

Isolation and characterization of acid-soluble collagen from the skin of *Rutilus Frisii Kutum* (Kamensky) of the Caspian Sea

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Received: January 2017

Accepted: April 2017

Abstract

This study proceeds to extract collagen from the skin of *Rutilus kutum*, one of the main and economical species of the Caspian Sea. Acid-soluble collagen (ASC) from the skin of *R. kutum* was extracted and characterized by SDS-PAGE, FTIR and UV spectrophotometry. Based on the data from the SDS-PAGE and the composition of amino acid, it was probable that the obtained collagen classified as type I collagen. Collagen consists of two different types of α chains, including α_1 and α_2 , and β components. Also, FTIR analysis revealed the presence of the helical arrangements of the collagen. Moreover, UV spectrophotometry exhibited this collagen has an absorbance at 220 nm. On the basis of dry weight, the yield of ASC was calculated as 15.6%. The amount of proline in ASC was 89.6 residues per 706.1 residues, and 25.7% of the collagen was composed of glycine. These results showed that *R. Kutum* skin could be considered as a possible source of collagen and can supply the theoretical basis for further research.

Keywords: *Rutilus Kutum*, Fish skin collagen, Characterization, Amino acid composition

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Introduction

Collagen is the most important protein of animal origin, including around 30% of the entire animal protein. There are 19 variations of collagen, called type I to XIX. Types I, II, III and V are the fibrous collagens (Aberoumand, 2012). Collagen is combined of three similarly sized triple helix polypeptide chains; while, the average length and diameter of each chain are 300 and 1.4 nm, respectively. Also, the number of amino acid residues in each chain is about 1000. Collagen has a repetitive primary sequence of which every third sequence can be described as Gly-X-Y, in which X and Y are often found to be proline and hydroxyproline forming a left-hand super helix with the other two chains (Whitford, 2005). Type I collagen is found in all connective tissues, including bones and skins, cartilage, tendons, ligaments, blood vessels, teeth, cornea, and all other organs of vertebrates (Aberoumand, 2012). Because of the specific features of collagen, such as biodegradability, biocompatibility, and very low antigenicity, it is considered as an important biomaterial for tissue engineering (Lee *et al.*, 2002).

Commonly, bovine skin, pig skin or chicken wastes are commercial collagen sources, the usage of which is occasionally improper because of some ethnic fundamental and religious beliefs. Furthermore, the collagen obtained from land animals may contain biological contaminants, infection and poisons, such as BSE (Mad Cow Disease), transmissible spongiform encephalopathy (TSE) and foot-and-

mouth disease (FMD). So, the need for new sources of collagen originating from seafood and fish supplies seems important (Aberoumand, 2010).

Collagen peptide extracted from fish skin consists of small peptide molecules. Its absorption in the small intestine is premier to other collagen products due to its smaller molecular size and leads to more efficacious collagen synthesis in different parts of the body like joint tissue, blood vessels, bone and skin dermis. Consequently, this product is used as supplements to lessen the pains and aches due to arthritis, arterio sclerosis and other signs (Jongjareonrak *et al.*, 2005). Thus, collagen as a novel type of biomaterial, has been applied in several areas like drug delivery, tissue engineering, regenerative medicine (Pachence, 1996; Friess, 1998), pharmaceutical food and cosmetic productions (Lee *et al.*, 2002; Kim and Mendis, 2006).

The Kutum, *Rutilus frisii kutum* (Kamensky), a member of the cyprinid family, is an endemic species to the Caspian Sea. This species is dispersed from the estuary of the Volga River up to Astrakhan Bay, but the main population is narrowed to the southwestern portion of the sea (Abdoli, 1999). *R. frisii kutum* is anadromous, lives the sea, and migrates to shallow river waters to spawn from March to April. Kutum is considered one of the most inestimable commercial species in the Caspian Sea region so that it comprises more than 70% of the total catch in the southern part of this sea (Ahmadian *et al.*, 2015)

Thus, it can be expected to extract economically collagen from this fish. In this study, we attempted to use *Rutilus frisii kutum* fish skin for collagen isolation, followed by purification and characterization of this obtained collagen. Eventually, different biomedical parameters have been compared.

Materials and methods

Extraction of collagen

Materials

Fresh fish skin was purchased from the fishers' market, and carried to the laboratory. In the laboratory the skin samples were first washed with tap water then the scales were cut with scissors and minced into small pieces (0.5×0.5 cm). They were then washed with cold distilled water, and the mixture of skin samples was packed in polyethylene bags and stored at -20 °C until the time needed to use.

Chemicals

B- mercaptoethanol (BME), pepsin from porcine gastric mucosa (Ec 3.4.23.1) powdered; 0.7 FIP mg⁻¹ dry matter, acetic acid, Butyl alcohol (Butanol), tris (Hydroxymethyl) aminomethane, sodium dodecyl sulfate (SDS), coomassie Brilliant blue R-250, all these materials obtained from Merck and Sigma Aldrich Company.

Alkali-extraction of non-collagenous proteins

All operations were performed following methods from Nagia and Suzuki (2000), at 4 °C. First, fish samples were washed one more time

with distilled water. Then, 10 volumes (v/w) of 0.1M NaOH was added to the sample. The suspension was stirred overnight for 2 days. The final precipitation was stirred overnight for 2 days. The final precipitation was washed completely with distilled water to reach a neutral pH. The solution was changed every 8 h.

Removal of fat from tissue

To remove fat from the tissues, 10% (v/v) butyl alcohol solution at a ratio of 1:10 (w/v) was used for 24 h with mild stirring, and the solution was changed every 8 h. Defatted tissues were thoroughly washed with cold distilled water.

Preparation of acid soluble collagen (ASC) from R. kutum fish skin

Fish collagen was extracted following our previous research work (Alizadeh Nodeh *et al.*, 2014). In summary, the prepared tissues were soaked in 0.5M acetic acid with a sample: Solution ratio of 1:15 (w/v) for 3 days continuously with gentle stirring on a shaker (device model: GFL, 3005). Two layers of cheese cloth was used to filter the mixture. Then, the supernatant was collected and stored at 4°C. The residue was re-extracted in the same way. Both collected supernatants were mixed and added to NaCl to reach a concentration of 0.9M. This solution stayed for 30 minutes. Then NaCl was added to obtain a final concentration of 2.6M in 0.05 M tris (hydroxyl methyl aminomethane), pH 7.0. The resultant precipitate was collected using a centrifuge (Sigma, 3-30K). The pellet

was dissolved in some volumes of 0.5M acetic acid. The solution obtained was dialyzed against 10 volumes of 0.1M acetic acid in a dialysis bag (dialysis tubing, D116, D117) for 24h, and then dialyzed with some volumes of distilled water. The dialysis water was changed till neutral pH was obtained. The resulting dialysate was freeze-dried and specified as acid-solubilized collagen (ASC).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed based on the method of Laemmli *et al.* (1970), using 7.5% gel containing 10% SDS at pH 8.8. Protein samples containing 50 μ l dialysis collagen, 10 μ l SDS 10% and 3 μ l 2-mercaptoethanol were added to boiling water for 5-10 minutes. Then 50 μ l of 20% glycerol and 0.005% bromophenol blue were added to it. The gel was stained for protein with coomassie brilliant blue R-250.

Amino acid analysis

Pico.Tag method was performed for amino acid analysis (Bidlingmeyer *et al.*, 1984). According to this method, amino acid derivatization and determination of the phenylthiocarbonyl derivative of amino acids were conducted using phenylisothiocyanate (PITC) and reversed phase High Performance Liquid Chromatography (HPLC), respectively. Dry collagen (10-20 mg) from skins of *R. kutum* were mixed with 6 M HCl (1 ml) containing 1% phenol (v/v). The mixture was evacuated,

blown with N₂ and vacuum-sealed before hydrolysis at 110 °C for 24 h. After hydrolysis, the samples were cooled and diluted to 5 ml with de-ionized water. A portion (25 μ l) was then dried and derivatised. Derivatisation included the addition of 10 μ l of a mixture of methanol, water and trimethylamine (2:2:1), mixing and then drying for 5 min. This was followed by the addition of 20 μ l of methanol, water, trimethylamine and phenylisothiocyanate (7:1:1:1). The samples were allowed to stay for 20 min at room temperature (20-25 °C), dried under vacuum and then disband in 200 μ l of pH 7.4 phosphate buffer and filtered with a 0.45 μ m filter. Portions (20 μ l) of the filtered samples were injected using an automatic loader (WISPTM) (Millipore Corp, Milford, MA, USA) into the Pico. Tag column (part no 88131, 3.9 mm \times 13 cm) (Millipore Corp, Milford, MA, USA) for amino acid analysis.

UV absorption spectrum

The UV-Vis absorption spectrum of ASC collagen in the wavelength ranges of 220 to 350 nm was conducted through a Shimadzu spectrophotometer. Purified collagen was dissolved in 0.5 M acetic acid to reach a concentration of 0.5 mg ml⁻¹. Then 200 μ l from the concentrate solution was dissolved in 800 μ l of 0.5 M acetic acid. Then the resulting homogenized solution was placed into a quartz cell to determine the absorption wavelength.

FTIR

Fourier transform infrared (FTIR) (Nexus 470 Fourier transform infrared spectrometer, Thermo Nicolet Co., USA) spectroscopy of collagens was analyzed based on the method of Cao *et al.* (2008) and Xu *et al.* (2012). The FTIR spectra were assessed at a resolution of 4 cm^{-1} in the range of $4000\text{-}400\text{ cm}^{-1}$ at room temperature. In this regards, KBr discs containing 2 mg samples in about 100 mg potassium bromide associated with a Fourier IR instrument were used to obtain FTIR spectra.

Results

Collagen

Collagen was prepared from *R. frisii kutum* skin. Based on the dry weight, the amount of extracting acid-soluble collagen from the skin of *R. kutum* was 15.6%. This result indicated that, the yield of ASC, from the skin of *R. frisii kutum* skin fish was much higher than from Brown stripe red snapper skin (9%) and lower than from the skin of Japanese sea-bass (51.4%), chub mackerel (49.8%), and bullhead shark (50.1%) (Nagia *et al.*, 2000).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The acid-soluble collagen from the skin tissue of *R. kutum* fishes were examined by SDS-PAGE using a 7.5% resolving gel (Fig. 1). This showed that ASC existed as trimmers consisting of two distinct α chains ($\alpha_1 + \alpha_2$). There were different positions in mobility in the α

region. The electrophoretic mobility position of α_1 was different from that of α_2 position and α_2 chain move more space. This result demonstrated that the molecular weight of the α_2 was smaller than the α_1 . Based on data obtained from electrophoretic mobility and subunit composition it is suggested that collagen from skin tissue of *R. kutum* fish were type I collagens and were composed of two α_1 and one α_2 chains.

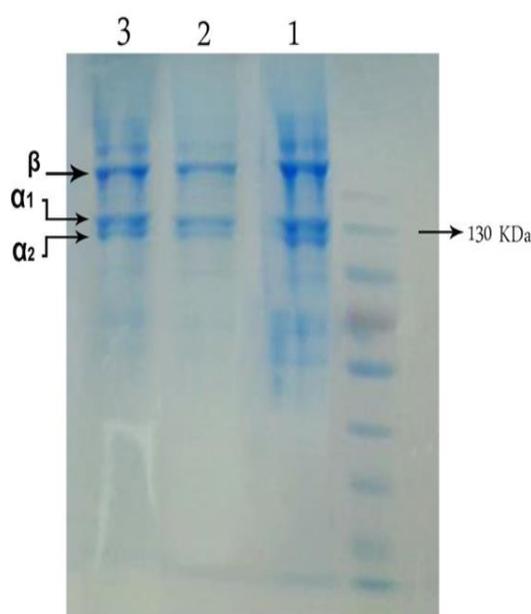


Figure 1: SDS-PAGE patterns of acid-soluble collagen from *Rutilus kutum* skin, 1- marker 1, 2, 3 – ASC.

Amino acid compositions of collagen

The amino acid composition of the acid-soluble collagen from *R. kutum* skin is shown in Table 1. The amino acid composition of ASC expressed as residues per 1000 total amino acid residues.

Table 1: Amino acid composition of collagen.

Alanine	73.1
Arginine	70.8
Aspartate	42.1
Cysteine	0
Glutamate	81.1
Glycine	182.5
Histidine	8.8
Isoleucine	10.7
Leucine	21.1
Lysine	31.1
Methionine	14.8
Phenylalanine	20.6
Proline	89.6
Serine	34.8
Threonine	20.6
Tyrosine	4.4

Values were given as mean \pm standard deviation of triplicate

Amino acid analysis revealed a much higher glycine content in collagen extracted which accounted for one third of the total amino acids. Besides, proline as a unique amino acid in ASC had the specific amount of 89.6 residues per 706.1 residues. The content of amino acids in ASC from *R. kutum* skin was very similar to that in Brown stripe red snapper skin collagen (Montero *et al.*, 1990; Nagai *et al.*, 2002) and higher than that of skins collagen from crap (157) and Ocellate puffer (170). Proline is correlated with species and their living habitat (Love *et al.*, 1976; Foegeding *et al.*, 1996). Higher contents of alanine, glutamate, arginine and proline which are characteristics of collagen could be obtained in the present study. In extracted collagens tryptophan or cysteine were not found. Their amino acid profiles revealed low in methionine, tyrosine and histidine.

UV-Vis Spectra

The UV spectrophotometry of *Rutilus kutum* fish collagen is shown in Fig. 2. In this study absorption wavelength of the *Rutilus kutum* fish collagen was observed at 240 nm. The UV absorption spectrum of ASC was at the wavelengths 220–350 nm. Most proteins have a maximum ultraviolet absorption at 280 nm, which is related to the numbers of tyrosine and tryptophane residues in proteins. But the amount of tyrosine in ASC was 4.4 residues per 1000 residues. UV spectrophotometry showed a maximum absorption at 240 nm. This may be related to the groups C=O, -COOH, CONH₂ in polypeptides chains of collagen (Edwards *et al.*, 1997).

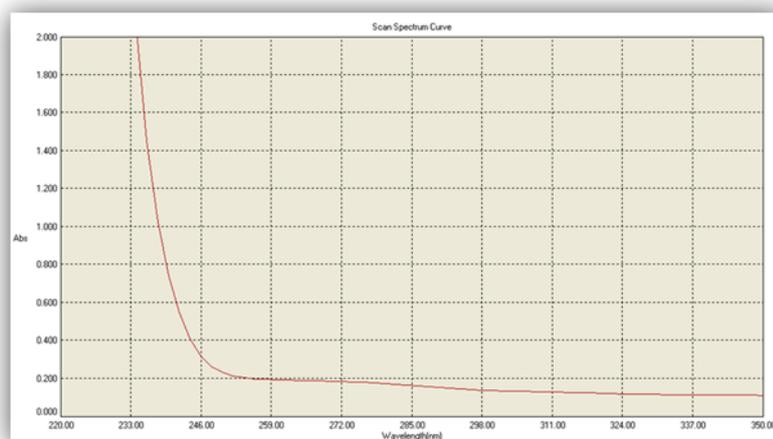


Figure 2: UV- Spectra of acid soluble collagen from *Rutilus kutum* skin.

Fourier transforms infrared spectroscopy

The FTIR spectra in the range of 400-4000 cm^{-1} of acid-soluble collagen from *R. kutum* are presented in Fig. 3. The amide A band is related to the N-H stretching frequency. A free N-H

stretching vibration occurs in the range of 3400-3440 cm^{-1} . The amide A band of *R. kutum* skin collagen was 3415 cm^{-1} . The amide B band of collagen was found at 2925 cm^{-1} associated with the asymmetrical stretch of CH₂ (Muyonga *et al.*, 2004).

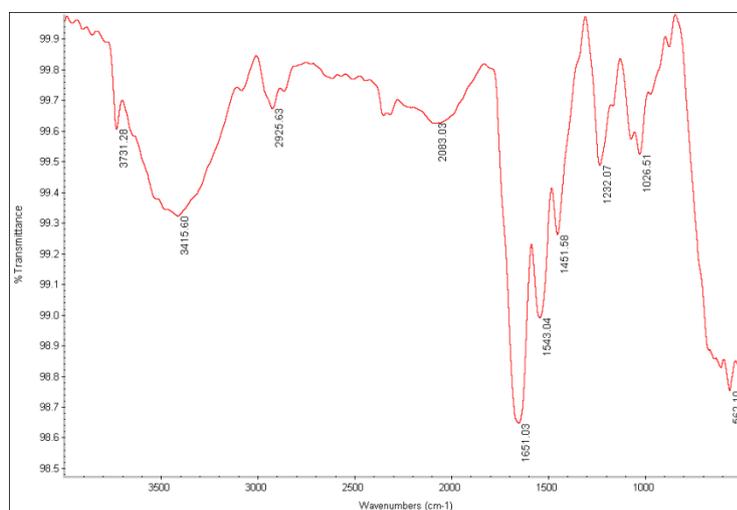


Figure 3: The FTIR spectra of Acid-soluble collagen.

The amide I band position was observed at 1651 cm^{-1} which was associated with C=O stretching vibration or hydrogen bond coupled with COO (Payne and Veis, 1998). It is associated with the secondary structure of the protein. The amide III peak of the collagens was observed at 1232 cm^{-1}

and 1451 cm^{-1} bands demonstrating the existence of a helical structure (Le *et al.*, 2007). The IR spectra recorded for thin films made of collagen from the fish *R. kutum* show typical bands for collagen type I: Amide A, Amide B, Amide I, Amide II and Amide III. The absorption characteristics of Amide A,

commonly associated with N-H stretching vibration, occurs in the wave number range 3400 ~ 3440 cm^{-1} . The maximum absorption peak of collagen from *R. kutum* was found at 3415 cm^{-1} . When the N-H group of a peptide is involved in a hydrogen bond, the position starts to shift to lower frequencies.

The wavenumber of characteristic absorption in Amide I bond is usually in the range of 1600 ~ 1700 cm^{-1} , which is generated by stretching vibration of C=O in polypeptide backbone of protein. This is the sensitive area of change of protein secondary structure, and is often used for protein secondary structure analysis. The absorption peak of Amide I was found at 1655 cm^{-1} .

The Amide II band was found at 1543 cm^{-1} . The Amide III peak is complex, with intermolecular interactions in collagen, consisting of components from C-N stretching and N-H in plane bending from amide linkages, as well as absorptions arising from wagging vibrations from CH₂. IR spectra as well as UV-Vis spectra (not shown) obtained for protein extracted from the skin of *Brama australis* clearly confirm that collagen was extracted.

Discussion

The ASC (acid-soluble collagen) has derived from the skin of *R. kutum* fishes that existed as trimmers, comprised of two different α chains ($\alpha_1 + \alpha_2$). In this regards, the electrophoretic mobility position of α_1 was different from that of α_2 and α_2 chain move more space. This revealed that the molecular weight of the α_2 was smaller than the α_1 . It was

recommended, based on subunit composition and electrophoretic mobility, that collagen from skin of *R. kutum* fish was type I and included two α_1 and one α_2 chains. We found our assessment similar to other studies such of Tylingo (2016), AlizadehNode (2014), Senaratne *et al.* (2006), Zhang *et al.* (2009), Yan *et al.* (2008), Wang *et al.* (2007), Duan *et al.* (2009), Ogawa *et al.* (2004), and Singh *et al.* (2010).

Amino acid analysis indicated higher content of glycine in extracted collagen and it was calculated as one third of the total amino acids of which the major amino acid was significantly lower than that of salmon fish (Tylingo *et al.*, 2016), but similar to that of Nile perch (Muyonga *et al.*, 2004) and channel catfish (Liu *et al.*, 2007). The reason that the glycine content was lower may be relevant to contamination by other proteins. Characteristics of collagen such as higher contents of alanine, amino acids -glutamate - arginine and proline, can be found in the present study. The collagen was very low in tyrosine, histidine, methionine and isoleucine, and similar to albacore tuna fish, rohu fish and lung fish skin, no cysteine or tryptophan was detected (Eastoe, 1956; Hema *et al.*, 2013). The distribution pattern of amino acid composition, similar to acid-soluble collagen from the skin of channel catfish (Liu *et al.*, 2007), proved that ASC could be categorized as type I collagen.

In this study absorption wavelength of the *R. kutum* fish collagen was observed at 240 nm which was closer to the absorption of other fish collagen

such as Nile tilapia, walleye pollock and black pomfret (Yan *et al.*, 2008; Zeng *et al.*, 2009; AlizadehNodeh *et al.*, 2014). Most proteins have a maximum ultraviolet absorption at 280 nm. Phenylalanine, tryptophane and tyrosine have absorption bands between 250 and 290 nm (Yan *et al.*, 2008; Zhanh *et al.*, 2011), while the absorption wavelength of this collagen was less. This may be due to the groups C=O, -COOH, CONH₂ in polypeptides chains of collagen (Zeng *et al.*, 2009). Therefore, it is compatible with the characteristics of a collagen.

The FTIR spectra in the range of 400-4000 cm⁻¹ of acid-soluble collagen from *R. kutum* is similar to walleye pollock, channel catfish, Nile perch fish collagen (Muyonga *et al.*, 2004; Liu *et al.*, 2008; Yan *et al.*, 2008). The amide A band is related to the N-H stretching frequency. A free N-H stretching vibration was in the range of 3400-3440cm⁻¹. The amide A band and amide B band of *R. kutum* skin collagen was 3415 cm⁻¹ and 2925 cm⁻¹, respectively which was attributed to the asymmetrical stretch of CH₂ (Muyonga *et al.*, 2004). The amide I band position was observed at 1651cm⁻¹ which was associated with C=O stretching vibration or hydrogen bond coupled with COO (Payne and Veis, 1998) and with the secondary structure of the protein. The amide III peak of the collagens was seen at 1232 cm⁻¹ and 1451 cm⁻¹ bands showing the existence of helical structure (Le *et al.*, 2007). Therefore, the FTIR investigations indicated the helical arrangements of *R. kutum* skin collagen.

In this study the *R. kutum* process waste collagens were extracted by 0.5M acetic acid or pepsin. The results showed that *R. kutum* contain higher amount of type I collagen.

The SDS-PAGE indicated the presence of this type of collagen in ASC and it was further confirmed by FTIR and UV-vis analysis. In the ASC α and β chains were found in the sample level. The analysis confirmed the fibrous nature of collagen. It was found that higher amount of *R. kutum* process wastes are expelled, but the results showed that it is probable to use these wastes as a significant and substitute collagen source from the Caspian Sea.

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