*Journal of Thermal Analysis, Vol. 45 (1995) 1255-1264*

# THERMAL DENATURATION OF BOVINE SERUM ALBUMIN AND ITS OLIGOMERS AND DERIVATIVES *pH* DEPENDENCE

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(Received February 13, 1995)

## Abstract

In a previous paper, we report a preliminary DSC study on bovine (BSA) and human (HSA) serum albumins. However, at accurate HPLC analysis the commercial proteins show three peaks: Fraction V-I, probably globulins (as declared by the producers), Fraction V-II (about 15–18% of the product) and Fraction V-III that represents pure BSA or HSA. A hypothesis is that the Fraction II is a covalent dimer, or trimer or a mixture of both, generated during the scalf-life of the commercial product.

Denaturation enthalpies of the purified Fraction V-III and Fraction V-II of BSA, have been determined calorimetrically, at changing the *pH*, and the results of both compared with those obtained on the untreated protein. Few calorimetric experiments have been also carried on a BSA monomer derivative with sulphidril group protected. Computer program have been developed for the deconvolution of exo- and endothermic effects and for the analysis of thermal denaturation profiles.

**Keywords:** bovine serum albumin, denaturation, *pH* dependence

## Introduction

BSA is a protein that exhibits its carrier function against an impressive lot of ligands of very different nature: polar, hydrophobic, charged or not. It is very rich of ionizable groups (59 Glu and 41 Asp *vs.* 59 Lys and 23 Arg on a total of 582 residues) and of –S–S– bridges (17 and 1 –SH free) [1]. In Fig. 1 are shown the BSA primary structure and its strict homology with other serum albumins: the human serum albumin (HSA) and the rat serum albumin (RSA). In spite of the fact that BSA can be easily crystallized for purification purposes, good and well ordered crystals, suitable for X-ray diffraction studies have not yet been obtained. The three-dimensional structure of human serum albumin however has been recently determined by a X-ray analysis at a resolution of 2.8 Å.

*0368–4466/95/ $ 5.00*

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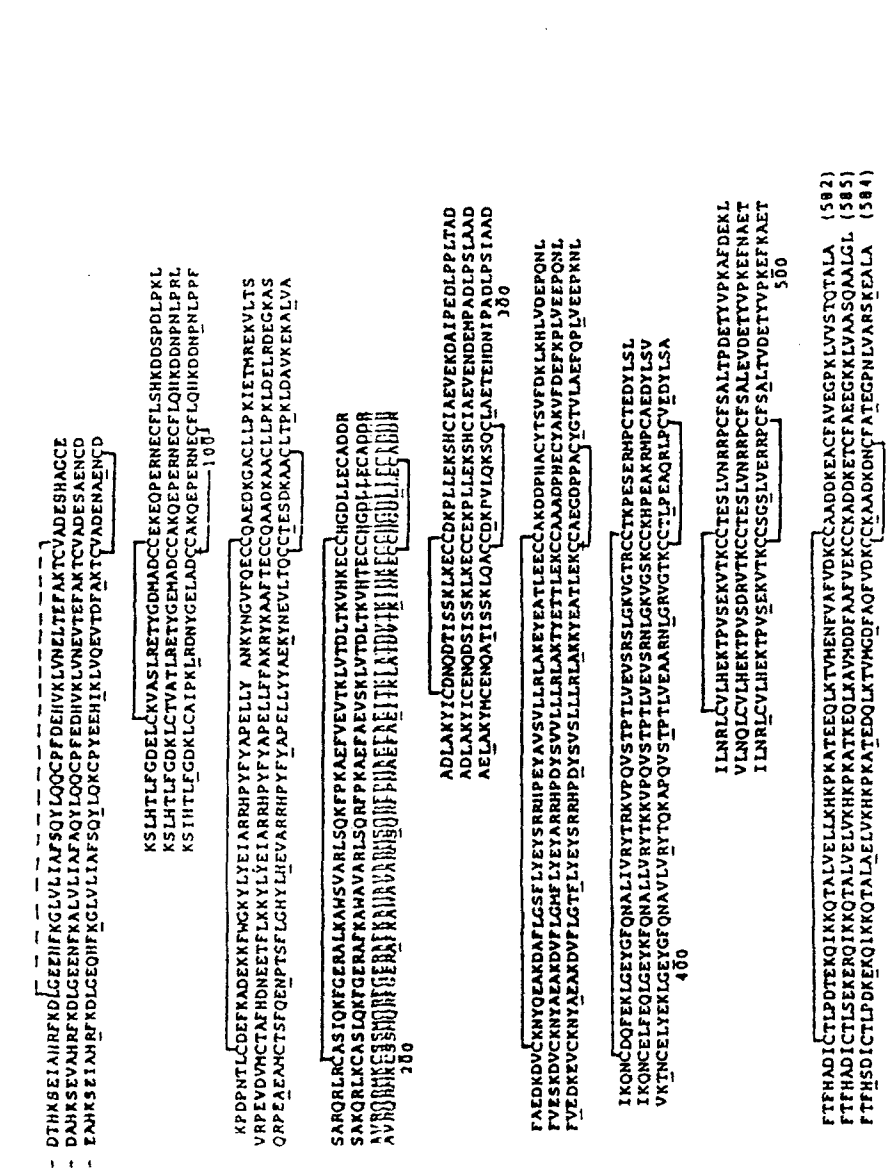


Fig. 1 Primary structure of bovine serum albumin (BSA) compared with those of human serum albumin (HSA) and rat serum albumin (RSA): from Ref. [1]

Its results formed by three highly homologous domains that assemble to form a heart shaped molecule [2].

The process of thermal denaturation of BSA monomer (Fraction V-III) has been investigated in the *pH* range 4.0–8.0 to emphasize the *pH* induced conformational transitions of this protein [3]. Indeed the profiles of the calorimetric curves show a marked dependence on solution *pH*; further, it is always present an exothermic phenomenon also *pH*-dependent which whose attributed to the aggregation of the macromolecules [4]. The last is overlapped to thermal transition at *pH* 4.0 and 4.5 and for this reason it is impossible to carry out a non questionable analysis of these particular curves. At higher *pH* values the aggregation occurs at the end of the denaturation or it is separated from the denaturation peak. A preliminary analysis of the experimental data in the range 5.0–8.0 shows that the transition is not well represented by a simple one-step mechanism.

## Experimental

The BSA was a SIGMA product (Fraction V) [5] of high purity, fatty acid content less than 0.005% [6], crystallized and lyophilized [7]. The commercial product was dissolved in 0.1 *M* ammonium acetate *pH* 5.0 and submitted to HPLC analysis, by means of a "Protein-pak 125" column (7.8×300 mm) from Waters for gel filtration. Rate of elution 0.6 ml/min. The chromatograms show a small peak (≈ 2.5% by weight) primarily attributed to the presence of the $\alpha_1$ and $\alpha_2$ globulins, as declared by the Producer. Other two peaks were attributed to an oligomer of BSA (17–18%) and to BSA monomer, respectively. Sufficient amounts of the sample were then passed through a preparative column with the similar package gel, to obtain material enough for the calorimetric measurement. After each run, the obtained fractions (V-II for oligomer and V-III for the monomeric BSA) were tested on the analytical column for ensuring the purity of each fraction after each separation.

Results of electrophoresis on polyacrylamide gel show that Fraction V-II is a mixture of trimeric and dimeric forms of BSA, while the Fraction V-III corresponds exactly to the molecular weight of BSA ($M_r$ = 66300). According to the HPLC analysis the Fraction V-I must be considered rather a supramolecular complex of proteins or a polymer of BSA (approximately an eptamer) than a mixture of pure globulins.

For testing the hypothesis that the free sulphidril group–SH of BSA is the responsible for both the irreversibility of denaturation and the aggregation phenomenon, we protected it with iodoacetamide according to literature procedure [8]. All measurements were carried out in 0.15 *M* NaCl and 0.01 *M* acetate buffer at *pH* between 4.0 and 5.5 and 0.01 *M* phosphate buffer at *pH* between 6.0 and 8.0.

Calorimetric measurements were carried out on a second generation Micro-DSC apparatus of the Setaram (Lyon), expressly designed for studies on dilute solutions of biological macromolecules.

## Results

In the Figs 2–4 are reported some characteristic thermal denaturation curves for the Fraction V-III (pure BSA monomer) and in Fig. 5 a curve for the Fraction V-II. The results are summarized in Table 1.

**Table 1** Experimental thermodynamic parameters for the thermal denaturation of BSA Fractions at changing *pH*

| *pH* | Fraction V-III | | Fraction V-III protected | | Fraction V-II | |
|---|---|---|---|---|---|---|
| | $T_d$/ °C | $\Delta_d H$/ kJ·mol$^{-1}$ | $T_d$/ °C | $\Delta_d H$/ kJ·mol$^{-1}$ | $T_d$/ °C | $\Delta_d H$/ kJ·mol$^{-1}$ |
| 5.0 | 69.7 | 759 | – | – | – | – |
| 5.5 | 70.3 | 855 | – | – | – | – |
| 6.0 | 68.4 | 991 | – | – | 64.6 | 644 |
| 6.5 | 69.5 | 1120 | – | – | – | – |
| 7.0 | 71.5 | 1130 | 67.7 | 1045 | 65.3 | 988 |
| 7.5 | 71.6 | 1175 | – | – | – | – |
| 8.0 | 68.8 | 1190 | 68.6 | 990 | 63.5 | 1025 |

In all cases the thermal denaturation results irreversible and an exothermic phenomenon, connected with an aggregation, occurs in the explored temperature ranges. It is reproducible and depends strongly on *pH* and only little on protein concentration. This exothermic peak is more narrow than the endothermic peak due to the denaturation. If the concentration is enough, the aggregation is revealed by the precipitation of BSA. The superposition of denaturation and aggregation peaks gives rise to some problems for their separation and for deconvolution of both the endothermic and exothermic phenomena. These problems were solved with the help of programs for the handling of the data, especially implemented [9].

The denaturation enthalpies evaluated from Fraction V-III are 25–30% higher than the corresponding values found by us for the unfractionated BSA [4]. Also the maxima of the apparent heat capacities fall at temperature higher than those found for unfractionated BSA.

At decreasing the *pH*, the complexity of the peaks seems to increase. However, while at *pH* 4.0 the unfractionated BSA seems to form a gel, in the case of Fraction V-III it is obtained a precipitate.

The exothermic peaks give thermal effects of the order of 20–25% with respect to the endothermic peaks. The temperature of the minimum seems to depend on both the *pH* and protein concentration.

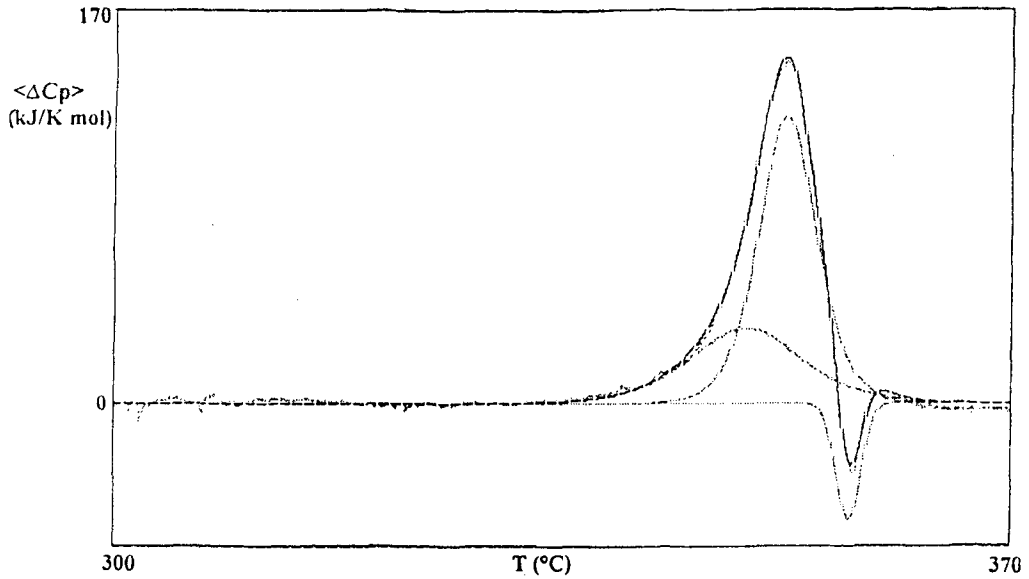


**Fig. 2** Experimental and simulated curve for BSA, Fraction V-III, at *pH* 6.0. At high temperatures an exothermic peak, due to the aggregation of denaturated protein is present

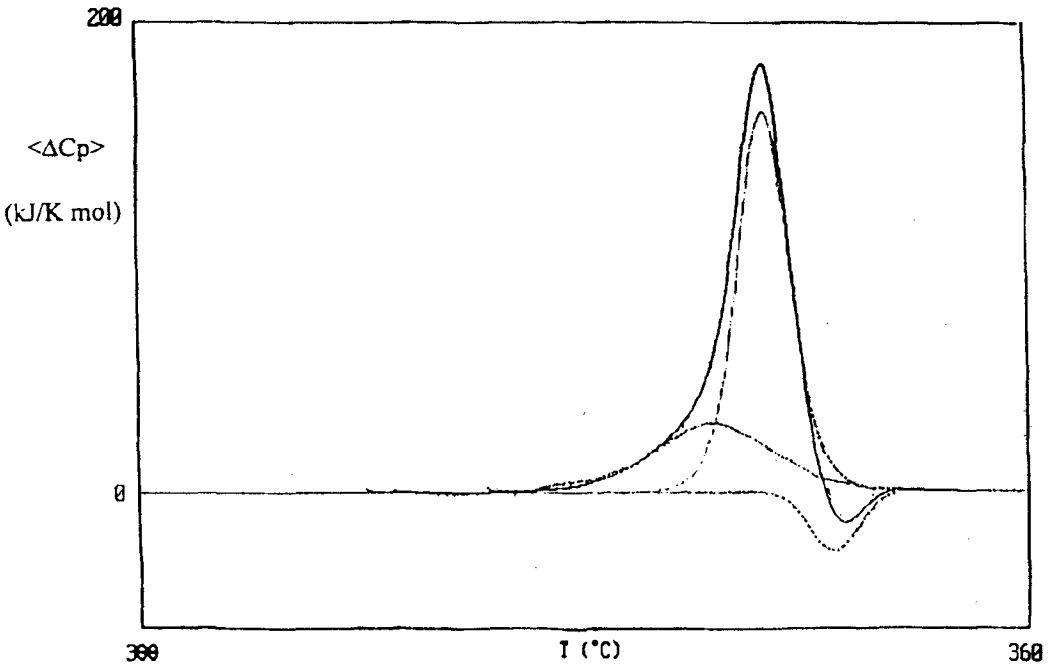


**Fig. 3** Experimental and simulated curve for BSA, Fraction V-III, at *pH* 7.0. At high temperatures an exothermic peak, due to the aggregation of denaturated protein is present

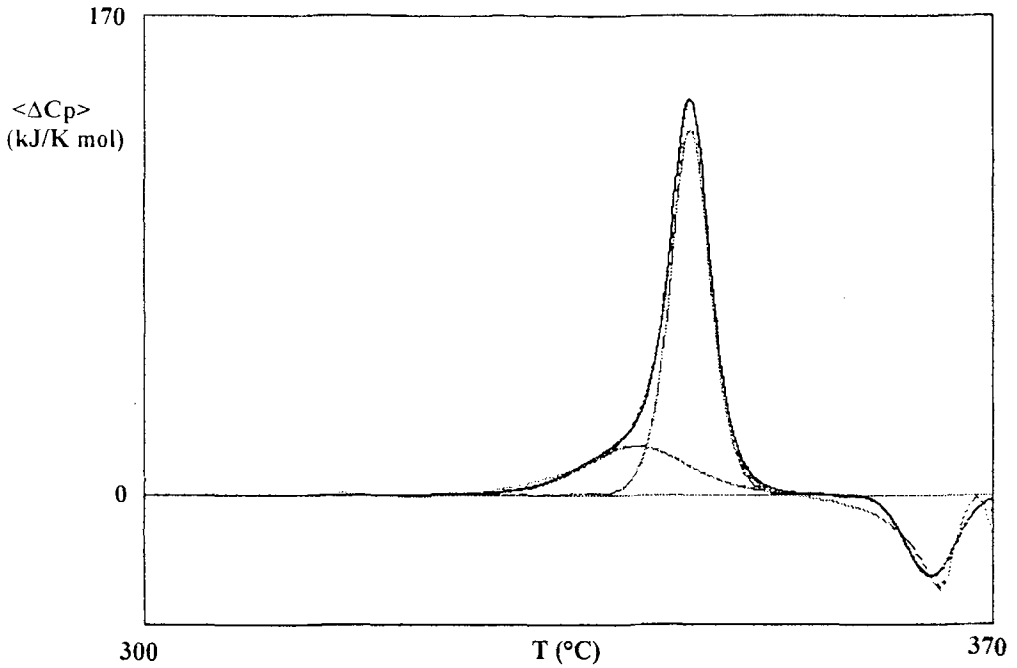


**Fig. 4** Experimental and simulated curve for BSA, Fraction V-III, at *pH* 8.0. At high temperatures an exothermic peak, due to the aggregation of denaturated protein is present

Referring to the same amount of monomeric units, and using the same extinction coefficient of unfractionated BSA ($\varepsilon_{279} = 44200\ M^{-1} \cdot cm^{-1}$) [10], comparative results are obtained for Fraction V-II. This is a confirm that this Fraction is an oligomer of BSA, due probably to exchanges between the free –SH (position 34) and disulphide bridge of another molecule or to the formation of a complex originating from a bridging ligand.

For Fraction V-II the $T_d$ are regularly lower then those of Fraction V-III but higher than those of the unfractioned BSA. The same occurs for the $\Delta_d H$. This effect can be rationalized assuming that the formation of dimer or trimer weakens the stability of domains (the $\Delta_d H$ is referred to the monomeric BSA). The $\Delta_d H$ values concerning the unfractionated BSA were averaged on those of the components. Then the commercial sample must contain, besides the native monomeric BSA, globulins (or some supramolecular complexes of globulins alone or with other proteins) oligomers and aggregates of BSA molecules, fractions of BSA, hydrolysis products etc.

Some measurements have been tried also on the protected monomeric BSA at *pH* 7.0 and 8.0, respectively (Table 1). These results are well reproducible. The denaturation profile is very similar to that of monomeric parent protein, but the corresponding parameters are a little smaller. This finding is different from

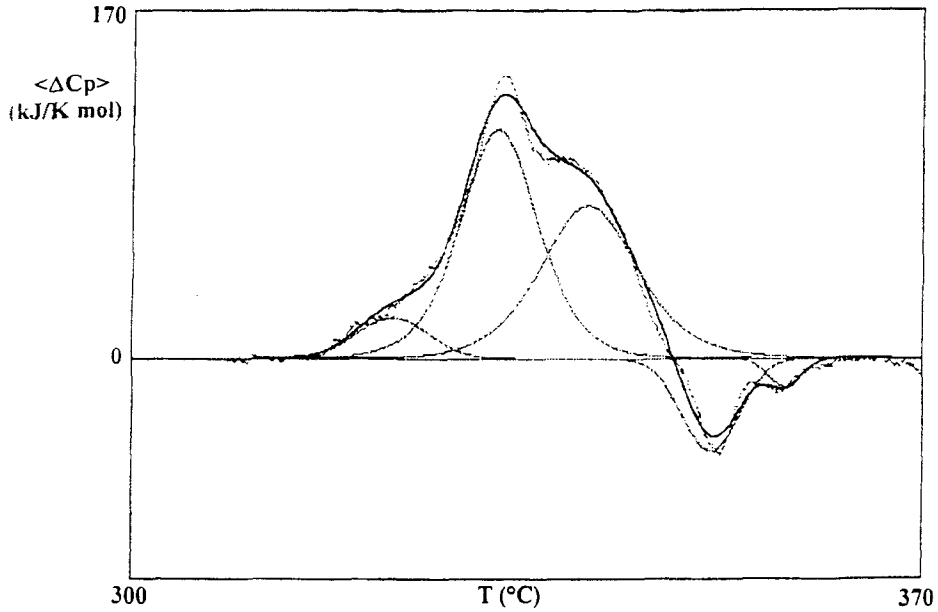


**Fig. 5** Calorimetric profile of BSA Fraction V-II, *pH* 7.0 (bold line) with superimposed the simulated curves obtained as reported in the text

the conclusions of Aoki [11]. The exothermic peak is still present and the denaturation results irreversible, this meaning that the irreversibility is due to aggregation of the denaturated albumin molecules rather than to secondary reactions involving the free –SH of BSA.

## Deconvolution

The endothermic peaks, after evaluation, by regression techniques, of the reference baseline delimiting the exothermic contribution, were deconvoluted in the range of *pH* 6–8 giving the results reported in Table 2.

In order to analyze accurately the calorimetric curves and to extract from them reliable thermodynamic values of denaturation enthalpies and significant mid-point temperatures, we have implemented computer programs for the deconvolution of thermal denaturation profiles. The DEDALUS program performs the analysis of complex curves according to the model of a series of independent one-step transitions, while the MINOS program deconvolves the peaks according to the model of sequential transition [12–17].

A preliminary test [12] on the reliability of the models, show that the more simple model (two independent transition) gives in this case, as in other particular situations, a result better than the more complicated model of the sequential

transitions. There is no large differences between the results of the two approaches because the probability that the more stable domain denature at lower temperature than the less stable is very low. So the formation of unique intermediate form is practically common to the two models.

**Table 2** Deconvolution in two main contributions of the thermodynamic parameters of Table 1, according to DEDALUS program

| *pH* | Fraction V-III | | | | Fraction V-III protected | | | |
|---|---|---|---|---|---|---|---|---|
| | $T_1$ / | $T_2$ / | $\Delta_d H_1$ / | $\Delta_d H_2$ / | $T_1$ / | $T_2$ / | $\Delta_d H_1$ / | $\Delta_d H_2$ / |
| | °C | | kJ·mol$^{-1}$ | | °C | | kJ·mol$^{-1}$ | |
| 5.0 | 61.4 | 69.0 | 210 | 535 | – | – | – | – |
| 5.5 | 63.8 | 69.8 | 297 | 623 | – | – | – | – |
| 6.0 | 65.4 | 68.2 | 370 | 730 | – | – | – | – |
| 6.5 | 66.4 | 69.5 | 396 | 780 | – | – | – | – |
| 7.0 | 67.7 | 71.5 | 340 | 810 | 62.9 | 67.7 | 372 | 672 |
| 7.5 | 67.4 | 71.6 | 385 | 820 | – | – | – | – |
| 8.0 | 64.0 | 68.7 | 325 | 880 | 63.9 | 68.3 | 320 | 690 |

For Fraction V-III it needs actually only of two deconvolution peaks. A third, small one, could be invoked, but it does not ameliorate the results. The peak at lower temperature has a $\Delta_d H_1$ about one half of the other peak, the last one becoming even more predominant at increasing the *pH*. It could be hypothesized that the first peak correspond to the collapse of the domain I, which bares the major unbalanced negative charge. This is confirmed by the fact that one of the two Tryptophane residues (that in position 212) is borderline of this domain and its fluorescence maximum undergos a red shift, due to the probable opening of the hydrophobic environment with the formation of a crevice in domain I (11). The other Trp 134 is probably less exposed to the solvent in both the native and denaturated conformations. At increasing the *pH*, the stability of the domains II and III (that seems to collapse together) increases relatively but that can depend on many effects: the increasing negative net charge, the distribution of positive and negative charges and also on the side-effects of the charged groups both on the hydration of next polar groups, and on the solvation cages of hydrophobic residues.

The denaturation process of the protected BSA monomer is complex and well represented as a sum of two-independent one-step transitions as for BSA monomer (Table 2).

Both the temperature $T_1$ and $T_2$ result lower than those of unprotected V-III Fraction, while the values of $\Delta_d H_1$ are practically the same and the values of $\Delta_d H_2$ are a little lower than the values found for the unprotected Fraction. On

this basis a possible rationalization that the N-therminal domain is less stable could be confirmed. Even some structural indications are not in contrast with this kind of hypothesis.

The presence of the bulky –SH-protecting group do not alter the $\Delta_d H_1$, but rather $T_1$ (the denatured chain has little more freedom degrees than the denatured parent).

A possibility to confirm is that the protected sulphidril group can induce a conformational perturbation that makes less stable in the overall the domains II and III. These domains in turn are probably stabilized by the higher content of hydrophobic amino acid residues: in fact it seems that the main binding regions for apolar ligands are located on the subdomains 4, 5 (II A according another nomenclature) and 7,8 (III A) [1, 2]. The spectral changes of Tpr however seem largely supporting this approach.

For Fraction V-II the deconvolution (not reported in Table 2) gives three components for the endothermic peak and two for the exothermic one, but it is not possible (in absence of structural evidences) to make an assignment, considering also that Fraction V-II seems more a mixture of oligomers then a pure substance. The values for the two major peaks are: $\Delta_d H_2 = 527$ kJ·mol$^{-1}$ (61°C) and $\Delta_d H_3 = 438$ kJ·mol$^{-1}$ (67°C).

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**Zusammenfassung** — In einer vorangehenden Mitteilung wurde über eine einleitende DSC-Untersuchung von Serumalbuminen beim Rind (BSA) und beim Mensch (HSA) berichtet. Bei

präziser HPLC-Analyse zeigen alle handelsübliche Proteine drei Peaks: Fraktion V-I mit wahrscheinlich Globulinen (wie vom Hersteller angegeben), Fraktion V-II (etwa 15-18 % des Produktes) und Fraktion V-III, welche reines BSA oder HSA darstellt. Laut einer Hypothese handelt es sich bei der Fraktion II um ein kovalentes Dimer oder Trimer oder ein Gemisch aus beiden.

Durch Änderung des pH-Wertes wurden die Denaturierungsenthalpien der gereinigten Fraktionen V-III und V-II von BSA kalorimetrisch bestimmt und die Resultate beider mit denen von unbehandelten Proteinen verglichen. Einige kalorimetrische Experimente wurden auch an einem mit einer Sulfhydrylgruppe geschützten BSA-Monomerderivat durchgeführt. Zur Dekonvolution von exo- und endothermen Effekten und zur Analyse der thermischen Denaturierungsprofile wurde ein Computerprogramm entwickelt.