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Biochemical Characteristics of Acid-Soluble Collagen from Food Processing By-Products of Needlefish Skin (*Tylosurus acus melanotus*)

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Abstract: The by-product of needlefish (*Tylosurus acus melanotus*) waste possesses important characteristics that could be used in food applications. Fish by-product collagen may be used in place of mammalian collagen due to ethical and religious considerations over environmental degradation. Different forms of acid-soluble collagen (ASC) were successfully extracted from needlefish skin. Based on dry weight, the collagen extracted using acetic acid (AAC), lactic acid (LAC), and citric acid (CAC) treatments was 3.13% with a significantly difference ($p < 0.05$), followed by 0.56% and 1.03%, respectively. Based on proximate analysis, the needlefish skin composition was found to be significantly different ($p < 0.05$) between compositions, with the highest moisture content at 61.65%, followed by protein (27.39%), fat (8.59%), and ash (2.16%). According to the SDS-PAGE results, all extracted collagen were identified as a type 1 collagen. Additionally, ATR-FTIR revealed that all collagens had amide A, B, amide I, II, and III peaks. AAC significantly outperforms LAC and CAC in terms of yield following physicochemical characterisation, including pH determination, colour (L* value), and hydroxyproline content. All collagens demonstrated strong heat resistance and structural stability with T_{max} above 38 °C. Collagen was most soluble at pH 5 for AAC, pH 3 for LAC, and pH 7 for CAC. The effect of collagen solubility on NaCl concentration was discovered to be significantly reduced to 50 g/L for all collagen samples. All collagens can be used as alternatives to terrestrial collagen in a diverse range of applications.

Keywords: by-product; needlefish (*Tylosurus acus melanotus*); acid-soluble collagen extraction; physicochemical; characterization

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1. Introduction

Nowadays, fishing is an important sector of the world economy, producing a wide range of by-products from fish processing, including over 196 million tonnes of fish expected to be processed by 2025, making fish production a potentially profitable industry [1]. As an outcome, needlefish (*Tylosurus acus melanotus*) can be found in abundance in Malaysia, particularly in Sabah and Sarawak. Generally, needlefish are processed into surimi and utilised to manufacture fish balls. Wastes from needlefish, such as skin, bones, fins, and heads are eliminated during the preparation of surimi. However, by-products can be recovered and turned into a range of useful substances, including collagen [2]. As collagen is made from fish wastes, the resulting collagen will be halal, as Muslims are barred from consuming pig collagen and its use is therefore prohibited [3]. According to Schmidt et al. [4],

fish by-products such as scales, bones, fins, and skin are the most secure sources for collagen production, and no fish-related diseases have been reported. Furthermore, crude protein levels in fish by-products can reach up to 35%, and depending on the species, age and season, collagen can constitute up to 70% of the fish's dry weight [2]. As a result, these have the potential to serve as a source of collagen, polyunsaturated fatty acids (PUFA), gelatine, enzymes, and essential amino acids [5]. Therefore, there is a need for collagen that can be made from marine sources that has been demonstrated to be comparable to traditional collagen made from mammalian sources in terms of amino acid content and biocompatibility [6]. Wastes often created by fish processing that have been utilised; include the skin, bone and muscle of leather jackets (*Odonus niger*) [7]; skin, scales and fins of *Catla catla* and *Cirrhinus mrigala* [8]; skin of tilapia (*Oreochromis niloticus*) [9]; and several other parts which have texture and are promising sources of collagen.

Due to its unique properties, collagen is important to many sectors, including those that provide healthy meals, cosmetics, tissue engineering, and wound-care products [10]. Collagen makes up nearly 30% of the total protein in mammalian skin and bone connective tissue [6] and governs tissue growth, supports structural integrity, and offers texture, shape, thickening, durability, and gel function [11]. Recently, Oslan et al. [2] discovered a range of techniques for extracting collagen from fish, including acid solubilization, enzyme solubilization, ultrasound, and the extrusion-hydro-extraction (EHE) approaches. In order to extract collagen, acids and enzymes are commonly used. Generally, collagen dissolves in acidic liquids; however, when exposed to a very acidic pH, its solubility diminishes, and certain chemicals can be hazardous or toxic [12]. By attaching an amine group (-COOH) on the collagen protein, weak acids or organic acids, such as acetic acid, citric acid, and lactic acid, are utilised to facilitate the collagen extraction process [13]. In addition, studies on collagen extraction from fish have revealed that the yield of collagen and the optimal conditions required vary depending on the type of raw material, type of acid, concentration, time, and temperature [14,15]. However, excessive acid concentrate destroys peptides, lowering the quantity and quality of the collagen [15]. In terms of thermal stability, fish collagen has less thermal stability than mammalian collagen, which may be attributable to the short amino acid (proline and hydroxyproline) residues [16]. However, hydroxyproline improves with heat stability by stabilising the triple helix structure of collagen. Fish with lower hydroxyproline concentrations have been shown to have lower denaturation temperatures than fish with greater hydroxyproline concentrations [2]. Furthermore, several strategies for enhancing cross linking and thermal stability of fish collagen have been used. The denaturing temperature of salmon atelocollagen (SC) has been enhanced from 18.6 °C to 47 °C after employing a modified approach in which a neutral buffer was combined with an acidic SC solution at 4 °C during collagen fibril development [16]. Therefore, the goal of this work was to extract and analyse acid soluble collagen from needlefish skin (*Tylosurus acus melanotus*) for the use in food and other industries.

2. Materials and Methods

2.1. Raw Materials Preparation

The needlefish (*Tylosurus acus melanotus*) was obtained at a fish market in Kota Kinabalu, Sabah. The needlefish were chilled and delivered to the laboratory within 40 min of purchase, then washed with cold distilled water. The fish were deboned using mechanical equipment (SFD-8, 137 Taiwan). The skins were thoroughly cleaned under running tap water before being trimmed into 0.5 cm pieces using a scalpel [11]. Additionally, skin samples were cleaned, placed in polyethylene bags, and stored at −20 °C until collagen extraction. Analytical-grade reagents were employed in all experiments.

2.2. Sample Pre-Treatment and Defatting Process

The needlefish skin was immersed in a 0.1 N NaOH (1:10) (*w*/*v*) solution at 4 °C for 6 h, with the solution being replaced every 3 h to eliminate non-collagen protein. Subsequently, they were rinsed with tap water to achieve a pH of 7.0 neutrality [17]. The samples were

then submerged in a 10% (1:10) butyl alcohol solution at 4 °C for 24 h. This solution was changed every 6 h to remove fat, and then the skin was washed again with cold distilled water until the pH level was neutralize.

Proximate Analysis

Proximate analysis of moisture, fat, protein and ash content of skin needlefish was performed using established techniques developed by the Association of Official Analytical Chemists (AOAC) [18].

2.3. Extraction of Collagen Using Different of Organic Acid

The preparation of the acid-soluble collagen (ASC) extraction method was based on Zaelani et al. [19] with slight modifications. Extraction was carried out utilising various forms of organic acids. After pre-treatment and defatting, skin samples were extracted with 0.5 M organic acid. This study utilized acetic acid, lactic acid, and citric acid. All procedures were conducted at 4 °C, with samples immersed in a different solution for 72 h at a ratio of 1:15 (w/v). Afterwards, the mixture was passed through two layers of gauze fabric in order to collect the supernatant. Next, the salting-out process was done by adding NaCl solution to the resultant supernatant until the supernatant's final concentration reached 2.5 M at a pH of 7.0. This was carried out in the presence of 0.05 M Tris–HCl. The mixture was centrifuged at 15,000× g for 30 min at 4 °C (Eppendorf, Centrifuge 5804, Hampton, VA, USA) in order to generate a precipitate. After removing the supernatant, the residue was collected, re-dissolved in a 0.5 M ratio of 1:9 (w/v) of organic acid and then washed. The residue was then dialyzed in 0.1 M acetic acid, lactic acid, or citric acid for 24 h and then cold distilled water for 48 h, changed every 12 h. The generated collagen was subsequently frozen at −80 °C and freeze-dried (Labconco, South Kansas City, KS, USA) for 72 h by following the freeze-dry technique. The produced collagen was classified as acetic acid collagen (AAC), lactic acid collagen (LAC), or citric acid collagen (CAC) and was stored at 4 °C until further examination. The collagen extraction yield was examined and characterised using a variety of methods.

2.3.1. Extraction Yield

The collagen extraction yield was estimated according to Normah and Maidzatul [20], its dry basis was used in the calculation of the percentage of the following equation:

$$\text{Yield } (\%) = \frac{\text{Weight of collagen (g)}}{\text{Weight of skin (g)}} \times 100 \quad (1)$$

2.3.2. Percentage of Swelling

The needlefish skin that had undergone the non-collagenous protein removal technique was filtered and allowed to rest for 15 min before being weighed on a digital scale. The following equation was used from Huda et al. [21] to determine the rate (%) of swelling:

$$\text{Swelling } (\%) = \frac{\text{Weight after immersed 0.1 N NaOH (g)}}{\text{Weight of raw material (g)}} \times 100 \quad (2)$$

2.4. Characterization of Collagen

2.4.1. Recognition of the Colour

The colour of all collagen samples was determined using the Huda et al. [22] technique using a colorimeter (Konica Minolta, Tokyo, Japan) based on the International Commission de I'Éclairage (CIE) scale. It was necessary to use the colour values of L*, a*, and b* as arguments. L* indicates brightness; 0 was darkest and 100 was the brightest. a* represented redness, from red +60 to green −60, while b* represented yellowness.

2.4.2. Hydroxyproline Content

The hydroxyproline concentration in the ASC powders of all extracted collagens was determined using a flexible method described by Nalinanon et al. [23]. Initially, collagen samples were dissolved in 6 M hydrochloric acid at 115 °C for 24 h at a concentration of 5 mg/mL. Then, the hydrolysed collagen was dissolved in distilled water, and the pH was adjusted until it reached 6–6.5. The mixture was then incubated for 20 min at room temperature after 2 mL of hydrolysate and 1 mL of chloramine reagent were added. Next, 1 mL of perchloric acid was added, and the mixture remained at room temperature for 5 min. Following the addition 1 mL of Ehrlich's reagent to the hydrolysate, the sample was incubated for 20 min at 60 °C. The sample was then analysed with a UV–Vis spectrophotometer at 558 nm wavelength.

2.4.3. Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR spectra were acquired using a previously described approach by Matmaroh et al. [24], with a few minor adjustments to identify the structural properties of collagen. A 30-mg collagen sample was combined with 100 mg potassium bromide (KBr) and shaped into a tablet, which was placed into the FTIR spectrometer's sample container. All spectra were captured using an infrared spectrophotometer (Nicolet, Thermo Electron, Waltham, MA, USA) with a resolution of 2 cm^{-1} between 400 to 4000 cm^{-1}.

2.4.4. Ultraviolet–Visible Spectroscopy (UV–Vis) Measurement

Based on the study by Chinh et al. [25], an ultraviolet–visible spectroscopy UV–Vis spectrophotometer (Agilent Cary 60) was used to measure the UV–Vis spectra of collagen solutions between 200 and 400 nm.

2.4.5. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyse the protein patterns of ASC samples using a modified approach by Jeong et al. [26]. Samples of collagen were dissolved in 0.1 M acetic acid with 5% sodium dodecyl sulphate before being heated for 1 h at 85 °C. The mixture was centrifuged at 5000 rpm for 5 min at room temperature to remove undissolved debris. After centrifugation, the supernatants were pipetted into a second tube. The sample buffers (with and without 5% mercaptoethanol) were mixed at 1:1 (v/v) with extracted collagens and heated at 85 °C for 3 min. After heat incubation, 15 µL of the mixture was put into a 5% stacking and 12% resolving acrylamide gel. Electrophoresis was set at a constant voltage of 120 V for 1.5 h, and the molecular weight markers were determined using a dual colour protein standard (10–250 kDa). The soluble sample was mixed 4:1 (v/v) with 60 mM Tris–HCl at pH 8 containing 10% sodium dodecyl sulphate, 25% glycerol, and 0.1% blue bromophenol. 25% glycine, 20% SDS, 5% mercaptoethanol, and 0.1% bromophenol blue were used to create loading dyes. Wide range molecular weight markers were used to estimate the molecular weight of proteins. The gel was stained with Coomassie Brilliant Blue R-250 staining solution after electrophoresis. The Mini-PROTEAN electrophoresis equipment was used to run the SDS-PAGE gel.

2.4.6. Thermal Transition Measurement

The temperature transition of collagen was evaluated using a differential scanning calorimeter (DSC Q200 V24.4 Build 116, TA Instruments, Water LLC, New Castle, DE, USA) following the method of Zeng et al. [27]. Lyophilized collagens were rehydrated in deionized water for 48 h at 4 °C. The rehydrated samples (5–10 mg) were carefully weighed into sealed aluminium pans (PerkinElmer, Waltham, MA, USA) and scanned over the temperature range of 20–50 °C at a rate of 10 °C/min. An empty sealed aluminium pan was utilised as a reference. The device was calibrated for temperature and enthalpy using indium as the standard, and samples were continuously purged at a rate of 50 mL/min with ultrahigh-purity nitrogen. The device was equilibrated at 20 °C for 5 min before scanning; ice water was employed as a cooling medium. The area on the DSC thermo-

gram was utilised to assess total denaturation enthalpy (H). The thermogram determined the transition temperature (T_{max}). The findings were analysed using TA software (TA Instruments, New Castle, DE, USA).

2.4.7. Effect of pH and NaCl on Solubility

The solubility of collagens at various pH and NaCl concentrations was investigated using the Jamilah et al. [28] technique with slight modified. All collagen was dissolved in 0.5 M acetic acid at 4 °C and the solution was stirred for 18 h to obtain a final concentration of 3 mg/mL. The solution was centrifuged at a speed of 10,000× *g* for 30 min (Eppendorf, Centrifuge 5804, USA) at a temperature of 4 °C before the pH was altered to 1, 3, 5, 7, 9, or 11 with 2 mol/L HCl or 2 mol/L NaOH. The amount of protein in the supernatant was determined, with bovine serum albumin as the standard. The relative solubility was estimated using the solubility value at each pH using the following Equation (3): Relative solubility (%) = (protein content of supernatant at current pH/total protein content in sample) × 100. In order to carry out the solubility test pertaining to the impact of NaCl concentration, 5 mL of collagen solution with a concentration of 6 mg/mL was mixed with 5 mL of NaCl solution. In this study, the NaCl solution 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 stirred continuously for 60 min at 4 °C, followed by centrifugation at 10,000× *g* for 30 min at 4 °C. Protein content in the supernatants was then determined. All samples were tested for protein using the Enhanced BCA Protein Assay Kit, with bovine serum albumin as the standard. The relative solubility at each NaCl concentration was measured and the relative solubility was calculated according to Equation (4): relative solubility (%) = (protein content of supernatant/total protein content in sample) × 100. The relative solubility was evaluated in terms of the highest pH and NaCl concentration values recorded. The relative solubility of both substances was estimated using the following Equations (3) and (4):

$$Relative\ solubilty\ (\%) = \frac{Current\ concentration\ of\ protein\ at\ current\ pH}{The\ highest\ concentration\ of\ protein} \times 100 \quad (3)$$

$$Relative\ solubilty\ (\%) = \frac{Current\ concentration\ of\ protein\ at\ current\ NaCl}{The\ highest\ concentration\ of\ protein} \times 100 \quad (4)$$

2.5. Statistical Analysis

The values for the various parameters examined are reported as the mean ± standard deviation, assayed in triplicate. One-way analysis of variance (ANOVA) was used for statistical analysis, and IBM SPSS Statistics version 27.0 software was used (IBM Corp., Armonk, NY, USA).

3. Results and Discussion

3.1. Proximate Analysis

According to Table 1, the highest proximate needlefish skin composition was moisture at 61.65%, followed by protein (27.39%), lipid composition (8.59%), and ash (2.16%) with a significant difference ($p < 0.05$) between the proximate compositions that were analysed. The proportion of moisture is a reliable measure for the relative content of energy, protein, and lipids. The higher the protein and lipid composition and the higher the energy density of the fish, the lower the percentage of moisture [29]. According to the previous analyses, fish moisture contributes for 70 to 80% of its weight. In this study, moisture content was below 61.65%. This may be influenced by differences in fish species, habitat, genetics, and dietary habits [3]. Furthermore, in terms of protein content, marine fish tend to outperform freshwater fish. Proteins and lipids are major nutrients in fish and their levels help define the nutritional status of particular organisms.

Table 1. Proximate composition of needlefish skin.

Composition (%)	Skins Sample
Moisture	61.65 ± 1.15 [d]
Protein	27.39 ± 0.08 [c]
Lipid	8.59 ± 0.07 [b]
Ash	2.16 ± 0.03 [a]

Values are reported as mean ± SD (n = 3). Different lowercase letters indicate significant difference ($p < 0.05$).

Moreover, a high protein content was recorded, which is regarded as a most crucial ingredient in the food sector. However, the presence of lipids in this component can prompt food producers to use these wastes while processing foods. Moreover, different parts of the fish have different composition to other sections, such as the scales, fins, and bones. According to Maktoof et al. [30], the amount of proteins and lipids in *Cyprinus carpi* fish scales ranged between 22.1–23.99% and 1.9–2.3%, respectively. The fish's protein, lipid, and calorie density affect its moisture content. This is because proteins have high biological value and contain essential amino acids, whereas fish lipids contain both fatty acids and omega-3 fatty acids [31]. Following this, the swordfish ash content of its skin sample was reported at 2.16%. A significant excess of essential minerals in the skin may result in high ash levels [32].

3.2. Extraction Yield, Hydroxyproline Content, pH, Colour and Thermal Transition Measurement

Table 2 shows that the collagen yields of needlefish skin samples treated with different organic acids AAC, LAC, and CAC, all of which were significantly different at ($p < 0.05$). AAC produced the most collagen (3.13%), followed by CAC (1.03%) and LAC (0.56%). Due to the use of different acids during the extraction, the interaction of aldehydes with lysine and hydroxylin at the telopeptide helix region may account for differences in collagen yield among AAC, LAC, and CAC [15]. This may be due to the fact that various types of acids were employed to extract collagen from fish skin, and the pH of the combination was not kept at the same level as it was at the beginning of the process. This resulted in a shift in the amount of collagen that was extracted from the fish skin. A major reason that organic acids are used so frequently in collagen production studies is that the acid–collagen molecule interaction can split collagen crosslinks and is able to boost extraction efficiency. For instance, homogenization, mixing, acid dissolution, and extraction time can affect collagen yield [7]. Furthermore, based on previous studies, AAC should extract more collagen than CAC because the acid employed has a high capacity for collagen extraction [33]. This may be connected to the excess salt left behind after cellulose-tube dialysis, which increases collagen mass. This is clear when CAC has a rough surface and removable crystals. In addition, Skierka and Sadowska [34] reported that synthesised collagen from Baltic cod skin (*Gadus morhua*) using various acids produced different percentages of collagen, with the greatest collagen content formed from acetic acid and lactic acid, with a maximum yield of 90%, followed by citric acid with a yield of 60%. In contrast, the production of collagen with hydrochloric acid yielded the lowest percentage (18%). The yield of collagen extracted from marine fish skin has been reported in ranges from 49.8% to 51.4% [35]. However, Govindharaj et al. [36] revealed a final yield of eel skin collagen was around 4.2%.

In collagen extraction, the swelling percentage is as significant as the collagen yield content. This is because collagen can swell when the pH is decreased to 4 or elevated to 10 due to its inability to connect between portions of its molecular structure [37]. According to Table 1, there was a significant difference at ($p < 0.05$), indicating that the largest swelling percentage for CAA (226.37%), followed by CAC (210.41%), and LAC (198.37%). This significant swelling percentage indicates that the weight of the needlefish skin expanded fourfold from its original weight of 50 g. Furthermore, swelling is an important factor to consider in collagen extraction because it can impact the ability of collagen's internal molecular structure to connect to other proteins and enhance protein fragmentation by breaking non-covalent interactions [38]. Furthermore, according to Table 1, the value of

hydroxyproline content for all collagens demonstrated a significant difference ($p < 0.05$). AAC had a slightly higher hydroxyproline content of 81.69 mg/g, than LAC (79.07 mg/g) and CAC (79.95 mg/g). Kittiphattanabawon et al. [39] determined the hydroxyproline concentration in the skin and bones of a giant eye fish (*Priacanthus tayenus*) to be 58.5 mg/g and 42.4 mg/g, respectively. Additionally, hydroxyproline is an amino acid marker that supports collagen's triple helical structure and a substance used to quantify tissue collagen [40]. The hydroxyproline component is likewise found exclusively in collagen and is in negligible quantities compared to other proteins. As a result, it can provide a quantitative estimate of the amount of collagen present. Furthermore, the more the hydroxyproline content value, the greater the extractable collagen content [2].

Table 2. Extraction yield and physicochemical properties of acid-soluble collagen from the skin of needlefish (*Tylosurus acus melanotus*) extracted using different acid extraction methods.

Parameters	AAC	LAC	CAC
Yield (% dry weight basis)	3.13 ± 0.02 ^{c}	0.56 ± 0.011	1.03 ± 0.03 ^{b}
Swelling (%)	226.37± 0.03 ^{c}	198.37± 0.03 ^{a}	210.41± 0.16 ^{b}
Hydroxyproline content (mg/g dry sample)	81.69 ± 2.01 ^{b}	79.07 ± 0.3 ^{a}	79.95 ± 0.09 ^{a}
pH	4.85 ± 0.49 ^{a}	4.81 ± 0.19 ^{a}	4.51 ± 0.07 ^{a}
Colour scale, L*	69.77 ± 4.04 ^{b}	71.89 ± 1.52 ^{c}	57.14 ± 4.13 ^{a}
a*	0.15 ± 0.16 ^{a}	0.92 ± 0.22 ^{b}	0.73 ± 0.09 ^{b}
b*	5.52 ± 1.23 ^{a}	5.20 ± 0.02 ^{a}	4.69 ± 0.65 ^{a}
T_{max} (°C)	39.00± 0.25 ^{a}	38.60± 0.19 ^{a}	38.15 ± 0.12 ^{a}
Enthalpy (ΔH) (mJ/g)	0.0398	0.0346	0.0218

Values are reported as mean ± SD (n = 3). Different lowercase letters indicate significant difference ($p < 0.05$). The colour scale: L* (brightness), a* (green-reddish), and b* (brightness) (blue-yellowish). AAC: acetic acid collagen; LAC: lactic acid collagen; CAC: citric acid collagen.

Table 1 shows the results of the pH test for acid-soluble collagen, which indicates that there were no appreciable differences between the various types of acid extraction. The pH value of AAC was 4.85, which was higher than that of LAC (4.81) and CAC (4.51). Typically, a pH measurement of between 6 and 7 is considered ideal for collagen [41]. In contrast, the measurements of the pH of AAC, LAC, and CAC in this study did not exceed pH 6, which indicates that these compounds are acidic. This means that during the dialysis process, salts and acids could be removed completely, so there are still salts and acids left [42]. Additionally, the type of acid utilised and the amount of acid during the extraction process may contribute to differences in the pH of collagen [30]. This could also be the result of a pH neutralisation process that is not working to its full capacity. Another factor that could contribute to the low pH is that the pH of the collagen in the fish varies according to species [15].

In terms of colour, the quality of collagen can be measured. There are three values on the colour scale: L* (brightness), a* (green–reddish), and b* (brightness) (blue–yellowish). Table 1 shows the L* a* b* values for AAC, LAC, and CAC collagen samples. LAC had a higher L* colour scale value than AAC and CAC, with significant differences at ($p < 0.05$) of 71.89, 69.77, and 57.14, respectively. The colour scale a* revealed that LAC had the greatest value of 0.92, followed by CAC (0.73) and AAC (0.15), with a significant difference of ($p < 0.05$) between them. Furthermore, b* colour showed that the AAC sample recorded a reading of 5.52 that was slightly higher than CAC (5.20) and LAC (4.69), with no significant difference at ($p < 0.05$). In the thermal conversion of collagen, when collagen is heated, its triple helix breaks up into a random coil through a series of changes in viscosity, sedimentation, diffusion, light scattering, and other properties [43]. Protein denaturation is associated with the breakdown of tertiary structure and the loss of biological activity. Collagen denatures by detaching from its alpha chain and melting from its triple helix crystal [44]. According to several studies, marine collagen has less heat stability than mammalian collagen. The thermal stability of collagen is also closely connected to the environment and body temperature [45]. According to Table 1, the maximal transition

temperature, T_{max}, and the enthalpy (ΔH) for collagen AAC, LAC, and CAC were 39.00 °C, 38.60 °C, and 38.15 °C, respectively, and producing (ΔH) 0.0398 J/g, 0.0346 J/g, and 0.0218 J/g, respectively. These were slightly higher than the maximum temperatures recorded for collagens from catla skin, which were 34.99 °C, and 35.19 °C for collagens from rohu skin. Collagen has different thermal properties because its molecular structure breaks down when heated to high temperatures [46]. In the DSC, collagen absorbs heat when the temperature rises, and at a certain temperature, it starts to break down [15]. This study showed that the T_{max} values were greater than those typically found in fish collagen, which are usually less than 30 °C. This reveals that AAC, LAC, and CAC have great heat resistance and structural stability and could be utilised to replace mammalian collagen [47]. Generally, the collagen of fish species that inhabit high-temperature regions contains a higher concentration of amino acids and is more resistant to heat than the collagen of fish species that inhabit high-temperature regions with low elevations [45,48]. Another possible explanation is the intramolecular hydrogen bonds that stabilise collagen's triple helix shape may also break into many levels in the presence of acidity, leading to the repulsion of collagen molecules in an acidic solution [49].

3.3. Characterization Structure of Collagen

3.3.1. UV-VIS Absorption Spectra

Figure 1 represents the ultraviolet (UV) absorption spectra of collagen after treatment with three different acids ranging 210 nm to 400 nm in wavelength: AAC, LAC, and CAC. The absorption of needlefish skin collagen was measured at 231.5 nm (Figure 1). This was aligned with the normal absorption of collagen. Therefore, triple helix collagen has a prominent absorption peak near 230 nm [11]. Through peptide bonds and side chains, protein molecules, particularly collagen, absorb UV light. This indicates that peptide bonds are incorporated into the collagen polypeptide chain via n* transitions of C=O, -COOH, and -CO-NH2-groups [50]. Moreover, as the principal functional component in collagen with the ability to absorb UV and visible light, collagen is a potent antioxidant [51]. All extracted collagen had no absorption peaks at 250–280 nm (Figure 1). This is related to type I collagen that lacks aromatic residues, such as tryptophan, tyrosine, and phenylalanine, which are detected at wavelengths of 280 nm in collagen samples [11]. Based on the UV–VIS spectrum of AAC, LAC, and CAC samples (231.5 nm), samples had an absorption peak that is about the same as the study by Liao et al. [52], in which skin samples from Barramundi and Tilapia had absorption peaks of 230.3 nm and 230.9 nm, respectively. Furthermore, these values were equivalent to those obtained from the skins of Horse Mackerels (*Magalaspis cordyla*), Croaker (*Otolithes ruber*) [50] and Shabout (*Arabibarbus grypus* Heckel, 1843) [53].

3.3.2. Fourier Transform Infrared Spectroscopy (ATR-FTIR)

Figure 2 shows the FTIR spectra absorption rate measurements for ASC in the range 4000–800 cm^{-1}, which are comparable. 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. Five major absorption peaks have been identified, amide A, amide B, amide I, amide II, and amide III [15].

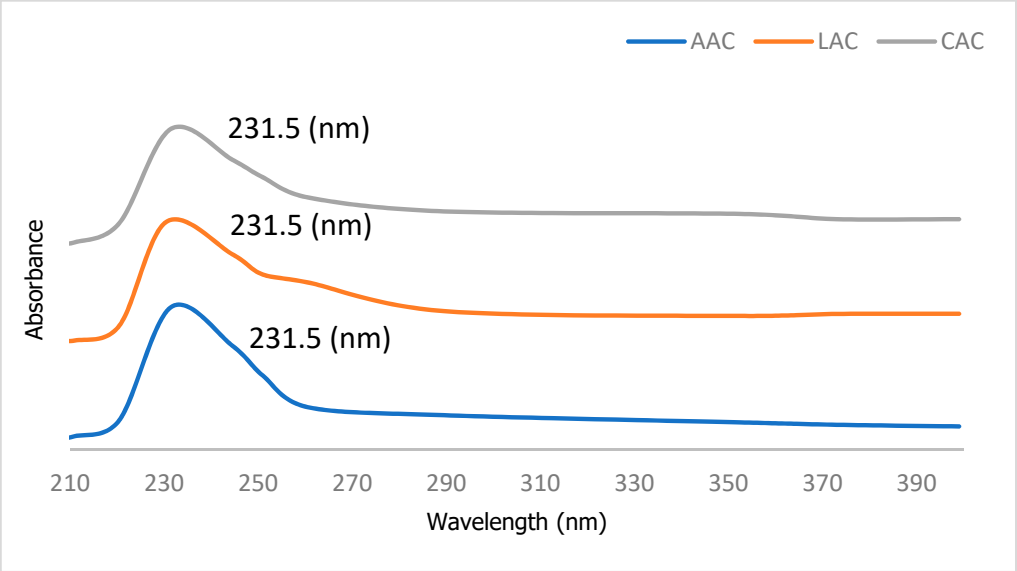

Figure 1. UV absorption of needlefish skin collagens extracted with different acids. AAC: acetic acid collagen; LAC: lactic acid collagen; CAC: citric acid collagen.

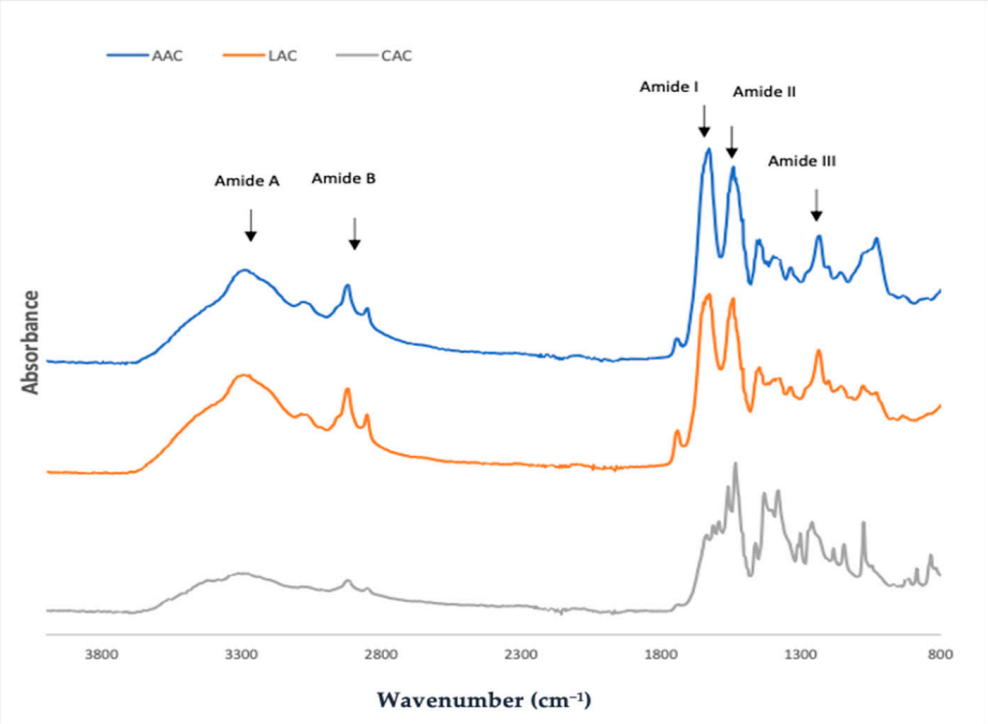

Figure 2. ATR-FTIR patterns of needlefish skin collagen extracted with different acids. AAC: acetic acid collagen; LAC: lactic acid collagen; CAC: citric acid collagen.

Previous studies have found that amide A peaks are typically recorded between 3400 and 3440 cm^{-1} and are frequently associated with N-H stretching vibrations. Nonetheless, in this study the amide A peak was the most prominent peak at (3289.46 cm^{-1}) for all collagen extractions, which were lower than previously reported. This is because the N-H stretching functional group of amide A interacts with amines of several proteins and complex molecules, shifting the amide A band to a lower frequency range than usual. It also suggests that hydrogen bonding exist in every collagen [39]. When the CH_2 group is stretched asymmetrically [36], amide B peaks are frequently observed in collagen. Amide B

readings for AAC were 2939.08 cm^{-1}, those for CAC were 2920.44 cm^{-1}, and the reading for LAC was marginally lower than those for AAC and CAC recorded at 2918.58 cm^{-1}. Amide I peaks are typically found between 1600 and 1700 cm^{-1} due to their strong absorption, which is principally caused by C=O stretching vibrations along the polypeptide spine [54]. Lower frequency number peaks are associated with a decrease in molecular organisation, making it a sensitive predictor of the secondary structure of peptides [55].

Amide I was found at the peak of this study, and the absorption rates of AAC, LAC, and CAC were all within the same range (1640.07 cm^{-1}). In addition, assessments of the amide II absorption rate for all extracted collagen remained within a similar range, 1541.30 cm^{-1}. The amide II peaks have wavelengths between 1500 and 1600 cm^{-1} and are associated with N-H bending and C-N stretching vibrations, as well as the helical shape of three collagens. Since the NH group in the peptide chain is involved in hydrogen bonding, CAC had a low value. As a result, the amide group's position moves to a lower intensity. When the wavelength decreases, the number of hydrogen bonds in collagen increases [54]. The amide peak of the peptide III bond indicates complicated interactions between molecules. As shown in Figure 2, it arises due to the C-N stretching and N-H bending vibrations of the peptide group. Amide III is detected for normal collagen range between 1200 cm^{-1} and 1350 cm^{-1} for maximum absorption. According to the data, the absorption rate of amide III was within the normal range for AAC (1231.92 cm^{-1}), CAC (1235.64 cm^{-1}) and LAC (1231.92 cm^{-1}). As amide III was in the standard range, a collagen triple helix structure could be observed [56].

3.3.3. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed to determine the protein pattern of collagen extracted with various acids. Figure 3 shows that the SDS-PAGE pattern demonstrated that all acid-treated collagens from swordfish skin were recognised as type I collagen. Figure 3 demonstrates collagen extracted using AAC, LAC, and CAC by a distinct 250 kDa band that is connected to two other bands to form a beta band, suggesting the presence of chain crosslinking links. Bands ranging from 130 to 100 kDa corresponded to the different chains—type I collagen containing two α1(I) chains and one α2(I) chain [57]. There were no variations in protein patterns found under reducing and non-reducing conditions (with or without beta-mercaptoethanol) for the collagen samples, demonstrating the absence of disulphide connections between chains. This is because cysteine and methionine are present in low levels in collagen type I [35]. Additionally, the lower bands show a lower molecular weight, which could be caused by non-collagenous proteins or collagen peptides made when only part of the collagen was broken down [30]. These results are comparable to previously reported for collagens taken from the skins of southern catfish (*Silurus meridionalis* Chen) [58], and hybrid sturgeon [30].

3.3.4. Effect of NaCl Solubility

Figure 4 shows the percentage relative solubility of all acid-soluble collagens (AAC, LAC, and CAC) with varied concentrations of sodium chloride (NaCl) ranging from 0 to 60 g/L. All collagens had a high relative solubility in the range of 0–10 g/L NaCl. This is because salt ions connect weakly to the surface of charged protein groups at low NaCl concentrations, and as a result, they do not upset any of the hydration loops on the collagen domain [51].

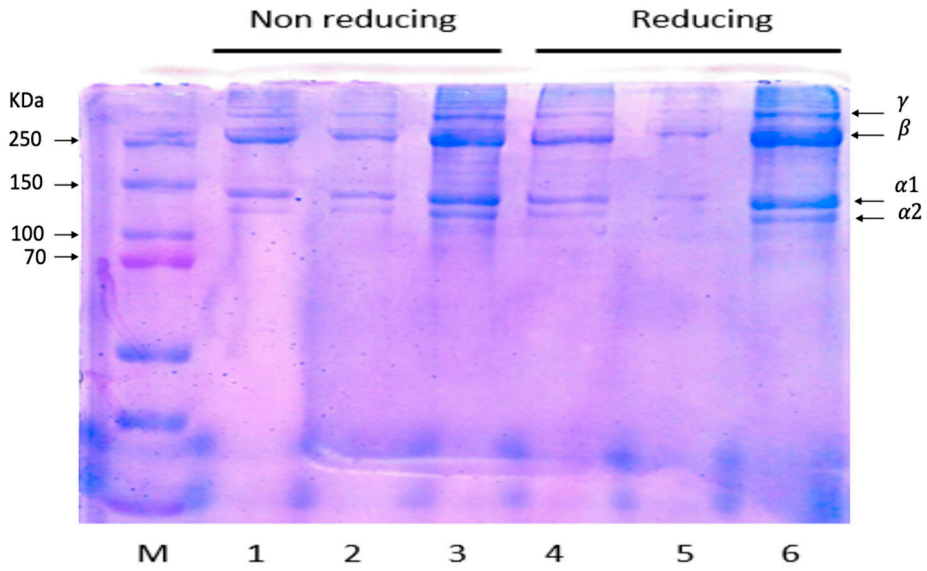

Figure 3. SDS-PAGE protein pattern of acids-soluble collagen for non-reducing and reducing conditions. Lane M: molecular weight marker; Lanes 1 and 4: acetic acid extraction (AAC); Lanes 2 and 5: lactic acid extraction (LAC); Lanes 3 and 6: citric acid extraction (CAC).

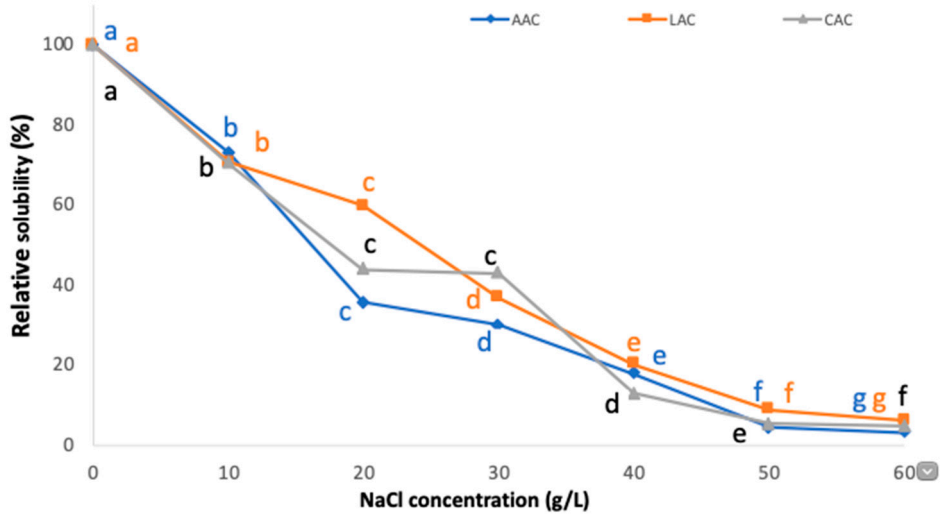

Figure 4. Effect of solubility of needlefish skin collagens extracted with different acids at different concentration of NaCl. AAC: acetic acid collagen; LAC: lactic acid collagen; CAC: citric acid collagen. Different letters indicated significant differences between the samples.

In most instances, the solubility of collagen in acetic acid at a concentration of 0.5 M decreased as the NaCl concentration increased [2]. The results were consistent to Pamungkas et al. [59] of collagen from haruan scales (*Channa striatus*) and Jamilah et al. [28] of collagen from barramundi skin (*Lates calcarifer*). At a NaCl concentration of 30 g/L, however, the graphs for all three acid-soluble collagens showed a substantial decrease in relative solubility to 50 g/L. Reduced collagen solubility may have resulted from the out-salting phenomenon, which occurs at relatively high NaCl concentrations. Increased salt concentration increases hydrophobic–hydrophobic interactions between protein chains and competition for water with salt ions [51]. In addition, this substantial decline can be attributed to the effects of salinization [24]. The solubility of collagen, for instance, is found to decrease with increasing salt chloride concentration. This results in a boost in ion strength, which in turn decreases protein solubility and precipitates stimulated proteins [55].

3.3.5. Effect of pH of Solubility

Figure 5 shows the effect of solubility of needlefish skin collagens extracted with different acids at different pH. The extracted collagen was examined by dissolving them in 0.5 M acetic acid before testing at pH 1,3, 5, 7, 9 and 11. It was found that all three samples were highly soluble at pH 1.

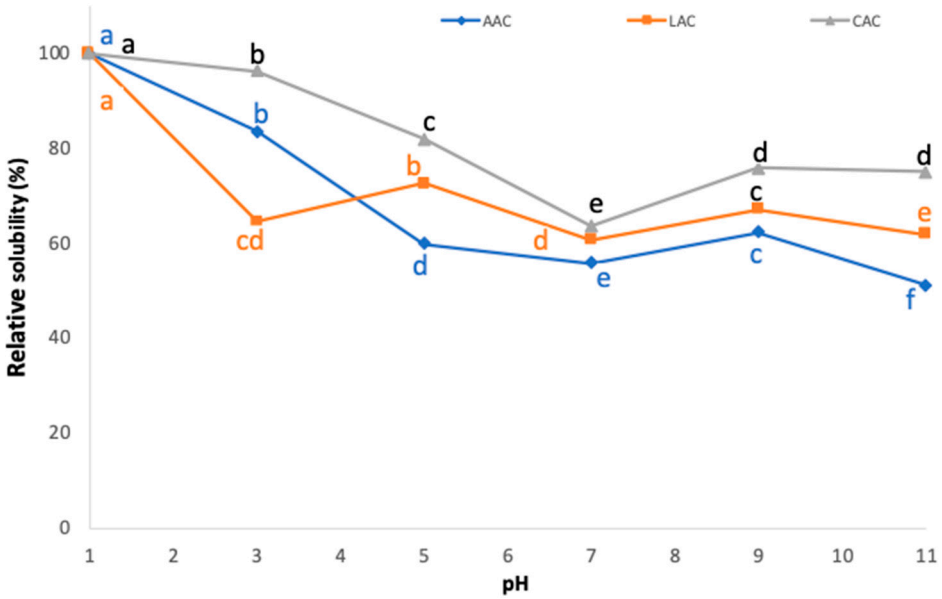

Figure 5. Effect of solubility of needlefish skin collagens extracted with different acids at different concentration of NaCl. AAC: acetic acid collagen; LAC: lactic acid collagen; CAC: citric acid collagen. Different letters indicated significant differences between the samples.

Generally, collagen protein is most soluble at pH values between 2 and 5, where it dissolves more than 80% [23]. Therefore, the solubility of AAC and CAC decreased as the pH rose over 3. Nurkhoeriyati et al. [60] reported pH 5–6 as the lowest solubility of myofibrillar protein. Due to hydrophobic interactions between collagen molecules, collagen becomes less soluble at neutral and alkaline pH values [17]. Based on the obtained solubility values, AAC indicated a drop in pH 5, 7, 9, and 11 when the relative solubility was less than 60%. Furthermore, the isoelectric point (pI) for all the extracted collagen was discovered at pH 7. According to Chen et al. [51], the increased hydrophobic–hydrophobic interactions between molecules in collagen causes the lower relative solubility. In addition, it was observed that the pH increased slightly from 7 to 9. This is due to the fact that relative solubility increases under situations with an acidic pH (pH 1). Moreover, the greater solubility can be attributed to a repulsive force that exists between the chains when the pH is either lower than or higher than the pI [17]. According to the Nalinanon et al. [23], collagen may be subject to molecular degradation, which can lead to reduced solubility.

4. Conclusions

The economical and ecological utilisation of fish skins resulting from industrial processing has attracted increasing interest. The variety of acids utilised influence the extraction of acid soluble collagen (ASC) from needlefish skin. In this study, pre-treatment and extraction with organic acids was found the acid–collagen molecular interaction can split collagen crosslinks and enhance extraction efficiency. The amount of hydroxyproline in each collagen of extracted from needlefish skin presented a significant difference ($p < 0.05$), with AAC (81.69 mg/g) having slightly more hydroxyproline than LAC (79.07 mg/g) and CAC (79.95 mg/g). It showed that extractable collagen was enhanced by the hydroxyproline content. The results revealed that lipid and pigment were eliminated by ASC extraction, and skin fibres were efficiently relaxed by the extraction methods. The yield and purity of

the extracted collagen were clearly increased, with yield percentages of 3.13%, 0.56%, and 1.03% for AAC, LAC, and CAC, respectively. Collagen has been fully characterised in terms of its structure, physicochemical properties, and biological roles. Analysis of the UV spectra, FTIR spectroscopy, and thermal stability identified the collagen obtained from fish skin as type I. Moreover, the collagen recovered from fish skins revealed that its denaturation temperature, T_{max} was comparable to that of collagen taken from terrestrial skin. These results show that needlefish skin collagen can be employed in industrial applications.

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