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Research Article

Isolation, characterization and biocompatibility evaluation of collagen from *Thunnus tonggol* skin

Samiei M.H.[1]; Jamili S.[2*]; Nikukar H.[3],[4]; Razban V.[5]

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Abstract

Acid-soluble collagen could be isolated from fish skin using acetic acid. In recent years, much attention has been paid to collagen from marine sources, mainly arising from the fact that there is no risk of contagious diseases. Moreover, by processing the fish, significant amounts of waste materials are produced which can be considered as a substitute for these collagen sources. *Thunnus tonggol* skin collagen was extracted by acid and base methods and evaluated by SDS-PAGE, FTIR and UV spectrophotometry and amino acid composition analysis. To determine biocompatibility, growth, and proliferation of extracted collagen, MTT and H&E staining methods were used. The results, predicated on SDS-PAGE and amino acid analysis, demonstrated that the fish skin collagen was of type I consisting of two α chains (α1 and α2) with molecular weight of 135 kDa and mass fraction of β. FTIR analysis also revealed helical compositions of both collagens, and UV spectrophotometry in *T. tonggol* skin collagen indicated a maximum absorption of 235 nm. The amount of collagen extracted from *T. tonggol* skin turned out to be 17.3% dry weight. Analysis of extracted collagen amino acids from the mentioned fish showed that glycine was the predominant amino acid. Human fibroblast culture presented a superior growth and good biocompatibility on extracted collagen. So, this type of collagen could be used in tissue engineering as a proper scaffold.

Keywords: Collagen, Fish skin, Amino acid composition, *Thunnus tonggol*

1-Department of Natural Resources and Environment, Science and Research Branch, Islamic Azad University, Tehran, Iran.
2-Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran.
3-Department of Advanced Medical Sciences and Technologies, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
4-Medical Nanotechnology and Tissue Engineering Research Center, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
5-Department of Molecular Medicine, School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz, Iran.
*Corresponding author's Email: Shahlajamili45@yahoo.com

Introduction

Collagen is a fibrous protein found in all multicellular animals and also an important component in the supporting structures (cartilage, tendons, etc.) of vertebrates and invertebrates. It is the most abundant protein in vertebrates that plays important role in maintaining biological and structural integrity of tissues and helps structure, shape and molecular mechanical properties of various connective tissues of the body (e.g., skin, bones, ligaments, tendons and cartilage). Approximately, 50% of total collagen in human body is present in skin, most of which is of type I (Nalinanon *et al*., 2011; Alves *et al*., 2017; Arumugam *et al*., 2018; Carvalho *et al*., 2018). It is major component in skin and bone and is the most abundant protein in mammals, accounting for about 25% and the highest amount, about 30%, of total protein in animal body. Collagen has shown excellent biological compatibility, biodegradation and regeneration and poor antigenicity. Such features and knowledge on this have led to mass production of collagen-based biomedical devices, including drug delivery systems, surgical stitches and tissue engineering applications. In addition, collagen has well-preserved amino acid structure and composition among species, which contributes to these properties when using non-human collagen in biomedical fields (Carvalho *et al*., 2018; Naderi Gharagheshlagh *et al*., 2020). Marine collagen, enjoying unique properties, such as no risk of disease transmission, less religious restrictions, cost-effectiveness, low molecular weight, easy biocompatibility and better absorption by human body, is progressively used compared with its mammalian type (Blanco *et al*., 2019; Jafari *et al*., 2020).

The main feature of a typical collagen molecule is its long, strong, three-stranded helical structure, which is made up of three polypeptide chains called α chains that are wrapped around each other (Nagai *et al*., 2001; Nagai, 2004). So far, 29 types of collagens have been identified, all of which exhibit a triple helical structure. Fish collagen is of type I and is different and unique compared to mammalian and poultry collagen due to its extremely high solubility in diluted acids. Collagen precursors are known for influencing cell growth characteristics and regulating various aspects of cellular behavior and function such as cell fusion, proliferation and differentiation. Previous studies reported that collagen composition of fish is similar to its mammalian counterpart (Nagai *et al*., 2001; Nagai, 2004; Bae *et al*., 2008). For commercial exploitation, available collagen is mainly obtained from land animals, i.e., cattle, pigs, chickens and various species of mice. Fish collagen is a good source for many applications. Collagen is used in cosmetics, and applicable parts of it are also employed in medicine. There is great interest and demand for use of collagen in medical applications, and collagen-based materials are widely used in restorative medicine. Collagen is also used in medicine to make creams, gauzes and bandages for dressing and improved wound healing, and in pharmacy to make medicine capsules and tablets (Nagai and Suzuki, 2000a; Safandowska and Pietrucha, 2013; Sionkowska and Kozlowska, 2014; Mahboob, 2015; Venkatesan *et al*., 2017).

Nowadays, skin and bones of cows and pigs are the main sources of collagen and gelatin extraction. But much attention is exercised to separation of collagen from marine organisms due to its unrestricted use in diet and low risk of infectious diseases, including bovine madness, swine flu, avian flu, as well as common gastrointestinal diseases in cows, pigs and buffaloes (Yamada *et al.*, 2014).

Despite careful screening for communicable diseases, risk of transmission and occurrence of bovine madness has led to anxiety among consumers of bovine collagen and gelatin. Also, prevalence of diseases, such as transmissible spongiform encephalopathy (TSE), swine influenza, foot and mouth diseases (FMD) in pigs and cattle, swine flu and bovine spongiform encephalopathy (BSE) in some countries as well as solving the problem by BSE is of great importance (Nagai, 2004; Nazeer *et al*., 2014; Bhagwat and Dandge, 2016, Sotelo *et al*., 2016; European Food Safety Authority, 2020; Nagai *et al*., 2020). Moreover, religious restrictions, especially in Islam, Judaism, and Hinduism (both Judaism and Islam forbid consumption of pork-related products and Hindus do not consume cow related yield) and religious slaughter, restrict use of mammalian-based collagen (Dayton, 2008; Nalinanon *et al*., 2012; Tamilmozhi *et al*., 2013; Fu *et al*., 2014; Sionkowska and Kozlowska, 2014; Mahboob, 2015; Beishenaliev *et al*., 2019, Lin *et al*., 2020). As the risk of disease transmission is not yet known, collagen obtained from fish could be a safe option. In addition, fish processing by-products, such as skin, bones and scales are fully available; while addressing related environmental pollution problems, they ensure sustainable exploitation of marine resources through residual valuation (Carvalho *et al.*, 2018).

Due to importance of this issue, scores of studies are carried out on collagen of fish and many marine organisms of different species as an easy source of biomass for collagen separation, either from a fundamental point of view or as a raw material for biopolymer production as well as examination of their physical and chemical properties. In these investigations, fish and its by-products have been identified as a safe source due to their low risk of disease transmission, easy access, lack of religious restrictions, and high collagen content (Nagai and Suzuki, 2000a; Aewsiri *et al*., 2008; Kaewdang *et al*., 2014; Sun *et al*., 2017; Arumugam *et al*., 2018; Naderi Gharagheshlagh *et al*., 2018; Lim *et al.*, 2019; Sun *et al.*, 2019; Claverie *et al.*, 2020; Ge *et al*., 2020; Li *et al*., 2020; Naderi Gharagheshlagh *et al*., 2020; Zhou *et al*., 2020).

Use of fish collagen, instead of mammalian collagen, has numerous rewards. During food processing, fish solids waste constitute 20–80% of original raw material, depending on the processes used and types of products (Jongjareonrak *et al*., 2005; Ghaly *et al*., 2013; Bhagwat and Dandge, 2016; Sousa *et al*., 2017). Fish by-products, especially skin, are abundant in collagen, constituting approximately 30% of the dry weight (Wasswa *et al*., 2007).

T. tonggol is one of the most valuable and important fish that lives in tropical to subtropical regions of Indian and Pacific oceans. It is found in coastal waters of various countries, including Indonesia, Pakistan, Malaysia, Oman, Yemen, India, Thailand and Iran. In Iran, industrial fishing takes place in Indian Ocean, Persian Gulf and Oman Sea, which is economically very important for Iran's fisheries industry. Catch data were extracted from IOTC (Indian Ocean Tuna Commission) Secretariat database for the period 1950–2018, given that total catches of *T. tonggol* were estimated as 135, 282t in 2018. Iran ranks the first in *T. tonggol* fishing from Indian Ocean. The average amount of *T. tonggol* caught in Iran over the period 2012-2018 reached a maximum of 60,000 tons (Dan, 2020). The annual catch of *T. tonggol* in 2011 was reported as 80877 t in southern Iranian waters (Persian Gulf and Oman Sea) (Sadough Niri *et al*., 2020). Given the main role of fisheries activities in countries located at the high seas, sustainable development of fisheries industry is of vital importance. In this respect, Iran is a good example, located beside most shores of Persian Gulf and Oman Sea, and hence gains access to Indian Ocean (Sistani *et al.*, 2021).

Materials and methods

Sample Preparation

In the present study, collagen sources including *T. tonggol* skins (100 gr) were prepared from Bandar Abbas fishmongers' market, and samples were frozen and transferred to the laboratory of Medical Nanotechnology and Tissue Engineering Research Center of Shahid Sadoughi University of Medical Sciences in Yazd.

Chemical reagents

The chemicals included caustic soda (sodium hydroxide), butyl alcohol (1-butanol), acrylamide, bisacrylamide, Sodium chloride, Tris (hydroxymethyl) aminomethane, Sodium Dodecyl Sulphate (SDS), ammonium sulfate, glycerol, mercaptoethanol, coomassie brilliant blue R-250 was prepared from Sigma Aldridge®, methanol and glacial acetic acid from Merck®, and protein markers from Sina Gene (SL7002).

To test, all materials were stored in a laboratory freezer at -25°C for one week. Throughout the research process, temperature conditions were set at 4°C, and pH was controlled to be constant with a value of 7.2.

Alkaline extraction of non-collagenous proteins

All steps were performed using Nagai *et al.* (2020) method with slight modification. First, the samples were washed several times using cold distilled water. Then 0.1 M NaOH (pH 12) was added to them in the ratio of 1:20 (W/V). The suspension was stirred by a magnetic stirrer for 24 h. The final solution was filtered and rinsed with cold distilled water to neutralize the pH.

Tissue degreasing

The samples were mixed and stirred in 10% butyl alcohol solution at ratio of 1:10 for 24 h. The solution was changed every 8 hours. The fat-free tissue was thoroughly washed

with cold distilled water (Nagai *et al.*, 2020).

Preparation of acid-soluble collagen from skin of T. tonggol

To extract collagen, the prepared tissue in 0.5 M acetic acid with ratio of 1:20 (W/V) was placed on a stirrer for 72 h. The mixture was filtered through two layers of filter cloth. The supernatant was then collected and stored at 4°C. The remainder was extracted as before. The obtained extract was then placed in a refrigerating centrifuge for 1 h at a rate of 10,000 rpm and extracted. Both supernatants were placed in 0.9 M NaCl for 30 min. It was then added to NaCl to obtain final concentration of 2.6 M in 0.05 Tris with pH: 7 (hydroxymethyl aminomethane) and stirred for 30 minutes. The resulting precipitate was collected by refrigerating centrifuge for 45 minutes at a rate of 10,000 rpm. The precipitate was dissolved in 0.5 M acetic acid for 6 h and then dialyzed in 0.1 M acetic acid at 0.1 M for 48 h (D117, D116) and finally in 72 M cold distilled water. The dialysis water was changed every 6 h until it reached normal pH. The dialyzed precipitate was dried by freeze drier at -55° C and pressure of P5 for 48 h and introduced as acid-soluble collagen (Nagai *et al.*, 2020).

Performance and efficiency of extracted collagen

The amount of acid-soluble collagen was calculated based on dry weight of raw material (Radhika Rajasree *et al.*, 2020).

Yield % = Weight of lyophilized collagen (g) / Weight of initial dry skin ×100

UV spectrophotometry

Atomic UV absorption of collagen in wavelength range of 200-400 nm was performed by Unico spectrophotometer (model: uv4802). Pure collagen was dissolved in 0.5 M acetic acid and dissolved in 0.5 mg/mL to obtain the appropriate concentration. Totally, 200 µL of solution was dissolved in 800 µL of 0.5 M acetic acid. Then 1 mL of the homogenized solution was poured into cuvette and placed in the spectrophotometer to determine absorption wavelength (Naderi Gharagheshlagh *et al.*, 2020).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) test

To perform this test, 30.8% polyacrylamide gel was used. At this stage, the gel lower than 8% and upper than 3.5% was used. *T. tonggol* collagen was dissolved in 4% acetic acid and placed in 95°C boiling water for 10 minutes. The samples were then transferred onto the gel and into the wells with a protein marker (Laemmli, 1970). Finally, electrophoresis was performed at 120 volts for 3 h. After electrophoresis, gel with 0.1% aqueous Coomassie (weight/volume) in 50% methanol (volume/volume), 10% acetic acid (volume/volume) and 40% distilled water (volume/volume) was stained for 20 minutes. Then the gel was placed onto a dye-removing shaker to remove additional dyes with a dye solution containing 10% methanol (volume/volume), 10% gallic acetic acid (volume/volume) and 80% distilled water (volume/volume) for 24 h.

Fourier-transform infrared spectroscopy (FTIR)

Infrared spectroscopy is used to determine the secondary structure of polypeptides and proteins. This technique has the capacity of non-destroying the sample. In this spectroscopy, the functional groups in different compounds have different power of absorption of infrared rays in various regions of the wave number. Infrared spectroscopy information is plotted under the structural unit vibrations of the functional groups. Amide I and amide II bonds are dominant bonds in structure of the protein and the polypeptide. The most sensitive region is amide I bond in the spectrum with wave number of 1600-1700 cm^{-1}, which belongs to tensile vibrations of O=C bond in peptide junctions of the polypeptide protein, while amide II bonds belong to vibrations of C-N bond (Kong and Yu, 2007; Naderi Gharagheshlagh *et al*., 2020).

In order to investigate infrared spectroscopy, FTIR test was performed based on method of Xu *et al.* (2012) FTIR spectra were examined at resolution of 4 cm at range of 550-4000 cm^{-1} at room temperature. In this regard, potassium bromide (KBr) tablets containing 2 mg of sample containing about 100 mg of KBr were prepared. Then, the resulting tablets were placed in a special tube for FTIR (Perkin Elmer Spectrum Two model LS55) and the relevant analyses were performed using OPUS software and the relating spectrum was prepared.

Amino acid analysis

Pico Tag method was used for amino acids analysis (Naderi Gharagheshlagh *et al.*, 2020). Based on this method, amino acid derivative of phenylthiocarbamyl and determination of amino acid derivative using phenyl isothiocyanate and reverse phase with high efficiency liquid chromatography were performed respectively. First, 10-20 mg of dry collagen isolated from fish skin was mixed with 6 M hydrochloric acid containing 1% phenol (v/v). The prepared mixture was then exposed to N2 gas and vacuumed for 24 h overnight at 110°C before performing hydrolysis. After this, the samples were dried and diluted with 5 ml of distilled water. Then 25 µL of protein was dried and derivate, and 10 µL of mixture of methanol, water and trimethylamine was added at 2: 2: 1 ratio. The sample was mixed and then dried for 5 minutes. Later, 20 µL of methanol, water, trimethylamine and phenyl isothiocyanate were added at ratio of 7: 1: 1: 1. The samples were dried for 20 minutes at room temperature of about 20 to 25°C under vacuum and then dissolved in 200 µl of phosphate buffer with pH of 4.7 which were then filtered with a 0.45-micron filter. Finally, 20 µL of the filtered samples were automatically injected into the Pico Tag column for amino acid analysis using an automatic loader (WISPTM) (Millipore Corp., Milford, MA, USA).

Cell activity and proliferation testing

To determine biocompatibility, growth, and proliferation of extracted collagen, MTT (methylthiazolyldiphenyl-tetrazolium

bromide) assay and H&E (Hematoxylin and Eosin) staining were used.

Human foreskin fibroblasts cells (HFF) kindly donated by Medical Nanotechnology and Tissue Engineering Research Center, Yazd Reproductive Sciences Institute of Shahid Sadoughi University of Medical Sciences were cultured at cell culture dishes and in standard condition, using DMEM (Dulbecco's Modified Eagle Medium) (Gibco, UK), supplemented with 10% fetal bovine serum (FBS) (Gibco, UK), with 1% penicillin/streptomycin (Gibco, UK), at density of 1×10^4cells per well on 24-well plates. The culture medium was changed every 48hr. On days 3, 7 and 12 of incubation, 300 µL of MTT solution (containing 10% MTT and 90% DMEM/10% FBS) was added to each well. The cultures were incubated at 37°C and 5% CO2 for an additional 3 h. 300µL of dimethyl sulfoxide (DMSO) was then added to each well to dissolve purple MTT formazan crystals. Then another 30min of incubation was done (Kumar *et al*., 2018). Absorbance of the solution was measured at 570-630 nm using enzyme-linked immunosorbent assay (ELISA) plate reader (STAT FAX -2100, USA).

Cell infiltration of various compositions on extracted collagen was investigated via histology technique (H&E staining). After seven days of cells seeding on extracted collagen, the samples were fixed via 2.5% Glutaraldehyde at 4°C. The samples were dehydrated by graded ethanol series (30, 50, 70, 80, 90, 95, and 100%) twice for 20 min each and stained by H&E staining method and then examined by an inverted microscope (Olympus, Tokyo, Japan) (Cardiff *et al*., 2014).

Statistical Analysis

Origin 8.0 software (Origin Lab Inc., USA) and GraphPad Prism 9 were used for statistical analysis of data and plotting. Data were expressed as mean±standard deviation. Statistical comparison was performed with one-way ANOVA. Tukey test was used to evaluate the statistical differences between the groups. Pvalue of less than 0.0001 was considered significant for the groups.

Results

Collagen was extracted from skin of *T. tonggol* by Nagai *et al.* (2020) method with some changes. Evaluation and amount of collagen extracted from *T. tonggol* skin was 17.3% dry weight.

UV spectrophotometry

T. tonggol skin collagen spectrophotometry was performed (Fig. 1). Acid-soluble collagen (ASC) UV uptake was examined at wavelength of 200-350 nm. Most proteins have a maximum UV absorption at 280 nm, which is related to the amount of tyrosine and tryptophan residues in the proteins. UV spectrophotometry revealed maximum absorption at 235 nm, which could be related to CONH2, -COOH and C=O groups in collagen polypeptide chain.

SDS-PAGE test

Collagen extracted from *T. tonggol* skin was tested by SDS-PAGE. The results of SDS-PAGE test indicated that *T. tonggol* skin collagen composed of two chains: α

(α1 and α2) and β. The test results also revealed that α1 molecular weight was about 135 kDa whereas α2 molecular weight amounted to around 125 kDa (Fig. 2).

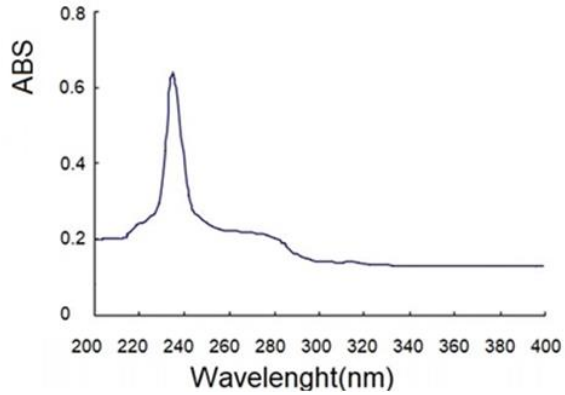

Figure 1: Spectrophotometry of *T. tonggol skin collagen* showing absorption from 200 to 350 nm (Maximum absorption at 235 nm).

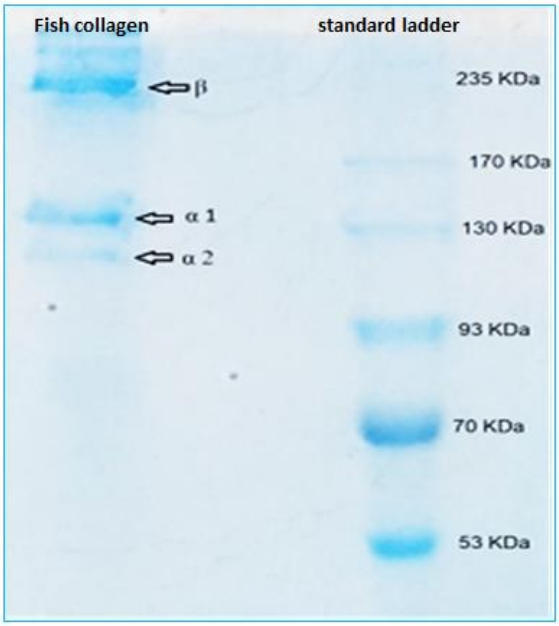

Figure 2: Collagen electrophoresis pattern of the *T. tonggol* skin shows it composed of two chains: α (α1 and α2) and β.

FTIR

FTIR spectrum at range of 550-4000 cm^{-1} in acid-soluble collagen from *T. tonggol* is presented in Figure 3. Amide group A corresponds to N-H tensile frequency. N-H free tensile vibration with hydrogen bonding occurs at range of 3300-3440 cm^{-1}. Amide A band was observed at 3300. B-bond amide group was identified at 2929 in association with asymmetric stretching of CH2. Peak of amide I uptake was observed at 1633, which is associated with C=O tensile vibration of the hydrogen bond linked with COO, related to secondary structure of the protein. This region is sensitive to changes in secondary structure of the protein and is often used to analyze secondary structure of the protein. The amide II bond was found at 1539 and peak of amide III bond was adsorbed at 1238, indicating a spiral structure of these bonds. Infrared spectra recorded for thin films made from *T. tonggol* collagen showed common bonds for type I collagen, amide A, amide B, amide I, amide II and amide III. The properties of amide A are usually related to tensile vibration of N-H, which occurs at range of 3300-3440 cm^{-1}. The maximum peak absorption obtained from *T. tonggol* collagen was 3300. When N-H group of a peptide contains a hydrogen bond, the situation begins to change direction to lower frequencies. The number of characteristic waves absorbed in amide I bond is usually at range of 1620-1800 cm^{-1}, which is produced by tensile vibration of C=O in the protein polypeptide. This region is sensitive to secondary changes in protein structure and is often used for protein (second) structural analysis. Absorption peak of amide I was obtained at 1633. N-H bending with C-N tension occurs at range 1590-1650 and is related to amide II bond, which was observed at 1539. Amide III peak is at range of 1200-1400 in complex

with internal reactions in collagen, which includes compounds of tensile C-N and N-H at the level of smoothness associated with amide as well as vibrational absorptions induced by CH2 shaking. Infrared spectra for the protein extracted from *T. tonggol* skin specifically confirm collagen extraction.

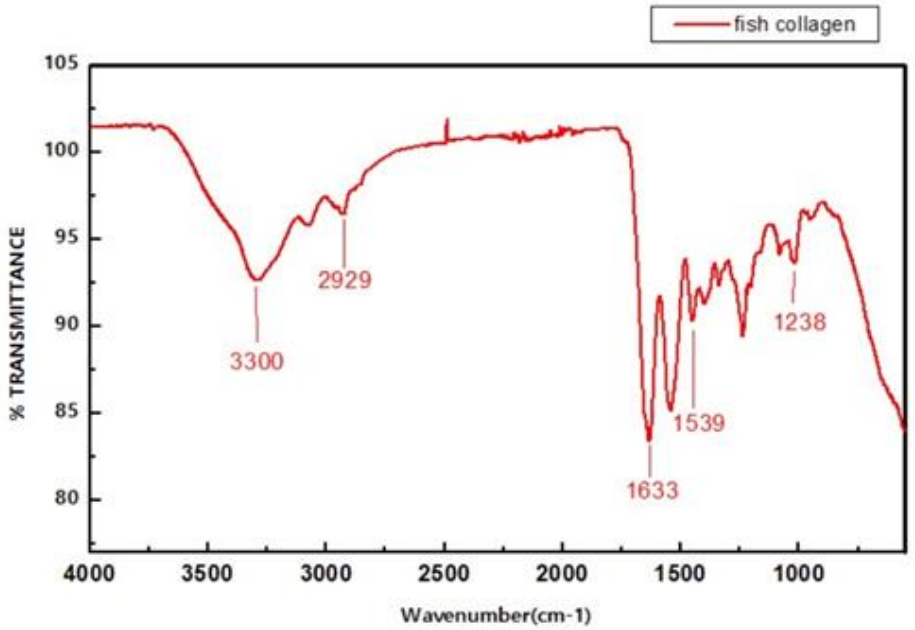

Figure 3: FTIR spectrum of acid-soluble collagen from *T. tonggol* skin showing all collagen amide bands were present at the range of 550-4000 cm^{-1}.

Amino acid composition of T. tonggol collagen

Amino acid composition of *T. tonggol* collagen based on residues in 1000 is set out in Table 1. Amino acid analysis revealed very high levels of glycine in collagen extracted from skin of *T. tonggol*, which accounts for about one-third of all amino acids. Moreover, proline amino acid is a unique type in ASC that bears a certain amount of residues (97.2 per 1000). Alanine, glutamate, arginine and proline compose the most abundant amino acids that can be used as properties of collagen obtained in this study.

Table 1: Composition of amino acids in *T. tonggol* skin in terms of residues per 1000.

Amino acid	Residue
Hydroxyproline	**69**
Asparagine	**62**
Threonine	**21**
Serine	**27**
Glutamine	**85**
Proline	**104**
Glycine	**309**
Alanine	**95**
Valine	**22**
Methionine	**15**
Isoleucine	**18**
Leucine	**28**
Tyrosine	**4**
Phenylalanine	**21**
Histidine	**9**
Hydroxylysine	**6**
Lysine	**32**
Arginine	**73**
Cystine	**0**
Total Amino acids	**1000** **173**

The extracted collagen did not contain any cysteine but there were the lowest levels of amino acids methionine, tyrosine and histidine. Total amino acids (proline and hydroxyproline) amounted to 173 residues per 1000.

The results of MTT assays are shown in Figure 4. No significant difference in viability of cultured cells on extracted collagen was detected on day 3. However, viability of cells increased significantly over the culture time from 3 to 7 and 12 days and showed significant difference compared to the control (on standard plate) on days 7 and 12 (p<0.0001). Results of H&E staining are shown in Figure 5. HFF was well attached and spread over the cell culture plate on days 3 and 7.

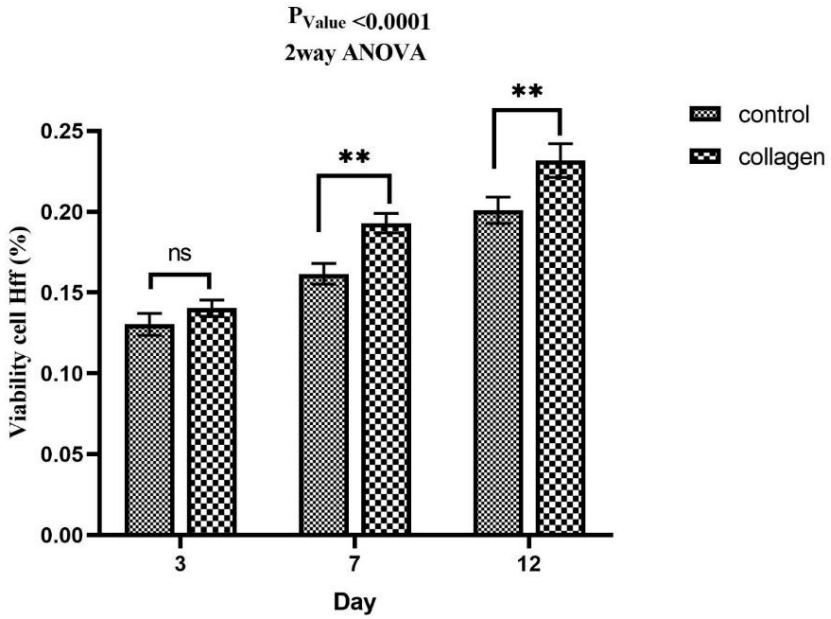

Figure 4: MTT results showed human fibroblast growth on the extracted collagen at days 3, 7 and 12 after the culture was more than the control (*p value*<0.0001). Error bars show standard deviation.**

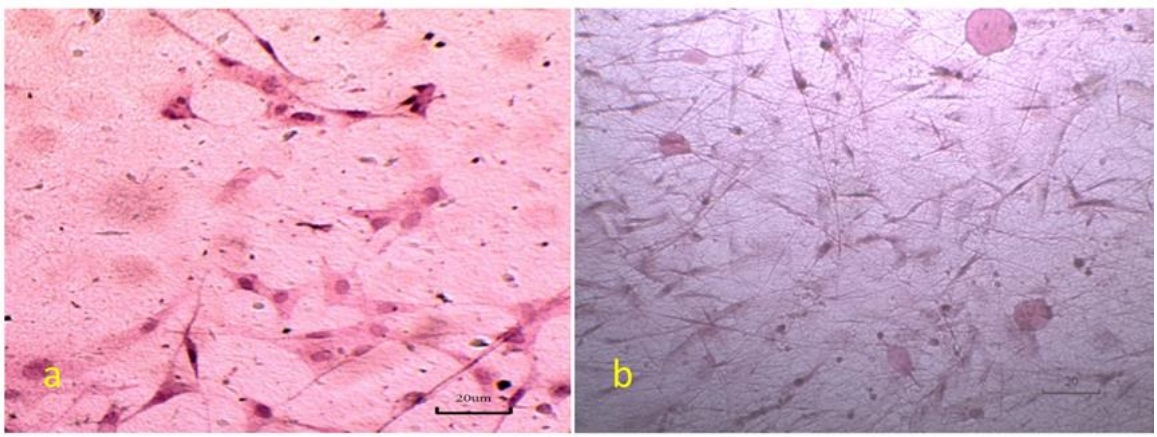

Figure 5: H&E staining of HFF on collagen extracts, (a) day 3 and (b) day 7 after culture (the scale is 20µm).

Discussion

The amount of collagen isolated from skin of *T. tonggol* fish was 17.3% dry weight, which was higher than the amount of collagen produced from skin of *Scomberomorus guttatus* by 14.5%, kutum or *Rutilus frisii* by 15.6 % (Naderi Gharagheshlagh *et al.*, 2020), brownstripe red snapper by 9% (Kiew and Mashitah, 2013), dusky spinefoot, Sea chub, eagle ray, red stingray and Yantai stingray respectively by 3.9, 3.4, 5.3, 5.7 and 5.5% (Bae *et al.*, 2008), balloon fish (*Diodon holocanthus*) by 4% (Huang *et al.*, 2011), and yellowfin tuna by 1.07% (Kaewdang *et al.*, 2014). On the other hand, it stood lower than collagen of Malaysian catfish 18.11% (Kiew and Mashitah, 2013), blue grenadier 20%, Japanese sea-bass 51.4%, chub mackerel 49.8%, bullhead shark 50.1% and ocellated puffer fish 44.7% (Nagai, 2004).

In this study, absorbed wavelength of *T. tonggol* collagen was at 235 nm (Figure 1), which was close to absorption of collagen of other fish, such as *Scomberomorus guttatus*, northern pike, black pomfret, balloon fish, walleye pollock, channel catfish and *Sphyrna lewini* (Lin and Liu, 2006; Liu *et al*., 2007; Bae *et al*., 2008, Huang *et al*., 2011, Adibzadeh *et al*., 2014, Kozlowska *et al*., 2015, Naderi Gharagheshlagh *et al*., 2018). Most proteins have the highest UV absorption at 280 nm. Phenylalanine, tryptophan and tyrosine have absorption bonds of 250-290 nm (Liu *et al*., 2007; Zhang *et al*., 2011; Fu *et al*., 2014; Naderi Gharagheshlagh *et al*., 2018). In general, tyrosine and phenylalanine are sensitive chromophores and absorb ultraviolet light at 283 and 251 nm (Lin and Liu, 2006; Nazeer *et al*., 2014). Phenylalanine and tyrosine have absorption bonds of 250-290 nm while collagen has no apparent uptake in this region. Separate uptake from *T. tonggol* skin collagen was obtained at about 235 nm, which was less than 280 nm and supported the collagen property and is the result of isolated protein collagen.

Collagen isolated from skin of this fish was examined by electrophoresis (Figure 2). Pattern of fish skin collagen electrophoresis demonstrated that fish skin collagen, being a natural polymer, was composed of two different α chains (α1 and α2). Due to this identity, vibrational position of alpha 1 electrophoresis was different from that of alpha 2. Alpha 1 chain had more vibrational space, which indicates molecular weight of alpha 2 being smaller than that of alpha 1. The results of this study were in line with those of other researchers (Lin and Liu, 2006; Yan *et al*., 2008; Zhang *et al*., 2009; Jeong *et al*., 2013; Adibzadeh *et al*., 2014; AlizadehNodeh *et al*., 2014; Nazeer *et al*., 2014; Liu *et al*., 2015; Tylingo *et al.*, 2016; Naderi Gharagheshlagh *et al*., 2018, 2020). Examination of electrophoretic pattern of collagen extracted from *T. tonggol* showed absence of disulfide bonds. In general, type I collagen contains lower amounts of cysteine (>0.2%) and methionine (1.24-1.33%), which play a key role in formation of disulfide bonds. However, type III and VI collagens contain amounts of oxidizable cysteine (Kittiphattanabawon *et al.*, 2005). As a result, based on analysis of electrophoresis patterns and composition of the constituent units, it can be stated that *T.*

tonggol skin collagen is of type I and as its main protein; this is in agreement with the results of other studies conducted in proving that skin collagen is of type I (Nagai *et al*., 2008; Jeong *et al*., 2013; Muralidharan *et al*., 2013; Yan *et al*., 2015; Krishnamoorthi *et al*., 2017; Arumugam *et al*., 2018; Carvalho *et al*., 2018; Sun *et al*., 2019; Fatemi *et al*., 2021).

The infrared spectrum is characteristic of any molecule, and certain groups of atoms form bonds that exist at about the same frequency, regardless of structure of the molecule. It is precisely these specific groups that provide access to useful structural information.

For different chemical groups, the wavelength absorbed and the natural need appear to be unique vibrations and depend on the type of the bond available (C=C, CH, C=O, NH and OH) (Silva Junior *et al.*, 2015). Medium infrared (wave number 400 up to 4000 cm^{-1}) is the range that is mostly used to describe organic compounds. Figure 3 projects the FTIR spectrum of acid-soluble collagen from *T. tonggol* skin at the range of 550-4000 cm^{-1}, which is in line with and very close to studies of other researchers on collagen of other fish species (Muyonga *et al*., 2004b; Liu *et al*., 2007; Yan *et al*., 2008; Kozlowska *et al.*, 2015; Silva Junior *et al*., 2015; Naderi Gharagheshlagh *et al*., 2020). Tensile frequency N-H is related to the amide group A bond. The bonds found for free tensile vibration N-H and O-H occur at the range of 3100-3400, and when N-H group of a peptide is involved in a hydrogen bond, the position shifts to a lower frequency, usually near 3300 (Yan *et al*., 2008; Naderi Gharagheshlagh *et al*., 2018). The amide A bond position in this study was found to be at 3300, indicating that NH bonds were involved in hydrogen bonds. Extracted collagen amide B bond was observed at 2929, which was related to asymmetric tension of CH2. Three main bonds of collagen were also observed. Position of the amide I bond was observed at 1633, which was related to secondary structure of the protein and was related to the tensile vibration C=O. Amide II and III bonds were observed at 1539 and 1238, respectively. This finding indicates tensile vibration C=O, flexural vibrations N-H, and C-H tension. The amide I bond, which is associated with secondary structure of the protein and the amide III bond, indicating presence of helical structure for collagen extracted from *T. tonggol* (Yan *et al*., 2008; Silva Junior *et al*., 2015; Krishnamoorthi *et al*., 2017; Naderi Gharagheshlagh *et al*., 2020).

The amino acid profile of *T. tonggol* collagen exhibited in Table 1 expressed as residual parts per 1000 residual. The results unveiled that the most abundant amino acid in *T. tonggol* skin was glycine (residual 309) followed by proline (104), hydroxyproline (69), and alanine (95) and glutamic acid (85). Glycine, hydroxyproline and proline are specific amino acids of collagen, thus indicating high content of collagen in fish skin, and it is quite obvious that glycine, alanine, proline and hydroxyproline are abundant in type I collagen. The least common types of amino acids in *T. tonggol* skin were tyrosine, histidine and hydroxylysine, all of which showed minor amounts of amino

acids in collagen. These results suggest that *T. tonggol* skin is a good source for type I collagen extraction (Sun *et al*., 2019; Jafari *et al*., 2020).

In general, glycine accounts for about one-third of all the sediments and is the major amino acid in collagen but low in other proteins (Jafari *et al.*, 2020). Examining amino acids of the collagen extracted from *T. tonggol*, a large amount of glycine was observed containing approximately 30% of total amino acids of glycine from the skin. The glycine content of *T. tonggol* skin collagen was lower than that of other species, i.e. turbot (Sun *et al.*, 2019), salmon, African catfish, Baltic cod (Tylingo *et al.*, 2016), seabass, bighead carp, *Argyrosomus japonicus* (Jafari *et al.*, 2020), albacore tuna (Hema *et al.*, 2013), and cuttlefish (Nagai *et al.*, 2001), but higher than that of *Scomberomorus guttatus*, *Rutilus frisii* (Naderi Gharagheshlagh *et al.*, 2020), *Gibelion catla*, *Cirrhinus mrigala* (Mahboob, 2015), Nile perch (Muyonga *et al.*, 2004a), channel catfish (Liu *et al*., 2007) and yellowfin tuna (Kaewdang *et al.*, 2014), thus being consonant with the results of other studies performed on sailfish (Tamilmozhi *et al.*, 2013), dog shark (Hema *et al.*, 2013), sea urchin (Nagai and Suzuki, 2000b), minke whale (Nagai *et al.*, 2008), and silver carp (Safandowska and Pietrucha, 2013).

Yan *et al*. (2008) reported that hydroxyproline content of walleye pollock skin remains at 69 per 1000, which is similar to the related results obtained in the present study on *T. tonggol* skin (residual 69). Alanine, glutamine, arginine and proline contain the highest amount of amino acids; this can be considered as findings and characteristics of collagen in this study. Unlike bighead carp (0.2) (Jafari *et al.*, 2020), Pacific cod (2) (Sun *et al.*, 2017) and turbot skin (4.7) (Sun *et al.*, 2019), extracted collagen from *T. tonggol* skin did not contain the amino acid cysteine. Cysteine has not been detected mainly in freshwater or marine sources (Zhang *et al.*, 2011). The result of this study is similar to results of other studies on the following species: *Argyrosomus japonicus*, *Scomberomorus guttatus*, *Rutilus frisii*, albacore tuna, dog shark, rohu, walleye pollock (Yan *et al*., 2008; Hema *et al*., 2013; Jafari *et al*., 2020; Naderi Gharagheshlagh *et al*., 2020). Collagen proved to have the lowest levels of hydroxylysine, tyrosine, and histidine amino acids. The amount of isoleucine obtained from *T. tonggol* collagen was low which was the same as *Argyrosomus japonicus*, bighead carp (Jafari *et al.*, 2020), salmon, Baltic cod (Tylingo *et al.*, 2016), albacore tuna and rohu (Hema *et al.*, 2013).

Level of amino acids (Amino acids include proline and hydroxyproline) vary significantly among fish species (Hema *et al*., 2013; Sotelo *et al*., 2016). Amino acids help stabilize collagen structure (Nalinanon *et al*., 2011). High content of these amino acids, especially hydroxyproline content is of great importance for them to affect functional properties as well as thermal stability of collagen. The amount of collagen amino acids in *T. tonggol* skin was found to be 173 per 1000 residual parts, which was very close to that of balloon fish (179) (Huang *et al.*, 2011), but higher than

that of Pacific cod (157) (Sun *et al.*, 2017), *Sphyrna lewini* (141.9) (Lin and Liu, 2006), salmon (161) and Boltic cod (156.5) (Tylingo *et al.*, 2016) it was, however, lower than that of turbot skin (233) (Sun *et al.*, 2019), and also lower than mammalian skin such as pigs and cows (Yan *et al.*, 2008). A group of researchers reported that fish collagen contains lower levels of amino acids than mammalian collagen, and that animal collagen levels are relevant to their habitat (Jongjareonrak *et al*., 2005; Kaewdang *et al*., 2014; Nazeer *et al*., 2014).

Type 1 collagen extracted from *T. tonggol* fish skins can influence cell growth indicated with MTT assay. Growth, proliferation and biocompatibility of human fibroblasts on fish collagen, as a natural biopolymer, showed that fish collagen like mammalian collagen could be used as available base for storing and culturing cells and in addition could replace pig and bovine collagen, which are primary sources of collagen production now. The results of this study were consistent with results of other research (Wang *et al*., 2011; Pati *et al*., 2012; Cao *et al*., 2015; El-Rashidy *et al*., 2015; Leong *et al*., 2015; Li *et al*., 2017).

Appropriate amount of collagen was successfully isolated from disposable skin of marine fish and confirmed by various tests and different analytical methods. SDS-PAGE analysis indicated that ASC product from *T. tonggol* was a type I collagen, and extracted collagen from fish skin has high viability against HFF fibroblast cells culture, which is suitable for tissue engineering. The findings demonstrate that this easy access, abundant collagen, as an alternative to its animal counterpart, can successfully be deployed in cosmetics, medical and pharmaceutical industries. Studies revealed that nutritional value of fish skin is relatively high and can be used by processing fish skin through acid-soluble technique. Fish skin which is often wasted away with the exception of its occasional use in some fish meals is abundant, available, and proper as an economic source material.

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