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Research Article

Extraction and characterization of collagen from the skin and bone of shabout (*Arabibarbus grypus* Heckel, 1843)

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Abstract

Acid soluble collagen (ASC) from the skin (ASC-S) and bone (ASC-B) of shabout (*Arabibarbus grypus* Heckel, 1843) were isolated and characterized. Both ASC-S and ASC-B from shabout contained glycine as the major amino acid and high amount of proline (Pro), hydroxyproline (Hyp), alanine and glutamic acid. On the basis of dry weight, yields of ASC-S and ASC-B were 6.79 and 2.57%, respectively. Furthermore, fourier transform infrared spectroscopy (FTIR) proved that both collagens were integrated and native. The denaturation temperature of ASC-S and ASC-B were 31.59 and 32.25°C, respectively. Additionally, the results of X-ray diffraction (XRD) proved that the two products retained their helical structures. These collagens had prominent absorptions at 230 nm by UV-Vis spectra. Additionally, the scanning electron microscope (SEM) studies have shown that ACS-S and ASC-B are porous and exhibited fibrous nature. According to the UV–Vis and FTIR results, extracted collagens were characterized as Type I collagen based on their amino acid profile. In the current study, the total amount of amino acid (Pro+Hyp) was 19.28% and 19.55% for ASC-S and ASC-B, respectively. The results of the current study suggested that the collagen isolated from shabout can potentially be an alternative source for use in the food, pharmaceutical and biomedical industries.

Keywords: Shabout (*Arabibarbus grypus*), Characterization, FTIR, SEM skin, bone, Type I collagen

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Introduction

Collagen is the major fibrous glycoprotein found mainly in the skin, bone, cartilage, tendon, and connective tissues of animals (Muyonga *et al.* 2004; Pataridis *et al.* 2008; Cheng *et al.* 2009; Huo and Zhao, 2009; Aberoumand, 2012; Liu *et al.*, 2012). The collagen monomer consists of three long cylindrical and coiled alpha chains, approximately 2800 Å in length and 14-15 Å in diameter (Foegeding *et al.*, 1996a). It consists of sequences with repeating amino acids in the form of glycine-X-Y in an alpha chain. Today, at least 29 collagen types have been identified according to the differences of α chain. Due to its resistance to stretching and its fibrous structure, collagen provides strength and elasticity of the skin as well as plays an important role in strengthening blood vessels and tissue development. Collagen's low immunogenicity and high biocompatibility make it a favorite biomaterial in both general use and biomedical applications. Leaving aside its industrial uses, there is a great interest in collagen's anti-aging effect in many medical fields such as plastic surgery, burn surgery, and even weight management (Buehler, 2006; Fratzl, 2008). Bovine, pig, and fish are main sources for collagen production. Cattle are more widely used in collagen production compared to pig and fish sources due to its relatively low price owing to the abundance of its skin and bones. However, there are increased concerns about the consumption of bovine-based products due to diseases transmission such as, bovine spongiform encephalopathy (BSE) to humans. Moreover, Jewish and Islamic beliefs prohibit the consumption of porcine products, such as porcine-derived collagens. For this reason, many studies have been done to find alternative collagen sources. Recently, there has been an increase in the trend towards collagen derived from aquatic sources (Felician *et al.* 2018, Khajavi *et al.* 2021). Fish-derived collagen is still under investigation as it has better properties for cosmetic and pharmaceutical applications than those obtained from mammals (Sadowska *et al.* 2003). In many studies, it has been reported that fish waste constitutes a potential source of collagen (Kiew and Don, 2012; Wang *et al.* 2013; Mahboob *et al.* 2015).

In the current study, shabout (*Arabibarbus grypus* Heckel, 1843) was investigated for its collagen resources. Shabout is found in many countries such as Iran, Turkey, Syria, and Iraq. It is the most important endemic species found in the Euphrates and Tigris rivers (Nikpey, 1996; Abdoli, 2000). Shabout is usually caught by local residents as a source of food and sold commercially. It can reach about two meters and over 50 kg (Coad, 1996) and is the most preferred fish by local people thanks to its delicious meat. There has been no previous study involving collagen extraction from its skin and bones, with this study it was tried to determine whether the skin and bones of shabout can be used as an alternative source of collagen.

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Materials and methods

Shabout (one fish) was purchased from the fish market in Kahta, Adıyaman Province, Turkey in May 2019. The total weight of the shabout was 2800 gr and was 66.5 cm in the total length. Then, it was brought to the Çukurova University Biotechnology Laboratory and cleaned, its skin and bones were separated from the body and they were frozen at -20°C until reuse. Fish skin and bones were thawed in the refrigerator at +4°C and later was brought to room temperature before extraction.

Sample preparation

The preparation of the collagen samples (two samples for both skin and bone) was performed with minor modifications according to the method that was previously described by Nagai and Suzuki (2000). All sample preparation procedures were carried out at no more than +4°C.

Extraction of acid soluble collagen (ASC) from the skin and bone

The fish skins (two samples) were cut into small square shaped pieces (approximately 0.5×0.5 cm) and the bones were minced and kept in 0.1 M NaOH solution for three days in order to remove any present non-collagen proteins. The solution was changed every 24 h. Subsequently, the samples were washed with distilled water until neutral pH was achieved. Minced bone samples were kept in 0.5 M Na_2EDTA solution for five days to eliminate the presence of calcium. The solution was changed at 24-hour intervals.

To remove the fat (lipid) from the protein free skin and bone samples were kept in 10% butyl alcohol at ratio of 1:10 (w/v) for three days and the solution was changed at 24-h intervals. For the collagen extraction, samples were kept in 0.5 M acetic acid solution for three days. The solution was also changed at intervals of 24 hand the residue was collected in a separate container. The combined extracts were subjected to salt precipitation. In this purpose, the final concentration of 2.5 M NaCl was added to precipitate the collagen found in the extract. Precipitated collagen samples were centrifuged at 10,000 rpm for 1 h at +4°C with a refrigerated centrifuge. Then, the precipitated collagen samples were dissolved in 0.5 M acetic acid, dialysis was performed first against 0.1 M acetic acid and then against distilled water. After dialysis, the obtained collagen gels were lyophilized (Labconco Freezone 2, 5). The extracted acid-soluble collagen from the shabout skin was named ASC-S, while the one extracted from bone was named ASC-B.

Characterization of collagens

Collagen yields

Collagen yield was calculated based on the dry weight of the material that was initially used (formula is given below):

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

Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis of collagen samples (two repeats) was performed by following the method that was previously described by Kittiphattanabawon *et al.* (2005). Lyophilized collagen samples were gelled with 0.05 M acetic acid at a solid/liquid ratio of 1:40 (w/v) and then stored at +4°C for two days. Measurements were done by using a Mettler Toledo, Model DSC 3, (Schwerzenbach, Switzerland) equipment. The gelled samples (5-10 mg) were weighed in an aluminum pan. Screening was performed in the temperature range of 10°C at an increasing rate of 1°C/min. Liquid nitrogen was used as cooling medium. An empty aluminum container was used as a reference and the temperature was calibrated using an indium thermogram. The maximum transition temperature (Tm) and total denaturation enthalpy (ΔH) were calculated from the DSC thermogram.

X-Ray diffraction analysis

Crystal structures of lyophilized collagen samples (duplicates) were determined at 0.5C°/min scan speed and 0.02°C step interval using an X-ray diffraction (XRD; PANalytical X'Pert High Score Empyrean, 45kV, 40mA) with CuKa (λ=1.54) radiation in the scanning range of 5°C to 45°C.

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) spectra of collagen were obtained from two mg collagen of each of the duplicate in about 100 mg of potassium bromide (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) at a data acquisition rate of 4 cm^{-1} from 4000 to 600 cm^{-1}. Analysis of spectral data was performed using Spectra Manager TM II cross-platform software program (JASCO).

Ultraviolet and visible light absorption spectroscopy analysis

Measurement

Ultraviolet spectra of collagen samples (two repeats) were obtained using a Cary 100 UV-Vis spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA, USA). The samples were dissolved in 0.5 M acetic acid at a concentration of 0.2 mg/mL. Readings were made against 0.5 M acetic acid (negative control) in the wavelength range of 200 – 400 nm.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed using the Quanta 650 model, FEI®, (Columbus, Ohio, USA). The surface of the samples (two repeats) was made conductive by coating them with Gold-Palladium (Au/Pd)

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(approximately 2Å/second). Samples were observed using 30 kV, and EDS technique was used to determine the major compounds of the samples surface regions.

Amino acid composition

Collagen samples were hydrolyzed under vacuum in 6 N HCl for 24 h at 110°C. Amino acid analysis was performed by pre-column derivatization with phenylisothiocynate. The phenylthiocarbomoylamino acids were analyzed using a Pico Tag column (3.9×150 mm) on a Waters HPLC system, equipped with a 1525 binary pump and Waters 2996-photodiode-array (PDA) detector set at 254 nm (Biddlingmeyer *et al.* 1984). Amino acid analysis was performed with a HPLC (Shimadzu model Nexera-X2 device, Japan). Two micro liters of the derivatized sample was injected into the Shimadzu shim-pack XR-ODS II column. Column oven temperature was adjusted to 40°C. The mobile phases used were (A) KH_2PO_4 solution (1 mM K_2HPO_4 in water); (B) Acetonitrile/Methanol/ Water (45/40/15). Amino acids were defined and calculated according to the retention times and peak areas of the standards.

Statistical Analysis

In the current study, the results are expressed as mean ± standard deviation. One Way ANOVA was used to determine the differences between groups. For pair comparison, the T-test was used. Statistical analysis was performed using the Statistical Package for Social Sciences SPSS (Pallant and Manual, 2010). Any P value below 0.05 (p<0.05) were considered statistically significant.

Results

Collagen yield

On dry wight basis, the yields of acid-soluble collagen extracted from the shabout skin (ASC-S) and bones (ASC-B) were 6.79±0.04% and 2.57±0.03%, respectively. Collagen yield obtained from the skin was found to be higher than the bone (p<0.05).

Thermal stability of collagen by (DSC)

The maximum transition temperatures (Tmax) of acid-soluble collagen extracted from the skin and bones dissolved in 0.5 M acetic acid are shown in Figure 1. Tmax and enthalpy (ΔH) values of ASC-S and ASC-B were found as 31.59°C, 0.358 J/g and 32.25°C, 0.452 J/g, respectively.

X-Ray diffraction

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 5.30º and 21.05º for ACS-S and 7.35º and 20.23º 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.

Fourier transforms infrared

Collagen showed similar spectral properties with five characteristic collagen absorption bands (amide A,

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amide B, and amide I, II, and III), indicating the presence of high proline and hydroxyproline amino acids in the collagen molecule, these are typical bands for collagen and they mean that the obtained collagen is type I collagen (Fig. 3).

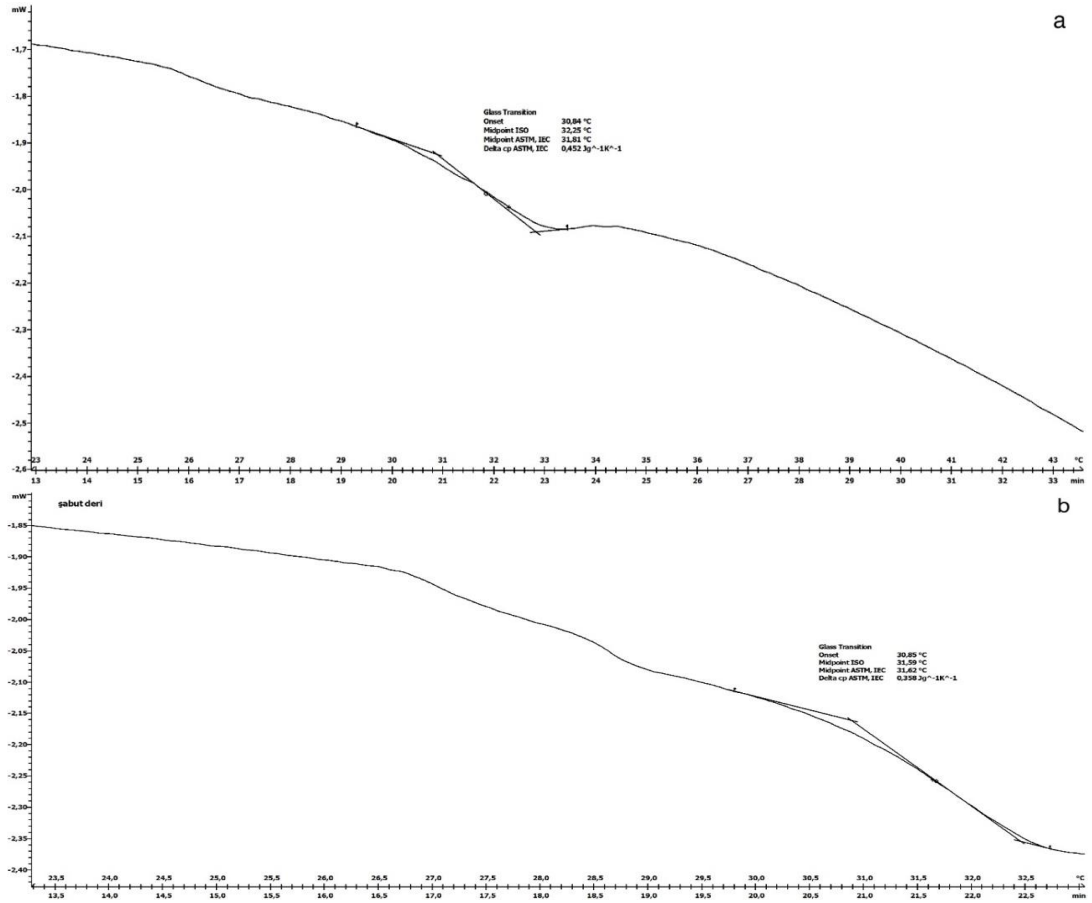

Figure 1: DSC thermogram of ASC-B (a) and ASC-S (b) from the skin and bone of shabout dispersed in 0.05 M acetic acid.

Ultraviolet and visible light absorption spectroscopy analysis

There is a single absorption peak and showed maximum absorbance at 230 and 232 nm, respectively (Fig. 4). This absorbance range is the characteristic absorbance of type I collagen. Generally, the maximum protein absorbance is seen at 280 nm; however, our results have shown maximum absorbance at 230-232 nm due to the absence of tryptophan amino acid and low tyrosine amino acid content in both ASC-S and ASC-B.

Scanning electron microscopy

The morphological structures of the extracted lyophilized (freeze dried) collagens (ASC-S and ASC-B) were visualized by scanning electron microscopy (SEM) under three different magnifications ×200, ×500, and ×2,000 (Figs. 5 and 6).

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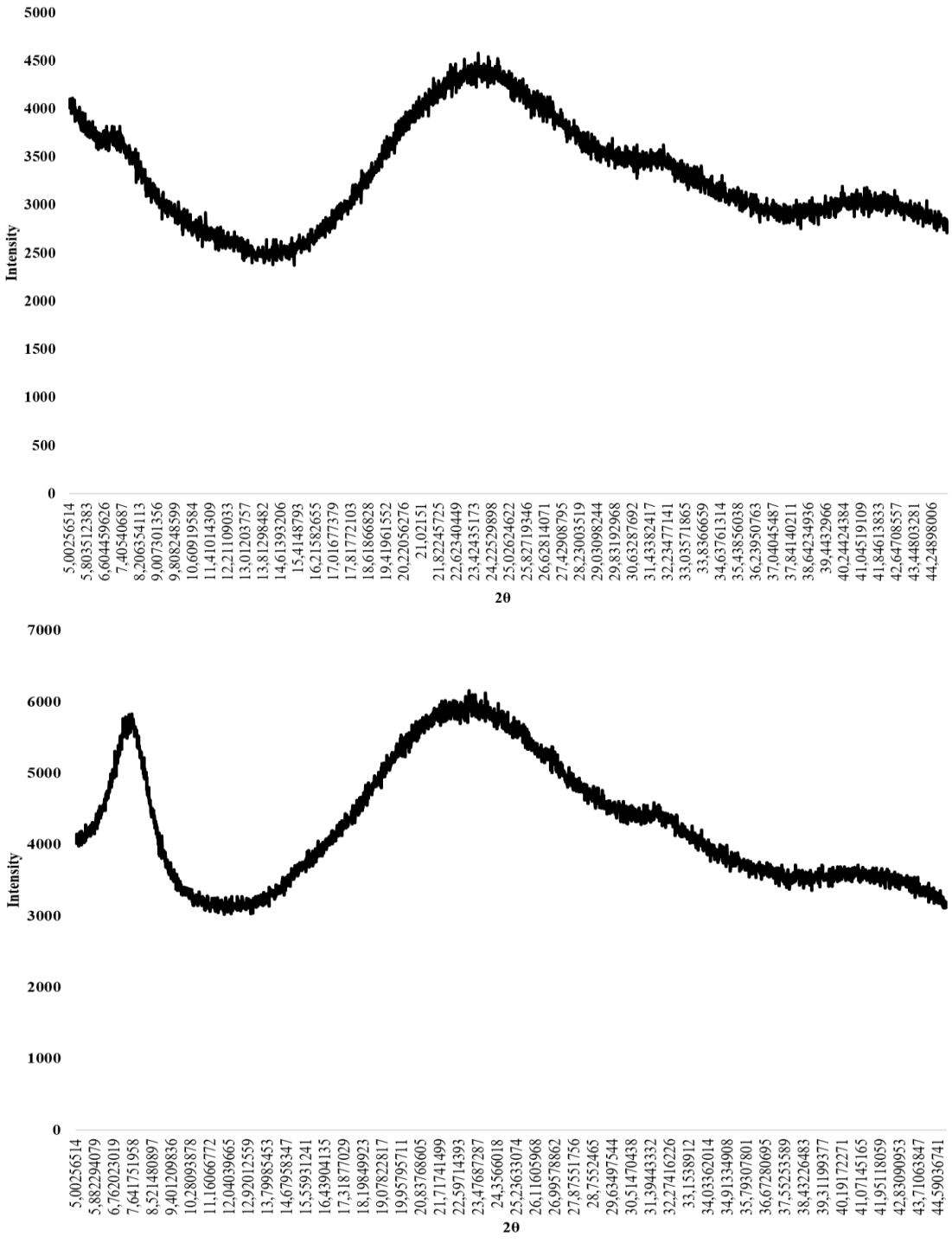

Figure 2: X-ray diffraction spectra of ASC-S (a) and ASC-B (b).

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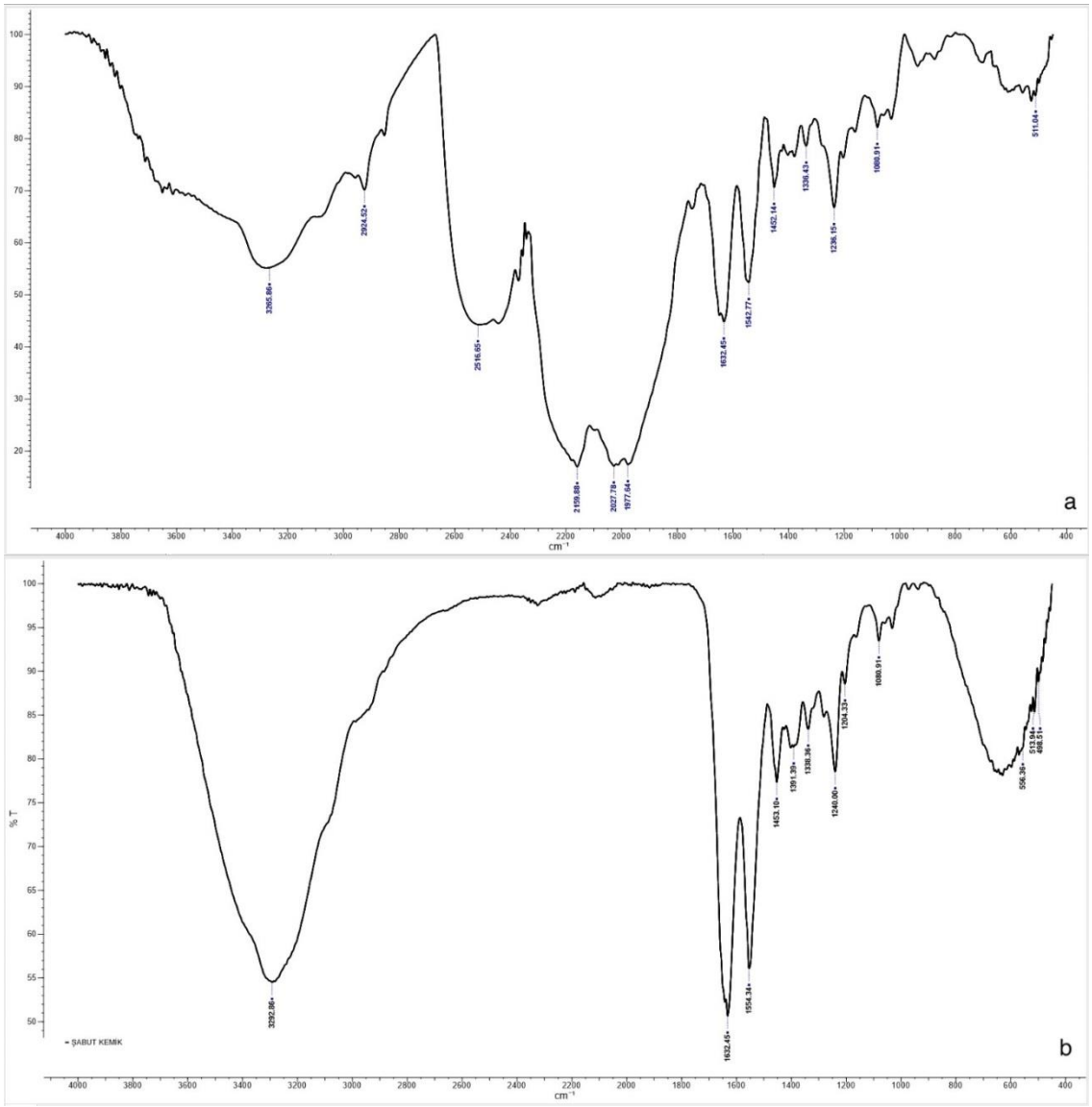

Figure 3: The FTIR spectra of acid-soluble collagens from skin (a) and bone (b) of shabout.

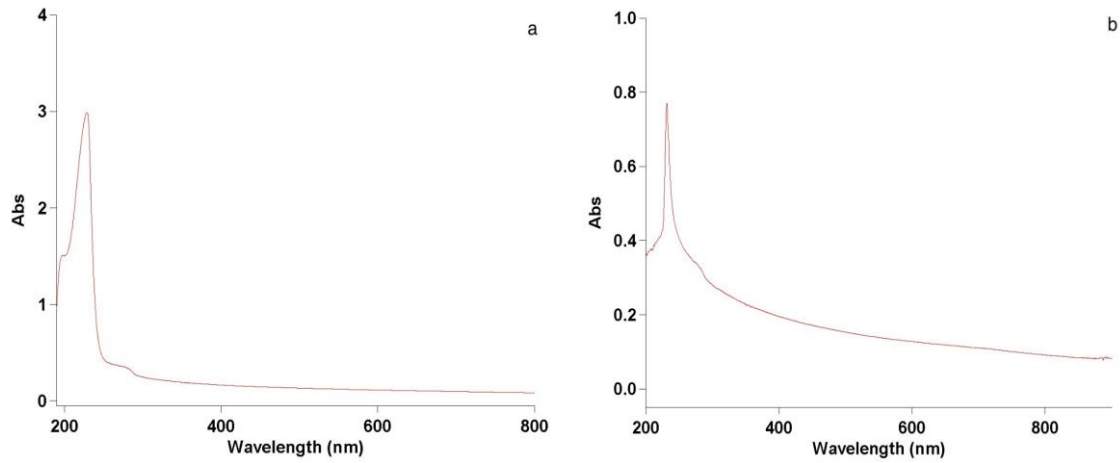

Figure 4: UV- Spectra of acid-soluble collagens from skin (a) and bone (b) of shabout

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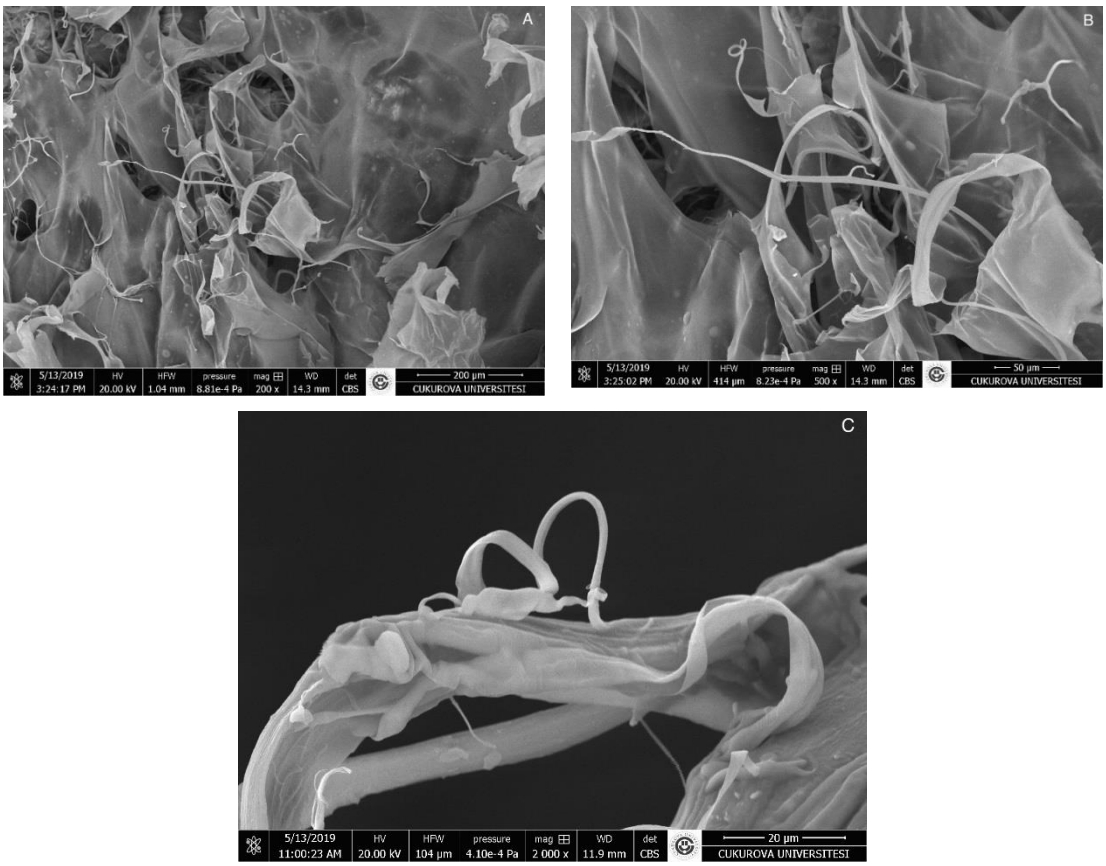

Figure 5: SEM images of acid-soluble collagen from skin of shabout A: ×200, B: ×500, C: ×2.000

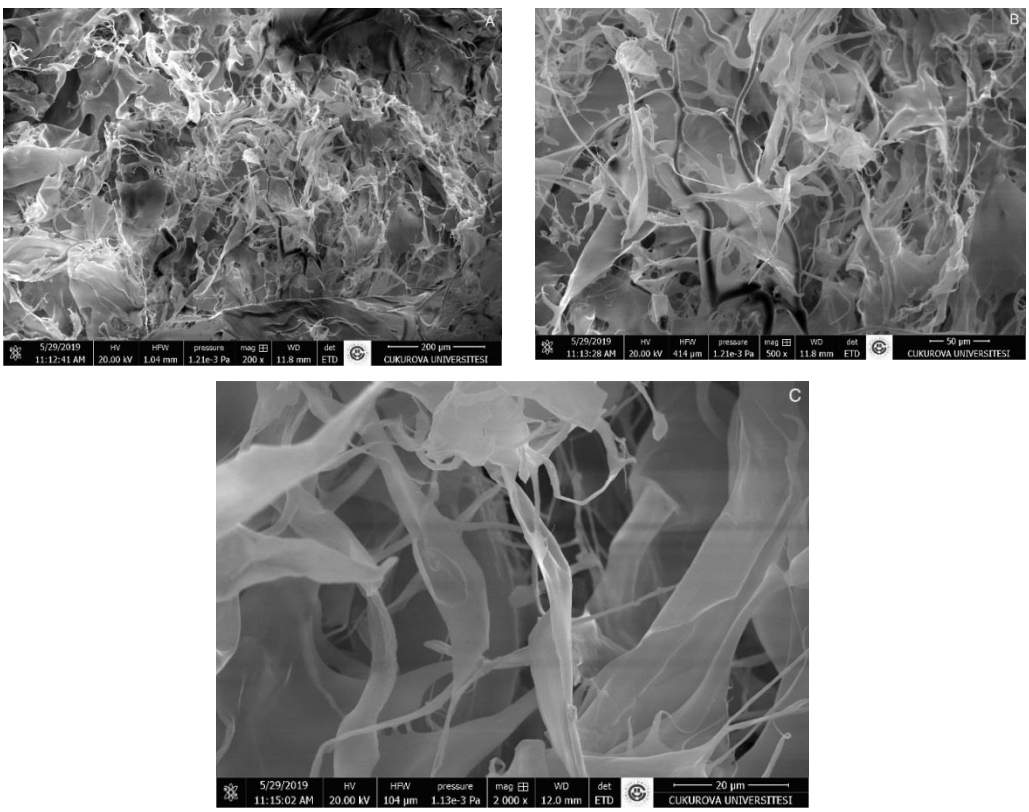

Figure 6: SEM images of acid-soluble collagen from bone of shabout A: ×200, B: ×500, C: ×2.000

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Amino acid composition

The amino acid compositions of acid-soluble collagen extracted from the shabout skin (ASC-S) and bones (ASC-B) are shown in the Table 1. Both extracted collagens have been determined to have similar amino acid compositions.

Table 1: Amino acid content (g/100g protein) of skin (ASC-S) and bone (ASC-B) collagens of shabout.

Amino Acid	ASC-S	ASC-B
Aspartic acid	4.75 ± 0.18	4.90 ± 0.16
Glutamic acid	8.78 ± 0.13[a]	9.14 ± 0.06[b]
Serine	3.51 ± 0.22	3.36 ± 0.13
Glycine	31.62 ± 0.20	31.00 ± 0.12
Threonine	2.06 ± 0.05	2.14 ± 0.06
Arginine	6.43 ± 0.17	5.79 ± 0.13
Alanine	9.35 ± 0.06[a]	8.73 ± 0.16[b]
Tyrosine	0.36 ± 0.08	0.25 ± 0.03
Cysteine	ND	ND
Valine	0.86 ± 0.04	1.22 ± 0.05
Methionine	1.05 ± 0.05	1.13 ± 0.05
Tryptophan	ND	ND
Phenylalanine	2.44 ± 0.12	2.69 ± 0.09
Leucine	2.55 ± 0.19	2.24 ± 0.13
Lysine	3.35 ± 0.08	3.23 ± 0.09
Hydroxyproline	9.39 ± 0.15[a]	9.97 ± 0.06[b]
Proline	9.89 ± 0.11	9.58 ± 0.22
Total amino acid (Hydroxyproline + proline)	19.28 ± 0.07	19.55 ± 0.16

±, represents standard deviations. The superscript letters [a] and [b] indicate to the statistical differences (p<0.05, n=6) between groups within the same line. ND, not determined.

Discussion

When collagen yields of the present study are compared with previous studies, one can see some differences as follows. A previous study suggested that the collagen yields of a defatted dry skin and wet skin of southern catfish (*Silurus meridionalis* Chen) were 78.57% and 23.14%, respectively (Xu *et al*., 2017). Similar to the results of the present study, Kittiphattanabawon *et al.* (2005) was found the yield of collagen obtained from big-eye snapper (*Priacanthus tayenus*) bone (1.59%) to be lower than the yield of collagen obtained from its skin (10.94%). Additionally, the results of the present study were found to be over than two-fold higher than the yield of collagen extracted from carp bone (1.06%) (Duan *et al.*, 2009). Unlikely, Doğdu *et al.* (2019) extracted collagen from silver cheeked pufferfish (*Lagocephalus sceleratus*) skin and the collagen yield was found to be 50.9%, which is much higher than the current study. In another study, Benjakul *et al.* (2010) was found the collagen yield (7.7% and 7.1%) extracted from *Priacanthus tayenus* and *Priacanthus macracanthus* skin. Likewise, Wei *et al.* (2019) isolated and characterized collagen from sturgeon fish and reported that the collagen yield was 5.73%. Both of Benjakul *et al.* (2010) and Wei *et al.*

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(2019) findings represent very close collagen yield results to the present study. In almost all of the mentioned studies including the current, the same method which was adopted without any modification or with minor modification of Nagai and Suzuki (2000). Since there are many different results in the collagen yield percentages within the reviewed research, all of these studies suggest that it could be species specific.

The thermal stability of collagen is affected by its amino acid composition, especially amino acids constitution. While proline and hydroxyproline provide the spatial structure of collagen with pyrrolidine rings, hydroxyproline increases the thermal stability of collagen by forming inter-chain hydrogen bonds that stabilize the triple helical structure of collagen (Gelse *et al*., 2003). Therefore, the Tmax value has a positive relationship with the amino acid content. As a matter of fact, the reason why the Tmax value of cattle skin collagen is higher related to its higher amino acid content (Foegeding *et al.*, 1996b; Komsa-Penkova *et al*., 1999).

As it was tested previously, cattle's amino acid content in ASC-S was 19.26%, while the ASC-B was 19.89% (Foegeding *et al.* 1996b; Komsa-Penkova *et al.* 1999). This value was found to be lower than the collagen obtained from many cold climate fish (Ciarlo *et al.* 1997). The collagen of shabout skin and bone had a hydroxyproline+proline content of 19.28% and 19.55%, whereas southern catfish skin had a hydroxyproline content of 39.1% (Xu *et al*., 2017), which was higher than that of collagen from bighead (*Hypophthalmichthys nobilis*) and grass carp (*Ctenopharyngodon idella*) skin (36.46% and 34.9%, respectively) (Liu *et al.*, 2014). This explains why collagen isolated from subtropical and tropical fish has better thermal stability (Hsieh *et al.,* 2016).

XRD is often used to analyze the crystal structure of polymers. When X-ray encounters crystalline particles, refraction occurs and the position and density of the diffraction peak reflects the structural properties of the crystals (Bigi *et al.,* 2001). The XRD results of the current study confirm that both of the collagens preserve the triple helix structure and is not denatured. Similar results have been obtained by several studies include carp scale collagen study by Zhang *et al.* (2007), *Oreochromis niloticus* skin collagen (Sun *et al.*, 2017a), *Gadus macrocephalus* skin collagen (Sun *et al.*, 2017b), Atlantic cod and Atlantic salmon skin collagen (Alves *et al.*, 2017).

According to the FTIR results, amide A absorption peaks of ASC-S and ASC-B were found to be 3265.86 and 3292.86 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}. However, Doyle *et al.* (1975) mentioned that when the NH group of a peptide is involved in the hydrogen bond, the position can shift to a low frequency, usually around 3300 cm^{-1}. Therefore, the shift of amide A towards lower wavelengths, as observed in this

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study, indicates that hydrogen bonded hydroxyl groups are present in both skin and bone collagens. Amide I bands of both collagen types were found to be 1632.45 cm^{-1}, which is consistent with the 1625-1690 cm^{-1} range that is the position of the general amide I bands of collagen. Amide II band was found to be 1542.77 cm^{-1} for ASC-S and 1554.34 cm^{-1} for ASC-B, amide II band is generally seen at wavelengths of 1550-1600 cm^{-1} (Krimm and Bandekar, 1986), 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 1236.15 and 1240 cm^{-1}, respectively. The ratio of the density between the absorption peak of Amide III and the absorption peak of 1450 cm^{-1} was 1.17 (ASC-S/ASC-B=1.17). Matmaroh *et al.* (2011) stated that a value approaching 1.0 indicates that collagen still has a triple helix structure.

When observed with the naked eye, both of the lyophilized collagens were soft, white, and spongy with a porous structure. However, when the observation was done with SEM, both of the collagens were found to have a dense, irregular, and partially wrinkled surface image bound by randomly wrapped filaments. This is probably due to the dehydration during lyophilization. Likewise, some similar results have been reported by various researches such as the observation of collagen obtained from Amur sturgeon skin (Wang et al. 2014) and *Istiophorus platypterus* skin (Tamilmozhi *et al.*, 2013).

In this study, both of the studied collagens were found to be similar in many properties. They represent poor organization, intersecting fibers, entangled bundles, and some fibrils have been found to have intricate meshes in contact with others. The fibrils of different thickness of both collagens were intertwined throughout the porous matrix. As a result, the SEM images of the collagens support that they have Type I collagen with fibrillar structure. As shown in the table, both products have been determined to have similar amino acid compositions. As expected, the ASC-S and ASC-B samples were high in glycine (Gly), proline (Pro), and hydroxyproline (Hyp) due to the characteristic (Gly-Pro-Hyp) triple-helix repeats of all collagens. As with other collagens, tryptophan and cystine were not detected (Yata *et al.* 2001; Muyonga *et al.* 2004; Jongjareonrak *et al.* 2005). Proline and hydroxyproline found in both ASC-S and ASC-B are important amino acids that ensure the structural integrity of collagen. The total amount of amino acid (Pro+Hyp) is 19.28% and 19.55% for ASC-S and ASC-B, respectively, and is statistically similar (p>0.05). This value is similar to the values reported for Nile tilapia (*Oreochromis niloticus)* (19.8-19.4%) (Potaros *et al.* 2009) and carp (19.4%) (Zhang *et al.* 2011); higher than the values reported for tilapia, (17.75%), grass carp (17.90%) and silver carp (17.78%) (Tang *et al.*, 2015); lower than

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the value reported for tilapia (25.4%) (Grossman and Bergman, 1992). The difference in amino acid content between different species is due to the different habitats of fish species, especially water temperature (Singh *et al.* 2011).

In summary, collagens were extracted and characterized from shabout skin and bone successfully. Both extracted collagens were Type I collagen, a typical amino acid composition. The FTIR and XRD analyses indicated that their triple helical structure remained intact following the extraction processes. Both extracted collagens showed maximum absorption at 230-232 nm with no absorption at 280. The SEM images of both collagens exhibited interconnective pores with lace-like fibers. As a consequence of the positive characteristics exhibited by the extracted collagens in this study, there is a high potential for use as a valuable collagen alternative in the food, pharmaceutical, and nutraceuticals industries.

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