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Two-dimensional infrared spectroscopic study on the thermally induced structural changes of glutaraldehyde-crosslinked collagen

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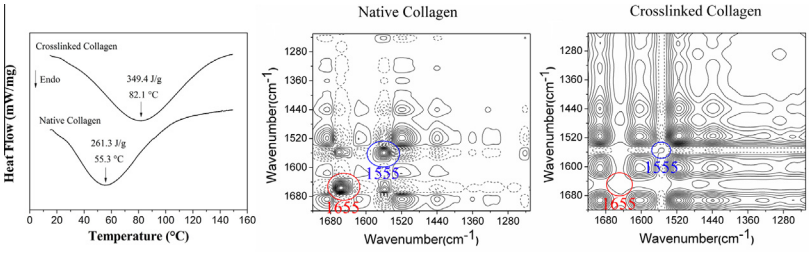
HIGHLIGHTS

- Thermal stability of native and crosslinked collagens was measured.
- FTIR spectra of native and crosslinked collagens at various temperatures were tested.
- 2D correlation analysis was applied in thermally induced collagen structural changes.
- Collagen triple helix was reinforced due to the introduction of crosslinking bonds.

GRAPHICAL ABSTRACT



The values of T_d (°C) and ΔH (J/g) increased.

The auto-peak at 1655 cm^{-1} was absent. The intensity of the auto-peak at 1555 cm^{-1} was diminished.

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ABSTRACT

The thermal stability of collagen solution (5 mg/mL) crosslinked by glutaraldehyde (GTA) [GTA/collagen (w/w) = 0.5] was measured by differential scanning calorimetry and Fourier transform infrared spectroscopy (FTIR), and the thermally induced structural changes were analyzed using two-dimensional (2D) correlation spectra. The denaturation temperature (T_d) and enthalpy change (ΔH) of crosslinked collagen were respectively about 27 °C and 88 J/g higher than those of native collagen, illuminating the thermal stability increased. With the increase of temperature, the red-shift of absorption bands and the decreased A_{III}/A_{1455} value obtained from FTIR spectra indicated that hydrogen bonds were weakened and the unwinding of triple helix occurred for both native and crosslinked collagens; whereas the less changes in red-shifting and A_{III}/A_{1455} values for crosslinked collagen also confirmed the increase in thermal stability. Additionally, the 2D correlation analysis provided information about the thermally induced structural changes. In the 2D synchronous spectra, the intensities of auto-peaks at 1655 and 1555 cm^{-1}, respectively assigned to amide I band (C=O stretching vibration) and amide II band (combination of N—H bending and C—N stretching vibrations) in helical conformation were weaker for crosslinked collagen than those for native collagen, indicating that the helical structure of crosslinked collagen was less sensitive to temperature. Moreover, the sequence of the band intensity variations showed that the band at 1555 cm^{-1} moved backwards owing to the addition of GTA, demonstrating that the response of helical structure of crosslinked collagen to the increased temperature lagged. It was speculated that the stabilization of collagen by GTA was due to the reinforcement of triple helical structure.

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1. Introduction

Collagen, the major structural component in connective tissue, has been widely used as drug carriers for tissue regeneration [1], injectants for tissue augmentation [2], implanted scaffolds for artificial organs [3] and sponges for wound healing [4] because of its advantages including low antigenicity, high biocompatibility, controlled biodegradability and good bioresorbability. These excellent bio-functionalities of collagen mainly owe to its typical right-handed triple helix, which is formed by three left-handed α-chains without interruption of the repeated tripeptide Gly-X-Y sequences [5,6]. Nevertheless, this structural hierarchy is susceptible to the damage and denaturation caused by a number of factors including heat, enzyme, strong acids or alkali and heavy metals, among which the denaturation due to heat treatment is always encountered [7]. In general, the most commonly used method to improve the thermal stability of collagen is the introduction of exogenous crosslinking bonds due to its plentiful functional groups such as —COOH, —NH_2, —OH and —CONH [8].

Glutaraldehyde (GTA) is a preferred crosslinking agent used to stabilize collagen against thermal and enzymatic degradation because of its low cost, high reactivity and high solubility in aqueous solution [9]. It is generally assumed that the primary reaction of GTA with collagen is the interaction between aldehyde groups of GTA and ε-amino groups of lysine or hydroxylysine of collagen, resulting in the formation of a Schiff base type compound [10,11]. With regard to the thermal stability of GTA-crosslinked collagen, the previous literatures reported that the shrinkage temperature (T_s) and denaturation temperature (T_d) of collagenous materials in solid state (such as rat tail tendon [12], dermal sheep collagen [10], pericardial tissue [13] and membrane [14]) increased by 20–30 °C due to the presence of GTA. The uneven distribution of GTA molecules through collagen matrix (solid state) due to the incomplete penetration can lead to an inadequately crosslinking in the inner core, which is a hidden danger in practical utilization [13,15]. As for the crosslinking reaction of collagen solution, this disadvantage does not exist [16–18]; nevertheless, the concentration of collagen solution is an important consideration. If the concentration is too low, the properties (such as thermal stability and mechanical strength) of collagen had no significant improvement; and it is inconvenient to convert the diluted collagen solution to solid and gel. If the concentration is too high, the inhomogeneous reaction will occurs because collagen and GTA molecules cannot be mixed uniformly in a short time. For these reasons, the collagen solution with a concentration of 5 mg/mL was prepared by the authors and the physicochemical properties (such as thermal stability and viscoelastic behavior) of the collagen solution crosslinked with various amounts of GTA [GTA/collagen (w/w) = 0–0.5] were investigated [17]. The results showed that at low GTA amounts [GTA/collagen (w/w) < 0.1], the crosslinking degree increased largely from 0% to 67.24%, while the T_d value did not change visibly and the fluidity of collagen samples was still retained. When the ratio of GTA to collagen exceeded 0.1, although the crosslinking degree only increased by 15.05%, the crosslinked collagen solution at the GTA/collagen ratio of 0.5 (crosslinking degree of 82.29%) displayed a clear loss of flow and a sudden rise (~2.0 °C) of the T_d value compared to the native collagen solution, illustrating that the collagen solution was converted into a gel with mature network observed in atomic force microscopic image.

Though it is known that GTA stabilizes collagen against thermal degradation and the early literatures reported that the stability of collagen was related to crosslinking degree, the crosslinking bonds (covalent bonds) among collagen molecules stay intact during the thermal denatured process [19]. In order to make a clear understanding of the stabilization of collagen solution by GTA, it is crucial to investigate the difference on the thermally induced structural changes between native and crosslinked collagens. Wang et al. [20] pointed out that the loss of α-helix structure involving in the thermally induced transition of ribonuclease A resulted in the collapse of secondary structure using two-dimensional (2D) correlation analysis, an effective tool to study the structural changes induced by the given external perturbation.

Therefore, in the present work, the crosslinked collagen was prepared via the reaction of collagen solution (5 mg/mL) with GTA [GTA/collagen (w/w) = 0.5], and the thermal stability of native and crosslinked collagens was measured using differential scanning calorimetry and Fourier transform infrared spectroscopy. Then, an attempt was made to explore the structural changes of native and crosslinked collagens with increasing the temperature by 2D correlation analysis.

2. Materials and methods

2.1 Materials

Collagen was extracted from calf skin using 0.5 mol/L acetic acid containing 1% pepsin (EC 3.4.23.1, 1:10,000, Sigma Chemical Co.) according to the previously described method of Zhang et al. [21]. The supernatant of extracted solution was collected by centrifugation (10,000 × g, 10 min) at 4 °C and salted out in 3 mol/L NaCl solution followed by centrifugation. Then the precipitated collagen was again dissolved in 0.5 mol/L acetic acid and salted out by adding NaCl to a final concentration of 0.7 mol/L. The precipitate was dissolved in 0.5 mol/L acetic acid, and then dialyzed against 0.1 mol/L acetic acid for 3 days to remove NaCl. The purity and molecular weight of the obtained collagen were judged by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The SDS–PAGE pattern of the sample displayed two α bands (~100 kDa for α1 and α2) and one β band (~200 kDa) which were typical electrophoretic bands of type I collagen, indicating the collagen with high purity preserved original triple helix and its molecular weight was about 300 kDa [17,21,22]. Finally, the collagen solution was lyophilized with a freeze dryer (Labconco Freeze Dryer FreeZone 6 Liter, USA) at −50 °C for about 2 days and stored at 4 °C for no more than 3 months.

2.2 Preparation of the crosslinked collagen solution

The crosslinked collagen solution was prepared using the method of Tian et al. [17] with some modification. Lyophilized collagen was cut into small pieces and dissolved in 0.2 mol/L sodium acetate-acetic acid buffer solution (pH 4.00) to obtain collagen solution with the concentration of 5 mg/mL. Then, 5% glutaraldehyde (GTA) solution was added dropwise to collagen solution and the final GTA/collagen weight ratio was 0.5:1. Excess glycine was added into the mixed solution to react the residual GTA after stirring incessantly for 24 h at room temperature (~20 °C). The resultant collagen solution was centrifuged at 9000×g for 5 min to remove entrapped air-bubbles, and then dialyzed against 0.5 mmol/L acetic acid for 7 days. The native collagen solution used as a control was prepared by the similar treatment except to add an equivalent volume of acetate buffer solution instead of GTA and glycine solution.

2.3 Differential scanning calorimetric measurements

The thermal stability of native and crosslinked collagens was carried out by differential scanning calorimetry (DSC) (Netzsch DSC 200PC, Germany). The samples for DSC measurement were