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STABILITY OF BOVINE SERUM ALBUMIN AT DIFFERENT pH

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The effect of pH on the thermal denaturation of BSA containing fatty acids was studied by use of differential scanning calorimetry (DSC). Thermal scanning of BSA aqueous solutions gave various types of DSC curves depending on the protein concentration and on the pH. The broad bimodal endothermic transition was suggested to be connected with loose protein structure in contradistinction to single peak for compact molecule structure. The propensity toward precipitation at pH conditions ranging from 3.8 to 5 was observed. A scan–rate independent and partly reversible behavior of the thermal heating of BSA was found. Deconvolution of DSC traces in non-two-state model with assumption of two- or three-component transition allowed to study the effect of pH on different parts of BSA molecule.

Keywords: *bovine serum albumin, DSC, protein denaturation, thermal stability*

Introduction

Serum albumin, a principal component of blood, the most multifunctional transport protein, is also chiefly responsible for the maintenance of blood pH. In solution, the different isomeric forms of serum albumin exist depending on pH value [1, 2]. The thermal characteristics of albumin are also pH-dependent. The effects of pH, ionic strength, SDS concentration and other environmental conditions on the thermal stability were reported mainly for essentially fatty acid free bovine serum albumin (BSA) [3–6]. However, the substantial differences in stability of defatted and carrying fatty acids BSA (circulating albumin normally contains 1–2 fatty acids per molecule) in aqueous solution were evidenced earlier [7]. Albumin protected by fatty acids is more stable and at constant conditions the endothermic peak connected with its unfolding is very well reproducible. Owing to the latest fact, the influence of different factors on protein stability can be investigated basing on the even subtle changes in the shape of DSC curves [8].

In this work, the effect of pH on thermal denaturation of non-defatted BSA has been studied by differential scanning calorimetry (DSC) in the range of pH 3.5–7. The 'N' (normal) form is predominant in the pH 4.5–7. Between pH 4.5 and 4.0 the N–F (Normal–Fast; 'F' – fast migrating) transition occurs and 'F' form is produced abruptly upon lowering the pH to <4. At pH lower than 3.5 the 'E' (expanded) form appears. The N–F transition involves a decrease in the content of ordered (secondary) structure. The albumin molecule in the N-form is more compact, while it becomes partly open in the F-state.

Barone *et al*. [3] found that thermodynamic characteristics of the thermal denaturation of BSA are related to protein concentration. Thus, in this study, DSC measurements have been performed at two BSA concentrations: 0.022 and 0.044 mM L^{-1}. The studies were performed in aqueous solutions for two reasons: 1) to enable the comparison of current results for undefatted BSA with those obtained earlier for defatted BSA [6, 7] in the same conditions, 2) the shape of DSC peaks was considerably better (for analysis) than in phosphate buffer, where appreciable protein aggregation occurred.

Materials and methods

Undefatted BSA as a crystallised and lyophilised powder (purity minimum 99%), essentially globulin free (023K7612 CAS 9048-46-8) was obtained from Sigma. It is likely to be not more than 1.5 mol of endogenous LCFA/mol of albumin molecule in this preparation. Nielsen *et al*. [9] determined the total amount of fatty acids bound to a commercial albumin preparation (fraction V, 96–99% pure, from Sigma) as 1.47 and 1.43 mol fatty acid/mol protein by the gas chromatographic and an enzymatic method, respectively. Quantitatively, palmitic, stearic, oleic and linoleic acid are the more important, and together they constitute about 86% of the total.

The BSA solutions (0.022 and 0.044 mM L^{-1}, which will be named below 'low' and 'high' respectively) were prepared using distilled and degassed water. The protein concentrations were additionally monitored spectrophotometrically.

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The pH of water in which BSA was dissolved, was adjusted to the desired value with 0.1 M NaOH or HCl solution. The measurements of pH were made with pHmeter model CI-316 (ELMETRON) with accuracy ±0.1.

DSC was carried out on a VP DSC ultrasensitive microcalorimeter (MicroCal Inc., Northampton, MA) with cell volumes 0.5 mL in the temperature range 20–100°C. A scan rate 1 K min^{-1} was chosen for the present study, except for the experiment on scan-rate dependence, where the scan rates ranged from 0.66 to 1.5 K min^{-1}. All other DSC experimental details were the same as described previously [7].

The calorimetric data were corrected for the calorimetric baseline (by subtracting water – water scan) and for the difference in heat capacity between the initial and the final state by using a sigmoidal baseline. All data manipulation was performed using the Origin software provided with the instrument. The fit of the curves of the theoretical model to the experimental data was achieved by the non-linear Levenberg–Marquardt method.

The experimental error was estimated by the standard deviation for measurements repeated several times on the sample with the same pH value.

Statistical analysis of the results was done with Statistica 5.1 using Kruskal–Wallis test.

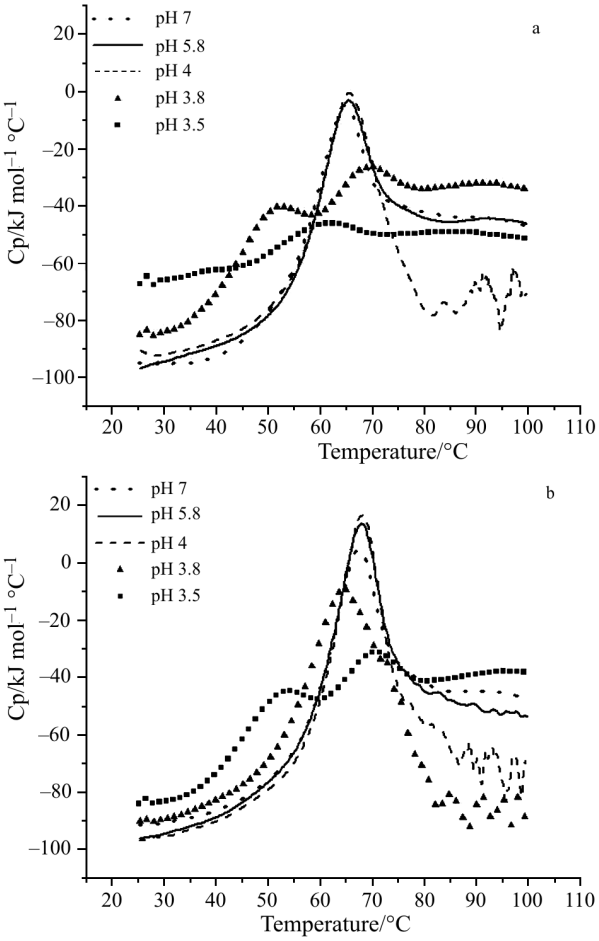

Fig. 1 Effect of pH on DSC curves of BSA solution; a – 0.022 mM L^{-1}, b – 0.044 mM L^{-1}

Results

pH dependence of DSC curves

Thermal denaturation of albumin in aqueous solution is observed as an endothermic transition on DSC curves. The profiles of calorimetric curves show a marked dependence on solution pH. The baseline – corrected and concentration – normalized curves in Fig. 1a, b show how the pattern of DSC curves vary with the changes in pH for 'low' and 'high' BSA concentration respectively. If the stability of albumin was independent on the concentration of the solution, the shape of DSC curves should be the same in Fig.1a and b at given pH value. However essential differences exist, particularly in the acidic pH region.

DSC curves are very similar at pH from 5.5 to 7.0. BSA has one melting temperature under these solution conditions. Decreasing of the pH causes precipitation of the protein, that is evident as the drop and the jaggedness of the right shoulder of the peak. (Such effect is not observed at pH 7). Next the transition becomes very broad and bimodal for 'low' con-

Table 1 Overall calorimetric parameters (mean±SEM) for the thermal denaturation of BSA at different pH values obtained for BSA concentrations: 0.022 mM L^{-1} (l) and 0.044 mM L^{-1} (h)

pH	ΔH_{cal}/kJ mol^{-1}		T_m/°C		$T_{1/2}$/°C	
	l	h	l	h	l	h
3.5	126±28	496±13	58.2±0.7	52.7*±0.5	15.1±0.8	15.7*±0.3
3.8	401±5		49.7*±0.1		13.6*±0.1	
4.0	994±36	999±38	65.1±0.4	67.5±0.1	12.8±0.5	10.2±0.1
5.8	803±39	833±34	64.7±0.1	67.3±0.1	11.6±0.4	10.3±0.1
7.0	708±19	745±64	63.8±0.1	66.2±0.3	11.3±0.1	10.8±0.1

SEM – standard error of the mean
* – parameters for the lower temperature peak in bimodal curve

centration at pH 3.8 and for 'high' concentration at pH 3.5. In strong acidic media (pH< 3.5) BSA shows only very small, broad endothermic peak. At 'low' protein concentrations this kind of transition is observed even at pH=3.5 (Fig. 1a).

Results of preliminary analysis

The results of preliminary analysis based on integration of data in selected temperature range (enthalpy change ΔH, the transition temperature, T_m (defined as the temperature at which a maximum occurred in the endothermic peak) and width of curve at half height, $T_{1/2}$) are displayed in Table 1. Reported values are the means of 3–10 independent replicates (the errors are given as standard errors of the mean–SEM).

The value of ΔH was maximum for pH 4. However this value could be somewhat overestimate on account of the effects caused by the strong precipitation under these acidic conditions. The smallest ΔH was determined for pH 3.5. The results of statistical analysis indicated essential differences in ΔH (p<0.01) between all pH, except the pair 5.8 and 7.0 for both 'low' and 'high' concentrations.

The T_m values were the highest at pH 4.0 and 5.8. The difference between T_m for these two pH values was not significant while each other T_m's for various pH differed essentially.

The biggest width of bimodal DSC curves is self-evident (Fig. 1). No essential differences were found for $T_{1/2}$ in the pH range 4.0–7.0.

The comparison of the transition parameters for two studied concentrations showed the statistically essential differences (p<0.01) between all T_m's at constant pH values. Additionally statistically essential differences were obtained between $T_{1/2}$ for 'low' and 'high' concentration at pH 4.0.

Reversibility

The usual interpretation of DSC data, with equilibrium models, presumes that equilibrium conditions are maintained during the scans. The lack of influence of scan rate on the unfolding midpoint temperature and reversibility of the transition are the criterions, which must be discussed [9]. We found that under the experimental conditions of our study there was no significant dependence of the heat capacity data on scan rate (in range 0.66–1.5 K min^{-1}), but the transition was only partly reversible. The reversibility of the transitions was checked in detail by the preliminary heat of albumin up to 60, 70, 80, 100°C and their cool up to 20°C with subsequent scanning. Figures 2a, b and c illustrate the results of this experiment for the 'high' BSA solution at pH 7 and 4 and for the 'low' concentration at

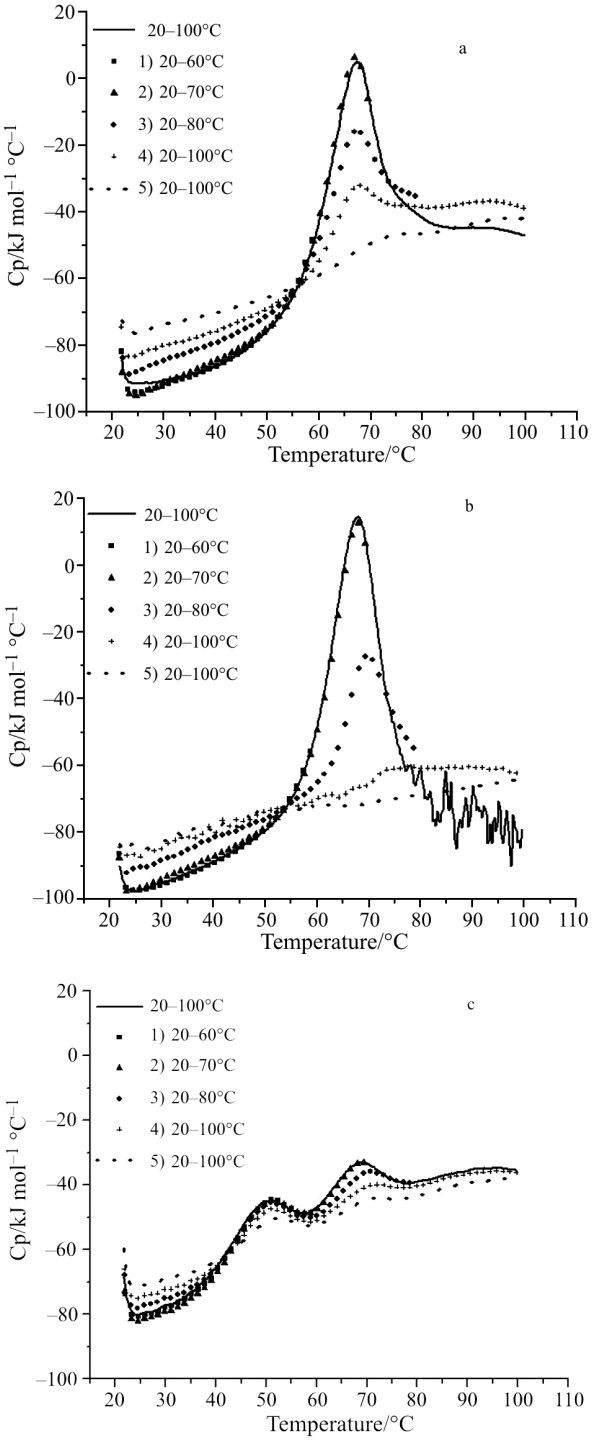

Fig. 2 DSC curves for: 0.044 mM L^{-1} BSA solution a – pH 7, b – pH 4 and c – 0.022 mM L^{-1} BSA solution, pH 3.8, obtained by successively scanning (scans 1–5) the protein up to different final temperatures in comparison with individual scanning from 20 to 100°C

pH 3.8. When the BSA solutions were heated up to 60°C, then cooled and re-heated again, the observed curves were superimposed (curves (1) and (2) in Fig. 2). It was true even in the case of 'low' concentration

Table 2 Reversibility of BSA denaturation process after preliminary heating to different temperatures

T/°C	Reversibility/%			
	c=0.022 mM L^{-1}		c=0.044 mM L^{-1}	
	pH 7	pH 4	pH 7	pH 4
60	100	100	100	100
70	78	76	67	57
80	56	48	39	20

at pH 3.8, where temperature 60°C was higher than the T_m of the first transition in bimodal curve. It is worth to point out the relatively big reversibility of this transition also after heat up to 100°C (Fig. 2c). In other cases, if the protein was heated up to the temperature higher than T_m, the peak obtained in subsequent scan was smaller. Percentage reversibility of denaturation process after preliminary heating to different temperatures is shown in Table 2. The estimated uncertainties in reversibility are ±5%.

The ascertained irreversibility, greater in more concentrated BSA solutions is probably caused by precipitation and aggregation. These phenomena take place with significant rate only at temperature above those corresponding to the transition. Equilibrium thermodynamic analysis is permissible in this case [9].

Deconvolution analysis

The fairly high reversibility of the BSA (Table 2 and Fig. 2) after heat up to 70°C (the temperature above T_m) and the scan-rate independence of the DSC curves allowed us to apply reversible denaturation models. We used the Origin software package supplied by MicroCal.

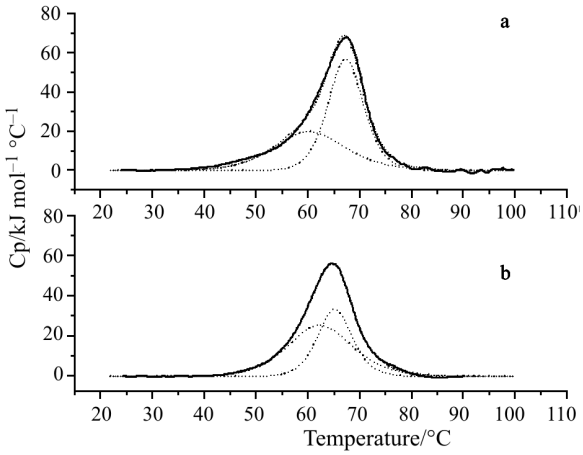

Fig. 3 The fitting of DSC curve with assumption of two-component transition; pH=5.8, a – 0.044 mM L^{-1}, b – 0.022 mM L^{-1} BSA solution

The dependence of the DSC data on protein concentration suggested an equilibrium based on association/dissociation occurring [9]. However the fittings of our experimental data to reversible two-state unfolding model with simultaneous dissociation into n subunits: $N_n \leftrightarrow nU$ where N and U are the native and unfolded states of the protein, respectively, were unsatisfactory. The best fittings (but generally not good, with high value of χ^2) was achieved for $2.6<n<3.8$.

At neutral pH good results were obtained with the three-component two-state model, but the attempts to apply a two-state function at the acidic pH region did not adequately account for the unfolding process.

Finally, for the whole studied pH range, the thermodynamic parameters were determined by fitting the DSC data to a non-two-state model with two- or three-component transitions. A better fit was achieved when three rather than two transitions were included, particularly in the case of 'high' BSA concentration. The results are shown in Figs 3, 4, Table 3,

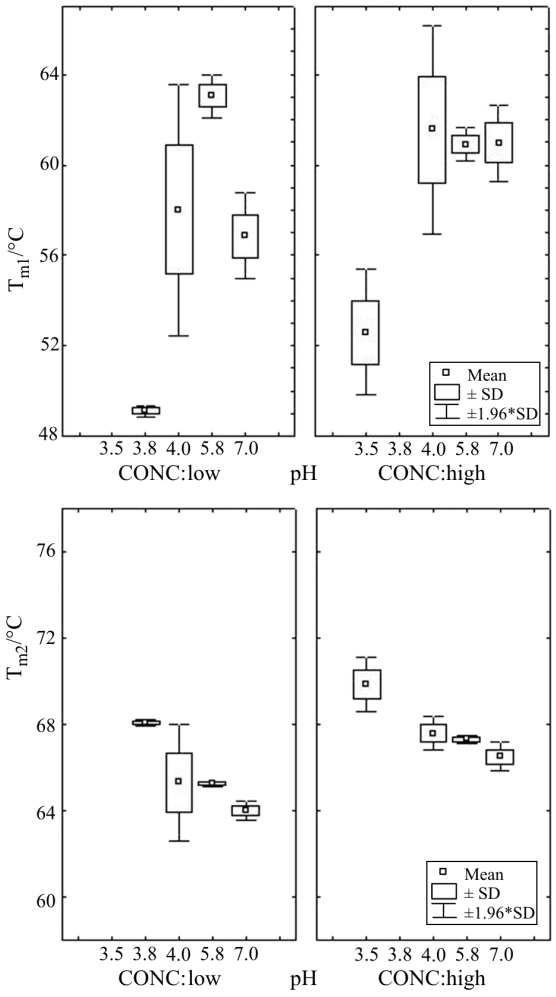

Fig. 4 Effect of concentration and pH on the transition temperatures T_{m1}, T_{m2}

Table 3 The thermodynamic parameters (±SEM*) obtained from the deconvolution procedures (two-component transition)

pH	ΔH_{1cal}/kJ mol^{-1}		ΔH_{1cal}/ $\Delta H_{1\gamma H}$		ΔH_{2cal}/kJ mol^{-1}		ΔH_{2cal}/ $\Delta H_{2\gamma H}$	
	l	h	l	h	l	h	l	h
3.5		323±24		1.5±0.2		181±17		0.5±0.1
3.8	266±1		1.1±0.1		138±2		0.4±0.1	
4.0	336±54	395±38	1.6±0.3	2.0±0.2	627±40	633±69	1.8±0.2	1.6±0.2
5.8	492±24	347±10	2.1±0.1	1.7±0.1	249±17	506±5	0.5±0.1	1.2±0.1
7.0	332±11	348±21	1.6±0.1	1.7±0.2	373±11	378±43	0.9±0.1	0.9±0.1

* SEM – standard error of the mean in columns l and h parameters obtained for BSA concentrations 0.022 and 0.044 mM L^{-1} respectively

Table 4 The thermodynamic parameters (±SEM*) obtained from the deconvolution procedures (three-component transition)

	Enthalpy change ΔH/kJ mol^{-1}					
pH	ΔH_{Ical}	$\Delta H_{Ical}/\Delta H_{I\gamma H}$	ΔH_{IIcal}	$\Delta H_{IIcal}/\Delta H_{II\gamma H}$	ΔH_{IIIcal}	$\Delta H_{IIIcal}/\Delta H_{III\gamma H}$
3.5	46±4	0.1±0.0	289±17	1.2±0.1	184±4	0.5±0.1
4.0	255±50	1.6±0.4	515±79	1.5±0.3	251±50	0.5±0.1
5.8	402±29	2.2±0.4	406±29	1.0±0.1	213±42	0.4±0.1
7.0	146±12	0.8±0.2	603±8	2.1±0.1	117±8	0.2±0.0

* SEM – standard error of the mean

and in Figs 5, 6, Table 4 for two- and three-component transitions, respectively.

For the both studied concentrations the transition temperature of the first from two fitted peaks (lower-temperature with T_{m1}) is significantly smaller at pH≤3.5 (when DSC curves are bimodal) and practically does not depend on pH in the range 4–7. Only between pH 5.8 and 7.0 at 'low' concentration significant difference ($p<0.02$) appears. In analyzed pH range (Fig. 4) the T_{m2} systematically decreases with increasing pH by 4.1 and 3.4°C for 'low' and 'high' BSA concentration, respectively. Such tendency in T_{m2} was unexpected. It is not correlated with behavior of the ΔH_{m2} presented in Table 3. Probably the increase of T_{m2} with acidity does not evidence the higher stability of BSA fragment, which unfolding is represented by higher-temperature peak. The possible explanation is an arising of the fraction of more stable molecules of albumin or its fragments due to the migration of fatty acids between protein molecules upon heating [1].

The proportion of heat effects connected with the first and the second transition is various at different pH (Table 3). The ΔH_{m1} value is somewhat greater at pH 5.8 (only for 'low' BSA concentration) but generally similar in whole studied pH range. The highest ΔH_{m2} (~630 kJ mol^{-1}) is obtained at pH 4.0. At pH≤3.5 this value is 3–4 times lower.

At constant pH (Fig. 4) both T_{m1} and T_{m2} are slightly higher for 'high' than for 'low' albumin concentration (with the exception of T_{m1} for pH 5.8). It is worth mentioning that in the case of 'low' concentration at pH 5.8 the contribution of the lower-temperature peak is bigger than the second one, while for 'high' concentration it is inversely (Fig. 3). These two facts suggest that the part of albumin unfolding in lower temperatures range is more stable in this pH media at lower BSA concentration. The average T_{m2} and ΔH_{m2} are bigger for 'high' than for 'low' concentration by 2°C and 260 kJ mol^{-1} respectively. Thus, the second fragment of albumin molecule seems to be more stable at pH 5.8 when BSA concentration is 'high'.

The results obtained at pH 7.0 in this work (T_{m1}=60.9±0.3°C, ΔH_{m1}=348±21 kJ mol^{-1} and T_{m2}=66.5±0.1°C, ΔH_{m2}=378±43 kJ mol^{-1}) are very similar to the reported by Kang *et al*. [10] (T_{m1}=60.9±0.4°C, ΔH_{m1}=289±8 kJ mol^{-1} and T_{m2}=66.4±1.0°C, ΔH_{m2}=310±46 kJ mol^{-1}) at pH 7.4.

The $\Delta H_{1cal}/\Delta H_{1\gamma H}$ ratio ($\Delta H_{\gamma H}$ – the van't Hoff enthalpy) is at each pH greater than 1 (Table 3), thus the unfolding involves unfolding intermediates or independent domains. The interpretation of $\Delta H_{2cal}/\Delta H_{2\gamma H}$ ratio is difficult because there is no regularity in its value.

DSC results obtained for concentration 0.044 mM L^{-1} were additionally fitted to three peaks. The contributions of transitions described by these peaks are dependent on pH (what is shown in Fig. 5). The middle transition (II) has the greatest ΔH value at each pH. One can see in Fig. 6 that the T_I and T_{II} are significantly lower at pH 3.5 than at other pH. The T_{III} values are similar at all pH, although they slightly decrease with increasing pH. The small difference (5°C) between T_{III} and T_I at pH 5.8 suggests the compact structure of al-

bumin molecule. At pH 3.5, when the structure is much more loose, this difference is about 24°C.

The average calorimetric enthalpy and $\Delta H_{cal}/\Delta H_{\gamma H}$ ratio for each transition are listed in Table 4. These last values seem to be very informative. Particularly interesting is the comparison between pH 7.0 and 5.8. At pH 7.0 this ratio is nearly 1 for the I transition and about 2 for the II one. At pH 5.8 it is inversely. These values suggest that at pH 7.0 peaks with T_I≈54°C and

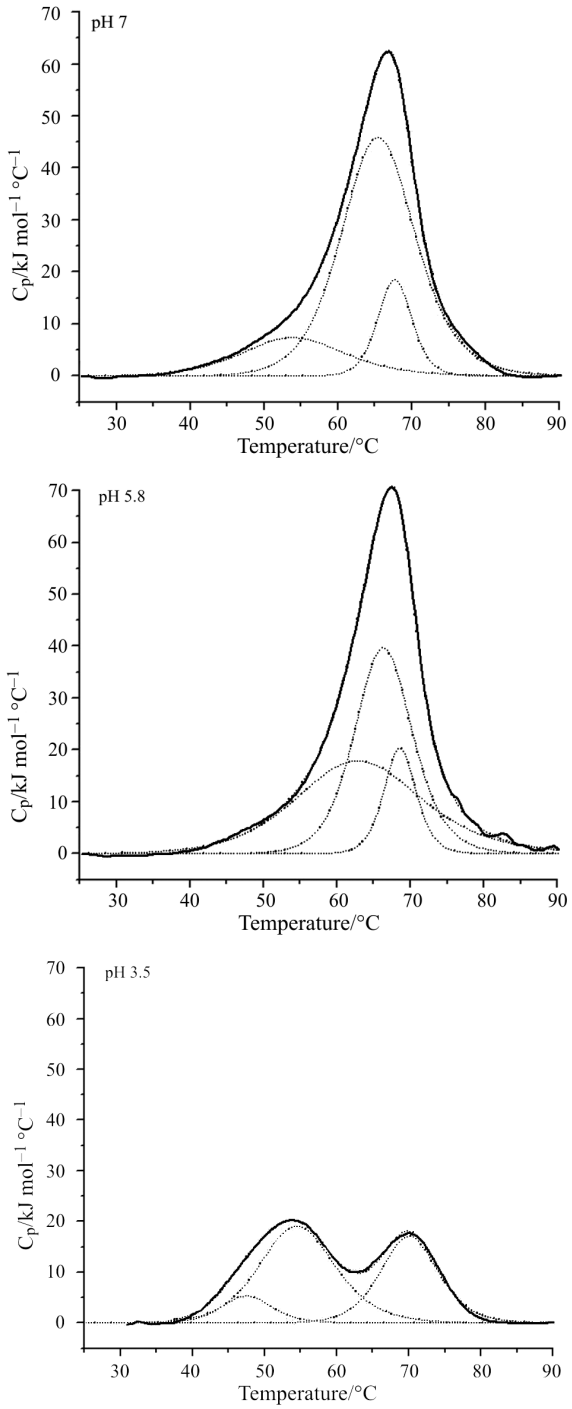

Fig. 5 The fitting of DSC curves with assumption of three-component transition

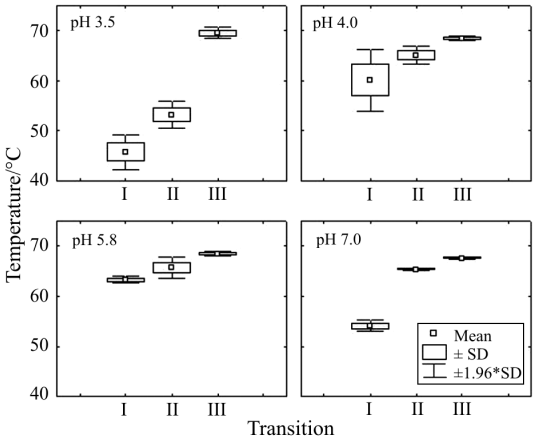

Fig. 6 Effect of pH on the transition temperatures T_{mI}, T_{mII}, T_{mIII}

T_{II}≈65.5°C represent unfolding transitions of one and two BSA domains, respectively, while at pH 5.8 peaks with T_I≈63°C and T_{II}≈66°C are connected with unfolding of two and one BSA domains, respectively (BSA is three-domain protein). At pH 3.5 $\Delta H_{IIcal}/\Delta H_{II\gamma H}$ is approximately 1 indicating 2-state transition of one protein fragment.

We suppose that the third (the highest-temperature) deconvoluted peak, which is very similar for all results obtained at pH range 4–7 (Fig. 5), represents the fraction of the albumin forms stabilized additionally by migrated (upon heating) fatty acids [1]. In every case the $\Delta H_{IIIcal}/\Delta H_{III\gamma H}$ ratio is less than 1 suggesting that intermolecular cooperation play a role in this transition. Another explanation for this result is related to the contributing enthalpies associated with aggregation and precipitation, which artificially sharpen the DSC peak.

Discussion

The effect of pH on the thermal denaturation of BSA was earlier studied by use of defatted BSA [3–6]. The pH-dependent unfolding has been found. Various types of DSC curves, depending on the pH and ionic strength, have been observed: single peak, peak having a shoulder on the lowest or the highest side of the main peak, two peaks. Yamasaki *et al*. [6] advanced the suggestion that two peaks appear in the DSC curve when some of the native conformation of BSA is broken. They showed that for 2% fatty acid free BSA two peaks appeared in the pH range 4.2–7.3 at an adequate ionic strength. Then BSA is separated into two thermodynamically independent units, because a crevice is formed in the vicinity of the tryptophan Trp 212 residue. These two cooperative units are different in the thermal stability, thus the DSC curves show two peaks.

It is reported that the structures of fatty acid-free HSA and the C14:0/HSA complex (with the eight myristic acid ligands) show conformational differences [12]. The large change in overall conformation is probably a consequence of the binding of multiple ligands. Under normal physiological conditions not more than 2 molecules of fatty acid are bound to each albumin molecule. Curry *et al*. [13] suggest that the high affinity binding site located in subdomain IIIB is most probably occupied under physiological conditions. It is not known whether the binding of one or two molecules of fatty acid causes the conformational changes, but the thermal stability of the non-defatted albumin differs from defatted one [7].

Our studies indicate that the occurrence of a single or bimodal transitions depends not only on the pH and ionic strength but also on protein concentration and fatty acids content. At some solvent conditions (e.g. in no modified distilled water) a single peak is observed for stabilized by fatty acids BSA and the curve with two peaks – for defatted albumin [7]. We obtained at pH 5.8 T_m=64.7°C, ΔH=803 kJ mol^{-1} and T_m=67.3°C, ΔH=833 kJ mol^{-1} for 'low' and 'high' concentrations, respectively. These values are somewhat lower than reported in earlier work [7] for another series of the same kind of albumin. The comparison of the thermodynamic parameters obtained in this work for non-defatted BSA with these related in the literature is possible (according to our knowledge) only for pH 7. We found that the denaturation temperature at this pH was 63.8°C for 'low' and 66.2°C for 'high' BSA concentration, while ΔH values were 708 and 745 kJ mol^{-1} respectively. Zhang *et al.* [14] reported slightly higher values: T_m=67.6°C, ΔH=213 kcal mol^{-1} (i.e. about 893 kJ mol^{-1}) for similar BSA concentration in phosphate buffer.

With increasing acidic conditions (at pH<4) the enthalpy of thermal denaturation decreases. It was reported that below pH 3.5 a thermal transition for defatted BSA is not observed anymore [6]. For non-defatted albumin studied in this work a small transition is still present. The profile of BSA thermal unfolding at pH 3.5 is dependent on protein concentration. The overall calorimetric parameters as well as these obtained from the deconvolution procedure indicate that BSA was more stable in solution with higher concentration in conditions of our experiment.

As we showed earlier [8], ageing of albumin in solution is manifested in stretch of DSC endothermic transition. A single peak in DSC curves (for fresh non-defatted albumin) transforms with time successively to a peak having a shoulder, two peaks and next to small, broad, again single peak with transition temperature intermediate between these for two peaks in bimodal curve.

Generally, bimodal, broad endothermic transition is observed when compact albumin molecule becomes more loose as a result of weakness of the interactions stabilizing native protein structure. When the N form is changed into the F and next to the E form with increasing acidic conditions, partial opening of the albumin molecule takes place. N–F transition leads also to major changes in hydration [15]. The changes in protein hydration in different media may be important for protein stability.

Our deconvolution results (model with three-component transitions) showed the difference in the stability of particular BSA fragments (or domains) at various pH. At pH 7 the peak at the lowest temperature represents melting of the smaller fragment of molecule (or one domain) while the middle peak – the melting of the bigger fragment of molecule (or two domains). At pH 5.8 the situation is completely inversely. Such observations are in agreement with earlier reports for defatted BSA that: 1) at pH 7 the N-terminal domain I unfolds at lower and the domain II and III melts together at higher temperatures [5], 2) at pH 5.8 (when the crevice is formed) the bigger C-terminal fragment containing domain III and the greater part of domain II melts at lower temperatures [7].

Conclusions

The detailed analysis of DSC curves allows to follow the thermal unfolding of BSA from initial state determined by environmental factors to denatured form. In the pH range 5.5–7.0 albumin containing fatty acids is in compact N form. Under increasing temperature it unfolds cooperatively and single endothermic peak in DSC curve appears. In spite of apparent likeness of endotherms in such conditions, deconvolution analysis indicates different fragmentation of BSA molecule at pH 5.8 and 7.0 under heating. The protein concentration influences the thermal stability of different regions of albumin also. At increased acidic conditions F form arises and its precipitation is visible in DSC curves as the drop and the jaggedness of the higher-temperature shoulder of the peak. Between pH 3.5 and 3.8 albumin stability is much lower and bimodal character of endothermic transition is observed. Stability of non-defatted albumin at those pH values is similar to the stability of defatted BSA in less acidic medium at low ionic strength. In strong acidic media (pH<3.5) containing fatty acids BSA shows very small, single peak while a thermal transition for fatty acid-free BSA is not observed anymore.

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