Extraction and characterization of type I collagen from skin of tilapia (Oreochromis niloticus) and its potential application in biomedical scaffold material for tissue engineering

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Tilapia & Aquatic origin collagen (Aqua-collagen) has been pursued as an alternative to mammalian origins. Acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) were successfully extracted from the skin of tilapia with the yield about 19–20% (basis of lyophilized dry weight), and examined for their physico-functional and structural properties. Both ASC and PSC containing \( \alpha_1 \), \( \alpha_2 \) chains were characterized to be type I collagen and had lower denaturation temperature compared to mammalian origins. PSC (low telopeptides collagen) was selected for biomedical scaffolds construction due to its low immunogenicity. SEM analysis of fish collagen scaffolds showed a wide range of pore size distribution, high porosity, and high surface area-to-volume ratios. The tilapia collagen microfiber matrix scaffolds were grafted beneath the dorsal skin in 96 mice towards tissue regeneration, with bovine collagen microfiber matrix scaffolds (Avitene™ UltraFoam™ sponge) serving as control. Biocompatibility evaluation in the dorsal tissue showed that implanted scaffolds degraded completely after 20 days with no pathological inflammatory tissue responses. These findings indicated that aqua-collagens microfiber matrix scaffolds were highly biocompatible in nature, exploring its feasibility for the development of scaffolds in tissue engineering.

1. Introduction

Collagen is one of non-branched chain fibrous proteins in the extracellular matrix (ECM) component, plays a pivotal role in maintaining tissue homeostasis, biological integrity and structural mechanics [1]. Collagen, up to 25% of the total proteins, being presence in skin, bone, muscle sheath, ligaments, cartilage and other connective tissues of animals [2], is contributing to unique physiological functions [3]. Generally, collagens are formed by polypeptide chains constituted by repeating triplets Gly-X-Y of Glycine and two other amino acids, where proline (Pro) and hydroxyproline (Hyp) are the most common ones among about 1000 total amino acids [4]. Currently, researchers have identified at least 27 variants of collagens on the basis of sequence homology and the molecular structure [5]. Type I was the most abundant and important collagen and was a natural macromolecular hydrocolloid with widespread use in the food, cosmetic, biomedical, and pharmaceutical industries because of its excellent biocompatibility and biodegradability, and weak antigenicity [6].

Type I collagen was most prevalent and found in connective tissues such as bone, skin, and cornea of the eyes [7], contains three \( \alpha \) chains such as \([\alpha_1(I)]_2, [\alpha_2(I)]_1\) and \([\alpha_3(I)]_3\). Every chain contains more than 1000 amino acids and has more than 1000 amino acid sequences of \( \alpha \) chains between different vertebrates. There is a repetition of glycine in every three amino acid sequence, such sequences make the distance of \( \alpha \) chain shortened and the space minimized, so the chains close together to form a more stable helical structure since glycine has the smallest side groups.

Marine and aquaculture capture fisheries are widely distributed in China, and they provided abundant fisheries with deep processing of aquatic products [8]. Large quantities of protein-rich fish processing by-products, accounting for as much as 40%–50% of the total weight of catch, were discard as waste [9]. Nowadays, huge quantities of by-products have been discarded which used for feed, fertilizer, even caused environment pollution [10]. On the other hand, the outbreak of bovine sponge encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD) [11], coupled...
with the reasons for religion, aqua-collagens are being considered highly attractive by the industry as an important alternative source [12]. In many countries, such as the United States, the government agencies issued specific rules to regulate the source and processing of collagen to reduce the potential risk posed by BSE [13,14]. Even though the scientific evidence does not show that collagens are BSE carrier materials, many consumers still have worried about the safety of these products [15]. Therefore, many researchers have focused on the collagen from aquatic origin, such as the skis from marine fishes, deepsea redfish [16], brown banded bamboo shark [17], some eyed on the freshwater fish, grass carp [18], ocellate puffer fish [19]. However, these papers are largely explored for food purposes; biomedical material applications are still poorly explored. The high potential utilizes of aqua-collagens have been the rational for intense research over the years.

Recent years, considering the collagen is the main component of the extracellular matrix, the application in biomedical material industries of collagen scaffolds for tissue engineering is a hot topic [20,21]. Aqua-collagens are used in various biomedical applications [22]. The number of papers on aqua-collagens used for bone tissue engineering and surgical dressing are continuous increase [23,24]. Aqua-collagen scaffolds normally exhibit excellent biocompatibility and tuning of cellular behavior. Nevertheless, compared with mammalian collagen, aqua-collagens matrix scaffolds are still insufficient on thermal stability, in vivo degradation stability, and in vivo material morphological stability: such as easily denaturation under body temperature conditions, the loss of inherent shape after absorb moisture, and rapid degradation in vivo, then the bio-function was lost [25]. Therefore, it is necessary to improve the mechanical properties of fish collagen by technical methods.

Nowadays, the common method to improve material performance is crosslinking [26]. But, the modification of collagen materials by cross linking technical methods may cause some problems. For instance, chemical treatments would lead to cytotoxicity, enhancement of calcification, and a mild inflammatory response. Physical cross-linking is inefficient than those cross-linked by chemical treatments and may destroy the collagen structures [27]. Therefore, fibrillar recombination technical method would be a novel approach. Fibrillar recombination refer to that collagens can form fiber which is similar to our body fiber by self-assembly nature [28] in the structure under suitable conditions in in vitro. After the fiber recombined, the bio-stability of the collagen scaffolds can be significantly improved.

In this work, ASC and PSC were extracted under the condition below denaturation temperature and physical-chemical and structural properties were analyzed. Besides, we provided a technical method to construct the biomedical scaffolds from type I fish skin collagen by self-assembling nature. The microfiber collagen matrix scaffolds prepared by this method owned high density and stability in vivo. This research will provide theoretical basis for the construction of collagen material and expand the application in the field of medical tissue engineering, and shedding some light on future perspectives for this material.

2. Materials and methods

2.1. Raw materials and reagents

Tilapia fish (Oreochromis niloticus) was purchased from the local fish market in Yantai, Shandong province, The People’s Republic of China. The skins were mechanically separated, and the residue of the adhering tissues was manually removed. They were washed with cold tap water, dried at 50 °C and stored at -20 °C. All reagents for extraction and isolation were of analytic grade.

2.2. Proximate composition of tilapia skin

The proximate composition of tilapia skin, including moisture, crude protein, ash, and crude fat, was determined according to the Association of Official Analytical Chemists (AOAC, 2012) methods [29]. The protein was calculated by multiplying nitrogen content with a factor 6.25. The fat was determined by Soxhlet method using petroleum ether as solvent. Moisture content was determined by the hot air oven method. The ash content was determined by using muffle furnace at 550–600 °C. The analyses were replicated three times.

2.3. Extraction of ASC

ASC was extracted from the fish skin as per reported method [30]. All of the procedures were carried out at below 10 °C with gentle stirring. Acellular environment used in the extraction process to reduce the exogenous pyrogen. The non-collagenous proteins were removed by soaking the skin with 0.1 mol/L NaOH at a solid-solution ratio of 1:20 (w/v) for 24 h. The alkali-treated skins were then washed with dH2O until the washed water became neutral. Fat was removed using 10% butyl alcohol with a sample/solution ratio of 1:20 (w/v) for 24 h, and thoroughly washed with dH2O. Extraction was initiated with addition of 0.5 M acetic acid at a sample/solution ratio of 1:50 (w/v) for 48 h, and the extract spun at 10,000 × g for 30 min at 4 °C. The supernatants were collected and stored at 4 °C. The precipitate was re-extracted in 0.5 M acetic acid with a sample/solution ratio of 1:10 (w/v) for 18 h, followed by centrifugation at 10,000 × g for 30 min at 4 °C. The supernatants obtained were combined with the first extract. The combined extracts were salted out by the method of gradient salting out, to give a NaCl concentration 0.9 mol/L. The resultant precipitates were collected by centrifugation at 10,000 × g for 30 min at 4 °C and then dissolved in 0.5 M acetic acid. The solution obtained was dialyzed (50 KD, 31 mm) against 0.1 M acetic acid for 48 h with a change of solution once per 24 h, followed by lyophilisation (LGJ-30H, China). Then, the ASC was sealed and exposed to 60Co γ-source (Delivered to Qingdao Irradiation Center), at dose rates of 6.5 and 14.5 kGy/h and irradiation exposure doses ranging from 1 to 25 kGy. Finally, ASC was obtained.

2.4. Extraction of PSC

The same quality of skin was used for the extraction of PSC. The pre-treatment process of PSC was followed the method of ASC. The pre-treated skin was solubilized in 0.5 M acetic acid containing 0.1% (w/v) pepsin for 48 h at below 10 °C with continuous stirring. The mixture was centrifuged at 10,000 × g for 30 min at 4 °C. Residue was re-extracted with 0.5 M acetic acid containing 0.1% (w/v) pepsin for 48 h, and the extract was centrifuged as described above. The combined extracts were salted out by the method of gradient salting out, to give a NaCl concentration 0.9 mol/L. Then, the supernatant was dialyzed (50 KD, 31 mm) against 0.02 mol/L Na2HPO4 for 12 h, with solution changed every 2 h in order to inactivate the pepsin. The rest of the extraction and sterilization process followed the ASC.

2.5. Extraction yield of collagen

Collagen (ASC and PSC) yield was calculated from the following equation:

\[
\text{yield (％)} = \frac{\text{weight of dried collagen (g)}}{\text{weight of dry tilapia skin used (g)}} \times 100
\]

2.6. Amino acids analysis

The ASC and PSC sample were hydrolyzed in the gaseous phase with 6 mol/L HCl at 110 °C for 24 h, respectively. The hydrolysates were analyzed on a Hitachi La Chrome liquid chromatography system (HITACHI 835–50 Amino Acid Analyzer, Tokyo, Japan).
2.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

ASC and PSC were characterized by SDS-PAGE refer the method of the Laemmli et al. [32] and Santos et al. [33], using 7.5% separating gel and 5% stacking gel. The ASC and PSC samples were re-solubilized in 0.01 M acetic acid solution (ratio 1:10 w/v) overnight under stirring (DF-101 T high power magnetic), at 4 °C [34]. Solubilized collagen sample was mixed with the same buffer (0.5 M Tris – HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v)β-ME) at 1:1 (v/v) ratio reached to 2 ml. The mixtures were heated at 100 °C in a boiling water bath for 10 min and centrifuged at 2000 x g for 10 min to remove undissolved collagen fiber. Samples (1:1) were loaded onto the polyacrylamide gel (7.5% running gel and 5% stacking gel) and subjected to electrophoresis at a constant voltage of 120 V using a Bio-Rad electrophoresis. After electrophoresis, the gel was stained with 0.05% (w/v) coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid, and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. High-molecular weight marker (Sigma, Shanghai, China) was used to estimate the molecular weights of collagen samples (ASC and PSC).

2.8 Determination of the denaturation temperature

The denaturation temperature was determined from the viscosity changes using an Ubbelohde viscosimeter refer the method of Zhang et al. [35]. ASC and PSC solution (0.1% (m/v) of collagen in 0.1 M acetic acid) were filtered through a filter funnel incubated at 5 °C for 15 min. Then the temperature was raised from 5 to 55 °C at increasing rate of 5 °C and maintained at each temperature for 10 min. The measurement was carried out three times at each point. Then, the fractional viscosity at the given temperature was calculated by the following equation:

\[ \eta_f(t) = \eta_0(t) / \eta_0(T) \]

where \( \eta_0 \) is the specific viscosity, \( \eta_0(t) = (\eta_0(t) - \eta_0(55°C)) / t_0 \) where \( t \) is the time of collagen sample solutions passing through capillary, and \( t_0 \) is the time of solvent passing through capillary at the same temperature. The denaturation temperature was determined as the mid-point temperature where viscosity changes reached 0.5.

2.9 Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of ASC and PSC were obtained according to the method of Muonyog and Cole [36]. Collagen sample was prepared with KBr (1:100), and the mixture then pressed into a slice by powder-compressing machine for subsequent IR spectrum recording. FTIR spectra were obtained from the slice using the equipment of Tensor 37 FTIR spectrometer (German, provided by BRUKER Co., Ltd., Beijing, China) with spectral range of 4000 to 400 cm\(^{-1}\) and were registered in the transmission mode with a 2 cm\(^{-1}\) resolution.

2.10 Construction of microfiber collagen matrix scaffolds

The construction of fish microfiber collagen matrix scaffolds applied the fibrillar recombination technology, refer to that collagens can forming fiber which similar to our body fiber by self-assembly nature in the structure under suitable conditions in vitro [37,38]. PSC is being recommended for biomedical applications. There are studies that claim atelocollagens, due to removal of telopeptides through pepsin digestion, present a negligible level of immunogenicity as the majority of the antigenic sites were believed to be present on the telopeptide chains [39]. Lyophilized PSC sample was dissolved in 0.1 M acetic acid by stirring at 4 °C to give a concentration of 6.0 mg/mL. Assembly process was initiated by mixing collagen solution with the same volume of Na-phosphate buffer (pH 6.8, 90 mM) including 210 mM NaCl at 4 °C in an ice bath, then the resulting mixtures were homogenized for 5 min and incubated in a water bath at 37 °C for 6 h, pH was adjusted to 7.0-7.6. Finally, the matrices were lyophilized. Then, the matrix scaffolds were sealed and exposed to 60Co γ-source (Delivered to Qingdao Irradiation Center), at dose rates of 6.5 and 14.5 kGy/h and irradiation exposure doses ranging from 1 to 25 kGy.

The bovine collagen matrix scaffolds (AviteneTM MCH, type 1 collagen) was purchased commercially (Davol Inc./C.R. Bard Inc., Warwick, RI). AviteneTM UltraFoam™ sponge, the core ingredient is microfibrillar collagen homestat (MCH), which is known as an active absorbable collagen hemostat, proven to accelerate clot formation [40]. It can effectively enhance platelet aggregation and the release of proteins to form fibrin, resulting in hemostasis. The MCH is an FDA approved medical material for in vivo application. It’s often applied for controlling bleeding in all surgical applications. Its safety and efficacy has been clinically tested over 30 years [41].

2.11 Scanning electron microscopy (SEM)

SEM was used to examine the fibrillar structure formed by fish collagen matrix scaffolds and the bovine collagen matrix scaffolds. The microfiber collagen matrices were fixed to an adhesive carbon stub and covered with gold using a sputter coater at 30 mA for 5 min [42]. SEM images were obtained at different magnifications (HITACHI S-4800 Scanning Electron Microscopy, Tokyo, Japan).

2.12 The histocompatibility tests in vivo implantation

All animal experiments were carried out in the Institute of Animal Experimentation, Shandong Quality Inspection Center for Medical Device. (Approval Number: 20170701-001). All procedures were approved by the Institutional Animal Care and Use Committee. PSC was selected to proceed with the study for evaluation of biomedical potential as component on the development of scaffolds in tissue engineering strategies as telopeptides have been associated with potential immune responses in in-vivo implantation [43]. Atelocollagen, collagen obtained after removal of telopeptides, has been investigated for a wide range of drug and gene delivery methods [39]. In order to evaluate the biological responses of the collagen matrix scaffolds, implantation tests were performed according to the guidelines for biological evaluation of the safety of biomaterials published by the China Food and Drug Administration. Ninety-six female BALB/c mice were randomly divided into three groups: fish skin collagen microfiber matrix scaffolds (group A), bovine skin collagen microfiber matrix scaffolds (group B), and those with the surgery without implants as the control group (group C). Surgical procedures were performed under sterile conditions as it could significantly affect the result of implantation. The facilities were treated with high-temperature sterilization (120 °C for 20 min).

Adsorption tests of different surgical dressings were experimented on the mice’s dorsal skin respectively, following a 12 h starvation. Surgery was performed under anesthesia with diethyl ether inhalation. 2 cm area of the ventral or dorsal skin was cleaned and shaved the site of implantation. The surface of the implant and the wound should be kept away from the hair. Briefly, the implants were inserted through a 0.5 cm incision in the lower right quadrant of dorsal, and secured to the inner dorsal wall using one non-absorbable nylon suture stitch. Finally, the mice were fed in a routine way with adequate feed and drinking water, and the whole body were under disinfection and the wounds were examined daily. During the period, the activities of the mice were carefully monitored and recorded. The graft and its surrounding tissues were harvested 3, 7, 10, 15, 20, 25, 28 and 30 days after cut off and fixed in 10% formalin for pathological observation. The microfiber collagen matrix scaffolds were recovered and the area of implantation was visually inspected for evidence of any tissue reaction or inflammation. The pieces of the muscles were fixed in 10% neutral-buffered formalin, embedded in paraffin, and then sliced with a microtome along the longitudinal axis of the implanted areas. The slices
were stained with hematoxylin and eosin, and observed with light microscopy (XF-15; Nikon).

2.13. Statistical analysis

All methods and analysis of collagen were replicated three times. The results were presented with mean ± standard deviations, and the value of P < 0.05 was used to indicate significant deviation.

3. Results and discussion

3.1. The proximate compositions of tilapia skin

The tilapia skin contained moisture (64.10 ± 1.04) as a major component followed by ash (11.70 ± 1.12), protein (21.89 ± 0.08), fat (2.27 ± 1.09) and others (0.04 ± 0.01), respectively. The present finding was in agreement with the previous report of Akter et al, analyzed proximate composition of tilapia skin and found moisture 71.73 ± 0.31%, protein 21.51 ± 1.17%, fat 1.55 ± 0.49% and ash 3.88 ± 0.44% [44]. In addition, shark and Nile perch skin contained moisture, protein, fat and ash of 61.96 and 68.4%, 24.75 and 21.6%, 71.73 ± 0.31%, protein 21.31 ± 1.17%, fat 1.55 ± 0.49% and ash 3.88 ± 0.44% [44]. But, the result was different from the eel fish skin 75.89% moisture, 90.05% protein, 1.23% lipid and 8.82% ash [47]. Brown backed toad fish skin 73.4 moisture, 90.3 protein, 1.3 lipid and 8.4 ash percent, respectively [48]. In the above studies, the protein content in eel fish and brown backed toad fish skins were comparatively higher than the tilapia skin.

Similarly, the ash content was greater than the skins of nile perch, eel fish and brown backed toadfish, but lower than the shark.Almost complete demineralization might cause the looser matrix of skin, which could be easier for collagen extraction [49]. Consequently, emulsion may have formed in the process of salting out precipitation if the supernatant contains plenty of fat, which decreasing the rate of collagen content [50]. Pal et al.found that the seafood raw materials devoid of non-collagenous proteins and minerals can be defatted with butyl alcohol treatment [51].

3.2. Yield of ASC and PSC from the skin of tilapia

The procedures were identical with the preparation of ASC and PSC, and they were successfully extracted from the skin of tilapia with the yield of 19.80% and 20.03% (basis of lyophilized dry weight), respectively. This is in agreement with literature, where the yield of collagen extraction from other aquatic species is proposed to be in the range 19–20% [52]. Among the collagen extracted compared with other species, ASCs were higher than extracted from the skin of bigeye snapper (1.59%) [53], deep-sea redfish (10.3%) [54], but significantly lower than Japanese sea-bass (40.7%), ayu (53.6%), yellow sea bream (40.1%), and horse mackerel (43.5%) [55,56]. PSCs were lower than extracted from the skin of grass carp with a yield of 46.6% [52], golden pompano skin was 64.68% [57]. Moreover, the results indicated that the use of pepsin increased the yield of collagen by 0.23%. Several researchers showed that use of limited pepsin to extract collagen from aquatic by-products increased the yield of collagen. The low yield of ASC might be attributed to the low solubility of cross-links formed through the reaction of aldehyde with lysine and hydroxylysine at telopeptide helical sites [58]. With further limited pepsin digestion, the cross-linked molecules in the telopeptide region were most likely cleaved, resulting in the increased collagen extraction efficacy [59].

3.3. Amino acid composition of collagen

The amino acids compositions of ASC and PSC from the skin of tilapia were shown in Table 1. Generally, the fish skin collagen had glycine as the major amino acid and that was nearly 23% of total amino acids (about 1/3 of total residues), which is the most abundant amino acid in type I collagen. Glycine (Gly) generally found as being third residue throughout the central region of the α chain in collagen [60]. Followed the amino acids are alanine (17.07% and 18.01%), proline (12.02% and 12.05%). Low contents of methionine, tyrosine were observed and cysteine amino acid was not detected.

Gly is the most dominant amino acid in collagen, and all members of the collagen family are characterized by domains with repetitions of the proline-rich tripeptides (Gly-X-Y) involved in the formation of the triple helix, except for the first 14 amino acid residues from the N-terminus and the first 10 amino acid residues from the C-terminus of the collagen molecules, where X is mostly proline and Y is hydroxyproline [60]. Additionally, the levels of imino acid (Pro and Hyp) are important for the structural integrity of collagen. There is no obvious difference on the imino acid (proline and hydroxyproline) content of ASC (20.03%) and PSC (19.15%), which was higher than the marine fish skin collagen (17.9% and 17.4%) [61]. The difference in imino acid content among the animals was related to the thermal stability of the collagen [62]. In particular, Hyp was believed to play a key role in the stabilization of the triple-stranded collagen helix due to its hydrogen bonding ability through its hydroxyl group.

3.4. Electrophoretic pattern of tilapia skin collagens

SDS-PAGE patterns of ASC and PSC were shown in Fig.1. Similar protein patterns were observed from PSC and ASC, which consisted of

| Amino acids     | ASC         | PSC         |
|----------------|-------------|-------------|
| Hydroxyproline  | 80.07 ± 3.99| 70.99 ± 1.28|
| Aspartic acid   | 50.26 ± 1.75| 50.21 ± 2.13|
| Threonine       | 20.18 ± 2.37| 20.15 ± 3.55|
| Serine          | 20.50 ± 2.82| 20.49 ± 1.81|
| Glutamate       | 90.96 ± 3.71| 90.73 ± 2.07|
| Proline         | 120.23 ± 1.23| 120.52 ± 0.79|
| Glycine         | 230.09 ± 15.42| 230.38 ± 9.99|
| Alanine         | 170.72 ± 8.29| 180.12 ± 8.46|
| Cysteine acid   | 0 ± 0.87    | 0 ± 0.94    |
| Valine          | 20.27 ± 1.35| 20.37 ± 1.05|
| Methionine      | 7.9 ± 0.74  | 7 ± 0.93    |
| Isoleucine      | 10.36 ± 1.04| 10.36 ± 2.35|
| Leucine         | 20.26 ± 3.93| 20.40 ± 2.04|
| Tyrosine        | 3.2 ± 1.44  | 3.5 ± 0.90  |
| Phenylalanine   | 10.86 ± 0.55| 10.84 ± 0.51|
| Lysine hydrochloride | 40.58 ± 0.33| 30.78 ± 0.77|
| Histidine       | 10.21 ± 0.81| 10.37 ± 2.28|
| Arginine        | 40.32 ± 0.65| 40.24 ± 1.06|

Table 1: Amino acids composition of ASC and PSC (residues / 1000 residues). ASC: Acid-soluble collagen extracted from the skin of tilapia; PSC: Pepsin-soluble collagen extracted from the skin of tilapia. The results are expressed as residues / 1000 total amino acid residues.

Fig. 1. SDS-PAGE patterns of collagen. 1: protein markers; 2: ASC; 3: PSC; Acid-soluble collagen extracted from the skin of tilapia; PSC: Pepsin-soluble collagen extracted from the skin of tilapia.
two α chains (α1 and α2) as the major constituents, and the band intensities of the α1 chain was approximately two-fold higher than those of the α2 chain. Additionally, high molecular weight components, β (dimers) and γ (trimers) components, as well as other cross-linked molecules with higher molecular weight, were also observed in both ASC and PSC. The result suggested that the isolated collagen from tilapia skin was characteristic of type I collagen, a heterotrimer containing two identical α1 chains and one α2 chain in the molecular form of [α1(I)]2α2(I) [63]. The molecular weights of α1 and α2 chains of ASC and PSC were approximately 110 kDa and 100 kDa. In fact, the latter exhibited an intense band at about 66 to 110 kDa being attributed to the presence of polypeptide chains. Pepsin is able to cleave the peptides localized at the telopeptide region, so almost all subunit components were converted to α chains and low molecular weight components [59]. On the other hand, sample electrophoresis was relatively clear and no impurity bands appeared. The results show clearly that the protein maintained its original structure, while the PSC gave extra bands showing the blunted staining normally attributed to degraded or partially hydrolyzed materials. These findings were in accordance with the previous report that the molecular weight of collagen from Spanish mackerel skin extracted with the aid of pepsin decreased slightly when compared with ASC [29].

3.5. Thermal denaturation temperature of collagen from the skin of tilapia

It is known that the triple helix structure of collagen organized by hydrogen bonds can be converted into the random coil configuration by the process of thermal depolymerization, which is accompanied by changes in physical properties, such as viscosity, sedimentation, light scattering, and optical activity [64]. Therefore, viscosity measurements are usually used to investigate the thermal stability of collagen.

The thermal denaturation temperature (T_d) of tilapia fish skin collagen was calculated using the thermal denaturation curves shown in Fig. 2. T_d is the temperature at which the triple-helix structure of collagen deforms to a random coil structure [65]. ASC and PSC showed transition curves with maximum denaturation temperatures of 34.5 °C and 30.0 °C, respectively, which is higher than that of type IV collagen from human placenta (28.5 °C). The result was corresponding to previous research result with the number of imino residues primarily formed by pyrrolidine rings of proline and hydroxyproline and hydrogen bond of hydroxyproline hydroxyl groups [66]. Apparently, the removal of telopeptides by pepsin was leading to unstable samples after freeze-drying, promoting their thermal structural change upon heating. Compared to the transition temperatures of the collagens from other fish skins, such as skins of carp (31.7 °C) [22], and higher than the brown backed toadfish (28 °C) [70], ocellate puffer fish (28 °C) [23], bigeye snapper (30.4 °C) [58]. The thermal stability of protein showed a direct positive correlation with imino acid contents [67-69] (proline and hydroxyproline). T_d of collagen from the skin of E. macrura was close to that of pig skin collagen (37 °C) [70]. Furthermore, a higher cross-linkage of marine eel fish skin collagen more likely contributed to the higher T_d of both ASC and PSC. Therefore, collagen modification will be a hot topic in the future.

3.6. Fourier transform infrared spectroscopy

FTIR spectra in the range of 4000 – 400 cm⁻¹ of ASC and PSC are presented in Fig. 3. Table 2 shows that the distribution of infrared spectroscopy of ASC and PSC. The secondary structure of proteins was closely related to the different types of hydrogen bond, and through Fourier Transform Infrared (FTIR) spectroscopy, we can obtain almost all the infrared spectra of biological materials under various conditions, to be one of the most efficient methods in the study of proteins hydrogen bond. In general, the absorption of amide A band is associated with the N–H stretching vibration occurs in the range 3400–3440 cm⁻¹, and when the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequency [71,72]. The amide A bands of ASC and PSC were at 3340.11 cm⁻¹ and 3424.96 cm⁻¹, respectively. The results indicated that more NH groups of ASC was involved in hydrogen bonding than those of PSC. – C=O symmetric stretching vibration absorption band of amide B was at 2920-2944 cm⁻¹. The amide B bands of ASC and PSC were at 2927.41 cm⁻¹. The wavenumbers of the amide I, amide II and amide III bands are directly associated with the configuration of collagen [73]. The absorption of the amide I band produced by C=O stretching vibration was at 1600-1700 cm⁻¹. The amide I bands of ASC and PSC were found at 1650.77 cm⁻¹ and 1646.91 cm⁻¹. This result was showed that the partial removal of telopeptides by pepsin during the extraction of PSC might affect the loss of reactive amino acids. The amide II bands of ASC and PSC were found at 1546.63 cm⁻¹, resulting from N–H bending vibration coupled with CN stretching vibration [71]. The amide III bands of ASC and PSC were

![Fig. 2. Thermal denaturation curves of ASC and PSC from tilapia skin. ASC: Acid-soluble collagen extracted from the skin of tilapia; PSC: Pepsin-soluble collagen extracted from the skin of tilapia.](image)

![Fig. 3. FTIR pattern of ASC and PSC. ASC: Acid-soluble collagen extracted from the skin of tilapia; PSC: Pepsin-soluble collagen extracted from the skin of tilapia.](image)

| Region     | Peak wave number (cm⁻¹) | Assignment          |
|------------|-------------------------|---------------------|
| Amide A    | 3440.11                 | NH stretch coupled with hydrogen bond |
| Amide B    | 2927.41                 | CH₃ symmetrical stretch |
| Amide I    | 1650.77                 | C = O stretch/hydrogen bond coupled with COO⁻ |
| Amide II   | 1546.63                 | NH bend coupled with CN stretch |
| Amide III  | 1241.93                 | NH bend coupled with CN stretch |
found at 1241.93 cm\(^{-1}\). The triple helical structures of ASC and PSC were confirmed from the absorption ratios between amide III and 1450 cm\(^{-1}\) bands, which was 1.05 and 1.04 [73], pointing out therefore that the integrity and the composition of the polypeptide chains have been kept.

3.7. Construction of microfiber collagen matrix scaffolds

From the above results concerning the higher yield and characterization of the PSC extracts, conjugated with the potential low immune responses upon in-vivo implantation when removed the telopeptides [74], this material was selected to proceed with the study for evaluation of biomedical potential as component on the development of implanted scaffolds for histological analyses in tissue engineering strategies. Additionally, there are studies that claim atelocollagens, due to removal of telopeptides through pepsin digestion, present a negligible level of immunogenicity as the majority of the antigenic sites were believed to be present on the telopeptide chains [39].

ThePSC was reconstituted to fibrils for the preparation of microfiber collagen matrix scaffolds. The bovine collagen matrix scaffolds (Avitene™ MCH, type I collagen) was purchased commercially (Davol Inc./C.R. Bard Inc., Warwick, RI). Scanning electron microscope images were made to observe the insert structure of microfiber collagen matrix scaffolds. The scaffold displayed a homogenous structure with regular network pores composed of fine linear fibers (Fig. 4 a, b). The tilapia collagen matrix scaffolds showed a porous structure composed of lamellae networks, -from the interconnectivity between pores, it may be exhibit good absorption characteristics, while the bovine skin microfiber collagen matrix scaffolds present a small patchy network structure (Fig. 4c, d). Other architectural features (fibril shape, wall morphology, and interconnectivity) between collagen fibers are reported to influence cell behaviors like cell seeding, cell adhesion, growth, migration, differentiation, gene expression, mass transport, and tissue formation [75].

3.8. The histocompatibility and tissue adaptation examinations

At 3–7 days, both the microfiber collagen matrix scaffolds of tilapia skin and bovine skin induced mild inflammatory responses. However, the control group was surrounded by fibrous tissue, including fibroblasts, macrophages, and a few neutrophils without any muscle necrosis, and a mild inflammatory response was observed (not shown in the picture). The inflammatory area decreased at 15 days. When scaffolds implanted at 20d, macroscopic inspection of the implantation site revealed no pathological inflammatory tissue responses to the collagen dorsal tissue implantation. According to the histological section research (Fig. 5), connective tissue matrix increased within the microfiber collagen matrix scaffolds implant pore spaces during the implantation period under investigation. The microfiber collagen matrix scaffolds of tilapia skin and bovine skin were first decreased into little pieces in 15 days. The implanted scaffolds can be completely degraded and absorbed after 20 days without redness and swelling occurred around the implantation site (data was not shown). This might be attributed to the combined effects of protease, collagenase and lysozyme.

4. Conclusion

There is a large quantity of tilapia skin, a by-product of processing, which could serve as a valuable source for collagen extraction and utilization. The present results show that there was no notable difference in the physicochemical properties of tilapia skin ASC and PSC. Both collagens are typical I collagens. The FTIR analyses indicate that their triple helical structure remained intact following extraction. The present study revealed the potential of aqua-collagen as an alternative to mammalian collagen in biomedical uses. Collagen microfibrillar structures into matrix scaffolds by its self-assembling nature gives the possibility to produce new biomaterials with potential applications in tissue engineering. There were no morphological differences observed between microfibres of tilapia and bovine skin collagen matrix implanted into mice tissue. Indicating that the tilapia skin collagen scaffolds can be valuable biomaterials for tissue regeneration. Overall, the findings suggest that aqua-collagen may be suitable for the development of implantable materials.

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Fig. 5. HE-stained histological of implanted sites in dorsal skins. A: microfiber collagen matrix scaffolds of tilapia; B: Avitene™ UltraFoam™ sponge (bovine collagen scaffold); C: control group. Original magnification: ×100.

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