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Temperature effect on the structural stability, similarity, and reversibility of human serum albumin in different states

Shun-Li Wang[a], Shan-Yang Lin[b,*], Mei-Jane Li[b], Yen-Shan Wei[b], Tzu-Feng Hsieh[b]

[a]*Department of Applied Chemistry, National ChiaYi University, ChiaYi, Taiwan*
[b]*Biopharmaceutics Laboratory, Department of Medical Research and Education, Veterans General Hospital-Taipei, Shih-Pai, Taipei, Taiwan, Republic of China*

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Abstract

In order to investigate the thermal stability of human serum albumin (HAS) in three different states (aqueous solution, cast film, and solid powder), Fourier transform infrared (FTIR) spectroscopy was applied to determine the protein secondary structural changes of these HSA samples under non-isothermal or isothermal condition. The structural similarity of HSA before and after thermal treatment was also studied to estimate the thermo-reversible property of the HSA in these different states. The results indicate that with the increase of temperature, the maximum peaks at 1652 and 1547 cm^{-1} (α-helix) shifted to 1647 and 1542 cm^{-1} (random coil), respectively. An additional peak at 1620 cm^{-1} assigned to intermolecular β-sheet structure clearly appeared with temperature. The α-helix content was found to be reduced in favor of the formation of intermolecular hydrogen-bonded antiparallel β-sheet structure beyond 60 °C in the heating process. From the data of structural similarity, HSA sample whether in solid powder or cast film form exhibited a better thermo-reversible property than HSA in aqueous solution even heating to 200 °C.

Keywords: HAS; FTIR; Thermal stability; Thermal reversibility; Structural similarity

1. Introduction

Most proteins in the solid state are unfolded or aggregated to show an inactive form. Once the protein is dissolved into a solution, its three-dimensional conformation may recover from an unfolded state to a folded one and exhibit its biological activity [1,2]. This three-dimensional conformation of protein is constructed by several secondary bonds such as ion-dipole, electrostatic or hydrophobic interaction, hydrogen bonding, and Van der Waals [3]. Although the protein is stable in a certain condition, the balance between folded and unfolded structures can be delicately changed by various factors such as temperature, pH, inorganic salts, organic solvents, detergents, and pressure [4]. Among these factors, temperature is the most important one to influence the stability of protein [5]. Although the protein in a solid state is more stable than that in a liquid state, it is not always true. The stabilization of protein in the solid state is still dependent on temperature, residual moisture, and the composition of formulation. Generally, higher temperature may cause protein to be unstable, since the weakened hydrogen bonding and the strengthened hydrophobic interaction may result in unfolding of protein [6].

Human serum albumin (HSA) has been extensively used as a model protein for protein folding and ligand-binding studies, because it is the most abundant protein constituent of blood plasma in human body [7,8]. HSA not only serves as a transporter or/and depot carrier for many substrates, but also maintains the osmotic pressure and plays a role in coagulation and thrombosis [9]. The structure of HSA is divided into three homologous helical domains, in which each domain has two subdomains with a common helical motif. These structural domains are

* Corresponding author. Tel.: +886 22 875 7397; fax: +886 22 873 7200.
E-mail address: sylin@vghtpe.gov.tw (S.-Y. Lin).

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covalent by peptide bonds into a single polypeptide chain with 585 amino acids [10]. Many studies have indicated the conformational changes of HSA after drug–protein binding, since the drug–HSA interaction might influence the absorption, distribution, metabolism, and excretion of drug [11]. Our previous studies have determined the effect of ethanol and/or captopril on the secondary structure of HSA before and after protein binding [12,13]. A cysteine-induced change in the secondary conformations of bovine serum albumin after UV-B irradiation has also been investigated [14].

Recently, Fourier transform infrared (FTIR) spectroscopy has become a very powerful tool for determining the structure of biological materials and for diagnosing the disease tissues [15,16]. The technique is also commonly used to monitor the conformational changes in the secondary structure of proteins and peptides [17,18]. The specific stretching and bending vibrations of the peptide backbone in amide I, II, and III bands provide the information of different types of secondary structure, since these vibrational modes are sensitive to hydrogen bonding and coupling between transition dipole of adjacent peptide bonds. It is well known that the amide I band is particularly sensitive to protein secondary structure than other amide bands, but the absorbance of water and water vapor near 1640 cm^{-1} in the amide I band is also a serious disturbance. Although the intensity in the amide III region is relatively small, it does not interfere OH vibrations from water. Thus, the amide III band can also be used to determine the secondary structure of protein [19,20]. By connecting with computerized software, the secondary conformation of proteins in aqueous solution via subtracting the water contribution from the protein aqueous solution can be easily estimated.

Protein can be sensitively denatured by temperature, but there exists little information about the thermal-induced structural changes of HSA in different states. Thus, the goal of this work was to compare the thermal-related conformation of HSA in aqueous solution, cast film, and solid powder determined by thermal FTIR spectroscopy with attenuated total reflection (ATR) or transmission technique. The structural similarity of HSA before and after temperature treatment in three different states was also investigated to estimate the thermostability and thermo-reversibility of HSA.

2. Materials and methods

2.1. Materials

Human serum albumin (HSA, A-1887, Lot. No. 1449319, fatty acid free) was purchased from Sigma Chem. Co. (St. Louis, MO, USA) and used as supplied without further purification. The KBr crystals for the pellets were obtained from Jasco Parts Center (Jasco Co., Tokyo, Japan).

2.2. Preparation of different HSA samples

(1) Two percentages (w/v) of HSA aqueous solution was prepared by dissolving HAS in distilled water, and then stored at 4 °C for 24 h.
(2) One drop of 2% HSA aqueous solution was dropped on the aluminum foil, and stored at 25 °C, 50% RH condition. After storage for 1 day, the cast film on the foil was formed.
(3) A trace amount of HSA powder was adhered on KBr pellet and pressed by an IR spectrophotometric hydraulic press (Riken Seiki Co., Tokyo, Japan) under 200 kg/cm^2 for 15 s to form a KBr disc.

2.3. Thermal ATR/FTIR spectroscopic study for HSA aqueous solution

2.3.1. Non-isothermal study

IR spectra of HSA in aqueous solution at various temperatures (30–80 °C) were, respectively, determined by using an FTIR spectrometer (IR 620, Jasco, Co., Japan) equipped with a deuterated L-alanine triglycine sulfate (DLATGS) detector and a horizontal zinc selenide ATR accessory (HATR, Pike Technologies, Inc., USA). The spectra were obtained at a resolution of 4 cm^{-1}, and generally 200 scans were accumulated to get a reasonable signal-to-noise ratio. Solvent spectra were also examined in the same accessory and instrument conditions. Each different sample spectrum was obtained by digitally subtracting solvent spectrum from the corresponding sample spectrum, according to our previous report [12,13]. Here, the 30 °C-treated HSA sample was defined as a native sample since there was no change in its IR spectra. Each sample solution was divided into three batches and analyzed separately. The individual spectrum of three determinations to each sample was obtained and averaged to produce a single spectrum for subsequent data processing.

2.3.2. Isothermal study

The same concentration (2%, w/v) of HSA aqueous solution was isothermally incubated at 80 °C at different prescribed intervals (1–15 min), and their FTIR spectra were determined after quick cooling to 30 °C, the IR spectra were determined again.

2.4. Thermal FTIR microspectroscopic studies for HSA in the solid powder or cast film state

The KBr disc or cast film on foil was put directly on a micro hot stage (DSC microscopy cell, FP 84, Mettler, Greifensee, Switzerland). This DSC microscopy cell was then placed on the stage of the microscope equipped in the FTIR microscopic spectrometer (Micro FTIR-200, Jasco Co., Tokyo, Japan) with MCT (mercury– cadmium telluride) detector. The system was performed in the transmission or reflectance mode [21,22]. The temperature of the DSC

microscopy cell was monitored with a central processor (FP 80HT, Mettler, Greifensee, Switzerland). The heating rate of DSC assembly was controlled at 3 °C/min from 30 to 200 °C. The DSC heating program and IR spectra could be simultaneously recorded. The temperature- dependent IR spectra at each point were determined with a resolution of 4 cm^{-1} and 20 scans. After heating to 200 °C, the sample was cooled to 30 °C and then reheated with the same procedure.

2.5. Data acquisition and handling

2.5.1. Spectral analysis

A software of spectral manager for window (Jasco Co., Tokyo, Japan) was used for data acquisition and handling. Second-derivative spectral analysis was applied to locate the position of the overlapping components of the amide I and II bands and assign them to different secondary structures. The protein secondary structure and the composition of each component in amide III band of these IR spectra were also estimated quantitatively by a least-square fitting program iterating the curve-fitting process with Gaussian function. The proportion of a component was computed to be the fractional area of the corresponding peak, divided by the sum of the areas of all the peaks.

2.5.2. Structural similarity

In order to estimate the thermo-reversibility of HSA sample after thermal treatment, the structural similarity among second-derivative spectra for the HSA samples was determined with the following formula [14,23]:

$$r = \frac{\sum (x_i - x^*)(y_i - y^*)}{\left(\sum (x_i - x^*)^2 \sum (y_i - y^*)^2\right)^{1/2}}$$

where x_i and y_i are the corresponding peak intensity of various wavenumber (i) in reference and sample spectra. A spectral correlation coefficient (r) obtained from the comparison of two baseline-corrected second-derivative spectra was used to evaluate the similarity of reference and sample. The x^* and y^* are the average intensity of reference and sample spectra in the range studied. Here, the reference sample means the native HSA in aqueous solution, cast film, or solid powder at temperature of 30 °C, respectively.

3. Results

Fig. 1 shows the original IR spectra of HSA in aqueous solution recorded at various temperatures in the amide I and II regions. The baseline-corrected second-derivative spectra are also indicated. Obviously, two absorbance maxima at 1652 (1655) and 1547 (1548) cm^{-1} were observed in both original and second-derivative IR spectra, indicating a predominantly characteristic of the α-helical structure [17,18,24]. With the increase of temperature, the maximum peaks at 1652 and 1547 cm^{-1} shifted to 1647 and 1542 cm^{-1}, respectively. An additional peak at 1620 cm^{-1} also appeared, which can be attributed to intermolecular β-sheet structure. By normalizing the peak intensity of amide II band, the thermal-induced intermolecular β-sheet formation of HSA is shown as in Fig. 2A. It clearly indicates that the dramatic increase of the β-sheet conformational structure beyond 60 °C was found.

Since the OH vibrations of water do not interfere the amide III band, the assignment of different secondary structural elements in amide III (1330–1220 cm^{-1}) can be used to estimate the overlapping component bands in this region. The assignment of each component in amide III band is α-helix (1330–1290 cm^{-1}); β-turn (1290–1270 cm^{-1}); random coil (1270–1250 cm^{-1}); and β-sheet (1250–1220 cm^{-1}) for protein [25,26]. The curve-fitted amide III band of HSA in aqueous solution at different temperatures is shown in Fig. 3. The assignment and composition of the secondary structure for HSA at 30 °C are as follows: 1315 cm^{-1}, 22% α-helix; 1294 cm^{-1}, 35% α-helix; 1268 cm^{-1},

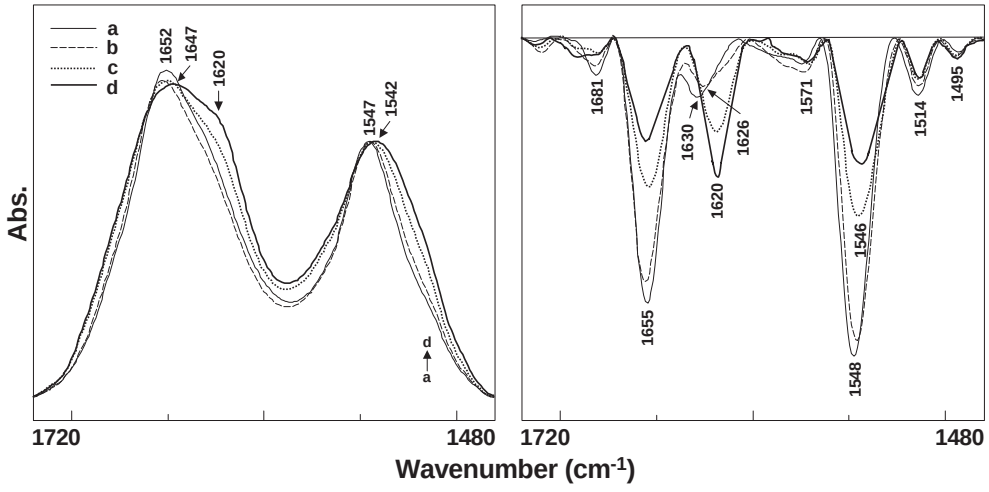

Fig. 1. The original and second-derivative FTIR spectra of HSA in aqueous solution at different temperatures. Key: a, 30 °C; b, 60 °C; c, 70 °C; d, 80 °C.

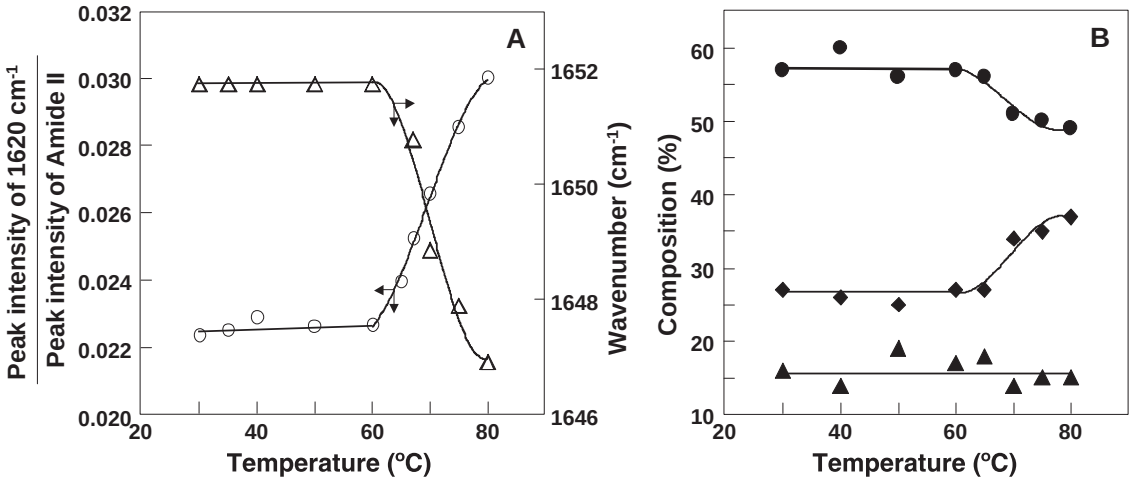

Fig. 2. The thermal-induced intermolecular β-sheet formation (A) and compositional changes (B) of HSA in aqueous solution. Key: ○, peak intensity ratio of 1620 cm^{-1}/amide II; △, peak shift from 1652 to 1647 cm^{-1}; ●, α-helix; ◆, β-sheet; ▲, random coil.

16% random coil; and 1243 cm^{-1}, 27% β-sheet. This native HSA in aqueous solution consisted of 57% α-helix, 27% β-sheet, and random coil 16% in their structure, which was consistent with other reports [27,28]. The secondary structural compositions of HSA almost maintained constant until 60 °C. Beyond that temperature, the contents of α-helix and β-sheet in HSA structure might change (Fig. 2B).

In order to estimate the thermo-reversibility of HSA in aqueous solution after heating, HSA aqueous solution was previously isothermal at 80 °C for a predetermined period of time, and quickly cooled to 30 °C for FTIR determination. Fig. 4 reveals the original and second-derivative FTIR spectra of 80 °C-preheated HSA aqueous solution at different heating intervals. The IR spectral peak at 1619 (1618) cm^{-1} assigned to intermolecular β-sheet structure appeared gradually with heating [29,30]. The longer heating time could easily induce the formation of β-sheet structure. The structural similarity between native and 80 °C-preheated HSA samples is compared in Table 1. Here, the criteria were made: the identical spectra gave a value of 1.0, but the less similar spectra exhibited a lower r value [23]. In this study, the structures of 80 °C-preheated HSA samples diversified with the increase of heating time. Thermal-dependent FT-IR spectra of solid powder and cast film of HSA via first- and second-heating processes were plotted three-dimensionally (Fig. 5). There was no significant change in IR spectra for both samples after repeated heating, suggesting the good thermal-stability for HSA in solid powder and cast film states.

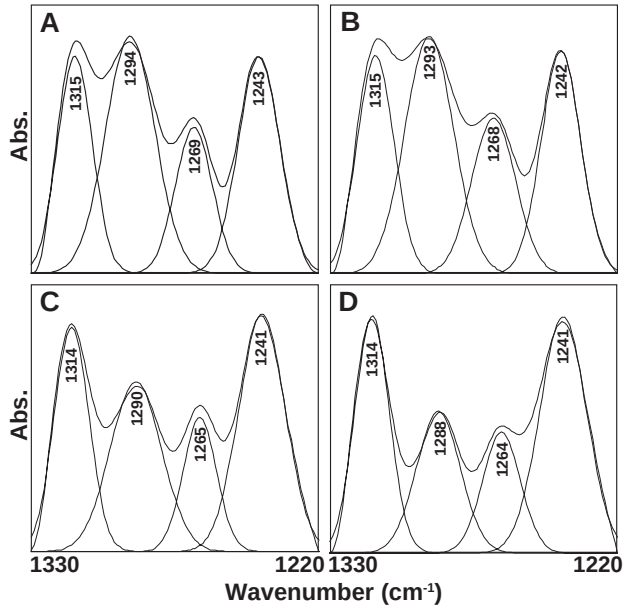

Fig. 3. The curve-fitted amide III band of HSA in aqueous solution at different temperatures. Key: (A) 30 °C; (B) 60 °C; (C) 70 °C; (D) 80 °C.

4. Discussion

It is important to maintain a native-like structure of protein in the biopharmaceutical products, prepared with whether methods, to fast recover the activity after a rehydration process or a long-term storage [31]. However, the stability of protein with folded conformation is easily disrupted by environmental conditions such as pH, pressure, temperature, and detergents to form an inactive unfolded conformation [32]. Denatured proteins are unfolded but make no change in their covalent structure.

A lot of studies have reported that the thermal liability of protein may be increased with temperature. The hydrogen bonding is weakened at a higher temperature but the hydrophobic interaction becomes strengthened [6]. As the process of heating continues, some of the cooperative hydrogen bonds that stabilized helical structure began to break and exposed the hydrophobic groups to the solvent to unfold the protein structure. The existence of water is also a critical point; it not only corrects the folding of proteins with

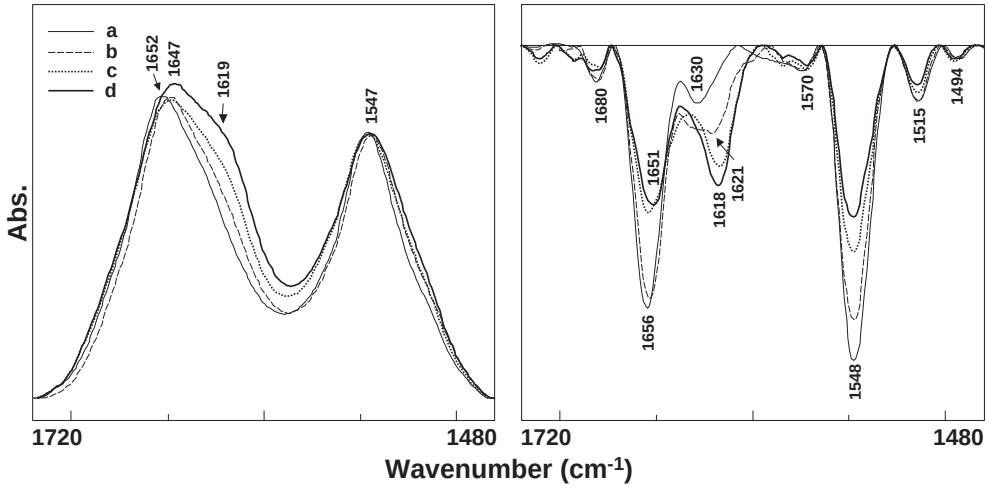

Fig. 4. The original and second-derivative FTIR spectra of 80 °C-preheated HSA aqueous solution at different isothermal intervals, then quick cooled to 30 °C. Key: isothermal interval: a, native (30 °C); b, 1 min; c, 5 min; d, 15 min.

three-dimensional structure but also maintains the structure to exhibit the protein activity [33]. The protein possesses a conformational flexibility in solution, but it is not seen in the crystal or in non-aqueous environments. The activity of protein is lacking in the absence of water. Water can markedly facilitate the thermally denaturing process of proteins; however, dry powder of protein is less sensitive to temperature. This suggests that temperature and water play an important role in influencing the thermal stability of protein.

The effect of temperature on the conformational stability of HSA in aqueous solution indicated the structural transformation from α-helix to random coil due to the shifting of the predominant peak from 1652 cm^{-1} to 1647 cm^{-1} (Fig. 1). Moreover, the second-derivative spectra of HSA also illustrated the shift of the peak at 1630 cm^{-1} (intramolecular β-sheet) to 1620 cm^{-1} (intermolecular β-sheet). The appearance of lower frequency band around 1620 cm^{-1} suggested the formation of an intermolecular hydrogen-bonded antiparallel β-sheet structure to cause thermally-induced protein aggregation [29,30]. The higher temperature might result in a partial heat denaturation of HSA, leading to the loss of α-helical structure but the formation of intermolecular β-sheet.

Table 1
The spectral correlation coefficients (r) of HSA samples after different treatments, as compared to native (30 °C) sample

Comparison	r	Comparison	r
(A) Non-isothermal study for HSA aqueous solution (from 30 °C to 80 °C)			
30 °C/40 °C	0.997	30 °C/50 °C	0.992
30 °C/55 °C	0.989	30 °C/60 °C	0.982
30 °C/65 °C	0.976	30 °C/70 °C	0.914
30 °C/75 °C	0.803	30 °C/80 °C	0.741
(B) Isothermal study for HSA aqueous solution (isothermal at 80 °C for 1–15 min)			
Native/1 min	0.974	Native/2 min	0.950
Native/5 min	0.898	Native/10 min	0.881
Native/15 min	0.833		
(C) Thermo-reversible study for different states of HSA			
(a) Solid powder			
30 °C/80 °C	0.896	30 °C/200 °C	0.781
30 °C/30 °C*	0.969		
(b) Cast film			
30 °C/80 °C	0.905	30 °C/200 °C	0.771
30 °C/30 °C*	0.944		
(c) Aqueous solution			
30 °C/30 °C*	0.741	30 °C/30 °C*	0.974

30 °C* indicated preheated to 200 °C (a, b) or 80 °C (c), then cooled to 30 °C.

The result of thermal-dependent structural similarity of HSA in aqueous solution is listed in Table 1. Using the IR spectra at 30 °C as a reference, we found that with the increase of temperature, the lower r value was obtained. The r value still larger than 0.982 before 60 °C, but became 0.741 for 80 °C-heated sample. Namely, the IR spectra were not so similar to the sample treated at higher temperature when compared with the native HSA as a reference. As shown in Fig. 1, the spectra obtained at 30 and 60 °C were essentially the same. The shape of IR spectra changed beyond 60 °C, and the formation of 1620 cm^{-1} peak was found (Fig. 2A). Furthermore, the content of α-helix structure of HSA decreased but its β-sheet structure increased beyond 60 °C. The heating process made no major alteration for random coil structure (Fig. 2B). In other words, the denaturation of HSA might explain the transformation from α-helix to β-sheet structure.

It is well known that the denaturation of protein is only partially reversible in many cases, in which the thermal denaturation often results in an irreversible inactivation [34]. In order to investigate the reversibility of the IR spectral changes of HSA, the HSA aqueous solution was preconducted at 80 °C from 1 to 15 min for the accelerated stability studies. At predetermined intervals, the HSA solution was then quickly cooled to 30 °C for IR

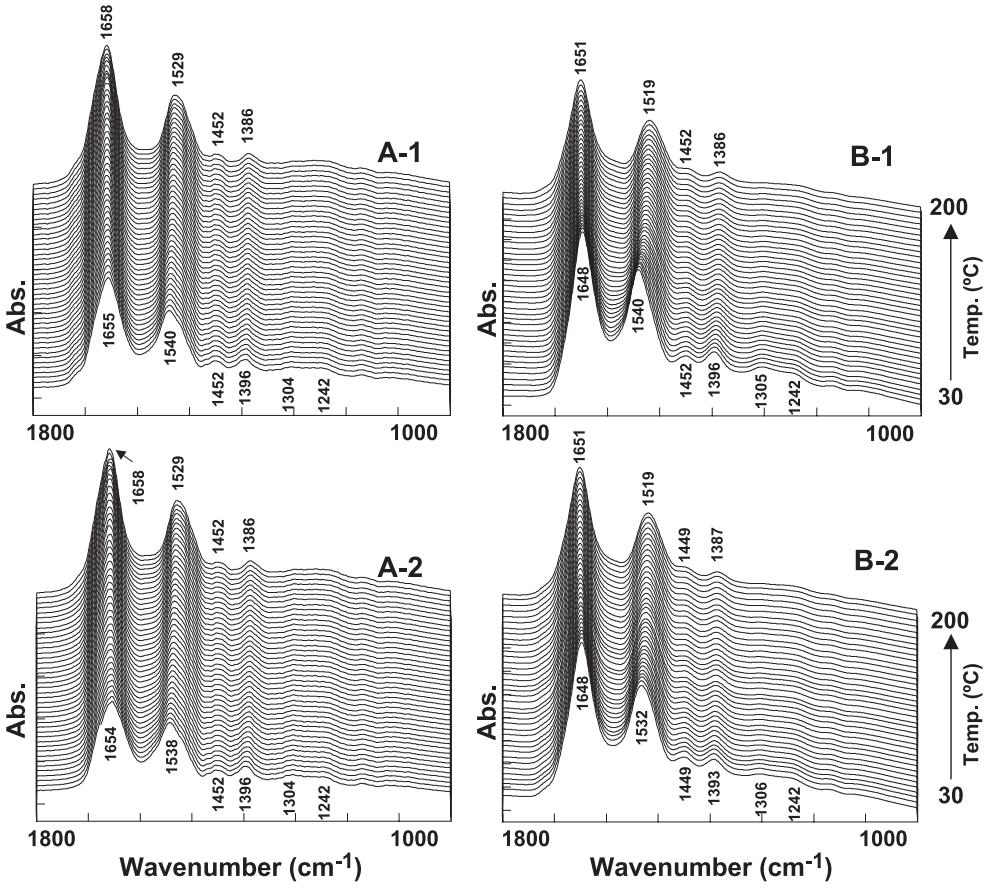

Fig. 5. The effect of temperature on the three-dimensional plots of FTIR spectra for HSA in the solid powder (A) and cast film (B) and within 1800 and 1000 cm^{-1}. Key: A-1, B-1: first heating; A-2, B-2: reheating.

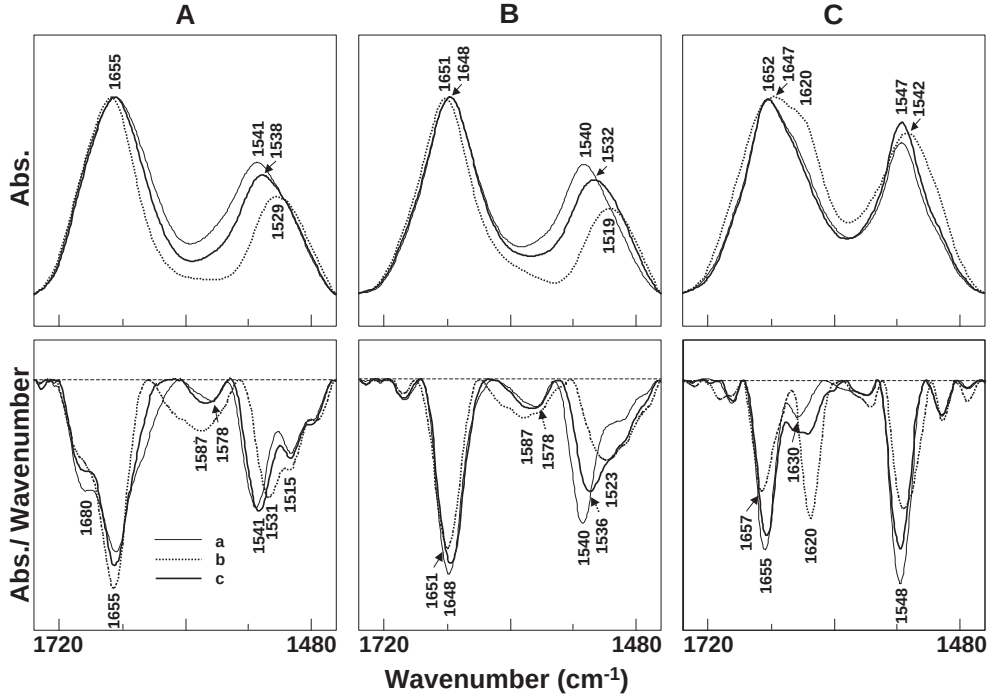

Fig. 6. The original and second-derivative IR spectra of HSA in three different states before and after heating treatment. Key: (A) solid powder; (B) cast film; (C) solution; a, native (30 °C); b, heating to 200 °C (A, B) or 80 °C (C); c, cooling to 30 °C.

determination. The results are shown in Fig. 4 and Table 1. The samples pretreated at 80 °C gave an r value against 1.0 and became 0.833 after 15 min heating, suggesting the poor reversibility of HSA in aqueous solution with heating. Temperature and water might result in this irreversible denaturation.

The original and second-derivative IR spectra of HSA in three different states (solution, cast film, and solid powder) are shown in Fig. 6. The spectral correlation coefficient (r) of structural similarity for these three different states is also listed in Table 1. It is interesting to note that the heated HSA sample, whether in powder or film form, exhibited a good thermo-reversible property even heated to 200 °C, in which their r values were 0.969 and 0.944, respectively. This confirms that a dry state of protein is less sensitive to temperature, due to the lack of water. If we compared the 80 °C- pretreated HSA samples with the native HSA sample, the r value was near 0.896–0.905 for both film and powder samples, but was 0.741 for HSA aqueous solution. This illustrates that water played an important role in the thermal stability of HSA. Although the 80 °C-pretreated HSA aqueous solution for 1 min seemed to exhibit a thermo-reversibility (r=0.974), the 15 min-heated sample became 0.833, suggesting the poor reversible property of HSA in aqueous solution with heating. Temperature and water might result in this irreversible denaturation.

5. Conclusions

HSA aqueous solution was non-isothermally or isothermally investigated to study its thermal stability and thermo-reversibility. The cast film and solid powder of HSA were also determined. The results indicate the reduction of the α-helix conformation in HSA aqueous solution might favor the formation of intermolecular β-sheet structure beyond 60 °C in the heating process. The structural similarity of two second-derivative spectra was used to estimate the thermo-reversibility of HSA samples. HSA sample, whether in powder or film form, exhibited a good thermo-reversible property even heated to 200 °C, compared with HSA in aqueous solution.

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