

Gelatin extraction from the indigenous *Pangasius* catfish bone using pineapple liquid waste

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ABSTRACT Gelatin extraction from fish bone has traditionally involved hydrogen chloride and/or sodium hydroxide during pre-treatment. However, these chemicals have begun to be abandoned because of their associated safety and environmental issues. Several studies have looked at the use of citric acid as a safer alternative in fish bone gelatin extraction. The aim of this research was to extract gelatin from the bone of *Pangasius* catfish with pineapple liquid waste. The extraction was performed in two steps: pre-treatment followed by main extraction at various times (24–56 h) and temperatures (45–75°C). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used as a confirmation test and showed a band for gelatin at ~120 kDa. Gelatin yields were calculated as the ratio of weight of dried gelatin to the total weight of fish *ossein*. The results indicated that pineapple liquid waste can be used for fish bone gelatin extraction. The recommended conditions for extraction of fish bone gelatin using pineapple liquid waste are 56 h of pre-treatment and 5 h of main extraction at a temperature of 75°C. The gelatin yield was 6.12% and the protein concentration 4.00 g/100 g.

KEYWORDS extraction; fish bone; gelatin; green solvent; pineapple waste

1. Introduction

Gelatin is a polypeptide (biopolymer) that has been used widely in food, pharmaceutical and cosmetic industries. In the food industries, gelatin is used both as food additive and functional food (Mariod and Fadul 2013) since gelatin has unique physical characteristic. Gelatin is thus used as a stabilizer, thickener, emulsifier, adhesive, as a biodegradable film, as well as a foaming, gelling, and microencapsulation agent for foods product such as confectionary, jelly, milk, yoghurt, ice cream, cheese, and canned foods (Koli et al. 2012). Gelatin also has bioactive properties including antimicrobial or antioxidant properties and is antihypertensive by inhibition of angiotensin converting enzyme (ACE) (Gómez-Guillén et al. 2011).

In the past, most gelatin came from pigs (41%), cattle skin (28.5%) and cattle bone (29.5%) (Jeya Shakila et al. 2012). Gelatin made from pigs is prohibited in both Islam and Judaism, while gelatin from cattle origin is unacceptable in Hinduism. In addition, the presence of cases of mad cow disease or bovine spongiform encephalopathy (BSE) as well as nail and mouth disease are increasingly pushing the need for new alternative sources of gelatin (Sanaei et al. 2013). The most potential as alternative source of gelatin is shown by skin and bone of fish (Nurul and Sarbon 2015). Around 30% of the total weight of fish

comes from its skin and bones (Shyni et al. 2014). Fish skin is sometimes still used for consumption and further processing, but fish bones are often disposed of as waste. Utilization of fish bones as a source of gelatin can minimize waste and provide value-added fishery products.

Gelatin produced from warm-water fish has better thermostability, rheological and viscosity properties compared to cold-water fish (Gómez-Guillén et al. 2009). *Pangasius* catfish has a life habitat in the rivers and estuaries in Indonesia. Mahmoodani et al. (2014) reported that the physical characteristics of gelatin extracted from *Pangasius* catfish bones resembles gelatin from cattle bone. Only the ash content of gelatin from *Pangasius* catfish bone that confirm with standard of gelatin in proximate parameter compared another warm-water fishes (Atma 2017). Therefore, it is necessary to explore method of gelatin extraction from bone of *Pangasius* catfish.

Most previous researchers used hydrogen chloride and or sodium hydroxide in gelatin extraction from fish processing by-product (Karim and Bhat 2009). However, because of safety and environment concerns, as well as industrial needs, citric acid could be of interest as it can be used together with hot water in fish bone gelatin extraction. Many researchers tried to extract fish bone and skin gelatin using a safer solvent (Karayannakidis and Zotos 2016). This research aimed to extract fish bone gelatin from Pa-

ngasius catfish using pineapple liquid waste. Pineapple liquid waste contains citric acid at approximately 0.18–0.32% (Hajar et al. 2012). Imandi et al. (2008) reported that 1 kg of pineapple waste produced around 202.35 g citric acid. This research therefore aimed to replace harmful chemicals and utilize a readily available waste in gelatin extraction technology, as well as to minimize the waste of fisheries and agriculture, enabling an eco-friendly process in the future.

2. Materials and methods

2.1. Raw material and preparation

Bones of Pangasius catfish were obtained from the waste of a filleting industry in Cikarang, Bekasi, West Java. This by-product of Pangasius catfish filleting was transported to the laboratory on ice. In the laboratory, the Pangasius catfish bones were separated from other waste material such as the head, fin, scale and viscera. The bones were scraped with a knife and washed in warm water (60–70°C) for 30 min to remove attached flesh. Then, the fish bones were washed using tap water and stored in a freezer (-20°C). The solvent, pineapple liquid, was prepared by peeling pineapple fruit (*Ananas comosus*). The peel and stems (central core) of the pineapple were chopped into small pieces and blended. The resulting pulp was filtered through cheesecloth, and the obtained liquid extract, termed pineapple liquid waste, was sterilized at 121°C, 2 atm for 15 min. The pineapple liquid waste was stored at 4°C prior to the experiment.

2.2. Gelatin extraction

Gelatin extraction was performed in two steps, the first step being pre-treatment and second main extraction. During the pre-treatment step, the cleaned bones were minced in a meat grinder (Fomac MGD-G31, Taiwan) and then soaked in the pineapple liquid waste (1:5 w/v) at room temperature with varying time periods (24–56 h) for demineralization. The leached bones (*ossein*) were separated from the pre-treatment solvent (pineapple liquid waste) by centrifugation for 10 min at 10,000xg at 4°C (Hitachi CR21GIII, Japan). The *ossein* was then neutralized by washing it with distilled water until reaching pH 7. The neutralized *ossein* was transferred to an Erlenmeyer flask, and then distilled water was added at a ratio of 1:5 (bone/water, (w/v)) for the main extraction step. The main extraction was carried out for 5 h at various temperatures (45–75 °C). Finally, the gelatin extracted was filtered with a filter paper and refrigerated at 4 °C. The resulting extracted gelatin was dried at 55 °C for 24–48 h and stored until subsequent analyses.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The extracted fish bone gelatin was prepared for SDS-PAGE analysis according to the discontinuous

Tris/HCl/glycine buffer system method described by Laemmli (1970) with some modification. The SDS-PAGE was performed using a 10% separation gel and a 3.9% stacking gel composed of 0.5–1.5 M Tris HCl pH 6.8–8.8, 40% acrylamide, 10% ammonium persulfate (APS), tetramethylethylenediamine (TEMED), and distilled water. The sample and molecular weight marker proteins (PM 2700 SMOBIO, Taiwan) were each loaded at 20 µL and then run in a Mini Electrophoresis Set (ATTO WSE-1500MW PageRunAce, Japan) for 25 min at a constant current of 15 mA/gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 for 24 h. Destaining was carried out in a methanol/acetic acid/water solution (5:1:4, v/v/v) until the zones on the blue background were clear.

2.4. Determination of extraction yield

Protein concentration of fish bone gelatin was determined using a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific). Bovine serum albumin (BSA) (Thermo Fisher Scientific) was used as protein standard in range 0–2 mg/mL. A 10-µL sample and nine standard concentrations respectively were loaded in a 96-well plate. Then, 200 µL BCA reagent solution, containing reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tartrate in 0.1 M sodium hydroxide) and reagent B (copper (II) sulfate), was added at the ratio of 50:1 (v/v). The mixture was mixed for 30 s and incubated for 30 min. Absorbance was measured against a blank at 560 nm using a ELISA Micro-plate Reader (Multiskan Ex, Thermo Electron Corp. USA). The analysis was replicated three times.

2.5. Determination of gelatin yield

The gelatin yield was calculated as the ratio of weight dried fish bone gelatin to the total weight of leached bone (*ossein*) on wet basis (Mahmoodani et al. 2014) using the formula below:

$$\text{Yield of fish bone gelatin (\%)} = \frac{\text{Dry weight of fish bone gelatin (g)}}{\text{Wet weight of ossein (g)}} \times 100\%$$

3. Results and discussion

3.1. Molecular weight distribution of fish bone gelatin

Gelatin is a mixture of different polypeptides, α -chains, β -chains (dimers of α -chain) and γ -chains (trimers of α -chain) (da Trindade Alfaro et al. 2009). The α -chain gelatin has a molecular weight of ~120 kDa, β -chain gelatin of ~250 kDa and γ -chain gelatin has a molecular weight of > 250 kDa (Zhou and Regenstein 2006). The molecular weight distribution of fish bone gelatin from Pangasius catfish was analyzed using SDS-PAGE. The

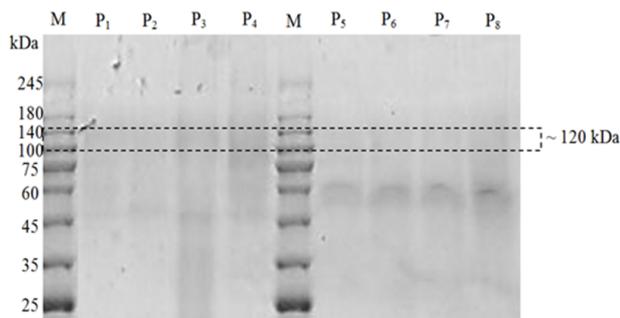


FIGURE 1 SDS-PAGE of fish bone gelatin from *Pangasius catfish* extracted using pineapple liquid waste. P₁, P₂, P₃, P₄ = pre-treatment 24, 36, 48 and 56 h, respectively at 45°C; P₅, P₆, P₇, P₈ = pre-treatment 24, 36, 48 and 56 h respectively at 55°C; M = broad protein marker.

broad molecular weight standard protein was used as the marker. These SDS-PAGE patterns of gelatin extracted with pineapple liquid waste treatment are shown in Figure 1 and Figure 2.

SDS-PAGE was used as a confirmation test for the presence of gelatin after extracting bones of *Pangasius catfish* using pineapple liquid waste. Figure 1 shows that gelatin molecular weight pattern was absent. This indicates that extraction with pre-treatment for 24–56 h and main extraction at 45–55°C was not successful. The longer pre-treatment time showed a strong protein band but not in the gelatin molecular weight range. Figure 2 shows that that the gelatin molecular weight pattern is noticeable when the extraction condition is 56 h pre-treatment and 75°C main extraction for 5 h. The extraction condition of 36 h pre-treatment and 75°C main extraction for 5 h also indicated a successful extraction. However, the band in the molecular weight gelatin area was stronger and more clearly visible at the extraction condition of 56 h pre-treatment.

A study by Zhang et al. (2011) revealed that the molecular weights of gelatin extracted from the grass carp fish scale were 117 kDa (α_1), 107 kDa (α_2), and 200 kDa

(β). The fish bone gelatin extracted from the king weakfish had a molecular weight ~100 kDa (α_2) and ~110 kDa (α_1). Gelatin with a higher content of α -chain usually gives a greater of yield compared with β -chain gelatin (da Trindade Alfaro et al. 2009). The fish bone gelatin extracted from the channel catfish had a molecular weight higher than 200 kDa, 100 kDa and less than 97 kDa (Liu et al. 2009). Lizardfish bones were found to have a higher molecular weight portion at 100–120 kDa, which was demonstrated by sharp bands (Taheri et al. 2009). The protein pattern of fish bone gelatin extracted from the red snapper and grouper was found at a molecular weight of > 200 kDa (β and γ chains) and around 130 kDa (α chain). Other authors or researchers have observed that lower molecular weights of gelatin were particularly influenced by high temperatures during extraction (Jeya Shakila et al. 2012). In addition, (Mahmoodani et al. 2014) optimized fish bone gelatin extraction from *Pangasius catfish* using chemical solvent. The electrophoresis pattern of fish bone gelatin from *Pangasius catfish* corresponding to α -chain gelatin as demonstrated by intense bands ~116 kDa. It also showed the presence of β -chain gelatin of ~200 kDa as well as some lower band in around 97 kDa (Mahmoodani et al. 2014).

3.2. Extraction yield

The extraction yield was used to determine both the gelatin recovery and the gelatin purity obtained from the extraction process. This determination is thus important because it will indicate whether fish bone extraction is successful and effective. Some factors that influence the extraction yield are prior treatment of the raw material, concentration of pre-treatment solvent, time of pre-treatment, as well as time and temperature of main extraction (Mariod and Fadul 2013; Sanaei et al. 2013). The high of protein concentration obtained in the process described here implies adequate and suitable extraction conditions. The extraction yield or protein quantification from this study is compared with other studies in Table 1.

TABLE 1 The extraction yield of fish bone gelatin with some kind solvent extraction.

No.	Source of fish bone gelatin	Solvent for extraction	Extraction yield/protein recovery	Reference
1	Indigenous <i>Pangasius catfish</i>	Pineapple liquid waste	4.00 g/100 g	This study
2	<i>Pangasius catfish</i>	Hydrogen chloride	68.75 g/100 g	Mahmoodani et al. (2014)
3	Catfish	Hydrogen chloride	60.54 g/100 g	Sanaei et al. (2013)
4	Red snapper	Sodium hydroxide	13.00 g/100 g	Jeya Shakila et al. (2012)
5	Grouper fish	Sodium hydroxide	15.00 g/100 g	Jeya Shakila et al. (2012)
6	Tiger-toothed croaker	Combined (sodium hydroxide, sulfuric acid, citric acid)	0.77 g/100 g	Koli et al. (2012)
7	Pink perch	Combined (sodium hydroxide, sulfuric acid, citric acid)	0.74 g/100 g	Koli et al. (2012)
8	Lizardfish	Sodium hydroxide	21.28 g/100 g	Taheri et al. (2009)
9	King weakfish	Sodium hydroxide	14.80 g/100 g	da Trindade Alfaro et al. (2009)

TABLE 2 Fish bone gelatin yield from different fish species.

No.	Fish bone gelatin source	Gelatin yield (%)	Reference
1	Indigenous Pangasius catfish	6.12	This study
2	Pangasius catfish	13.86	Mahmoodani et al. (2014)
3	Catfish	17.52	Sanaei et al. (2013)
4	Red snapper	9.14	Jeya Shakila et al. (2012)
5	Grouper fish	13.66	Jeya Shakila et al. (2012)
6	Tiger-toothed croaker	4.57	Koli et al. (2012)
7	Pink perch	3.55	Koli et al. (2012)
8	Lizardfish	8.90	Taheri et al. (2009)
9	King weakfish	8.20	da Trindade Alfaro et al. (2009)
10	Channel catfish	8.43	Liu et al. (2009)
11	Nila perch	2.40	Muyonga et al. (2004)

The extraction yield of Pangasius catfish and catfish was measured by the hydroxyproline assay method and then predicted using response surface methodology (RSM) (Sanaei et al. 2013; Mahmoodani et al. 2014). The extraction yield of fish bone gelatin from red snapper and grouper fish was determined by Kjeldahl conventional method using Kel plus Analyzer (Jeya Shakila et al. 2012). (Koli et al. 2012) measured extraction yield of fish bone gelatin from the tiger-toothed croaker and pink perch by the hydroxyproline assay method. The extraction yield of fish bone gelatin from lizardfish and king weakfish was determined with the Kjeldahl method by Assn. of Official Analytical Chemist (AOAC) (da Trindade Alfaro et al. 2009; Taheri et al. 2009). The extraction yields illustrated in Table 1 were thus obtained using different method for analysis. Nevertheless the same goal was to obtain protein concentration. A slight effect of the methods used, which should not significant though, is undeniable.

3.3. Gelatin yield

The gelatin yield indicates the weight of real gelatin, which is obtained from the raw material, in this case fish

bone. The fish bone gelatin yield of Pangasius catfish extracted using pineapple liquid waste in this research was 6.12%. Fish bone gelatin yields have been reported to vary among difference species. It is due to difference of gelatin or collagen content between species and diverse pre-treatment and main extraction methods (Muyonga et al. 2004; Jongjareonrak et al. 2006). The comparison of fish bone gelatin yield is presented in Table 2.

According to da Trindade Alfaro et al. (2009), gelatin yield increases as temperature, time and solvent extraction concentration increase. While these conditions are favorable for higher gelatin yields, they also cause greater chain degradation and hydrolysis. Chain degradation and hydrolysis will in turn decrease the gel strength, which is an important physical characteristic of gelatin (Sanaei et al. 2013). In addition, the variation of gelatin yield obtained from different fish bone gelatin sources may be due to a loss of gelatin or collagen during extraction, leaching during washing phase and incomplete protein or collagen denaturation or hydrolysis during the extraction process (Jeya Shakila et al. 2012). Koli et al. (2012) observed and reported that the optimal swelling of bone during the pre-

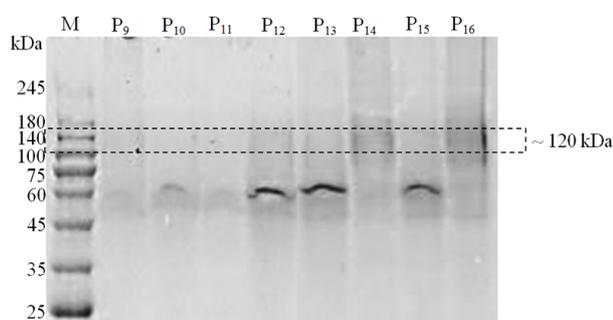


FIGURE 2 SDS-PAGE of fish bone gelatin from Pangasius catfish extracted using pineapple liquid waste. P₉, P₁₀, P₁₁, P₁₂ = pre-treatment 24, 36, 48 and 56 h, respectively at 65°C; P₁₃, P₁₄, P₁₅, P₁₆ = pre-treatment 24, 36, 48 and 56 h, respectively at 75°C; M = broad protein marker.

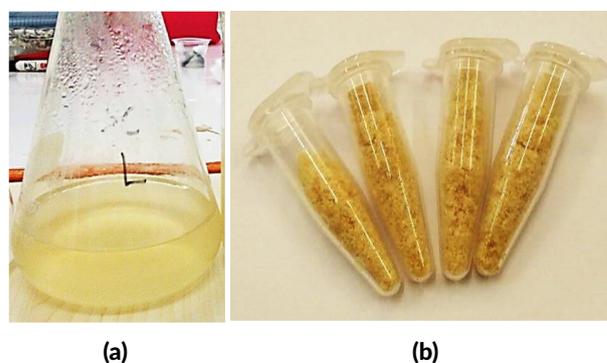


FIGURE 3 The liquid fish bone gelatin filtered after extraction (a) and dried powder of fish bone gelatin (b) from Pangasius catfish extracted using pineapple liquid waste.

treatment step correlates with higher yields of fish bone gelatin due to opening the cross-linking of collagen or protein. A high degree of cross-linking by covalent bonds in the collagen or protein source can cause low solubility and extractable gelatin.

4. Conclusions

The optimal extraction conditions to extract fish bone gelatin from *Pangasius catfish* bones using pineapple liquid waste were 56 h pre-treatment, and 5 h of main extraction at a temperature 75°C. The gel electrophoresis pattern showed that fish bone gelatin had a noticeable band at a molecular weight of ~120 kDa, indicating the α -chain. The extraction yield of fish bone gelatin using pineapple liquid waste was 4.00 g/100 g. In addition, the gelatin yield of fish bone gelatin from *Pangasius catfish* was 6.12%. Pineapple liquid waste can thus be recommended as a solvent for gelatin extraction that can contribute to a safer and greener environment.

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Authors' contributions

YA and HR designed the study. YA conducted all the laboratory work, data analysis and wrote the manuscript. HR reviewed the study and manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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