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Comparison of dynamic denaturation temperature of collagen with its static denaturation temperature and the configuration characteristics in collagen denaturation processes

Yang Li [a], Yanwen Li [b], Zongliang Du [b], Guoying Li [a,*]

[a] *The Key Laboratory of Leather Chemistry and Engineering of Ministry of Education, Sichuan University, Chengdu 610065, PR China*
[b] *College of Light Industry, Textiles and Foods, Sichuan University, Chengdu 610065, PR China*

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Abstract

Exposing collagen to a sinusoidally fluctuating strain, dynamic denaturation temperature (T_{dd}) of collagen was obtained by measuring its dynamic viscoelasticity changes depended on temperature using a dynamic rheometer, and it was compared with static denaturation temperature (T_{sd}), which was determined both by differential scanning calorimeter (DSC) and measuring specific viscosity changes of collagen only depended on temperature using an Ubbelohde viscosimeter. The results showed that T_{dd} was 31.1 °C and was about 4 °C lower than T_{sd}. The changes of apparent viscosity caused by increasing shear rate were reversible when collagen was sheared at 28.5 °C, but they were irreversible when sheared at 33 °C. The results of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), circular dichroism (CD) and fibril formation experiments showed collagen sheared at 28.5 °C could keep its triple helical structure but the triple helix of collagen sheared at 33 °C was transformed to random coils, indicating it was heat-denatured. However collagen only heated at 33 °C did not denature and kept its native configuration. The results revealed that shearing could induce a decrease of denaturation temperature of collagen.

Keywords: Collagen; Dynamic denaturation temperature; Static denaturation temperature; Configuration characteristics

1. Introduction

Collagen exists as a triple helical configuration, consisting three α-chains. Each collagen chain adopts a left-handed helical configuration, and the three strands intertwine with a right-hand superhelical twist. Inter- and intra-molecular hydrogen bonds are responsible for the stability of the triple helix of collagen. In recent years, collagen has been widely used in various products and fields. As a biomaterial, collagen exhibits biodegradability and has weaker antigenecity and better biocompatibility than do other natural polymers [1]. However, when being treated above its denaturation temperature, collagen would denature and transform into gelatin. Many good physicochemical properties of collagen would also disappear due to the collapse of the triple helical structure [2]. In order to make good use of native collagen, it should be treated and stored under the denaturation temperature to keep its native configuration. Consequently, denaturation temperature is an important parameter of collagen in its utilization.

Differential scanning calorimeter (DSC) [3–5], circular dichroism (CD) measurement [6] and specific viscosity (η_{sp}) measurement [7,8] are often used for determining denaturation temperature of collagen. Collagens used in these methods are not exposed to any strain/stress and the change of configuration is only caused by increasing temperature, thus the temperature determined by these methods is static denaturation temperature (T_{sd}). However, collagen not only can be extracted into an aqueous solution, used as an injectable hydrogel for the controlled delivery systems [9] and as a standard raw material in cosmetic formulations [10], but also can be molded into various forms for biomedical application, such as fibers [11,12], pellets [13], nanoparticles [14], etc. Collagen is usually exposed to a certain strain/stress during the molding process. Therefore, it is necessary to study the thermal stability of collagen being sheared or extruded and to elucidate the influence of the strain/stress on the helix-coil transition of collagen.

* Corresponding author. Tel.: +86 28 85462568; fax: +86 28 85400530.
E-mail address: liguoyings@163.com (G. Li).

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In this paper, a new method of determining denaturation temperature of collagen by measuring its dynamic viscoelasticity was used. Exposing collagen to a sinusoidally fluctuating strain at a constant frequency of 1 Hz, its dynamic viscoelasticity depended on temperature was obtained using a dynamic rheometer. The temperature determined by this method was dynamic denaturation temperature (T_{dd}). Additionally, changes of the configuration of collagen in the static and the dynamic thermal denaturation processes were compared.

2. Materials and methods

2.1. Preparation of collagen

Collagen was obtained from calf skins by known procedures [15] and lyophilized in a freeze dryer (Labconco Freeze Dryer FreeZone 6 Liter, USA) and stored at −20 °C until needed.

2.2. Determination of static denaturation temperature

The static denaturation temperature (T_{sd}) was determined both by differential scanning calorimetry (T_{sd1}) and measuring specific viscosity changes of collagen only depended on temperature using an Ubbelohde viscosimeter (T_{sd2}).

Collagen was dissolved in 0.5 M acetic acid with a concentration of 1.0% (w/w). Approximately 6 mg samples were sealed in an aluminium pan and an empty pan was used as the reference. The endothermal curve of the sample was recorded from 20 to 50 °C at a heating rate of 1.5 °C/min in a nitrogen atmosphere (Netzsch DSC 200 PC, Germany). T_{sd1} was measured at the mid-point of the transition peak [16,17].

T_{sd2} was measured by the method described by Muyonga et al. [18]. Collagen was dissolved in 0.5 M acetic acid with a concentration of 0.05% (w/w) and the solution was filtered through a filter funnel (40–80 μm). A 10-ml collagen solution was heated from 25 to 45 °C, and was held for 30 min prior to efflux time (t) determination at the designated temperature. The measurement was carried out three times at each point. The efflux time of 0.5 M acetic acid solution (t_0) was also determined under the same conditions. The fraction was calculated with the equation: $F(T) = (\eta_{sp(T)} - \eta_{sp(45\,^\circ C)})/(\eta_{sp(25\,^\circ C)} - \eta_{sp(45\,^\circ C)})$, where η_{sp} is the specific viscosity and is calculated by $(t - t_0)/t_0$. The fraction was plotted against the temperature and T_{sd2} was taken to be the temperature where the fraction was 0.5.

2.3. Determination of dynamic denaturation temperature

The dynamic viscoelasticity of collagen depended on temperature was measured by a Rheometer System Gemini 200 (Molvern Instruments, UK). Collagen was prepared with the same method as used for the measurement of T_{sd}. Dynamic viscoelasticity measurement was performed using a 4° cone/plate system (40 mm diameter). The collagen sample was heated from 20 to 40 °C at a heating rate of 0.5 °C/min. The constant frequency and strain were 1 s^{-1} and 5%, respectively during the measurement [19]. The curves of storage modulus (G'), loss modulus (G'') and complex viscosity (η^*) of collagen depended on the temperature were obtained at the same time, and the dynamic denaturation temperature (T_{dd}) was determined as the temperature where η^* changes reached 50%.

2.4. Apparent viscosity measurements

The measurements of apparent viscosity (η_a) of collagen were performed on a coaxial viscometer RV-II. The collagen samples were prepared as the same as that used for the measurement of T_{dd}. The temperature was controlled by a circulating water bath, and the experiments were carried out at 28.5 and 33 °C, respectively. The measurements were performed over a shear rate ($\dot{\gamma}$) range of 0.5–13.5 s^{-1}. The shearing of the solution was intermittently twice. After the first shearing from 0.5 to 1.35 s^{-1}, the shearing was stopped and the sample was held over an hour in order to recuperate its strain introduced by the first shearing, and then the sample was sheared under the same shear rate for the second time.

2.5. Preparation of collagen samples for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), circular dichroism and AFM experiments

Sample I: collagen was dissolved in 0.5 M acetic acid.
Sample II: collagen solution was sheared at 28.5 °C for 30 min at a shear rate of 1 s^{-1}.
Sample III: collagen solution was sheared at 33 °C for 30 min at a shear rate of 1 s^{-1}.
Sample IV: collagen solution was heated at 33 °C for 30 min.
Sample V: collagen solution was heated at 37.5 °C for 30 min.

2.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed using the discontinuous Tris–HCl/glycine buffer system with 7.5% separation gel and 4% stacking gel. All the samples were all mixed with 0.5 M Tris–HCl buffer (pH 6.8) containing 1% SDS, 10% glycerol and 0.01% bromophenol blue and then heated at 100 °C for 5 min. 15 μl of each treated sample was injected into the gel well and run for approximately 120 min. The gel was stained for 40 min with 0.25% Coomassie Brilliant Blue R-250 solution and de-stained with 7.5% acetic acid/5% methanol solution until the bands were clear.

2.7. Circular dichroism

All the collagen solution samples were diluted to 0.05% and centrifuged at 10,000 × g for 15 min under a temperature of 4 °C before circular dichroism analysis. The CD spectra of the samples were recorded at 25 °C from wavelength of 190–250 nm using a circular dichroism apparatus (Jasco J-800C, Japan).

2.8. Scanning electron microscopy (SEM)

All the collagen samples were dialyzed against McIlvaine buffer (0.1 M Na_2HPO_4, 0.05 M citric acid, pH 7.2) in the pres-

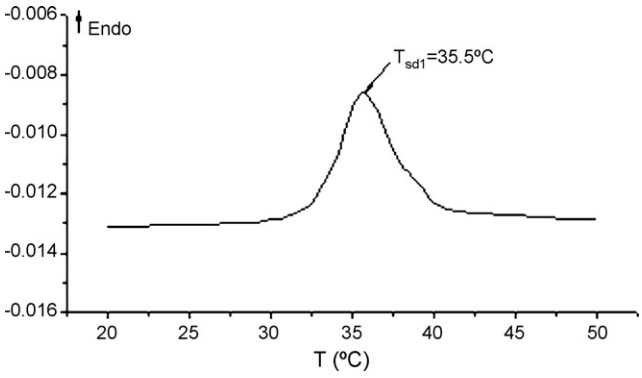

Fig. 1. Thermal transition curve of collagen, as shown by DSC.

ence of NaCl (final concentration adjusted to 0.15 M) at 4 °C for 3 days. The concentrations of the samples were adjusted to 0.05% using the same buffer solution. The samples were incubated to 33 °C to initiate fibril formation, and the turbidities were recorded at 313 nm using a UV spectrometer (PerkinElmer Lambda 25, USA). The resulting collagen fibrils were collected by centrifugation at 3000 × *g* for 15 min at room temperature and were lyophilized. The morphology was observed by scanning electron microscopy (JEOL JSM-5900LV, Japan). The SEM specimen was prepared by the method described by Li et al. [15].

3. Results and discussion

3.1. Static and dynamic denaturation temperature of collagen

The heat transformation of collagen is interpreted as disintegration of collagen triple helical structure into random coils. This is accompanied by a change in physical properties, such as viscosity, elasticity, sedimentation, diffusion light scattering and optical activity [20]. The thermal transition curve of collagen in 0.5 M acetic acid is presented in Fig. 1, and the T_{sd1} was approximately 35.5 °C.

The fraction depended on the temperature is presented in Fig. 2. It was shown that the fraction was observed to decrease with an increase of temperature. It decreased sharply from 32.5 to 37.5 °C and was almost steady after 37.5 °C. According to the determination of denaturation temperature of collagen in previous reports [18], T_{sd2} was approximately 35.1 °C. The helix-coil transition of collagen involves the breakage of hydrogen bonds between the adjacent polypeptide chains of collagen molecules and the changes of intact trimers into individual chains or dimers, causing the sharp decrease of η_{sp} at the same time.

Dynamic viscoelasticity measurement gives information on the dynamic property of polymer materials exposed to the stain/stress [21]. The storage modulus (G', elastic modulus) describes the elasticity of the collagen whereas the loss modulus (G'', viscous modulus) reflects the dissipated energy as a characteristic of the viscous properties [22]. Fig. 3 shows the curves of η^*, G' and G'' plotted against temperature, which were

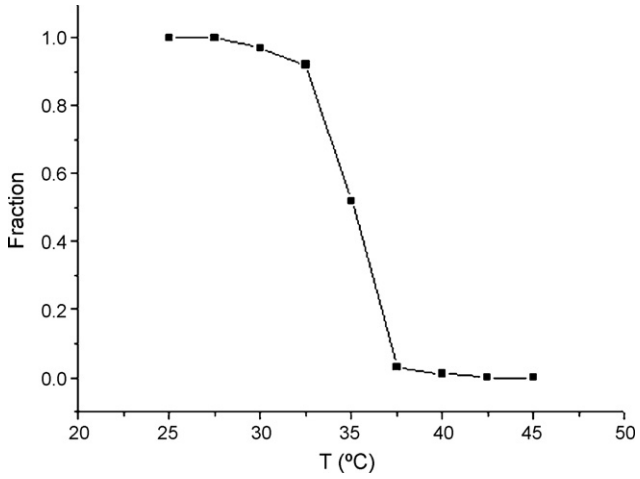

Fig. 2. Temperature dependence of collagen as shown by changes of fraction.

obtained by measuring dynamic viscoelasticity of collagen. As a natural polymer, the changes of dynamic viscoelasticity of collagen involve the transformation of the specific structure. With an increase of temperature, η^*, G' and G'' all slightly decreased originally and fell suddenly at about 30 °C and then reached a plateau at 32.5 °C. The viscoelastic curves in Fig. 3 reflected clearly the denaturation process of collagen and T_{dd} was determined to be about 31.1 °C in this method. The helix-coil transition of collagen involves the breakage of hydrogen bonds between the adjacent polypeptide chains of collagen molecules and the changes of intact trimers into individual chains or dimers, causing the sharp decrease of η^*, G' and G'' at the same time.

The results showed that the thermal stability of collagen changed when it was exposed to a sinusoidally fluctuating strain. This suggested the strain caused inter- and intra-molecular hydrogen bonds more sensitive and partial transformation of the triple helix to random coils. So the cleavage of the hydrogen bonds resulted in a decrease of the denaturation temperature. T_{dd} measured by this method was 4 °C lower than T_{sd}.

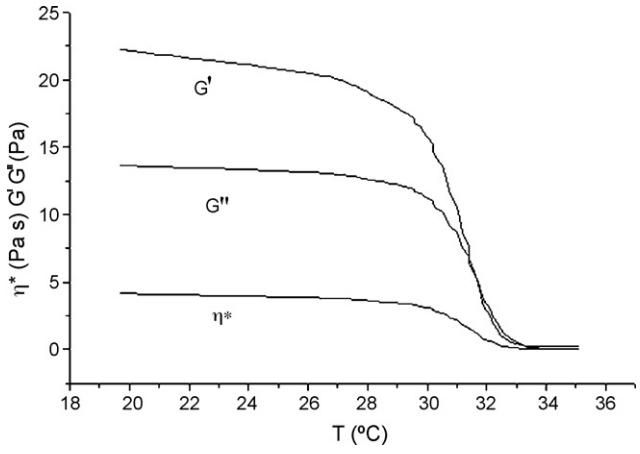

Fig. 3. Temperature dependence of storage modulus G', loss modulus G'', and complex viscosity η^* of collagen.

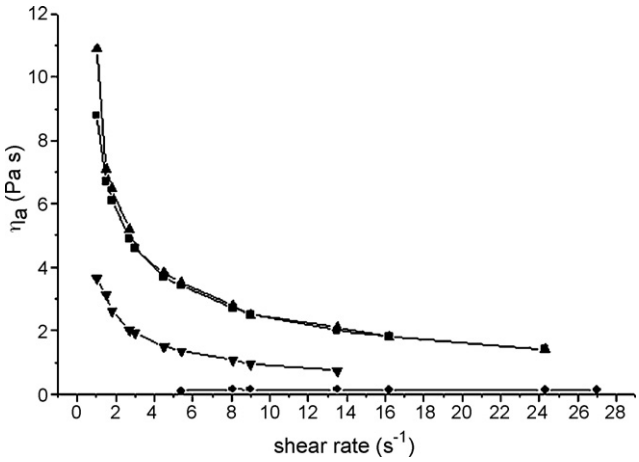

Fig. 4. Shear rate dependence of η_a of collagen at 28.5 °C (▲, the first shear and ■, the second shear) and 33 °C (▼, the first shear and ●, the second shear).

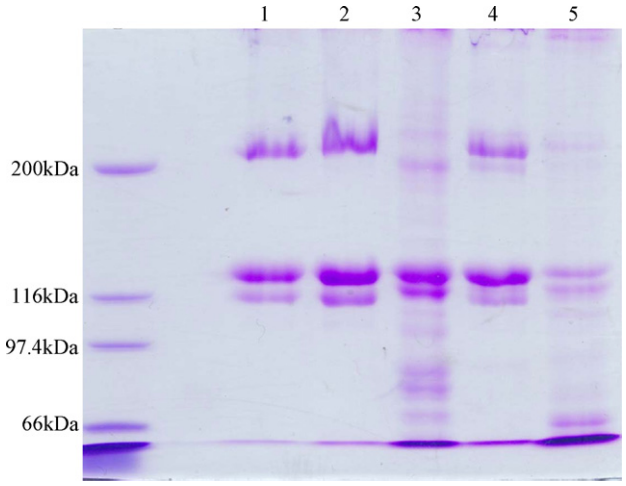

Fig. 5. SDS-PAGE analysis of different collagen samples (lane 1, sample I; lane 2, sample II; lane 3, sample III; lane 4, sample IV; lane 5, sample V).

3.2. Apparent viscosity of collagen depended on shear rate

Fig. 4 shows the relations between η_a and at the temperatures of 28.5 and 33 °C, respectively. η_a was observed to decrease with an increase of and collagen solution exhibited a shear-thinning flow behavior that is one of non-Newtonian fluid characteristics. η_a curves of the first and the second shearing at 28.5 °C were almost overlapped, illustrating the changes of η_a caused by the increase of $\dot{\gamma}$ were reversible and η_a of collagen could recuperate when the collagen solution was held over an hour after the shearing stopped. However, η_a curves of the first and the second shearing at 33 °C could not overlap any more, demonstrating the changes of η_a caused by the increase of $\dot{\gamma}$ were irreversible. η_a of collagen decreased to a very small value when collagen was sheared for the second time, which was quite different from the curve of the first shearing at 33 °C. Strain caused by stress could mostly recuperate in general after eliminating the stress due to the viscoelasticity of polymer. As a natural polymer, collagen has very good viscoelasticity. So the changes of η_a caused by the increase of $\dot{\gamma}$ should be reversible and the irreversible changes of η_a might involve the transformation of the collagen-specific structure. When sheared at 28.5 °C under T_{dd}, collagen could keep its triple helical structure as native collagen, so η_a exhibited good recuperation. Nevertheless, when collagen was sheared at 33 °C above T_{dd}, η_a could not recuperate any more. The results showed that the configuration was changed when collagen sheared above its T_{dd}.

3.3. Configuration characteristics in denaturation processes

The electrophoresis patterns of the samples on sodium dodecyl sulphate-polyacrylamide gel are shown in Fig. 5. Sample I (lane 2), sample II (lane 3) and sample IV (lane 5) displayed two α bands (α_1 and α_2) and one β band. These patterns were similar to those of type I collagen and their molecular weights were about 300 kDa. Sample III (lane 4) and sample V (lane 6) had different migration patterns, with smaller molecular weights and wider distributions. There were obvious decreases in the β band intensities in the electrophoresis pattern of sample III and these components were degraded into peptide fragments, with molecular weights ranging from 116 to 66 kDa. The β-component of sample V almost entirely digested and most α-components (α1 and α2) hydrolyzed, with the appearance of peptide fragments of molecular weight less than 66 kDa.

The CD spectra of collagen samples are given in Fig. 6. Native collagen is a sort of optically active protein and adopts the polyproline II-like helical configuration, having a characteristic CD spectrum with a positive maximum peak at 220 nm and a negative minimum peak appeared at 195–197 nm [23]. As seen in Fig. 6, the CD spectrum of sample II showed a positive extreme at 220 nm and a negative peak at 195 nm, which were typical triple helical configuration of collagen. However, the CD spectrum of sample III revealed the positive maximum value of molar ellipticity at 220 nm decreased with an increase negative minimum value at 195 nm, showing that a part of the triple helix of native collagen transformed to the random coil configuration. But the CD spectrum of sample IV showed it had typical triple helical configuration as native collagen. The positive peak in CD spectrum of sample V disappeared, suggesting that the triple helical configuration collapsed when collagen was heated at 37.5 °C.

Native collagen can aggregate into collagen fibrils under the physiological conditions in an in vitro system [24] and turbidity–time curves have been used to study collagen samples fibrillogenesis in vitro. According to the study of Gelman et al. [25] fibril formation of collagen in vitro requires at least three steps. The first step is a lag phase in which there is no detectable change in turbidity, the second step is a growth phase in which turbidity changes rapidly, and the third step is a plateau phase in which turbidity again remains constant. The fibril formation profiles are shown in Fig. 7. The solutions of samples II–IV became turbid with increasing time, showing fibril formation in these solutions. However, the optical intensity of sample

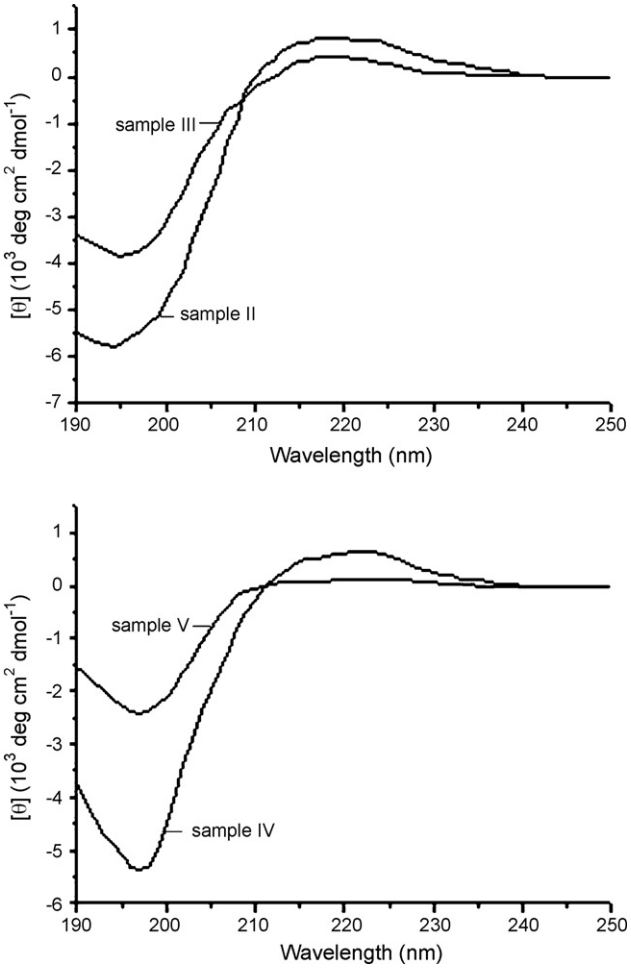

Fig. 6. CD spectra of different collagen samples.

V was stable, suggesting no fibrillogenesis. Moreover, the plot of sample IV showed a curve similar to that of sample II, but the curve of sample III had longer lag and growth phases and weaker intensity. In addition, the SEM image of the resulting fibrils of samples II–IV presented obvious fibril morphology (Fig. 8). The

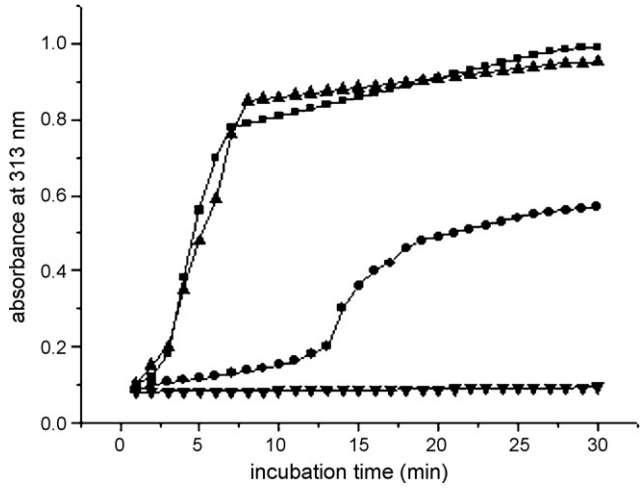

Fig. 7. Turbidity–time curves for collagen samples (■, sample II; ●, sample III; ▲, sample IV; ▼, sample V).

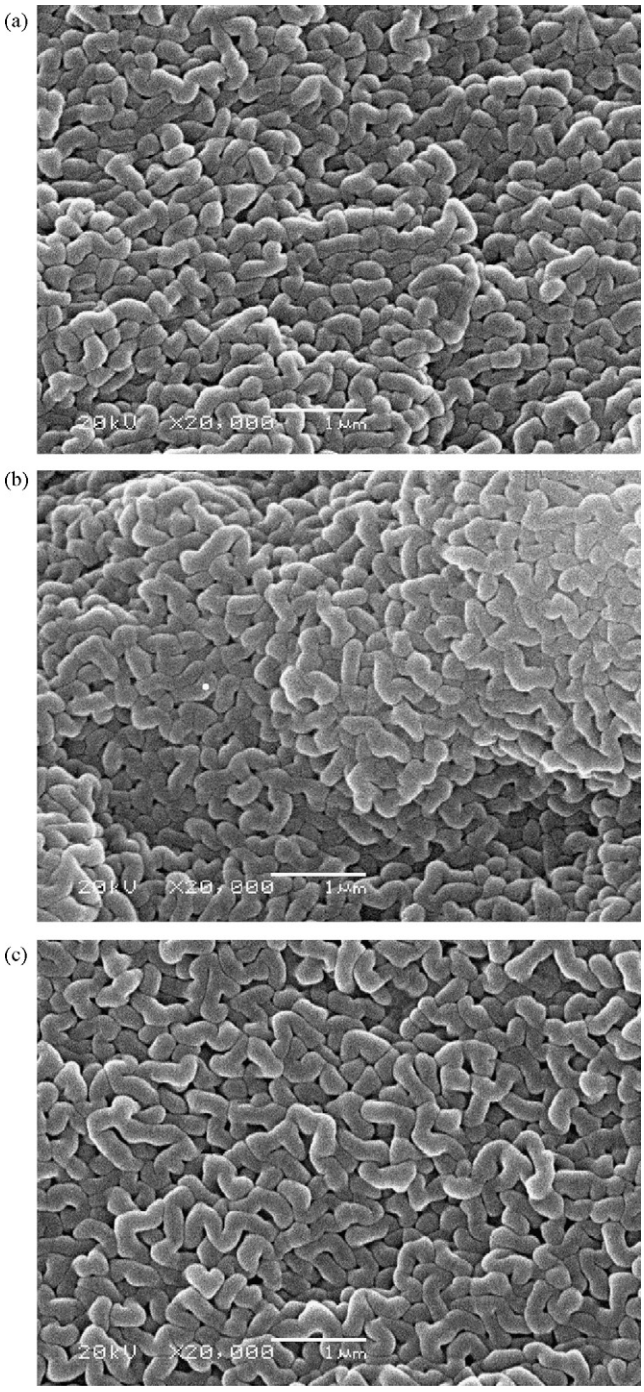

Fig. 8. SEM images of different collagen samples (a, sample II; b, sample IV; c, sample III).

diameters of fibrils were uniform, and the fibrils were entwisted together.

The collagen fibrillogenesis involved different collagen types, different extraction methods and self-assembly conditions (buffer, temperature, proteins concentration and so on). In our study, collagen was extracted in acidic solution containing pepsin, which attacked only the non-triple helical domain of native collagen. But the triple helical structure of sample V was disappeared and its configuration was disorder and the

molecular weight distribution was very wide, which made fibrillogenesis impossible. In addition, self-assembly of collagen monomers into fibrils involved hydrophobic and electrostatic interactions between collagen chains, which were changed during the denaturation. Thus sample V lost its ability to form fibrils under the physiological conditions. The self-assembly curves of samples II and IV were similar to that of native collagen reflecting that shearing at 28.5 °C under T_{dd} and heating at 33 °C under T_{sd} did not destroy their triple helical structure. Sample III still presented triple helical structure as indicated by the experiments of collagen fibril formation, however, a part of the triple helix transformed to the random coil configuration and the molecular weight distribution of sample III was also wide. As a result, the self-assembly curve of sample III had longer lag and growth phases and the fibrils of sample III were less. The self-assembly curves of samples III and IV were different, indicating the triple helical configuration of the collagen sheared at 33 °C was changed by shearing.

The results of SDS-PAGE, CD and SEM all showed collagen could keep its triple helical configuration as native collagen when it was only heated under T_{sd}, but the configuration changed and collagen denatured when it was sheared under T_{sd} but above T_{dd}. These results suggested that shearing could involve the breakage of inter- and intra-molecular hydrogen bonds, which are responsible for the stability of the triple helical configuration, and induce a decrease collagen thermal stability.

4. Conclusion

The thermal helix-coil transition of collagen was very sensitive to strain/stress and dynamic denaturation temperature of collagen was 31.1 °C that was 4.4 °C lower than static denaturation temperature. It could be found a decrease of the denaturation temperature when collagen was exposed to strain/stress. A part of inter- and intra-molecular hydrogen bonds of collagen were destroyed and the triple helical configuration was partially transformed into the random coil configuration by shearing. Collagen sheared was less tolerant to heat-denaturation than collagen unsheared.

Acknowledgements

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