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Isolation and Characterization of Acid-Soluble Collagens from the Bone and Fins of the Barracuda (*Sphyraena spp.*) as Marine Collagen Sources

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***Abstract* - Barracuda fish (*Sphyraena sp.)* bone and fins could be a source of aquatic collagen. Marine collagen has recently gained popularity due to its lack of infectious infections. This collagen extraction yields 1.99 % acetic acid-soluble collagen (AAC), 2.36 % lactic acid-soluble collagen (LAC), and 3.26 % citric acid-soluble collagen (CAC). AAC has a high L* value compared to LAC and CAC, indicating great brightness in color. For hydroxyproline content, the amount of collagen was 82.70, 81.31, and 80.93 for AAC, LAC, and CAC. AAC and LAC have maximum collagen solubility at pH 3, and CAC at pH 5. The effects of collagen solubility on NaCl concentrations drop substantially at 30 g/L for all collagen samples. All extracted collagen structures are type I collagen consisting of two chains (α1 and α2) based on SDS-PAGE analysis and possessing a complete triple helical structure based on UV absorption (229.5 nm) and Fourier Transformation Infrared Spectrometry (ATR-FTIR) showed all collagen samples had amide A, B, amide I, II, and III peaks. All collagens demonstrate strong heat resistance and structural stability as Tmax is above 30°C. LAC demonstrated higher absorption of water (0.50 0.01±mL/mg) and oil (0.70±0.07 mL/mg) than AAC and CAC. At pH 7, CAC and AAC reduced foam and foam case capacity. In emulsion properties, only AAC does not demonstrate important emulsion stability. AAC showed superior collagen than LAC and CAC based on physicochemical and functional qualities. Therefore, all collagen samples can be employed as replacements for terrestrial collagen in diverse applications.**

***Keywords*—Barracuda fish (*Sphyraena sp.);* bone and fins; acid-soluble collagen; physicochemical; functional qualities**

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I. INTRODUCTION

Over 196 million tons of fish are expected to be processed by 2025, making fish production a potentially lucrative industry [1]. The average yearly production of barracuda in Malaysia, as reported by MYAgro [2], is between 7,000 and 8,000 metric tons. In accordance with their religious prohibitions, Muslims are not permitted to consume porcine collagen. Thus, its application is prohibited [3]. In addition, fish by-products contain a significant quantity of crude protein, with levels ranging from 8% to 35% of the total, and they are a potential resource for collagen, gelatin, polyunsaturated fatty acids (PUFA), enzymes, and important amino acids [4], [5]. Solid wastes comprise around 50 to 70 % of the original raw material, depending on the processing technique. This material includes collagen-rich heads, viscera, skin, bone, scales, and fins [5]. Underutilized barracuda bio-resources can be used as a starting material for collagen manufacturing.

According to Harianti [6], the percentage of a barracuda's total weight that is comprised of fish skin and head bone is roughly 0.025 % and 0.01 %, respectively. This is in comparison to the overall weight of the fish. In addition, Jaziri et al. [7] found that barracuda had a moisture content of

between 55.76 and 79.86 %, 18.46 to 27.29 % protein, 0.05 to 2.55 % fat, 1.22 to 24.36 % ash, and 0.41-0.88 % carbohydrate content. Recently, Oslan et al. [4] revealed a variety of methods for extracting collagen from fish, including acid solubilization, enzyme solubilization, ultrasound, and the extrusion-hydro-extraction (EHE) procedure. Even though the enzymatic method is convenient, it is not good for processing large amounts of raw materials because enzymes are expensive. Despite the fact that chemical reagents are more affordable, certain chemicals can be hazardous or toxic [8]. Furthermore, recent research on collagen extraction derives from various sources prior to different technique extractions. These sources include the skin of *Thunnus tonggol* [9], the skin of salmon [10], the bone of lizardfish [11], the scales of tilapia [8], and the scales of *Sardinella longiceps [12]*. However, only a few studies have been reported about the extraction of collagen from fin fish. Recently, it was reported by Kuwahara [8] that collagen could be extracted from tilapia scales by using 0.1 M acetic acid and ultrafine bubbles of carbon dioxide solution for 5 hours. This process resulted in a yield of 1.58%. Furthermore, Truong et al. [13] discovered that the yield of fish skin recovered from snakeheads was 13.6%, and the yield of the scale was 12.09% using the ASC extraction process.

Collagen is a structural protein that is prevalent in connective tissue, including skin, bone, and internal organs. [14,15]. There are at least 29 different forms of collagen, each with distinct molecular characteristics and composed of a subunit called tropocollagen [4]. It has found its way into the food industry, as well as the cosmetics industry, the biomedical product industry, and the pharmaceutical industry [5]. Collagen type I is the most common pattern in fish, consisting of bands of -chains (α-1 and -2) and their dimers (ß-components) [3]. The acid-collagen interaction destroys crosslinks in the collagen helix, boosting collagen extraction efficiency [16]. Recent evidence from amino acid composition, analysis, electron microscopy, X-ray diffraction analysis, and physiochemical testing of collagen solutions established its molecular structure [11]. As consumer demand for fish rises, including for Barracuda (*Sphyraena* sp.), effective utilization of their wastes after filleting, particularly investigation of their potential use as a vital resource of collagen, could be profitable. Consequently, this research aimed to extract and characterize ASC collagen from the bone and fins of barracuda, which would be of excellent significance in related industries. Therefore, information on collagen and characterization approaches, particularly to fish collagen's composition, characteristics, and structure, is explored.

II. Materials and Methods

A. Materials

The 20kg Barracuda (*Sphyraena* sp.) fish was purchased in the Kota Kinabalu fish market. The barracuda fish were placed in a mechanical debone machine (SFD-8, 137 Taiwan) to remove the fins, bones, and fill from the fish [17]. Clean the bones and fins and cut them into small pieces (0.5cm long) [18]. All bone and fin samples were rinsed with tap water. After that, samples were packed and stored in polyethylene bags at 20 °C until they were utilized.

B. Pre-treatment Process

The pigment elimination process may increase lightness [4]. Barracuda fish bones and fins were immersed in 0.1M sodium hydroxide (NaOH) solution for six hours to remove non-collagen proteins with the sample-to-solution ratio 1:10 (w/v) and a continuous stirring of the mixture solution. Following that, the fish bones and fins were washed in cold distilled water until the desired pH of 7.0 was reached [18]. Later, in a sample-to-solution ratio of 1:10 (w/v), the bones and fins of the barracuda fish were submerged in 0.5 M EDTA-2Na at pH 7.4 for a period of 48 hours. In order to thoroughly mix the samples, a magnetic stirrer was utilized, and the solution was replaced after every 24 hours. The demineralized bone and fin samples were rinsed with distilled water until the pH of the water became neutral, which was necessary for the extraction of collagen.

C. Extraction of Collagen Using Different Types of Acid

Acid-soluble collagen was extracted with minimal modifications using the approach described by Yu et al. [18], Khong et al. [19], and Hukmi and Sarbon [20] by using acetic acid, lactic acid, and citric acid. All procedures were conducted for 72 hours at 4 °C with constant stirring. The precipitate was obtained by filtering the mixture through two layers of gauze fabric. In the presence of 0.05 M Tris(hydroxymethyl) aminomethane, pH 7.0, the precipitate was salted out of the supernatants by adding NaCl (crystal) until its final concentration was 2.5 M. The precipitate that formed, as a result, was collected by centrifuging the mixture at 4 ℃ for 30 minutes at 15,000 x g (Eppendorf, Centrifuge 5804, USA). The pellet was then mixed with 0.5 M acetic acid and dialyzed against 50 volumes of 0.1 M acetic acid for 24 hours. Afterward, the pellet was dialyzed against the same volume of distilled water for another 24 hours. Finally, the generated collagen was frozen at a temperature of -80 °C for 72 hours before being freeze-dried (Labconco, USA). The produced collagen can be classified as acetic acid collagen (AAC), lactic acid collagen (LAC), or citric acid collagen (CAC) and stored at 4°C pending further examination. The collagen yield was examined for its characteristics and described using a variety of techniques.

D. Extraction Yield

The yield of each collagen sample under various acid extraction conditions was determined. Based on Khong et al. [19], the yield of collagen findings was determined using the following equation:

$$Yield\ (\%) = \frac{Dry\ Weight\ of\ collagen}{Weight\ early\ of\ sample} \quad X\ 100 \qquad (1)$$

E. Hydroxyproline Content

The hydroxyproline content of all collagen samples was determined using a slightly modified method reported by Nalinanon et al. [21].

F. Determination of pH

The pH of collagen samples obtained using the procedures of Shon et al. [22] and Martinez-Ortiz et al. [23] was determined using a pH meter (Eutech pH 700, USA).

G. Determination of Color

The color determination of bone and fish fin collagen was based on the method of Ismail et al. [24] utilizing Colorimeter (Konica Minolta, Japan) based on the International Commission de I'Éclairage (CIE) scale. The color values of L*, a*, and b* are the parameters that are employed. The value indicated by L* is the lightness; the value represented by L* = 0 is the black, and the value indicated by L* = 100 is the diffuse white. The value indicated by a* is the redness; its range is from red +60 to green -60, and the value represented by b* is the yellowish value.

H. Differential Scanning Calorimetry (DSC)

The sample was hydrated by introducing 0.05M acetic acid with a solid-to-solution ratio of 1:10 (w/v). The combination was then incubated for two days at 4 ℃. After carefully measuring out 5 to 20 mg of the sample onto an aluminum pan, sealing it, and running it through a DSC (Perkin-Elmer, Model DSC7, Norwalk, CA, USA) at a temperature of 1 ° C / min across the range of 25 to 50 ° C, and the results were recorded. Empty pans were utilized as a reference, and indium was employed to calibrate the temperature. By measuring the area in the DSC thermogram, the total enthalpy of denaturation was calculated. Finally, the thermogram is used to calculate the maximal transition temperature (T_{max}) [11].

I. Proximate Analysis

The approximate composition of ash, moisture, fat, and protein in the bones and fins of Barracudas fish (Sphyraena sp.) was analyzed by using the Association of Official Analytical Chemists' standard protocols [25].

J. Ultraviolet (UV) Absorption Measurements

The UV ray absorption spectra was measured with a UV-Vis Spectrophotometer (Agilent Cary 60) according to the procedure described by Wang et al. [26]. 20 mg of samples collagen was dissolved in 20 mL of 0.05 mol/L acetic acid with a final concentration of 0.3 mg/mL. The spectrum was measured at wavelengths ranging from 200nm to 400nm. The data was recorded straight to load data in accordance with the wavenumber set.

K. Fourier Transform Infrared (FTIR) Spectra

Using Fourier Transformation Infrared Spectroscopy (FTIR), the structural properties of the produced collagen were analyzed (Agilent Technologies, USA). The infrared spectrum employed was 650 to 4000 cm-1 (Nicolet, Thermo Electron, USA). A total of 2 mg of collagen was applied to the crystal cells prior to scanning at 2cm-1 resolution for 32 scans versus the spectrum obtained from crystal cells at 25 ° C that were free of collagen [27].

L. Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was utilized to assess the protein patterns of ASC samples using a slightly modified method described by Jeong et al. [28]. Collagen samples were dissolved in 0.1 M acetic acid with 5% sodium dodecyl sulfate before being heated at 85°C for 1 hour. To remove unresolved debris, the mixture was centrifuged at 8,000 x g (Eppendorf, Centrifuge 5804, USA) for 5 minutes at room temperature (MERCK, Sigma-Aldrich, USA). The soluble sample was combined 4: 1 (v/v) with a buffer sample of 60mM Tris-HCl at pH 8.0, which contained 10% Sodium dodecyl sulfate, 25% glycerol, and 0.1% blue bromophenol. The following ingredients were used to make three sample loading dyes: 25% glycine, 20% SDS, 5% β-mercaptoethanol, and bromophenol blue. Gel was stained with Coomassie Brilliant Blue R-250 after electrophoresis. Mini-PROTEAN electrophoretically separated SDS-PAGE gel.

M. Solubility of Collagens

The solubility of collagens at various pH and NaCl concentrations was determined according to the method of Jaziri et al. [11] with slight adjustments. All collagen was dissolved in 0.5 M acetic acid at 4 ℃ to achieve final concentrations of 3 mg/mL and 6 mg/mL, and the solution was agitated for 18 hours. A total of 8 mL of collagen solution at a concentration of 3 mg/mL was used to investigate the solubility of collagen against the influence of pH. Before the pH is changed to 1, 3, 5, 7, 9, or 11, the solution is centrifuged at a speed of 10,000 x g for 20 minutes at a temperature of 4 ° C (Eppendorf, Centrifuge 5804, USA). In order to conduct the solubility test regarding the influence of the NaCl concentration, five mL of collagen solution containing a concentration of 6 mg/mL was combined with five mL of NaCl solution. The NaCl solution employed in this study was dissolved in 0.5 M acetic acid at concentrations of 0, 10, 20, 30, 40, 50, and 60 (g/L). The mixture of collagen solution and NaCl solution was constantly agitated for 60 minutes at 4 ° C followed by centrifugation (Eppendorf, Centrifuge 5804, USA) at 10 000 x g for 30 minutes at 4 °C. All samples were analyzed for protein content using the Lowry method, with bovine serum albumin serving as the standard. The relative solubility was determined in contrast to the highest pH value and greatest sodium chloride concentration value observed. The following equations (2) were used to estimate the relative solubility of the compounds:

$$\text{Relative solubilty (\%)} = \frac{\text{Current concentration of protein}}{\text{The highest concentration of protein}} \text{ X } 100 \quad (2)$$

N. Water Absorption Capacity (WAC) and Oil Absorption Capacity (OAC)

50 mg of collagen was dissolved in 1 mL of clean water and centrifuged for 10 minutes at 25 °C at a speed of 10,000 x g (Eppendorf, Centrifuge 5804, USA). Next, the samples were then left for ten minutes at room temperature. The samples were then centrifuged at a speed of 15,000 x g for 20 minutes (Eppendorf, Centrifuge 5804, USA). The supernatant volume was carefully dehydrated, and the residual pellets were weighed. For instance, a total of 50 mg of collagen was dissolved in soybean oil to test oil absorption ability. According to the approach proposed by Chen et al. [28], the formula for calculating water absorption capacity and oil absorption is as equation (3) below:

$$\text{WAC or OAC} = \frac{\text{Supernatant volume}}{\text{Volume of early water or oil before centrifuge}} \text{ X } 100 \quad (3)$$

O. Foaming Characteristics

Collagen foaming capacity (FC) and foam stability (FS) were measured by techniques developed by Chen et al. [29]. Collagen was dissolved in distilled water to a solution

concentration of 0.5 %. Each 20 mL of collagen solution produced was tested for pH, particularly pH 4 (acid), pH 7 (neutral), and pH 10 (alkaline). A 15mL volume of collagen solution was measured with a measuring cylinder. To assess the foam's ability, the collagen solution was homogenized (Ultra Turaxx®, USA) at a speed of 15,000 x g for two minutes at room temperature for foam capacity. The homogenized collagen solution was then allowed at room temperature for 1 hour before a volume reading was taken to determine the foam stability. The following are the formulas for foaming capacity (FC) and foam stability (FS) as equations (4) and (5) below:

$$FC\ \% = \frac{\text{Vol after homogenized} - \text{vol before homogenized}}{\text{Volume after homogenized}} \text{ X } 100 \quad (4)$$

$$FS = \frac{\text{Vol before homogenized} - \text{vol after homogenized (60 min)}}{\text{Vol after homogenized} - \text{vol before homogenized}} \text{ X } 100 \quad (5)$$

P. Emulsion Capabilities

The emulsion activity index (EAI) and emulsion stability index (ESI) of the isolated collagen were assessed using the method described by Akram and Zhang [30]. Collagen was dissolved in distilled water to a concentration of 0.5%. Each 6mL of the resultant collagen solution was tested for pH using 1 M HCl or 6 M NaOH at pH 4.0 (acid), pH 7.0 (neutral), and pH 10 (alkaline). The collagen solution was then added to 2mL of soybean oil before being homogenized at 16,000 x g for 2 minutes at room temperature using a homogenizer (Ultra-turrax®, USA).

Q. Statistical Analysis

All statistical analyses were carried out using SPSS version 23.0 of the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA). The study was performed in duplicate (n = 2) and triplicate (n = 3) using one-way ANOVA and the Duncan Multiple Range Test (DMRT) to compare the mean values of each sample. A p-value of (<0.05) was used to indicate a significant difference between samples.

III. Result and Discussion

A. Yield, Hydroxyproline Content, pH, Color, and Thermal Properties of Collagen

The percentages of collagen for the three acid treatments AAC, LAC, and CAC for bone and fin samples of Barracudas fish are presented in Table 1 based on significant differences at (p <0.05). AAC produced collagen at a rate of 1.99%, followed by LAC at 2.36 % and CAC at 3.26 %. The interaction of aldehyde with lysine and hydroxyproline at the telopeptide helix location may account for variations in collagen yield between AAC, LAC, and CAC [28]. According to a study conducted by Duan et al. [31], the acid-soluble collagens (ACS) yield from carp bones (1.06 %) was comparable to the ASC yield in the study but different from skipjack tuna (42.3 %) and yellow sea bream (40.1 %). According to prior research, AAC should have a greater collagen content than CAC since the acid employed has a high collagen extraction capability [16]. This may be related to the extra salt left behind during cellulose-tube-based dialysis, which increases collagen mass. This is evident when the surface of CAC is abrasive and contains easily detachable crystal components.

TABLE I
YIELD AND PHYSICOCHEMICAL PROPERTIES OF ACID-SOLUBLE COLLAGEN FROM BONE AND FINS OF BARRACUDA FISH EXTRACTED USING DIFFERENT ACID EXTRACTION METHOD

Parameters	AAC	LAC	CAC
Yield (%, dry weight basis)	1.99 ± 0.01a	2.36 ± 0.01b	3.26 ± 0.02c
Hydroxyproline content (mg/g dry sample)	82.70 ± 2.01a	81.31 ± 1.30a	80.93 ± 0.54a
pH	5.86 ± 0.09b	4.85 ± 0.07a	5.23 ± 0.20a
Color scale, L*	74.70 ± 7.7b	61.91 ± 4.05a	84.72 ± 1.04b
a*	1.42 ± 0.6b	1.18 ± 0.07b	0.20 ± 0.14a
b*	6.94 ± 2.63a	8.32 ± 0.64a	4.72 ± 1.42a
T_{max} (°C)	33.68	33.21	33.08
(Δ H) (mJ/g)	0.0787	0.0741	0.0107

Values are reported as mean ± SD (n=3). $^{a-c}$ Subscript with the different letter is significantly different at ($p < 0.05$).

Table 1 further shows that there was no significant difference between the three collagens (AAC, LAC, and CAC) at (p> 0.05). AAC had a slightly greater hydroxyproline concentration of 82.70 mg/g than LAC (81.32 mg/g) and LAC (80.93 mg/g). Hydroxyproline is a chemical that is used to determine the amount of collagen in a given tissue [32]. Kittiphattanabawon et al. [33] determine giant eye fish's hydroxyproline concentration in the skin and bones (*Priacanthus tayenus*) to be 58.5 mg/g and 42.4 mg/g, respectively. In contrast to this study, the hydroxyproline concentration of the bone and fin mixture was greater, ranging from 80 mg/g to 83 mg/g depending on the kind of acid treatment. This is attributed to the high protein level in collagen arising from a combination of bone and fin samples rather than only bone, which is consistent with the assertion that an increase in protein content often accompanies a rise in hydroxyproline concentration in collagen samples. Furthermore, f fish collagen hydroxyproline levels are lower than mammalian collagen [34], and hydroxyproline levels were determined to evaluate collagen concentrations in fish bones and fins [35].

According to Nalinanon et al. [21], the higher the hydroxyproline level, the more collagen can be recovered. According to Table 1, AAC had a pH value of 5.86, followed by LAC at 4.85 and CAC at 5.23, with statistically significant variations amongst collagen (p<0.05). Sionkowska et al. [36] state that excellent collagen has a pH of 6 to 7. Martinez-Ortiz et al. [23] conducted a study in which they synthesized collagen from rabbit skin with pH readings ranging from 6.2 to 6.4. In this study, however, the pH measurements of AAC, LAC, and CAC did not exceed pH 6. This means that salts and acids cannot be eliminated completely throughout the dialysis process, leaving residual salts and acids [23]. Furthermore, the kind and strength of acids utilized during the extraction technique may contribute to changes in collagen pH [22]. This study employs a low concentration of acetic acid of 0.5M, which corresponds to the pH results reported in the skin of silver catfish (Pangasius spp.) in the study of Hadfi and Sarbon [37]. Another factor contributing to low pH is that the pH of collagen in fish bones and fins varies depending on the fish species [22].

Color can be used to measure the quality of collagen. There are three values on the color scale: L* (lightness), a* (green reddish), and b* (blue-yellowish). The color features of fish collagen for three forms of acid treatment (AAC, LAC, and CAC) are shown in Table 1 using the color scale L*, a*, and b*. CAC has a higher L* color scale than AAC and LAC, with significance differences ($p < 0.05$) of 84.72, 74.70, and 61.91, respectively. The color scale a* revealed that AAC had the greatest value of 1.42, followed by LAC (1.18) and CAC (0.20), with a significant difference of ($p<0.05$) between them. In contrast, the b* scale shows that LAC has the highest value of 8.32, followed by AAC (6.94) and CAC (4.72), with no significant difference at ($p>0.05$).

According to Table 1, the maximal transition temperature, Tmax, and (ΔH) for collagen AAC, LAC, and CAC are 33.68 ° C, 33.21 ° C, and 33.08 ° C, respectively, and 0.0787 J/g, 0.0741 J/g, 0.0107 J/g. Differential calorimetry (DSC) scanning is used to examine collagen's thermal properties because collagen's molecular structure is solved when heated at high temperatures; collagen absorbs heat when the temperature rises in the DSC, and it begins to dissolve at a particular temperature [16]. These three Tmax values are greater than what is typically found in fish collagen, which is less than 30 °C. This reveals that AAC, LAC, and CAC have great heat resistance and structural stability and could be utilized to replace mammalian collagen [38]. This is also consistent with the ASC investigation on bigeye snapper fish bones (30.80 °C) [33]. According to previous year's reports, the Tmax for each collagen varies based on imino acid content, habitat temperature, season, tissue type, and species. For example, ASC from balloon fish skin (29.64 °C) and bigeye snapper fish skin (28.68 °C) [32,38]. Generally, the collagen of fish species that live in areas with high temperatures has a higher concentration of amino acids and is also more resistant to heat than the collagen of fish species that live in locations with high temperatures but low altitudes [26,39,40].

B. Proximate Analysis

According to Table 2, the protein content of bones and fins was high (34.95% and 33.86%, respectively, above the total value of proximate analysis (27%) [41]. This could be attributed to the low moisture found in the bones (49.35%) and the fins (50.75%). Because the moisture content of fish samples is high, the protein quantity will be low, as evidenced by other minerals in the fins relative to the bones, which causes excessive ash levels [42].

In contrast to fat composition, bone (7.40%) had a higher value than fins (7.29%). Barracuda fish is a fatty fish (fatty fish) similar to salmon that stores fat in the muscles and causes fat in the bones to be high. These results coincide with the expression of fat in animal bones ranging from 1 to 27% [43]. In contrast, for the bone and fin mixture results, the composition of moisture (51.45%), ash (2.61%), fat (10.07%), and protein (35.35%) had higher values compared to the bone and fin samples alone. Generally, the content of energy, protein, and fat is determined by the moisture percentage. The higher the protein and lipid composition of the fish and the higher the energy density for the fish, the lower the moisture percentage. The mixture of bones and fins has a high protein content suitable for healing and processing. This is because proteins have a high biological value and contain all the necessary amino acids, while fish lipids also contain fatty acids and omega-3 fatty acids [41]. A mixture of bone and fin samples has been used in this study to obtain excellent and high acid-soluble collagen results.

TABLE II
PROXIMATE ANALYSIS OF BONES AND FINS OF BARRACUDA FISH

Composition (%)	Sample		
	Bones	**Fins**	**Bones and Fins**
Moisture	49.35 ± 0.02^{a}	50.75 ± 0.03^{b}	51.45 ± 0.01^{c}
Ash	2.33 ± 0.03^{a}	6.95 ± 0.01^{c}	2.61 ± 0.02^{b}
Fat	7.40 ± 0.04^{a}	7.29 ± 0.03^{b}	10.07 ± 0.01^{c}
Protein	34.95 ± 0.02^{b}	33.86 ± 0.03^{b}	35.35 ± 0.02^{c}

Data are shown as mean ± standard deviation (n = 3). $^{a-c}$ Subscript with the different letter is significantly different at ($p < 0.05$).

C. UV-VIS Absorption Spectra Analysis Structure

Figure 1 reveals the UV absorption spectrum of collagen treated with three different acids at 210nm and 400nm wavelength range: acetic acid collagen (AAC), lactic acid collagen (LAC), and citric acid collagen (CAC). Collagen UV scanning is a basic and straightforward method for testing collagen purity because the triple helix structure has the highest absorption peaks in the 210–240 nm range due to the presence of carbonyl groups (C = O), carboxyl groups (COOH), and amide groups (CONH2) in the polypeptide chain. Due to the overall presence of aromatic amino acids, proteins have a maximum absorption wavelength of 280 nm in general [44]. It can be noted that AAC, LAC, and CAC have different maximum absorption peaks, thus at 229.5 nm, rather than the maximum UV absorption peaks found in most other protein types, which were at 280 nm.

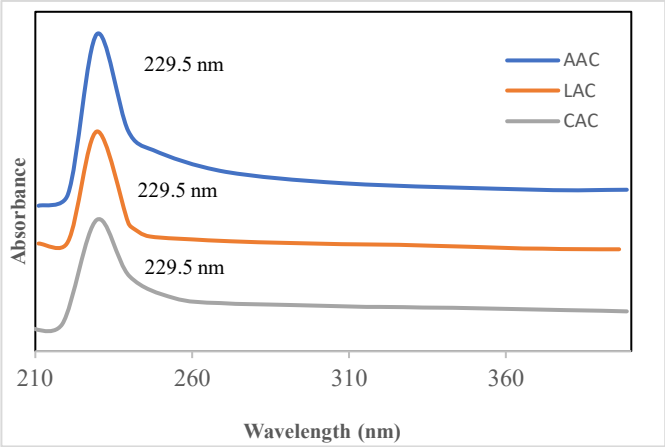


Fig. 1 UV absorption spectrum of collagen treated with acetic acid, lactic acid, and citric acid

This is related to this collagen's lack of aromatic residues such as tryptophan, tyrosine, and phenylalanine [26]. The findings of this study are consistent with earlier research, specifically collagen isolated from mackerel and horsetail (230nm) and collagen extracted from black pomfret (220nm) [42,45]. As a result, as indicated by the UV absorption

spectrum at 280 nm, this method of collagen extraction from these three acid treatments is well appropriate to generate collagen of high purity, with minimal quantities of tryptophan, tyrosine, and phenylalanine [46].

D. Fourier Transform Infrared Spectroscopy (ATR-FTIR)

Figure 2 demonstrates the absorption rate readings for collagen that was subjected to various acid treatments, specifically acetic acid collagen (AAC), lactic acid collagen (LAC), and citric acid collagen (CAC).

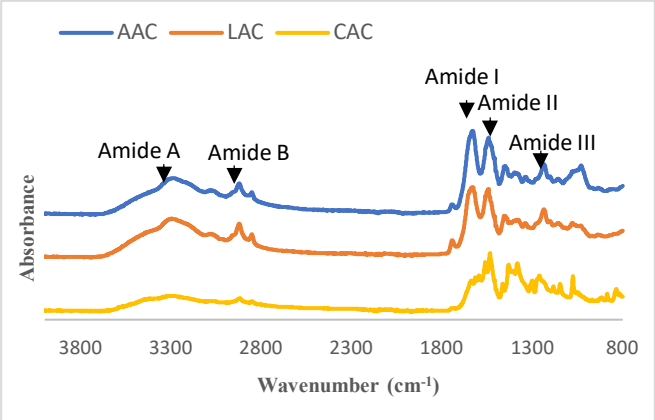


Fig. 2 ATR-FTIR spectra for collagen treated with different acid.

The FTIR is among the most popular methods for determining the chemical structure of proteins since it involves minimal sample preparation, saves time, and does not require the use of solvents [23]. Figure 2 exhibits the FTIR spectra of AAC, LAC, and CAC in the range 4 000-800 cm^{-1}. All collagens contain a primary absorption band in the amide band region, however, the FTIR spectra for AAC, LAC, and CAC differ slightly, suggesting that their secondary structures varied. After three separate acid treatments, the FTIR spectrum for mixed bone and fin collagen reveals distinct peaks of amide I, amide II, amide III, amide A, and amide B with varied peak assignments.

According to prior research, Amide A peaks are commonly observed between 3400 and 3440 cm-1 and are frequently connected with N-H stretching vibrations. Furthermore, Herath et al [47] obtained comparable results for fish bone collagen at 3426.7 cm^{-1}. The Amide A absorption rate readings for AAC, LAC, and CAC were 3283.87 cm-1, 3296.91 cm-1, and 3315.55 cm^{-1}, respectively, which were lower than reported in this study. This is due to the peptide's N – H group engaging the H bond, causing the A amide band to shift to a lower frequency range than typical. It also implies that hydrogen bonds occur in each collagen [48]. According to the results of the study, LAC has a higher absorption value of 2922.31 cm^{-1} than AAC and CAC, which have absorption values of 2920.44 cm^{-1}. Amide B peaks are commonly found in collagen and are generated when the CH_2 group is stretched asymmetrically [17]. The wavelengths of the amide I, amide II, and amide III bands are also tightly related to the collagen arrangement. Amide I peak are frequently found between 1600 and 1700 cm^{-1} due to their significant absorption, mostly attributable to C=O stretching vibrations along the polypeptide spine [18,20]. In this study, Amide I was detected in AAC and LAC with a reading of 1628.9 cm^{-1}, which is within the range of Amide I, and not in CAC, which has a slightly lower reading of 1533.84 cm^{-1}. Lower wave number peaks are related to a decrease in the molecular organization, making it a sensitive indicator of peptide secondary structure [18].

The Amide II peak for AAC and LAC is the same at 1541.29 cm^{-1}, whereas it is lower for CAC at 1431.33 cm^{-1}. The amide II peaks are related to N-H bending and C-N stretching vibrations, as well as the helical structure of three collagens, and have wavelengths between 1500 and 1600 cm^{-1}. The NH group is engaged in hydrogen bonding in the peptide chain; hence CAC has a low value. As a result, the location of the amide group shifts to a lower frequency. When the wavelength changes to a lower wavelength, collagen has a greater amount of hydrogen bonds [20]. The C – N stretching and bending components in the N – H plane of the amide link generate an amide III peak that commonly appears at wavelengths 1320– 1220 cm^{-1}. This amide III peak identifies the collagen triple helix structure [49]. The Amide III peak for AAC, LAC, and CAC is 1235.64 cm^{-1} and 1261.74 cm^{-1}, respectively. These three forms of collagen are in the same Amide III peak range as the study by [47], which revealed amide III peaks in the 1278.8 cm^{-1} range for collagen from fish bones.

E. Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

The extracted collagen protein profile was analyzed using SDS-PAGE. According to Figure 3, there is no variation in electrophoretic pattern for the three acid treatments (AAC, LAC, and CAC) in non-reducing and reducing conditions (with or without beta-mercaptoethanol), showing the absence of disulfide interaction between chains [17]. This is due to the amino acids cysteine and methionine forming disulfide bonds, however, cysteine and methionine exist in low amounts in collagen type I [33].

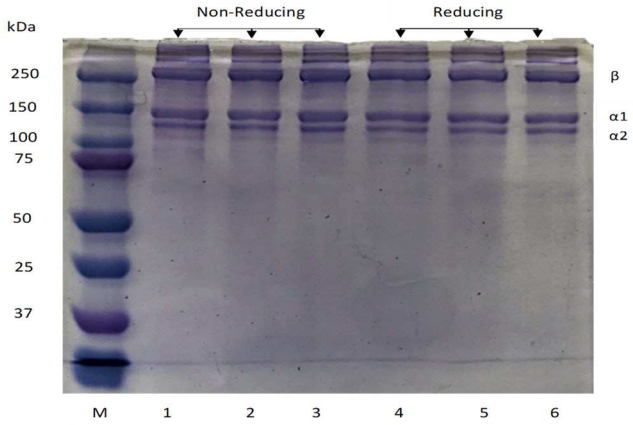


Fig. 3 SDS-PAGE pattern for collagen treated using different acids under non-reducing and reducing conditions. Lane M: Molecular weight marker; Lane 1 and 3: Acetic acid soluble collagen (AAC); Lane 2 and 5: Lactic acid soluble collagen (LAC) & Lane 3 and 6: Citric acid soluble collagen (CAC)

There are various bands found on collagen extracted using different acid treatments (AAC, LAC, and CAC), including two for chains (α1 and α2) and two for cross-linked chains (dimer) and (trimer). The 1 chain has a molecular weight of 158 kDa and the 2 chain has a molecular weight of 126. Due to having a molecular weight comparable to that of the α1

chain, the α3 chain is not recognizable on SDS-PAGE gels. This is because the α1 chain may include α3 chains [50]. The subunit has a molecular weight of 251 kDa. The presence of two distinct chains demonstrates that the primary collagens AAC, LAC, and CAC from the bones and fins of barracuda fish are type I collagen. A previous study based on electrophoretic patterns containing α1 and α2 chains in different types of tissues and fish revealed similar results, such as collagen from catfish skin [51], bones and skeletons of skipjack tuna [18] and bones from two marine fish *Magalaspis cordyla* and *Otolithes ruber* [44]. Type I collagen is a component of all connective tissues, including bone and skin, and performs functions such as mechanical protection of tissues and organs as well as physiological management of the cell environment [52].

F. Effect of Solubility of Collagen on NaCl Concentration

Figure 4 indicates the percentage of relative solubility for three acid treatments, particularly AAC, LAC, and CAC, using varying amounts of sodium chloride (NaCl) (0-60 g/L). All collagens exhibit a high relative solubility of more than 40% at concentrations ranging from 0 to 20 g/L. This is because salt ions adhere poorly to the charged group on the protein surface at low NaCl concentrations without affecting the domain's hydration shell. In most cases, the solubility of collagen in acetic acid at a concentration of 0.5 M will decline as the concentration of NaCl in the solution rises [53]. In contrast, at NaCl concentrations of 30 g/L, all collagen extraction showed a drop in relative solubility, with values ranging from 10% to 40%. Reduced collagen solubility can be due to the out-salting phenomena that happens when NaCl concentrations are rather high. Increased salt concentration causes an increase in hydrophobic-hydrophobic interactions between protein chains, as well as increased competition for water with salt ions [54]. This generates an increase in ion strength, which reduces protein solubility and precipitates stimulated proteins [18].

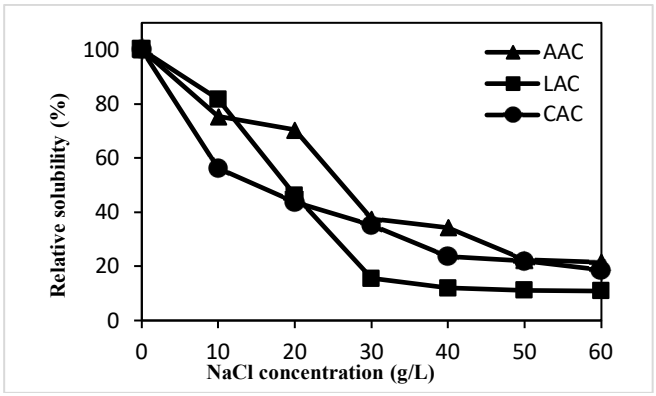


Fig. 4 Effect of collagen solubility on different NaCl concentrations

G. Effect of Solubility of Collagen on pH

In this study, the solubility of AAC, LAC, and CAC against pH was determined by being dissolved in 0.5M acetic acid before being tested at pH 1,3, 5, 7, 9, and 11, as in Figure 5. The results of this study showed that AAC, LAC, and CAC were dissolved in the range of pH 1 to pH 3, with the highest solubility at pH 3. Similar results were also recorded for ASCs from Spanish Mackerel skin and bone [55]. According to Nalinanon et al. [21], collagen has the highest solubility reading at pH between 2 to 5, with a relative solubility rate exceeding 80%. According to Figure 5, the results of this study, all collagen extractions were soluble between pH 1 and 3, with the highest solubility at pH 3. ASCs generated from Spanish Mackerel skin and bone produced comparable outcomes [55]. Collagen has the highest solubility reading at pH values spanning from 2 to 5, with a relative solubility rate of more than 80%, according to Nalinanon et al. [21]. Therefore, the solubility of all collagens reduces when the pH is greater than 3. This is because collagen loses solubility at neutral and alkaline pH values due to hydrophobic interactions between collagen molecules [56]. AAC, LAC, and CAC also showed a relative collagen reduction below 60% when pH 7 and pH 9 approached the isoelectric point (pI). All collagen's pI values were discovered at pH 9, which is compatible with studies that show collagen's pI ranges from 6 to 9. Yu et al. [18] The solubility of AAC and LAC increased marginally at pH 11 compared to pH 9 due to the higher residual net charge of the protein molecules. The increased solubility is due to the repulsive force between the chains when the pH is lower or higher than pI [56]. However, there is no increase in CAC at pH 11, which could be related to variations in collagen solubility or differences in collagen properties and conformation molecules.

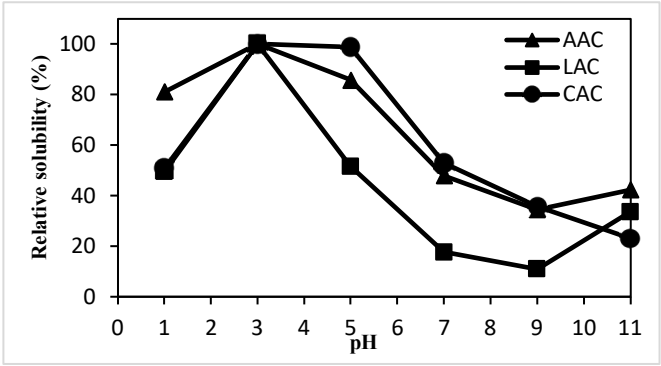


Fig. 5 The effect of collagen solubility on pH levels

H. Water Absorption capacity (WAC) and Oil Absorption Capacity (OAC)

The water and oil absorption capability of three types of acid treatment ASCs is shown in Table 3. The amount of water and oil absorbed per gram of collagen powder (protein) was calculated using the water and oil absorption capacity (WAC) and oil absorption capacity (OAC). WAC is determined through the direct interaction of collagen molecules with water and solvents [30]. Oil absorption in OAC is affected by protein source, processing conditions, protein concentration, and temperature. In this specific study, a total of 50 milligrams (mg) of collagen derived from three different kinds of acids (AAC, LAC, and CAC) was utilized to ascertain the water and oil absorption rate.

TABLE III
WATER AND OIL ABSORPTION CAPACITY OF COLLAGEN

Type of collagen	Capacity (mL/mg)	
	Water absorption	Oil absorption
AAC	0.44 ± 0.06^{a}	0.30 ± 0.14^{a}
LAC	0.50 ± 0.01^{a}	0.70 ± 0.07^{b}
CAC	0.45 ± 0.07^{a}	0.35 ± 0.08^{c}

Data are shown as mean ± standard deviation (n = 3). $^{a-b}$ Subscript with the different letter is significantly different at ($p < 0.05$).

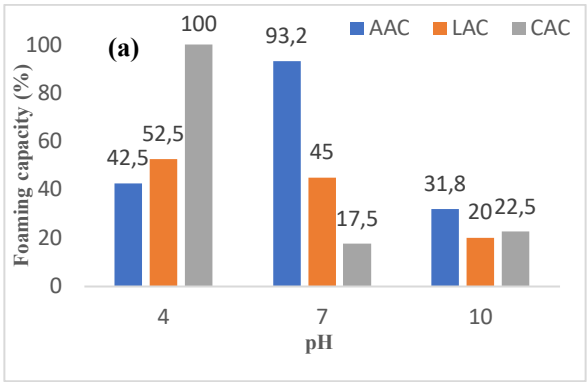
LAC has a higher water absorption capacity (WAC) of 0.50 mL/mg than AAC and CAC, which had readings of 0.44 mL/mg and 0.45 mL/mg, respectively. There was no difference in confidence between AAC, LAC, and CAC ($p > 0.05$). According to Chen et al. [29], these reading inconsistencies are caused by changes in protein solubility, particle size, micromorphology, and physicochemical environment. LAC is excellent for usage in food items since the WAC value is vital for controlling moisture in food goods.

For OAC, the AAC, LAC, and CAC concentrations were 0.30 mL/mg, 0.70 mL/mg, and 0.35 mL/mg, respectively. Despite the fact that there was not significantly different ($p > 0.05$) between the three acid treatments, LAC (0.70 mL/mg) had higher values than AAC (0.30 mL/mg) and CAC (0.35 mL/mg). Generally, a protein's non-polar amino acid residue defines the OAC since the hydrophobic interaction between the non-polar amino acid of the protein molecule and the oil hydrocarbon chain dictates the protein. Based on OAC values, it can be determined that LAC possesses more non-polar amino acid residues than AAC and LAC [29].

I. Foaming

Figures 6 (a) and 6 (b) show the percentages of foaming capability (FC) and foam stability (FS) for three acid treatments at pH 4, pH 7, and pH 10. In general, foaming qualities are comparable to emulsion properties [57]. Foam expansion, capacity, and stability were all parameters in foam analysis that were influenced by protein structure and composition. Transport, penetration, and rearrangement of protein molecules at the air-water interface are substantially responsible for the creation of foam. Therefore, the ability of the foam (FC) will be deemed good if the protein can rapidly and effectively dissolve the water's surface [58].

Figure 6 (a) indicates that CAC has a high FC measurement at pH 4 and pH 10 as a result of a drop at pH 7. A similar outcome can be seen in the FC reading for AAC. This drop is brought on by the low solubility level of collagen molecules as well as their poor electrostatic repulsion, which leads to the formation of collagen aggregation molecules. This reduces the interaction between protein and water required to produce foam and lowers collagen's FC and FS. Similar findings were achieved in research with red stingray skin collagen [28] and chicken sternal cartilage collagen [30]. FC readings for AAC and LAC were irrelevant due to inconsistent increases and decreases at all three pHs and for LAC and CAC FS readings.



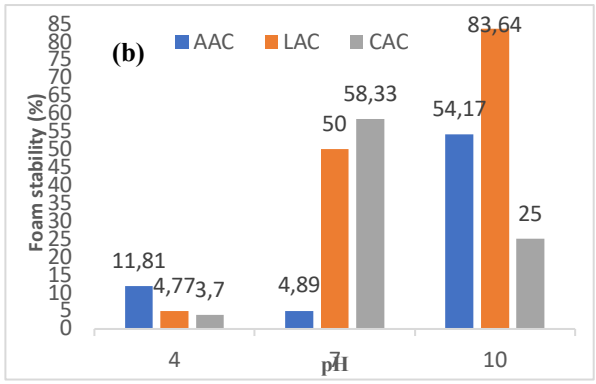


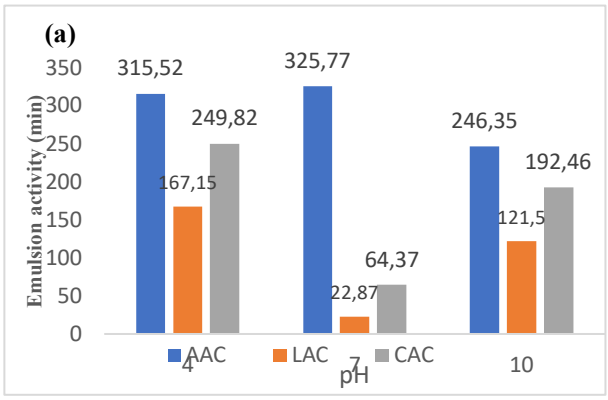
Fig. 6 (a) Foaming capacity and (b) foam stability for acid soluble collagen at different level of pH.

J. Emulsion

Figures 7 (a) and 7 (b) illustrate the emulsion activity index (EAI) and emulsion stability index (ESI) for three acid treatments: acetic acid collagen (AAC), lactic acid collagen (LAC), and citric acid collagen (CAC) at pH 4, pH 7, and pH 10. The emulsion properties were investigated using EAI and ESI. EAI is used to calculate the amount of oil that can be emulsified per unit of protein, whereas ESI is used to track emulsion resistance over time. This is because protein properties such as hydrophobic-hydrophilic ratio, protein folding, and presentation can significantly impact collagen emulsion ability [59].

In general, at pH 6–7, the EAI and ESI of acid-soluble collagen will drop because collagen solubility and electrostatic charge on collagen molecules decrease at the isoelectric point. Oil droplet aggregation may develop due to decreasing repulsive intensity [28,29]. When oil droplets are aggregated, the interaction required between oil and water to produce foam is reduced, decreasing the EAI and ESI of collagen [29]. This statement is corroborated by studies on red stingray skin by Chen et al. [29] and chicken sternal cartilage [30] that exhibit higher EAI and ESI values at pH 4 and pH 10 and low at pH 7.

According to Figure 7 (a), the EAI for AAC is very high at pH 4, pH 7, and pH 10. In contrast, LAC and CAC exhibit high values at pH 4 and 10, but low values at pH 7, which is consistent with the prior findings. Meanwhile, in Figure 7 (b), the pH 7 readings for LAC and CAC are insignificant because they are higher than pH 4 and pH 10.



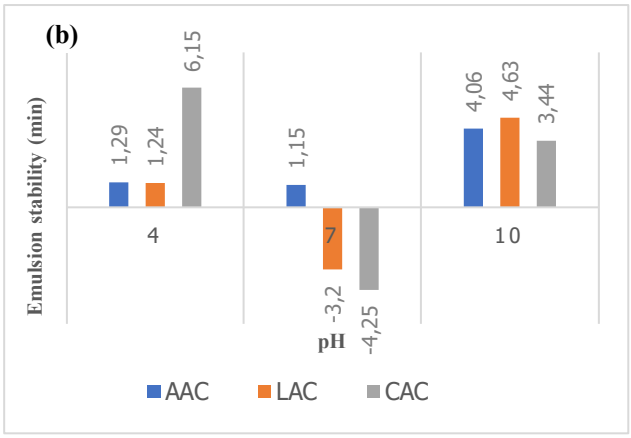


Fig. 7 (a) Emulsion activiity and (b) emulsion stability for acid soluble collagen at different level of pH.

This could be due to a sudden increase in absorption value caused by barriers encountered throughout the analysis, in which case changing values should be obtained at minute zero and minute ten. Based on the findings of EAI and ESI, it can be stated that AAC can potentially replace terrestrial protein sources.

IV. Conclusion

In conclusion, the extraction of acid-soluble collagen from the bones and scales of the Barracuda fish is influenced by the types of acids used. This extraction yields three forms of collagen: AAC, LAC, and CAC, with respective collagen yield percentages of 1.99%, 2.36%, and 3.2%. Characterization of structural, physicochemical, and functional properties has been carried out on all collagens. The results have demonstrated that the collagen properties are suitable for numerous applications and can replace collagen sources derived from terrestrial collagen sources.

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