

The Characterization of Acid Soluble Collagen from Sheep Tail Tendon

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ABSTRACT

The sheep (*Ovis aries*) tail tendons are the major by-products after being slaughtered for food consumption. A tendon is a powerful band of fibrous connective tissue that is composed of parallel bundles of collagen fibers and connects muscle to bone, due to the transmit forces and tolerate tension during muscle contraction. The tendon collagen structure is found as the main molecule of dense fibrous tissue and forms approximately 70% of dry weight. The collagen type is largely composed of Type I (60%) and other types. Type I collagen is by far the most abundant molecules in vertebrates, and it is a particularly mechanical scaffold in bone, skin, and connective tissue. This study was conducted to extract and characterize acid-soluble collagens (ASC) from sheep tail tendons. The tendon collagen was confirmed as collagen by different physicochemical techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Zeta Potential Analyzer, and Fourier transform infrared (FTIR) spectra analysis. The yield of type I ASC from sheep tail tendon was about 9.7% on the dry weight of raw material. The collagen's α_1 , α_2 , and β chain bands are observed in SDS-PAGE for both ASC and pepsin soluble collagen. The zeta potentials of ASC had a positive charge when pH from 2 to 4.5, while the negative charge in pH range from 6 to 11. But the electric potential of ASC was zero at pH 5. The results of FTIR spectra analysis detected the presence of triple superhelical structure in acid-soluble collagens, presenting isolation procedure did not interrupt the triple helical structure from the sheep tail tendon. Therefore, the study showed that it is a potential reference for collagen extraction and application of sheep tendon tails.

Keywords: Tendon, Type I collagen, Acid-soluble collagen, Pepsin soluble collagen

1. INTRODUCTION

The most abundant protein in connective tissue is collagen. Collagen is largely present in the mammalian body such as skin, bone, and tendon. Tendons are functional connective tissue, have a rich extracellular matrix, while the cellular structure constitutes approximately 20% of total tissue volume, the remaining cells form 80% of the extracellular matrix. The cellular matrix is mainly composed of

collagen fibers and other connective proteins such as proteoglycan, elastin, and other proteins [1, 2].

The main structure of tendon fibers is collagen, which makes up about 70% of the dry weight. Collagen molecules are stabilized by hydrogen bonds and intermolecular bonds, which are about 280 nm long, with a molar mass of 360000 Da. There are 29 collagen types that have been identified, varying considerably in their amino acid composition, amino acid sequence, spatial structures, and function [3].

Type I collagen is mostly contained (60%) in the tendon compare with other types, namely type III, IV, V, and VI [4]. Among all these different types, type I collagen is the most common in mammals and fish, widely distributed in bones, skins, and tendons.

Heterotrimers of two identical $\alpha 1(I)$ (~126 kDa) and one $\alpha 2(I)$ (~110 kDa) chains are the dominant isoforms of type I collagen. Collagen three chains, evolve a triple right-handed superhelical structure stagger one another manage in left-handed helix form by itself. The triple helical structure is flanked by two short N- and C- peptides, called telopeptides, which determine the intermolecular interactions and cross-link [3, 5, 6].

A major abundant component of functional connective tissue protein is collagen type I from tissues such as bone, skin, or tendon extracted by acid using acetic or citric acid large quantity [7] enzymatic digestion using pepsin [8], neutral salt extraction using disodium phosphate [9] organic extraction using urea and combinations of these methods [10]. Collagen has been widely studied as a natural material in the field of wound dressing [11] tissue engineering [12], drug delivery, health, and beauty [13].

In the present study, we extracted a long-term storable pre-purified collagen sponge and ready-to-be-transformed acid soluble (ASC) collagen and pepsin soluble collagen (PSC) from the Mongolian sheep tail tendon. Type I collagen was fractioned from the pre-purified collagen sponge. Generally, pepsin is used to interrupt telopeptides without destroying the triple helix structure of collagen. However, we used PSC only for the gel electrophoresis system, further use other results exclusive to ASC.

2. EXPERIMENTAL

2.1. Preparation of Sheep Tendon

The fresh sheep tail obtained from a butcher shop was transported to the laboratory on ice. Sheep tail bone with tendon was soaked in the distilled water to wash out resident cells and blood content at 5 minutes, and hand-dried to remove excess water before isolation of tendons. The tail bones were cut off using sterile surgical clamps and isolated 1.651 g wet tendons from between bone and muscles of sheep tail. Tendons are collected in PBS solution, transferred to the beaker with pure acetone for 5 minutes. After transferring the fibers to 70% (v/v) isopropanol for another 5 min. The isolated tail

tendons were stored at -20°C until used and kept at 4°C for 24 h to soften in PBS for collagen extraction.

2.2. Acid Soluble and Pepsin Soluble Collagen Extraction

2.2.1. Acid Soluble Collagen

Sheep tail tendons were purified with 0.02 N acetic acid solution [7]. A total of 0.02 N acetic acid was used to extract acid-soluble collagens from sheep tendons with a solid/solvent ratio of 1:10 (w/v) for 2 days. Poured the solution into the plastic container to form a liquid film 5 cm in thickness and freeze the container at -20°C until the solution is completely frozen in 3 days. The frozen collagen-containing solution was lyophilized using a freeze dryer (SJIA-10 N 50A, Ningbo Shuanjia, China). 1.488 g of dried collagen sponge dissolved in 140 mL of cold 0.02 N acetic acid solution for fractioning type I collagen. The solution was centrifuged at 4°C at 6000 rpm for 45 min. After centrifugation, collected the supernatant in the beaker on ice and de-aerated. The collagen solution was poured for the dialysis onto 14 kDa molecular weight cut-off tube (D9402, Sigma Aldrich, US) in 0.02 N acetic acid solution, and gently stirred for 1 h at 4°C . After transferred the dialysis bags from the acetic acid beaker to the chloroform beaker for sterilization, stirred gently for 1 h in 4°C . Discarded chloroform solution and transferred dialysis bags in 0.02 N acetic acid solution; stirred 7 days in 4°C . Finally collected sterile 15 mL tube from dialysis bags and collagen solution prepared aliquots 1.5 mL tubes, always stored at 4°C . Type I collagen yield was determined the final concentration by Bradford protein assay.

2.2.2. Pepsin Soluble Collagen

Sheep tail tendon collagen was extracted in 0.02 N acetic acid containing pepsin (P7000, Sigma Aldrich, US) with a solid/solvent ratio of 1:10 (w/v) at 4°C for 14 days and 24 h with stirring. The PSC was then treated as stated above for the extraction of ASC to obtain PSC. PSC was only used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis to compare ASC.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis of proteins was performed as previously described (Laemmli 1970). Samples were dissolved in 5% SDS. Protein concentration was determined by the Bradford protein assay.

A volume of collagen solution was added with the same volume of loading buffer and boiled for 20 min. Electrophoresis was performed using a 7.5% resolving gel (BlueGel™ electrophoresis system, mini PCR Biotechnology Company, California, USA). A high molecular weight marker was obtained from Thermo Fisher Scientific (Waltham, USA, #26630). After electrophoresis, Coomassie Brilliant Blue R-250 (Amresco, USA) was used to stain overnight and then destained.

2.4. Zeta Potential Analysis

Samples were re-dissolved completely in 0.5 M L⁻¹ acetic acid to obtain a final concentration of 0.05% (w/v). Zeta potential was measured using Zeta Potential Analyzer (Zeta potential meter ZetaCAD, CAD instruments, France).

2.5. Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The secondary structure of both ASC was obtained by FTIR spectra at 25°C using an FTIR spectrometer (IR Prestige 21 Shimadzu, Kyoto, Japan).

3. RESULTS AND DISCUSSION

3.1. Yield of Sheep Tendon Collagen

Acid soluble collagen (ASC) was extracted from the sheep tail tendon. The yield of ASC 0.144 g (9.7%) was extracted from 1.651 g (dry weight) of the tendon. ASC yield was shown in Table 1.

Table 1. Yield of type I collagen from sheep tail tendon

Indication of tendon	Amount
Tendon weight (g)	1.651
Moisture (%)	9.860
Tendon dry weight (g)	1.488
Type I Collagen (g)	0.144
Yield (%)	9.7

3.2. SDS-PAGE Patterns and Protein Identification of Sheep Tail Tendon Collagen

The SDS-PAGE analysis of ASC and pepsin treated soluble tendon collagen (PSC) from sheep tail tendon was shown in Figure 1. Both ASC and PSC from the sheep tail tendon had similar electrophoretic

patterns but some differences in molecular weight when compared with type I collagen. Sheep tail tendon collagen was composed of two different α -chains ($\alpha 1$ and $\alpha 2$). The band intensity of $\alpha 1$ -chain was approximately double heavier than that of $\alpha 2$ -chain for both ASC and PSC. The β -chain commonly, which belong to high molecular weight comparing to α -chains components, were also observed [14]. The electrophoretic patterns of sheep tendons collagen comparable to type I collagen from other sources such as rat tail collagen, bone, and skin collagen [15, 16, 17]. Thus, it was logical to infer that sheep tail tendon collagen was mainly a type I collagen. The molecular weights of $\alpha 1$ and $\alpha 2$ chains of ASC were ~115 and ~129 kDa, respectively, and those of PSC were ~110 and ~125 kDa, respectively. In addition, the molecular weight of both $\alpha 1$ and $\alpha 2$ chains of PCS were lower than compared with ASC, that result counterpart with study result from Gao Ling *et al.*, [3]. Therefore, our result is pretty similar to collagen extracted from the bone using other methods.

Type I collagen contains a triple helical structure, namely the collagen domain, and two telopeptides (N- and C-), namely non-collagenous domains [18].

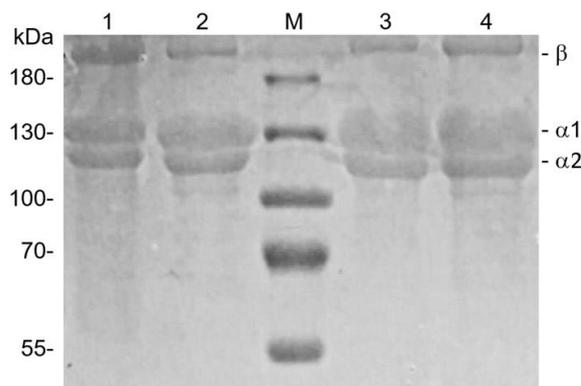


Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of collagen from sheep tail tendons. Lane 1- acid-soluble collagens; lane 2 - acid-soluble collagens: $\alpha 1$, $\alpha 2$ chains (~115 kDa and ~129 kDa) and dimeric β chain of α chains (~240 kDa); lane 3- pepsin treated tendon collagen for 14 days; lane 4- pepsin-treated tendon collagen for 24 hours: $\alpha 1$, $\alpha 2$ chains (~110 kDa and ~125 kDa) and dimeric β chain of α chains (~240 kDa). M Page Ruler Prestained protein ladder 10 to 180 kDa, Thermo Fisher Scientific)

3.3. Zeta Potential Analysis

The sample was re-dissolved completely in 0.02 N acetic acid to obtain a final concentration of 0.05% (w/v). The collagen was positively charged at pH=3 to 5, whereas negatively charged at pH=6 to 11 [3]. Thus we considered that the collagen zeta potential, isoelectric point is pH=5 causing collagen zeta potential meaning near to zero.

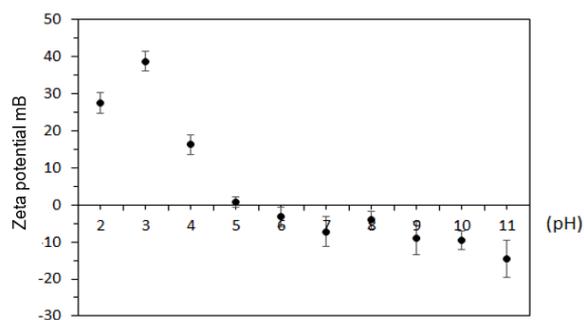


Figure 2. Zeta potential of acid soluble sheep tail tendon collagen

However, collagen clearly charged and solubility was increased when pH meaning not equal to isoelectric point Figure 2.

3.4. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectrums of sheep tail tendon collagen in the range of 400–4000 cm^{-1} were shown in Figure 3. The absorption characteristic of amide A is described as N-H stretching vibration. Generally, absorption in the wave number range of 3400–3440 cm^{-1} means a free N-H group stretching vibration. Amide A bands of ASC from sheep tail tendon were indicated at a wave number of $\sim 3200\text{--}3500$ cm^{-1} , which suggested that N-H groups of sheep tail tendon collagen contained rich hydrogen bonds. In addition, absorption in the wave number range of amide B bands found $\sim 2100\text{--}2140$ cm^{-1} [19]. The amide B band was found the asymmetrical stretch of CH_2 , and the wave number of the amide B bands of sheep tendon collagen was at ~ 2100 cm^{-1} means sheep tail tendon collagen included amide B band.

The amide I band was mostly associated with the stretching vibrations of the carbonyl group ($\text{C}=\text{O}$ bond) through the polypeptide backbone, the characteristic frequencies ranging from 1600 to 1700 cm^{-1} [20]. Amide I band is barely changed by the conformation of the side-chain, also it is largely applied to analyze the secondary structure of proteins [21]. The maximum absorbance in the amide I band of different secondary structures in the order: α -helix,

1645 to 1659 cm^{-1} ; β -sheet or non-stranded extended structure, 1620–1640 cm^{-1} ; β -turn, 1660–1700 cm^{-1} ; irregular structure, 1640–1644 cm^{-1} ASC from sheep tail tendon were found at the wave number of 1600 cm^{-1} , which suggested that the secondary structure of sheep tail tendon collagen was α -helix. The amide II band characteristic frequencies ranging from 1500–1600 cm^{-1} [22]. Our FTIR spectrums result is comparable to the result of the type I collagen spectrum obtained for membranes washed with Milli-Q water (hydrated) [23].

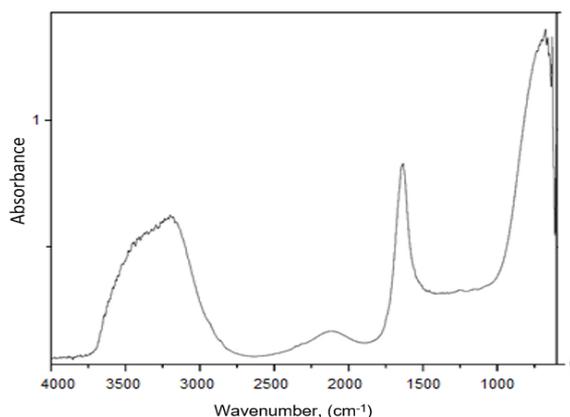


Figure 3. Fourier transforms infrared spectra of acid-soluble collagen

Type I collagen is the major constituent of different tissues such as skin, tendon, bone, ligament, and cornea [24, 25]. It has been used extensively for biomedical applications in medicine, cosmetology, pharmacology, molecular biology, and tissue engineering. Most of the collagen type I for biomedical applications is extracted from mammalian sources, especially bovine or porcine skin and tendon as they have the most similar properties to humans [16]. Collagen has been extensively used to study primary and stem cell-based experiments in 3D scaffolds such as a gel. Esdale and Brad were reporting a method to produce a cell-embedded collagen gel [26]. Many studies were reported to use rat tail collagen for cell culture scaffold, furthermore becoming commercial rat tail collagens [27, 28, 29]. Apparently, sheep tail tendon collagen must be able to use the cell culture 3D matrix.

4. CONCLUSION

In this study, we were extracted ready-to-use sterile collagen according to the method of Navneeta Rajan *et al.*, and which can be used in cell culture studies such as stem and primary cell culture [7]. Therefore, the type I collagen yield was 9.7%, and the collagen triple helix was proved by SDS-PAGE

and FTIR. Fortunately, the extraction method involves a freeze-drying step for improved long-term storage and reproducibility of future collagen solutions. In the future, we will be able to compare collagen from different sources of collagen and different collagen extraction methods.

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