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Effect of different acids during collagen extraction the bone and fins from purple-spotted bigeye (*Priacanthus tayenus* Richardson, 1846) and their physicochemical properties

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Abstract

This study was carried out to determine the effectiveness of different acids (acetic, lactic and citric)-on the extraction of collagen from the bone and fins of purple-spotted bigeye (*Priacanthus tayenus* Richardson, 1846) particularly on the yield and physicochemical properties. The physicochemical properties were characterized by electrophoretic pattern, X-ray diffraction, FT-IR, colour and pH. The citric acid-extracted collagen (CAC) has a higher percentage of yield (1.93±0.57), followed by lactic acid-extracted collagen (LAC) (1.43±0.42) and acetic acid-extracted collagen (AAC) (0.83±0.18) although those collagens did not differ significantly ($p>0.05$), and those acid solubilized collagens (ASC) contained about 96.82 - 96.89 mg/g of hydroxyproline. The ASCs obtained showed similar electrophoretic patterns due to the presence of identical α chains (α1 and α2) and classified as type I collagen. UV absorption spectrum in all ASCs was approximately around 231.0 - 231.5 nm. For diffraction angle analysis, first diffraction was detected at 7.23 - 7.41° and second diffraction was at 19.41 - 20.29° in all ASCs. Major absorption peaks of FT-IR spectrum, *viz*. amide A, amide B and amide I-III were present in the collagens with different wavenumbers. The results indicated that the physicochemical properties of ASCs extracted in the present study were comparable with collagens reported from other fish species. The findings suggested that ASCs from purple-spotted bigeye bones and fins could serve as alternative source of collagen in functional food, pharmaceutics and tissue engineering production, particularly found in acetic acid-extracted collagen because of its properties.

1. Introduction

Collagen is a fibrous protein and composed mainly of the amino acids (glycine, proline and hydroxyproline). These amino acids form three strands, which make up the triple helical structure of collagen (León-López *et al.*, 2019). As a structural protein, collagen is the most abundant found in the extracellular matrix of connective tissues that provides strength, facilitates cell addition, improves chemotaxis and migration, and direct tissue development (Rozario and DeSimone, 2010). Over 28 types of collagens have been identified and every type is differed based on protein structure and amino acid sequence (Huda *et al*., 2013a; Sorushanova *et al.,* 2019). Among these types, type I collagen is extensively applied in food, cosmetic, nutraceutical, and biomedical fields due to high availability and excellent characteristics distributed mainly in the skin, bone, and tendon tissue (Huda *et al.,* 2013b; Salvatore, 2020). Traditionally, the major sources of collagen were derived from land-based animals, such as cows and pigs. However, the cattle-extracted collagen raised anxiety due to the outbreak of foot-and-mouth disease (FMD), bovine spongiform encephalopathy (BSE) and transmissible spongiform encephalopathy (TSE) (Coppola *et al.,* 2020). Beside disease issues, there are also religious obstacles, particularly for Muslim and Jews are prohibited to consume porcine-derived collagen (Jaziri *et al.,* 2020).

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Fish collagen has attracted significant attention amongst researchers and processors as a promising source of collagen. Numerous studies have reported that fish collagen had similar properties to collagen extracted from terrestrial animal origin after being modified during extraction (He *et al.,* 2011; Zhang *et al.,* 2020). Additionally, it is considered as safer and more acceptable to most religious groups. Many research have extracted and characterized collagen from fish species, such as bigeye tuna (*Thunnus obesus* Lowe, 1839), threadfin bream (*Nemipterus japonicus* Bloch, 1791), spotted golden goatfish (*Parupeneus heptacanthus* Lacepède, 1802), giant grouper (*Epinephelus Ianceolatus* Bloch, 1790.), tilapia (*Oreochromis niloticus* Linnaeus, 1758), channel catfish (*Ictalurus punctatus* Rafinesque, 1818), rohu (*Labeo rohita* Hamilton, 1822), carp (*Cyprinus carpio* Linnaeus, 1758) and lizardfish (*Saurida wanieso* Shindo and Yamada, 1972) (Nalinanon *et al*., 2011; Matmaroh *et al*., 2011; Chen, Li, Yi *et al*., 2016; Zhang *et al.,* 2016; Savedboworn *et al*., 2017; Upasen *et al*., 2019; Ahmed *et al*., 2019; Moniruzzaman *et al.,* 2019). More interestingly, most of the collagen was extracted from by-products of fish processing activities (skin, bone and scale). Extraction procedure is a vital process to effectively isolate collagen from fish by -products. Acid-assisted extraction is the most common technique in obtaining collagen from the tissues (Jongjareonrak *et al.,* 2005), and organic acids such as acetic, citric, and lactic acid were usually used to extract fish collagen. According to the research from Skierka and Sadowska (2007), organic acids were more effective in solubilizing non-collagen chains, as well as some internal chains of collagen in comparison to inorganic acids such a hydrochloric acid. Moreover, the use of organic acids could produce higher yields, result in shorter extraction time, has a lower cost and relatively safer (Schmidt *et al.,* 2016). Previous reports have explored successful extractions of collagens using organic acids from several fish species (tilapia, catfish, conger, lizardfish, carp, rohu and grouper).

Purple-spotted bigeye (*Priacanthus tayenus*), also known as lolong bara among the locals in Malaysia, is a commercially important marine fish for surimi processing. It belongs to the family Priacanthidae, ranging from 29 to 35 cm in size with clear purple or black spots on its pelvic fins (McGrouther, 2019). As a raw material for surimi, the average production of this fish in Malaysia was around 23,152 tons from 2015 to 2019 (Department of Fisheries Malaysia, 2021). Approximately 60-75 percent of by-products is produced after processing, and some by-products would be utilized for low value-added products and/or sometime dumped as waste and causing serious problems in environment. On the other hand, the poor-utilized by-products not only led to loss of potential revenues but also resulted in extra cost when disposing of these products (Jayathilakan *et al.,* 2012). An effective utilization of fish by-products is necessary to generate high-value products which could bring financial gain and reduce environmental problem. In the context of utilization, previous studies on the extraction of collagen from purple-spotted bigeye have been reported by Kittiphattanabawon *et al.* (2005) and Nalinanon *et al.* (2007) from fish skin and bone using acetic acid-aided extraction on fish from Thailand. This paper presents the effectiveness of various organic acids on the extraction of collagen from purple-spotted bigeye bone and fins from Sabah, Malaysia and the characterization of the physicochemical and structural properties.

2. Materials and methods

2.1 Materials

Around 15 kg of purple-spotted bigeye was purchased from a local market in Kota Kinabalu, Sabah, Malaysia. The bone and fins were automatically separated using the mechanical deboner machine (SFD-8, Taiwan). The collected samples were cut into 1.0 × 1.0 cm^2 pieces /cubes with scissors and washed with running tap water. The samples were then put into polyethylene boxes and kept at -20°C until further experiment. Molecular weight markers were procured from Bio-Rad Laboratories (Hercules, CA, USA). Sodium dodecyl sulphate (SDS), *N, N, N', N'*-tetramethyl ethylene diamine (TEMED), Coomassie Blue R-250, Lowry reagent and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Tris (hydroxymethyl) aminomethane hydrochloride was supplied from Sigma Chemical Co. (St. Louis, MO, US). A pre-stained natural protein standard (dual colour standards) for SDS-PAGE was purchased from Bio-Rad Laboratories (Hercules, CA, US). Other chemicals were of analytical grade.

2.2 Preparation of acid solubilized collagen

Collagen extraction from the combination of bone and fin samples was carried out as described by Matmaroh *et al*. (2011) with some modifications. Prepared samples (around 100 g) were soaked in 0.1 N NaOH at a ratio of 1:10 (w/v) for 6 hrs with continuous stirring to remove non-collagenous proteins and the solution was changed every 3 hrs. Treated samples were washed with cold distilled water to reach a pH of 7. Demineralization process was conducted using 0.5 M EDTA-2Na solution (pH 7.4) at the ratio of 1:10 (w/v) for 48 hrs and the solution was replaced every 24 hrs. Demineralized samples were then subjected to acid solubilized collagen (ASC) extraction by treating with

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different acids solution, *i.e.,* 0.5 M acetic, 0.5 M lactic and 0.5 M citric acids at the same ratio of 1:15 (w/v) for 72 hrs. After extraction, samples were filtered using a 2-layer of cheese cloth. The supernatants were salted out by adding NaCl to a final concentration of 2.5 M. The precipitates were collected after centrifugation at 15,000×*g* for 30 mins. The precipitates were dissolved in 0.5 M acids previously used for extraction at the ratio of 1:9 (w/v) and then dialyzed using dialysis tubing cellulose membrane (flat width 43 mm, Sigma) in 20 volumes of 0.1 M acetic, lactic and citric acids, followed by distilled water. All process was run at 4°C. ASC was lyophilized using a freeze-dryer (Labconco, US). The fish collagen was stored at -20°C until use. Collagen yield was calculated based on the initial weight of prepared samples (bone and fin) with the weight of freeze-dried collagen using the following formula:

$$\text{Yield}(\%)=\frac{\text{freeze dried collagen(g)}}{\text{Initial weight of bones \& fins (g)}}\times 100$$

2.3 Determination of protein content

The protein content of the bone, fins and both combined was determined according to the method described by the Association of Official Analytical Chemist (AOAC, 2000). The protein content determination was carried out by Kjeldahl method using a Kjeltec 2300 Auto Distillation Unit (FOSS Tecator, Sweden). The content of protein was measured based on the amount of nitrogen obtained in the prepared samples, and calculated with the following equation:

$$\text{N}\ (\%) = \frac{(\text{mL HCl-mL blanko}) \times 14.007 \times \text{N HCl}}{\text{w}} \times 100$$

where a nitrogen-to-sample conversion factor of 6.25 was used for the calculation of protein present in the tested samples.

2.4 Hydroxyproline content

Hydroxyproline is used as a method to quantify the amount of collagen in a particular tissue (Kittiphattanabawon *et al.,* 2005). The hydroxyproline content was measured according to Hofman *et al.* (2011) with modifications. ASCs were hydrolyzed in 6 M HCl for 24 hrs at 115°C. The hydrolyzed collagens were diluted with distilled water at the ratio of 1:100 (w/v) and pH were adjusted to pH 6-7. Samples (2 mL) were then mixed with 1 mL of chloramine T reagent and then incubated for 20 mins at room temperature. After incubation, 1 mL of perchloric acid was added and incubated for 5 mins at room temperature. Around 1 mL of Ehrlich reagent was later added to the mixtures and heated at 60°C for 20 min. Upon completion of heating, the mixtures were cooled, and absorbance was measured at 550 nm using a UV-Vis Spectrophotometer (Agilent Cary 60, US). The standard curve of hydroxyproline was prepared in advance at 1 to 10 µg/mL and the standard curve obtained was Y = 0.1637x + 0.0643 with R^2 value of 0.995. The hydroxyproline and collagen content were calculated using the following equation:

$$\text{Hyp content (mg/g)} = \left[\frac{\text{Hyp (µg)}}{\text{Hydrolysate volume (mL)}}\right] \times \text{df}$$

$$\text{Collagen content (mg/g)} = \left[\frac{\text{Hyp (mg/g)}}{10}\right] \times 7.7$$

where, df is dilution factor, 10 refers to initial concentration of hydrolysate and 7.7 is the conversion factor.

2.5 pH value

The pH of ASC samples was measured using a pH meter (Eutech pH 700, US) as indicated by Martínez-Ortíz *et al.* (2015) with modification. Around 1.0 mg of samples were dissolved in 10.0 mL of distilled water using a homogenizer (Ultra-Turrax®, Germany). The pH meter was previously calibrated at pH 4.0 and pH 7.0 using prepared buffer solutions. After calibration, solubilized samples were measured and then recorded.

2.6 Analysis of colour

The analysis of colour in ASC samples was carried out according to the method reported by Huda *et al.* (2013b) using a colourimeter (HunterLab, US). Lyophilized collagens were placed into the clear glass cup and then recorded the colour parameters. The parameters used were *L**, *a** and *b** values, which *L** value represents the brightness, while *a** value is redness and *b** is yellowish.

2.7 UV absorption spectrum

The UV absorption spectrum of ASC was measured using a UV-Vis Spectrophotometer (Agilent Cary 60, US). Around 10 mg of each collagen sample were dissolved into 10 mL of 0.5 M acetic acid at the ratio of 1: 1 (w/v), and the sample solution was dropped in a quartz cell. The spectrum was measured within 200 nm to 400 nm wavelengths. The base line was set with 0.5 M acetic acid.

2.8 X-ray diffraction

The crystal structures of ASC samples were evaluated using an X-ray diffraction (XRD) instrument (Rigaku Smart Lab®, Japan) with Copper K-α as a source of X-ray. Tube voltage and current were adjusted to 40 kV and 40 mA, respectively. Scanning range was determined between 10° to 50° (2θ) with a speed was 0.06° per second.

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2.9 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) analysis was used to characterize secondary structure of collagen extracted from the purple-spotted bigeye bone and fins. FT-IR spectra of ASCs were determined using total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) (Agilent Cary 630, US). About 1.0 mg of ASC samples were placed on the crystal cells. The spectra were set within the range of 4000-600 cm^{-1} with a resolution of 4 cm^{-1} for 32 scans against a background spectrum recorded from the clean empty cells at room temperature. The resultant spectral was analyzed using Agilent Microlab software program.

2.10 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to Laemmli (1970) procedure with slight modification. Approximately 2.5 mg of purple-spotted bigeye collagens were dissolved in SDS (5%) and the solutions were incubated at 85°C for 1 hrs in a water bath. After thermal incubation, the sample solutions were centrifuged at 8,500×*g* for 5 mins at room temperature. The supernatants were mixed with the prepared sample buffers (with and without 10% β- mercaptoethanol) at the ratio of 1:1 (v/v) and then heated at 85°C for 3 mins. ASC samples were loaded into an acrylamide gel containing 5% stacking gel and 12% resolving gel. Then, run electrophoresis at a constant voltage of 120 V/gel employing a Mini Protein II unit (Bio-Rad laboratories, US). After electrophoresis, gel was stained with 0.1% (w/v) Coomassie blue R-250 in 30% (v/v) methanol and 10% (v/v) acetic acid and de-stains with 30% (v/v) methanol and 10% (v/v) acetic acid. The molecular weight of ASCs was determined using a pre-stained natural protein standard (dual colour standards) (Bio-Rad laboratories, US).

2.11 Statistical analysis

Data are presented as mean ± standard deviation (SD). Data were subjected to one-way analysis of variance (ANOVA). The differences of means were done by Duncan multiple range test and performed using IBM SPSS Statistics version 27.0 (IBM Corp., Armonk, New York). Average comparisons were accepted at confidence of $p<0.05$.

3. Results and discussion

3.1 Protein content of bone and fins sample, yield and hydroxyproline content of acid solubilized collagen samples

In this study, we used fins, bones and combination of fins and bones of purple-spotted bigeye as substrates to extract the collagen. These parts were selected due to the high protein content in them. As presented in Table 1, the protein content of combined bones and fins (19.51±0.35, based on wet matter basis) was significantly higher ($p < 0.05$) compared to bone (12.46±0.21) or fins (17.49±0.14) only. The reason might be due to the higher content of moisture observed in the combined bones and fins (58.0%), compared to the fins (59.81%) and bones (66.78%). The protein level is an indication of the portion of collagen in selected sample as collagen is a main structural protein found in skin, bone, fin and scale of fish (Chen, Li, Yi *et al.,* 2016; Nagai and Suzuki, 2000).

Yield of ASCs is also outlined in Table 1. The results showed that citric acid-extracted collagen (CAC) has a dominant percentage of yield (1.93±0.57), followed by lactic acid-extracted collagen (LAC) (1.43±0.42) and acetic acid-extracted collagen (AAC) (0.83±0.18) although those collagens did not differ significantly ($p>0.05$). The yield of collagen was comparable to previous studies from different fish species, such as carp (1.06%) (Duan *et al*., 2009), grass carp (0.7%) (Wang *et al.,* 2014), tilapia (0.5%) (Liu and Huang, 2016), bigeye tuna (0.1%) (Ahmed *et al.,* 2019) and bigeye snapper (1.6%) (Kittiphattanabawon *et al.,* 2005). The lower yield found in most fish bone collagens extracted using acid indicates that the degree of cross-linking among collagen molecules might be stronger in bone as suggested by Ahmed *et al.* (2019).

Hydroxyproline content is used to quantify amount of collagen in the ASCs (Bhuimbar *et al.,* 2019) since

Table 1. Protein content of raw materials, yield, hydroxyproline and collagen contents.

Samples	Protein content (%)	Collagen yield (%)	Hydroxyproline content (mg/g)	Collagen content (mg/g)
Bone	12.46±0.21[a]	-	-	-
Fins	17.49±0.14[b]	-	-	-
Bone and fins	19.51±0.35[c]	-	-	-
AAC	-	0.83±0.18[a]	96.89±0.46[a]	746.02±3.52[a]
LAC	-	1.43±0.42[a]	96.83±0.57[a]	745.59±4.38[a]
CAC	-	1.93±0.57[a]	96.82±0.62[a]	745.49±3.68[a]

Values are presented as mean±standard deviation (n = 3). Values with different superscripts within the same column are statistically significantly different.

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this amino acid is present almost exclusively in collagen. The higher the hydroxyproline content, the more collagen was obtained in ASC. Table 1 shows the contents of hydroxyproline detected in ASCs in the present study. The amount of hydroxyproline in AAC sample was recorded at around 96.89 mg/g, while LAC and CAC had approximately 96.83 mg/g and 96.82 mg/g, respectively and not significantly different ($p>0.05$). Our results are comparable of hydroxyproline content in collagen from marine eel-fish (94 - 98 mg/g) (Veeruraj *et al*., 2013). However slightly lower hydroxyproline content in some fish collagens, including tilapia (76 - 80 mg/g) (Liu and Huang, 2016), bigeye tuna (82 - 87 mg/g) (Ahmed *et al.,* 2019) and cobia (84 - 99 mg/g) (Zeng *et al.,* 2012) has been demonstrated. Differences among the hydroxyproline contents reported could be due to variation in species, size, age, structure and composition of tissue, as well as the extraction procedures (Regenstein and Zhou, 2007).

3.2 pH value

The values of pH in ASCs extracted are presented in Table 2. AAC showed the highest pH reading (4.17±0.02) compared to LAC (3.68±0.05) and CAC (3.52±0.03). Our results are in agreement with pH of silver catfish collagen (3.79±0.88 to 4.04±0.06) as reported by Hadfi and Sarbon (2019). The lower pH of extracted fish collagens was probably due to the less effective process of neutralization during extraction process. This statement is supported by Alhana *et al.* (2015) who reported that the ineffective neutralization process is one of the factors causing the pH value of collagen becoming lower in the final product. Besides that, a rapid maturation process also contributes to the decrease in collagen pH (Sionkowska *et al.,* 2020). However, the pH of commercial collagen is supposed to be higher around 6.52±0.04 (Hadfi and Sarbon, 2019). Similarly, Sionkowska *et al.* (2020) stated that the better-quality collagen possesses a pH value between 6.0 to 7.0. However, collagens with a lower pH are still acceptable and could be used in food, cosmetic and drug systems (León-López *et al.,* 2019).

3.3 Colour analysis

Colour is one of the most important quality factors attracting consumers including collagen (Sliburyte and Skeryte, 2014). Colour attributes (L^*, a^* and b^*) of all ASCs are showed in Table 2. The results indicate the AAC sample has the highest L^* score (89.69±0.29) ($p < 0.05$) compared to other ASC samples. This indicates that collagen extracted using acetic acid was brighter than when using lactic or citric solutions. The ingredient with a brighter colour is usually preferred due to easy incorporation with other material and does not interfere with the original colour of product (Huda *et al.,* 2013b; Pal *et al.,* 2015). The L^* value of the purple-spotted bigeye collagens analyzed in the present study was higher compared to the L^* value (44.76 - 65.41) of barramundi skin collagen (Bakar *et al.,* 2013), but this L^* value was similar to the H_2O_2-treated snakehead (*Channa argus*) skin collagen (L^* = 89.49). Meanwhile, there was no significant difference ($p>0.05$) in the a^* value (0.80±0.05 and -0.10±0.05) for all *P. tayenus* collagens. On the other hand, LAC and CAC samples were significantly more yellowish than AAC.

3.4 UV absorption spectrum

The triple helical collagen has a maximum absorption peak between 210 to 240 nm (Wang *et al.,* 2017). Therefore, to identify collagen from *P. tayenus* bone and fins absorption from 200 nm to 400 nm by UV spectrophotometer was applied. The spectrum of all ASCs is depicted in Figure 1. AAC and LAC had identical maximum absorption peak at 231.0 nm, whilst CAC at 231.5 nm. The peak maximum absorption is mainly correlated with the carbonyl group (C = O), carboxyl group (COOH) and amide group ($CONH_2$) in the collagen chain (Yu *et al.,* 2014). The maximum absorptions peak observed in this study was in line with other studies including catfish skin (232 nm), barramundi skin (230.3 nm), tilapia skin (230.9 nm) and black ruff skin (232 nm) (Liu *et al.,* 2007; Liao et al. 2018; Bhuimbar *et al.,* 2019). In addition, no maximum absorption peaks were recorded from 250 nm to 280 nm in the extracted samples. This is due to the lack of concentration of aromatic amino acids such as phenylalanine, tyrosine and tryptophan found in the collagen (Liu *et al.,* 2007; Wang *et al.,* 2017). On the other hand, these findings indicate that various acids used in isolating collagen from purple-spotted bigeye are effective, resulting in similar peak absorption as investigated in commercial collagen (Chen *et al.,* 2019).

Table 2. The pH value and colour attributes of ASC samples.

Samples	pH value	Colour parameters		
		L^*	a^*	b^*
AAC	4.17±0.02^{c}	89.69±0.29^{c}	-0.80±0.05^{a}	5.48±0.34^{a}
LAC	3.68±0.05^{b}	88.03±0.46^{b}	-0.10±0.03^{b}	9.73±0.22^{b}
CAC	3.52±0.03^{a}	86.93±0.29^{a}	-0.82±0.05^{a}	9.09±0.52^{b}

Values are presented as mean±standard deviation (n = 3). Values with different superscripts within the same column are statistically significantly different.

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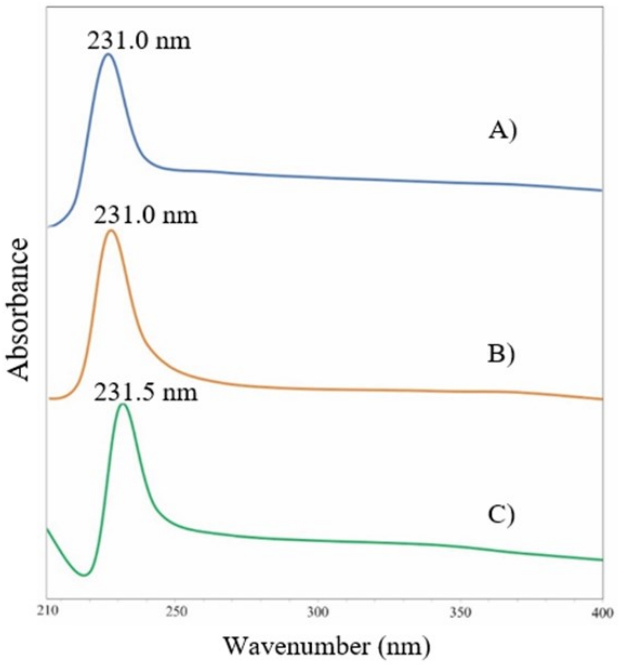

Figure 1. UV absorption spectra of collagens from the bone and fins of purple-spotted bigeye with three different acids. A) AAC: acetic acid-extracted collagen, B) LAC: lactic acid-extracted collagen, C) CAC: citric acid-extracted collagen.

3.5 X-ray diffraction

X-ray diffraction (XRD) serves to identify the secondary structure of collagen. Generally, collagen has two diffraction peak angles, viz. the first diffraction peak angle is between 7° to 8° and the second is between 19° to 25° (Chen *et al.,* 2019). Diffraction peak angles of the bone and fin collagens from this study are illustrated in Figure 2. The XRD of different acids treatment exhibited two peaks: the first peaks around 7.23° to 7.41° and the second peaks 19.41° to 20.29°. Similarly, Giraud-Guille *et al*. (2000), reported the first diffraction peak (approximately 7°) represents the distance between the molecular chains of collagen fibrils (d). The d value is calculated using the Bragg's formula ($d= n \lambda/2\sin\theta$) and the results present the d values of AAC, LAC and CAC samples were 1.19 nm, 1.20 nm and 1.22 nm, respectively. From those findings, CAC had the highest d value compared to LAC and AAC. This suggests that citric acid selectively cleaves the terminals of peptide arrangement on collagen fibers, then weakens the interaction between molecules, resulting in the enhancement of the distance between molecular chains

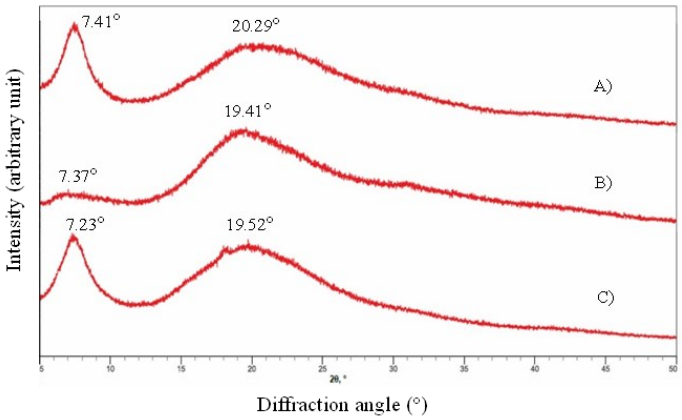

Figure 2. X-ray diffraction diagram of collagens from the bone and fins of purple-spotted bigeye with three different acids. A) AAC: acetic acid-extracted collagen, B) CAC: citric acid-extracted collagen, C) LAC: lactic acid-extracted collagen.

(Zhang *et al.,* 2007). The second diffraction peak (around 20°) reflects diffusion scatter caused by the various layers of the collagen fibril structure. All collagen samples showed a wide diffraction peak and had d values of around 0.43 nm, 0.45 nm and 0.46 nm, respectively. The difference in values d from all extracted collagens is small and presented the triple helix formation of the ASC samples were well-maintained.

3.6 Total reflectance-Fourier transform infrared spectroscopy spectra

ATR-FTIR spectra of all treated collagen samples are depicted in Figure 3 and Table 3. The ASCs s showed the same pattern for each spectrum. However, the absorption rate for all ASCs were slightly different. In general, the FTIR spectra of collagens were categorized into five major absorption peaks, i.e., amide A, amide B, amide I, amide II and amide III (Chen *et al.,* 2019). Amide A indicates the presence of protein compounds, and the results show amide A in *AAC*, *LAC* and *CAC* were 3295.05 cm^{-1}, 3295.05 cm^{-1} and 3298.78 cm^{-1}, respectively. These readings were slightly lower compared to barramundi (3378.63 cm^{-1}) dan tilapia collagen (3434.23 cm^{-1}) (Liao *et al.,* 2018); however, amide A is associated with N-H stretching frequency. Amide B peaks for AAC, LAC and CAC were 2933.49 cm^{-1}, 2933.49 cm^{-1} and 2927.90 cm^{-1}, respectively. CAC sample showed a slightly lower absorption value compared to AAC and LAC samples. Amide B

Table 3. Absorption rate by peak area and peak assignments for all collagen samples.

Region	Wavenumber (cm^{-1})			Peak assignments
	AAC	LAC	CAC	
Amide A	3295.05	3295.05	3298.78	Mainly N-H stretching coupled hydrogen bond
Amide B	2933.49	2933.49	2927.90	CH_2 – asymmetric stretching
Amide I	1640.07	1640.07	1634.48	C=O stretching / hydrogen bond with COO^-
Amide II	1541.29	1543.16	1543.16	N-H bond coupled with C-N stretching
Amide III	1241.23	1228.19	1196.51	N-H bond coupled with C-H stretching

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illustrates the asymmetric stretching of the CH_2 vibration (Chen *et al.,* 2019).

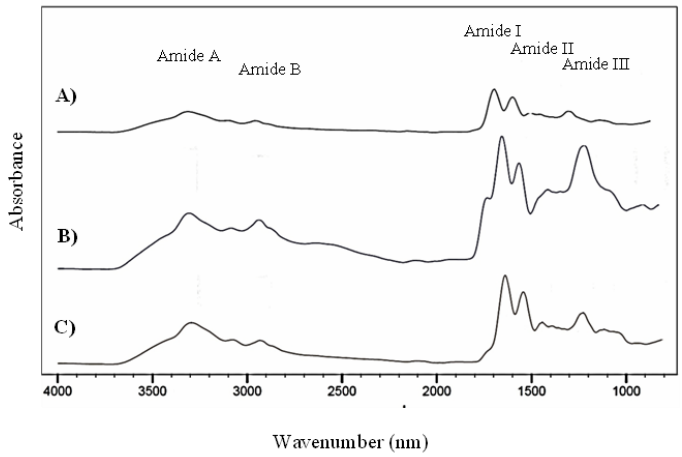

Figure 3. Fourier transform infrared spectrum of collagens from the bone and fins of purple-spotted bigeye with three different acids. A) AAC: acetic acid-extracted collagen, B) CAC: citric acid-extracted collagen, C) LAC: lactic acid-extracted collagen.

Amide I is associated with the stretching vibrations of the carbonyl group (C=O). The absorption range for amide I range from 1600 cm^{-1} to 1700 cm^{-1} (Abedin *et al.,* 2012). The absorption rates for AAC and LAC were slightly higher (1640.07 cm^{-1}) than CAC (1634.48 cm^{-1}). According to Liao *et al*. (2018), amide I is a sensitive marker of peptides secondary structure because the formation of hydrogen bonds between N-H and C=O stretches is responsible for the introduction of the collagen triple helix. On the other hand, amide II is responsible for the combination of N-H and C-N stretching. Normally, the absorption range for amide II is between 1500 cm^{-1} and 1600 cm^{-1} (Abedin *et al.,* 2013). LAC and CAC samples shared the same wave number of around 1543.16 cm^{-1} and slightly higher than *AAC* (1541.29 cm^{-1}). Amide III represents the combination of C-N stretching and N-H (Matmaroh *et al.,* 2011). Amide III for standard collagen has an absorption peak between 1200 cm^{-1} and 1350 cm^{-1} and acts to identify the triple helical structure of collagen (Abedin *et al.,* 2013). The formation of triple helix for AAC, LAC and CAC were identified at wavenumber of 1241.23 cm^{-1}, 1228.19 cm^{-1} and 1196.51 cm^{-1}, respectively.

3.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Protein pattern of ASCs from purple-spotted bigeye bone and fins combination was determined by electrophoresis under reducing and non-reducing condition as depicted in Figure 4. The patterns of all ASCs consist of two different α chains (α1 and α2), a β and a γ components. Due to the presence of 2 identical subunits of α1 and α2, ASCs in the present study are classified as a type I collagen. The β- mercaptoethanol treatment was performed to check disulfide bonds in the ASCs. The results show that there was no difference in electrophoretic patterns observed in reducing and non-reducing treatments for all ASC samples. This indicates that ASCs do not contain any disulfide bonds (Nalinanon *et al.,* 2007), reflecting a low amount of cystine and methionine, which are essential in the formation of disulfide bonds (Kittiphattanabawon *et al*., 2005). Tamilmozhi *et al.* (2013), Bhuimbar *et al.* (2019) and Liao *et al*. (2018) also reported type I collagen from sailfish (*Istiophorus platypterus*), black ruff fish (*Centrolophus niger*), barramundi (*Lates calcarifer*) and tilapia (*Oreochromis niloticus*). From the literature, a type 1 collagen is found to be suitable for food, cosmetic, pharmaceutical and medical industries (Lim *et al*., 2019).

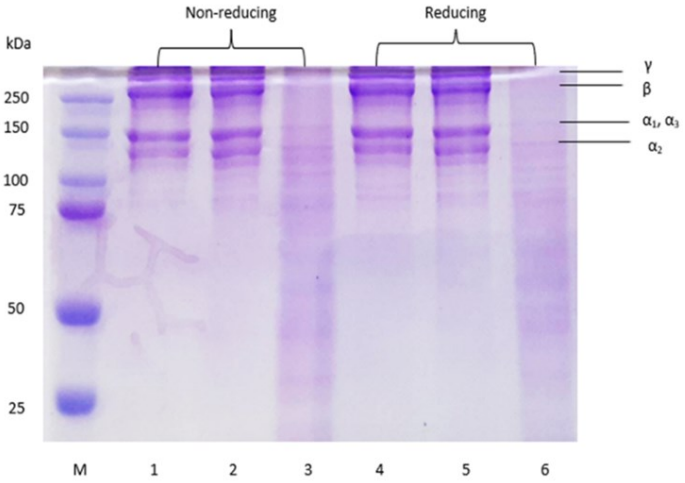

Figure 4. SDS-PAGE of collagens from the bone and fins of purple-spotted bigeye with three different acids. Lane M: Molecular weight marker; Lanes 1 and 4: Acetic acid-extracted collagen (AAC), Lanes 2 and 5: Lactic acid-extracted collagen (LAC), Lanes 3 and 6: Citric acid-extracted collagen (CAC).

4. Conclusion

Collagens from the combined bones and fins of purple-spotted bigeye were successfully extracted by different acids treatment. Citric acid-aided extraction showed a higher yield of ASCs, but not significantly different ($p>0.05$) with lactic and acetic acids treatments. FTIR and XRD analysis showed the triple helical structure of ASCs was maintained, and UV spectrum confirmed that ASCs were in accordance with collagen isolated from other fish species. Under and SDS-PAGE study, all ASCs were classified as type I collagen due to the presence of identical α1 and α2 chains. These findings suggest that ASCs from the combined bones and fins of purple-spotted bigeye could be potentially utilized as a substitute source of collagen in food or biopharmaceutical industries.

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Conflict of interest

The authors declare no conflict of interest.

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