STREAMLINING BUFFER OPTIMIZATION FOR BIOLOGICS: A LYSOZYME CASE STUDY

Introduction

Protein stability is a critical consideration throughout biopharmaceutical development, formulation optimization, and quality control. Buffer composition can significantly influence protein folding, aggregation, shelf life, and overall performance. Rapid screening of buffer systems enables researchers to identify conditions that maximize protein stability while minimizing formulation development time.

Dynamic Light Scattering (DLS) is widely used to assess protein size, homogeneity, and the presence of aggregates in solution. By measuring the hydrodynamic diameter and polydispersity of protein formulations, DLS can provide early indications of changes formulation quality.

Differential Scanning Fluorimetry (DSF) provides a label-free approach for assessing protein thermal stability by monitoring changes in intrinsic tryptophan fluorescence during thermal unfolding. Simultaneously, aggregation onset can be detected through Static Light Scattering (SLS), providing a comprehensive assessment of protein behavior under stress.

In this study, DLS was used to characterize the size distribution and homogeneity of lysozyme in different buffer systems prior to thermal analysis, while DSF and SLS were employed to evaluate thermal unfolding and aggregation behavior. The combined approach enables a comprehensive assessment of protein stability and formulation performance. This study investigates the effect of four commonly used buffer systems and a sodium chloride solution on the stability of lysozyme at a concentration of 1 mg/ml.

Materials

The following buffers were prepared using analytical-grade reagents and deionized water: acetate buffer (pH 4.0), 50 mM HEPES buffer (pH 7.0), and 10 mM Tris-HCl buffer (pH 7.0). A 10 mM NaCl solution was also prepared. Buffer pH values were adjusted using HCl or NaOH as appropriate and verified using a calibrated pH meter prior to use. All solutions were prepared fresh or filtered through a 0.02 µm membrane before use when required. Lysozyme from hen egg white was prepared at a concentration of 1 mg/ml in all the buffers as well as phosphate-buffered saline (PBS).

Method

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.

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%.

Results

DLS measurements were initially performed to characterize the size and homogeneity of lysozyme in the different buffer systems. The results presented in Table 1 showed excellent repeatability, with all formulations exhibiting average hydrodynamic diameters at around 3.5 nm and low polydispersity indices. These data indicate that lysozyme remained predominantly monomeric and monodisperse under all investigated conditions.

Table 1: Hydrodynamic diameter and polydispersity index (*PI*) of lysozyme in different buffers

Buffer	Average diameter, nm	*PI*
PBS	3.89 ± 0.08	0.07
Acetate buffer	3.76 ± 0.09	0.04
HEPES	3.16 ± 0.05	0.19
Tris	3.67 ± 0.07	0.03
NaCl	3.70 ± 0.08	0.07

Among the tested formulations, the HEPES sample exhibited the smallest average protein size (3.16 ± 0.05 nm) but also displayed the highest polydispersity index (0.19) suggesting a broader particle size distribution than the other buffer systems.

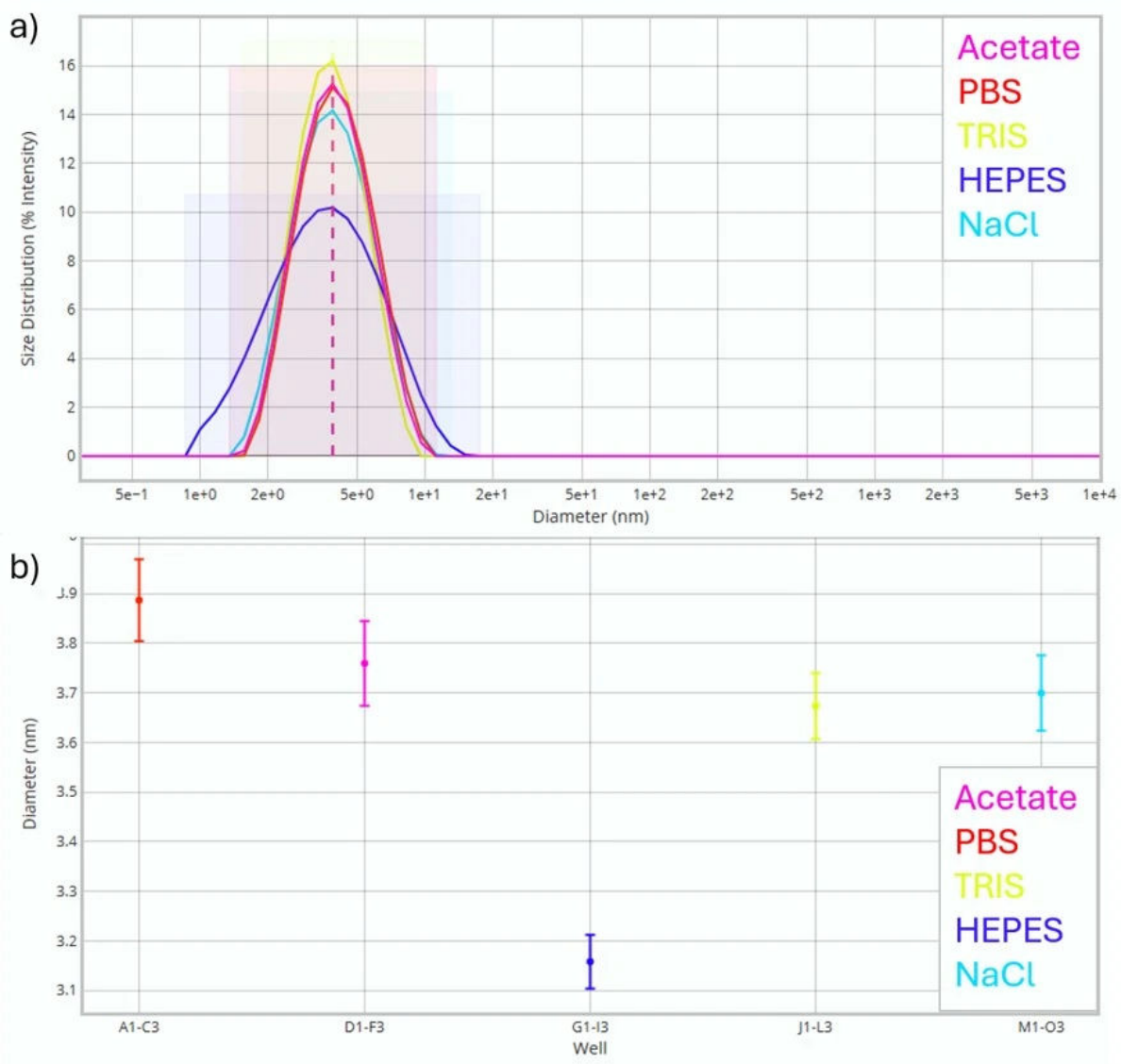

Figure 1: a) Intensity based size distributions and b) comparison of average hydrodynamic diameter for all the different buffer conditions.

As it can be seen the size distribution of lysozyme in HEPES buffer is broader compared to the others as it was also reflected by the *PI*. In contrast, acetate, Tris, PBS and NaCl formulations exhibited narrow distributions and low *PI* values (< 0.1). This is an early indication that lysozyme is less stable in 50 mM HEPES compared to the other buffers.

To further evaluate formulation stability, thermal unfolding was measured using Differential Scanning Fluorimetry (DSF). Representative thermal unfolding profiles are shown in Figure 2, while the extracted thermal transition temperatures are summarized in Table 2.

Table 2: Thermal unfolding and aggregation parameters of lysozyme measured by DSF and SLS

Buffer	T_{m1} (°C)	T_{on1} (°C)	T_{agg} SLS (°C)
PBS	72.1 ± 0.3	67.7 ± 0.2	69.6 ± 1.0
Acetate buffer	77.2 ± 0.3	72.8 ± 0.1	-
HEPES	73.8 ± 0.2	69.5 ± 0.1	-
Tris	75.2 ± 0.3	70.6 ± 0.1	-
NaCl	76.0 ± 0.2	71.5 ± 0.3	-

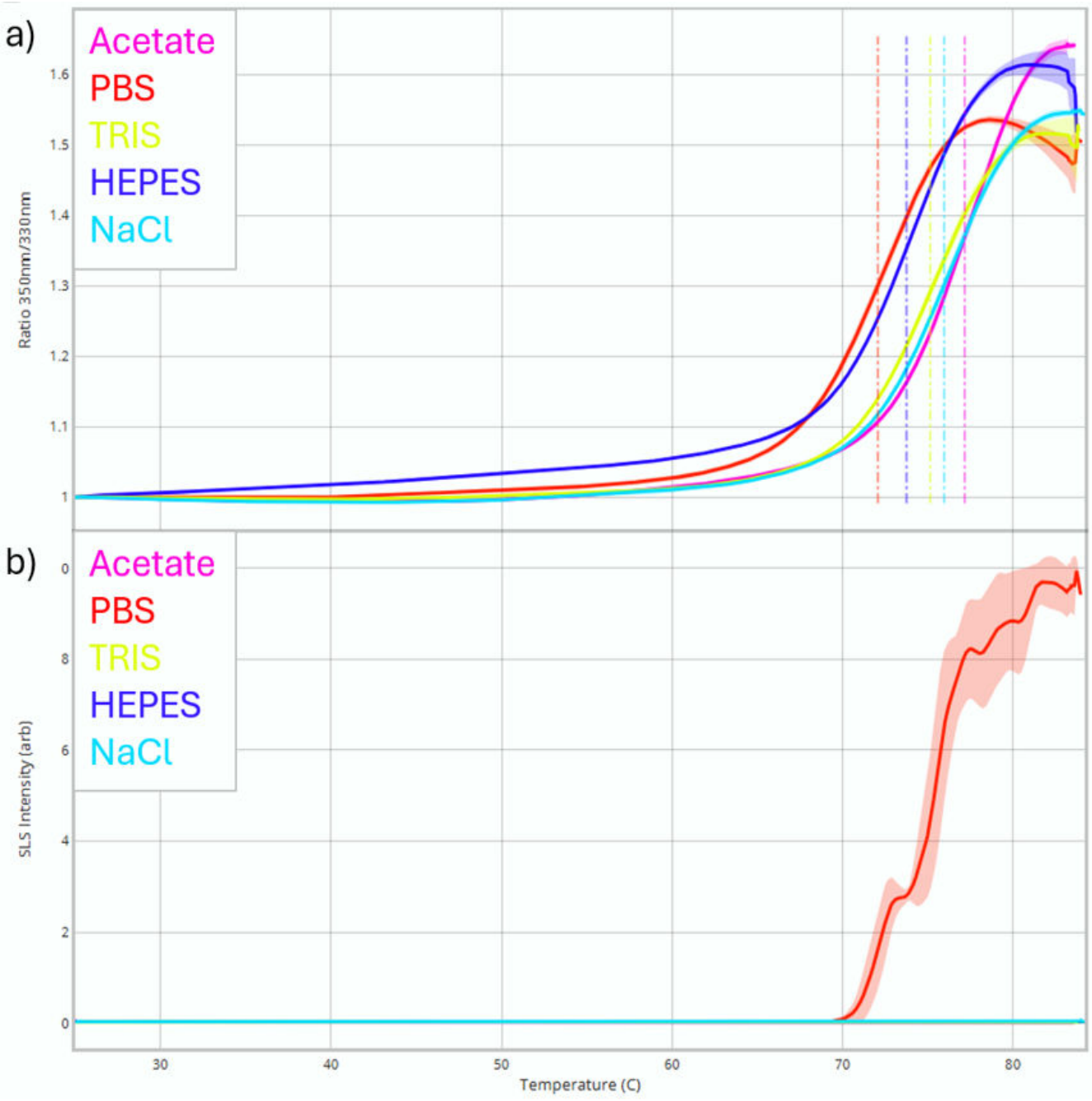

Figure 2: Thermal stability profiles of lysozyme in different buffers obtained by a) DSF and b) SLS. Dotted lines represent the T_m points.

It can be seen from Table 2 that the onset temperature of unfolding (T_{on1}) and melting temperature (T_{m1}) were found to be highly reproducible across all replicate measurements, with standard deviations below 0.3 °C.

Significant differences in thermal stability were observed between the buffer systems. Lysozyme formulated in acetate buffer exhibited the highest thermal stability, with a T_{m1} of 77.2 ± 0.3 °C and a T_{on1} of 72.8 ± 0.1 °C. These values were approximately 5 °C higher than those measured in PBS, indicating a substantial stabilizing effect of the acetate formulation. Since lysozyme is known to be stable under mildly acidic conditions, the enhanced thermal stability observed in acetate buffer is consistent with its expected solution behavior.

The NaCl and Tris formulations also improved stability relative to PBS. The lower thermal stability was observed in HEPES and combined with the broader size distribution measured by DLS, suggests that this formulation was less favorable for maintaining lysozyme stability under the conditions investigated.

Static light scattering measurements were acquired simultaneously with DSF to monitor thermally induced aggregation. However, clear aggregation transitions were not consistently observed for all formulations, preventing reliable determination of aggregation temperatures, apart from lysozyme in PBS. This behavior suggests that unfolding and aggregation were not strongly coupled under the investigated conditions and highlights the importance of combining multiple orthogonal techniques when assessing protein stability

Conclusion

The combined DLS and DSF measurements demonstrated that buffer composition significantly influences lysozyme stability. Among the formulations investigated, acetate buffer (pH 4) provided the greatest thermal stabilization, resulting in the highest unfolding onset and melting temperatures. Tris and NaCl formulations also improved stability relative to PBS, whereas HEPES showed the lowest thermal stability and exhibited a broader particle size distribution, indicating increased sample heterogeneity.

DLS measurements confirmed that all formulations remained predominantly monodisperse prior to thermal stressing, while DSF provided highly reproducible measurements of thermal unfolding. Together, these techniques provide complementary information on protein size, thermal stability, and aggregation behavior, enabling rapid identification of optimal formulation conditions. For lysozyme, acetate buffer was identified as the most favorable condition among those tested, demonstrating the value of high-throughput biophysical screening during early-stage formulation development.

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