Characterization of Biomolecules by Means of DSC: Lysozyme

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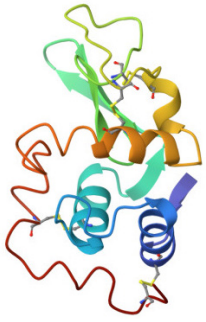
Introducton

Lysozyme, or muramidase, is the name of a group of enzymes that hydrolyzes the peptidoglycans, a structural macromolecule composed of sugars and amino acids, forming a protective layer on the external wall of bacterial cells. Lysozyme is widespread in nature, being present in animals, plants, bacteria, and also in bacteriophage viruses. It is part of the innate immune system acting against bacterial infection. It can be found in body secretions like saliva and tears, tissues, and also in organs. Due to its antibacterial and antifungal activity, lysozyme has a potential in clinical, feed, and food applications [2]. It is also widely applied as a model molecule for the investigation of the protein structure, stability and function in several research areas [3].

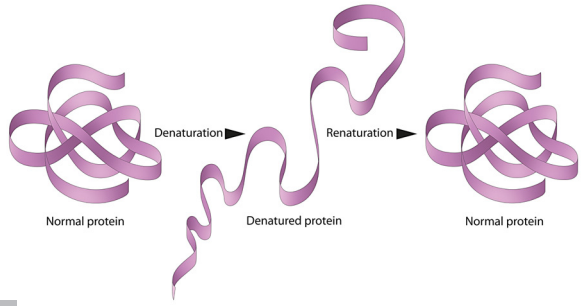
Lysozyme is a small globular protein with a similar chemical structure in the different living beings in which it is present, see figure 1. The different types of lysozymes are classified into three main families: chicken-type, goose-type and invertebrate-type. Human and chicken lysozymes are classified as chicken-type and are almost 60% identical in their amino acid sequence, while chicken lysozyme is composed of 129 amino acids residues (14.3 kDa), human lysozyme has 130 (14.7 kDa). Chicken egg white is the main commercial source of lysozyme [2,3]. The so-called hen egg white lysozyme (HEWL) is active in a large range of pH (6 – 9) and represents a melting/transition temperature, T_m, of 72°C at pH 5.0 [4].

DSC is largely applied to study the thermal stability of proteins and protein formulations. Unfolding a protein is an endothermic effect resulting from the exposure of its hydrophobic groups to the aqueous medium. Therefore, for proteins in solutions, a heat absorption peak is often observed in the DSC curve, and its peak maximum is referred in literature as the melting/transition temperature (T_m). The thermal denaturation (unfolding of the 3-dimensional structure of the protein) can be reversible or irreversible, depending on the protein characteristics and on the conditions of the medium, figure 2 [5]. Medium conditions that influence the reversibility of the denaturation include, for example, protein concentration, pH, ionic strength, and temperature. It is therefore expected that changes in the protein structure or in the formulation medium may influence the thermostability of proteins, which is reflected in the T_m measured.

DSC measures directly the heat absorption associated with the unfolding process. It is a reliable method for determining the thermodynamic characteristics of a native protein to characterize proteins that underwent structural modifications or to access the thermal stability of protein formulations for therapeutic use.



1 3D structure of hen egg white lysozyme [1].



2 Schematic example of denaturation and renaturation of proteins.

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Experimental

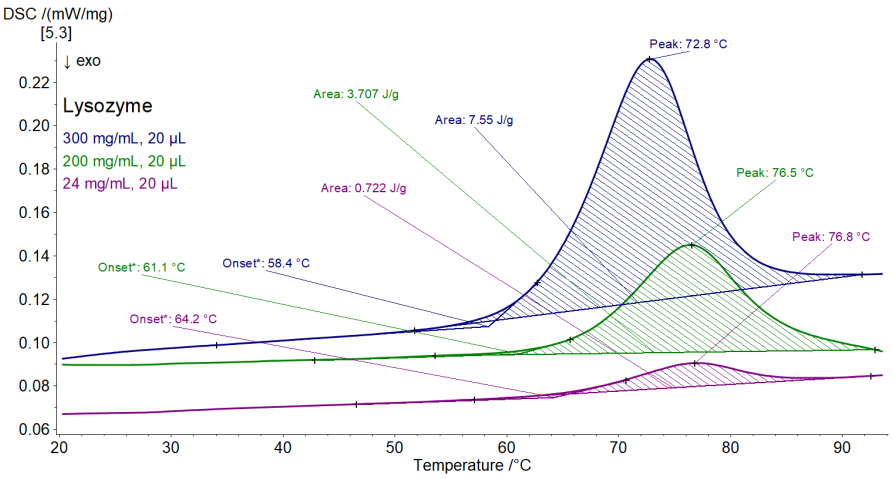
Sample Preparation Method

Lysozyme[1] was solubilized in distilled and filtered[2] water at concentrations of 300 mg/ml, 200 mg/ml, 24 mg/ml, and 5 mg/ml. 20 µl of each concentration was pipetted into *Concavus*® crucibles[3] that were immediately sealed. For the solution at 24 mg/ml, a volume of 5 µl was also analyzed. At least three measurements on each sample were carried out. The reference crucible was filled with the same volume of distilled filtered water. The measurements were performed under an inert atmosphere (dynamic N_2, 40 ml/min) at a heating rate of 10 K/min.

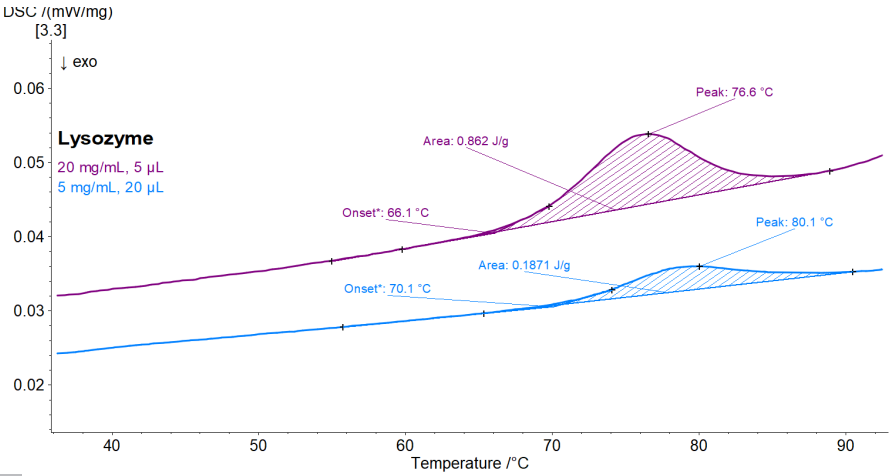
[1] Hen egg white lysozyme, ≥ 45 000 FIP U/mg, lyophilized, 14 kDa, Carl Roth GmbH + Co KG
[2] Polyether sulfone - PES membrane filter, 450 µm
[3] *Concavus*® 40 µl aluminum crucibles, NETZSCH-Gerätebau GmbH

Measurement Results and Discussion

The DSC curves of lysozyme aqueous solutions show the typical single endothermic effect in the range of 75°C for all measured concentrations. Figure 3 depicts typical curves of solutions at concentrations of 300, 200 and 20 mg/ml. The extrapolated onset temperature, peak temperature (T_m) and area under the curve (enthalpy) vary with the concentration. The higher the mass sample in the crucible, the broader the endothermic effect. The broadening effect is observed during variation of the extrapolated onset and peak temperatures as well as the enthalpy. The chosen concentrations are representative of ordinary therapeutic protein drugs, which are usually highly concentrated, with the protein dosage given in mg/kg of body weight. Figure 4 shows the influence of the sample volume by displaying the DSC curves of solutions at 20 mg/ml (5 µl) and at 5 mg/ml (20 µl).



3 DSC measurement on lysozyme at 300 mg/ml (blue curve), 200 mg/ml (green curve), and 20 mg/ml (purple curve). Sample volume: 20 µl.



4 DSC measurement on lysozyme at 20 mg/ml (purple curve), 5 mg/ml (light blue curve). Sample volume: 5 µl and 20 µl, respectively.

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Table 1 Characterization of lysozyme by means of DSC: concentration, protein mass, volumes of measured samples and the respective transition temperatures (peaks) and enthalpies (areas)

Concentration (mg/ml)	Sample Volume (µl)	Concentration (mM)	Protein Mass (mg)	Area (J/g)	Peak (°C)
300	20	21.4	6.37 ± 0.34	7.41 ± 0.12	73.0 ± 0.2
200	20	14.3	4.26 ± 0.14	3.56 ± 0.14	76.2 ± 0.4
20	20	1.7	0.51 ± 0.0	0.69 ± 0.05	77.4 ± 0.5
20	5	1.7	0.10 ± 0.0	0.78 ± 0.11	76.6 ± 0.2
5	20	0.36	0.10 ± 0.0	0.33 ± 0.19	79.3 ± 0.5

The respective masses were 0.13 mg and 0.10 mg. The results of all measurements are summarized in table 1.

Summary

In this study, the DSC 300 *Caliris*® was used to investigate the transition temperature of lysozyme in a wide range of concentrations, from 5 to 300 mg/ml, which is representative of commercially available protein formulations. Although high-concentration solutions were used, measurement on volumes as small as 5 µl allowed for saving the expensive formulations with high reproducibility.

The sensor sensitivity and the possibility of using small volumes in the range of a few microliters, along with the possibility of having an automated sample changer, make DSC a valuable technique for the analysis of biomolecules. Depending on the heating/cooling rate, the throughput can be as high as 3 samples per hour.

References

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