Near Infrared and Two-dimensional Correlation Infrared Spectroscopic Study on the Heat Denaturation of Collagen in Aqueous Solution

by

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Abstract

The thermal behaviors of collagen in aqueous solution were studied by *in situ* near infrared spectroscopy (NIR) and two-dimensional correlation infrared spectroscopy (2D-IR). The spectral data of water molecule and Amide I region fairly provided the dynamic details about the changes of water molecules and imino groups (–NH) in collagen under a temperature evolution from 25 to 54 °C. The splitting of combination bands of water molecule separately at 1455 nm and 1395 nm revealed that there are two different states of water molecules in collagen molecule, the surrounding free water molecules and the structural participating water molecules. Additionally, the two-step phase shift of the combination band of water molecule suggested that the interaction between collagen and water molecule experience two significant changes during the process of thermal denaturation transition. Based on the study of NIR and 2D-IR, a novel molecular model about the micro-changes of collagen from native to thermally denatured state was proposed. In this model, the breaking of hydrogen-bonded –NH groups occurs at first upon heating and the released –NH groups form hydrogen bonds with water molecules immediately to build a new stable water-mediated hydrogen-bonding structure simultaneously. With the increase of temperature, the collagen triple helix collapses gradually, accompanied by the breaking of water-mediated hydrogen bonds of inter-triple helix at 36 °C, and the breaking of intra-helix hydrogen bonds at 48 °C.

Resumen

Los comportamientos térmicos del colágeno en solución acuosa fueron estudiados *in situ* por medio de espectroscopia infrarroja cercana (NIR) y con espectroscopia infrarroja de correlación en dos dimensiones (2D-IR). Los datos espectroscópicos de las moléculas de agua y la región de la Amida I casi proveyeron los detalles dinámicos acerca de los cambios sufridos por las moléculas de agua y los grupos iminos (-NH) en colágeno bajo una evolución térmica entre 25 y 54°C. La separación de las bandas combinadas de 1455nm y 1395nm demostraron que hay dos diferentes estados de las moléculas de agua en el colágeno, las moléculas de agua participando en la estructura colagénica y las moléculas libres rodeando el colágeno. Adicionalmente, el desplazamiento en dos pasos de las fases de la banda combinada de las moléculas de agua sugiere que la interacción entre el colágeno y el agua experimenta dos cambios significativos durante el proceso de transición en el proceso de denaturación térmica. Basado en el estudio de las NIR y 2D-IR, un nuevo modelo molecular de los micro cambios del colágeno de estado nativo a térmicamente desnaturalizado, se propone. En este modelo, la ruptura de enlaces de hidrógeno de los -NH grupos se produce en un primer momento después del calentamiento y los grupos -NH liberados forman enlaces de hidrógeno con las moléculas de agua inmediatamente para construir una nueva estructura estable de agua por medio de puentes de hidrógeno simultáneamente. Con el aumento de la temperatura, la triple hélice del colágeno colapsa gradualmente, acompañada por la ruptura de los puentes de hidrógeno del agua de la inter triple hélice a 36°C, y la ruptura de los enlaces de hidrógeno dentro de la hélice a 48°C.

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INTRODUCTION

Collagen is an important protein in the body of the high-ranking vertebrates. As the major structural components of the extracellular matrix (ECM), collagen provides the main mechanical support and structural organization of connective tissues such as skin, tendon, cartilage, and bone.[1] These specific functions of collagen are mainly related to its triple helix structure. However, this characteristic structure of collagen is very sensitive to the temperature variation.[2] The collagen denaturation transition crucially affects the pharmacological activity and drug distribution in pharmacology.[3,4] On the other hand, the collagen denaturation transition also has relations to many human diseases and important physiological processes, because both of them may originate from the temperature-dependent changes of collagen.[5] Therefore; there is a great interest in understanding the thermal stability of collagen and its structural changes in the denaturation transition.

So far, a great deal of experimental and theoretical research has been done to investigate the temperature-dependent change of collagen, and numerous techniques such as circular dichroism (CD),[6,7] fluorescence depolarization,[8] light scattering (LS),[9] differential scanning calorimetry (DSC),[10-15] X-ray diffraction,[16] NMR,[17] various vibrational spectroscopy,[18-21] and molecular dynamics (MD) method[22-24] were used. However, there still remain some unsolved problems about the thermal stabilization of the collagen due to the limitation of research techniques.

It has been well documented that collagen undergoes a thermally denaturational transition from the triple helix structure to a randomly coiled conformation upon heating, in which the N−H···O=C hydrogen bonds were broken and the triple helix structure was collapsed.[25] However, the precise details about the behavior of the change of N−H···O=C hydrogen bonds in collagen are still lacking. Subsequently, it is showed in recent studies that the water-mediated hydrogen bonded bridge also plays an important role in the thermal stability of collagen.[26-31] A variety of studies have indicated that the structure of water absorbed in collagen is different from that in bulk. In order to explain the structure of water in collagen, two models have been proposed. Mainly based on the NMR results, Migchelsen et al.[26-29] proposed a two-state model. In their model the hydrogen-bonded water molecule bridges between N−H and O=C groups of collagen, and a part of the hydrogen-bonded water molecules can exchange rapidly with free water molecules. On the other hand, based on the dielectric and heat capacity measurements, Hoeve et al.[30,31] have proposed a one-phase model. This model still consists of hydrogen-bonded water molecule chains. However, it is emphasized that these water molecules in this model are not fixed in the collagen, and they diffuse through the interstices to preserve their mutual hydrogen bonding. So far, the viewpoints about the existence state of water molecules in collagen are still in dispute.[26-31]. Additionally, it has been reported that the collagen solution exhibit multiple denaturational transitions.[32] Privalov *et al.* suggested there was some pre-denaturational conformational changes before the denaturation, but no further detail is elucidated.[32] Wallace *et al.* proposed that such multiple transitions resulted from sequential changes of distinct classes of molecules in the reconstituted collagen.[33] However, such sequential changes have not been directly observed. It is noted that the origin of the multiple denaturational transitions is important for our understanding the denaturation mechanism and the stabilization of collagen.

Recently, we employed a newly technique, near infrared (NIR) diffused reflection (NIR DR) spectroscopy to further understanding the mechanisms of three kinds of classical tannages, which provided some new information to get insight into the tanning mechanisms.[34] The NIR spectroscopy covers the wavelength range from vicinity of the visible region to the mid-infrared (MIR), which is extremely sensitive to the functional groups of -NH_2, -NH-, -OH and -COOH involved in the formation of hydrogen bond.[35] Thus, NIR spectroscopy is especially effective in the study of the hydration and the hydrogen bond in the macromolecules with amide structure such as nylons[34] and PNIPAM[35], which implies that NIR may provide precise details of structural change of collagen. On the other hand, the absorption bands arising from water solvent appear in the amide I band region in the MIR region, often adding noise to the amide I bands that are key bands for exploring the secondary structure of protein. However, in the NIR region the intensity of water solvent bands is much weaker, and their effect on the bands arising from protein is relatively small[36], which suggests that it is possible to investigate the structural change of collagen and the state of water molecule simultaneously in the range of NIR spectrum. In connection with our previous and ongoing research, we herein intend to use the near infrared (NIR) spectroscopy to further understanding the thermally induced micro-dynamic changes of collagen.

Generalized two-dimensional correlated infrared spectroscopy (2D-IR), a technique originally proposed by Noda and Ozaki,[37] is a mathematical improvement over the techniques in vibrational spectroscopy in recent years. It can simplify overlapped features in the conventional one-dimensional (1D) spectra through spreading the spectral bands over the second-dimensional (2D) spectra. Additionally, it is also very important to point out that the dynamic sequential order change of various chemical structures can be concluded by this method.

In the present work, we employed NIR and 2D-IR to investigate the temperature-dependent micro-dynamic

change of collagen in aqueous solution. The important feature of this research is that the detailed structural basis and the sequential-order dynamic changes of water molecules and collagen are elucidated in aqueous solution for the first time by the 2D-NIR correlation spectroscopy.

Experimental

Materials

Collagen used in this study was isolated from the skin of pig. After successive rinsing with acetone to remove the grease, the pigskin was extracted with 0.5 mol·L^{-1} acetic acid at 4°C for 2 days under stirring and then salted out with sodium chloride. Subsequently, the collagen was collected by refrigerated centrifugation and further dialyzed by ultrapure water (heavy metal ion < 0.1ppb, UPH-1, Ultrapure Technology Co., Ltd., P. R. China) at 4°C for 2 days. Finally, the collagen was freeze-dried and kept in refrigerator under the temperature of -20°C.

Near Infrared Spectroscopy Measurements

Collagen solution (5.0×10^{-4} g/mL) was prepared by dissolving the freeze-dried collagen in the ultrapure water. The original 1D NIR spectra were measured on the UV-Vis-NIR spectrophotometer (UV-3600, Shimadzu, Japan) in the range of amide I band from 1000 to 1770 nm. The spectra were collected from 25°C to 54°C in two stages which consist of first phase from 25 and 32 °C with an increment of 6 °C and second phase from 32 and 54 °C with an increment of 2 °C. The time at each temperature increment while heating the collagen is about 30 s. A quartz cell with a length of 1 cm was employed for the denaturation experiments. The cell was put into a cell holder whose temperature was controlled by electronic temperature controlled water-bath. To measure and control the bath temperature, a digital thermometer and heating unit were dipped into the water-bath with a temperature fluctuation less than ± 0.5 °C. After a temperature was reached, the quartz cell stored with collagen solution was immediately transferred into the holder of NIR spectrometer and the determination of the NIR spectra was initiated simultaneously.

Temperature-dependent Two-dimensional Correlation Analysis

The generalized 2D correlation analysis was performed by using the software of 2D Shige ver. 1.3 (developed by Shigeaki Morita in Professor Yukihiro Ozaki's group at Kwansei-Gakuin University, 2004-2005). With inputting the data of original 1D NIR spectra into the software of 2D Shige, the correlated 2D spectra can be successfully calculated and outputted based on the Noda's mathematical calculation procedure. In our analysis, the 2D spectra were further plotted in the type of contour maps by the software of Origin 8.0 (OriginLab Corporation) to obtain good quality and clarity spectroscopy. In the 2D maps, the warm-colored (yellow-red) regions are defined as the positive correlation intensity, and the cold-colored (cyan-blue) regions are regarded as the negative correlation intensity.

Results and Discussion

Temperature-dependent Near Infrared Spectroscopy Analysis

Figure 1 show NIR spectra of collagen solution in the spectral region of 1000 to 1770 nm measured under the temperature range of 25 to 54 °C. The spectral region of 1770 to 2500 nm was not measured because of an extremely strong absorption arising from the combination band of O-H stretching and bending vibration of water solute. In the format of spectral overlap in **Figure 1(a)**, the broad band at 1450 nm is the contribution primarily from O-H stretching vibration of water, as well as the band from amide I N−H···O=C hydrogen bond of collagen backbone that is the key band for exploring the secondary structure of protein.[38] The three-dimensional perspective in **Figure 1(b)** showed that the intensity and position of certain peaks exhibited some slight

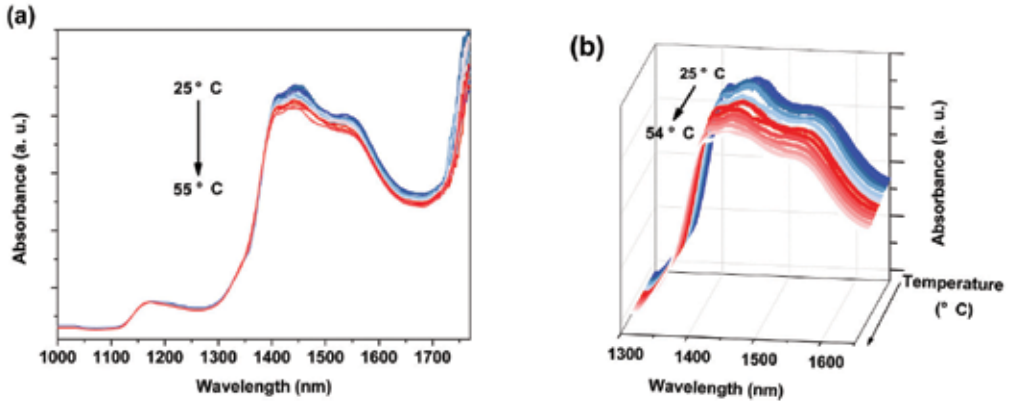


Figure 1. Temperature-dependent NIR spectra of collagen solution in the temperature range of 25 - 54°C

changes upon heating, but no more detail information can be obtained visually from **Figure 1.** Thus, we carried out 2D correlation analysis for this target spectral region.

Two-Dimensional Correlation Analysis of the Temperature-dependent Micro-dynamic Changes

Figure 2 shows the synchronous (a) and asynchronous (b) 2D contour maps of collagen aqueous solution in the temperature range of 25 - 54°C. It can be found that the 2D correlation spectroscopy provides two kinds of spectra, commonly referred as the 2D synchronous (a) and 2D asynchronous (b) contour maps. Both of these 2D correlation spectroscopy contour maps are characterized by two vertical "wavelength" axes (λ_1, λ_2), and the correlation intensity is represented by the contour color. In the 2D synchronous contour map, some peaks appearing along the diagonal are called the autopeaks which represent the autocorrelation of chemical group vibrations. In this study, the vibrational peak where the autopeak appears would change significantly with the temperature perturbation. Additionally, the off-diagonal peaks, named cross-peaks (marked as Φ (λ_1, λ_2)), represent two types of chemical groups measured by λ_1 and λ_2, and the spectra intensity of them exhibits a simultaneous change. Moreover, the positive cross-peaks demonstrate that the intensity of these two chemical groups separately at λ_1 and λ_2 increases or decreases simultaneously under the temperature perturbation; while the negative cross-peaks indicate that the intensities of these two chemical groups change conversely (one increases, while the other decreases) under the temperature perturbation.

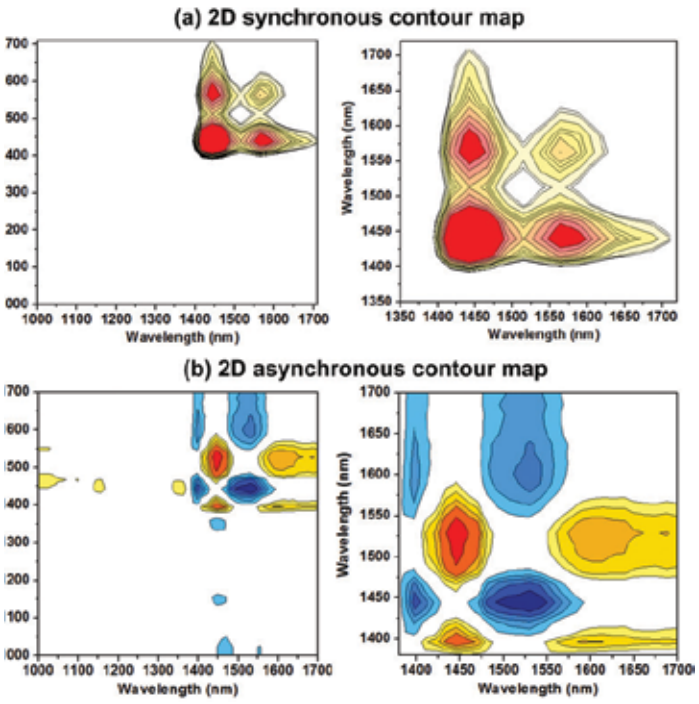


Figure 2. 2D NIR correlation contour maps of collagen solution under the temperature perturbation from 25 to 54°C

On the other hand, the bands that are significantly decoupled, or whose transition moments respond out of phase at different rates under the external temperature perturbation will appear in the 2D asynchronous contour map, as shown in **Figure 2 (b).** Unlike synchronous spectra, only off-diagonal cross-peaks appear in asynchronous spectra, and these cross-peaks are also either positive or negative. The intensity of the asynchronous spectrum (marked as Ψ (λ_1, λ_2)) represents sequential-order change of two chemical group vibration observed at λ_1 and λ_2. According to the Noda's rule,[37] when Φ (λ_1, λ_2) > 0, Ψ (λ_1, λ_2) > 0 or Φ (λ_1, λ_2) < 0, Ψ (λ_1, λ_2) < 0, band λ_1 will vary prior to band λ_2. If Φ (λ_1, λ_2) > 0, Ψ (λ_1, λ_2) < 0 or Φ (λ_1, λ_2) < 0, Ψ (λ_1, λ_2) > 0, band λ_1 will vary after λ_2 under the temperature perturbation.

Figure 2 (a) shows the synchronous contour map of collagen under the temperature perturbation from 25 to 54°C, it can be found that an intense autopeak centered at 1450 nm arises primarily from the combination band of O-H symmetric and antisymmetric stretching vibrations ($\nu_1+\nu_3$) of water molecule.[38] Free and different kinds of hydrogen bonded water molecules can co-exist in the collagen solution, and thus the band at 1450 nm is widely-spreading. It also can be observed that there is a relatively wide band at about 1560 nm and a relatively weak band at 1520 nm which is assigned to the first overtone of the bonded N-H vibration $\nu(NH_b)$ and free N-H vibration $\nu(NH_f)$.[39] Upon the heating, the intensity of peak centered at 1560 nm decreases drastically as shown in the NIR second derivative spectra in the wavelength region of 1500-1680 nm (see **Figure 3)**. These results indicated that upon the increases of temperature, the amide hydrogen bond breaks which correspond to the increasing of free -NH group. This observation is agreed with the prior results in the investigation of collagen structure using synthetic poly-tri-peptides and poly-hexa-peptides as models.[40] Additionally, a remarkable positive crosspeak Φ(1450, 1550) at 1450 and 1550 nm can be found in the synchronous 2D contour map, as shown in **Figure 2 (a)**. It indicated that the intensity of $\nu(NH_b)$ and the combination band of ($\nu_1+\nu_3$) of water decrease in synchronization with same direction in the temperature evolution. It means that the decreasing amount of hydrogen-bonded -NH in collagen molecule is accompanied by the change of existing state of water molecule during the thermal denaturation transition.

Figure 2 (b) shows the asynchronous contour map of collagen in the temperature range of 25 to 54°C. The crosspeaks Ψ (1450, 1550) centered at 1450 and 1550 nm revealed that the conformation change of water molecules and the increase of the free -NH groups occur at different rates. Moreover, the sign of these crosspeaks Ψ (1450, 1520) is negative, which indicated that the conformation change of water molecules occurs earlier than the formation of free -NH groups. This result revealed that the intermolecular hydrogen bonding of -NH groups was gradually weakened upon heating, and the

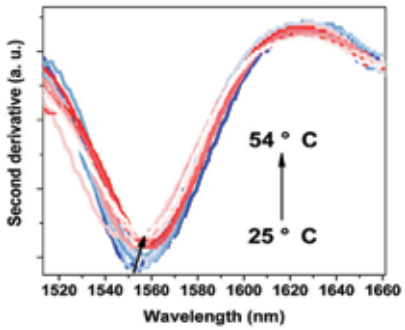


Figure 3. Second derivatives of NIR spectra for the first overtone of the bonded N-H vibration $\nu(NH_b)$ in the wavelength region of 1500-1680 nm.

change of triple helix conformation was in the wake of the conformational change of water molecules. Furthermore, the asynchronous crosspeaks Ψ (1520, 1560) implied that the forming and breaking of the hydrogen bonded -NH occur out-of-step, and the breaking process of hydrogen-bonded -NH groups is prior to the increase of population of the free -NH groups. This result is also supported by the observation in the synchronous 2D contour maps in **Figure 2 (a)**, where no crosspeak between 1520 nm and 1560 nm attributed to the vibrations of free -NH groups and hydrogen-bonded -NH groups can be observed. In addition, the asynchronous crosspeaks (1395, 1455) suggested that the wide-spread 1450 nm band in the synchronous 2D contour maps attributed to the combination band of (ν1+ν3) in water molecule has two origins. The positive sign of these crosspeaks confirmed that the dynamic change of intensity at 1455 nm band proceeds earlier than that at 1395 nm band. The specific assignment of these two bands has not been fully understood yet. It is possible that the splitting of the two bands separately at 1455 and 1395 nm arises from two states of water molecules. One is the structural participating water molecule which participates in the formation of hydrogen-bonded N−H···O=C bridges in collagen molecule, and the other is the surrounding hydrogen bonded water net which are not fixed in the intra-helix hydrogen bonds of collagen molecule. The results of 2D NIR provide new persuasive evidences for the suggestion of "two-state" model about the existence form of water molecule in collagen.

Figure 4 shows the peak position of combination band of the O-H symmetric and antisymmetric stretching vibrations ($\nu_1+\nu_3$) of water molecule in second derivative of NIR spectra. It is interesting to see that there are two large shifts at ≈ 38°C and ≈ 48°C, respectively. Therefore, it can be indicated that the interaction between collagen and water molecules experience two sudden changes from 25 to 54°C. According to the literature,[41] the bio-modal thermally induced denaturation of collagen exhibits multiple phase transitions, which are observed in the present study indeed. The origins of these two peak top frequency shift are different. The first change at ≈ 38 °C is probably associated with the breaking of inter-triple helix hydrogen bonds of collagen, and the second change at ≈ 48 °C is probably from the collapse of intra-helix hydrogen bonds.[42] These two shifts suggested that the collapse of collagen triple helix is accompanied with the change of existence state of water molecule, which demonstrate that water molecule can participate in the formation of both the inter- and the intra-helix hydrogen bonds. These results are in good agreement with the conjectural water mode proposed by Bella, J. *et al* [43], obtained from the structure factors of collagen-like peptide.

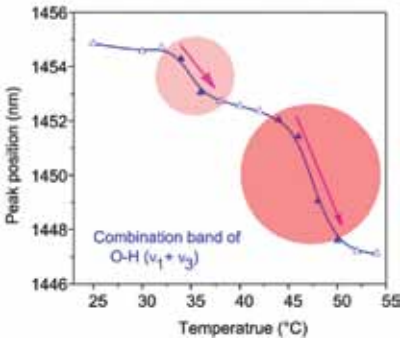
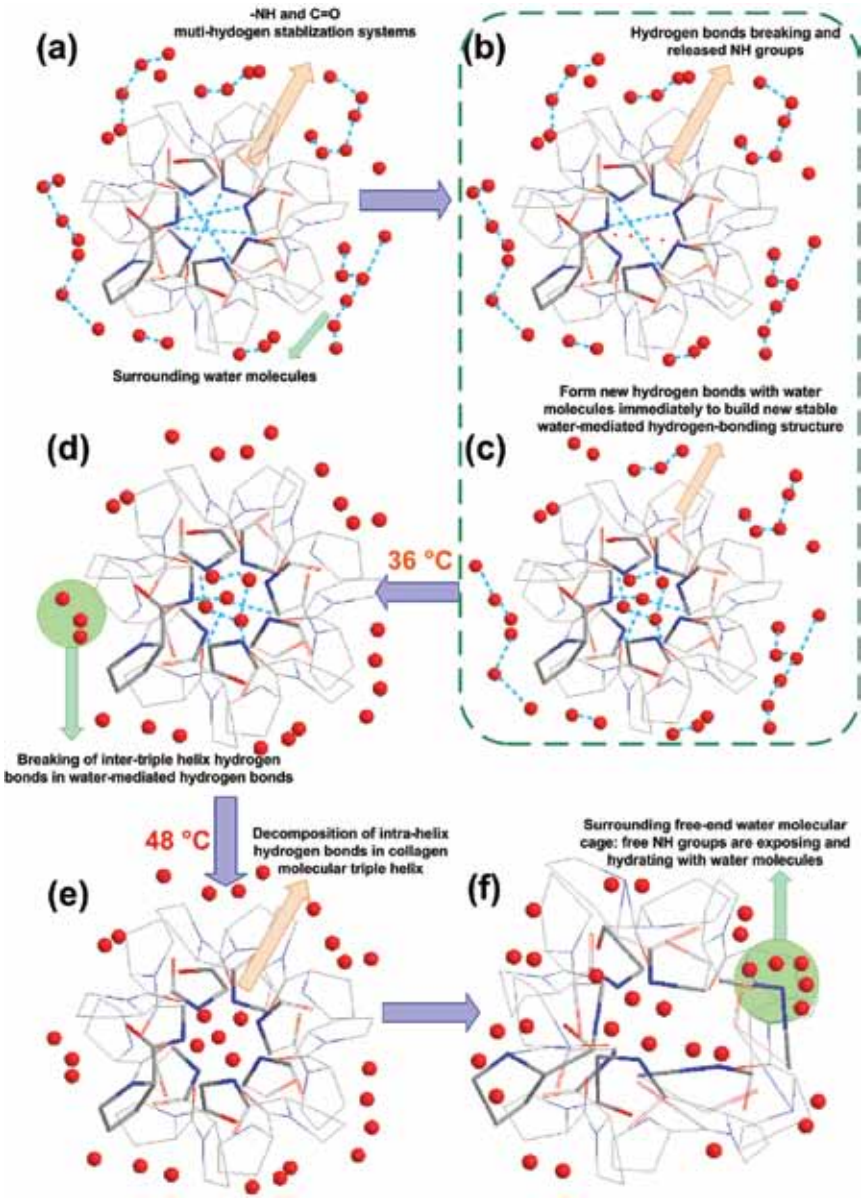


Figure 4. Peak position of the combination band of the O-H symmetric and antisymmetric stretching modes of water molecule as a function of temperature.

Herein, to make a summary, the sequential order of intensity changes of different chemical groups in collagen gained above in the 2D correction analysis can be linked as the sequence:

1570nm (NH_b) 1455nm (structural participating water molecules) 1520nm (NH_f) 1395 nm (surrounding water molecules

We have drawn an approximate figure in **Scheme 1** to show the integrated process of the thermally induced micro-dynamic changes in collagen solution. As the temperature increases, the intra- and inter-chain amide hydrogen bonds break at first (b), and then the released -NH groups form new hydrogen bonds with water molecules immediately to build a new stable water-mediated hydrogen-bonding structure (c). When the temperature reaches 36 °C, the unfolding of collagen triple helix starts, which leads to great change in hydration associated with the breaking of water-mediated inter-triple helix hydrogen bonds (d). With further increase of temperature, the breaking of intra-helix water-mediated hydrogen bonds leads to the collapse of collagen at 48°C (e). As more and more -NH groups released, the free -NH groups are hydrated to form a surrounding water layer. Finally, some surrounding water molecules disentangle at higher temperature (f).



Scheme 1. Sketch map for the possible mechanism of the thermally induced micro-dynamic changes in collagen aqueous solution under temperature evolution, where the collagen molecule is shown in the vertical section of stick model, and red balls stand for the water molecules, as well as the blue dotted lines represent the hydrogen bonds.

CONCLUSIONS

Our findings show that the NIR spectra and newly developed technique, generalized two-dimensional correlated infrared spectroscopy (2D-IR), are powerful techniques to investigate the dynamic thermal change of collagen in aqueous solution. It also provided strong evidence that there are two different states of water molecules in collagen, which are the surrounding free water molecules and the structural participating water molecules, respectively. The two-step phase shift of the combination band of water molecule suggested that the interaction between collagen and water molecule experience two significant changes during the process of thermal denaturation transition. Based on the analysis of NIR and 2D NIR results, a novel model for the thermally induced micro-dynamic changes of collagen in aqueous solution is proposed. The study helps us to deeply understand the fundamental properties of collagen molecule and the role of "special" water molecules in it, which may be helpful for further investigating the tanning mechanism of the leather manufacture process. It was suggested that the tanning process is greatly related with those "special" water molecules. Moreover, those findings also have a significant guideline for the study of physiological process of collagen and the design of newly drug delivery systems.

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