Journal of Thermal Analysis and Calorimetry, Vol. 88 (2007) 2, 449–454

EFFECT OF ETHANOL ON THE THERMAL STABILITY OF HUMAN SERUM ALBUMIN

Anna Michnik and Zofia Drzazga*

University of Silesia, A. Chełkowski' Institute of Physics, Department of Medical Physics, ul. Uniwerytecka 4
40-007 Katowice, Poland

The effect of ethanol on human serum albumin stability in aqueous solution was studied by use of differential scanning calorimetry. A deconvolution of DSC traces in 2-state model with $\Delta C_p=0$ and $\Delta C_p\neq 0$ was performed and analysed to obtain information on the interaction of ethanol with different parts of albumin molecule both fatty acid containing and fatty acid free. The differences in ethanol binding affinity for both kinds of albumin were found. At very low concentrations ethanol was observed to be a stabilizer of the folded state of albumin contrary to the higher concentration where its binding to the unfolded protein predominates.

Keywords: *albumin–ethanol interactions, DSC, human serum albumin, thermal stability*

Introduction

The study of albumin–ethanol interaction has been vital to the understanding of how alcohols can affect biological processes, particularly these in which proteins play an important role. It is known that the structure of a protein dictates its biological activity. The structure, stability and thus the function of protein can be altered by interaction with alcohol. Two main effects of alcohols on proteins have been reported: the destabilization of their tertiary structure and the stabilization of the helical secondary structure [1]. These combined effects of alcohols may induce partially folded intermediates.

Human serum albumin, that forms about 60% of the mass of plasma proteins, is characterised by a repeating pattern of three α-helical homologous domains numbered I, II, III, starting from the amino terminus. Each domain is divided into two sub-domains, A and B. At present, not only a detailed structural but also ligand-binding properties of albumin as well as dynamic changes within its molecular structure have been relatively well known [2, 3]. Regarding alcohol-albumin interactions, the direct binding of ethanol to bovine serum albumin (BSA) has been suggested [4] and ethanol induced transition from β-sheet to an α-helical structure was reported for human serum albumin [5]. Recently it has been proposed that the solvent-mediated effects are dominant and the binding component is very small to the overall interaction of trifluoroethanol with BSA [6]. From differential scanning calorimetry (DSC) studies we concluded previously [7] that ethanol promotes albumin unfolding and decreases its thermal stability due to the binding to the unfolded state of protein to a higher degree than to the native state.

In current study we have used DSC to investigate the effect of ethanol on the thermally induced unfolding transition of human albumin. Taking into account human physiology, the most interesting ethanol concentration range would be that which corresponds to the values of blood ethanol concentration after the alcohol consumption, i.e. from about 0.1 to 0.5% *v*/*v*. However, such low ethanol concentrations could cause only very small modifications of albumin DSC curves. The lowest ethanol concentration (1% *v*/*v*) used in this study has resulted in hardly appreciable changes. The ethanol concentration of tenth part of M and the protein concentration of tens μM (suitable for microcalorimetric measurements) lead to the ethanol/albumin molar ratio much greater than that which can happened in human blood. Nevertheless, at similar supersaturated experimental conditions for the binding of ethanol to albumin, the interesting results on the effects of ethanol on HSA/warfarin interaction have been obtained [8]. In this study, higher than physiologically possible, but generally low (having in mind denaturational consequences) ethanol concentrations were used to clarify the effect of ethanol on albumin thermal unfolding. The thermal behaviour of two kinds of albumin: fatty acid containing (HSA) and fatty acid free (HSAf) in aqueous ethanol solutions has been compared basing on deconvolution analysis of DSC curves.

* Author for correspondence: michnik@us.edu.pl

1388–6150/$20.00

Akadémiai Kiadó, Budapest, Hungary
Springer, Dordrecht, The Netherlands

Experimental

Materials and methods

Two kinds of human serum albumin: 1) containing endogenous fatty acids (HSA, lot 111K7612) and 2) defatted (with fatty acid content below 0.0005%; HSAf, lot 113K7601), essentially globulin free (purity minimum 99%) were purchased from Sigma. DSC measurements were carried out for albumin concentration $2.25 \cdot 10^{-5}$ M in the temperature range 20–100°C with the scan rate 1°C min^{-1} by using the ultrasensitive microcalorimeter (MicroCal VP DSC). Albumin solutions were prepared by direct dilution of the lyophilised protein with sterile water or ethanol-water solutions. The low ethanol concentrations (up to 6% *v*/*v*) were applied to avoid general conformational destabilization of the protein. 2–4 independent experiments for every ethanol concentration were done. pH of the albumin solutions 6.0±0.5 was not affected by addition of ethanol. All other DSC experimental details were the same as described previously [9].

Curves analysis was performed with MicroCal Origin software. The signal obtained with the albumin solution was corrected for by the signal of an identical solution without the protein and for the difference in heat capacity between the initial and the final state by using a sigmoidal baseline.

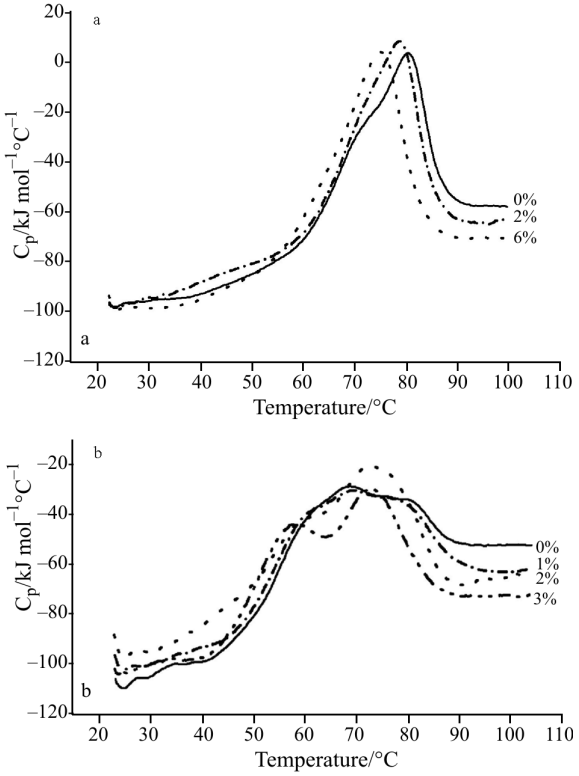

Fig. 1 DSC curves for a – HSA and b – HSAf in the presence of various concentration (in % *v*/*v*) of ethanol

Results and discussion

Effect of ethanol on albumin DSC curves

The effect of increasing ethanol concentration on HSA and HSAf DSC denaturation endothermic peak is shown in Fig. 1a and b, respectively. The HSA transition temperature T_m (temperature at the maximum excess molar heat capacity $C_{p,max}$) decreases with ethanol concentration increase. In the case of HSAf the changes caused by ethanol are more complex. T_m shifts to higher temperatures up to 2% *v*/*v* ethanol and subsequently, above this concentration, DSC curve becomes clearly bimodal. The temperature of the maximum of lower-temperature peak T_{mI} decreases successively with increasing ethanol concentration while that of the higher temperature peak T_{mII} remains practically constant up to 10% *v*/*v* (not shown here but documented in earlier paper, where the effect of higher ethanol concentration was investigated [7]).

An interesting effect of ΔC_p diminution in the presence of ethanol is visible in Fig. 1 (the post-transition heat capacity base lines move down with increasing ethanol concentration). The disappearance of ΔC_p on unfolding in aqueous ethanol solutions can be probably interpreted similarly to the case of ubiquitin in aqueous methanol [10] where a ΔC_p value fell close to zero above about 30% *v*/*v* methanol.

Reversibility

The equilibrium criterion usually applied is the reproducibility of the trace in a second heating of the sample, the so-called calorimetric reversibility. We checked (results not shown) that after scanning up to 100°C the albumin unfolding transitions were not reproducible on reheating a sample both in aqueous and ethanol solutions. The reversibility was better for HSA than for HSAf but not greater than 15%. Figures 2a and b illustrates the results of experiment done for more detailed determination of the transition reversibility in 3% *v*/*v* ethanol. Both kinds of albumin were successively heated up to 60, 70, 80, 100°C with inter-scan cooling to 20°C. In Table 1 the percentage reversibility (±3%) in water and 3% *v*/*v* ethanol solutions has been compared. It should be noted that the reversibility in pure aqueous solutions is somewhat better than in aqueous–ethanol mixture.

Deconvolution analysis

The lack of reversibility and scan rate effects are the features of DSC transition that restrict the application

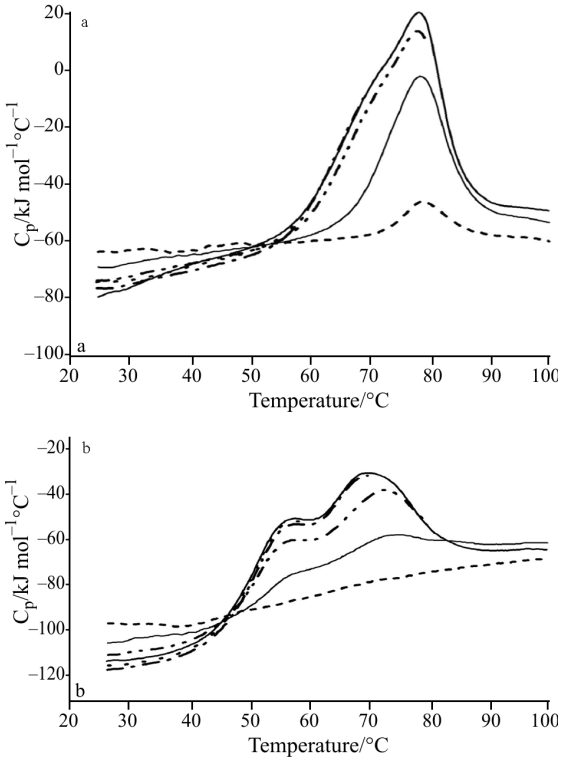

Fig. 2 DSC curves for a – HSA and b – HSAf obtained by successively scanning up to different final temperatures in comparison with single (individual) scanning to — – 100°C. The final temperatures for protein heating were: ····· – 60, – · – · – · – 70, – ·· – ·· – ·· – 80, — – 100°C the first time, - - - – 100°C the second time

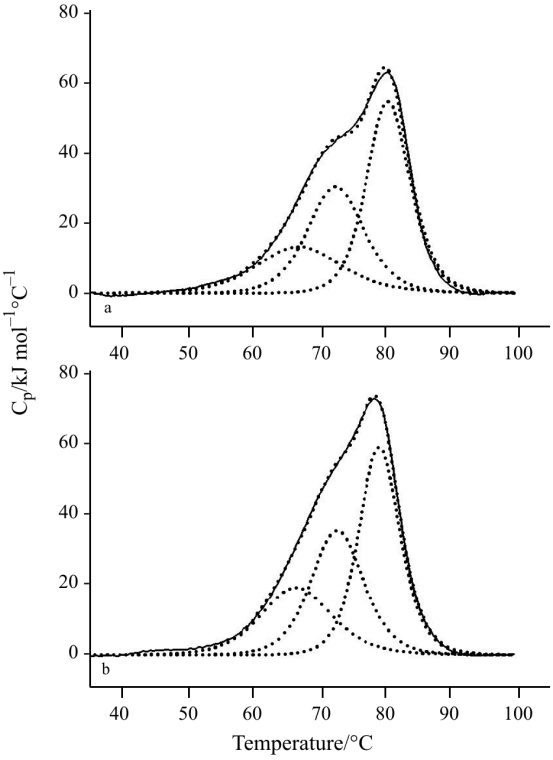

Fig. 3 The curve fitting of HSA DSC profile for a – aqueous and b – 3% *v*/*v* ethanol solution; 2-state model with ΔC_p=0

of equilibrium thermodynamic to its analysis. Our investigations show that the denaturation of albumin is only partly reversible, probably due to aggregation of unfolded molecules at high temperatures. A slight scan rate effect has also been found [11] indicating an occurrence of some kinetic distortion. However, the applicability of reversible thermodynamic to apparently irreversible processes for the case of reversible unfolding followed by a rate limited irreversible step might be permissible as it has been previously discussed [12]. We find that the process analysed in this study falls into this category of permissibility. Thus, an attempt of DSC curve fitting in 2-state model was undertaken. A deconvolution of HSA traces were performed assuming ΔC_p=0 and the three-component transition. The same model has been applied successfully for the description of bovine serum albumin (BSA) denaturation transition [9] and the three components have been correlated to the domain structure of albumin. The representative results of DSC curve fitting in 2-state model are shown in Figs 3a and b for HSA aqueous and 3% *v*/*v* ethanol solution, respectively. The only visible differences are the narrowing of two lower temperature peaks (decrease of HHW, the width in half height) and decreasing of transition temperature of the third peak. Figure 4 illustrates the ethanol concentration dependencies of component transition temperatures T_i and denaturation enthalpies ΔH_i (i=1, 2, 3). One can see an increase of T_1 and T_2 with increasing ethanol concentration but only up to 2

Table 1 Reversibility of HSA and HSAf denaturation process in aqueous and 3% v/v ethanol solutions after preliminary heating to different temperatures

	Reversibility/%			
	HSA		HSAf	
T/°C	water	3% *v*/*v* ethanol	water	3% *v*/*v* ethanol
60	100	100	100	100
70	95	85	90	81
80	70	58	42	31
100	13	15	7	5

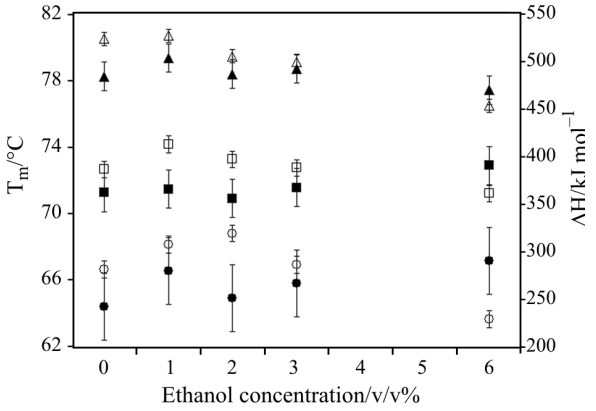

Fig. 4 The thermodynamic parameters (±SEM) of the three-component denaturation transition for HSA in aqueous ethanol solutions obtained from 2-state model with ΔC_p=0 (circles, squares and triangles for I, II and III peak, respectively; open – transition temperatures T_i, solid – ΔH_i)

and 1% *v*/*v*, respectively. T_3 remains constant in the same ethanol concentration range. An increase of transition temperatures with increasing ethanol concentration indicates ethanol binding to the native form of albumin. At ethanol concentration above 2% *v*/*v* (0.3 M), all T_i decrease with increasing ethanol concentration, pointing out ethanol binding to the unfolded albumin molecules. The lack of essential ΔH_i changes (within the experimental errors) was observed except a decrease of ΔH_3 in 6% *v*/*v* ethanol.

It was not possible to obtain good fitting of HSAf DSC curves in 2-state model with ΔC_p=0 neither at assumption of two nor three components transitions, particularly in the case of aqueous ethanol mixtures. We applied another 2-state model (also available in MicroCal Origin software), with $\Delta C_p \neq 0$. The obtained results of fitting for HSA and HSAf in aqueous and 3% *v*/*v* ethanol solutions are shown in Fig. 5 and the corresponding thermodynamic parameters: T_i, ΔH_i, ΔC_{pi} (i=1, 2, 3) accompanying the component thermal transitions in the presence of increasing ethanol concentration are presented in Figs 6a and b. A pre-transition slope of 0.6 kJ mol^{-1} °C^{-2} has been obtained for the C_p value of both albumins in water and slightly higher for HSA in ethanol solutions. The finding that the sum of three two-state transitions describes well denaturation of HSA suggests that these transitions can be correlated with three albumin domains. There are no essential differences between T_i and ΔH_i values received in model with ΔC_p=0 and $\Delta C_p \neq 0$ for HSA.

Based on the results presented in Figs 6a and b, one can compare the effect of ethanol on nondefatted and fatty acid free albumin. In the case of HSAf the pronounced increase of T_2 (5.6°C) is observed in the presence of 0.3 M ethanol suggesting the ethanol binding to the native albumin domain (or part) unfolding in the middle temperature range. The maximal T_1 and T_2 increase for HSA, indicating ethanol binding to the native state of two first albumin domains (going from lower to higher temperatures), is only 2.1 and 2.6°C, respectively. It means the weaker binding effect for HSA than for HSAf and confirms the Avdulov *et al.* [4] suggestion about the interaction of ethanol with fatty acid binding sites on albumin. Because a primary site for shorter-chain fatty acids is lo-

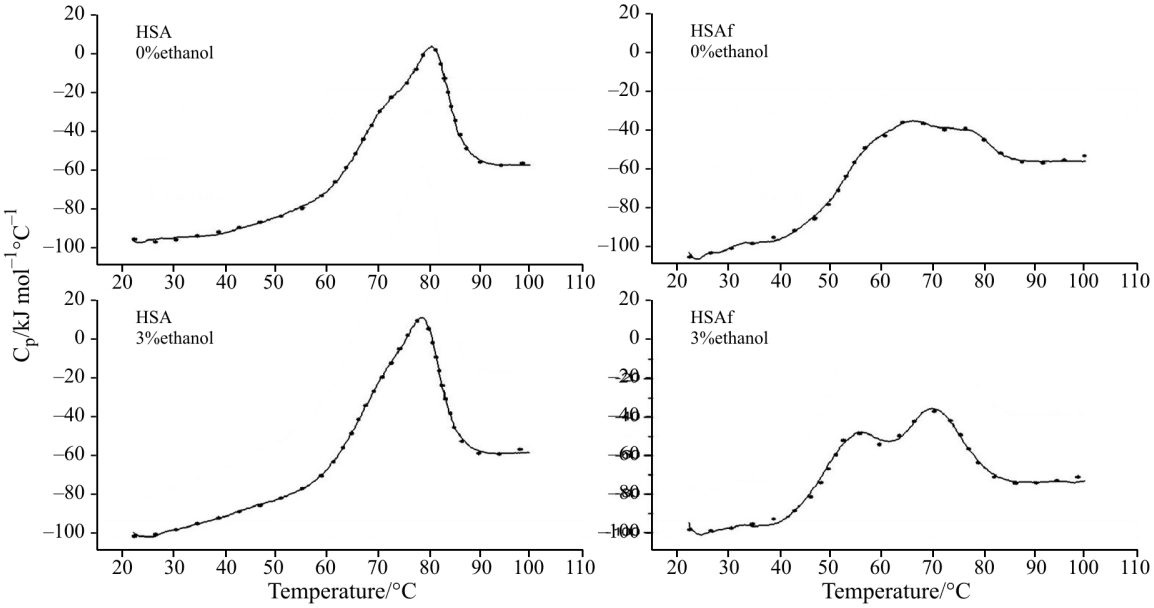

Fig. 5 The curve fitting of HSA and HSAf DSC profiles for aqueous (0%) and 3 *v*/*v*% ethanol solutions; 2-state model with $\Delta C_p \neq 0$ (— – experimental, ····· – theoretical curve)

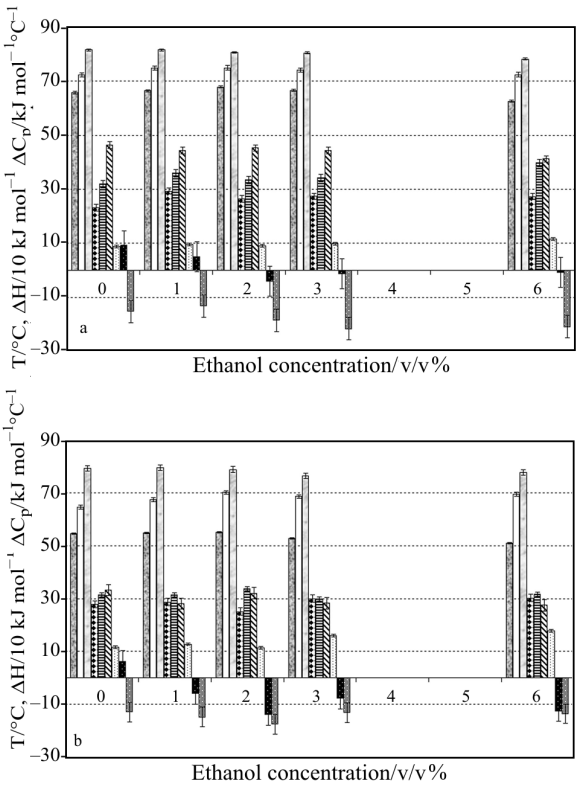

Fig. 6 The thermodynamic parameters (±SEM) of the three-component denaturation transition for a – HSA and b – HSAf in aqueous ethanol solutions obtained from 2-state model with $\Delta C_p \neq 0$ (set of columns from left to right for each ethanol concentration corresponds subsequently to: T_1, T_2, T_3 (in °C), ΔH_1, ΔH_2, ΔH_3 (in 10 kJ mol^{-1}), ΔC_{p1}, ΔC_{p2}, ΔC_{p3} (in kJ mol^{-1} °C^{-1})

cated in subdomain IIA [3], it is probable that ethanol binds favourably to this site.

At the higher ethanol concentration (above 0.3 M) all T_i for HSA and T_1 for HSAf decrease due to the binding of ethanol to the unfolded parts of protein. The denaturation enthalpies corresponding to component transitions I, II, III increase with theirs increasing transition temperatures in aqueous as well as in ethanol solutions in the case of HSA while for HSAf, all ΔH_i are very similar. The interpretation of the results for HSAf is difficult due to the bimodal character of unfolding. For a mixture of protein plus ligand one might observe in DSC endotherm two peaks corresponding to ligand-bound and free protein, particularly in the case of very tightly binding ligands [13]. Because the binding of ethanol to albumin is rather weak [4, 7], in this case the bimodality may arise from the separate unfolding of different fragments of albumin molecule and ethanol binding to both native and unfolded state of albumin with different affinities. At high concentration ethanol may cause the loosening of defatted albumin structure acting as denaturant. The unfolding of more expanded molecule may proceed in wider temperature range and can be bimodal. The similar bimodal character of defatted bovine serum albumin appearing also in aqueous solutions at certain pH and ionic strength was discussed earlier [9, 14].

The heat capacity change of protein unfolding ΔC_p provides us with important information about the changes of exposure of polar and non-polar amino acids residues to the aqueous solvent [15]. This is demonstrated in Fig. 6 that ΔC_{p1} is positive for both HSA and HSAf, practically does not depend on ethanol concentration up to 2% *v*/*v* and slightly increases above this concentration but only for HSAf. ΔC_{p2} is positive in aqueous solution, close to zero for HSA and negative for HSAf in ethanol solution. ΔC_{p3} is always negative. The heat capacity of a system depends on the number of excited states that are thermally accessible under the prevailing conditions. Its value is different for a solution in which water is free compared with one in which it is interacting with a biomolecule. The heat capacity increases when thermally labile water structuring occurs around dissolved non-polar groups while dissolution of polar groups causes a substantial decrease in heat capacity [10, 16]. It is difficult to dissect the various contributions to the ΔC_p from the folded and partially folded structures themselves and from the changes in solvent ordering around non-polar and polar groups. The present data do not permit more detailed analysis of the particular ΔC_{pi} values, however the conclusion that differences between HSA and HSAf thermal unfolding in the presence of ethanol occur, may be drawn out. Particularly, the differences concern the middle temperature component transition which is probably connected with the unfolding of central (II) albumin domain.

Conclusions

The results suggest that at the very low concentration ethanol is a stabilizer of the folded state of albumin due to its binding to the native state of protein. The maximal increase of transition temperature, greater for HSAf than for HSA, has been observed for the middle albumin domain after addition of 0.3 M ethanol. A primary site for short-chain fatty acids located in subdomain IIA may be considered as preferred binding site for ethanol. At higher concentration, above about 0.5 M ethanol acts as denaturant, binds to unfolded state of albumin and decreases its stability.

References

1 M. Sundd, S. Kundu and M. V. Jagannadham, J. Protein Chem., 19 (2000) 169.

2 R. Artali, G. Bombieri, L. Calabi and A. Del Pra, Il Farmaco, 60 (2005) 485.
3 A. A. Bhattacharya, T. Grüne and S. Curry, J. Mol. Biol., 303 (2000) 721.
4 N. A. Avdulov, S. V. Chochina, V. A. Daragan, F. Schroeder, K. H. Mayo and W. G. Wood, Biochemistry, 35 (1996) 340.
5 S. Y. Lin, Y. S. Wie, M. J. Li and S. L. Wang, Eur. J. Pharm. Biopharm., 57 (2004) 457.
6 T. Banerjee and N. Kishore, Biopolymers, 78 (2005) 78.
7 A. Michnik, J. Therm. Anal. Cal., (2007) in press.
8 Ch. E. Ha, Ch. E. Petersen, D. S. Park, K. Harohalli and N. V. Bhagavan, J. Biomed. Sci., 7 (2000) 114.
9 A. Michnik, J. Therm. Anal. Cal., 71 (2003) 509.
10 D. N. Woolfson, A. Cooper, M. M. Harding, D. H. Williams and P. A. Evans, J. Mol. Biol., 229 (1993) 502.
11 A. Michnik, K. Michalik, A. Kluczewska and Z. Drzazga, J. Therm. Anal. Cal., 84 (2006) 113.
12 J. M. Sanchez-Ruiz, Biophys. J., 61 (1992) 921.
13 A. Cooper, M. A. Nutley and A. Wadood, Protein-Ligand Interactions: Hydrodynamic and Calorimetry, S. E. Harding and B. Z. Chowdhury, Eds, Oxford, University Press, Oxford, New York 2000, p. 287.
14 M. Yamasaki, H. Yano and K. Aoki, Int. J. Biol. Macromol., 12 (1990) 263.
15 J. E. Ladbury and B. Z. Chowdhury, Biocalorimetry, Wiley and Sons, Chichester 1998, p. 166.
16 P. L. Privalov and G. I. Makhatadze, J. Mol. Biol., 224 (1992) 715.

DOI: 10.1007/s10973-006-8072-6