

The Evaluation of the Suitability of Fish Wastes as a Source of Collagen

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Abstract. Fish wastes such as skin, scales, bones and fins are major by-products in the fishery and aquaculture industries which have high collagen content. Therefore, an investigation into making more effective use of under-utilized resources, acid-solubilised collagen (ASC) was extracted from fish skin, scales bones and fins. As a result, the yields of skin, scales, bones and fins collagens are 70.67%, 13.03%, 38.03% and 40%, respectively. SDS-PAGE pattern showed that ASCs of fish skin, scales, bones and fins are all type I collagen, which are composed β , α_1 and α_2 chains. The molecular weight of fish skin, scale and fin α_1 , α_2 and β chains are 132.044kDa, 120.065kDa and 220.673kDa, whereas the molecular weight of fish bone α_1 , α_2 and β chains are 139.798kDa, 124.72kDa and 229.229kDa. Denaturation temperatures (T_d) of ASCs from skin, scales, bones and fins were 32.4°C, 35.8°C, 37.8°C and 32°C, respectively. Fourier transform infrared spectroscopy proved that ASCs are integrated and native. The results suggest that collagen of fish waste skin, scales, bones and fins have the potential to be an alternative source of collagen for various application in the future

Keywords: Collagen, fish waste, amino acids, protein

1. Introduction

Collagen is the most abundant animal protein polymer and comprises of about 30% of the total protein in the animal body. In its purified form, collagen is a bio material. Generally, collagen has a wide range of applications in cosmetic, biomedical, pharmaceutical, leather and film industries (Ogawa et al., 2004; Kittiphattanabawon et al., 2005). The soluble form of collagen is useful in various fields such as food, cosmetics and pharmaceutical fields due to its special characteristics including biodegradability and weak antigenicity (Kolodziejska et al., 1999). The physical and chemical properties of fish collagen are different from those of mammalian collagen (Zhang et al., 2007). The collagen from the fish waste is unlikely to be associated with infections such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot and mouth disease (FMD). Therefore fish waste also may be an effective alternative source for collagen production (Nagai et al., 2001; Jongjareonrak et al., 2005). In addition, the collagens extracted from bovine sources are prohibited for Sikhs and Hindus, whilst porcine collagen cannot be consumed by Muslims and Jews, both of whom require bovine to be religiously prepared. As a consequence, the alternative sources of collagen, especially from aquatic animals including freshwater and marine fish and mollusks have received increasing attention (Shen et al., 2007). Therefore, this study is aimed to evaluate the suitability of Malaysian fish waste as a source of collagen.

2. Material and Methods

2.1. Raw Materials

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Fish waste such as skin, scales and bones were collected from the local wet market at Jalan Genting Kelang, Kuala Lumpur, Malaysia. The wastes were then washed and kept at -20°C before used.

2.2. Chemical Reagents

All reagents were analytical grade. Bornstein and Traub Type I human collagen was purchased from Sigma-Aldrich company (St. Louis, MO, USA).

2.3. Preparation of Collagen from Fish Skin, Scales, Bones and Fins

The collagens were prepared using the methods of Nagai and Suzuki (2000a) with slight modifications. All preparation procedures were carried out at 4 °C.

2.3.1. Skin Collagen

The skins were first cut into small pieces of about 0.5cm in length, then deproteinised with 0.1M NaOH at a sample:alkaline ratio of 1:8 (w/v) to remove non-collagenous proteins. The mixture was stirred for 6 hours with changes of solution every 3h. Next the samples were washed with distilled water, until neutral pH was achieved. The deproteinised skins were then defatted with 10% butyl alcohol at sample:alcohol ratio of 1:10 (w/v) overnight and washed with distilled water thoroughly before extraction with 0.5M acetic acid for 1 day. Skin was discharged after that and the remaining solution was subjected to salting-out procedure by adding NaCl to a final concentration of 2.5M in the presence of 0.05M tris(hydroxymethyl) aminomethane, pH 7.0. The precipitates were then collected by centrifuged at 20,000 x *g* for 30 min and dissolved in 0.5M acetic acid. The fraction was dialyzed against 0.1M acetic acid and distilled water, respectively. Lastly, this acid-soluble collagen was lyophilized.

2.3.2. Bone Collagen

The fish head was broken by a hammer, and soaked in distilled water for 5 hours. Next, the bones were immersed in 0.1M NaOH at sample:alkali ratio of 1:5 (w/v) to remove non-collagenous proteins for 24 hours. Alkaline solution was changed every 6 hours. Subsequently, the bones were washed with distilled water until a neutral pH before adding 0.5M EDTA 2Na solution, pH 7.5 for 5 days and defatted with 10% butyl alcohol at a sample:alcohol ratio of 1:10 (w/v), overnight. The de-fatted alkaline treated bones were then washed with distilled water thoroughly, followed by extraction with 0.5M acetic acid at a sample:acid ratio of 1:4 (w/v) for 1 day. Extraction solution is filtered and the insoluble matter was re-extracted with the same solution at sample:acid ratio of about 1:2.5 (w/v) for another 2 days. Both the extraction solution was then combined and salted-out as described in (i).

2.3.3. Scale Collagen

Non-collagenous proteins were removed as stated in (i), after that the scales were decalcified with 0.5M EDTA-2Na of pH 7.5 at sample:EDTA ratio of 1:10 (w/v), 24 hours. Next, the scales were washed with distilled water and subsequently extracted with 0.5M acetic acid at sample:acid ratio of 1:2.5 (w/v) for 2 days. The scales were removed and the solution was salted-out as described in (i).

2.3.4. Fin Collagen

Fins were immersed in 0.1M NaOH to remove non-collagenous proteins. Next, it was washed thoroughly with distilled water and lyophilized. The insoluble matter was then extracted with 0.5M acetic acid for 3 days. The extract was centrifuged 20,000 x *g*, 1h. Two fractions were collected, namely acid-soluble and acid-insoluble fractions. Salting out procedure was then carried out for acid-soluble fraction as described in (i). The acid-insoluble fraction was washed with distilled water and decalcified with 0.5M EDTA (pH 7.4) for 5 days (fresh solution was changed daily). The decalcified fraction was then washed with distilled water and defatted in 10% butyl alcohol for 1 day. The defatted fraction was subsequently centrifuged (20,000 x *g* for 1h), dissolved with 0.5M acetic acid and dialyzed against 0.1M acetic acid. The dialysate was collected and freeze dried until used.

2.4. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein patterns of the collagen samples were analysed using SDS-PAGE according to the method of Laemmli (1970). Collagen samples were dissolved in 0.1M acetic acid together with a 5 times sample buffer

(0.5M Tris-HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) β -mercaptoethanol). The samples were heated to 93°C-95°C for 4 min prior loading onto the 7.5% gel. The gel was electrophoresed at a constant current of 70V/gel using a Compact-PAGE apparatus (Atto Corp., Japan). The proteins were visualized with Coomassie blue R-250. High-molecular-weight markers (212 kDa to 66.4 kDa) (Sigma Chemical co., St. Louis, Mo, USA) were used to estimate the molecular size of proteins. Commercial collagen, Bornstein and Traub Type I human collagen was used as positive control.

2.5. Determination of Denaturation Temperature (T_d)

Denaturation temperature (T_d) was determined by using the method of Zhu and Kimura (1991). A total of 2.5% collagen solution was prepared in 0.1M acetic acid solution and subjected to a rheometer (Anton Paar, USA). The rheometer was allowed to decrease to 9°C before placing the samples. The temperature was then increased stepwise up to 50 °C and maintained at every interval for 1min. The fraction change was calculated from the viscosity measurement obtained with the below equation. Where, C is the collagen concentration (mg/mL), ϵ_1 is the viscosity at 9°C, ϵ_2 is the viscosity at measured temperature (°C) and ϵ_3 is the viscosity at 45 °C.

$$\text{Fraction change} = [(\epsilon_2/C) - (\epsilon_3/C)] / [(\epsilon_1/C) - (\epsilon_3/C)]$$

The denaturation temperature was taken to be the temperature at which fractional viscosity was 0.5.

2.6. Fourier Transform Infrared Spectroscopy (FTIR)

All spectra were obtained using a FTIR (Omicron spectra) spectroscopy (Perkin Elmer, USA). A total of 0.5g collagen was fixed onto the crystal and knob of the Nicole i50 and placed on the FTIR apparatus. Each sample was subjected to 32 scans from 4000 to 400 cm^{-1} at a resolution of 4.0 cm^{-1} with an interval of 1.0 cm^{-1} . Triplicate samples of skin, bones and scales' collagen were analyzed respectively.

2.7. Results and Discussion

2.7.1. Preparation of Collagen from Fish Skin, Scales, Bones and Fins.

Collagen obtained from skin, scales, bones and fins collagens are 70.67%, 13.03%, 38.03% and 40%, respectively on a wet weight basis. In which, most collagen is distributed on the skin. As compared to other reported collagens, skin demonstrated higher collagen yield although it depends much on the fish species. The extractability of collagen is due to the construction of collagen which consists of two non helical terminal ends that assist the cross-linked structure of the collagen. When the telopeptide regions of collagen molecules are highly cross-linked, the collagen becomes less soluble in acid solutions (Foegeding et al., 1996), thus, affects the yield.

2.7.2. Electrophoresis

The protein pattern of the extracted collagens was observed with SDS-PAGE together with the commercially available Bornstein and Traub Type I human collagen (Fig. 1). The SDS-PAGE pattern show that all collagens had a doublet pattern for α_1 (approximately 132kDa to 139kDa) and α_2 chains (approximately 120kDa and 124kDa position) and a β chain (approximately 220kDa to 229kDa). The density for α_1 is twice as much as α_2 , (α_1 , upper; α_2 , lower).

Collagens of skin, scales bones and fins of fish waste matches the band patterns of the human skin collagen. Based on the α_1 and α_2 pattern, suggesting the fish collagens might have a composition of $(\alpha_1)_2\alpha_2$ heterotrimer, a type I collagen. Similar results were observed in carp fish (Duan et al., 2009), deep-sea redfish (Wang et al., 2007), Nile perch (Muyonga et al., 2004), black drum (Ogawa et al., 2004) and brownstripe red snapper (Jongjareonrak et al., 2005). Fish skin, scale, bone and fin collagen subunit molecular weights (α_1 , α_2 and β chains) revealed to be slightly lower than human collagen. This difference could be due to the dissimilar source of the collagens, namely resources from aquatic animals and mammalian animals.

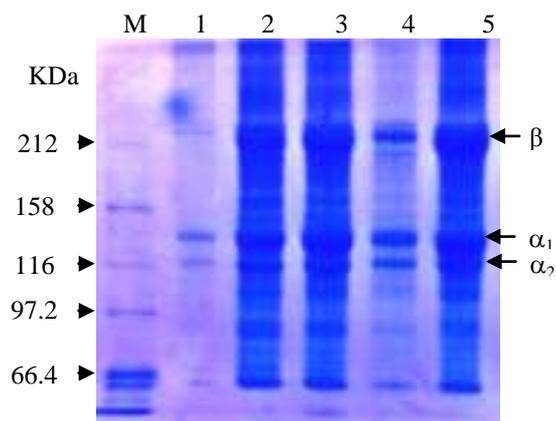


Fig. 1: SDS-PAGE pattern of collagen extracted from different part of the fish (skin, bone, scale and fin) on 7.5% gels. Lane M, molecular weight marker; lane 1, human collagen; lane 2, fish skin collagen; lane 3, fish scale collagen; lane 4, fish bone collagen; lane 5, fish fin collagen.

2.7.3. Denaturation Temperature

The denaturation temperature (T_d) was determined by measuring the viscosity of the collagens. The T_d value of each collagen is defined as the temperature at which the change in viscosity was half completed (Fig. 2). The T_d of fish waste skin, scale, bone and fin were 32.4°C, 35.8°C, 37.8°C and 32°C, respectively. Results obtained were higher than carp skin, scale and bone (around 28 °C) (Duan et al., 2009) and Alaska Pollack skin (17 °C) (Kimura & Ohno, 1987). As compare to the mammalian source, T_d of fish skin, scales and fins are slightly lower to porcine collagen (37°C) (Nagai & Suzuki, 2000b) except fish bone collagen (37.8°C). Generally, T_d collagen of fish living in cold environments is lower than the fish species living in warm climate (Takahashi & Yokoyama, 1954; Duan et al., 2009). This is because collagen from fishes in cold environment has lower hydroxyproline content (Tabarestani & Mahoonak, 2012, Sadowska et al., 2003). Hydroxyproline contains inter-chain hydrogen bonding that stabilizes the collagen's triple helical structure, thus improve the thermal stability of collagen.

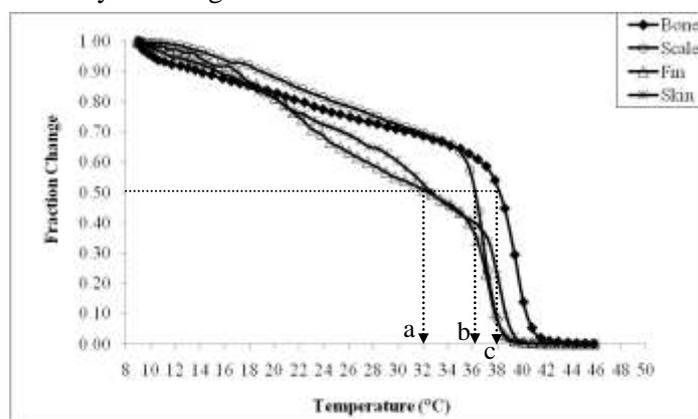


Fig. 2: Changes of viscosity of fish skin, scales, bones and fins collagen in response to the temperature change. a, T_d of skin and fin; b, T_d of scale; c, T_d of bone.

2.7.4. Fourier Transform Infrared Spectroscopy

The FTIR spectra (data not shown) of skin, scales, bones and fins demonstrated the absorption band at 1547.93 cm^{-1} to 1557.94 cm^{-1} , 1632.79 cm^{-1} to 1653.58 cm^{-1} and 3295.02 cm^{-1} to 3320.34 cm^{-1} region, indicated the existence of amide II, amide I and N-H stretching vibrations, respectively (Li et al., 2004; Duan et al., 2009). In terms of IR absorption ratio, collagen from fish bones, skin, scales and skin demonstrated equal ratio between amide III band (1236.83 cm^{-1} to 1455.98 cm^{-1}) and 1454.65 cm^{-1} to 1455.98 cm^{-1} bands. These results confirmed the helical structure of the collagen which is reserved in good conditions.

2.8. Conclusion

Collagen was successfully obtained from fish waste with promising yield from the fish skin. The collagens demonstrated a type I collagen structure from the SDS-PAGE pattern. Denaturation temperature

(T_d) obtained was higher as compare to collagen extracted from fishes in climate country, which might diversify the application in the future.

3. Acknowledgement

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4. References

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