ACCELERATING PROTEIN FORMULATION DEVELOPMENT: PH-DEPENDENT STABILITY ANALYSIS OF LYSOZYME USING THE ZETASIZER CORE

Introduction

The development of stable protein formulations is a critical step in biopharmaceutical research and development. Protein stability can be strongly influenced by formulation parameters such as pH, buffer composition, ionic strength, and excipient selection. Identifying optimal formulation conditions is therefore essential to maintain protein integrity, prevent aggregation, and maximize shelf life. However, screening multiple formulation conditions can be both time-consuming and resource-intensive, requiring significant sample preparation and analytical effort.

The Zetasizer Core streamlines protein formulation screening by enabling high-throughput analysis in a standard microplate format. By combining particle sizing and thermal stability measurements in a single automated workflow, the system allows researchers to rapidly evaluate multiple formulations while minimizing sample consumption and hands-on time. Automated plate-based measurements and controlled temperature ramping at 1 °C/min facilitate efficient comparison of protein behavior across a range of conditions.

In this study, lysozyme was used as a model protein to investigate the impact of formulation pH on protein size and stability. Lysozyme samples were prepared in a series of buffer conditions spanning different pH values and analyzed using Dynamic Light Scattering (DLS) to assess hydrodynamic size. Consecutively, Static Light Scattering (SLS) and Differential Scanning Fluorimetry (DSF) were used during a temperature ramp to monitor aggregation onset and thermal unfolding behavior. The thermal stability of each formulation was characterized by determining both the melting temperature (T_m), which reflects protein unfolding, and the aggregation temperature (T_{agg}), which indicates the onset of aggregation.

Materials

25 mM sodium acetate buffer solution was prepared by dissolving 0.3402 g of sodium acetate trihydrate in 100 mL of 10 mM NaCl. Acetic acid stock solution was prepared by diluting 0.1500 g of 17.4 M acetic acid to a final volume of 100 mL with 10 mM NaCl.

The acetic acid solution was used to set the buffer at pH values 3.16, 4.04, 5.06, 6.05 and 7.3.

Lysozyme was prepared at a concentration of 1 mg/mL by weighing 4 mg of lysozyme into a sterile glass tube and adding 4 mL of the prepared acetate buffers of different pH values. The sample was gently inverted until no visible powder remained on the tube walls. The solution was then placed on a mixer and mixed at 30 rpm for 4 hours to ensure complete dispersion.

Following dispersion, the lysozyme solution and the buffers was filtered through a 20 nm syringe filter. The initial filtrate was discarded to minimize contamination from the filter membrane, and the remaining filtrate was collected for subsequent analysis.

Methodology

Each formulation was prepared in eight replicate wells to enable assessment of measurement repeatability. Aliquots of 20 µL were dispensed into individual wells of a 384-well Aurora microplate with round-bottom wells. The plate layout used in this study is shown in Figure 1.

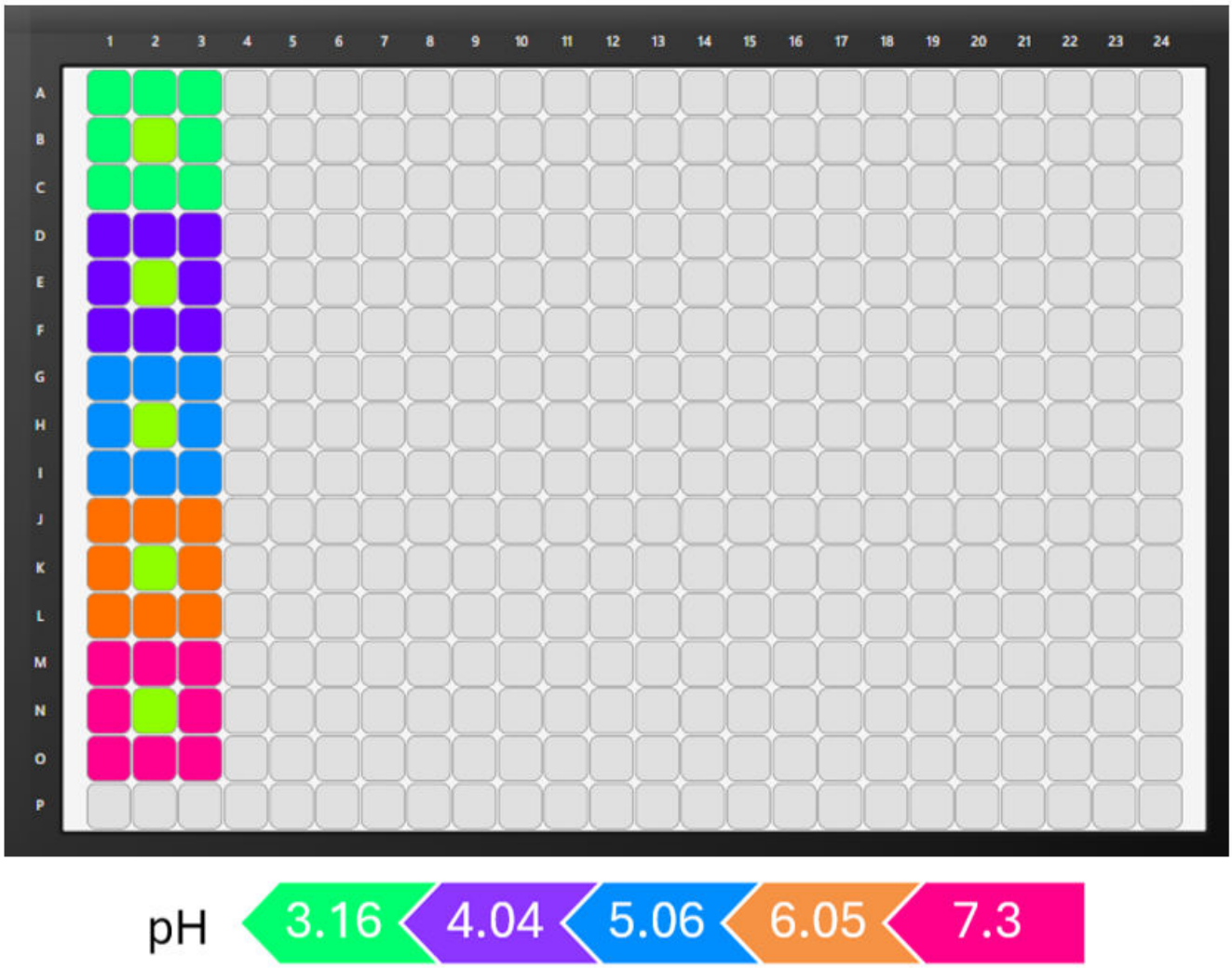

Figure 1. Plate map showing the distribution of lysozyme samples across the 384-well microplate (the green box in the middle of each sample cluster is the blank for each sample).

DLS measurements were performed in backscatter detection angle (158°). Measurements were acquired using the recommended settings of three acquisitions per well, with an acquisition time of 1 s per acquisition.

Thermal stability measurements were performed using a temperature ramp from 25 °C to 85 °C at a heating rate of 1 °C/min, corresponding to a temperature increment of 1 °C between successive measurements. Intrinsic fluorescence measurements were collected using an excitation wavelength of 280 nm at 100% UV intensity. Simultaneously, Static Light Scattering (SLS) was measured at 830 nm using a laser intensity of 100%. The run took approximately 70 minutes.

Results

Effect of pH on Lysozyme Size and Colloidal Stability

The measured hydrodynamic diameter of lysozyme remained consistent across the investigated pH range (Table 1), with average particle sizes between 3.77 and 3.94 nm. The narrow distribution of measured diameters indicates that the protein remained predominantly monomeric and that no significant aggregation occurred under any of the tested conditions (Figure 2). The repeatability of the measurements was excellent for all formulations.

Table 1: Average hydrodynamic diameter and polydispersity index (*PI*) of lysozyme at different pH values

pH	Average Diameter [nm]	StdDev of Average Diameter [nm]	*PI*
3.16	3.77	0.18	0.07
4.04	3.83	0.10	0.04
5.06	3.94	0.09	0.03
6.05	3.93	0.10	0.05
7.3	3.88	0.09	0.07

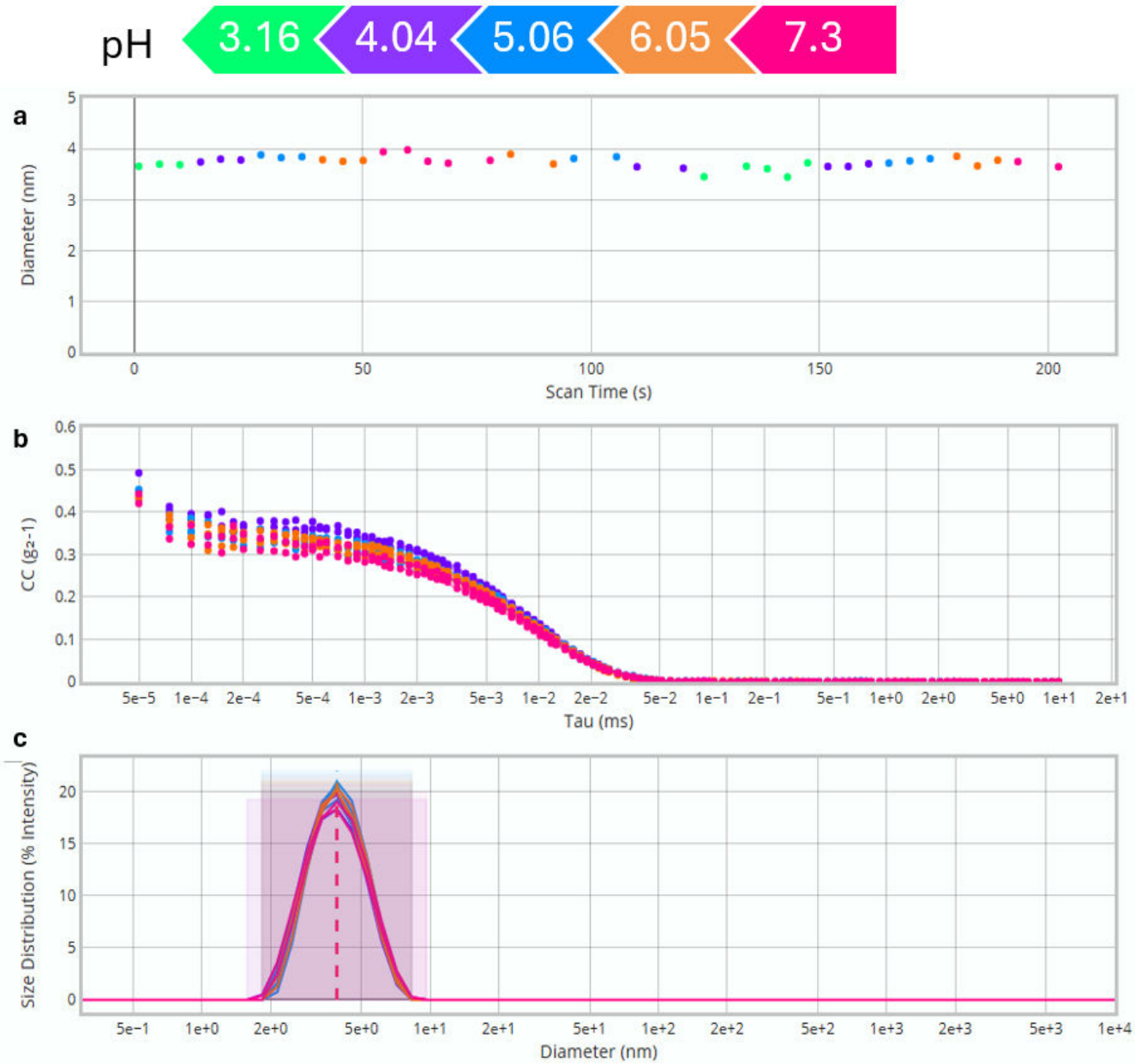

Figure 2. (a) Average diameter, (b) correlograms, and (c) intensity-based size distributions of 1 mg/mL lysozyme prepared in sodium acetate buffers of pH 3.16, 4.04, 5.06, 6.05, and 7.30. The plots represent eight replicate measurements for each sample. The excellent repeatability of the results in overlap between replicates, causing some data points to be obscured.

Correlation functions collected for all buffer conditions (Figure 2) showed excellent overlap and indicated good measurement quality. Likewise, the intensity-weighted size distributions exhibited a single, narrow population centered around 4 nm, with

no evidence of larger aggregated species (Figure 2).

The low polydispersity index (*PI*) values obtained for all samples (<0.1) further confirm that the lysozyme preparations were highly monodisperse and free from detectable aggregates.

Overall, the DLS measurements demonstrate that lysozyme maintains a consistent size across the pH range investigated. As particle size alone does not distinguish between formulations, additional thermal stability measurements were performed to determine whether differences in protein unfolding and aggregation behavior could be observed under the various pH conditions.

Effect of pH on Lysozyme Thermal Stability

While DLS measurements demonstrated that lysozyme remained monomeric and maintained a consistent hydrodynamic diameter across all investigated pH conditions, DSF revealed clear differences in thermal stability. The thermal unfolding profiles showed that the choice of buffer pH had a measurable impact on both the melting temperature (T_m) and the onset temperature of unfolding (T_{on}), highlighting the importance of formulation conditions when developing stable protein preparations.

The DSF thermograms are shown in Figure 3. All samples displayed a characteristic sigmoidal unfolding transition as the temperature increased, with excellent repeatability, but the transition temperatures varied between formulations. The highest thermal stability was observed at pH 4.04 and pH 5.06, where lysozyme exhibited T_m values of 76.7 °C and 76.3 °C, respectively (Table 2). These conditions also produced the highest T_{on} values, indicating that protein unfolding was initiated at higher temperatures compared with the other formulations.

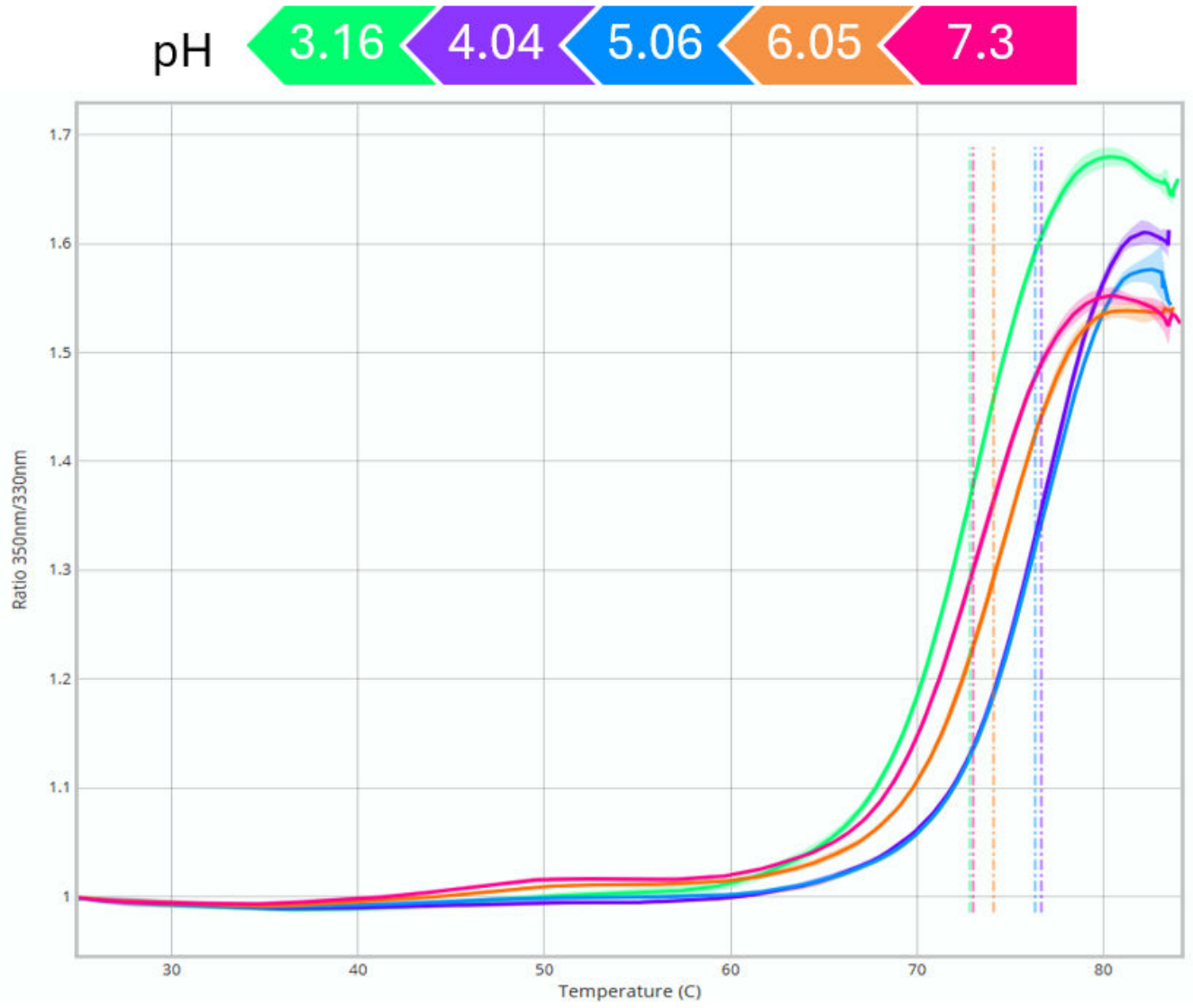

Figure 3: a) DSF of 1 mg/ml lysozyme prepared in sodium acetate buffers of pH 3.16, 4.04, 5.06, 6.05, and 7.30. The dotted lines represent the T_m points for each sample.

Table 2: Melting temperature (T_m) and onset temperature (T_{on}) of lysozyme at different pH values

pH	T_m [°C]	T_m StdDev [°C]	T_{on} [°C]	T_{on} StdDev [°C]
3.16	72.7	0.3	68.4	0.2
4.04	76.7	0.3	72.2	0.2
5.06	76.3	0.2	71.9	0.2
6.05	74.1	0.1	69.9	0.2
7.3	72.9	0.3	68.7	0.1

In contrast, the lowest thermal stability was observed at pH 3.16, where the T_m was reduced to 72.7 °C and the T_{on} was 68.4 °C. Similar behavior was observed at pH 7.30, which exhibited a T_m of 72.8 °C and a T_{on} of 68.7 °C. Both formulations therefore showed reduced resistance to thermal unfolding relative to the intermediate pH conditions.

The formulation prepared at pH 6.05 displayed intermediate stability, with a T_m of 74.1 °C and a T_{on} of 69.9 °C. Although more stable than the pH 3.16 and pH 7.30 samples, it did not achieve the thermal stability observed at pH 4.04 and pH 5.06.

Although the DSF measurements revealed clear differences in the thermal stability of lysozyme across the tested pH conditions, the SLS measurements showed little to no evidence of aggregation. Consequently, the SLS data were not included in the results section.

Conclusion

By comparing size measurements and thermal stability parameters across the investigated buffer conditions, this study demonstrates how the Zetasizer Core can rapidly identify formulation environments that maintain protein stability and minimize aggregation. Such information can be used to guide formulation development and accelerate the selection of optimal conditions for biotherapeutic proteins.

Overall, the results show that while lysozyme maintained a similar size distribution across all tested pH conditions, its thermal stability was strongly influenced by formulation pH. The highest stability was observed between pH 4 and 5, where thermal unfolding occurred approximately 4 °C later than at pH 3.16 and pH 7.30. These findings highlight the value of the Zetasizer Core as a rapid screening tool for assessing the impact of formulation conditions on protein stability, enabling the identification of optimal buffer environments early in the development process and supporting more efficient biotherapeutic formulation design.

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