

Microstructure, extractability and physicochemical properties of shortfin scad (*Decapterus macrosoma*) bone collagen as influenced by acetic acid concentration

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Abstract

Collagen from shortfin scad (*Decapterus macrosoma*) bone was extracted using varying concentrations of acetic acid. Yields of extracted collagen were $1.01 \pm 0.09\%$ and $1.31 \pm 0.07\%$ for 0.5 M and 0.7 M acetic acids, respectively. The pH values of commercial collagen and shortfin scad bone collagen extracted using acetic acids (0.5 M and 0.7 M) were 6.52 ± 0.03 , 4.99 ± 0.04 , and 5.32 ± 0.01 , respectively. The functional group analysis for collagen showed that the Amide A, Amide II and Amide III bands have been detected. The microstructure study showed that the isolated collagen had a porous fibril network. Maximum solubility for the commercial collagen was observed at pH 3, while the collagens extracted with 0.5 M and 0.7 M acetic acids were similar at pH 8. Meanwhile, the relative viscosities for all collagens indicated increasing values with increasing temperatures. In conclusion, the shortfin scad bone collagen showed potential for use as an alternative collagen. Although the values for shortfin scad bone collagen extracted by 0.5 M and 0.7 M acetic acids were lower than the commercial one, the collagens exhibited similar properties.

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Keywords

Acid soluble collagen (ASC)
Shortfin scad (*Decapterus macrosoma*)
Fish collagen
Acetic acid
Extraction

Introduction

Collagen is the most abundant protein in vertebrates and the main structural protein in the connective tissue of animal skin and bone. About 30% of total proteins are collagen (Bhattacharjee and Bansal, 2005; Li *et al.*, 2013). Originally, the collagen used in profitable production processes was mostly limited to that of land-based animals such as bovine and porcine sources. However, recent years have seen the proliferation of infectious diseases such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), foot-and-mouth disease (FMD) and avian influenza (Yan *et al.*, 2012; Li *et al.*, 2013). In addition, there are also Halal issues and dietary restrictions, as porcine collagen is prohibited to some religious and ethnic groups such as Jews and Muslims (Yu *et al.*, 2014). Fish collagen may be the best alternative collagen source. Fish-based collagen is undoubtedly viable as a safer and consumer-friendly collagen. To date, most of the studies have utilized by-products such as skins, bones, and fins during processing of fishes as the raw materials for collagen extraction that helps in minimizing the environmental pollution (Kiew and

Don, 2013; Li *et al.*, 2013). An individual collagen polypeptide chain has many repeating amino acid sequences, most often glycine-X-Y, where X is often proline and Y is often hydroxyproline. Marine species can be used as an alternative and safe source for the extraction of collagen. The extraction of collagen from fish bone and skin has been carried out from many fish species (Vika and Theodoropoulou, 2012; Arumugam *et al.*, 2018).

As the collagen market becomes huge, the precise method of isolation of collagen is needed to meet the increasing demand. Therefore, there are several methods of collagen isolation now available, each with their own strengths and weaknesses. The four common methods of isolation of collagen are salting out method, alkaline method, acid method and enzyme method (Yang and Shu, 2014). The best isolation methods are the acid and enzyme methods (Mocan *et al.*, 2011). The acid soluble method uses an acid solubilization process that commonly used to extract collagen by using acid solution in the preparation of acid-soluble collagen (ASC) (Matmaroh *et al.*, 2011). Ordinarily, the acids used include organic acids such as acetic, citric and lactic acids, and inorganic acids such as hydrochloric acid (Normah and Suryati,

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2015). The solubility of a collagen in acid solution will play a key role in extraction efficiency (Kiew and Don, 2013).

Several researchers have studied the physicochemical characteristics of the isolated alternative collagen, especially from marine sources (Li *et al.*, 2013; Tang *et al.*, 2015). The chemical characteristics of collagen include its chemical composition, amino acid composition, protein concentration and pH (Ortiz *et al.*, 2015). The physical characteristics of collagen include its functional group, viscosity, solubility and molecular weight (Safandowska and Pietrucha, 2012; Chen *et al.*, 2015). Collagen in food supplements can be used as an ingredient to enhance muscle gain, reconstruct damaged structures and improve cardiovascular performances. Therefore, the good quality of the collagen extracted can be explained by characteristics such as structural properties which demonstrate the collagen's molecular structure, amino acid composition and internal linkages (Jeevithan *et al.*, 2014).

At present, low value pelagic fishes such as the shortfin scad (*Decapterus macrosoma*) are widely used in the production of a popular snack food in Malaysia known as "keropok lekor". The production of this product is predominantly in the east coast of Peninsular Malaysia, mainly in the states of Kelantan, Terengganu and Pahang (Yeap and Denise, 2010). In relation to the production of "keropok lekor", a large amount of waste materials is discarded. Poor disposal of this by-product may create serious environment pollution with unpleasant odors (Li *et al.*, 2013). Consequently, the increasing level of fish waste serves as the driving factor for alternative utilization, in this case, as a potential alternative source of collagen. Therefore, the aim of the present work was to isolate and characterize the acid-soluble collagen (ASC) from shortfin scad bone extracted by varying concentrations (0.5 M and 0.7 M) of acetic acid.

Materials and methods

Materials

Shortfin scad was purchased from Maperow Sdn. Bhd., Kuala Terengganu, Terengganu, Malaysia, and transported to the laboratory in ice. The commercial collagen from tilapia scale used for comparison was purchased from the Umathy Industries Sdn. Bhd., Bandar Baru Bangi, Selangor, Malaysia. All reagents used were of analytical grade unless otherwise stated.

Sample preparation

The shortfin scad bone was removed manually

and washed with chilled water before being cut into pieces. All the bone was packed in a polyethylene bag and stored at -20°C until further use. The storage time was three months or less. All preparations were performed at 4°C (Minh Thuy Le *et al.*, 2014).

Pre-treatment

Before the isolation of collagen, the pre-treatment processes were conducted to ensure that unwanted materials and residues were removed from the sample. The process also ensured that the collagen was fully extracted without any interference that might affect the characteristics of the collagen produced.

Removal of non-collagenous proteins

According to Minh Thuy Le *et al.* (2014), all preparation was carried out below 4°C. The bone was dissolved in 0.1 M sodium hydroxide (NaOH) for 6 h at a sample to NaOH ratio of 1:8 (w/v) in order to remove non-collagenous proteins, followed by washing in cold distilled water until a neutral pH of 7 was achieved.

Demineralization

The demineralization process of the bone was achieved by treating the samples with 0.5 M ethylenediaminetetraacetic acid disodium salt (EDTA) solution (pH 7.5) at a sample/EDTA solution ratio of 1:5 (w/v) for 24 h. Then, the residues were cleaned with cold distilled water (Minh Thuy Le *et al.*, 2014).

Isolation of Acid soluble collagen (ASC)

In acid soluble collagen (ASC) extraction, 0.5 M and 0.7 M acetic acids were treated to the sample at sample to solution ratio of 1:5 (w/v) for 24 h with continuous stirring. Then, the extracts were centrifuged (Hitachi CR22N, Tokyo, Japan) at 8,000 g for 30 min at 4°C, and the supernatant obtained were separated. The sample residues were re-extracted with 0.5 M acetic acid with sample to solution ratio of 1:5 (w/v) for 12 h before centrifuged at 8,000 g for 30 minutes at 4°C. Both supernatants were pooled, and sodium chloride (NaCl) was added for salting out until final concentration of the supernatant was 0.7 M for the precipitation to occur. The supernatants were centrifuged again at 8,000 g to obtain precipitate. The precipitates were then lyophilized (Huang *et al.*, 2011). The collagen yield of ASC was calculated using the following formula:

$$\text{Yield (\%)} = \frac{\text{Dry weight collagen}}{\text{Weight of raw bone}} \times 100$$

Analysis

pH

Measurement of pH was conducted according to the methodology described by Ortiz *et al.* (2014) using a pH meter (inoLab pH 7110 bench-top pH meter) previously calibrated with buffer solutions at pH 4 and pH 7. Dialyzed collagen samples at 20°C were placed in a beaker with gentle stirring for homogenization. Then, the glass electrode was dipped into the solution. After each measurement, the reading was registered. Readings were taken in triplicate.

Structural properties

The structural properties of isolated collagen were examined by using Fourier Transform Infrared Spectroscopy (FTIR) following Hamdan and Sarbon (2019) with slight modification. The infrared spectrum was set in the range from 4000 to 400 cm^{-1} using an infrared spectrophotometer (Nicolet, Thermo Electron, USA). The lyophilized collagens were mixed with KBr with ratio 1:100 and molded into a disc. The background spectrum without the sample was collected. The KBr samples were then placed on the plate and the sample spectrum was collected. From the peak produced at the certain wavelength and absorbance, the functional group (Amide A, Amide II and Amide III) and mode of vibration were identified. Analyses were conducted in triplicate.

Morphological analysis

Morphological determination on extracted collagen was conducted as referred to Saharuddin (2013) with some alteration. The freeze-dried collagen was mounted on aluminum cylinder stubs (5 mm × 12.5 mm) and sputter-coated with Auto fine coated (JEOL JFC 1600, Tokyo, Japan). The microstructure of the powder was examined using a scanning electron microscope (JEOL JSM-6360LA, Tokyo, Japan) at an acceleration voltage of 10 kV (Hamdan and Sarbon, 2019).

Solubility determination

The solubility levels of extracted collagens were determined by using different pH levels according to the method described by Jongjareonrak *et al.* (2005) and Huang *et al.* (2011) with slight modification. Approximately 240 mg lyophilized collagens were dissolved in 80 mL 0.5 M acetic acid with a gentle stirring for 12 h to obtain final concentration of 3 mg/mL. Then, about 8 mL sample was transferred to a centrifuge tube and the pH was adjusted across a pH range of 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 min at 4°C, then centrifuged at 10,000 g for 30 min. The dissolved collagen and dissolved protein were determined as protein content in a supernatant by using Lowry's Method and the bovine serum albumin as a protein standard. The concentration of protein content was determined by using the standard curve. The relative solubility of collagen was calculated as follows:

Relative solubility (%)

$$= \frac{\text{Current concentration of protein at current pH}}{\text{The highest concentration of protein}} \times 100$$

Viscosity

Samples of 0.3% collagen in 0.1 M acetic acid were prepared by dissolving 0.15 g collagen in 50 mL 0.1 M acetic acid and subjected to viscosity measurements using a Brookfield DV-I viscometer (USA) with spindle No. 1 and speed of 100 rpm. Collagen solution was heated from 4 to 50°C with heating rate of 4°C/min. At each chosen temperature, the solution was held for 30 min prior to viscosity determination. The relative viscosity was calculated in comparison to that obtained at 4°C. The analysis was done in triplicate.

Statistical analysis

All experiments were run in triplicate and data was presented as mean ± standard deviation (± SD). The probability value of $p < 0.05$ was considered significantly different. Analysis of variance (ANOVA) was performed and means comparisons were done by Tukey's test. Analysis was performed using MINITAB 14.

Results and discussion

Yield of extracted collagen

Collagen from the bone of shortfin scad was successfully extracted using two different acetic acid concentrations which were 0.5 M and 0.7 M. The yields of the extracted collagen of 0.5 M and 0.7 M acetic acids from shortfin scad are presented as in Table 1. The results show that the yield of extracted collagen by 0.7 M acetic acid was significantly higher than extracted collagen by 0.5 M acetic acid. Hence, the yield of extracted collagen increased as the concentration of the acetic acid increased.

The differences in yields of the extraction collagen, varying according to concentration of acetic acid employed, were probably due to different solubility of collagen in the acidic extracting medium (Kiew

and Don, 2013). The collagen extracted in 0.5 M acetic acid might undergo incomplete solubilization as the inter-molecular cross-links still exist in collagen molecules. For collagen extracted in 0.7 M acetic acid, the electrostatic repulsive force between the one-nominal charged groups was much stronger. This then resulted in loosening of the structure of collagen fibers and the ability to form bonding with water rose, thus increasing the solubility of collagen in the medium (Kiew and Don, 2013). These findings agree with a previous report by Kiew and Don (2013), on hybrid *Clarias* sp. skin collagen, which found that the collagen extracted by 0.7 M acetic acid (26.69%) was the optimum extraction of acetic acid. However, the yield of extracted collagen from the shortfin scad bone in the present work was lower as compared to that of hybrid *Clarias* sp. skin collagen. Acetic acid has great extractability towards collagen and the capability of maintaining the structure of collagen so that the collagen can be fully extracted (Cheng *et al.*, 2009; Hamdan and Sarbon, 2019).

pH

The pH value of commercial collagen and collagen extracted by 0.5 M and 0.7 M acetic acids are presented in Table 1. There were significant differences ($p < 0.05$) between all the collagen samples. This shows that the higher the concentration of acetic acid used to extract the collagen, the lower the acidity of the collagen. The difference in pH values of the collagen extracted using 0.5 M and 0.7 M acetic acids might be due to the pH of the extraction solvent that plays an important role in the effects on polyelectrolyte assembly. The pH values of the 0.5 M and 0.7 M acetic acids were 2.53 and 2.45, respectively. The low pH value was detrimental to the physical properties as it produces more degradation and proliferation of lower molecular weight peptides (Huda *et al.*, 2013). The findings of the extracted collagen agree with the study by Huda *et al.* (2013) on duck feet collagen, which showed an acidic pH value (2.76), and similar with chicken feet collagen with an acidic pH value (3.47) (Liu *et al.*, 2001).

Table 1: The yield percentages, pH values and protein concentrations of extracted collagen from different acetic acid concentrations and commercial collagen.

Characteristics	Extracted Collagen		Commercial Collagen
	Acetic Acid		
	0.5 M	0.7 M	
Yield (%)	1.01 ± 0.09 ^b	1.31 ± 0.07 ^a	-
pH	4.99 ± 0.04 ^c	5.32 ± 0.01 ^b	6.52 ± 0.03 ^a

Standard deviation (± SD) is included for each average. Means with different letter within the same rows are significantly different ($p < 0.05$) ($n = 3$).

Structural properties

Table 2 shows the presence of Amide A, Amide II and Amide III in the collagen extracted from the shortfin scad bone (0.5 M and 0.7 M acetic acids) and commercial collagen. The FTIR range of frequency that detected Amide A, Amide II and Amide III were 3208 cm^{-1} – 3447 cm^{-1} , 1553 cm^{-1} – 1560 cm^{-1} and 1245 cm^{-1} – 1291 cm^{-1} , respectively. The Amide A provided the N-H stretching between molecules. The findings show that the wavelength for the Amide A for the commercial collagen was lower (3208.42 ± 5.20 cm^{-1}) and significantly different ($p < 0.05$) from the other two collagens. This may be due to the N-H group of a peptide in the commercial collagen involved in the hydrogen bond that shifted the position to a lower frequency. The Amide A wavelength from the collagen extracted using the 0.5 M and 0.7 M acetic acids were 3443.09 ± 0.32 cm^{-1} and 3451.45 ± 5.79 cm^{-1} , respectively. Hence, the findings show that the triple helical structure of collagen was not affected by the concentration of acetic acid treatment at telopeptide region as there were no significant differences ($p > 0.05$) for the both of extracted collagen on Amide A bands (Hamdan and Sarbon, 2019). This was due to Amide A indicating the stability of the triple helical structures. This finding on the Amide A FTIR spectra of extracted collagen agrees with a previous study by Chen *et al.* (2015) on skin and scales of tilapia collagen that showed a free N-H stretching vibration of Amide A occurring in the range of 3400 cm^{-1} – 3440 cm^{-1} .

Then, Amide II CH_2 forms formed the combination of N-H bending and C-N asymmetric stretching vibration group –CO-NH- in the trans form. For the Amide II, the wavelength for the collagen extracted using 0.5 M and 0.7 M acetic acids and commercial collagen were 1556.47 ± 4.86 cm^{-1} , 1559.96 ± 0.05 cm^{-1} and 1543.64 ± 2.88 cm^{-1} , respectively. There were significant differences ($p < 0.05$) between the collagen extracted using 0.7 M acetic acid with the commercial collagen on the Amide II value. This may be due to the commercial collagen that had more and/or stronger hydrogen bonds which shifted the position of spectra to a lower frequency (Hamdan and Sarbon, 2019). This is also supported by the higher finding of the protein concentration of the commercial collagen, so that the hydrogen bond in the commercial collagen was stronger. Moreover, this finding is also similar with Duan *et al.* (2009) who found a similar stretching pattern (1550 cm^{-1} – 1600 cm^{-1}) for Amide II of collagen from the skin, scale and bone of common carp. However, both extracted collagens (0.5 M and 0.7 M) showed that the concentration of acetic acid treatment did not

Table 2: The FTIR spectra of commercial collagen and the extracted collagen

Samples	Wavelength (cm ⁻¹)		
	Amide A	Amide II	Amide III
	N-H stretching	N-H bending + C-N stretching	C-H stretching
Commercial collagen	3208.42 ± 5.20 ^b	1543.64 ± 2.88 ^b	1245.71 ± 0.53 ^a
0.5 M acetic acid	3443.09 ± 0.32 ^a	1556.47 ± 4.86 ^{ab}	1291.10 ± 0.40 ^a
0.7 M acetic acid	3451.45 ± 5.79 ^a	1559.96 ± 0.05 ^a	1247.49 ± 1.75 ^a

Standard deviation (± SD) is included for each average. Means with different letter within the same column are significantly different ($p < 0.05$) ($n = 3$).

affect the Amide II band as there were no significant differences ($p > 0.05$) between them.

The Amide III was complex with the intermolecular interaction that correlated by the C-N stretching and N-H in plane deformation. The wagging vibrations from CH₂ groups from the glycine backbone and proline side chains will increase absorption (Wang *et al.*, 2014). According to Chen *et al.* (2015), the absorption ratio that increased was associated with triple helical structure. The FTIR wavelength for Amide III, collagen extracted using 0.5 M and 0.7 M acetic acids and commercial collagen showed wavelengths of 1291.09 ± 0.36 cm⁻¹, 1245.71 ± 0.53 cm⁻¹ and 1247.49 ± 1.75 cm⁻¹, respectively. From these findings, all the collagens (collagen extracted using 0.5 M and 0.7 M acetic acids and commercial collagen) showed similar spatial triple helical structures which are important for collagen backbones as there were no significant differences among all the collagens ($p > 0.05$) on the structural properties of Amide III. Then, the findings also showed that there were hydrogen bonds present in both extractions as the Amide III also indicated the existent of hydrogen bonds. These results agree with Chen *et al.* (2015) who found that the Amide III bands for tilapia scales to be 1242.83 cm⁻¹ and tilapia skin collagen (12442.3 cm⁻¹). In short, the difference in those range and number of Amide bands and structural group of all the collagen indicates the differences in their molecular structure (Benjakul *et al.*, 2012).

Morphology of the collagen

Figures 1 (a) and (b) show that the collagen extracted from 0.5 M and 0.7 M acetic acids had fibrils, porous and interconnective structures (connection among pores and fibrils). There were also agglomerate particles present in both collagens which could be NaOH (sodium hydroxide) used during the salting out step. Meanwhile, the structure of commercial collagen was in thin-layered sheets, flaky and the fibril could not be identified and much different from both extracted collagens (Figure 1 (c)).

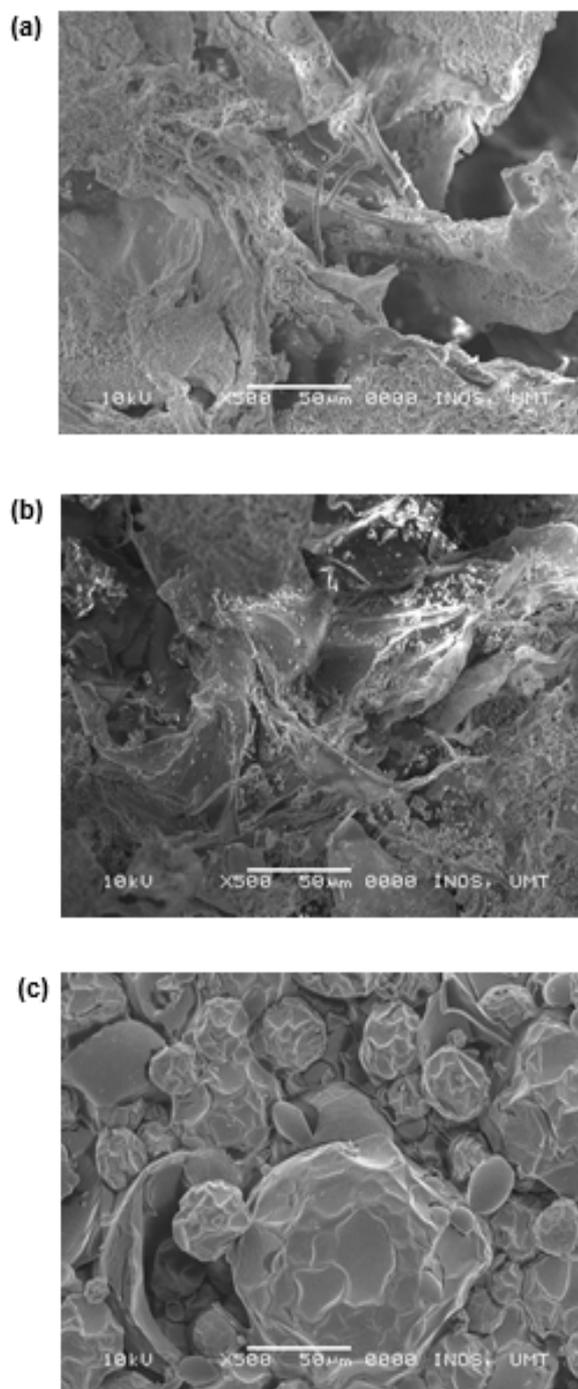


Figure 1. The microscopic structures of (a) 0.5 M acetic acid collagen, (b) 0.7 M acetic acid collagen, and (c) commercial collagen.

The results of extracted collagen agree with findings by Veeruraj *et al.* (2013) from a study of skin eel-fish collagen that also showed similar morphology, namely a fibril and porous structure in three dimensional forms.

The collagen extracted with 0.5 M acetic acid showed a similar morphology with the collagen extracted with 0.7 M acetic acid. However, the fibril of collagen extracted with 0.7 M acetic acid was clearer and the interconnected structure was more distributed. Hence, the higher the acetic acid concentration, the clearer the fibrils formations of collagen.

The findings for extracted collagen agree with analysis conducted by Tang *et al.* (2015) which showed the micrographs of the upper and lower surface of ASC films from three fish skins, namely tilapia, grass carp and silver carp. The results showed that the characteristics of the upper surface of collagen films seemed to relate to the fibril formation ability. Collagen features such as pore sizes, pore shape and pore wall morphology play important roles in cell seeding, growth, gene expression, migration, mass transport and new tissues formation; therefore the determination of the microstructure of each collagen is important (Jeevithan *et al.*, 2014).

Solubility of collagen

Figure 2 illustrates the findings for the relative solubility of the collagen in different pH's. There are fluctuating trends for commercial collagens. Meanwhile, for shortfin scad collagens extracted with 0.5 M and 0.7 M acetic acids showed similar trends in solubility at different pH's, increasing from pH 1 to pH 5 and becoming flat beyond that. The maximum solubility for the commercial collagen

were observed at pH 3, while the collagen extracted with 0.5 M and 0.7 M acetic acids was similar at pH 8. There were significant differences ($p < 0.05$) between the collagen samples at pH's 1, 2, 3, 5 and 7, while pH 6 displayed no significant difference ($p > 0.05$) between all the collagen samples. Meanwhile, there were significantly higher ($p < 0.05$) values of relative solubility for collagen extracted with 0.7 M acetic acid at pH 9 and 10. The overall result shows that the extracted collagen was soluble in alkaline condition.

Commercial collagen showed maximum solubility at pH 3. The findings of the commercial collagen agree with Minh Thuy Le *et al.* (2014), Matmaroh *et al.* (2011) and Singh *et al.* (2011) that showed most collagen in the increasing trends of solubility in acidic medium. This is because in an acidic pH, the charge will be positive, leading to strong electrostatics repulsion between the collagen molecules. This in turn will cause disrupting effects on the intrinsic stability of collagen preventing the droplets to form aggregates and coalescent. However, both collagens extracted with 0.5 M and 0.7 M acetic acids showed maximum solubility at pH 8. However, the 0.7 M acetic acid extracted collagen showed higher solubility than 0.5 M acetic acid extracted collagen. These findings agree with a report by Jongjareonrak *et al.* (2005) on the collagen of bigeye snapper which showed high solubility at pH levels ranging from 8 to 10. The instability of collagen at certain pH was due to the isoelectric point (pI) (pH 6-9). At certain pH levels, the net negative or positive charge residue of protein molecules will rise, and the solubility will be increased by the repulsive forces between the chains (Jongjareonrak *et al.*, 2005).

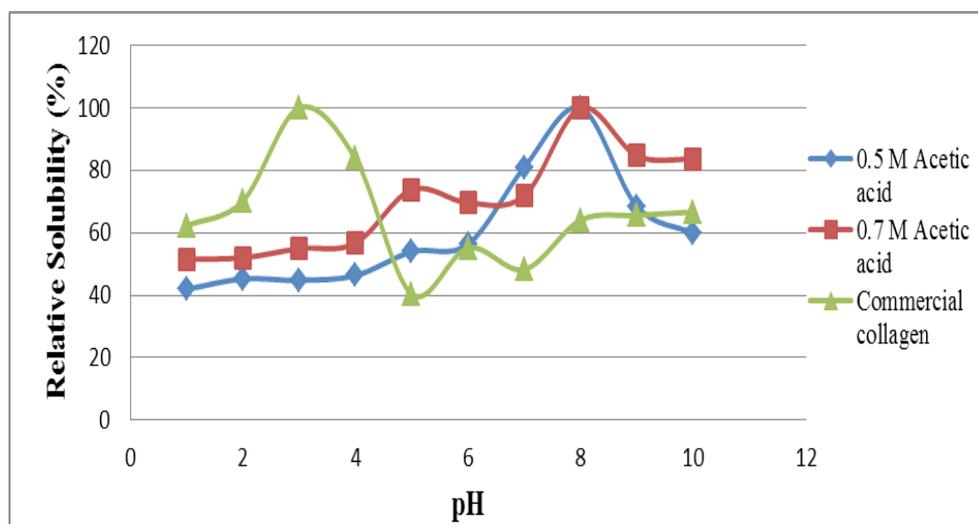


Figure 2. The relative solubility of 0.5 M acetic acid collagen, 0.7 M acetic acid collagen, and commercial collagen at different pH's.

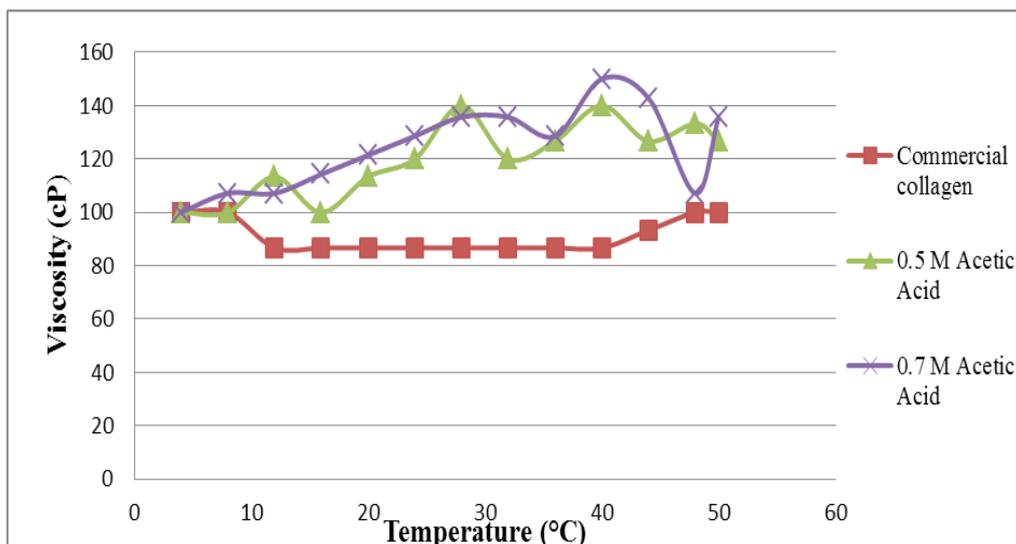


Figure 3. The viscosity of 0.5 M acetic acid collagen, 0.7 M acetic acid collagen, and commercial collagen at different temperatures.

Viscosity of collagen

The viscosity of the collagen extracted with 0.5 M and 0.7 M acetic acids (Figure 3) showed increasing values with increasing temperature, which might be explained by the fact that heat energy could break the hydrogen bonds of collagen molecule, leading to the conversion of collagen triple helix structure into random coil configuration through a process of thermal depolymerization. The breaking down of the collagen structure will lead to several physical changes in viscosity, sedimentation, diffusion, light scattering and their optical activity (Wang *et al.*, 2014). Moreover, the collagen extracted with 0.7 M acetic acid was more viscous than the collagen extracted with 0.5 M acetic acid. This indicates that the concentration affected the viscosity of the collagen. This behavior could be due to the higher amount of hydrogen bonds present in the 0.7 M acetic acid extracted collagen.

Conclusion

In conclusion, collagen extracted from shortfin scad using 0.5 M and 0.7 M acetic acid has been successfully isolated, and the characteristics were compared to commercially available collagen. The yield for the collagen extracted by using 0.7 M was higher, and exhibited a better physicochemical characterization and more viscous, which indicates stable collagen structure. Hence, 0.7 M acetic acid is more effective to be used as the extraction medium. Although the yield of fish bone collagen is lower than that of mammalian collagen, it has similar properties to commercial collagen and has potential for use due to its specific properties.

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