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Author: Anna Michnik, Agnieszka Kiełboń, Klaudia Duch, Ewa Sadowska-Krępa, Ilona Pokora

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Comparison of human blood serum DSC profiles in aqueous and PBS buffer solutions

Anna Michnik[1] · Agnieszka Kiełboń[1] · Klaudia Duch[1] · Ewa Sadowska-Krępa[2] · Ilona Pokora[2]

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Abstract
The results of studies of physiological fluids by differential scanning calorimetry (DSC) for the purpose of diagnosis and monitoring of diseases are promising. Before the DSC method is accepted in medical diagnostics, it is worth experimenting with various environmental conditions at the research stage. Among other things, it is important to choose an appropriate solvent to dilute the tested biological fluids. In this work, human blood sera DSC profiles in aqueous and PBS (phosphate-buffered saline) solutions have been compared. Visibility of haptoglobin in the DSC profile of human blood serum is much better in water solution. In addition, contributions from albumin and haptoglobin are well separated in contrast to the PBS serum solutions. The peak or shoulder at about 83 °C which represents contributions from the C_H3 domain of immunoglobulin IgG1 and/or transferrin is more clearly visible in PBS solution. The results show that the type of solvent is essential when interpreting the serum DSC profile.

Keywords DSC · Haptoglobin · Human blood serum · Protein thermal denaturation

Introduction

Differential scanning calorimetry (DSC) is an appropriate method for assessing protein thermal stability and conformational changes. Recently, it has been recognized as having potential in diagnostics and monitoring of diseases. DSC has been used to characterize thermal denaturation transitions in various biological fluids, in particular, blood plasma [1–9], serum [10–16], synovial fluid [17–19], cerebrospinal fluid [20, 21]. This highly sensitive method allows detecting, registering and analysis the changes taking place in various diseases, during treatment, after high-intensity exercise or because of other efforts and stressors [1–26]. The thermal denaturation heat capacity changes measured as a function of temperature include information about the structural changes in the molecular constituents of the fluid.

DSC profiles of human blood plasma/serum show the complex endothermic transition, which represents the weighted sum of denaturation profiles of major protein components and reflects proteome modifications in variety of diseases and conditions. The shape of this transition and the thermal parameters of unfolding events occurring during DSC scan are dependent on the health status of blood donor, physicochemical properties of the serum/plasma solution, and may be markedly modified by environmental and experimental factors.

To properly interpret the changes occurring in the highly complex calorimetric profile of the blood plasma/serum proteome for diagnostic purposes, the experimental conditions should be optimally selected and set. One of the important experimental factors is the choice of the solvent. Phosphate-buffered saline (abbreviated *PBS*) is a buffer solution frequently used in biological applications. Dilution of serum samples in PBS is the most common practice as it provides the closest to physiological conditions. Most publications showing the diagnostic power of DSC have analyzed plasma profiles in pH 7.4 buffer solutions [1–9]. We also used this buffer in some of our earlier research [14, 15, 24]. It seems that if medical diagnostics based on DSC profiles

✉ Anna Michnik
anna.michnik@us.edu.pl; aemichnik@gmail.com

[1] Faculty of Science and Technology, Institute of Biomedical Engineering, The Silesian Centre for Education and Interdisciplinary Research, University of Silesia in Katowice, ul. 75 Pułku Piechoty 1A, 41-500 Chorzow, Poland

[2] The Jerzy Kukuczka Academy of Physical Education in Katowice, Institute of Sport Sciences, ul. Mikołowska 72a, 40-065 Katowice, Poland

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of physiological fluids are formally accepted, then probably PBS buffer will be the standard. However, it seems worth experimenting at the research stage. Thus, our earlier DSC studies of athletes' sera [22, 23, 25, 26], aimed at supporting sports training, were performed using distilled water as the solvent.

The shapes of heat capacity profiles presented for aqueous solutions of athletes' blood serum (pH 6.5–7.0) distinctly differed from serum/plasma profiles reported for healthy persons when the final pH of buffer solution was in the range 7.2–7.5. To show these differences more clearly, in current study we made duplicated measurements: in water and in buffer for each sample of serum acquired from the same portion of blood taken from a healthy person. This work compares the thermal denaturation profiles of human blood serum samples in aqueous and PBS buffer solutions obtained under identical other experimental conditions.

Materials and methods

Blood serum samples

The human blood serum samples were obtained from 18 healthy volunteers, 15 men, 3 women (the age range of 28–68 years). The blood from the antecubital vein was drawn into clot activator tubes (BD Vacutainer, UK) and processed using routine procedures to separate serum. Serum samples were stored at − 20 °C. Immediately before the DSC measurements, each serum sample was thawed out at room temperature. Then, 20-fold diluted serum solution was prepared using the redistilled and degassed water or the degassed PBS buffer (0.01 M, pH 7.4). The pH values of aqueous serum solutions were within the range 6.5–7.0. Total protein content in serum samples was determined by the 2,20-bicinchoninic acid method using Sigma Protein Assay Kit BCA1.

The informed consent from volunteers was obtained before the peripheral blood samples were taken. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki was approved by the Institutional Ethics Committee at the Jerzy Kukuczka Academy of Physical Education in Katowice (certificate of approval No. 4/2013).

Pure protein samples

Transferrin (Tr), from human blood plasma (Product Number 90190, Lot # BCBG3191V) and Human Haptoglobin (Hp), phenotypes Type 1–1 (Catalog Number H0138, Lot # SLBP9714V) and Type 2–2 (Catalog Number H9762, Lot # SLBN4983V) 98–100%, essentially salt–free, lyophilized powder were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Haptoglobin Type 2–1 was unavailable. Protein samples were diluted with the redistilled and degassed water to the concentration suitable for DSC (1.5 mg mL^{-1} for Tr and 0.4 mg mL^{-1} for Hp).

Differential scanning calorimetry

DSC measurements were conducted using the VP DSC MicroCal instrument (Northampton, MA) in the temperature range 20–100 °C with the heating rate 1 °C min^{-1} and a pre-scan equilibration time 15 min. A constant pressure of about 1.7 10^5 Pa was exerted on the liquids in the cells. Two scans were obtained for every sample. The calorimetric data were corrected by the subtraction of a water–water or a buffer–buffer scan as the instrumental baseline. DSC curves were normalized for the gram mass of protein and next a linear baseline was subtracted. An apparent excess heat capacity Cp^{ex} (J $^oC^{-1}$ g^{-1}) versus temperature (^oC) has been plotted.

The following parameters of observed serum denaturation DSC transitions have been determined: temperatures of local peak maxima T_m (m = 1, 2, 3), excess heat capacities at these temperatures Cp_m, the enthalpy (ΔH) of serum denaturation (calculated as the area under the endothermic peak, expressed in J g^{-1}) and the width of peak in its half height (*HHW*).

Results and discussion

Both in aqueous and buffer solutions, the denaturation of human blood serum proceeds in a similar temperature range of about 40–90 °C. In Fig. 1, the mean normalized DSC data for serum samples in aqueous (pH within the range 6.5–7.0) and buffer (pH 7.4) solutions are compared. The difference

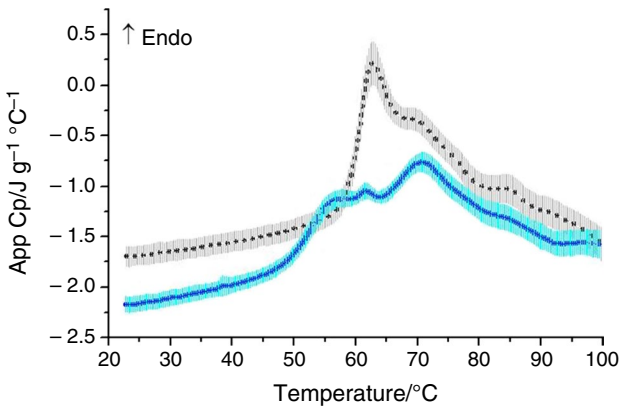

Fig. 1 The averaged normalized heat capacity data for aqueous (solid blue line), and buffer (dotted black line) solutions of blood serum. The shaded area represents the standard deviation at each temperature

in the shape of the complex endothermic transition is easily noticeable. Additionally, a shift in relation to the heat capacity axis is observed.

For sera denaturation in buffer solution, peaks around 63 °C and 70 °C reflect the thermal denaturation of the most abundant serum proteins, albumin and immunoglobulins, respectively. In aqueous solution, three relatively well-resolved transitions can be usually observed. The first one, at about 58 °C, mainly represents the contribution from unliganded albumin, while the third one, at about 70 °C, comes from denaturation of immunoglobulins. The middle sharp peak at about 62 °C is not always visible. It has its origin mainly in haptoglobin (Hp), the acute-phase protein belonging to alpha-2 globulins fraction. The peak or shoulder at about 83 °C, better visible for buffer serum solution, also represents an important component of the complex plasma/serum denaturation transition, usually attributed to contributions from IgG and transferrin [2]. Sometimes a clear peak in this temperature range is observed (see e.g., Fig. 1a in Todinova et al. [16]—calorimetric profiles of blood sera of MM patients with secretion of IgG–cases 1, 2).

The main source of the differences described above and well visible in Fig. 2 is probably the thermal denaturation profile of unligated form of albumin, the most abundant serum protein. DSC profiles of aqueous and buffer solutions of this protein have been compared in Fig. 1a of ref. [27] by Michnik A & Drzazga Z. DSC transition representing unfolding of ligand-free fraction of albumin molecules starts at higher temperatures and is more sharp (so the thermal unfolding is more cooperative) in buffer than in water solution. The denaturation temperatures Tm corresponding to the unfolding of an un-liganded human albumin in buffer solution (pH 7.2) is about 62 °C (62.5 ± 0.4), while in aqueous solution even higher (68.3 ± 1.2), but a large part of the transition there is below this temperature. The shapes of DSC curves and the denaturation temperatures for nondefatted albumin have been found very similar in both solutions [27].

As mentioned, under physiological conditions, i.e., pH 7.4, the main peak (centered at about 63 °C) in serum/plasma denaturation transition corresponds to albumin with a trace of haptoglobin. It follows from DSC curves shown by Garbett et al. [1] for individual pure plasma proteins in such conditions. According to the Supplemental Fig. 1 [Garbett Suppl.], haptoglobin denaturation is represented by a narrow, sharp peak at about 62 ÷ 63 °C. A similar peak is observed in the case of an aqueous solution of haptoglobin (Fig. 3). This specific peak has also been well detected in presented earlier DSC curves of α, β-globulins fraction in buffer as well as in water solutions (Fig. 1c in [27]). The DSC curve of this serum protein fraction displays multiple peaks and shoulders associated with contributions from different constituent proteins. The whole denaturation transition together with the most specific peak coming from haptoglobin denaturation is shifted to higher temperatures in buffer in comparison with water solution.

In current work, two from three major Hp phenotypes (Type 1–1, Type 2–1, and Type 2–2) have been studied. In Fig. 3 DSC curves for Hp 1–1 and Hp 2–2 in aqueous solutions are presented. The denaturation temperatures Tm for Hp 1–1 is 61.8 °C while for Hp 2–2 60.8 °C. An additional difference between these two Hp phenotypes is that the thermal transition of Hp 1–1 is partially reversible while that representing Hp 2–2 denaturation, irreversible (not shown). It follows that the denaturation temperature of haptoglobin is slightly dependent on phenotype.

Under normal conditions, haptoglobin is present at very low levels (accounts for 0.4–2.6% of the total plasma proteins). Then the contribution from Hp is small (see Fig. 2 in ref. [1]). However, it can increase significantly in response to acute infection, inflammation or trauma. Then, the intensity

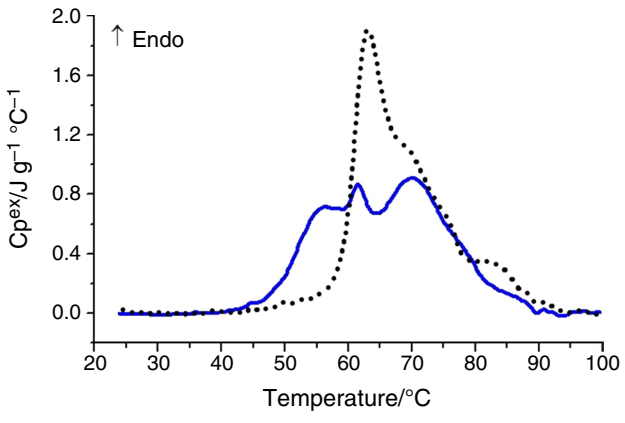

Fig. 2 The comparison of mean DSC curves of sera in buffer (dotted black line) and water (solid blue line) solutions

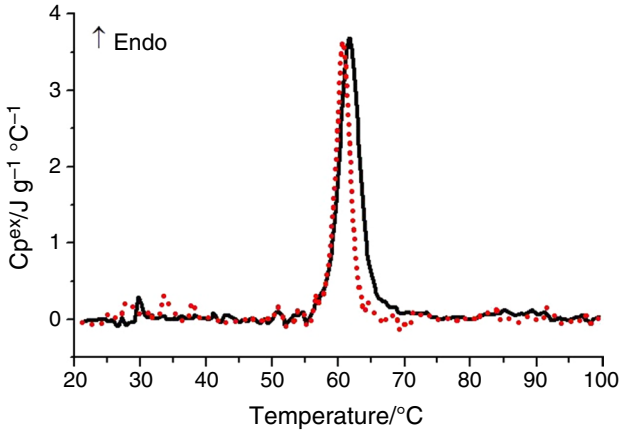

Fig. 3 DSC curves for Hp 1–1 (solid black line) and Hp 2–2 (dotted red line) in aqueous solutions

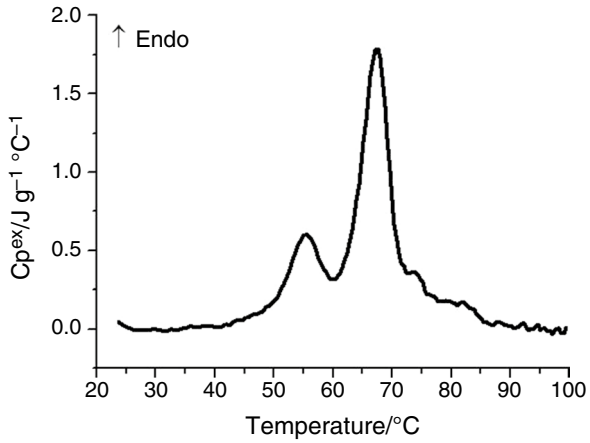

Fig. 4 DSC curve of transferrin in aqueous solutions

of the peak representing the unfolding of Hp also increases significantly. The advantage of DSC profiles observed for aqueous serum solutions (with pH below the physiological value) is the separation of contributions from albumin and haptoglobin. The rise in serum or plasma haptoglobin and the continuous monitoring of this during the acute-phase response gives valuable information to the clinician or researcher.

The main contributions from immunoglobulins (primary from IgG, IgA, IgM) are revealed in the heat capacity profile of healthy human blood serum at about 70 °C in both kind of solutions. However, they appear as a peak in aqueous solution and as a hump in a buffer solution (Fig. 2). Another difference between the two curves in Fig. 2 is revealed at about 83 °C. The peak or shoulder visible around this temperature has its source in denaturation of the C_H3 domain of immunoglobulin IgG1 [28, 29] and/or transferrin [1]. The comparison of the DSC curve of transferrin in aqueous solutions shown in Fig. 4 with the DSC curve of transferrin in PBS solution presented in the Supplemental Fig. 1 by Garbett et al. [1] allows to explain the greater intensity of heat capacity changes in this temperature range for serum in PBS solution. Regarding immunoglobulin, Schaefer, Sedlak et al. [29] have reported that the thermal as well as the colloidal stability of IgGs and their fragments are also affected by the solvent composition. This all means that it is not easy to distinguish between contributions from the IgG and transferrin in this area of serum thermal transition, regardless of the solvent used.

Conclusions

DSC results are affected by different preparation conditions and this must be remembered when interpreting data. When considering the use of information from DSC profiles of physiological fluids in medical diagnostics, these conditions should be established, and the data collection procedures carefully defined. Before implementing such applications, it is worth checking the advantages and disadvantages of various approaches at the level of scientific research. The standardization reduces potential errors thus improves the quality of patient care. However, standardization of practices may vary depending on the considered case or disease entity and should be as flexible as possible to meet all predefined goals. The difference found in the shape and interpretation of the denaturation transition of serum in aqueous and buffer solutions can be used in establishing such standardization procedures. For example, in serum aqueous solutions contributions from albumin and haptoglobin can be separated and an elevated level of haptoglobin, an acute-phase protein can be observed even without undergoing a deconvolution process. This can be very valuable when it comes to diseases where it is important to track elevated haptoglobin levels. Moreover, concerning the dilution of serum with distilled water, instead of diluting with PBS, it is easier to track changes in ligand-free fraction of albumin. So, when looking at a special effect, an important advantage of a given solvent appears in some types of research.

Author contributions A.M. was involved in conceptualization; formal analysis; investigation; methodology; supervision; validation; visualization; writing—original draft; writing—review & editing. A.K. contributed to investigation; data curation; formal analysis; visualization; K.D. was involved in investigation; data curation; formal analysis; visualization; E.S.-K. contributed to methodology; data curation; supervision; writing—review & editing. I.P. was involved in conceptualization; investigation; project administration; writing—review & editing. All authors critically reviewed the manuscript and approved the final version.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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