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Thermal denaturation of mixtures of human serum proteins

DSC study

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Abstract Differential scanning calorimetry (DSC) has been employed to study the thermal denaturation processes of the main protein fractions of blood serum. These processes have been compared for albumins (nondefatted (HSA) and fatty acid free (HSAf)), α,β-globulins, γ-globulins, and their mixtures in aqueous (pH 6.5) and buffer (pH 7.2) solutions. The results have indicated that α,β-globulins inhibit γ-globulins' aggregation in buffer solutions. The decrease of stability of HSA and HSAf aqueous solutions has been observed in the presence of γ-globulins. The mixtures of albumins and γ-globulins have revealed the tendency to ready aggregation in water. Moreover, the results have suggested that neither γ-globulins nor albumins severely change the stability of α,β-globulins.

Keywords Albumin · α,β-globulins · Differential scanning calorimetry · γ-globulins · Human serum · Protein denaturation

Introduction

Serum and serum proteins are commonly used for disease diagnosis and in the clinical therapeutic studies. Their elevated or decreased levels, the alterations of protein structure or polymerization state, abnormal denaturation and glycosylation, may serve as indicators of disease [1–4]. The differences between serum from normal and diseased individuals investigated by differential scanning calorimetry (DSC) can also provide signatures for specific diseases [5–7]. Presumably, modifications in the protein composition accompanying pathological conditions are not the sole origin of alterations caused by disease state in serum thermogram [7]. Whether human plasma (or serum) is indicative for specific disease or only for disease in general remains an open question. The profound studies of protein–protein interactions may help in understanding of the above problems. The recognition of properties of serum proteins mixtures would be the first step of such studies.

The thermal properties of serum proteins (for instance the ability to form gels during heating) and the knowledge regarding interactions that change their thermal behavior in the presence of other proteins are important in the food industry, where proteins are widely used. An inhibitory effect of albumin on globulin aggregation was observed in mixtures of albumin and globulins when albumin was the dominant fraction, although no major changes in the proteins' molecular structure were observed in FT-Raman spectra [8].

The studies concerning heat-induced effects in proteins are relevant for the development of protein pharmaceuticals, because a thermal treatment is applied in the processing of pharmaceutical products. These studies contribute to understanding of instabilities of liquid protein pharmaceuticals. Kinetic and thermodynamic models are developed to explore the stabilization effect of additives i.e. sugars and to analyze the aggregation mechanism of protein [9, 10].

In this study, the main protein fractions of blood serum: albumins (nondefatted (HSA) and fatty acid free (HSAf)), α,β-globulins, γ-globulins, and their mixtures were studied by DSC. The aim of the study was the characterization of the thermal denaturation processes of serum proteins as well as their mixtures in buffer and in aqueous solutions.

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Two kinds of solvent were used due to the earlier serum studies in similar experimental conditions.

Materials and methods

Materials

Essentially globulin free serum albumins (purity minimum 99%): HSAf-defatted (Product A3782, ~0.005% fatty acids), HSA-containing endogenous fatty acids (Product A8763), α,β-globulins (Product G3637) and γ-globulins (Product G4386) from human blood were obtained from Sigma Chemical Co.

Aqua pro injection (Fresenius Kabi, Poland) and buffer KH_2PO_4/Na_2HPO_4 (pH 7.2; FIXANAL, SIGMA-ALDRICH) were used as the solvents.

Sample preparation

Protein solutions at concentration 1.5 and 0.75 g L^{-1} were prepared by dissolving the proteins in degassed water (pH $\approx$ 6.5) or buffer (pH = 7.2). The exact protein concentration was determined spectrophotometrically using absorption spectra recorded in the wavelength range of 200–400 nm on JASCO V-530 spectrophotometer using 1.0 cm path length quartz cuvettes. The absorbance at 279 nm for the concentration of 1 g L^{-1} in a 1-cm cuvette was taken as 0.531, 1.20, and 1.38 for albumin, α,β-globulins, and γ-globulins, respectively.

Mixtures of protein solutions at fractions ratio 1:1 with an effective concentration of 0.75 g L^{-1} for each of them were made.

DSC measurements

DSC measurements were carried out on a VP DSC ultrasensitive microcalorimeter (MicroCal Inc., Northampton, MA) with cell volumes 0.5 mL. Heat capacity versus temperature profiles were obtained in the temperature range 293–373 K at a programmed heating rate of 1 K min^{-1}. The constant pressure of about 1.8 atm over the liquids in the cells was applied.

The calorimetric data were corrected for the instrumental baseline water–water or buffer–buffer depending on solvent. DSC curves were normalized for the gram concentration of protein. All DSC experiments were repeated 2–5 times. DSC curves were analyzed with MicroCal Origin software.

The denaturation temperature T_m corresponding to the maximum heat capacity, the heat of transition, or enthalpy change ΔH (determined as the area framed by the heat capacity peak and transition baseline) were computed from each thermal curve.

Results and discussion

Thermal denaturation of individual serum proteins

Figure 1a–d shows the DSC curves of four serum proteins: HSAf, HSA, α,β-globulins, and γ-globulins in buffer and water solutions. The thermal denaturation of these proteins is represented by endothermic peaks connected with unfolding events. For γ-globulins in buffer solution at pH 7.2 the endothermic transition has been additionally followed by an exothermic one, corresponding to the protein aggregation (inset in Fig. 1d).

Figure 1a indicates that in case of fatty acid free albumin the transition is more sharp, so the thermal unfolding of HSAf is more cooperative, in buffer than in water solution. The shape of HSA curves is very similar for its water and buffer solutions (Fig. 1b). The thermal curve of α,β-globulins solution (Fig. 1c) displays multiple peaks and shoulders associated with contributions from different constituent proteins of this serum fraction. The most specific peak is the one at about 333 K mainly coming from haptoglobin denaturation [7]. This peak together with the whole denaturation transition curve is shifted to higher temperatures in buffer in comparison with water solution. The endothermic transition for γ-globulins proceeds alike in aqueous and buffer solutions up to about 345 K (Fig. 1d). However, above this temperature protein rapidly aggregates in buffer solution while the unfolding process is going on up to about 360 K in water solution.

The thermodynamic parameters presented in Table 1 allow the comparison of thermal stability of albumins, α,β-, and γ-globulins in aqueous and buffer solutions. It was not possible to indicate the proper values of T_m and ΔH for unfolding of γ-globulins in buffer solution due to the overlapping of endo- and exothermic processes. The highest T_m has been observed for HSA (353.3 K in buffer and 352.6 K in water). The most substantial heat effect has been associated with unfolding of γ-globulins in aqueous solution (ΔH = 23.5 J g^{-1}). The results obtained here are in agreement with those reported earlier for thermal denaturation of albumins and immunoglobulins [8, 11–16]. The small differences are mainly connected with the kind of buffer used as protein solvent.

Thermal denaturation of protein mixtures

Figure 2 shows the averaged raw DSC curves for α,β- and γ-globulins buffer solutions (protein concentration 0.75 g L^{-1}) and the $\alpha,\beta/\gamma$ mixture (ratio 1:1, at 1.5 g L^{-1} final

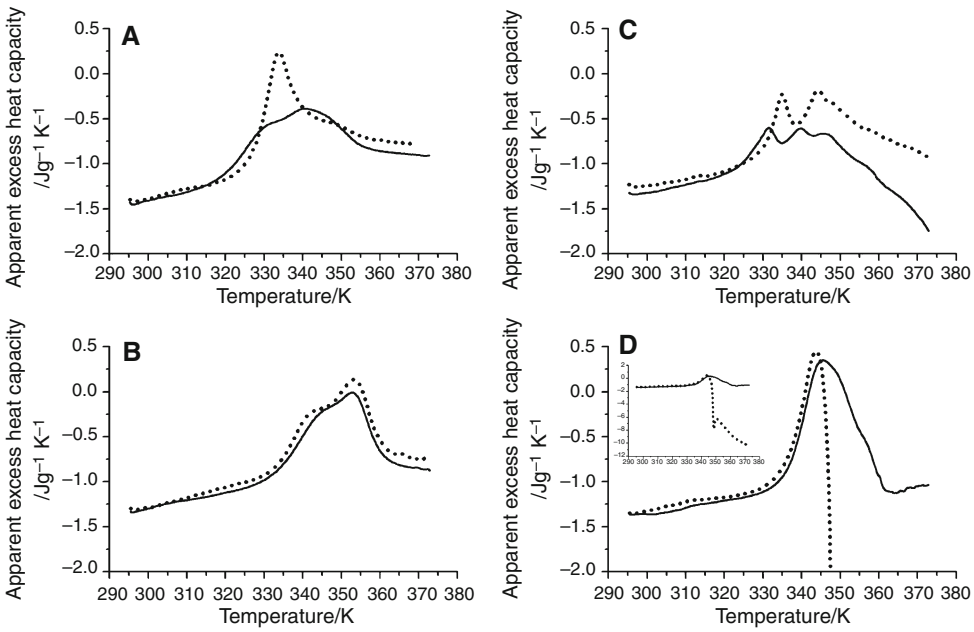

Fig. 1 DSC curves of HSAf (**a**), HSA (**b**), α,β-globulins (**c**) and γ-globulins (**d**) in buffer (*dots*) and water (*solid line*) solution. The inset in **d** shows exothermic aggregation

Table 1 The thermodynamic parameters (mean ± standard deviation) of serum proteins thermal denaturation in water and buffer solutions

Parameter	T_m/K		ΔH/J g^{-1}	
Protein solution	Water	Buffer	Water	Buffer
HSAf	341.4 ± 1.2	333.5 ± 0.4	12.8 ± 1.6	12.6 ± 0.8
HSA	352.6 ± 0.6	353.3 ± 0.7	16.8 ± 1.0	13.8 ± 1.2
αβ-globulins	339.9 ± 0.3	335.1 ± 0.2	16.3 ± 0.8	13.4 ± 1.9
γ-globulins	345.5 ± 0.3	–	23.5 ± 1.9	–

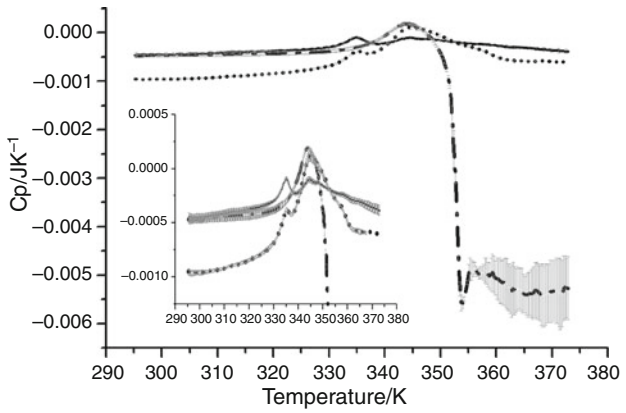

Fig. 2 The averaged raw heat capacity data for buffer protein solutions (protein concentration 0.75 g L^{-1}) of: α,β-globulins (solid line), γ-globulins (*dash-dot line*) and the α,β/γ mixture (*dotted line*) (ratio 1:1, at 1.5 g L^{-1} final protein concentration of mixture). The inset—the enlargement of the region of endothermic transitions. The *shaded area* represents the standard deviation at each temperature

protein concentration of mixture). Since the heat effect attributed to γ-globulin aggregation masks the other transitions, so the region of endothermic transitions is shown in the inset. The shaded area represents the standard deviation at each temperature. Due to good repeatability of measurements, the shade is hardly visible except the case of γ-globulin curve in the temperature range above 355 K. This observation suggests that the heat capacity of aggregated protein is not distinctive (specific) because various aggregated states of γ-globulin are probably possible.

On the contrary to γ-globulins denaturation, where both the endothermic and exothermic transitions occur, no exothermic events have been observed during heating of α,β/γ mixture. Thus, α,β-globulins inhibit γ-globulins aggregation in buffer solutions.

Similar protective effect toward γ-globulin has been revealed for both albumins: HSA and HSAf. The thermal denaturation of HSAf/γ as well as HSA/γ mixture in buffer solution proceeds as an endothermic transition without clear aggregation step (Fig. 3).

The profiles of thermal denaturation for mixtures of albumins with γ-globulins in aqueous solutions are shown in Fig. 4. The comparison of DSC curves in Fig. 1a, b and in Fig. 4 shows that the endothermic peaks connected with unfolding of HSA and HSAf in the presence of γ-globulins are not fully developed. The drop of heat capacity in higher than ~345 K temperature range points out an aggregation

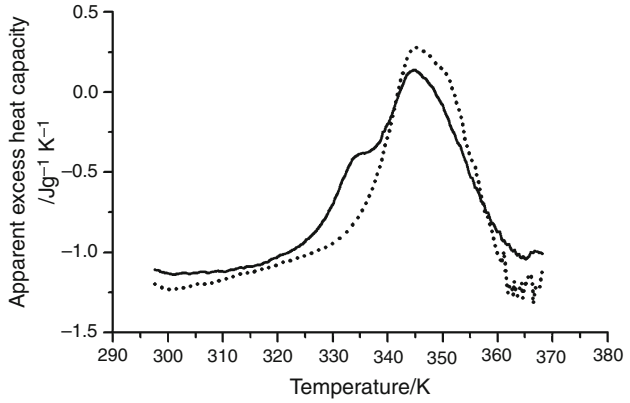

Fig. 3 The representative DSC curves of mixtures: HSA/γ (*dots*) and HSAf/γ (*solid line*) in buffer solutions

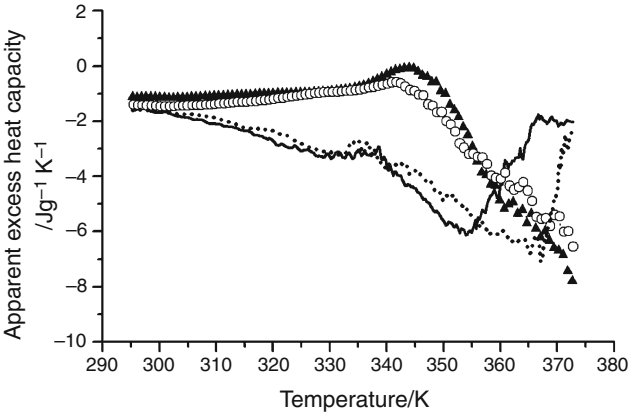

Fig. 4 The representative DSC curves of albumins–γ-globulins aqueous mixtures heated from 293 to 373 K: HSA/γ (*triangles*), HSAf/γ (*open circles*) and after cooling to 293 K heated again to 373 K: HSA/γ (*solid line*), HSAf/γ (*dots*)

of unfolded protein chains. Thus, the stability of aqueous HSA and HSAf solutions decreases in the presence of γ-globulins. The thermal transitions observed for aqueous mixtures of albumins and γ-globulins are completely irreversible. It is well visible in Fig. 4, where the second (reheating) scans of the same samples are shown. These DSC profiles for repeated heating are interesting because they show the properties of already denatured protein molecules. After cooling and reheating, the exothermic aggregation transitions have been observed for denatured forms of both albumins and γ-globulins in mixture.

Figure 5 illustrates the thermal unfolding of α,β-globulins, HSA, and their mixture in buffer solution. The DSC curve of mixture is very similar to the sum of DSC curves obtained when the individual proteins were heated separately. It suggests the lack of clear interactions between component proteins of α,β-globulins/HSA mixture. The same is true in case of HSAf/HSA aqueous and buffer mixtures (not shown).

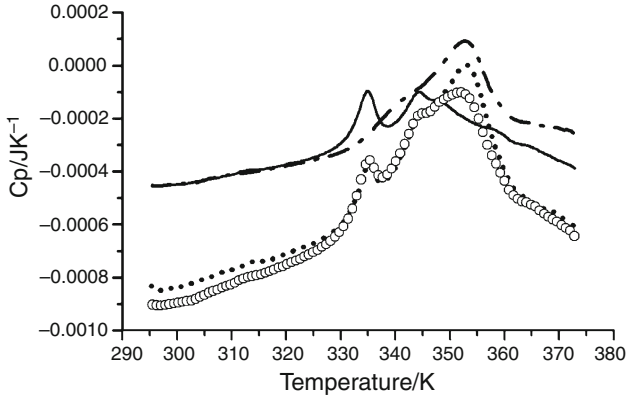

Fig. 5 The raw heat capacity data for α,β-globulins (*solid line*), HSA (*dash-dot line*) and the α,β/HSA mixture (*dotted line*) in buffer solutions (α,β-globulins and HSA concentration—0.75 g L^{-1}, total protein concentration of mixture—1.5 g L^{-1}).The sum of DSC curves of α,β-globulins and HSA (*open circles*)

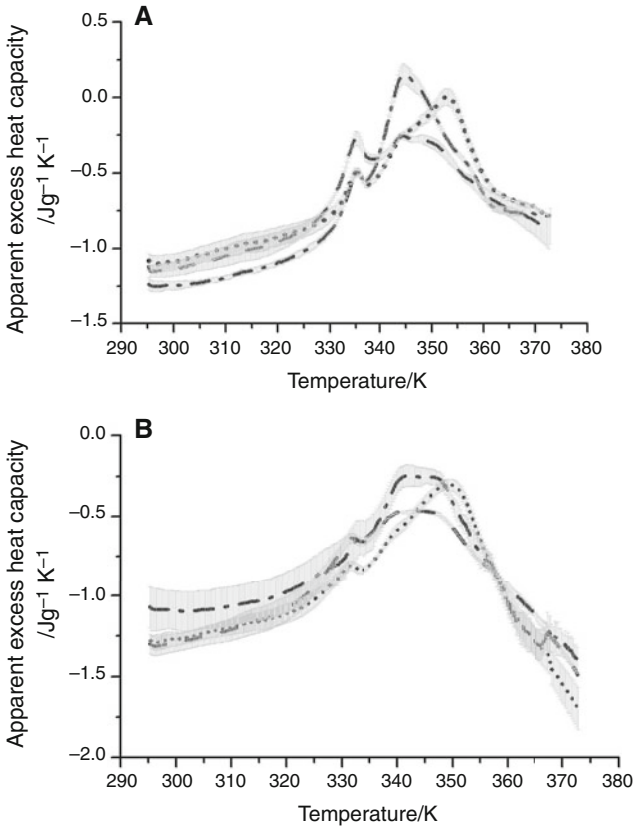

Fig. 6 The averaged DSC curves of mixtures: α,β/γ (*dash-dot*), α,β/HSA (*dots*) and α,β/HSAf (*dashes*) in buffer (**a**) and in water (**b**) solutions. The *shaded area* represents the standard deviation at each temperature

The averaged thermal denaturation profiles of α,β-globulins mixtures with γ-globulins, HSA, and HSAf in buffer and aqueous solutions are shown in Fig. 6a and b, respectively. Neither γ-globulins nor albumins severely change the stability of α,β-globulins.

Table 2 The heat effect (ΔH) of protein mixtures denaturation in buffer (b) and water (w) solutions

Mixture			αβ/HSAf	αβ/HSA	αβ/γ	HSAf/γ	HSA/γ	HSAf/HSA
ΔH/J g^{-1}	b	1)	13.8	13.4	17.3	24.7	24.6	14.7
		2)	13.0	13.6	–	–	–	13.2
	w	1)	14.5	18.6	22.0	–	–	16.5
		2)	14.6	16.6	19.9	18.2	20.2	14.8

1) determined from averaged DSC curves of protein mixtures, 2) calculated as a sum of denaturation enthalpies for constituent proteins of mixtures

The heat effects (ΔH) for thermal denaturation of protein mixtures in water and buffer are shown in Table 2 (The values of ΔH for αβ/γ, HSAf/γ, and HSA/γ mixtures in buffer solution have not been calculated due to the lack of ΔH estimation for γ-globulins; ΔH for HSAf/γ and HSA/γ mixtures in water have not been determined on account of the overlapping of endo- and exothermic processes). The results indicate that the enthalpy changes connected with denaturation of protein mixtures differ insignificantly from the sum of denaturation enthalpy found for constituent proteins of mixtures. It confirms the lack of clear interactions between component proteins of αβ/HSAf, αβ/HSA, and HSAf/HSA mixtures.

Conclusions

The thermal denaturation process of proteins from main serum fractions: albumins, α,β-globulins, γ-globulins, and their mixtures proceed in a wide temperature range from about 320–360 K. Each protein unfolds in the specific way with endothermic heat effect. The protein aggregation phenomena coming along with this step of denaturation or followed it can be in some cases clearly observed as exothermic transition. The comparison of serum proteins denaturation in aqueous and buffer solutions indicates that significant differences occur for both solvents. Only in case of HSA, DSC profiles are similar for its water and buffer solutions.

The most meaningful difference has been observed for γ-globulins. In water solution just the endothermic transition is present while in buffer solution the endothermic unfolding of γ-globulins is followed by the exothermic aggregation. The distinct difference between γ-globulins thermal behavior in water (pH $\sim$ 6.5) and buffer solution (pH $\sim$ 7.2) does not probably arise from the pH variation. It is rather specific for the buffer system. The similar exothermic aggregation has been observed for γ-globulins and albumin in Tris–NaCl–glycerol buffer (pH $\sim$ 7.0) [17] as well as in 0.9% NaCl solution (pH $\sim$ 6.5) (own experimental data).

The complex endothermic transition connected with the unfolding of different constituent proteins of α,β-globulins fraction is shifted to higher temperatures in buffer when compare with water solution. However, enthalpy change for denaturation of α,β-globulins is bigger in water solution.

More sharp endothermic transition observed for fatty acid free albumin in buffer indicates that protein thermal unfolding proceeds in more cooperative way in this solvent. The heat effects connected with HSAf thermal unfolding in water and buffer are similar, because the denaturation enthalpy does not differ significantly in both solvents.

The comparison of DSC curves of mixtures with the sum of the data obtained for separately heated constituent proteins allows to receive an information regarding protein–protein interactions. Davila et al. [8] reported the inhibitory effect of albumin on globulin aggregation when albumin was the dominant fraction in mixtures of albumin and globulins. The results of our study confirm those observations for buffer (pH 7.2) protein solutions. The strong exothermic transition connected with γ-globulins aggregation, clearly visible in DSC curves of γ-globulins buffer solutions, is absent in thermal curves of this protein mixtures with albumins (HSAf and HSA). No exothermic transition has appeared also for γ-globulins and α,β-globulins 1:1 mixture. Thus, albumin as well as α,β-globulins have revealed an inhibitory effect on γ-globulin's aggregation.

Unlike effect has been observed in aqueous solutions. The presence of γ-globulins has decreased the stability of HSA and HSAf. The aqueous mixtures of albumins and γ-globulins have disclosed the tendency to ready aggregation.

Moreover, it follows from the presented results that neither γ-globulins nor albumins severely change the stability of α,β-globulins. The unaffected value of the transition enthalpy, as well as of the peak profile, support the lack of clear interactions between these proteins. However, more detailed analysis of differences between DSC curves of protein mixtures and the sum of thermal profiles of their component proteins will be addressed in following studies to preclude or confirm the occurrence of protein–protein interactions.

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