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Comprehensive analysis of collagen: unveiling the distinctive characteristics and amino acid profiles from skin and bone of Mesopotamian spiny Eel, *Mastacembelus mastacembelus* (Banks & Solander, 1794)

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Abstract

Collagen, a pivotal extracellular matrix biomolecule ubiquitous in connective tissues, drew substantial attention due to its widespread presence, notably in skin and bone. This pioneering study delves into the extraction, characterization, and amino acid profiling of acid-soluble collagens (ASC) obtained from the bone (ASC-B) and skin (ASC-S) of the Mesopotamian spiny eel, *Mastacembelus mastacembelus* (Banks & Solander, 1794). Notably, this research marks the inaugural exploration of this species as a collagen source.

Both ASC-S and ASC-B from spiny eel skin and bone exhibited glycine as the predominant amino acid, constituting 29.88 and 29.77 g/100g of collagen for ACS-S and ACS-B, respectively. Fourier transform infrared spectroscopy (FTIR) confirmed the integrated and native nature of both collagens, while X-ray diffraction (XRD) results indicated the preservation of helical structures in both skin and bone collagens. UV-Vis spectra highlighted prominent absorptions at 230 nm. SEM studies revealed the porous and fibrous structure of both ACS-S and ASC-B.

Collectively considering UV–Vis and FTIR results alongside the amino acid composition, the extracted collagens were characterized as type I collagen. The collagen isolated from the spiny eel emerges as a potential alternative source of vertebrate collagens with prospective applications in diverse industries, including diet, medical, and pharmaceutical sectors.

Keywords: Spiny eel; ASC; XRD; FTIR; Type I collagen; Characterization

1. Introduction

According to fish base [1] *Mastacembelus mastacembelus* [2] is one of the species under the *Mastacembelus* genus, which is native to Asia and Africa and characterized by its elongate, anguilliform bodies, and belongs to *Mastacembelidae* family. The species can reach about 46 cm in length and weigh up to 600 g [2]. The *Mastacembelidae* family is spread worldwide mainly tropical Africa, Middle East, Southeast Asia, and China [3]. The only species belonging to this family found in Turkey is the *Mastacembelus mastacembelus* species [4]. *Mastacembelus mastacembelus*, inhabits the Euphrates and Tigris River basin in the Middle East, particularly in Iraq, Iran, Syria, and Turkey. Notably, it is known as the Mesopotamian spiny eel due to its habitat and is the sole representative of the order Synbranchioformes in Turkish freshwaters. It is usually caught by locals as a source of food.

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Collagen is the main component of connective tissue and is a major fibrous glycoprotein found primarily in the skin, bone, cartilage, tendon, and connective tissues of mammals and fish [5,6]. Collagen makes up to 85% of mammalian skin and 25-35% of total protein in their bodies. However, it accounts for up to 96% of fish skin and 19-38% of the total protein composition of the fish [7]. There is a wide range of collagen products available on the market, but not all collagens are derived from the same source, nor are they appropriate for all dietary requirements. Many of these collagens come from farm-raised livestock such as cattle, pigs, and chickens, while others come from aquatic sources such as fish, shellfish, jellyfish, and crustaceans. Cattle are more widely used in collagen production than porcine and fish sources due to their lower cost and abundance of skin and bones [8,9]. However, there is growing concern about the transmission and spread of diseases such as bovine spongiform encephalopathy (BSE) to humans through the consumption of bovine-derived products [10].

Collagen is crucial to many industries because of its special qualities, including those that produce nutritious foods, cosmetics, tissue engineering, and products for treating wounds [11,12]. Due to its resistance to stretching and fibrous structure, collagen gives skin strength and flexibility. It also plays a significant role in tissue formation and blood vessel strengthening [13,14]. Since collagen has a good biocompatibility and low immunogenicity, it is a desirable biomaterial in biomedical applications. In addition to its industrial uses, collagen's anti-aging effects are highly sought after in a variety of medical specialties, including plastic surgery, burn treatment, and even weight loss [15,16]. As a result, numerous studies to identify alternative collagen sources have been conducted. The use of collagen derived from aquatic sources has recently grown in popularity. Fish-derived collagen peptides have a higher level of bioavailability because they are easily digested, absorbed, and distributed throughout the body up to 1.5 times faster than collagen derived from bovine or porcine sources [17,18]. In many studies, it has been reported that even fish waste constitutes a high potential source of collagen [19–22]. Therefore, it has been known that it can be used to supplement the diet.

In the current study, the spiny eel (*Mastacembelus mastacembelus,* Banks & Solander 1794) was investigated for its collagen resources and their amino acid profiles. *Mastacembelus mastacembelus* (Banks & Solander 1794) is found in some Middle Eastern countries such as Iran, Türkiye, Syria, and Iraq. In Türkiye, this species found in the Orontes, Euphrates, and Tigris River system. Given that the spiny eel is one of the least encountered freshwater fish in Adiyaman province, and to the best of our knowledge, no prior research has been conducted on collagen extraction from its skin and bones, this study aims to determine whether the skin and bones of the spiny eel can serve as a viable substitute source of collagen.

2. Material and methods

2.1. Materials

A spiny eel (*Mastacembelus mastacembelus,* Banks & Solander 1794) was purchased from a local market in Kahta, Adiyaman in April 2019. Its total length was 61 cm and weighed 566 gr. Then the spiny eel was brought to the Cukurova University Biotechnology Laboratory and prepared for research after cleaned. The skin and bones were taken apart from the body and frozen at -20 °C until use. The skin and bones of the eel were thawed in the refrigerator at +4 °C and later was brought to room temperature before performing the extraction procedure.

2.2. Methods

2.2.1. Sample Preparation

The preparation of the collagen samples was performed with minor modifications of Nagai and Suzuki's method [23]. All procedures were carried out at not exceeding +4 °C.

2.2.2. Characterization of Collagens

Collagen Yields

Collagen yield was calculated using the dry weight of the material as specified in the formula.

$$Collagen\ Yield\ \left(\frac{g}{100g}\right) = \frac{Weight\ of\ lyophilized\ collagen}{Initial\ weight\ of\ lyophilized\ fish\ skin} \times 100$$

Differential Scanning Calorimetry (DSC)

The differential scanning calorimetry (DSC) analysis of collagen samples followed the methodology outlined by Kittiphattanabawon et al. (2005) [24]. Lyophilized collagen samples were gelled with 0.05 M acetic acid at a solid/liquid ratio of 1:40 (w/v) and subsequently stored at +4 °C for a duration of two days. Measurements were conducted using the Mettler Toledo, Model DSC 3 (Schwerzenbach, Switzerland). The gelled samples (5–10 mg) were weighed in an aluminum pan, and the screening involved a 10 °C temperature range with temperature increases of 1 °C/min. Liquid nitrogen served as the cooling medium. An indium thermogram was utilized for temperature calibration, employing an empty aluminum container as a reference. The DSC thermogram facilitated the calculation of the maximum transition temperature (Tm) and total denaturation enthalpy (H).

X-Ray Diffraction Analysis

X-ray diffraction (XRD) analysis, employing the PANalytical X'Pert High Score Empyrean with CuKa (=1.54) radiation, was conducted to identify the crystal structures of lyophilized collagen samples. The scanning range spanned from 5 °C to 45 °C, utilizing a scan speed of 0.5 °C/min and a step interval of 0.02 °C.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of collagen were obtained using 2 mg collagen in about 100 mg of KBr under dry conditions. All spectra were performed using a JASCO ATR Pro One Model 6700 FT/IR spectrometer (JASCO International Co. Ltd., Hachioji, Tokyo, Japan). The data acquisition rate of 4 cm^{-1} from 4000 to 600 cm^{-1}. The cross-platform program Spectra Manager TM II was used to analyze spectral data.

UV-Vis Measurement

Using an Agilent Technologies Cary 100 UV-Vis Spectrophotometer, UV spectra of collagen samples were collected and recorded. The samples were dissolved at a concentration of 0.2 mg/mL in 0.5 M acetic acid. Readings were taken in the 200–400 nm range against 0.5 M acetic acid (negative control).

Scanning Electron Microscopy (SEM)

The Quanta 650 model, FEI® (Columbus, Ohio, USA), was used for scanning electron microscopy (SEM). The samples' surfaces were made conductive by coating them with Gold-Palladium (Au/Pd) (about 2Å/sec). Samples were observed at 30 kV, and the EDS technique was used to determine the major compounds of the surface regions of the samples.

Amino Acid Composition

Samples of collagen were hydrolyzed in 6 N HCl for 24 hours at 110 °C while under vacuum. HPLC was used to analyze amino acids (Shimadzu model Nexera-X2 device). The derivatized sample was injected into the Shimadzu shim-pack XR-ODS II column in 2 µl. The temperature of the column oven was changed to 40 °C. Acetonitrile/Methanol/Water (45/40/15) and KH_2PO_4 solution (1 mM K_2HPO_4 in water) were the mobile phases used. The retention times and peak areas of the standards were used to define and calculate amino acids.

2.3. Statistical Analysis

The current study's findings are presented as mean standard deviation. The differences between groups were determined using One-Way ANOVA. Any P value less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Collagen Yield

Based on the wet weight, the yields of acid-soluble collagens extracted from spiny eel skin (ASC-S) and bones (ASC-B) were 9.38% and 3.71%, respectively. Collagen yield obtained from the spiny eel skin was found to be higher than its bone ($p < 0.05$).

3.2. Thermal Stability of Collagen by Differential Scanning Calorimeter (DSC)

The maximum transition temperatures (Tmax) of acid-soluble collagen extracted from template skin and bones dissolved in 0.5 M acetic acid are shown in Figure 1. Tmax and enthalpy (ΔH) value of ASC-S was found as 32.62 °C, 0.230 J/g. However, there were three different Tmax and enthalpy (ΔH) values for ASC-B and they were found as 1st

32.66 °C, 0.253 J/g, 2nd 38.12 °C, 0.452 J/g, and 3rd 41.37 °C, 0.320 J/g. The amino acid composition of collagen, particularly the imino acid composition, influences its thermal stability. While proline and hydroxyproline provide the spatial structure of collagen with pyrrolidine rings, hydroxyproline increases collagen's thermal stability by forming inter-chain hydrogen bonds that stabilize collagen's triple helical structure [25]. Therefore, the Tmax value has a positive relationship with the imino acid content. In fact, the reason why the Tmax value of cattle skin collagen is higher than the value we obtained at 40.8 °C is the higher amount of imino acid it contains [26].

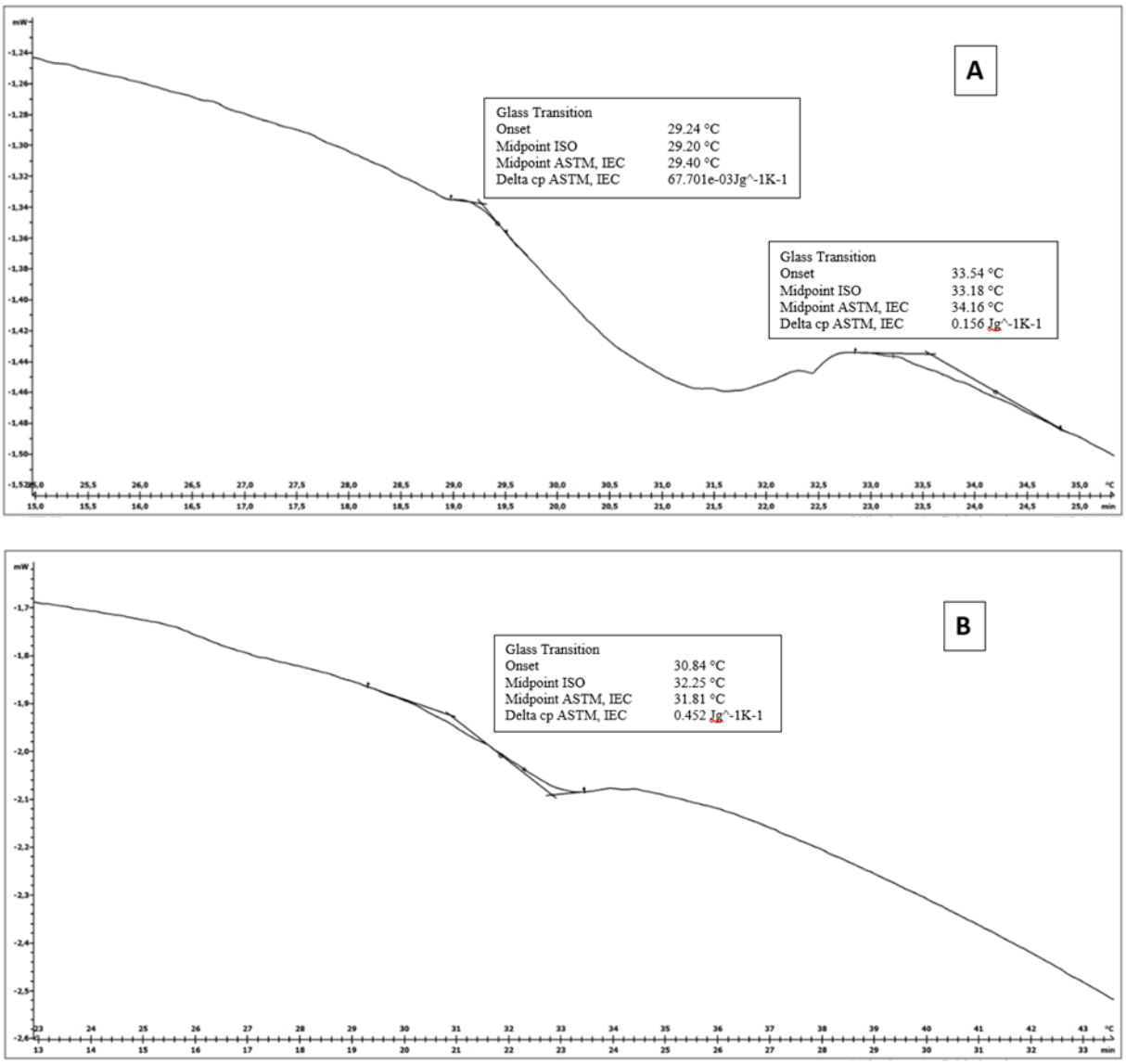

Figure 1 DSC thermogram of ASC-B (A) and ASC-S (B) from the skin and bone of spiny eel dispersed in 0.05 M acetic acid

3.3. X-Ray Diffraction (XRD)

As shown in Figure 2, the XRD curve for both ASC-S and ASC-B has characteristic two break peaks at diffraction angles (2θ) of approximately 8.11º and 22.96º for ACS-S and 7.86º and 22.89º for ASC-B. The first sharp peak is related to the triple helix structure of the collagen, while the second large peak indicates the distance between the chains. These results confirm that both of the collagens preserve the triple helix structure and is not denatured.

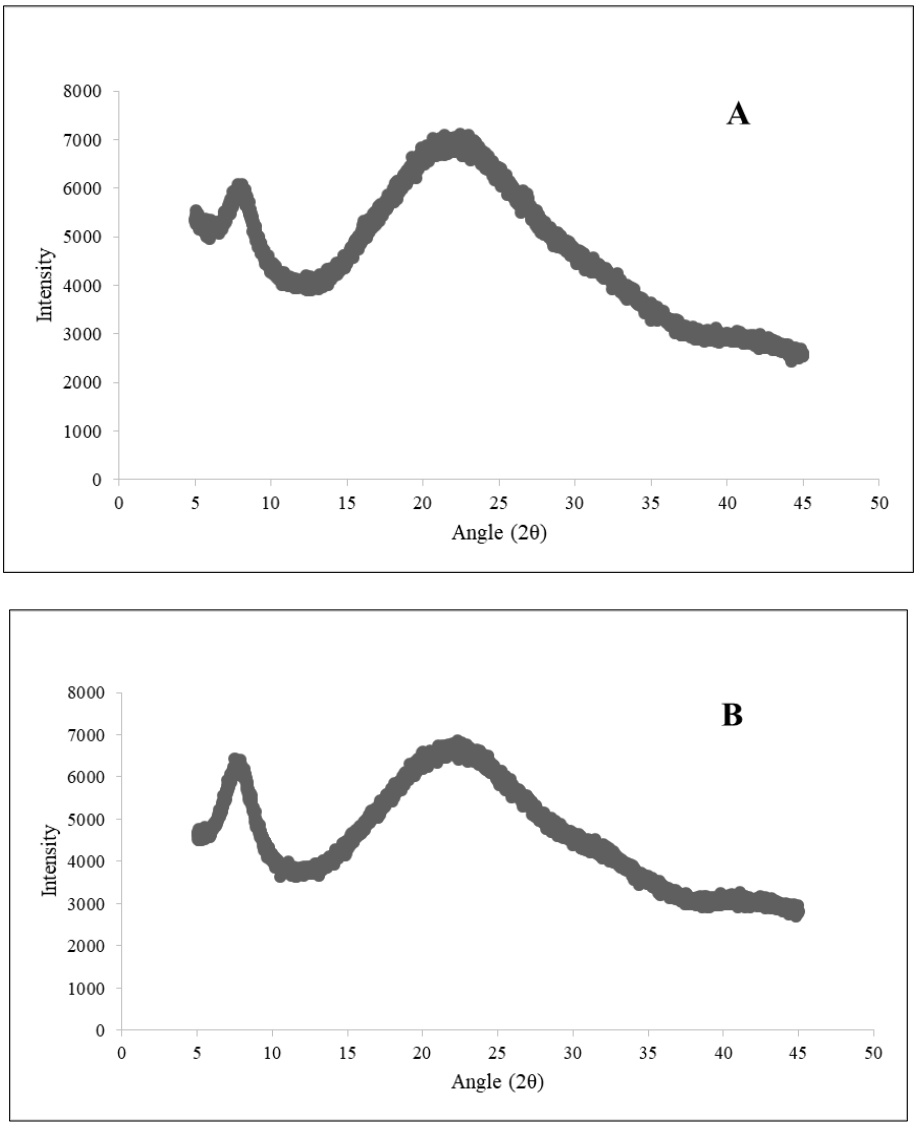

Figure 2 X-ray diffraction spectra of spiny eel's ASC-S (A) and ASC-B (B)

3.4. Fourier Transform Infrared (FTIR)

FTIR spectra of collagen extracted from spiny eel skin and bones are shown in Figure 3. Collagen displayed similar spectral characteristics with five distinct collagen absorption bands (amide A, amide B, and amide I, II, and III), indicating the presence of high proline and hydroxyproline amino acids in the collagen molecule. These distinctive collagen absorption bands indicate that the obtained collagen is type I collagen. The most common pattern in fish is collagen type I, which consists of bands of -chains (α-1 and α-2) and their dimers (β-components) [27]. Cao et al. characterized both the acid solubilized collagen (ASC) and pepsin solubilized collagen (PSC) extracted from European eel (*Anguilla anguilla*) muscles and found that there was not any apparent difference in the thermal stability of both collagens, probably due to the synergistic effect of molecular weight and hydrogen bonds [28].

The current study results suggested that amide A absorption peaks of ASC-S and ASC-B were found to be 3263.93 and 3283.21 cm^{-1}, respectively.

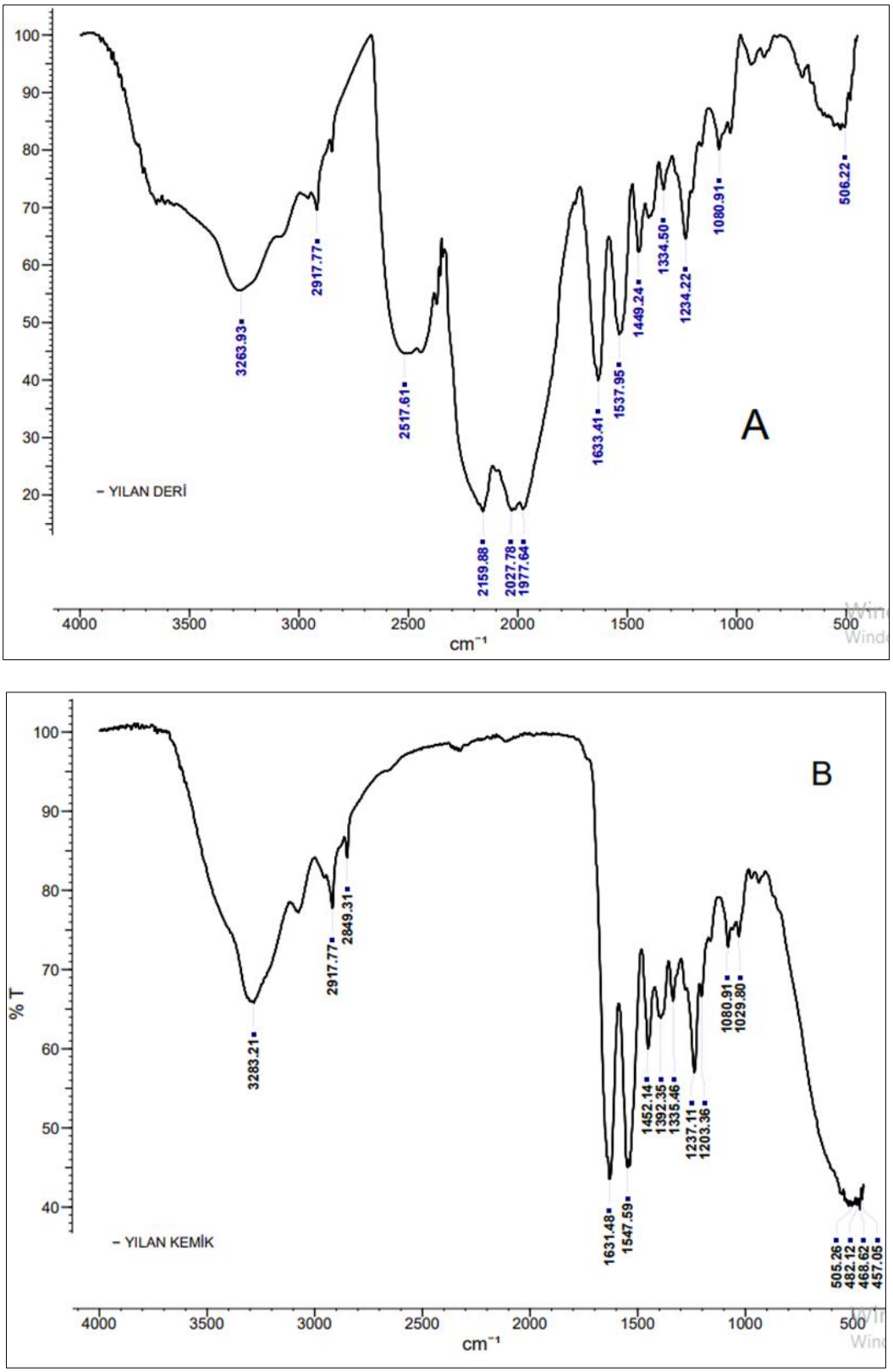

Figure 3 The FTIR spectra of acid-soluble collagens from skin (A) and bone (B) of spiny eel

3.5. Ultraviolet and Visible Light (UV-Vis) Absorption Spectroscopy Analysis

The purity of collagen can be evaluated using UV-Vis spectroscopy [29]. Figure 4 illustrates the UV-Vis measurement results for ASC-S and ASC-B. A solitary absorption peak is observed, with maximum absorbance recorded at 230 nm and 232 nm, respectively.

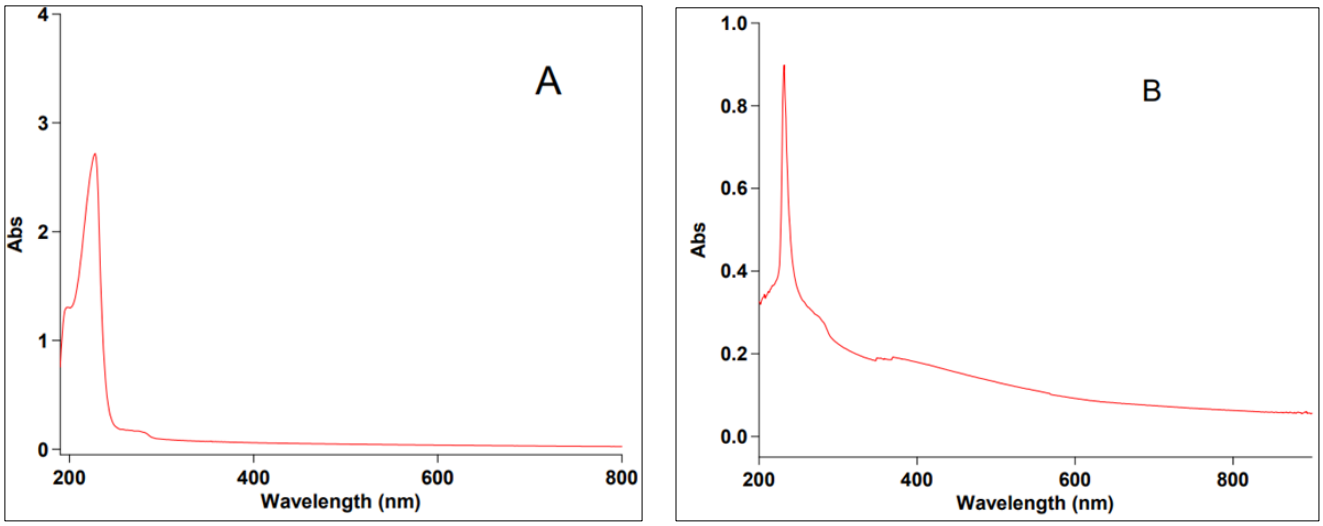

Figure 4 UV- Spectra of acid-soluble collagens from skin (a) and bone (b) of spiny eel

3.6. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was employed to examine the morphological structures of the extracted lyophilized collagens (ASC-S and ASC-B) at three different magnifications (×200, ×500, ×1000, and ×2,000), as depicted in Figures 5 and 6. Although both lyophilized collagens appeared soft, white, and spongy with a porous structure upon naked-eye observation, SEM analysis unveiled a dense, irregular, and partially wrinkled surface image bound by randomly wrapped filaments. This surface characteristic is likely attributed to dehydration during the lyophilization process, aligning with observations in other studies, such as collagen obtained *Amur sturgeon* skin [30] and *Istiophorus platypterus* skin [31].

In this investigation, both collagens exhibited similar characteristics, featuring poor organization, intersecting fibers, entangled bundles, and fibrils with intricate meshes in contact with others. The fibrils of varying thickness were intertwined throughout the porous matrix in both collagens. Consequently, the SEM images indicate that both collagens share a type I collagen classification with a fibrillar structure.

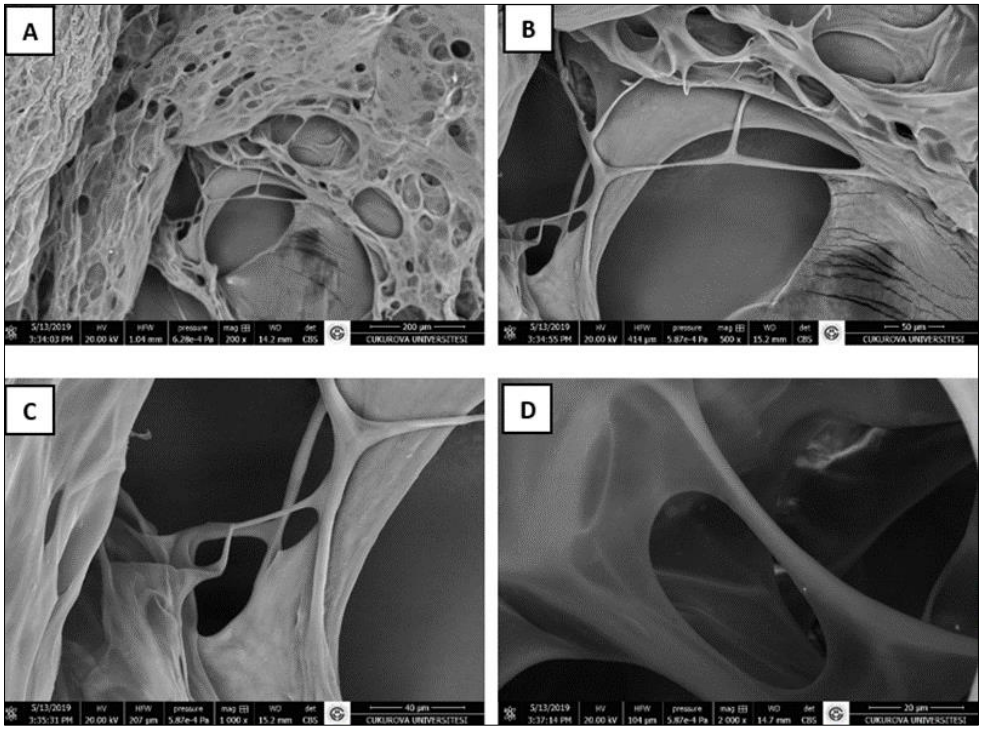

Figure 5 SEM images of acid-soluble collagen from skin of spiny eel A: ×200, B: ×500, C: ×1.000, D: ×2.000

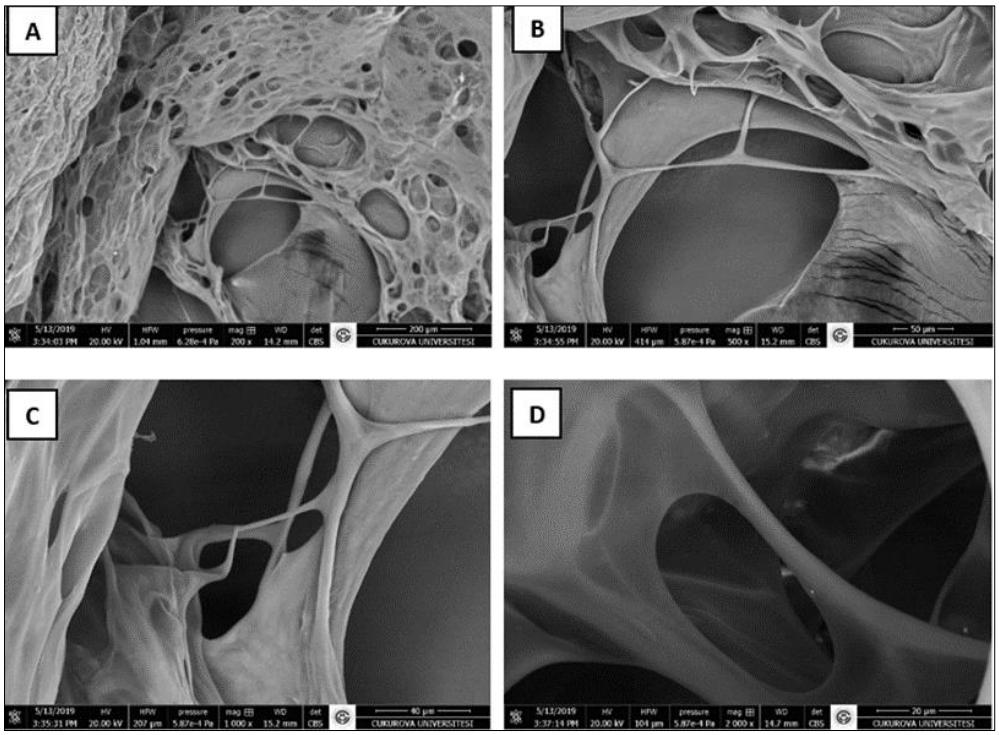

Figure 6 SEM images of acid-soluble collagen from bone of spiny eel A: ×200, B: ×500, C: ×1.000, D: ×2.000

3.7. Amino Acid Composition

Table 1 Amino acid profiles (g/100g protein) of skin and bone collagens of spiny eel

Amino Acid	Skin	Bone
Aspartic acid	5.00 ± 0.13	4.64 ± 0.11
Glutamic acid	8.39 ± 0.22	8.86 ± 0.14
Serine	3.64 ± 0.32	3.82 ± 0.11
Glycine	29.88 ± 0.39	29.77 ± 0.22
Threonine	3.18 ± 0.12	3.18 ± 0.05
Arginine	7.03 ± 0.13	7.20 ± 0.26
Alanine	9.51 ± 0.24	9.56 ± 0.29
Tyrosine	0.52 ± 0.03	0.44 ± 0.03
Cysteine	ND	ND
Valine	1.27 ± 0.06	1.13 ± 0.08
Methionine	0.93 ± 0.02	0.98 ± 0.03
Tryptophan	ND	ND
Phenylalanine	2.14 ± 0.14	2.00 ± 0.05
Leucine	2.76 ± 0.06	2.40 ± 0.23
Lysine	3.43 ± 0.30	3.30 ± 0.11
Hydroxyproline	9.33 ± 0.45	9.99 ± 0.10
Proline	10.38 ± 0.23	10.17 ± 0.20
Total imino acid (Hydroxyproline + proline)	19.72 ± 0.62	20.15 ± 0.30

±, standard deviations. ND, not determined.

The amino acid compositions of ASC-S and ASC-B extracted from spiny eel are depicted in the Table, showing similar compositions for both collagens. With the characteristic triple-helix repeats of all collagens, the samples were high in glycine, proline, and hydroxyproline, as expected. Notably, both ASC-S and ASC-B were found to contain high amount of glycine (Gly), proline (Pro), and hydroxyproline (Hyp), essential imino acids crucial for maintaining the structural integrity of collagen. This emphasizes the significance of these amino acids in the collagen extracted from spiny eel. The total amount of imino acid (Pro + Hyp) is 19.72% and 20.15% for ASC-S and ASC-B, respectively, and is statistically similar (p> 0.05).

4. Discussion

4.1. Collagen Yield

Similar to the present study, Kittiphattanabawon et al. (2005) reported a lower yield of collagen obtained from *Priacanthus tayenus* bone (1.6%) compared to collagen extracted from its skin (10.9%) [24]. The authors also suggest that their ASC-B yields were lower than the current study, whereas their ASC-S yields were higher. The authors noted that their ASC-B yields were lower than those in the current study, whereas their ASC-S yields were higher. Furthermore, the results of this study indicated that the collagen yield surpassed that of collagen extracted from carp bone (1.06%) [32] by more than 3.5 folds. In contrast, Doğdu et al. (2019) extracted collagen from silver cheeked pufferfish *Lagocephalus sceleratus* skin, reporting a much higher collagen yield of 50.9% compared to the present study [33]. In another investigation, Benjakul and colleagues (2010) discovered collagen yields of 7.7% and 7.1% extracted from the skin of *Priacanthus tayenus* and *Priacanthus macracanthus*. These results closely paralleled the collagen yield obtained in the current study [34].

4.2. Thermal Stability of Collagen by Differential Scanning Calorimeter (DSC)

The cattle's imino acid content in ASC-S was 19.26%, while the ASC-B was 19.89%. This value was found to be lower than the collagen obtained from many cold climate fish [35]. This explains why collagen isolated from subtropical and tropical fish have better thermal stability [36]. However, this study suggests that the spiny eel's imino acid content in ASC-S was 19.72%, while the ASC-B was 20.15%. These results were similar to the cattle's imino acid content found by Ciarlo et al. 1997 [35]. This could be due to the spiny ell's thermal stability.

4.3. X-Ray Diffraction (XRD)

As previously mentioned, Figure 2 illustrates the X-ray diffraction (XRD) curve for both ASC-S and ASC-B, revealing characteristic twin peaks at diffraction angles (2θ) of approximately 6.93º and 22.78º for ASC-S, and 7.68º and 22.17º for ASC-B. The initial sharp peak corresponds to the triple helix structure of collagen, while the subsequent larger peak signifies the spacing between the collagen chains. These findings provide confirmation that both collagens maintain their triple helix structure and have not undergone denaturation. Similar results have been obtained by several studies include carp scale collagen study by Zhang et al.[37], *Oreochromis niloticus* skin collagen [38], *Gadus macrocephalus* skin collagen (Sun et al. 2017b), Atlantic cod and Atlantic salmon skin collagen [39], and skins and bones of *Arabibarbus grypus* [40], *Luciobarbus esocinus* [41], and *Alburnus tarichi* [42].

4.4. Fourier Transform Infrared (FTIR)

FTIR spectra of collagen extracted from spiny eel skin and bones showed that Amide A absorption peaks of ASC-S and ASC-B were 3263.93 and 3283.21 cm^{-1}, respectively. According to Sai and Babu (2001), Amide A band generally originates from N-H stress vibration and occurs in the wavelength range of 3400-3440 cm^{-1} [43]. However, Doyle and colleagues (1975) noted that in the presence of a hydrogen bond involving the NH group of a peptide, the position of the band can shift to a lower frequency, typically around 3300 cm^{-1} [44]. Consequently, the shift of the amide A band towards lower wavelengths, as evidenced in this study, suggests the presence of hydrogen-bonded hydroxyl groups in both skin and bone collagens. Amide I band of ASC-S and ASC-B were 1637.27 and 1632.45 cm^{-1}, respectively. These results align with the general range of 1625-1690 cm^{-1}, which is the characteristic position of amide I bands of collagen. Similar results were reported by Shalaby et al. [45]. Amide II band was found to be 1541.81 cm^{-1} for ASC-S and 1548.56 cm^{-1} for ASC-B, amide II band is generally seen at wavelengths of 1550-1600 cm^{-1} [46], its shift to lower wavelengths represents the formation of hydrogen bond. The triple helix structure of collagen can also be presented by the ratio of the density between the absorption peak of amide III and the absorption peak of 1450 cm^{-1}. In our study, the Amide III absorption peaks of ASC-S and ASC-B were 1231.33 and 1236.15 cm^{-1}, respectively. The ratio of the density between the absorption peak of Amide III and the absorption peak of 1450 cm^{-1} was 1.18 (ASC-S/ASC-B=1.17). As described previously, a value approaching 1.0 indicates that collagen still has a triple helix structure [47].

4.5. Ultraviolet and Visible Light (UV-Vis) Absorption Spectroscopy Analysis

This spectroscopy is commonly employed for evaluating the purity of collagen [29]. As depicted in Figure 4, a single absorption peak is evident, with maximum absorbance observed at 230 nm and 232 nm for ASC-S and ASC-B, respectively. This specific absorbance range is indicative of type I collagen. Typically, proteins exhibit their highest absorbance around 280 nm. However, our results demonstrate a maximum absorbance at 230-232 nm, attributed to the absence of tryptophan amino acid and low tyrosine amino acid content in both ASC-S and ASC-B.

4.6. Scanning Electron Microscopy (SEM)

Upon close examination, the morphological structures of the extracted and lyophilized ASC-S and ASC-B revealed some interesting characteristics. Both were observed to have a soft, white, and spongy appearance with a porous structure when viewed without magnification. However, when analyzed using scanning electron microscopy, an entirely different picture emerged. The SEM results showed that both collagens exhibited a dense, irregular, and partially wrinkled surface, bound by randomly wrapped filaments. These observations could potentially be attributed to the dehydration process during lyophilization, a phenomenon that has been documented in similar studies on collagen obtained from *Salmo salar* L. [48], *Amur sturgeon* skin [30], *Istiophorus platypterus* skin [31], *Arabibarbus grypus* [40], *Luciobarbus esocinus* [41], and *Alburnus tarichi* [42].

4.7. Amino Acid Composition

The amino acid compositions of ASC-S and ASC-B collagen of spiny eel presented results as expected. As with other collagens, tryptophan and cystine were not detected [40,49–51]. Proline and hydroxyproline found in both ASC-S and ASC-B are important imino acids that ensure the structural integrity of collagen. The total amount of imino acid (Pro + Hyp) is 19.72% and 20.15% for ASC-S and ASC-B, respectively, and is statistically similar (p> 0.05). This value is similar to the values reported for *Oreochromis niloticus* (19.8 - 19.4%) [52] and Carp (19.4%)[53]; higher than the values reported for tilapia, (17.75%), grass carp (17.90%) and silver carp (17.78%) [54]; lower than the value reported for tilapia (25.4%) [55]. The difference in imino acid content between different species is due to the different habitats of different species, especially to the difference in temperature [56]. In another study, European eel's skin was searched for amino acid composition and Glycine was found to be the most abundant amino acid while the predominant essential amino acid was leucine [57]. The alterations in amino acid ratios among different fish species could be due to type of the diet of the fish and its habitat.

5. Conclusion

In summary, the successful extraction and characterization of collagens from spiny eel skin and bone were achieved. Both extracted collagens were identified as type I collagen, displaying a typical amino acid composition. Importantly, their triple helical structure remained intact post-extraction, as evidenced by FTIR and XRD analyses. The maximum absorption observed at 230–232 nm for both collagens, with no absorption at 280 nm, further confirmed their type I collagen nature. SEM images revealed interconnective pores and lace-like fibers in both collagens.

In conclusion, the positive attributes exhibited by the extracted collagens suggest a high potential for use as a valuable collagen alternative in various applications, including diet, medical, and pharmaceutical industries. Their strength and flexibility may contribute to skin repair and regeneration, as well as find applications in the nutraceuticals sector.

Compliance with ethical standards

Disclosure of conflict of interest

Authors declares no conflicts of interests

Author contributions

Concept: Yasemen Yanar, Mustafa Göçer, and Muhsin Aydın; Sample Collection: Mustafa Göçer; Methodology and applications: Yasemen Yanar and Mustafa Göçer; Literature review, data collection or processing: Yasemen Yanar, Mustafa Göçer, and Muhsin Aydın; Writing: Mustafa Göçer and Muhsin Aydın.

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