EXTRACTION OF COLLAGEN AND GELATIN FROM THE SCALES OF DIFFERENT FRESHWATER FISH

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ABSTRACT

Introduction: Collagen and gelatin are essential protein in vertebrates and extensively used in various industries. **Methods:** In this study, acid-solubilized collagen and gelatin were extracted from the scales of three different species of freshwater fish namely Kelah (*Tombroides*), Tilapia (*Oreochromis niloticus*) and Snakehead fish (*Channidae*) and then further quantified using Bradford assay and separated by molecular weight using SDS-PAGE. **Results:** The extracted collagen in Tilapia fish scale was found to be the highest with 0.018 of protein absorbance among the other three fish; Kelah fish (0.017) and Snakehead fish (0.011). For gelatin, Snakehead fish scales showed the highest amount of total protein concentration followed by Tilapia and Kelah fish with 0.467, 0.144 and 0.037 µg/µL per g, respectively. Based on the SDS-PAGE results, collagen from all the three freshwater fishes were identified as a type 1 (molecular weight approximately from 95 to 130 kDa) collagen. As for gelatin, only gelatin from Snakehead fish scale was identified to be a type 1(molecular weight approximately from 95 to 130 kDa) while the other two freshwater fishes showed no clear band due to high viscosity of the gelatin produced. **Conclusion:** It can be said that the fishes investigated in this study have a potential to be the alternative source of collagen and gelatin.

KEYWORDS: collagen, gelatin, SDS-PAGE

INTRODUCTION

Collagen and gelatin are essential protein in vertebrates which are widely used in food industries, cosmetic, pharmaceutical, photographic industry, and biomaterials applications due to its excellent characteristic in biocompatibility, biodegradability and weak antigenicity (Pati, Dhara and Adhikari,

2010). The annual world output of collagen and gelatin is increasing throughout the years and it is mostly come from cow and pig skins and bones. The outbreak of the transmissible disease in pigs and cattle such as bovine spongiform encephalopathy and foot and mouth disease has resisted the use of collagen and gelatins from both sources in recent year (Minh Thuy et al., 2014). Besides health and safety concern from these land-based animal resources, religious constraint for Muslim consumers have led to the other alternative source of collagen production such as from fish's scales, skins and bones (Binsi et al., 2009). With the increase of demand for the processed fish product and the growing of fish processing industries in Malaysia, enormous amount of fish waste has been generated such as skin, scales and bone. Utilization of those fish wastes has a huge advantage of adding value to something which is currently discarded into another valued product such as fishmeal, fertilizer, fish collagen and fish gelatin. The maximum utilization of the fish waste is needed to fulfill the consumers concern about reducing waste and to avoid environmental problem in viewpoint of environmental conservation and protection. This is also contributing to the fisheries sustainability by getting more value from each catch. Therefore, collagen and gelatin from fish scales waste are realistic alternative to the mammalian animal.

In recent years, present studies on extraction of collagen and gelatin from fresh water fish have been reported from various sources. Zhang et al., (2011) has reported the successful of collagen extraction from scales of fresh water carp fish and its characteristic. Besides, Liu et al (2014) succeeded to extract and compare acid-soluble collagen from the skin and scales of four carp species. On the other hand, Ratnasari et al., (2013) has extracted gelatin from the skin of different type of freshwater fishes such as *"pangas catfish"(Pangasius pangasius), "Nile tilapia" (Oreochromis nilocitus), "Asian redtail catfish" (Hemibagrus nemurus)* and "Striped snakehaead" (*Channa striata*). Sanaei et al., (2013), has also reported the successful of optimizing the gelatin extraction of catfish *(Clarias gariepinus)*. However, the extraction and analysis procedure used in the previous studies were varied from sample to sample. It was a debatable issue that the difference result of the extraction and analysis of collagen and gelatin resulted from the difference method used for the extraction of different fish parts; skin scale and bones. Therefore, the result interpretation and comparison from different research and their application study need to be done properly. Thus, this research is important to compare the extracted collagen and gelatin from different species of freshwater fish scales by using the same extraction and analysis procedure.

MATERIALS AND METHODS

Extraction of Acid-Solubilized Collagen

The types of fish used were Kelah (*Tombroides*) fish, Tilapia (*Oreochromis niloticus*) and Snakehead fish (*Channidae*). The unwanted non-collagenous protein on the surface of fish scales was washed out twice in 10 wt% of NaCl by stirring the solution for 24 hours. Demineralization of the fish scale was done by treating with 0.4mol/L HCl solution (dry scales: solution = 1:15) for 90 minutes and then washed them three times with cold distilled water (Zhang *et al*., 2011).

The extraction process of collagen was followed the method proposed by Nagai and Suzuki (2000) with some modifications. All the procedures were done at 4°C. The fish scales were cut into small pieces and extracted with 0.5M acetic acid in (1:1 ratio) for 2 day. After that, the extracts suspensions were centrifuged at 10,000×g for 30 minutes. The supernatant were collected. Then the residues were re-extracted with the same solution for another 1 day and centrifuged under the same condition. The supernatant of bot extracts were combined and salted out by adding NaCl to a final concentration of 0.7M. The precipitated collagen were collected by centrifugation at 10,000×g for 30 minutes and re-dissolved in 0.5M acetic acid to precipitate with NaCl again. The resulting precipitated was dialyzed against water and lyophilized. The collagen powder obtained was diluted with 100 µL 0.1 M acetic acid. The diluted sample was kept at -80°C.

Extraction of Gelatin

After the mineralization process of fish scales, the extraction of gelatin was carried out in distilled water at control temperature of 70°C for 5 hours. The ratio of 1:8 was used (1g of scales to 8 mL of distilled water). The solids were filtered out with filter cloth and followed by Whatman No.1 filter paper. Then the filter solution was dried under dryer at 40°C until brittle sheets were found. The gelatin sheets were diluted with 2 mL distilled water and was kept at -80°C.

Protein Quantification

Protein quantification is a method to quantified total protein contents according to Bradford, 1976 by using Bovine Serum Albumin (BSA) as standard. BSA stock solution of 0.5 mg/mL was prepared by dissolving 0.5 mg of BSA in 1mL of protein extraction buffer. The concentration of standard dilution of BSA (0.000, 0.005, 0.01, 0.04, 0.07, 0.10, 0.13, 0.15µg/µL) was prepared by mixing the BSA stock solution and 0.5M of NaCl to a final volume of 100µL in microcentrifuge tubes. After that, 1 mL of Bradford reagent was added to each tube of sample extract. The mixture was vortexed to mix them well and incubated for 5 min at room temperature. The mixture solutions were pipetted into the 2 mL cuvette then were measured at 595 nm using a spectrophotometer. The standard curve was plotted as reference to determine the concentration of sample extract protein.

Total protein content in samples extract was quantified using similar method. First, 20µL of sample extract was mixed with 80µL 0.15M NaCl to the total volume to 100µL. After that, 1mL of Bradford reagent was added to the mixture and vortexed to mix them well. Then, the mixture solutions were incubated for 5 min at room temperature. The absorbance was measure at 595nm using a

spectrophotometer. A blank solution was prepared containing 100µL of 0.15M NaCl. The protein concentrations of samples extract were determined based on the equation obtained from the standard curve.

Protein separation by SDS-PAGE

The SDS-PAGE of the sample extract was running on 5% stacking gel to 7.5% resolving gel according to Mini-Protean® 3 Cell Instruction Manual from Bio-Rad, Hercules. First, 7.5% resolving gel was prepared. An amount of 100 µL freshly prepared 10% APS and 10 µL of TEMED were added into the mixture at the end before the mixture was pipette into the cassette to initiate gel polymerization. Then the mixture was mixed and immediately pipette into the cassette until 1-2 cm from the top of the short plate. Distilled water was added on top of the gel right after the mixture being pipette to the cassette to create a smooth surface. Once the gel has solidified, the distilled water layer was removed by using tissue paper.A 5% stacking gel was prepared by according to Appendix C. An amount 50 µL of 10% APS which was freshly prepared and 5 µL of TEMED were lastly added into the mixture to initiate polymerization of the acrylamide. The mixture was mixed well and pipetted on top of the polymerized resolving gel. A comb was place into the cassette to create wells and let the gel to solidify. An amount of 20 µL of sample protein was mixed with sample buffer with equal ratio 1:1. A sample buffer used was prepared from 0.5 M Tris-HCl at pH 6.8, 10% (w/v) SDS, glycerol, 0.5% (w/v) bromophenol blue, β-mercaptoethanol and distilled water. The mixture was incubated at 95°C for 5 min by using PCR machine. After that, the mixture was loaded into the well on the gel by using 1-10 µL pipette. A known molecular weight protein ladder from Vivantis named Chromatein Prestained Protein Ladder was mixed with the sample buffer until the total volume of 20 µL. The mixture of protein ladder was loaded to the first well of the gel carefully. The electrophoresis was carried out in 1x electrode buffer at 20mA for 90 min. The run was stopped once the protein marker approached the bottom of the gel. The gel was stained with Coomasie Blue G-250 solution overnight with gentle shaking. After that, the gel was destain with destaining solution 1 prepared for 10 min, followed by destaining solution 2.The gel was left overnight on the shaker to remove the background color of the gel. The protein gel was viewed and analyzed by using Molecular Imager GS-800 Calibrated Densitometer. The gel was kept in 1% acetic acid at 4°C.

RESULTS AND DISCUSSIONS

Protein Quantification

The total protein contents of collagen and gelatin from Tilapia, Kelah and Snake head fish sample calculated by using the standard curve of Bovine Serum Albumin (BSA) as reference.

Table 1 The absorbance of collagen from different species of freshwater fish scales

Sample	Absorbance
Tilapia fish	0.018
Kelah fish	0.017
Snake head fish	0.011

The result in Table 1 shows Tilapia fish has highest protein absorbance compared to Kelah fish and Snake head fish. However, the absorbance value of collagen for all samples found to be lower than the minimum value in standard curve of Bovine Serum Albumin (BSA) compared to those that have been reported by Zang et al,. (2011); Minh Thuy et al,. (2014), collagen extracted from fish scale had abundance of amino acid composition.

Referable to the lower absorbance value of protein content, the protein concentrations of collagen are unable to be calculated. There are many factors which can affect the protein content of collagen. This might be due to the low initial weight of the fish scale which was used for collagen extraction and incomplete mince of the scale which reduce the extractability of collagen to be extracted out. A study conducted by Foegeding et al., (1996) state that collagen molecule consist of two terminal ends which are non-helical parts. They play an important role in the cross linked structure. If the molecules are highly cross-linked at the telepeptide region, the collagen's solubility in acid solution will decrease. Thus, the degree of cross-linking among collagen molecules at telepeptide region of fish scales tested might be stronger from the other fish. On the other hand, the age of animal also has influence to the connective tissue and its basic protein quantity and qualitative. Earlier study had found that tissue muscle from younger animals contains more collagen and easy to solubilize compared to the tissue of older animal. Collagenous tissue from older animals with more cross-linkage would be predicted to be more resistant to swelling and have a lower water holding capacity (Zayas, 1997).

The other reason that might support the lower absorbance of protein is the extractability of acid-solubilized collagen in the scale compared to other solubilized collagen. The yields of acid-solubilized collagens for scale and bone were lower than those of pepsin-soluble collagen (Duan et al., 2009).

The Table 2, shows the total protein concentration of three different species of freshwater fish. All the three freshwater fish species has very distinct value in total protein concentration. Based on the total protein concentration and extraction yield % calculated, Snakehead fish has the highest for both total protein concentration and extraction yield followed by *Tilapia* fish and *Kelah* fish which was the lowest. Snakehead fish was found to have achieved a significant value of extraction yield predicted for lizardfish (Wangtuei et al., 2009), after the optimization of some parameter that was 10.7%.

There are many factors that can influence the total protein concentration of the fishes. The most notably factors are the selection of the sample. Species and age of the fish have a great influence on the concentration yield and quality of gelatin produced (Karim and Bhat, 2009). Besides the environment condition of the fish contribute to the total protein obtained. As mentioned by Gudmundson, (2002), the amino acid content in gelatin is dependent on the origin of raw materials. Many earlier studies found that gelatin extracted from warm water fish has more amino acids compared to cold water fish. In addition, extraction components and condition used for gelatin extraction also have a significant effect on the extraction yield. The previous optimization study on gelatin extraction from lizard fish scales determined that the concentration of alkaline solution (NaOH), extraction temperature (°C) and extraction time (h) have highly effect to the extraction yield (Wangtuei et al., 2009).

Table 2: The protein content of gelatin from different species of freshwater fish scales

Sample	Total Protein Concentration (µg/µL per g)	Extraction yield (%)
Snakehead fish	0.467	18.7
Tilapia fish	0.144	5.8
Kelah fish	0.037	1.5

Protein Profile of Fish Scale Collagen on SDS-PAGE

The protein sample of Tilapia, Kelah and Snakehead fish scale were separated on polyacrylamide gels on the basis of molecular weight of the protein. Due to the very low total amount of protein obtained for all fish scale sample, the amount of protein loaded into a well was fixed to be 20µL. Figure 3.1 illustrates the protein profile of collagen from Tilapia, Kelah and Snakehead fish scale. SDS-PAGE was carried out to determine the quality, size and type of collagen from fish scale. Referring to Figure 1, it can be assumed that the protein extraction process was much efficient for Tilapia and Snakehead fish as the bands from both protein samples were observed even though the bands were not very clear and some smears were presence compared to Kelah fish which has too much smear. Possible reason that might affect the bands separation is the multiple steps used in the extraction process which cause the protein lost and degrade from their intact form. Protein marker which comprises of seven precisely sized proteins (42, 51, 62, 70, 95, 130, 175 kDa) was used as reference to the size of protein bands resolved on the gel.

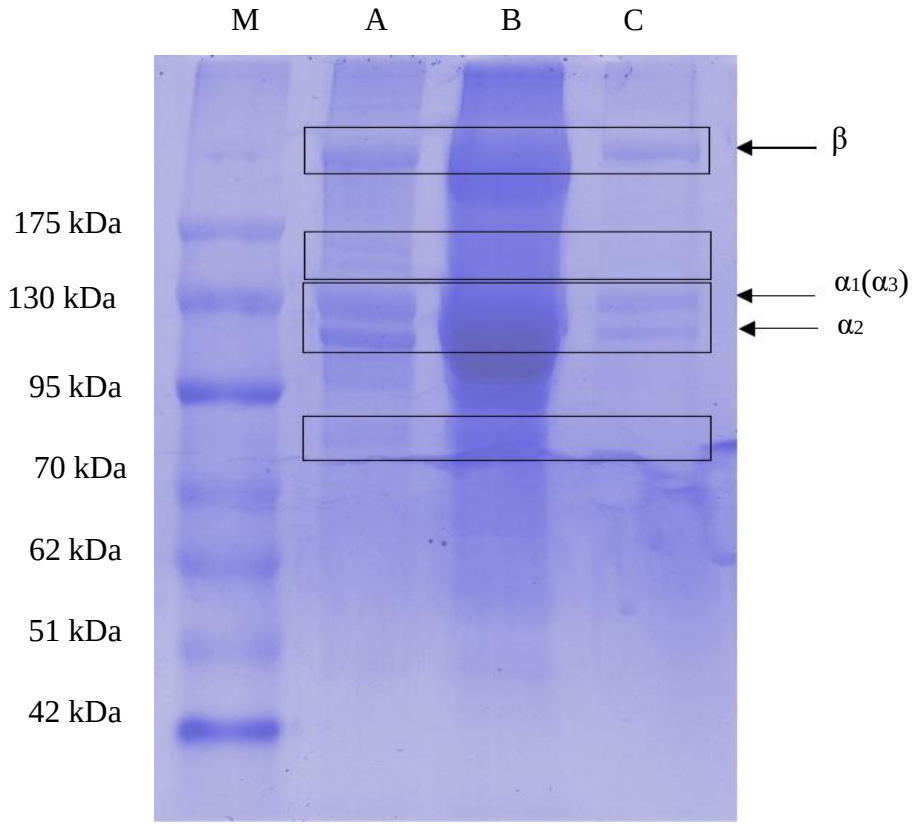

Figure 1: Protein profiles of extracted collagen from different freshwater fish that were separated by SDS-PAGE. M: protein marker; A, Tilapia fish; B, Kelah fish; C: Snakehead fish.

As shown in Figure 1, acid-solubilized collagen (ASC) from Tilapia fish and Snakehead fish had quite similar electrophoretic pattern compared to Kelah fish. This is because of the presence of smear had covered the band pattern in Kelah fish to be clearly observable. The band pattern of the collagen from Tilapia, Kelah and Snakehead fish scale were compared to the previous studies results done by Liu et al., (2014) to determine their collagen type. Therefore, from the band pattern obtained, acid-solubilized collagen of Tilapia, Kelah and Snakehead fish scales were found to be classified as type 1 collagen (reference to type 1 collagen of bovine calf) as they consist at least two different α chains (α_1 and α_2) with the molecular weight approximating 95 kDa to 130 kDa. This result supported by Kimura et al., (1991) which identified that soluble collagen type 1 from scale and bone of lathyritic carp consisted of two molecular forms, $(\alpha_1)_2$ α_2 as a main form and $\alpha_1\alpha_2$ α_3 as minor one. However, in this study, the α_3 chain existence was not clearly seen as the α_3 chain had a similar molecular weight to α_1 chain (Kimura and Ohno, 1987) as it was not separated from the corresponding α_1 chain under electrophoretic condition. Besides, Matsui et al., (1991) reported that the α_3 chain was deposited between the α_1 and α_2 as shown by the SDS-PAGE pattern. Yet, the existence of α_3 chain can be identified by using chromatographic method which was done by Kimura (1992).

Moreover, a small amount of β chain which was the dimmers from α chain component was obtained for all the three fish species. The molecular weight of β chain was assumed to be more than 175 kDa. This was proven by earlier research conducted by Ogawa et al., (2004); Zhang et al., (2011); Liu et al., (2012) who claimed that β chain was found higher in acid-solubilized collagen compared to pepsin-solubilized collagen.

Protein Profile of Fish Scale Gelatin

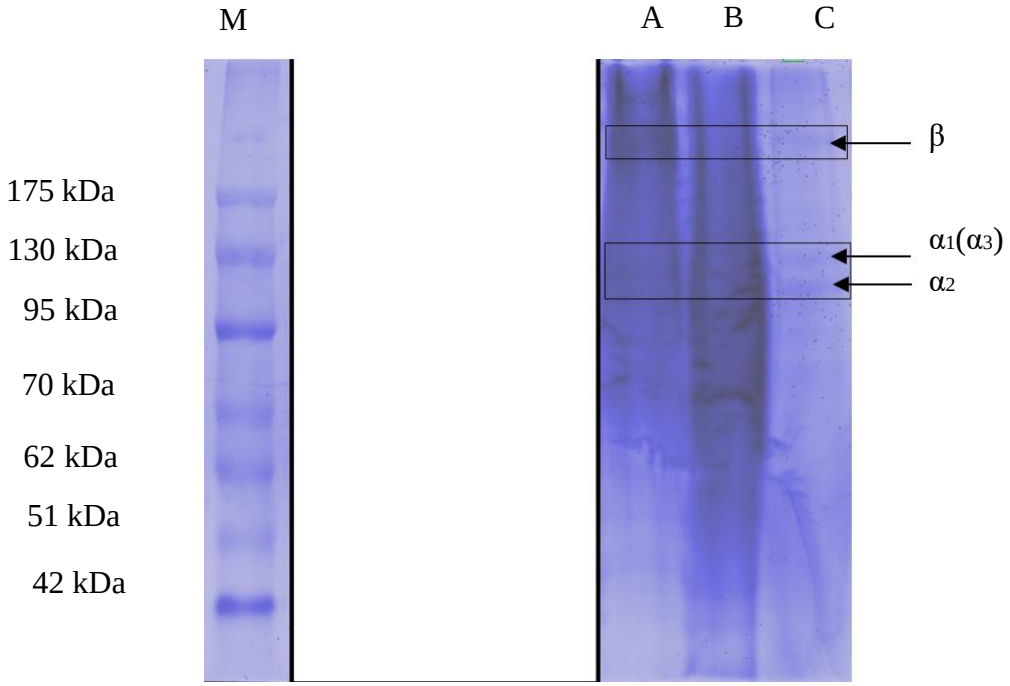

Figure 2: Protein profiles of extracted gelatin from different freshwater fish that were separated by SDS-PAGE in 5% stacking gel and 7.5% resolving gel. M, protein marker; A, *Tilapia* fish; B, *Kelah* fish; C, Snakehead fish.

The SDS-PAGE of gelatin extracted from Tilapia, Kelah and Snakehead fish were presented in Figure 2. From the figure, it can be said that gelatin extraction process was not very efficient since the protein extract were not separated well and a lot of smear were observed except for Snakehead fish. The possible interference for the protein bands to be well separated is from the higher viscosity of crude gelatin extract for Tilapia and Kelah fish compared to Snakehead fish. All the interferences occurred might cause from the quality of the raw sample used. The fish scale used for gelatin extraction might possibly from an old aged fish. According to Muyonga et al., (2004) gelatin extracted from adult *Nile* Perch aged more than 80 days old showed higher viscosity compared to young fish aged less than 80 days old. On the other hand, the protein band from Snakehead fish shows promising result as the band separated accordingly to their molecular weight. The extracted gelatin from Snakehead fish scale show a band approximately from 70 kDa to 175 kDa. With regard to the molecular weight distribution, gelatin from Snakehead fish scale consist a mixture of α chain (α_1 and α_2) and β chain at

molecular weight range from 95 kDa to 130 kDa and more than 175 kDa respectively. The presence of α chain frequently presenting a band pattern distribution of typical of type 1 collagen. This result supported by Zhang et al., (2011) who found the same band pattern of α and β chains with the molecular weight approximating 117 kDa (α1), 107 kDa (α2) and 200 kDa (β) in a grass carp fish (*Ctenopharyngodon idella*) scales gelatin. However, the gelatin type from Tilapia and Kelah fish scale were assume to be type 1 collagen although no clear band were identified. This is due to the result of collagen from the same representative fish which were clarified as type 1 collagen. Since gelatin is also known as denatured protein derived from collagen by thermo hydrolysis process (Cho et al., 2014), it give an idea that both collagen and gelatin have same type of collagen.

CONCLUSION

Generally, the collagen and gelatin content and type in the scale of different freshwater fish were successfully compared. A different amount of collagen and gelatin content were obtained in Tilapia, Kelah and Snakehead fish scales although the protein content were slightly lower compared to other researches done before. Nevertheless, the type of collagen and gelatin for all three freshwater fish were mainly identified as type 1 containing three different α and β chains which is most distributed in skin, bone and scale region. Thus the hypothesis of this research that mentioned there are differences in collagen and gelatin type was rejected. Based on the result analysis, the Snakehead fish was suggested having a potential for collagen and gelatin since it has shown a promising result compared to other freshwater fish tested.

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