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Characterization of Acid-soluble Collagen (ASC) from Milkfish (*Chanos chanos*) Heads

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As global demand for fish continues to escalate alongside the growing population, fish production also surges – resulting in an increase in various fish wastes, including fish heads. To address this challenge, this study aims to extract and characterize collagen from milkfish heads, paving the way for upcycling fish heads. The milkfish heads underwent a series of treatments: demineralization, deproteinization, and delipidation. The milkfish head waste samples were processed with 0.5 M acetic acid to extract acid-soluble collagen, resulting in a yield of 1.8%. The visual assessment revealed a soft, spongy texture with an off-white color. Scanning electron microscopy further showed a sheet-like, fibrillar morphology. Additionally, Attenuated total reflectance–Fourier transform infrared analysis confirmed the presence of characteristic collagen absorption bands. Ultraviolet-visible spectroscopy detected a characteristic peak at a wavelength of 235 nm. Moreover, differential scanning calorimetry analysis showed a melting temperature of 113.02 °C. This study contributes to sustainable resource utilization and waste reduction. Further research is recommended to refine extraction techniques and explore the biomedical potential of collagen derived from milkfish.

Keywords: acid-soluble collagen, extraction, fish waste, milkfish, yield

INTRODUCTION

The increasing global demand for fish underscores its critical role as a primary protein source. This has resulted in increased fish production, with the Philippines being one of the largest contributors, producing a total of 4.3 million tons as of 2022 (BFAR 2023). However, 20–30% of the country's fish production ends up as waste, posing a challenge to sustainability efforts (FAO 2021). Consequently, there is an urgent need for efficient utilization of these waste materials.

Fish waste, also known as residual raw materials (RRMs), contains valuable nutrients, yet only 13% of it is utilized for human consumption (Grefstad 2022). Among these RRMs, fish heads stand out due to their rich composition of valuable components such as collagen. Additionally, marine collagen derived from fish sources has garnered attention due to its unique properties, including a smaller molecular size and higher absorption rate compared

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to collagen from land animals. Marine collagen also contains specific amino acids essential for collagen synthesis and skin health, further accentuating its appeal for numerous applications. The utilization of collagen extracted from fish heads can significantly enhance the nutritional value of various food products and contribute to the development of high-value goods. Collagen also plays a vital role in providing structural support to tissues and organs. Extracting collagen from fish heads, rich sources of type I collagen, opens numerous avenues for applications across various fields, including medicine and food science (Silva *et al*. 2014). The extraction of collagen from fish waste has gained attention due to concerns over religious restrictions and disease transmission risks associated with collagen sourced from mammalian origins (Oslan *et al*. 2022).

The objective of this study was to characterize the collagen extracted from fish head waste – focusing on its histological, chemical, and thermal properties – and to determine the extraction yield. This research provides significant insights into the potential biomedical applications of fish head waste collagen, as well as highlighting the possibility of upcycling fish waste into valuable resources.

MATERIALS AND METHOD

Sample Collection and Preparation

A total of 42 milkfish head waste, weighing a combined 450 g, were collected from a local fish landing in Iligan City, Lanao del Norte, the Philippines. Proper storage and prompt processing are crucial for preserving the biochemical and structural properties of collagen, thereby preventing degradation and maintaining its functional attributes. To ensure this, the samples were placed in an ice cooler during transport, thoroughly washed, and subsequently rinsed in the laboratory to remove any remaining tissue residue. The head bones were manually separated, rinsed with distilled water, and mechanically reduced in size using an industrial grinder (Strand, S1002DS, China). The head bone samples were then washed with phosphate-buffered saline solution (1x PBS) to maintain viability. Following this, the pre-treatment and extraction methods from the study by Grefstad (2022) were employed with slight modifications.

Pre-treatment

Demineralization. Demineralization was conducted using a 0.5 M hydrochloric acid (HCl) solution with a solid-to-solvent ratio of 1:6 (w/v), continuously stirred at 300 rpm for 24 h at 4 °C. After demineralization, the head bone (DHB) samples were rinsed with distilled water until a neutral pH was achieved.

Deproteinization. The DHB samples were subjected to deproteinization using a 0.1 M NaOH solution at a solid-to-solvent ratio of 1:6 (w/v). The mixture was stirred at 300 rpm for 12 h at 4 °C, with the solution replaced every 4 h. Subsequently, the deproteinized head bone (DPHB) samples were washed three times with distilled water at a solid-to-solvent ratio of 1:10.

Delipidation. After deproteinization, the DPHB samples were subjected to a dilipidation process. The samples were immersed in a 10% ethanol solution for 12 h at 4 °C, with a ratio of 1:6 (w/v), and stirred constantly at 300 rpm. Subsequently, the resulting delipidized head bone (DLHB) samples were washed three times with a solid-to-solvent ratio (1:10) with distilled water.

Extraction of Collagen

The DLHB samples were subjected to collagen extraction using 0.5 M acetic acid at a 1:6 (w/v) solid-to-solvent ratio. This extraction process involved continuous stirring at 300 rpm for 12 h using a magnetic stirrer at 4 °C. After extraction, the resulting solution was filtered through a fine mesh sieve and double-layered cheesecloth.

Precipitation of Collagen

After the extraction process, sodium chloride (NaCl) was added to the filtrate to achieve a final concentration of 2.6 M, precipitating the collagen. Centrifugation was then performed at 9000 rpm for 30 min to collect the precipitate. The precipitated collagen was redissolved in 0.5 M acetic acid before purification *via* dialysis. The resulting acid-soluble collagen (ASC) was lyophilized and stored at –20 °C. The ASC was characterized using ultraviolet-visible spectroscopy (UV-Vis) for optimal wavelength detection, scanning electron microscopy (SEM) for morphological analysis, Fourier transform infrared spectroscopy (FTIR) for chemical structure analysis, and differential scanning calorimetry (DSC) for thermal properties.

Characterization of Collagen

Ultraviolet-visible spectroscopy (UV-Vis). The collagen samples (1 mg/ml) dissolved in 0.5 M acetic acid were recorded using a Thermo Fisher Genesys 10-S UV-Vis spectrophotometer. Spectra were scanned from 200–800 nm at 2 nm/min (Atef *et al*. 2020).

Scanning electron microscopy (SEM). An SEM was used to evaluate the cross-sectional structure of acid-soluble collagen. The lyophilized samples were mounted onto aluminum stubs using sticky carbon tabs and were

sputter-coated (JEOL Smart Coater, Tokyo, Japan) with gold prior to observation using SEM (JSM-IT200, Tokyo, Japan).

Attenuated total reflectance–Fourier transform infrared (ATR-FTIR). The samples were on the spectrum plate of the QATR-10 single reflection FTIR instrument (Shimadzu, Kyoto, Japan). The absorbance spectra obtained were recorded using LabSolutions IR software with a resolution of 4 cm^{-1}. Subsequently, these absorbance spectra were analyzed to identify the chemical bonds and functional groups present in the samples.

Differential scanning calorimetry (DSC). Approximately 5.0 ± 0.5 mg of collagen samples were placed in a metal pan to prepare the samples for analysis. The samples were then analyzed using a DSC 4000 instrument (Perkin Elmer, Waltham, MA, USA), heating from 15 to 150 °C at 10 °C/min to monitor heat flow (Jaziri *et al*. 2022).

Calculation of Yield

The collagen yield, expressed as a percentage, was also determined. For calculation, the yield was calculated as:

$$\text{Yield (\%)} = \frac{\text{Weight of collagen extracted (g)}}{\text{Weight of dry raw materials (g)}} x\ 100$$

This formula quantifies the amount of collagen obtained relative to the initial dry weight of the raw material (Lino-Sánchez *et al*. 2023). Additionally, a visual assessment was conducted to evaluate the overall appearance and quality of the extracted ASC.

RESULTS AND DISCUSSION

The extraction of ASC from milkfish heads resulted in a percent yield of 1.8% on a dry basis. The standard deviation of 0.17487 indicates the percent yields are consistent and have low variability, reflecting a high degree of consistency in the extraction process. This yield is slightly lower compared to the highest percentage yields observed in previous studies such as those conducted by Grefstad (2022), who reported a total yield of 2.3% from the heads of pollock (*Pollachius virens*) and 1.9% from the heads of cod (*Gadus morhua*), as well as by Göçer (2022), who achieved a total yield of 2.57% from the bones of shabout (*Arabibarbus grypus*). The differences in percent yield obtained are attributed to variations in tissue composition and the specific species utilized (Oslan *et al*. 2022).

On the other hand, the ASC exhibited an off-white color and a soft, spongy texture (Figure 1A), along with a fibrous, porous, sheet-like microstructure in its scanning electron images (Figure 1B). Additionally, the fibrillary structures observed showed interconnected patterns, underscoring their suitability as scaffolds for cell attachment and proliferation, highlighting their significance in biomedical applications. The porous structure of ASC allows for efficient nutrient and oxygen diffusion, crucial for maintaining cell viability and promoting healing. Furthermore, the sheet-like formation of ASC offers structural integrity and flexibility, similar to findings in previous studies. These results suggest its potential use as biofilm components or scaffolds for wound healing applications (Amirrah *et al*. 2022).

The FTIR spectrum of ASC derived from milkfish head waste confirms the presence of characteristic absorption bands of collagen – including Amides A, B, I, II, and III – as depicted in Figure 2A. Notably, the presence of the Amide A peak at 3309 cm^{-1}, which falls within the lower range of a free N-H stretching vibration, signifies intact hydrogen bonds. Additionally, the Amide B peak at 2924 cm^{-1} corresponds to CH_2 asymmetrical stretching,

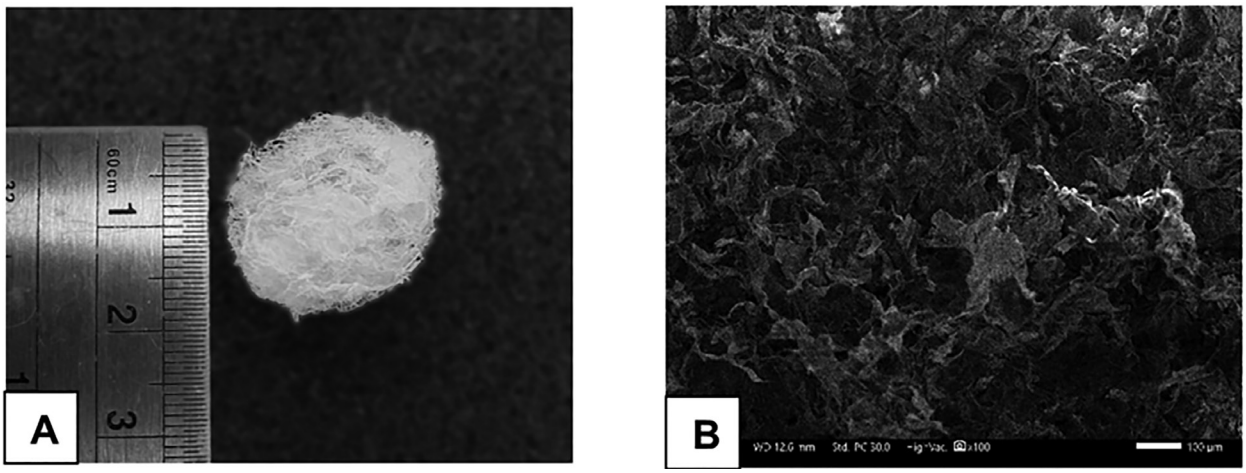

Figure 1. Morphology of ASC from milkfish head A. ASC sponge, B. SEM image (100 µm)

Table 1. Functional group assignments and peak wavelengths for ASC from milkfish head.

Functional group	Peak wavelength (cm^{-1})		Assignment	Reference
	ASC	Range		
Amide A	3309	3400–3440	NH stretch coupled with hydrogen bond	Jeong *et al*. (2013)
Amide B	2924	2920–2922	CH_2 asymmetrical/symmetrical stretch	
Amide I	1633	1633–1641	C=O stretch/hydrogen bond coupled with COO-	
Amide II	1550	1536–1544	NH bend coupled with CN stretch, CH2 bend, COO- symmetrical stretch	
Amide III	1389	1234–1235	NH bend coupled with CN stretch, C-O stretch	

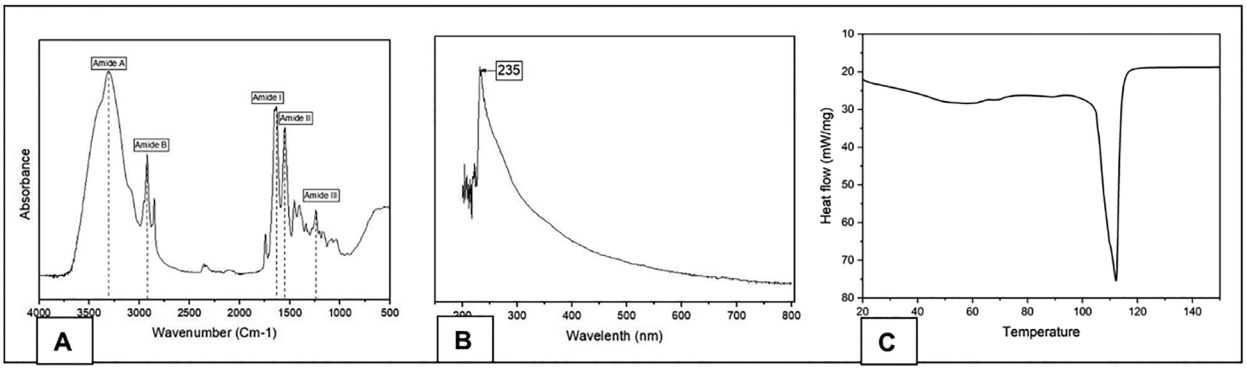

Figure 2. A. ATR-FTIR spectrum, B. UV-vis absorption spectrum, and C. DSC thermogram of the ASC from milkfish head.

whereas the peaks for Amides I, II (at 1633 and 1550 cm^{-1}, respectively), and III (at 1389 cm^{-1}) are associated with CH_2 bending, COO^- symmetrical stretching, and N-H bending with C-N and C-O stretching within Amide III (Jeong *et al*. 2023). The analysis of the spectrum indicates the preservation of the components and structural integrity of the collagen, which is crucial for biomedical applications in promoting cellular activities that mitigate inflammation and facilitate tissue regeneration. Moreover, the observed wavenumber difference between Amides I and II, which is less than 100 cm^{-1}, suggests the presence of an intact collagen triple helical structure. Specifically, the ASC exhibits a delta of 83 cm^{-1} between Amides I and II, indicating the preservation of collagen's triple helical structure (Nikoo *et al*. 2013).

Additionally, the absorption ratio between the Amide III band and the CH_2 bending band at 1450.53 cm^{-1} was approximately 1.0, further confirming the integrity of the native collagen triple helix (Sun *et al*. 2017). Similar results were observed in the study by Carpio *et al*. (2023) on the skin of the Amazonian freshwater fish pirarucu. Meanwhile, the study by Jeong *et al*. (2013) showed that the difference between Amides I and II in their samples was slightly higher at 88 cm^{-1} for the extracted collagen from the bones of bigeye tuna, with a ratio of 1.1.

In the UV-Vis analysis of collagen, the peak was within 220–240 nm, and the optimal wavelength of ASC from the milkfish head was detected at 235 nm (Figure 2B) (Nurubhasha *et al*. 2019). This suggests the presence of characteristic peptide bonds attributed to functional groups such as C=O, -COOH, and $CONH_2$ in the polypeptide chains of collagen. Furthermore, the presence and integrity of peptide bonds within the collagen suggest that the protein's primary structure, particularly the peptide linkages, is intact. This is crucial for the protein's functional and structural roles in various purposes, including biomedical applications (Han *et al.* 2023). These results show similarities to the studies of Jaziri *et al.* (2022) and Wang *et al.* (2013), with wavelength measurements of 230 nm in the bone of Pacific cod and 231.5 nm in the bone of lizardfish, both within the expected range.

The DSC thermogram of the extracted collagen reveals a melting temperature of 113.02 °C, indicating the point at which its natural three-dimensional structure begins to break down. The high melting temperature is significant for maintaining the functional integrity of collagen, enhancing its suitability for biomedical applications such as scaffolds and biomedical devices. Interestingly, the melting temperature of the extracted ASC closely aligns

with findings from previous studies, which reported melting temperatures of around 120.23 °C for collagen derived from the muscle of Amur sturgeon (Wang *et al*. 2013), and 110.49 °C for collagen extracted from the skin of hybrid sturgeon (Wei *et al*. 2019). These insights into collagen's thermal stability may be influenced by factors such as hydration levels and covalent cross-linking, which, in turn, are affected by both the organism's internal temperature and the surrounding environmental conditions (Amirrah *et al*. 2022).

CONCLUSION

In conclusion, the study has effectively extracted collagen from milkfish heads, as evidenced by the results obtained from UV-Vis and FTIR analyses, which confirm the presence of characteristic wavelength and functional groups of the extracted collagen. However, further research is necessary to refine and optimize extraction methods, thereby expanding its applicability across various fields.

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STATEMENT ON CONFLICT OF INTEREST

The authors have no competing interests to declare that are relevant to the content of this article.

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