Investigation on Thermally-Induced Conformation Transition of Soy Protein Film with Variable-Temperature FTIR Spectroscopy

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ABSTRACT: Soy proteins are important and versatile foodstuffs, and are potentially very valuable natural materials as replacements for synthetic polymers if mechanical properties and water sensitivity could be improved by changing its conformational structure during processing. As part of such investigations, the thermally-induced conformation transitions of soy protein films were studied in detail with variable-temperature FTIR spectroscopy and two-dimensional correlation analysis. The results show that during the heating process, relatively mobile random coil segments in the soy protein chains reconfigure to a relatively stable β-turn conformation as part of major structural changes. The observations in heated-cooled-heated experiments reveal that a fraction of such a conformation transition is reversible. As part of this analysis, we identify for the first time that the peak at 1500 cm^{-1} in the FTIR spectra can be assigned to the characteristic absorption of β-turn, which is also associated with the peak at 1700 cm^{-1} and is seen in other important structural proteins such as silk fibroin. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 2838–2845, 2012

Key words: proteins; conformational analysis; FTIR; biopolymers

INTRODUCTION

Soy protein isolate, existing as dehydrated storage proteins with globular structure in soybean (more than 90% protein contents),[1] attracts more and more interest because it is a potential replacement for petroleum-based products due to its sustainability, abundance, low cost, biocompatibility, and biodegradability.[2] In fact, soy proteins are quite heterogeneous with two major components: glycinin (11S, ~ 52% of the total protein content) and β-conglycinin (7S, ~ 35% of the total protein content).[1] Glycinin has a hexameric structure with a molecular mass of 300–380 kDa and is composed of five major subunits that have been identified and classified into two groups according to their amino acid sequences. Each subunit consists of an acidic polypeptide (α-polypeptide) with a molecular mass of about 32 kDa and a basic polypeptide (β-polypeptide) with a molecular mass of about 20 kDa, which are connected by a single disulfide bond forming the αβ subunit.[3,4] β-conglycinin has a trimeric structure and is composed of three kinds of subunit α (~ 67 kDa), α′ (~ 71 kDa), and β (~ 50 kDa).[5] To date, besides of the traditional food applications, soy proteins have also been widely applied in industrial materials, such as plastics,[6,7] gels,[8–10] films,[11–13] and coatings or adhesives.[14,15] However, most of these soy protein-based materials are not suitable for investigating secondary structure and functional properties, because they need to be modified chemically or blended with other synthetic/natural polymers to overcome poor physical properties, such as water sensitivity, poor processability, and low mechanical strength.[6,16,17] As we know, secondary structure determines the behavior of proteins during preparation, processing, storage, and consumption. Thus, a detailed knowledge of the conformational transitions of soy proteins is essential for the preparation and application of natural protein materials in the future, as well as the more direct application to soy-based foodstuffs and environmental friendly structural polymers.

FTIR spectroscopy has frequently been used to study the secondary structure in proteins as well as the conformation transition processes during the

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development of a controlling perturbation.[18,19] However, there are few studies on the conformation transition in soy protein owing to its complex structure within soy-based blend materials. In our previous work, we provided dynamic data using time-resolved FTIR spectroscopy to study the conformation transition of soy protein films induced by temperature under isothermal conditions in the range 40–100°C.[20] We believe that, this system is appropriate for understanding conformation transitions in practical applications, where heating is thought to be involved in the preparation of these protein materials under unstable conditions to control properties such as stiffness and water sensitivity. Although, we proposed a mechanism for the conformation transition *in vitro* based on the kinetics data, several details remained to be investigated including the assignment of the 1500 cm^{-1} absorption band.

The generalized two-dimensional (2D) correlation spectroscopy technique, originally proposed by Noda in 1993,[21] has received great attention, which emphasizes spectral features not readily observable in conventional one-dimensional spectra and also probes the specific order of certain events taking place with the development of a controlling physical or chemical variable, such as temperature,[22,23] time,[24] pressure,[25,26] and concentration,[27]etc. In the present article, the conformation transitions of soy protein films when heated gradually from 30 to 160°C were studied by combining variable-temperature FTIR spectroscopy and 2D correlation spectroscopy. We discuss the assignments of the absorption bands and the appearance of oxidation processes involved in the conformation transition induced by temperature in soy protein films. We also discuss the thermally-induced conformation transitions of soy protein films in detail to propose the corresponding mechanisms of conformation changes.

EXPERIMENTAL

Preparation of soy protein films

Six grams of soy protein isolate powder (Shanghai Shenyuan Food) was dissolved in 260 mL, 6 mol/L guanidine hydrochloride aqueous solution and then stirred at room temperature for 3 h while adding 2-mercaptoethanol (its concentration in the protein solution was 0.01 mol/L). After dialysis against NaOH aqueous solution (pH = 11.5) for 2 days and deionized water for another day at room temperature, the solution was transferred to a 7 mL centrifuge tube (8×60 mm^2) and centrifuged at a speed of 9000 r/min (relative centrifugal force of 5886 *g*) for 10 min to obtain a clear supernatant. The concentration of soy protein solution was about 1.6% (w/w) analyzed by gravity method. To prepare films, the solution was first diluted to 0.8% (w/w). Then, 0.8 mL of this soy protein solution was transferred to a polystyrene weighting boat (3×3 cm^2) and allowed to dry overnight at about 25°C and 50% relative humidity. All the resulted films were dried under vacuum for another two days. The thickness of the soy protein films was about 6 μm. This solution process has been described in detail elsewhere,[28] and results in a very stable protein solution with almost no degradation of the protein chains relative to more conventional alkaline processes, and is important for ongoing work on improved structural mechanical properties of soy proteins, for example.

FTIR measurements

The FTIR spectra were recorded using a Nicolet Nexus 470 FTIR spectrometer. To eliminate spectral contributions due to atmospheric water vapor, the instrument was continuously purged with pure nitrogen gas (40 mL/min). All spectra were recorded in a variable-temperature cell with an accuracy of ±0.1°C. For each measurement, 64 interferograms were coadded and Fourier-transformed employing a Genzel–Happ apodization function to yield spectra with a nominal resolution of 4 cm^{-1}.

The thin soy protein film was placed between a pair of NaCl windows in a variable-temperature cell. The FTIR spectra were collected after maintaining 10 min at each temperature point between 30 and 160°C with a temperature increment of 10°C. Absorbance spectra at each temperature point were generated by dividing the single beam spectrum by a background spectrum and converting to absorbance using OMNIC 5.1 (Microcal). The difference spectra were calculated by the subtraction of the first absorbance spectrum collected at 30°C. The data shown in the figures were from a single experiment, but closely similar results were obtained in replicates. All data reported in the text were the means and standard deviations for at least three separate runs.

2D correlation analysis

The data treatment was performed using the software 2D Pocha written by Daisuke Adachi (Kwansei Gakuin University). During the calculation, we applied the generalized 2D correlation formalism developed and modified by Noda.[21,29] The temperature-averaged FTIR spectrum was applied as a reference for the different spectral series. The 2D correlation spectra were characterized by two independent wavenumber axes (ν_1, ν_2) and a correlation intensity axis. Two types of spectra, 2D synchronous and asynchronous spectra are obtained in general. The correlation intensity in the 2D synchronous and asynchronous spectra reflects the relative degree of

in-phase and out-of phase response, respectively. Since 2D correlation is a function of two independent wavenumbers, the correlation peaks are represented as pairs in synchronous spectrum $\Phi(\nu_1, \nu_2)$ and asynchronous spectrum $\Psi(\nu_1, \nu_2)$. Throughout this article, the unshaded and shaded areas in the 2D correlation contour maps represent positive and negative cross-peaks, respectively. The peaks in 2D correlation spectra that developed before the noise peaks appear are considered as significant. The cross-peaks we discuss are in the upper left part in both synchronous and asynchronous spectra.

RESULTS

Figure 1 shows the FTIR spectra and the related difference spectra of soy protein film as a function of the heating temperature from 30 to 160°C in the range of 1400–1800 cm^{-1}. The thermal treatment induced an intensity decrease of the amide I band centered around 1645 cm^{-1} and a new increasing shoulder band at 1700 cm^{-1}. The amide II band around 1550 cm^{-1} showed a trend similar to that of the amide I band, i.e., a decrease in intensity with the increase of the heating temperature, and in addition, a shift to low wavenumbers.

The changes in the absorption spectra can be visualized and examined more readily using difference spectra [Fig. 1(b)] and the positive bands are attributable to developing structures, whereas negative intensities are due to vanishing structures. As the temperature increased gradually from 30 to 160°C, the band absorption around 1645 and 1550 cm^{-1} decreased considerably, corresponding to the vanishing random coil structures. In the meantime, the coupled band around 1700 and 1500 cm^{-1} increased corresponding to the developing new structures. Thus, in the heating process, some volatile random coil structure was transformed into new stable conformations. However, when the temperature was at 140°C or above, a new oxidation peak at 1716 cm^{-1} appeared that was similar to the silk fibroin films during heating.[30]

Figure 2 shows the effect of different heating temperatures on the normalized Δabsorbance-temperature curves of the soy protein films at all of the typical wavenumbers measured (1700, 1645, 1550, and 1500 cm^{-1}) to illustrate the conformation changes. We excluded the data from the FTIR spectrum at 160°C because the obvious oxidation peak at 1716 cm^{-1} adversely affected the absorbance at 1700 cm^{-1}. With the increase of the temperature, the bands at 1645 and 1550 cm^{-1} decreased, while the bands at 1700 and 1500 cm^{-1} increased gradually from 30 to 140°C. The rate of change in the band at 1645 cm^{-1} was found to be faster than that in the band at 1550 cm^{-1} [Fig. 2(a)], while it was almost

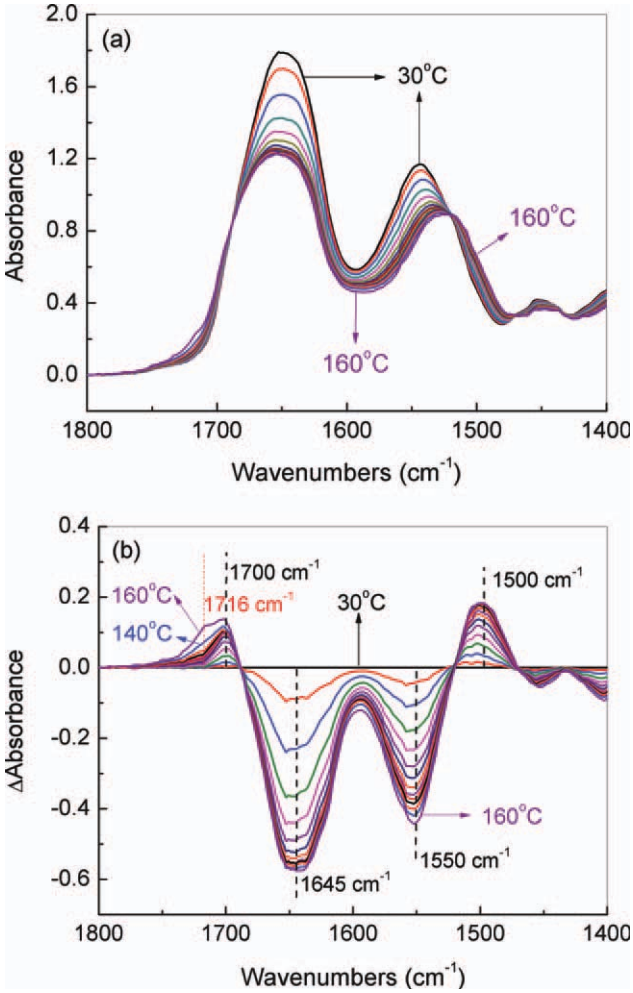

Figure 1 Original FTIR spectra (a) and difference spectra (b) of soy protein film as temperature increased from 30 to 160°C. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

the same in the band at 1700 and 1500 cm^{-1} within the experimental error [Fig. 2(b)]. It should be noted that the absolute Δabsorbance at 1700 and 1500 cm^{-1} were rather small compared to those at 1645 and 1550 cm^{-1} band, so the experimental errors enlarged in some extent when the Δabsorbance value was normalized.

Two-dimensional infrared correlation spectroscopy has been further applied to investigate the conformation changes of soy protein films in the amide I and II region induced by the thermal treatment. Figure 3 shows the contour maps of the synchronous and asynchronous 2D correlation spectra obtained from the FTIR spectra recorded *in situ* at different temperature range.

Firstly, we generated the 2D correlation spectra using the FTIR spectra recorded from 30 to 160°C. In the synchronous spectrum [Fig. 3(a)], two distinct auto-peaks of different intensities were observed in the diagonal of the contour map, at the wavenumbers

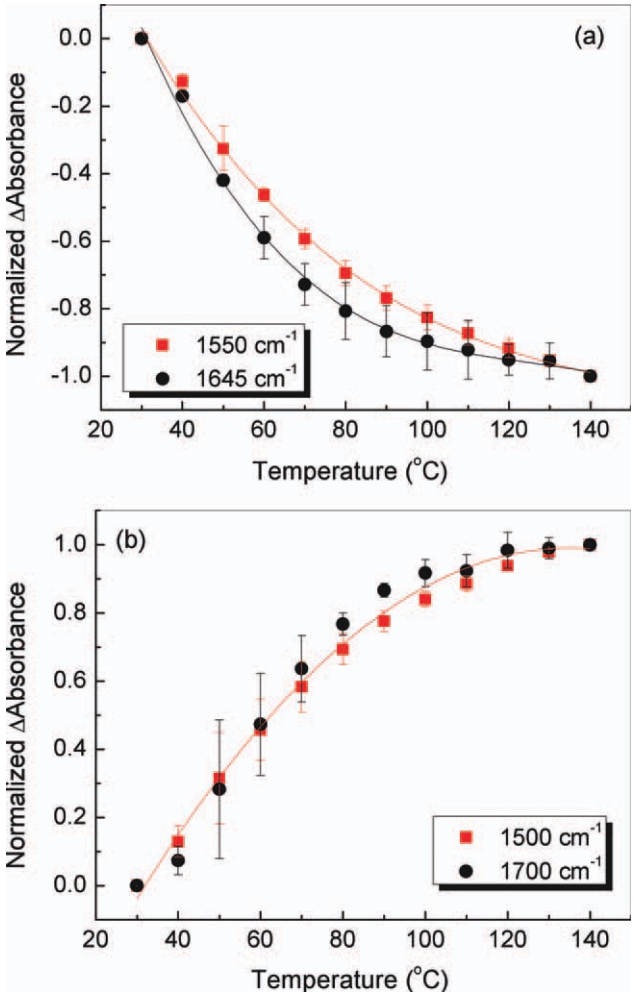

Figure 2 Normalized Δabsorbance of soy protein films as a function of temperature: (a) at 1645 and 1550 cm^{-1} band, (b) at 1700 and 1500 cm^{-1} band. The line is added as an aid to the eye. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

1645 and 1550 cm^{-1}. In addition, there were one positive cross-peak at (1645, 1550 cm^{-1}) and four negative cross-peaks at (1700, 1645 cm^{-1}), (1700, 1550 cm^{-1}), (1645, 1500 cm^{-1}), and (1550, 1500 cm^{-1}), respectively. In the asynchronous spectrum [Fig. 3(b)], four positive cross-peaks at (1716, 1645 cm^{-1}), (1716, 1550 cm^{-1}), (1645, 1550 cm^{-1}), and (1645, 1621 cm^{-1}) and four negative cross-peaks at (1716, 1500 cm^{-1}), (1645, 1500 cm^{-1}), (1550, 1500 cm^{-1}), and (1670, 1650 cm^{-1}) were observed. Two new characteristic peaks at 1621 and 1670 cm^{-1} that were not found in the normal FTIR spectra (Fig. 1) appeared here, but a major peak at 1700 cm^{-1} disappeared instead.

As the oxidation peak at 1716 cm^{-1} was quite strong at 160°C, so we think the cross-peaks containing 1700 cm^{-1} band may be covered up by the cross-peaks with 1716 cm^{-1} band in the asynchronous spectrum [Fig. 2(b)]. Therefore, we generated two other asynchronous spectra in the temperature intervals from 30 to 140°C [Fig. 3(c)] and 30 to 100°C [Fig. 3(d)]. In Figure 3(d), two distinct positive cross-peaks at (1700, 1645 cm^{-1}) and (1700, 1550 cm^{-1}) were observed, while the cross-peaks with 1716 cm^{-1} band was not seen. It was also found that the corresponding cross-peaks in Figure 3(c) are between 1700 and 1716 cm^{-1}. This indicates that the oxidation does not occur at 100°C, but become noticeable at 140°C and above. The asynchronous spectrum generated from 30 to 100°C shows four positive cross-peaks at (1700, 1645 cm^{-1}), (1700, 1550 cm^{-1}), (1645, 1550 cm^{-1}), and (1645, 1621 cm^{-1}) and three negative cross-peaks at (1645, 1500 cm^{-1}), (1550, 1500 cm^{-1}), and (1670, 1650 cm^{-1}), which is similar to that from 30 to 160°C but with the major difference between 1700 and 1716 cm^{-1} band.

DISCUSSION

Assignments for the conformations of soy protein films

The FTIR spectra in the 1400–1800 cm^{-1} range containing the amide I and amide II bands of soy protein were observed and documented in the earlier "Results" section. From our previous work on silk fibroin,[18,19,31,32] and noting that soy protein is also a nonphysiologically active protein similar to silk fibroin, we could assign the absorption bands in soy protein according to the literature on conformations of silk fibroin.

In the amide I region, the 1645 cm^{-1} band is assigned to random coil and/or helical conformation. However, for the shoulder band in the 1690–1700 cm^{-1} region, though it is classically assigned to weak β-sheet vibrations,[33–36] we have provided strong evidence, that it is better to be assigned to β-turns according to our previous works.[19,31,32]

In the amide II region, the band of 1550 cm^{-1} is commonly assigned to random coil conformation,[35,37–39] however, the origin of the 1500 cm^{-1} band was unknown. Hu et al. reported their FTIR analysis on the conformation transition of *Bombyx mori* silk fibroin film by heating from 65 to 165°C.[40] They found a very similar phenomenon as ours, in which the amide II band shifted to 1515 cm^{-1} during the heating process. In their difference spectra, the corresponding wavenumber is 1500 cm^{-1} (though they have not mentioned in the text), which is exactly the same as ours in soy protein films. They also pointed out that such a band could appear completely before the silk fibroin sample formed β-sheet structure, so it may not be assigned to an additional sign for β-sheets crystallization of silk fibroin as in the earlier literatures. Their finding is similar to our results on the analysis of 1690–1700 cm^{-1} band (which is classically assigned to high wavenumber

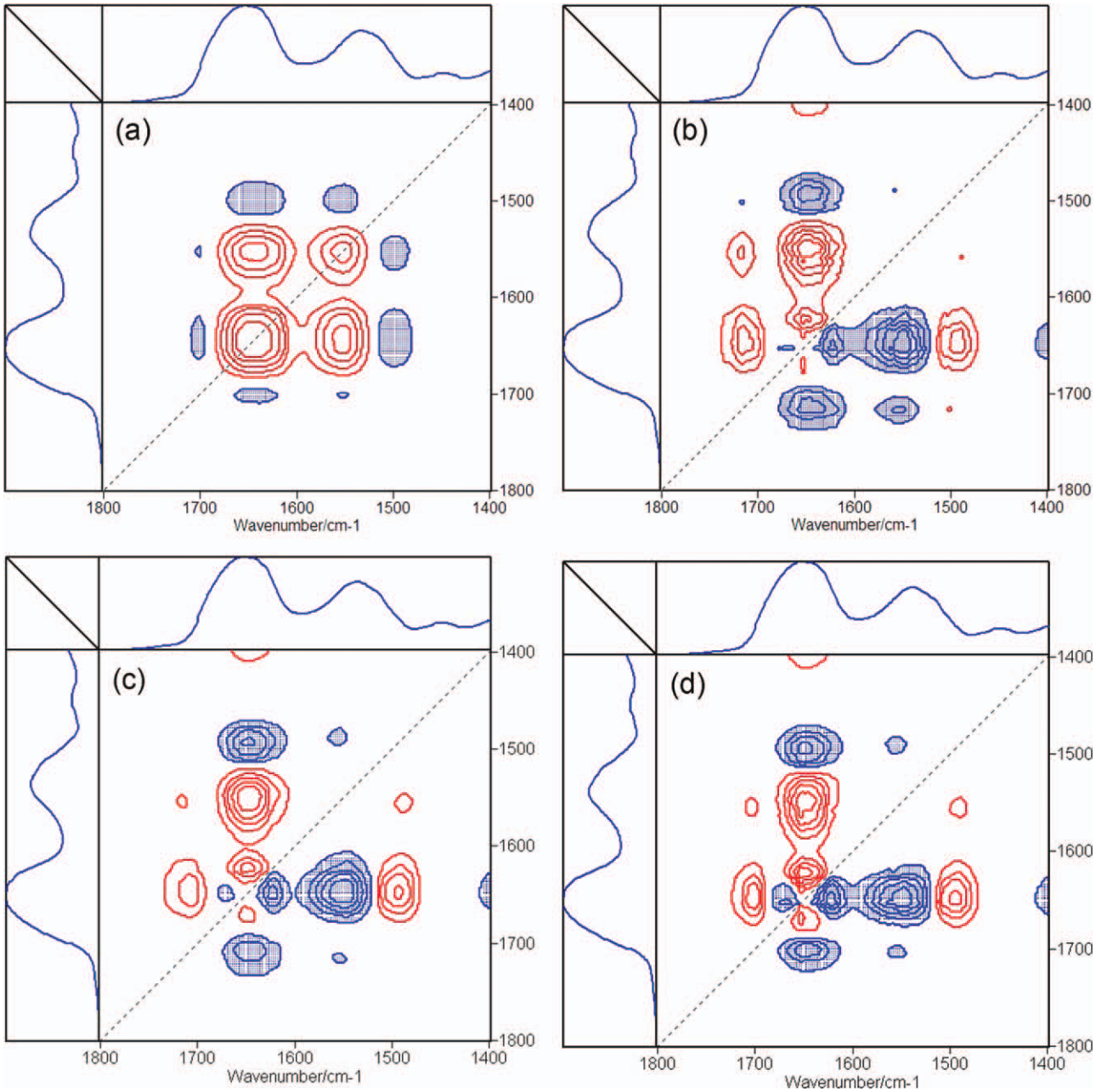

Figure 3 Two-dimensional correlation spectra of soy protein films: (a) synchronous spectrum as temperature increased from 30 to 160°C, (b) asynchronous spectrum as temperature increased from 30 to 160°C, (c) asynchronous spectrum as temperature increased from 30 to 140°C, (d) asynchronous spectrum as temperature increased from 30 to 100°C. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

β-sheet) in silk fibroin with time-resolved FTIR spectra, in which its formation kinetics was rather different from that of β-sheet and was assigned to β-turn[32] as mentioned earlier.

From the decreased intensity of the band at 1645 cm^{-1} and the increased intensity of the band at 1700 cm^{-1} [Fig. 1(b)], we thought that some unstable random coil structure disappeared and then a relatively stable β-turn conformation formed in the heating process. Thus, with the similar situation of the decreased intensity of the band at 1550 cm^{-1} and the increased intensity of the band at 1500 cm^{-1}, we considered that the amide II band at 1500 cm^{-1} was in accord with the amide I band at 1700 cm^{-1} (at least a considerable part of the same source). Therefore, based on the results of soy protein in this study and silk fibroin reported by Hu et al. mentioned earlier, the band at 1500 cm^{-1} could be assigned to β-turn. However, such a β-turn formation process may be related to the movements of tyrosine (Tyr) side chains (also see later "Discussion" section).[40–42]

The conformation transitions process of soy protein films

The major conformation transitions of soy protein film were observed in the FTIR difference spectra [Fig. 1(b)], that is the decrease of 1645 and 1550 cm^{-1} bands (assigned to random coil) and the increase of 1700 and 1500 cm^{-1} bands (assigned to β-turn). Two auto-peaks at 1645 and 1550 cm^{-1}, one positive cross-peak at (1645, 1550 cm^{-1}), and four negative cross-peaks at (1700, 1645 cm^{-1}), (1700, 1550 cm^{-1}), (1645, 1500 cm^{-1}), and (1550, 1500 cm^{-1}) in the 2D synchronous spectrum [Fig. 3(a)] agreed well with the FTIR data. Unlike the synchronous spectra, the asynchronous spectra can provide more information not seen in conventional FTIR spectra. As pointed out in the "Results" section, two new characteristic peaks at 1621 and 1670 cm^{-1} were found in the asynchronous spectra [Fig. 3(b–d)]. These two absorption bands are assigned to β-sheet and β-turn, which indicates that β-sheet and another kind of β-turn changes might also be involved in the conformation transitions of soy protein films, though these changes were weak.

From Figure 2, we found that the rate of change of β-turn formation represented by amide I (1700 cm^{-1}) or amide II band (1500 cm^{-1}) was identical, but rate of decrease in random coil conformation represented in amide I band (1645 cm^{-1}) was faster than in amide II band (1550 cm^{-1}), which was rather abnormal. This implies there is another contribution in the band of 1500–1550 cm^{-1} besides the amide II. Based on the role of Tyr in the conformation transition process of silk fibroin films[42] and the fact that soy protein has the similar Tyr content as silk fibroin,[43,44] we suppose such a contribution is from Tyr amino acid residues in the soy protein. The large Tyr side chain made it move relatively slower than the normal amide bond, which resulted in an apparently slow rate of changes in "amide II" band than that in amide I.

Two-dimensional correlation analysis is another powerful tool to determine the specific order of the spectral intensity changes taking place while the sample is subjected to an environmental perturbation. According to Noda's rule,[21,45] if the change of cross-peak (ν_1,ν_2) in the synchronous and asynchronous spectra is in the same direction (both positive or both negative), band ν_1 varies prior to band ν_2. However, if the change of cross-peak (ν_1,ν_2) in the synchronous and asynchronous spectra is different direction (one positive, and the other one is negative), band ν_1 varied after band ν_2. From the cross-peaks in asynchronous spectrum of soy protein film when heated from 30 to 100°C [Fig. 3(d)], i.e., three positive cross-peaks at (1700, 1645 cm^{-1}), (1700, 1550 cm^{-1}), and (1645, 1550 cm^{-1}) and two negative cross-peaks at (1645, 1500 cm^{-1}), (1550, 1500 cm^{-1}), we obtained the order of characteristic band changes as follows. The 1645 cm^{-1} band varied prior to the 1550 cm^{-1} band, agreeing well with the data from normal FTIR spectra [Fig. 2(a)], as the cross-peak at (1645, 1550 cm^{-1}) were both positive in the synchronous and asynchronous spectra. Similarly, both the 1645 and 1500 cm^{-1} bands varied prior to the 1500 cm^{-1} band because the cross-peaks at (1645, 1500 cm^{-1}) and (1550, 1500 cm^{-1}) were both negative in the synchronous and asynchronous spectra. In addition, the 1700 cm^{-1} band varied after the 1645 and 1550 cm^{-1} bands from the fact that the cross-peaks at (1700, 1645 cm^{-1}) and (1700, 1550 cm^{-1}) were negative in the synchronous spectrum but positive in the asynchronous spectrum. However, no cross-peak at (1700, 1500 cm^{-1}) was observed in the asynchronous spectrum, which means that the development of 1700 and 1500 cm^{-1} are simultaneous. This also agrees with the data from normal FTIR spectra [Fig. 2(b)] very well, which supports the assignment of 1500 cm^{-1} band to β-turn. Overall, these cross-peaks in 2D correlation spectra help to conclude the changing sequence of the four major bands observed in the FTIR spectra as $1645 > 1550 > 1500 \approx 1700$ cm^{-1} (the symbol ">" means "change prior to"). From such a sequence, we deduce that the random coil conformation was first destroyed in the heating process since it is unstable in the soy protein film, together with the Tyr side chain movement. Then, more stable β-turn conformations were formed by the partial adjustment or rearrangement of protein chains with the heating energy.

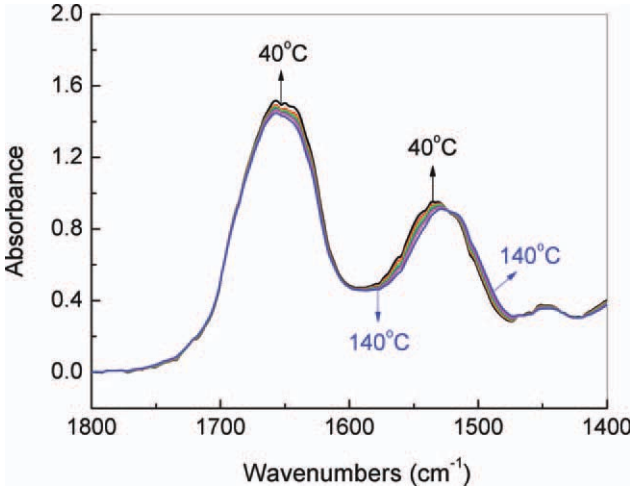

Figure 4 FTIR spectra of soy protein film as temperature decreased from 140 to 40°C. [Color figure can be viewed in the online issue, which is available at wileyonline library.com]

The partial reversibility of thermally-induced conformation transition of soy protein films

During the heating process, soy protein film underwent marked conformation changes. However, to

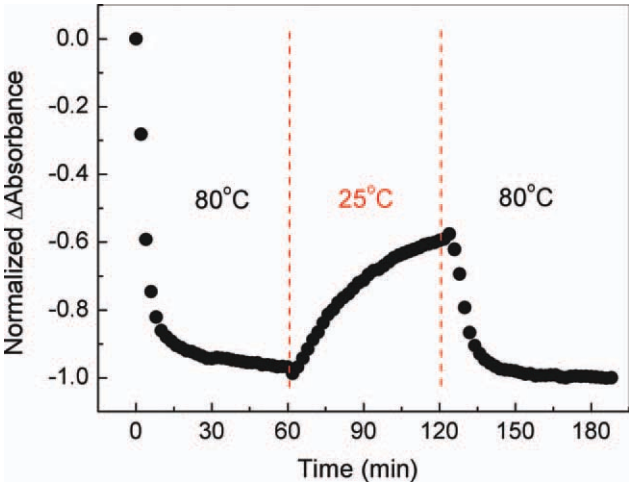

Figure 5 Normalized Δabsorbance-time curves at 1550 cm^{-1} for soy protein film heated at 80°C about 60 min then cooled to the room temperature, and heated at 80°C for 60 min secondly showing the effect of up-down-up temperatures on the random coil conformation transition kinetics. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

verify whether these conformation changes were reversible, we first raised the temperature gradually and then decreased back. Figure 4 is the FTIR spectra of soy protein film as temperature decreased from 140 to 40°C. It clearly shows that the conformation transition of soy protein film was partially reversible when cooling down the temperature.

This partial reversibility suggested that we needed to look at this effect in more detail in a series of heating-cooling cycles. We chose the temperature range from room temperature (about 25°C) to 80°C to make sure there was no effect on oxidation. Figure 5 shows a typical normalized Δabsorbance-time curve probed at 1550 cm^{-1} (change of random coil conformation) for soy protein films first heated at 80°C for 60 min then cooled down to room temperature at about 25°C for another 60 min, and then heated at 80°C for 60 min for a second time, indicating the effect of up-down-up temperature cycles on the conformation transition kinetics. In this case, the conformation of soy protein film was changed considerably during the first heating stage under constant temperature at 80°C and reached equilibrium in about 30 min. The conformation change of soy protein film was then partially reversible when cooling from 80 to 25°C in the second stage and the reversible fraction was about 40%. In the second round of the heating process (the third stage), the partially recovered random coil fraction in the second stage changed again to the final status like that in the first stage, but the time to reach the equilibrium was getting shorter. This indicates that during the heating process, part of the random coil changes to more stable conformation (β-turn) permanently, but part of them is reversible.

CONCLUSIONS

In this article, we used variable-temperature FTIR spectroscopy and 2D correlation analysis to monitor the conformation transition of soy protein films. From FTIR spectra, it was found that the bands at 1645 and 1550 cm^{-1} decreased, but the bands at 1700 and 1500 cm^{-1} increased during the heating process. The rate of change in the band at 1645 cm^{-1} was found to be faster than that in the band at 1550 cm^{-1}, but it was almost identical in the band at 1700 and 1500 cm^{-1}. From the 2D correlation analysis, it was also found that the changing sequence of the four major bands observed in the FTIR spectra was 1645 > 1550 > 1500 ≈ 1700 cm^{-1} (the symbol ">" means "change prior to"). Therefore, we suggest that the peak at 1500 cm^{-1} could be assigned to the characteristic absorption of β-turn, which is associated with the peak at 1700 cm^{-1}. We also suggest the reason for the rate of change in amide I at 1645 cm^{-1} looks faster than the band of 1550 cm^{-1} is the later band combining the vibration mode of amide II and the Tyr side chain. In conclusion, we suggest the following hypothesis for the conformation transition of soy protein film. In the heating process, molecular chain segments of the soy protein become mobile and some volatile random coil structure transforms into a relatively stable β-turn conformation, however, such a conformation transition is partially reversible.

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