

## Isolation and characterization of acid soluble collagen (ASC) and pepsin soluble collagen (PSC) extracted from silver catfish (*Pangasius sp.*) skin

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### Abstract

The aims of this study are to isolate and characterize acid soluble collagen (ASC) and pepsin soluble collagen (PSC) extracted from silver catfish (*Pangasius sp.*) skin. Isolated ASC and PSC collagen were characterized in terms of chemical composition (moisture, protein, fat and ash content), protein concentration, functional group, solubility, and morphological properties as compared to commercial collagen. Yields of ASC and PSC were 4.27% and 2.27%, respectively. The chemical compositions of raw skin were 34.64%, 2.81%, 3.68%, and 0.31%, while the chemical compositions of ASC and PSC were 94.21%, 3.48%, 0.81%, 59.15%, and 88.25%, 3.46%, 0.92%, and 29.24%, for moisture, protein, fat, and ash, respectively. ASC and PSC had protein concentrations of 2.27 mg/mL and 2.70 mg/mL, respectively. Functional group analysis revealed that both isolated collagens exhibited Amide A, II and III as a fingerprint for collagen structure. The highest solubility was found at pH 4 for ASC, pH 1 for PSC, and pH 5 for commercial collagen. The morphology of the isolated collagens was porous and they contained fibril. In conclusion, the characteristics of the isolated ASC and PSC from silver catfish (*Pangasius sp.*) skin indicate that value-added collagen can be produced from the alternative source of freshwater fish.

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### Keywords

Collagen, acid soluble collagen (ASC)

Pepsin soluble collagen (PSC)

Extraction method

Silver catfish.

### Introduction

Collagen is the most abundant protein in animal bodies. The word 'collagen' comes from the Greek word 'kola' for glue. Collagen constitutes approximately 30% of the total protein in animal bodies. Collagens are largely isolated from land-based animals, typically bovine and porcine bone or skin (Jose *et al.*, 2014). However, collagen can also be isolated from the skin, bone, scales, and fins of marine-origin animals.

The abundance of fish waste from fish processing industries is seen as an opportunity by the researcher to investigate alternative sources of collagen. Bone, skin, scales, and fins are solid wastes from fish (Sujithra *et al.*, 2013). Solid waste is 50-70% of the original portion of raw materials, depending on processing. Hence, improper disposal and management of waste may lead to pollution and an offensive odour in the surrounding environment. The optimal utilization by transforming this waste into a source of collagen is a recommending method to increase its value and to minimise the cost of disposal. So far, most studies examining the minimization of fish waste have focused only on skin and bone.

There are many studies conducted in extracting collagen from Coldwater fish (Wang *et al.*, 2008;

Kaewdang *et al.*, 2014). However, few studies have been conducted on collagen extraction from freshwater fish in Malaysia. *Pangasius sp.*, or fresh silver catfish, is among the most popular freshwater fish in Malaysia. It is also a fish which could lead to increased waste from the fishery industry. Silver catfish is a fast-growing fish which is widely cultured in ponds, floating cages, and pens (Normah *et al.*, 2014). The widespread availability of *Pangasius sp.* makes it a potential alternative source for the production of collagen and a replacement for mammalian collagen.

There are four isolation methods used to isolate collagen from animals, which are the salting-out, alkaline, acid, and enzyme methods (Yang and Shu, 2014). The preferred methods among researchers are the acid and enzyme methods. Collagen extraction by acid method can be carried out using organic acids such as chloracetic, citric, or lactic acid (Skierka and Sadowska, 2007). To produce pepsin soluble collagen (PSC), undissolved matter obtained from acid soluble collagen (ASC) isolation is used (Wang *et al.*, 2014). PSC is commonly applied in combination with 0.5M of acetic acid (Wu *et al.*, 2014; Kaewdang *et al.*, 2014). Pepsin is a common enzyme as it is able to maintain a collagen structure by cleaving to the N-terminal of protein chain and non-helix peptide

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chain (Nalinanon *et al.*, 2008). However, the pepsin-soluble method is very time consuming.

The isolated collagens are further characterized in terms of chemical and physical characteristics to determine the properties of extracted collagen as compared to available commercial collagens. Chemical characteristics include protein concentration, moisture, ash, protein, and fat content. Meanwhile, physical characterizations include thermal stability (Wang *et al.*, 2014), functional group (Matmaroh *et al.*, 2011), viscoelasticity, solubility (Jeevithan *et al.*, 2014) and morphological properties (Wang *et al.*, 2014). Previous studies have found that ASC and PSC collagens from marine-origin sources show similarities with mammalian collagen (Yd *et al.*, 2013).

Therefore, the objectives of this study were to isolate ASC and PSC from the skin of silver catfish (*Pangasius* sp.) and characterize their chemical and physical properties.

## Materials and Methods

### Materials

Fresh silver catfish (*Pangasius* sp.) with average weight of 1.5 kg and size of 20 cm were purchased from a local supplier in Kuala Terengganu, Malaysia. Commercial collagen from tilapia scales was purchased from the Umathy Industries Sdn. Bhd. All other chemicals used were of analytical grade and purchased from Sigma Aldrich.

### Methods

#### Sample preparation

The skins of the silver catfish were removed manually and thoroughly washed. All skins were packed in a polyethylene bag and stored at -20°C for further use.

#### Removal of non-collagenous proteins

Samples were soaked in 0.1 M sodium hydroxide (NaOH) solution for 6 hours with a ratio of sample to solution of 1:8 (w/v). The NaOH solution was changed every 3 hours. The samples were then thoroughly washed with cold distilled water until the rinse water become neutral (pH, 7) (Minh Thuy *et al.*, 2014).

#### Defatting

The skins were then soaked in 20 volumes of 10% butyl alcohol with a solid to solvent ratio of 1:10 (w/v) for 24 hours with continuous stirring. The solution was changed every 12 hours. Then, the

defatted skins were washed with cold water (5-8°C) (Nagai and Suzuki, 2002).

#### Isolation of Acid soluble collagen (ASC)

In acid soluble collagen (ASC) extraction, 0.5 M acetic acid with a sample to solution ratio of 1:10 (w/v) was applied to the samples for 24 hours with continuous stirring. Then, the extracts were centrifuged (Gyrozen 158R, Deejan, Korea) at 10,000×g for 30 mins at 4°C and the supernatants obtained were separated. The sample residues were re-extracted with 0.5 M acetic acid with a sample to solution ratio of 1:10 (w/v) for 12 hours before centrifuging at 10,000×g for 30 mins at 4°C. Both supernatants were combined and NaCl was added to salt-out until the final concentration of the supernatant reached 0.7 M for precipitation to occur. The supernatants were centrifuged again at 2,500×g to obtain the precipitate. The precipitates were then lyophilised (Huang *et al.*, 2011).

#### Isolation of pepsin soluble collagen (PSC)

To isolate pepsin soluble collagen (PSC), the undissolved matter which obtained from acid soluble collagen (ASC) extraction were used for further extraction using 2 volumes of 0.5 M acetic acid containing 1.5% (w/w) pepsin for 30 hours at 4°C with continuous stirring. The extracts were then centrifuged at 10,000×g for 30 mins at 4°C and the supernatants were separated. The residues were re-extracted with 0.5 M acetic acid containing 1.5% (w/w) pepsin for 12 hours before being centrifuged at 10,000×g for 30 mins at 4°C. Both supernatants were combined and NaCl was added to salt-out until the final concentration of the supernatant reached 0.7 M for precipitation to occur. The supernatant was centrifuged again at 2,500×g to obtain the precipitate. The precipitate was then lyophilised (Huang *et al.*, 2011). The collagen yields of ASC and PSC were calculated as follows:

$$\text{Yield of collagen (\%)} = \frac{\text{weight of freeze-dried collagen} \times 100}{\text{wet weight of raw skin}}$$

#### Chemical composition of raw silver catfish skin and isolated collagen

The chemical compositions of silver catfish skin and isolated collagen were analysed. The chemical compositions, including moisture, protein, fat, and ash content, were determined according to AOAC (2000). The results obtained were compared with commercial collagen.

## Characterisation of silver catfish skin extracted collagen

### Protein concentration

The protein concentration of acid soluble collagen (ASC), pepsin soluble collagen (PSC), and commercial collagen were determined using Lowry's method (Lowry *et al.*, 1951). Approximately 100 mg of bovine serum albumin was dissolved into 10 ml of distilled water to be used as a protein standard. About 8 ml of biuret reagent was added and left for 30 minutes at room temperature. Absorbance at 570 nm was measured via spectrophotometer (Shimadzu, United States). A graph of absorbance was plotted at 570nm against the concentration of protein standard solution. Then, collagen powder was diluted with distilled water at a ratio of 1:5. Thus, the final protein concentrations in the range of the calibration curve were determined.

### Structural properties of extracted collagen

Structural properties of extracted collagens were determined using Fourier Transform Infrared Spectroscopy (FTIR) per Rosli and Sarbon (2015). The infrared spectrum used was in the range from 4000 to 400cm<sup>-1</sup> using an infrared spectrophotometer (Nicolet, Thermo Electron, USA). The collagens were prepared by mixing with potassium bromide (KBr) at a ratio of 1:10, ground in a mortar and pestle and pressed into pellets by hydraulic press. The spectrum background was collected. The transmission readings were collected with 32 scans. Functional groups of interest such as Amide A, Amide II and Amide III were determined.

### Solubility determination

The solubility of extracted collagens was determined using varying pH levels according to the method by Jongjareonrak *et al.* (2005) and Huang *et al.* (2011) with slight modifications. The lyophilised collagens were dissolved in 0.5 M acetic acid with gentle stirring for 12 hours to obtain final concentration of 3 mg/mL. Approximately 8 mL of the sample was transferred to a centrifuge tube. The pH levels were adjusted across the pH range from 1 to 10 with 6 N NaOH or 6 N HCl. The volume was made up to 10 mL with distilled water. The solutions were stirred for 30 mins at 4°C, and then centrifuged at 10,000 × g for 30 mins at 4°C. Protein concentrations in the supernatant were measured using Lowry's method (Lowry *et al.*, 1951). Protein solubility was calculated in terms of that obtained at the pH level exhibiting the highest protein concentration. The relative solubility of collagen was calculated as

follows:

$$\text{Relative solubility (\%)} = \frac{\text{Protein concentration of supernatant} \times 100}{\text{The highest protein concentration}}$$

### Morphological properties

The microstructures of the extracted collagen were viewed at 25,000 magnification via scanning electron microscopy (SEM) (Hitachi S-4300SE, Hitachi Science System Ltd., Japan) at an accelerating voltage of 5.0 kV. The collagen powder was mount on aluminium cylinder stubs (5 mm x 12.5 mm) and sputter-coated with Auto fine coater (JFC 1600, Tokyo, Japan). The samples were observed in a superficial position at 100x magnification (Hanani *et al.*, 2011).

### Statistical analysis

Minitab 14.0 was used to characterize the chemical and physical properties of ASC and PSC silver catfish collagen in comparison to commercial collagen. Results were expressed as a mean (±SD) for each analysis. Comparative statistical analyses between mean and ANOVA were conducted with Minitab 14.0 to assess any significant differences (p<0.05).

## Results and Discussion

### Yield of extracted silver catfish (*Pangasius sp.*) skin collagen

The yields obtained for acid soluble collagen (ASC) and pepsin soluble collagen (PSC) of silver catfish skin were 4.27±0.06% and 2.27±0.16%, respectively. The yield of ASC was higher than PSC; however, there was no significant difference (p<0.05). The yield of ASC obtained was higher than PSC, perhaps due to silver catfish skins collagen undergoing incomplete solubilisation in 0.5 M acetic acid extraction during the acid soluble method. Then, when the residue was treated with pepsin in 0.5 M acetic acid during the pepsin soluble method, PSC was completely solubilized. However, when the skin was soaked in the 0.5 M acetic acid, it showed a high amount of cross-linking by covalent bonds through the condensation of aldehyde group at the telopeptide region, as well as the inter-molecular cross-linking, leading to a decrease of solubility of the collagen (Singh *et al.*, 2011). This finding was in agreement with a study on collagen extracted from rohu (*Labeo Rohita*) skin with an ASC yield of 4.13% (Hema *et al.*, 2013). The difference in collagen yield may be due to discrepancies in the content of collagen among different fish species and preparation methods (Yao *et al.*, 2012).

Table 1. Chemical composition and protein concentration (mg/mL) of acid soluble collagen (ASC), pepsin soluble collagen (PSC) and commercial

Samples	Chemical composition (%)				Protein concentration (mg/mL)
	Moisture	Protein	Fat	Ash	
Raw skin	65.36± 2.31 <sup>a</sup>	2.81± 0.21 <sup>c</sup>	4.38± 0.07 <sup>a</sup>	0.31± 0.03 <sup>d</sup>	
ASC	5.79± 0.08 <sup>c</sup>	3.48± 0.23 <sup>b</sup>	0.81± 0.32 <sup>b</sup>	59.15± 0.09 <sup>a</sup>	2.72± 0.17 <sup>b</sup>
PSC	11.57± 0.09 <sup>b</sup>	3.46± 0.38 <sup>b</sup>	0.92± 0.08 <sup>b</sup>	29.74± 0.15 <sup>b</sup>	2.70± 0.25 <sup>b</sup>
Commercial collagen	10.12±0.11 <sup>b</sup>	9.37± 0.51 <sup>a</sup>	0.03± 0.01 <sup>c</sup>	4.42± 0.01 <sup>c</sup>	3.99± 0.56 <sup>a</sup>

Different superscripts (<sup>a-d</sup>) in the same column of collagens denote the significant differences ( $p < 0.05$ ).

### Chemical composition

The chemical compositions of raw skin, ASC, PSC and commercial collagen are presented in Table 1. There were significant differences ( $p < 0.05$ ) between raw skin of silver catfish and extracted collagen, as well as commercial collagen, in terms of moisture content. However, there was no significant difference ( $p > 0.05$ ) between moisture content of extracted PSC and commercial collagen. The extracted collagen showed higher protein content compared to raw skin. There was no significant difference ( $p > 0.05$ ) between ASC and PSC in terms of protein content; however, these values were observed to be lower than commercial collagen. The fat content levels for ASC and PSC of extracted collagen were  $0.81 \pm 0.32$  and  $0.92 \pm 0.08\%$ , respectively. The low fat content in the isolated collagen showed an efficient defatting process. A study conducted by Shahiri *et al.* (2012) found only  $0.31 \pm 0.07\%$  (ASC) fat content in rainbow trout (*Onchorhynchus mykiss*) collagen. However, ash levels were quite high at  $59.15 \pm 0.09$  for ASC and  $29.74 \pm 0.15\%$  for PSC. The high value of ash may be due to the samples not undergoing demineralisation during pre-treatment.

### Characterization of extracted silver catfish (*Pangasius sp.*) skin collagen

#### Protein concentration

The protein concentrations of isolated collagen from silver catfish skin via acid method (ASC) and enzyme method (PSC) were  $2.72 \pm 0.17\%$  and  $2.70 \pm 0.25\%$ , respectively as compared to commercial collagen ( $3.99 \pm 0.56\%$ ). There were no significant differences ( $p > 0.05$ ) between ASC and PSC with commercial collagen in terms of protein concentration. Additionally, there were no significant ( $p > 0.05$ ) differences between ASC and PSC in terms of protein concentration. The chemical composition determination showed that commercial collagen had higher protein concentration than ASC and PSC. According to Veeruraj *et al.* (2013), glycine is a

major amino acid that present in collagen. One of the factors that affecting the protein concentration is the amount of imino acid (proline + hydroxyproline) content (Matmaroh *et al.*, 2011). Imino acid consists of pyrrolidine rings associated with hydrogen bonds to hold the structure of the alpha chains in both ASC and PSC, facilitating the intra-inter molecular cross-linking in strengthen the triple helix structure of collagen (Matmaroh *et al.*, 2011). The results obtained in this study were higher than protein concentration of collagen extracted from *Fringescale sardinella* waste conducted by Hamdan (2015). Thus, the higher protein concentrations in commercial collagen may be due to higher levels of glycine, proline and hydroxyproline contained in the collagen. The difference in the protein concentrations between commercial collagen and extracted collagen in this study may be due to the type of extraction used and also the different of raw material used.

#### Structural properties

Commercial collagen showed amide A bands at  $3423.22 \text{ cm}^{-1}$ , while the ASC and PSC of silver catfish skin collagen were at  $3457.07 \text{ cm}^{-1}$  and  $3447.86 \text{ cm}^{-1}$ , respectively (Figure 1). Amide A is a band usually associated with the N-H stretching vibration and the existence of hydrogen bonds. It commonly occurs in the range of  $3400-3440 \text{ cm}^{-1}$ . However, amide A may also be in the range of  $3318-3550 \text{ cm}^{-1}$  (Sujithra *et al.*, 2013). According to Vidal and Mello, (2011), when the N-H group of a peptide is involved in the hydrogen bond, the peak position is shifted to a lower frequency. In comparison, the amide A peaks of ASC and PSC silver catfish skin collagen were higher than those of bighead carp skin collagen (Yao *et al.*, 2012).

The amide II bands of commercial collagen were observed to be  $1545 \text{ cm}^{-1}$  while the ASC and PSC of silver catfish skin collagen were at  $1541.78 \text{ cm}^{-1}$  and  $1541.73 \text{ cm}^{-1}$ , respectively (Figure 1). Only a slight difference could be identified between the samples. Commonly, the amide II bands are within the range of  $1500-1600 \text{ cm}^{-1}$  (Wu *et al.*, 2014). This band is

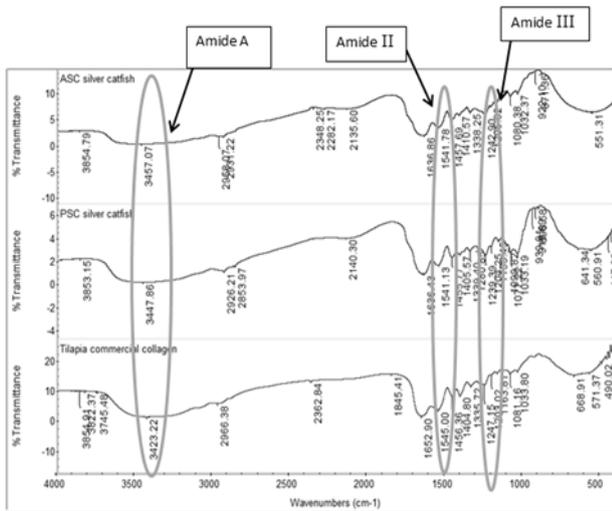


Figure 1. Functional group of ASC, PSC and commercial collagen as determined by Fourier Transform Infrared Spectroscopy (FTIR).

associated with the combination of N-H in-plane bending and the C-N stretching vibration. Amide II levels for silver catfish skin collagens are lower than in commercial collagen. This shows that the isolated collagens are more and/or stronger hydrogen bonds than the commercial collagen. The position of amide group shifts to lower frequencies when the NH group is involved with hydrogen bonding in a peptide chain. The wavenumber shifted to lower wavenumber as compared to commercial collagen showed isolated collagen contained stronger and higher number of hydrogen bonding. This was mainly due to the hydrolysis of telopeptide region by pepsin treatment which causes the destruction of hydrogen bonding (Kaewdang *et al.*, 2014). Additionally, at a lower frequency peak there is more hydrogen bonding in the triple helical structure, as well as a higher molecular order of collagen (Shanmugam *et al.*, 2012). This result was similar to that of amide II bands for ASC from scale and bone of deep sea redfish ( $1541\text{ cm}^{-1}$ ) (Wang *et al.*, 2008).

In addition, amide III is responsible for a combination of N-H deformation and C-N stretching vibrations, together with the triple helix of collagens (Zhang *et al.*, 2009). It usually occurs at  $1320\text{-}1220\text{ cm}^{-1}$ . From the results, the amide III levels for commercial collagen, ASC and PSC of silver catfish skin collagen were  $1247.15\text{ cm}^{-1}$ ,  $1242.90\text{ cm}^{-1}$  and  $1239.39\text{ cm}^{-1}$ , respectively (Figure 1). These findings were in agreement with a study by Huda *et al.* (2013) on duck feet collagen. In general, the structure obtained from Fourier transform infrared spectroscopy (FTIR) analysis shows that the extracted collagen has the fingerprint of the collagen structure, as in commercial collagen.

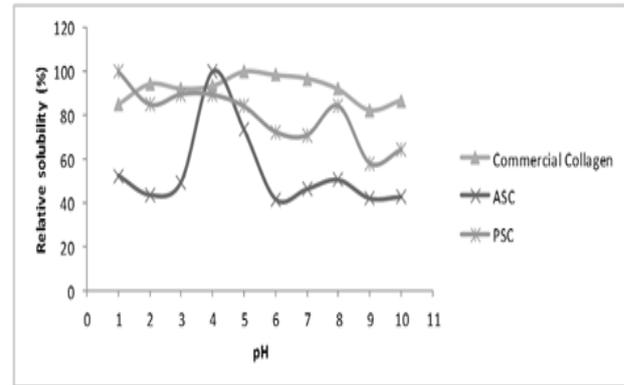


Figure 2. Relative solubility of different collagen (ASC, PSC and commercial collagen) against pH (pH 1-10).

### Solubility

The highest solubilities of ASC, PSC and commercial collagen were at pH 4, pH 1 and pH 5, respectively (Figure 2). All collagens showed the highest solubility in an acidic pH range. Both ASC and PSC present a similar pattern in terms of solubility. This was supported by Foegeding *et al.* (1996), who stated that both ASC and PSC are generally more soluble in the acidic pH. The commercial collagen showed relative solubility between 82.35-100% for pH 1-10, followed by PSC relative solubility between 58.15-100% and ASC relative solubility between 41.7-100%, respectively. Based on the isolated collagen from the skin of silver catfish, PSC showed higher solubility than ASC. This was due to the occurrence of partial hydrolysis by pepsin during the enzyme extraction process. This indicates that the acid extraction and enzyme extraction methods may affect the solubility rate of collagen. In contrast, the lowest solubilisation of collagen was in the alkaline range. The lowest solubilisation point for ASC was at pH 6 with  $41.75 \pm 7.28$ ; PSC at pH 9 with  $58.15 \pm 2.99$ ; and commercial collagen at pH 9 with  $82.35 \pm 7.79$ . These showed that solubilisation rate of ASC was significantly different ( $p > 0.05$ ) between PSC and commercial collagen. This finding is in agreement with a study by Singh *et al.* (2011) which found that low collagen solubility was found in the neutral and slightly alkaline pH range.

### Morphology properties

In microstructure measurements, all of the isolated collagens investigated depict different structures (Figure 3). The ASC was flaky and porous in nature. The collagens contain fibrils that connect the collagen. Besides that, on the surface of ASC, some agglomerate particles can be observed. These unknown particles could be a salt, which was used during the salt-out extraction process. Thus, according to Schuetz *et al.* (2013), those free

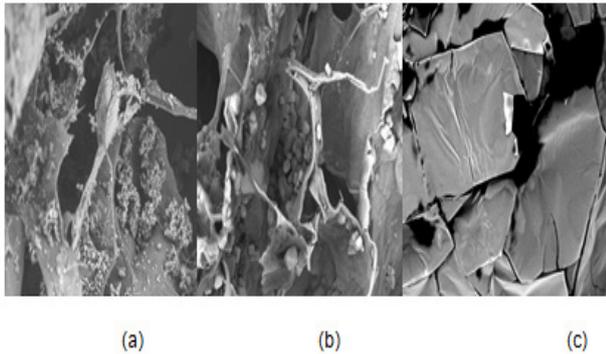


Figure 3. Microscopic structure of (a) ASC, (b) PSC and (c) commercial collagen at magnification of 100x.

particles at the surface of the collagen scaffold likely indicate minor damage to collagen during the sample preparation process. PSC also has a flaky and fibril texture. Similar to ASC, the agglomerate particles appeared on the surface of collagen. However, the particles are larger than those of ASC.

In addition, ASC and PSC have different pore sizes. PSC has a larger pore size than ASC. According to Joseph *et al.* (2008), a moderate pore size of collagen is suitable for the *in vivo* studies. Pore size was affected by the water content during the preparation, in which the higher the water content during preparation, the bigger the pore size (Veeruraj *et al.*, 2013). Meanwhile, the structure of commercial collagen was observed dry and flaky structure. Besides that, the burst-cells are in thin-layered sheets. However, these burst-cells are still linked. A similar structure was observed from the muscle of amur sturgeon collagen in a study conducted by Wang *et al.* (2014), which reported a dense, irregular sheet-like film linked by random-coiled filaments. In addition, the surface of the collagen is slightly wrinkled. These wrinkles are due to dehydration during lyophilization (Schuetz *et al.*, 2013).

## Conclusion

In conclusion, ASC demonstrates higher yields than PSC for silver catfish skin collagen. Different extraction methods resulted in different chemical and physical properties for extracted collagen. ASC and PSC were high in moisture but low in fat content. Meanwhile, the protein concentrations of both isolated collagens were quite low as compared to commercial collagen. Extracted collagens with functional groups amide A, II, III represent a fingerprint for collagen structure. Additionally, the collagens have a higher solubilisation at the acid pH ranges, and PSC is more soluble than ASC. However, the morphological structures of ASC and PSC differ from commercial collagens, as ASC and PSC are fibril and porous in nature, whereas commercial collagens have flaky

structures. In short, the characteristics of the isolated collagens obtained from this study are similar to those of commercial collagen, indicating that a value-added product could be produced from silver catfish (*Pangasius sp.*) skin.

## References

- AOAC. 2000. Official Methods of Analysis, 17<sup>th</sup> ed. Washington, DC: Association of Official Analytical Chemists.
- Foegeding, E. A., Lanier, T. C. and Hultin, H. O. 1996. Characteristics of edible muscle tissues. In O. R. Fennema (Ed.), Food chemistry, p. 902–906. New York: Marcel Dekker Inc.
- Hamdan, F. S. (2016). Isolation and characterization of collagen from Fringescale sardinella (*Sardinella fimbriata*) waste materials. Terengganu, Malaysia: Universiti Malaysia Terengganu, thesis.
- Hanani, Z. N., Roos, Y. and Kerry, J. 2011. Fourier Transform Infrared (FTIR) spectroscopic analysis of biodegradable gelatin films immersed in water. In 11th International Congress on Engineering and Food, ICEF11. Athens, Greece.
- Hema, G. S., Shyni, K., Mathew, S., Anandan, R., Ninan, G. and Lakshmanan, P. T. 2013. A simple method for isolation of fish skin collagen- biochemical characterization of skin collagen extracted from Albacore Tuna (*Thunnus Alalunga*), Dog Shark (*Scoliodon Sorrakowah*), and Rohu (*Labeo Rohita*). Annals of Biological Research 4(1): 271–278.
- Huang, L. P., Chai, X. L., Cheng, S. A. and Chen, G. H. 2011. Evaluation of carbon-based materials in tubular biocathode microbial fuel cells in terms of hexavalent chromium reduction and electricity generation. Journal of Chemical Engineering 166: 652–661.
- Huda, N., Seow, E. K., Normawati, M. N. and Aisyah, N. M. N. 2013. Preliminary study on physicochemical properties of duck feet collagen. International Journal of Poultry Science 12(10): 615–621.
- Jeevithan, E., Bao, B., Bu, Y., Zhou, Y., Zhao, Q. and Wu, W. 2014. Type II Collagen and Gelatin from Silvertip Shark (*Carcharhinus albimarginatus*) Cartilage: Isolation, Purification, Physicochemical and Antioxidant Properties. Marine Drugs 12: 3852–3873.
- Jongjareonrak, A., Benjakul, S., Visessanguan, W., Nagai, T. and Tanaka, M. 2005. Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of brownstripe red snapper (*Lutjanus vitta*). Food Chemistry 93: 475–484.
- Jose, H. M. P. M., Murugesan, P. and Kumar, K. M. 2014. Isolation and characterization of acid and pepsin - solubilised collagen from the muscle of mantis shrimp (*Oratosquilla Nepa*). International Journal of Pharmacy and Pharmaceutical Sciences 6(1): 1–4.
- Joseph, G., Jun, O. and Teruo, M. 2008. Biodegradable honeycomb collagen scaffold for dermal tissue engineering. Journal of Biomedical Materials Research Part A 87: 1103–1111.

- Kaewdang, O., Benjakul, S., Kaewmanee, T. and Kishimura, H. 2014. Characteristics of collagens from the swim bladders of yellowfin tuna (*Thunnus albacares*). *Food Chemistry* 155: 264-27.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with folin phenol reagent. *Journal of Biological Chemistry* 193: 265-275.
- Matmaroh, K., Benjakul, S., Prodpran, T., Encarnacion, A. B. and Kishimura, H. 2011. Characteristics of acid soluble collagen and pepsin soluble collagen from scale of spotted golden goatfish (*Parupeneus heptacanthus*). *Food Chemistry* 129(3): 1179-1186.
- Minh Thuy, L. T., Okazaki, E. and Osako, K. 2014. Isolation and characterization of acid-soluble collagen from the scales of marine fishes from Japan and Vietnam. *Food Chemistry* 149: 264-270.
- Nagai, T., Araki, Y. and Suzuki, N. 2002. Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). *Food Chemistry* 78: 173-177.
- Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2008. Improvement of gelatin extraction from bigeye snapper skin using pepsin-aided process in combination with protease inhibitor. *Food hydrocolloids* 22(4): 615-622.
- Normah, I., Nur Ain, H. and Effaniza, E. H. 2014. Physicochemical Properties of Silver Catfish (*Pangasius sutchi*) skin gelatin produced as affected by different extraction time. *Journal intelekt* 9: 7-15.
- Rosli, N. and Sarbon, N. M. 2015. Physicochemical and structural properties of Asian Swamp Eel (*Monopterus albus*) skin gelatin as compared to bovine gelatin. *International Food Research Journal* 22(2): 699-706.
- Schuetz, T., Richmond, N., Harmon, M. E., Schuetz, J., Castaneda, L. and Slowinska, K. 2013. The microstructure of collagen type I gel cross-linked with gold nanoparticles. *Colloids Surf B Biointerfaces* 101:118-25.
- Shahiri Tabarestani, H., Maghsoudlou, Y., Motamedzadegan, A., Sadeghi Mahoonak, A. R. and Rostamzad, H. 2012. Study on some properties of acid-soluble collagens isolated from fish skin and bones of rainbow trout (*Onchorhynchus mykiss*). *International Food Research Journal* 19(1): 251-257.
- Shanmugam, V., Ramasamy, P., Subhapradha, N., Sudharsan, S., Seedeivi, P., Moovendhan, M. and Srinivasan, A. 2012. Extraction, structural and physical characterization of type I collagen from the outer skin of *Sepiella inermis*. *African Journal of Biotechnology* 11(78): 14326-14337.
- Singh, P., Benjakul, S., Maqsood, S. and Kishimura, H. 2011. Isolation and characterisation of collagen extracted from the skin of striped catfish (*Pangasianodon hypophthalmus*). *Food Chemistry* 124(1): 97-105.
- Skierka, E. and Sadowska, M. 2007. The Influence of Different Acids and Pepsin on the Extractability of Collagen from the Skin of Baltic Cod (*Gadus morhua*). *Food Chemistry* 105: 1302-1306.
- Sujithra, S., Kiruthiga, N., Prabhu, M. J. and Kumeresan, R. 2013. Isolation and determination of type I collagen from Tilapia (*Oreochromis niloticus*) waste. *International Journal of Engineering and Technology* 5(3): 2181-2185.
- Veeruraj, A., Arumugam, M. and Balasubramanian, T. 2013. Isolation and characterization of thermostable collagen from the marine eel-fish (*Evenchelys macrura*). *Process Biochemistry* 48(10): 1592-1602.
- Vidal, B. C. and Mello, M. L. S. 2011. Collagen type I amide I band infrared spectroscopy. *Micron* 42 (3): 283-289.
- Wang, L., An, X., Yang, F., Xin, Z., Zhao, L. and Hu, Q. 2008. Food Chemistry Isolation and characterisation of collagens from the skin, scale and bone of deep-sea redfish (*Sebastes mentella*). *Food Chemistry* 108: 616-623.
- Wang, Z., Wangm, L., Lin, S., Liang, Q., Shi, Z., Xu, J. and Ma, H. (2014) Isolation and Characterization of Collagen from the Muscle of Amur Sturgeon (*Acipenser schrenckii*). *Biotechnology and Bioprocess Engineering* 941: 935-941.
- Wu, G. P., Wang, X. M., Lin, L. P., Chen, S. H. and Wu, Q. Q. 2014. Isolation and Characterization of Pepsin-Solubilized Collagen from the Skin of Black Carp (*Mylopharyngodon piceus*). *Advances in Bioscience and Biotechnology* 5: 642-650.
- Yang, H. and Shu, Z. 2014. The extraction of collagen protein from pigskin. *Journal of Chemical and Pharmaceutical Research* 6(2): 683-687.
- Yao, P., Wang, H., Wang, H., Li, Y., Wang, M. and Zhang, H. 2012. Biochemical and physiological characterization of collagen from the skin of bighead carp (*Aristichthys nobilis*). *Journal of Food, Agriculture and Environment* 10(3&4): 92-98.
- Yd, S., Em, A., Yusoff, A., Ssa, H., Ty, N. and Mys, A. 2013. Extraction, Purification and Physical Characterization of Collagen from Body wall of Sea cucumber (*Bohadschia bivitatta*). *Journal of Environmental Health* 4(2): 53-65.
- Zhang, M., Liu, W. and Li, G. 2009. Isolation and Characterization of Collagens from the Skin of Largefin Longbarbel Catfish (*Mystus macropterus*). *Food Chemistry* 115: 826-831.