Isolation and characterisation of collagen from fringescale sardinella (*Sardinella fimbriata*) waste materials

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Abstract

The present work was aimed to isolate and characterise collagen from fringescale sardinella (*Sardinella fimbriata*) waste materials using two extraction methods; acid soluble collagen (ASC) and pepsin soluble collagen (PSC). The physical and chemical properties of extracted collagens were characterised in terms of protein concentration, structural, solubility and morphological properties. The yields of extracted collagen were $7.48 \pm 4.84\%$ for ASC and $0.94 \pm 0.22\%$ for PSC. The protein concentrations for ASC and PSC were significantly ($p < 0.05$) lower than commercial collagen (from tilapia scale). Functional groups for extracted collagens such as amide A (3400-3440 cm^{-1}), amide II (1400-1600 cm^{-1}), and amide III (1200-1500 cm^{-1}) bands were in the same range as commercial collagen. A higher solubility of commercial collagen and ASC was observed under acidic conditions while PSC was highly soluble under alkaline conditions. Morphological study showed that the collagen had a flaky and fibrillary structure. ASC and PSC exhibited similar properties to the commercial collagen, albeit being of lower values. Therefore, the collagen extracted from fringescale sardinella demonstrated potential for use as an alternative collagen from marine sources

Introduction

Collagen is an important protein in animal bodies, and mostly found in connective tissues and bones. It contains specific amino acids such as glycine, proline, hydroxyproline and alanine (Muyonga *et al*., 2004) which are also a major insoluble fibrous protein in extracellular matrix and connective tissue (Muyonga *et al*., 2004; Jongjareonrak *et al*., 2005). Approximately 29 different types of collagen have been identified (Liu *et al*., 2015). At present, collagen is typically extracted from skins and bones of porcine and bovine; however, complications might arise due to certain issues or diseases. Therefore, some researchers have attempted to find a new source of collagen from fish or fish waste materials by isolating two types of collagen; acid soluble collagen (ASC) and pepsin soluble collagen (PSC).

For collagen extraction, several methods have been employed such as acid extraction, alkali extraction and enzyme extraction. However, the acid and enzyme extractions are the most common (Tamilmozhi *et al*., 2013; Veeruraj *et al*., 2015). For acid extraction, acetic acid at 0.5 M is typically used due to its effectiveness as compared to other acids while for enzyme extraction, pepsin is commonly used as it is efficient in maintaining the structure of collagen during digestion (Kiew and Don, 2013). In addition, alkali extraction has strong hydrolysis effect on protein collagen which produces a high amount of hydrolysed collagen. Generally, sodium carbonate and magnesium oxide are used to extract collagen from leather waste (Yang and Shu, 2014).

Many studies have been conducted in order to determine the characteristics of the isolated collagen from different sources in terms of physical properties such as thermal stability and solubility (Huang *et al*., 2011; skin of balloon fish), viscosity (Wang *et al*., 2008; skin, scale and bone of deep-sea redfish) and morphological properties (Jeevithan *et al*., 2014; skeletal and headbone of silvertip shark). Studies have also examined chemical properties such as chemical composition and structural properties (Pati *et al*., 2010; fish scale) and amino acids composition (Yu *et al*., 2014; spines and skulls of skipjack tuna). Those studies show that alternative collagen (fish sources) have high potential in applications similar to commercial collagen (bovine and porcine). As for the amino acid content of alternative collagen, most of them are even higher (22.3-22.5%) (Veeruraj *et al*., 2014) than in calf skin (21.5%) (Herbage *et al*., 1977; Jongjareonrak *et al*., 2005).

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Fringescale sardinella (*Sardinella fimbriata*) is a pelagic fish with ray finned bones. It can be found in Indo-West Pacific from Southern India and Bay of Bengal to the Philippines and also eastern tip of Papua New Guinea. It usually forms schools along the coast and in the deep sea (Kudale and Rathod, 2015). Usually, this underutilised fish is processed to make fish crackers or '*keropok lekor*' and salted dried fish. Because of this processing, a large amount of waste materials are produced. Even though the waste material of this fish is high in protein, it has high ash content which makes it unsuitable to be processed as fish meal (Silva *et al.*, 2014). However, it has been proven by previous studies that fish waste materials are rich in collagen. For this reason, many researchers have studied the collagen from waste materials of fish (Kittiphattanabawon *et al.*, 2005). Extraction of collagen from waste materials of fish processing industries such as fringescale sardinella can contribute in decreasing the pollution causes by the fish processing industries such as bones, scales and inner part of the fish. Instead of using collagen that is extracted from bovine or porcine to avoid certain diseases and religious restrictions, new source for collagen can therefore be obtained. Therefore, the objectives of the present work were to isolate and characterise the acid and pepsin soluble collagen from fringescale sardinella waste materials followed by characterising its protein concentration, structural properties, solubility and morphological properties.

Materials and methods

Materials

Waste materials of fringescale sardinella were obtained from a local supplier at Kuala Terengganu, Terengganu, Malaysia. It was brought to the laboratory in ice bucket, and stored at -20°C. The commercial collagen extracted from the scales of tilapia was purchased from Umaty Industries Sdn. Bhd., Selangor, Malaysia. Pepsin from bovine was purchased from Sigma-Aldrich, Germany. All chemicals used in the present work were of analytical grade unless otherwise stated.

Methods

Sample preparation

The waste materials from fringescale sardinella were washed, rinsed in excessive tap water, and coarsely grinded. It was then packed in a polyethylene bag and stored at -20°C until further analysis (Minh Thuy *et al.*, 2014).

Pre-treatment of sample

The removal of non-collagenous protein and demineralisation were conducted using a method described by Minh Thuy *et al.* (2014) and Wang *et al.* (2008) with slight modification. Briefly, the sample was soaked in 0.1 M NaOH solution with a ratio of 1:2 (w/v) for 6 h. The NaOH solution was changed every 3 h, and then washed thoroughly with cold distilled water until the rinsed water became neutral (pH 7.0).

Demineralisation was performed by soaking the waste materials in 0.5 M EDTA (pH 7.4) for 24 h with continuous stirring. The solutions were changed every 12 h. Then, it was washed with distilled water three times.

Extraction of acid soluble collagen

The pre-treated samples were extracted by following the method described by Huang *et al.* (2011) with slight modification in 0.5 M acetic acid with sample to solution ratio of 1:2 (w/v) for 24 h with continuous stirring. The extracts were then centrifuged (150R Gyrozen, Korea) at 10,000 g for 15 min at 4°C, and the supernatant was separated. The pellet was re-extracted with 0.5 M acetic acid with sample to solution ratio of 1:2 (w/v) for 12 h, and centrifuged at 10,000 g for 15 min at 4°C. Both supernatants were pooled, and NaCl was added to salt out until the final concentration of the supernatant was 0.7 M for precipitation to occur. The solution containing precipitate was centrifuged again at 2,500 g for 15 min in order to separate the precipitate from solution. The precipitates were then freeze-dried.

Extraction of pepsin soluble collagen

The undissolved matter from the ASC extraction was used for further extraction by following the method described by Huang *et al.* (2011) with slight modification in two volumes of 0.5 M acetic acid containing 1.5% (w/w) pepsin for 30 h at 4°C with continuous stirring. The extracts were then centrifuged at 10,000 g for 15 min at 4°C, and the supernatant was separated. The pellet was re-extracted with 0.5 M acetic acid containing 1.5% (w/w) pepsin for 12 h, and was centrifuged at 10,000 g for 15 min at 4°C. Both supernatants were pooled, and NaCl was added to salt out until the final concentration of the supernatant was 0.7 M for the precipitation to occur. The solutions containing precipitates were centrifuged again at 2,500 g for 15 min in order to separate the precipitate from the solution. The precipitates were then freeze-dried.

Protein concentrations

The concentrations of protein in collagens were determined using the Lowry's method (Lowry, 1951) which used bovine serum albumin (BSA) as the protein standard to construct the standard curve to determine the concentration of protein in each collagen samples. Approximately, 100 mg of BSA was dissolved into 10 mL distilled water. The protein standard was pipetted with distilled water into the test tubes. Approximately, 8.0 mL of biuret reagent was added and left for 30 min at room temperature. Using a spectrophotometer, the absorbance at 570 nm was determined. A graph of absorbance at 570 nm against concentrations of protein standard solutions was plotted. Next, the collagen powder was diluted with distilled water at a ratio of 1:5 so that the final concentration of protein fell within the range of the constructed calibration curve.

Structural properties

Functional groups of extracted collagens were determined by using Fourier Transform Infrared Spectroscopy (FTIR) by following the method described by Rosli and Sarbon (2015) with slight modification. The infrared spectra used were from 4,000 to 400 cm^{-1} using an infrared spectrophotometer (Nicolet, Thermo Electron, USA). The sample was prepared by mixing the freeze-dried sample with potassium bromide (KBr) with a ratio of 1:100, and moulded into a disc. As the peak of interest is produced at certain wavelength and absorbance, the functional group (amide A, amide II, amide III) and mode of its vibration could be identified.

Solubility of collagens

The solubility levels of extracted collagens were measured 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 freeze dried collagen powder was dissolved in 80 mL 0.5 M acetic acid with gentle stirring for 12 h to obtain a final concentration of 3 mg/mL. Next, 8 mL sample was transferred to a centrifuge tube, and the pH was adjusted across the pH range from 1 to 10 with NaOH and HCl. Then, the volume was made up to 10 mL with distilled water. The solutions were stirred for 30 min at 4°C, and were centrifuged (150R Gyrozen, Korea) at 10,000 g for 30 min at 4°C. The dissolved collagen was determined in terms of protein content in a supernatant using the Lowry's method with BSA as the protein standard. The concentration of protein content was determined using the standard curve. The relative solubility of collagen was calculated as follows:

$$\text{Relative solubility (\%)} = \frac{\text{current concentration of protein at current pH}}{\text{The highest concentration of protein}} \times 100$$

Morphological properties

For the morphological determination of extracted collagens, the method used was that of Jeevithan *et al.* (2014) with slight modification. Briefly, the freeze-dried collagen powders were mounted on aluminium cylinder stubs (5 mm × 12.5 mm) and sputter-coated with auto fine coater (JFC 1600, Tokyo, Japan). The microstructure of the powder was examined using a tabletop microscope (TM-1000, Hitachi, Tokyo, Japan) at an acceleration voltage of 10 kV.

Statistical analyses

All experiments were performed in triplicate, and data were presented as mean ± standard deviation. The probability value of $p < 0.05$ was considered significant. The normality and homogeneity test was conducted by using Chi-Square test before Analysis of Variance (ANOVA) was performed, and mean comparisons were measured via Tukey's test. Analysis was performed by using MINITAB 14.

Results and discussion

Yield of extracted collagens

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from waste materials of fringescale sardinella were successfully extracted (Table 1). The yield of ASC was higher than PSC. This might be due to the efficiency of acetic acid in maintaining the structure of collagen thereby ensuring complete/ higher extraction. The effectiveness of acid extraction depends on the concentration of acetic acid which is usually low. Usually, 0.5 M at pH 2 to 3 of acid has higher efficiency as compared to higher concentration because it can destroy the salt bonds between molecules and Schiff bases which cause the collagen to swell and dissolve in solvent (Muyonga *et al.*, 2004; Kiew and Mat Don, 2013). The low yield of PSC might be due to the low efficiency of pepsin in collagen extraction. During extraction, pepsin has the smallest reaction against collagen which is good in maintaining the collagen structure. Because of this, extraction by using pepsin also needs a longer time to complete. In the present work, the enzyme extraction used the substrate from acid extraction which was lesser in volume as compared to the raw materials. This result is in agreement with a study by Veeruraj *et al.* (2015) which found higher ASC than PSC. It has been reported that there was incomplete solubilisation of collagen in 0.5 M acetic acid. The limited pepsin digestion rendered the cross linked

molecules at the telopeptide region cleaved without totally damaging the structure of collagen's triple-helix, which contributed to the lower yield of PSC (Jongjareonrak *et al*., 2005).

Table 1. Yield of extracted collagen of commercial collagen, acid soluble collagen (ASC) of fringescale sardinella, and pepsin soluble collagen (PSC) of fringescale sardinella.

Samples	Yield (%)	Protein concentration (mg/mL)
Commercial collagen	-	4.21 ± 0.26[a]
ASC	7.48 ± 4.48	1.67 ± 0.08[b]
PSC	0.94 ± 0.22	1.61 ± 0.25[b]

The data represents (mean ± standard deviation). Different superscript letters within column show significance difference ($p < 0.05$).

Protein concentrations

Protein concentrations of commercial collagen were significantly ($p < 0.05$) higher as compared to those of ASC and PSC (Table 1). However, there was no significance difference ($p > 0.05$) between ASC and PSC in terms of protein concentration. High concentrations of protein might indicate that the commercial collagen was high in amino acids (proline and hydroxyproline) and the high content of triple helix structure of collagen contributed to the stability of the collagen. Amino acids content is important as it determines the solubility, cross linking ability and thermal stability of collagen (Gomez-Guillen *et al*., 2002; Hashim *et al*., 2015). The amino acids detected were glycine and alanine (Matmaroh *et al*., 2011). Approximately one-third of total amino acids at 1,000 residues are glycine. This amino is important to reduce the steric hindrance and provide an inter-chain hydrogen bond perpendicular to the helix axis of the collagen (Matmaroh *et al*., 2011). Furthermore, amino acid content also has an influence on the stability of collagen fibre, shrinkage and denaturation temperature of the collagen (Pati *et al*., 2010; Hashim *et al*., 2015).

Structural properties

Fourier Transform Infrared Spectrophotometer (FTIR) was used to detect the spectra of collagen for commercial collagen and extracted collagens (ASC and PSC). The amide A, amide II and amide III bands were detected at wavelength range of 3,417-3,416 cm^{-1}, 1,600-1,500 cm^{-1} and 1,450-1,400 cm^{-1}, respectively. Table 2 shows significant differences ($p < 0.05$) for amide II and amide III levels between the commercial, ASC and PSC samples, respectively. However, there was no significant difference ($p > 0.05$) among them for amide A. It was found that amide A, amide II, and amide III bands for PSC

have shifted to the lower wavelength as compared to commercial collagen and ASC, which shows that there were strong hydrogen bonds involved in amide II of PSC. However, slight differences between ASC and PSC for amide II and III bands might indicate that the pepsin hydrolysis at the telopeptide region apparently did not have any effect on the triple helical structure of PSC.

Table 2. Functional groups investigated (amide A, amide II and amide III) of commercial collagen, acid soluble collagen (ASC) of fringescale sardinella, and pepsin soluble collagen (PSC) of fringescale sardinella.

Samples	Wave length (cm^{-1})		
	Amide A	Amide II	Amide III
	N-H stretching	N-H bending + C-N stretching	C-H stretching
Commercial collagen	$3,416.65 \pm 4.65$[a]	$1,545.21 \pm 0.15$[b]	$1,456.13 \pm 0.07$[a]
ASC	$3,416.90 \pm 4.70$[a]	$1,589.77 \pm 0.52$[a]	$1,414.06 \pm 0.04$[b]
PSC	$3,423.64 \pm 0.17$[a]	$1,400.79 \pm 0.02$[c]	$1,264.47 \pm 0.16$[c]

The data represents (mean ± standard deviation). Different superscript letters within column show significance difference ($p < 0.05$).

These functional groups are important to confirm that the extracted samples were protein and collagen. Amide A (N-H stretching) is associated with hydrogen bonding and is an indication for the existence of protein (Tamilmozhi *et al*., 2013). This free N-H stretching vibration usually occurs in the range of 3,400-3,440 cm^{-1} and when the hydrogen bonds are involved in peptide groups of collagen, the position of bands of functional groups might be shifted to the lower frequencies, usually around 3, 300 cm^{-1} (Veeruraj *et al*., 2014). Amide II and amide III (N-H bending and C-N stretching) are an indication for the presence of triple helical structure of collagen (Plepis *et al*., 1996; Pati *et al*., 2010).

However, the position of peaks of functional groups of collagens was not exactly the same as some of the peaks shifted to a lower wavenumber associated with a decrease in the molecular order of collagen (Payne and Veis, 1988; Kittiphattanabawon *et al*., 2010). This result is in agreement with Sujithra *et al*. (2013) (collagen from waste of tilapia), Pati *et al*. (2010) (collagen from fish scales), Tamilmozhi *et al*. (2013) (collagen from skin of sailfish) and Kittiphattanabawon *et al*. (2010) (collagen from cartilage of brownbanded bamboo shark and blacktip shark), as there were slightly different IR spectra among extracted collagens.
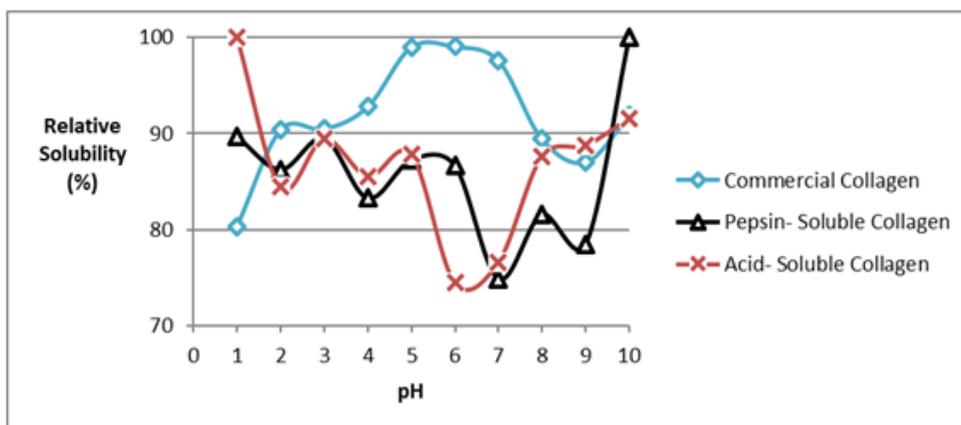
Figure 1. Relative solubility of acid soluble collagen and pepsin soluble collagen of fringescale sardinella, and commercial collagen at different pH levels (pH 1-10).

Solubility

Figure 1 shows the solubility of the collagens in different pH levels (pH 1 – 10). This shows fluctuation for ASC and PSC but no significant difference ($p < 0.05$) between extracted collagens (ASC and PSC) and commercial collagen. Relative solubility of collagen shows that commercial collagen's solubility was maximum at pH 5 with 99.06% solubility and minimum at pH 1 with solubility of 80.29%, while solubility for ASC was maximum at pH 1 (100.00%) and minimum at pH 6 (74.45%). For PSC, it shows unstable solubility with the maximum solubility was at pH 10 (100.00%) and the minimum solubility was at pH 7 (74.88%). From the results, the pI obtained for ASC and PSC were at pH 6 and pH 7, respectively, which are consistent to the previous report by Ahmad *et al*. (2010). That report stated that the pI levels of the collagens were at pH 6 to 9. The fluctuation of results in the solubility of ASC and PSC was also because of the instability of the collagen at the certain pH due to isoelectric point (pI) of collagens. When the pH value of the solution is near the pI of the collagen, the hydrophobic interaction within the molecules of collagen increases, thereby causing precipitation (Jongjareonrak *et al*., 2005; Ahmad *et al*., 2010).

These findings have indicated that PSC might contain a lower degree of cross-linking or predominance of weaker bonds than ASC (Yu *et al*., 2014). Therefore, PSC is easier to be dissolved than ASC. The bond possessed by PSC resulted from the pepsin hydrolysis that hydrolysed the peptides bonds in telopeptide regions (Yu *et al*., 2014). The differences in the solubility of collagen might also be due to the differences in molecular properties and conformations of collagen. This result is in agreement with Minh Thuy *et al*. (2014), Matmaroh *et al*. (2011) and Singh *et al*. (2011) which showed that most of collagen increased in solubility at acidic condition.

Other than that, solubility is also effected by amino acid content, which is also related to the cross-linking ability and thermal stability of collagen (Hashim, Ridzwan and Bakar, 2014). It is proven that the solubility of collagen increases at lower pH because of repulsive force between molecules of collagen (Ahmad *et al*., 2010). To conclude, this solubility study shows that the extracted collagens are suitable to be applied in beverages industry such as juices and yogurt drinks which have a pH 4.0 to 4.6 for yogurt drink, and pH 2.0 to 5.6 for fruit juices.

Morphological analysis

Figure 2 shows the morphology characteristics of the extracted collagens (ASC and PSC) and commercial collagen. It shows that commercial collagen (Figure 2a) have a flaky and multilayer structure, while PSC (Figure 2b) has a fibrillary structure with interconnective structure (connection amongst pores by fibril), and ASC (Figure 2c) has both a flaky and fibrillary structure. The flaky structure of collagen might be due to the extraction which compressed the collagen. Furthermore, the fibrillary structures of PSC and ASC also show that there are interconnective structures which have the potential to be used as biofilms or scaffolds for wound healing purposes.

The microstructure of collagen plays an important role in the determination of functional properties of collagen. Collagen with moderate size of pores like PSC is suitable to be used for *in vivo* studies. The pore size of collagen is influenced by the water content during the preparation (Jeevithan *et al*., 2014). The best structure of PSC has been obtained due to the selective digestion of pepsin; therefore, the structure of collagen can be maintained as compared to ASC, which caused unselective digestion or hydrolysis in the extraction of collagen. Since amino acids are
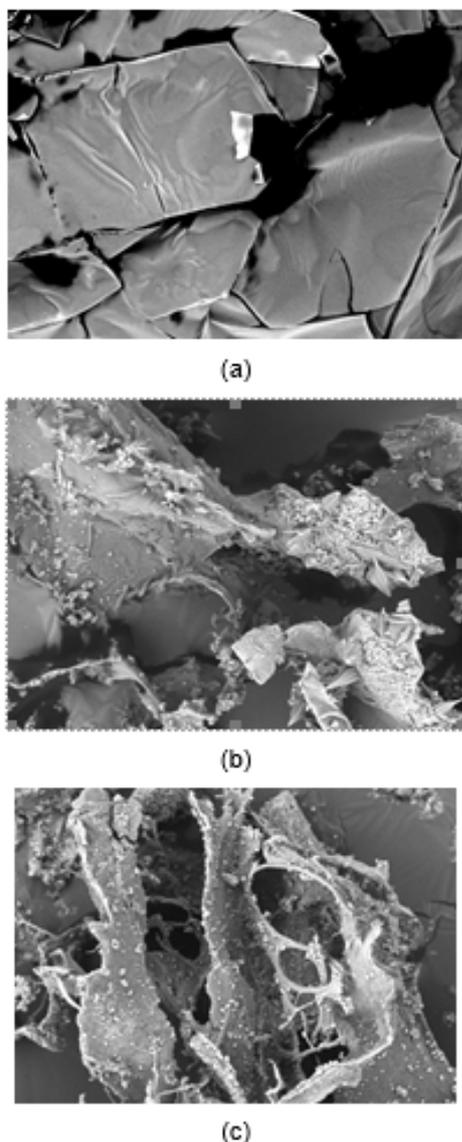
diameter of collagen fibre from skin of bovine was greater than collagen from fish skin. It is assumed that the stronger entanglement effect amongst molecules of bovine collagen does promote and stabilise the lateral and longitudinal assembly of microfibers (Zhang *et al*., 2010; Tamilmozhi *et al*., 2013). Other than that, Jeevithan *et al*. (2014) reported that the features of collagen such as pore size, pore shape and pore wall morphology have an important influence on cell seeding, growth, gene expression, migration, mass transport and new tissue formation.

Conclusion

In conclusion, collagen from fringescale sardinella was successfully extracted via the acid soluble collagen (ASC) and pepsin soluble collagen (PSC) methods. The yield of ASC was higher than PSC. The results obtained show that commercial collagen has good properties because it was high in protein content and protein concentration as compared to ASC and PSC. Commercial collagen and extracted collagens also have good physical properties due to the high intensity of amide A, amide II and amide III, showing that the collagens were high in protein and in the triple-helical structure of collagen. The ASC was better than PSC in terms of chemical properties, while PSC was better than ASC in terms of morphological structure. ASC was high in protein content and PSC had a more interconnective structure, which shows strong potential for biomedical use as compared to commercial collagen, which has flaky structure.

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Figure 2. Microscopic structure of (a) commercial collagen, (b) acid soluble collagen of fringescale sardinella, and (c) pepsin soluble collagen of fringescale sardinella.

easily destroyed and racemised; the non-selective hydrolysis causes the continuous distribution of molecular weight of collagen protein. The flaky structure of commercial collagen shows that this might be due to the compression of triple helical structure of collagen due to the penetration of crystal of apatite into internal fibrillary (Zhang, 2011). Therefore, the raw materials of commercial collagen should be demineralised effectively.

The structure of collagen obtained in the present work fits with that found by Sujithra *et al*. (2013) and Jeevithan *et al*. (2015) after observation under a scanning electron microscope (SEM), which showed that collagen fibres were formed in highly ordered 3D structure and consisted of many layers; fibril and flake in random orientation. However, the length and

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