios (Sahubawa and Putra, 2011). Collagen from pangasius skin is a beta chains, which indicates cross-linked components that are difficult to be broken by acetic acid (Ogawa *et al*., 2004).

4. Conclusion

Different immersion time significantly affected the yield of collagen from pangasius skin ($P<0.05$), where P3 (72h of immersion time) showed the highest yield with wet basis 89.93 % and dry basis 6.15 %. Different immersion time did not significantly affect the ash content of collagen from pangasius skin ($p>0.05$), but the moisture content of P1 differed significantly. The immersion for 72 hours at chilling temperature (P3) showed the highest protein content among all treatments. The pangasius skin is a potential raw material to produce collagen for pharmaceutical industry. The treatment of 72h immersion time (P3) is suggested to produce pangasius skin collagen with high yield and protein content. The information gained from this study is beneficial to develop different methods used to extract skin collagen using organic solvent, and longer time is suggested to gain optimum yield and protein of collagen.

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Authors' Contributions

Clara Amelia Kusumawinahyu, Sharilla Aryananti Abidin; writing the article, analysis, collecting data. Patmawati, Mochammad Amin Alamsjah, Laksmi Sulmartiwi, Dwi Yuli Pudjiastuti, Dwitha Nirmala, Raseetha Vani Siva Manikam; supervision, devised the main conceptual ideas, writing the article, critical revision of the article.

Conflict of Interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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