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Technical Note

Multiple simultaneous measurements provide improved characterization of protein stability

Introduction

Protein stability is relevant to a range of fields, such as the biopharmaceutical industry, where biologic therapeutics are required to remain in specific structural conformations in order to exert their therapeutic effects and withstand stresses of manufacture, storage and transport. Optimizing stability of proteins is achievable by manipulating the primary sequence or more readily, by altering the solution conditions *via* a process called formulation. High throughput methods for testing stabilities of proteins in multiple formulations can accelerate this process and ideally these should be rapid and use low sample volumes. The UNit is ideal for protein stability studies, enabling analysis of up to 48 samples in a matter of hours, requiring just 9 µl of sample. An added advantage of the UNit is that it employs fluorescence spectroscopy and static light scattering (SLS) to simultaneously measure changes in tertiary structure and presence of aggregates, respectively. Here, we use UNit to analyses α-chymotrypsinogen A in different formulations and demonstrate the advantage of being able to assess both conformational and colloidal stability.

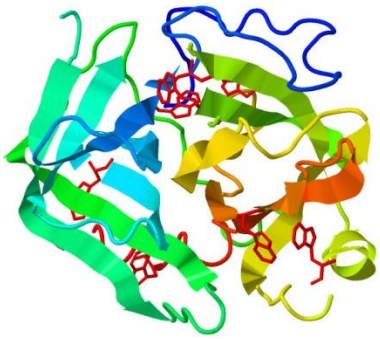
Methodology

α-chymotrypsinogen A was dissolved at concentrations of 1 mg/ml in 25 mM sodium phosphate or phosphate citrate between pH 2 – 9 at low ionic strength. Some additional proteins (bovine serum albumin (BSA) and β-lactoglobulin) were also analyzed and prepared in the same way. 9 µl of each sample was loaded into a UNi and analyzed in triplicate on the UNit. A linear temperature ramp was applied between 15 and 95 °C at a rate of 0.3 °C/min. The barycentric mean (BCM) and SLS signals at 266 nm were plotted against temperature in order to obtain melting temperatures (T_m) and aggregation onset temperatures (T_{agg}). BCM reflects the shift in tryptophan fluorescence emission wavelength due to changes in the polarity of its environment. SLS signal intensity is proportional to the mean solute particle mass. Data were analyzed using the UNit Analysis software v. 2.1.

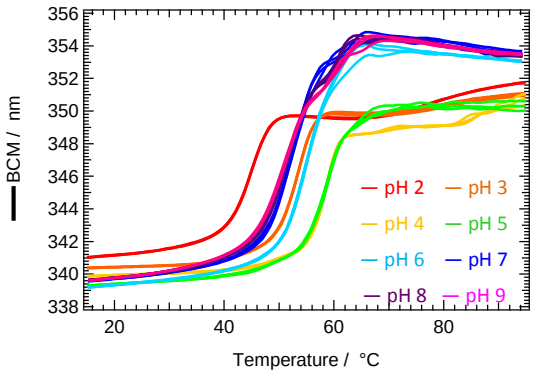
Results

α-chymotrypsinogen is a zymogen of the digestive enzyme chymotrypsin, and is itself enzymatically inactive. It is a relatively small protein (25.6 kDa) consisting of 246 amino acid residues, cross-linked by five disulfide bonds. The structure is shown in Figure 1a, where the eight tryptophan residues have been highlighted. The BCM *versus* Temperature plots for α-chymotrypsinogen A across the pH conditions are shown in Figure 1b. The profiles at pH 7, 8 and 9 become a little more complex at ~52 °C, which is a consequence of aggregates falling out of solution. This can be seen in the SLS (266 nm) *versus* Temperature plots (Figure 1c) as the signals peak and then drop, resulting in a 'bell shaped' curve. In this instance it was useful to have the orthogonal SLS data to explain the complexity in the intrinsic fluorescence data. The BCM data show a single unfolding transition at all pH and the SLS (266 nm) data show that aggregation occurs in all buffers, except pH 2 and 3. The T_m and T_{agg} values are shown in Table 1 and are plotted against pH in Figure 2. According to the T_m values, α-chymotrypsinogen A is most conformationally stable in the pH 4 and 5 formulations and there is little difference between them. However, the T_{agg} values allow the pH 4 and 5 formulations to be differentiated in terms of stability. T_{agg} is lower than T_m for the pH 5 formulation, whereas for pH 4, and the other formulations, the T_{agg} and T_m values are reasonably similar. This can be seen in plots of the overlaid BCM and SLS data (Figure 3), where the aggregation event clearly coincides with the unfolding event at pH 4, whereas at pH 5 the protein appears to aggregate before it unfolds.

a)



b)



c)

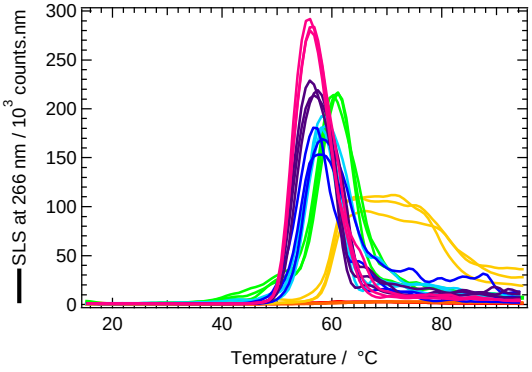


Figure 1: a) Structure of α-chymotrypsinogen with the tryptophan residues displayed in red wireframe (Protein Data Bank (PDB) ID: 1CHG[1]); b) BCM and c) SLS (266 nm) *versus* Temperature plots for α-chymotrypsinogen A in pH 2 - 9 buffers.

Therefore, the UNit has revealed that whilst the pH 5 formulation has the highest conformational stability (comparable to the pH 4 formulation) it has the lowest colloidal stability of all the formulations. For some other examples similar to this see BSA and β-lactoglobulin in 'Other Examples'.

pH	2	3	4	5	6	7	8	9
T_m (°C)	44.9 ±0.1	53.3 ±0.1	58.4 ±0.0	58.4 ±0.2	54.9 ±0.1	52.5 ±0.1	51.8 ±0.4	51.1 ±0.2
T_{agg} (°C)			57.0 ±0.6	47.1 ±0.8	50.3 ±1.6	49.4 ±1.0	49.3 ±0.4	49.6 ±0.1

Table 1: T_m and T_{agg} values for α-chymotrypsinogen A.

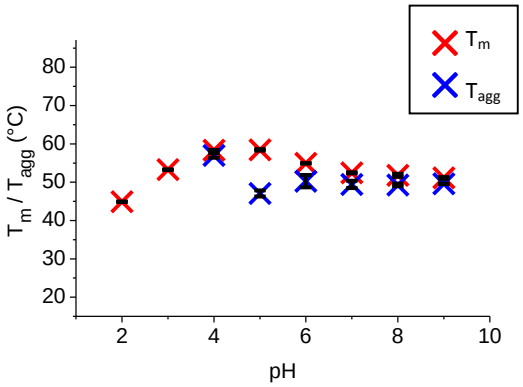


Figure 2: T_m/T_{agg} *versus* pH for α-chymotrypsinogen A.

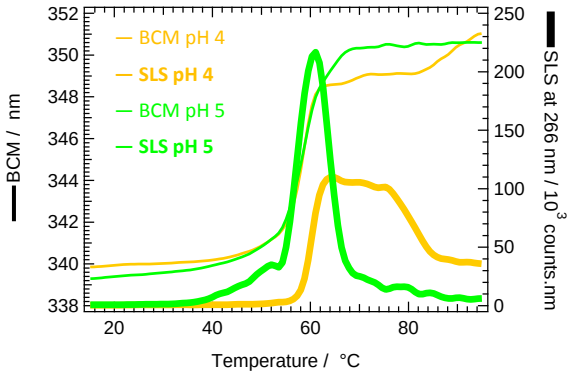


Figure 3: BCM and SLS (266 nm) *versus* Temperature for α-chymotrypsinogen A in pH 4 and 5 buffers.

Other Examples

Figure 4a shows overlaid BCM and SLS (266 nm) curves for BSA at pH 4, 5 and 6. At pH 4 there are two unfolding transitions and at pHs 5 and 6 there is one. The T_{agg} values show that aggregation occurs after the second transition at pH 4 and after the single transition at pH 6 (Figure 4b). However, at pH 5 T_{agg} is lower than T_m, indicating that this formulation has the lowest colloidal stabiliy, despite having the greatest conformational stablity.

β-lactoglobulin aggregated in the pH 4 and 5 buffers only, with a higher T_{agg} at pH 5, contradicting the T_m values (

Table 2). This indicates that despite being more conformationally stable, β-lactoglobulin aggregates more readily at pH 4 than pH 5.

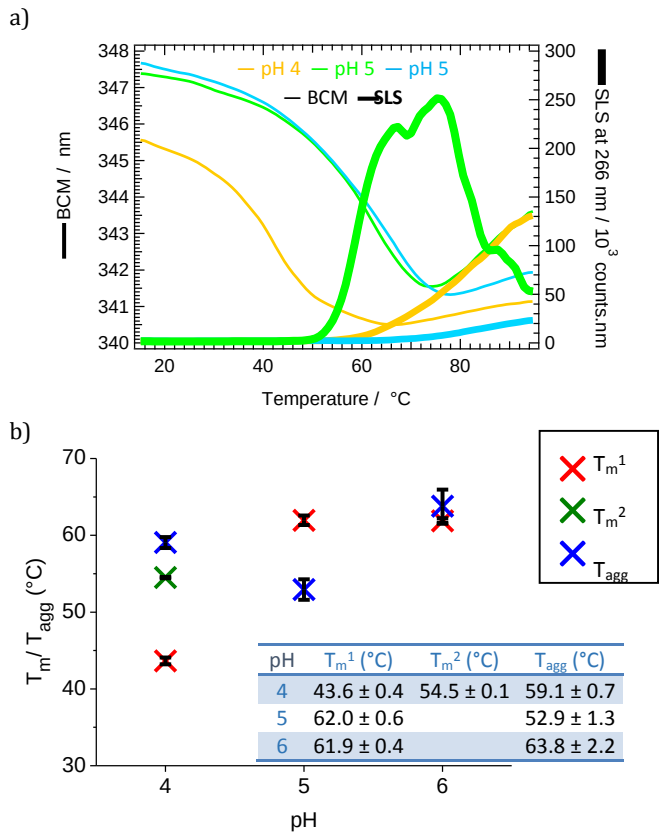


Figure 4: a) BCM and SLS (266 nm) *versus* Temperature and b) T_m/T_{agg} *versus* pH for BSA in pH 4, 5 and 6 buffers.

pH	T_m^1 (°C)	T_{agg} (°C)
4	75.5 ± 1.7	63.8 ± 0.6
5	73.5 ± 3.1	69.1 ± 0.9

Table 2: T_m and T_{agg} for β-lactoglobulin at pH 4 and 5.

Conclusions

This Technical Note has presented a number of case studies where obtaining both intrinsic fluorescence and SLS data was important for making a complete assessment of protein stability. In the cases of α-chymotrypsinogen A, BSA and β-lactoglobulin, the formulation that would have been identified as the most stable if only T_m values from fluorescence spectroscopy had been obtained, was found to have the lowest colloidal stability of all the formulations based on SLS data. These findings illustrate the importance of collecting multiple parameters in order to make a more comprehensive assessment of protein stability, a concept which has been discussed elsewhere previously[2]. This work endorses the use of the UNit which can provide several measures without increasing analysis time.

References

[1] Freer, *et al.*, *Biochemistry* (1970) **9**, 4767-4772.

[2] Garidel, *et al.*, *Spectrosc. Eur. Asia* (2014) **26**, 06Aug2014.

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