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RESEARCH ARTICLE

The characterization of acid and pepsin soluble collagen from ovine bones (Ujumuqin sheep)

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Abstract

Ovine bones are the major by-products after slaughtered. The present study was conducted to extract and characterize acid soluble collagens (ASC) and pepsin soluble collagens (PSC) from ovine bones (Ujumuqin sheep). Ovine bones collagen were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) as type I collagen. The results of Fourier transform infrared (FTIR) spectra analysis testified the existence of triple superhelical structure in both ASC and PSC, showing pepsin did not disrupt the triple helical structure of ovine bones collagen. Glycine, accounting for one-third of total amino acids, was the major amino acid for ovine bones collagen. Higher imino acid content was responsible for higher thermal denaturation temperature of ovine bones collagen compared to fish collagens. The isoelectric point of ASC was lower than PSC due to the higher content of acidic amino acids. Therefore, this study provides the potential reference for collagen extraction and application of ovine bones by-procduct.

Keywords: ovine bones, collagen, characterization, amino acid composition, denaturation temperature

1. Introduction

Bones are the major by-products after sheep are slaughtered. With the rapidly increasing production of sheep in recent years, a large sum of ovine bones are being produced in China (Zhou *et al*. 2012; Toldrá *et al*. 2016). It was estimated that over 400 000 tons ovine bones were produced in China annually. However, only a small fraction is used as animal feed with low-market-value, but the rest majority ends up in landfills, resulting in wasting of resources and causing environmental problems (Toldrá *et al*. 2016). Ovine bones are rich in collagen, which has been widely used in food, pharmaceuticals, cosmetic and tissue engineering (Veeruraj *et al*. 2015). Therefore, the research and utilization of collagen from ovine bones not only improve environmental quality, but also enhance additional value and economical benefit of bones.

Collagen, as the most abundant structural protein in living bodies, constitutes approximately 30% of the total proteins (Liu *et al*. 2015). Currently, at least 29 collagen types have been identified, varying considerably in their amino acid composition, amino acid sequence, spatial structures and function (Liu *et al*. 2012; Li *et al*. 2013; Chen *et al*. 2016). Among all these different types, type I collagen is the most

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common in mammals and fish, widely distributed in bones, skins and tendons. Type I collagen is composed of two identical α1 chains and one α2 chain in the molecular form of $[\alpha1(I)]_2\alpha2(I)$. Three chains, existing in left-handed helix form by itself, stagger one another to develop a triple right-handed superhelical structure. The triple helical structure is flanked by two short N- and C- peptides, called telopeptides, which determine the intermolecular interactions and cross-link. -Gly-Xaa-Yaa- repeating triplets are the main feature of the helix domain, and the Xaa and Yaa positions are predominant proline and hydroxyproline residues, respectively (Engel and Bächinger 2005; Shoulders and Raines 2009). Traditionally, collagen mainly came from cows and pigs, which were suffered from religious restrictions. In recent years, collagen from fish by-products also studied extensively (Karayannakidis *et al*. 2014; Liu *et al*. 2014; Chen *et al*. 2015; Kittiphattanabawon *et al*. 2015; Veeruraj *et al*. 2015), but the practical application of fish collagen was limited because of its low thermal stability. Ovine collagen is religiously and traditionally allowed to be used in almost all regions and by multifarious religionary groups compared to collagen from pigs. In addition, ovine bones collagen is expected to be type I collagen and has higher thermal stability compared to fish collagen as sheep are mammals. However, the structure characteristics and properties of collagen from ovine bones are not well characterized so far.

Pepsin is usually used to facilitate extraction of collagen by cleaving the telopeptides and doesn't destroy the triple helical structure. Therefore, for better application of ovine bones collagen, the present study extracted acid soluble collagens (ASC) and pepsin soluble collagens (PSC) from ovine bones. Structures and properties of ovine bones collagen were analyzed by protein identification, amino acid composition, secondary structure analysis and determination of denaturation temperature, etc., and further provided a synchronous comparison of two bones collagens due to different extraction methods.

2. Materials and methods

2.1. Preparation of ovine bones

The fresh ovine bones (Ujumuqin sheep aged 8-month-old) were obtained from a slaughterhouse in Xilingol League, Inner Mongolia Autonomous Region of China. Bones were transported to laboratory on ice. The two terminals of bones were cut off using a saw and bone marrows were removed. Bones were then broken into small pieces (0.5 cm in length) and shattered using an ultra-high speed grinder (FW100, Taisite, Tianjin, China). The shattered bones were freeze-dried and stored at –20°C until used.

Ovine bones were pretreated at 4°C. The prepared bones were first soaked in 0.1 mol L^{-1} NaOH with a sample/alkali solution ratio of 1:10 (w/v) for 48 h to remove non-collagen proteins, and NaOH solution was changed every 12 h. Then 10 volumes of 10% (v/v) butyl alcohol was used for 72 h to remove fat, which the butyl alcohol solution was replaced every 12 h. After being washed with distilled water, the defatted bones were decalcified with 10 volumes of 0.5 mol L^{-1} EDTA-2Na solution (pH=7.5) for 5 days, and the solvent replaced every 12 h. Bones were freeze-dried and ready for collagen extraction.

2.2. Collagen extraction

A total of 0.5 mol L^{-1} acetic acid was used to extract ASC from sheep bones with a solid/solvent ratio of 1:10 (w/v) for 3 days. The extracting solution was centrifuged at 10 000×g for 30 min at 4°C, and the pellet was extracted again under the same conditions. The two filtrates were combined together. NaCl with a concentration of 2.0 mol L^{-1} was used to salt out collagen. The sequent precipitate was centrifuged at 10 000×g for 30 min. The sediment was redissolved in 0.5 mol L^{-1} acetic acid, then salted out and centrifuged again. The sediment was suspended in 0.5 mol L^{-1} acetic acid and dialysed against 0.1 mol L^{-1} acetic acid for 1 day and ultrapure water for 2 days with dialysate changed every 12 h and lyophilize.

For the extraction of PSC, sheep bones were soaked in 0.5 mol L^{-1} acetic acid containing porcine pepsin (Amresco, USA) (20 U g^{-1} bones) with a solid/solvent ratio of 1:10 (w/v) at 4°C for 3 days with stirring. The extract was then treated as stated above for the extraction of ASC to obtain PSC.

2.3. UV absorption spectrum

Samples were redissolved in 0.5 mol L^{-1} acetic acid and subjected to a wavelength scan from 200 to 350 nm using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) at room temperature. The scan speed was 50 nm per min.

2.4. 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 BCA assay (Pierce Chemical Co., Rockford, IL, USA). A volume of collagen solution was added with a same volume of 2× loading buffer and boiled for 5 min. Electrophoresis was performed using 7.5% resolving gel (Mini-PROTEAN Tetra electrophoresis system; Bio-Rad, Hercules, CA, USA). High molecular weight marker was obtained from Thermo Fisher Scientific (Waltham, USA, #26630). After electrophoresis,