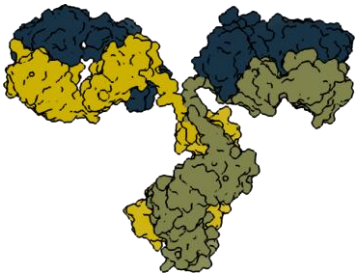
Protein Stable

Applied Innovations in Protein Characterisation



SUPR-DSF Full Spectrum Recording Facilitates Easy Thermal Melt Analysis of Proteins Lacking Tryptophan Residues

Introduction

SUPR-DSF measures protein stability by monitoring the intrinsic fluorescence of proteins as they unfold with increasing temperatures. Intrinsic protein fluorescence arises from both tryptophan and tyrosine residues, but proteins containing tryptophan are easier to measure because tryptophan emits 10 to 20 times more fluorescence due to its higher molar extinction coefficient and quantum yield.**[1]** This phenomenon makes it more challenging to measure intrinsic fluorescence of proteins lacking tryptophan residues.

This technical note investigates how the full spectrum capabilities of the SUPR-DSF make it easy for researchers to measure unfolding curves for proteins without tryptophan. The protein we are using for this study is Protein A, a 42 kDa surface protein originally found in the cell wall of the bacteria *Staphylococcus aureus*. It has found several uses in biochemical research due to its ability to bind to the Fc region of immunoglobulins. Protein A does not contain any tryptophan residues but does contain four tyrosine residues. The tyrosine fluorescence will be utilized to analyze Protein A's thermal stability on the SUPR-DSF.**[2]**

Fluorescence Spectra

Figure 1 illustrates the normalized folded and unfolded spectra generated with the SUPR-DSF for Protein A containing only tyrosine residues (***Figure 1a***) and Lysozyme containing tryptophan residues (***Figure 1b***). The spectral shape for each residue is very different with the folded spectrum of tyrosine having a peak fluorescence wavelength at 315 nm while tryptophan's folded spectrum peak fluorescence wavelength is much longer at 335 nm. The difference in spectral shape can make it challenging to measure intrinsic fluorescence in proteins containing only tyrosine residues using instruments equipped with two filter sets optimized for tryptophan fluorescence. The SUPR-DSF is optimized for proteins containing tryptophan or tyrosine residues by measuring the entire spectrum from 310 nm to 420 nm, providing the best results for a wider range of proteins.

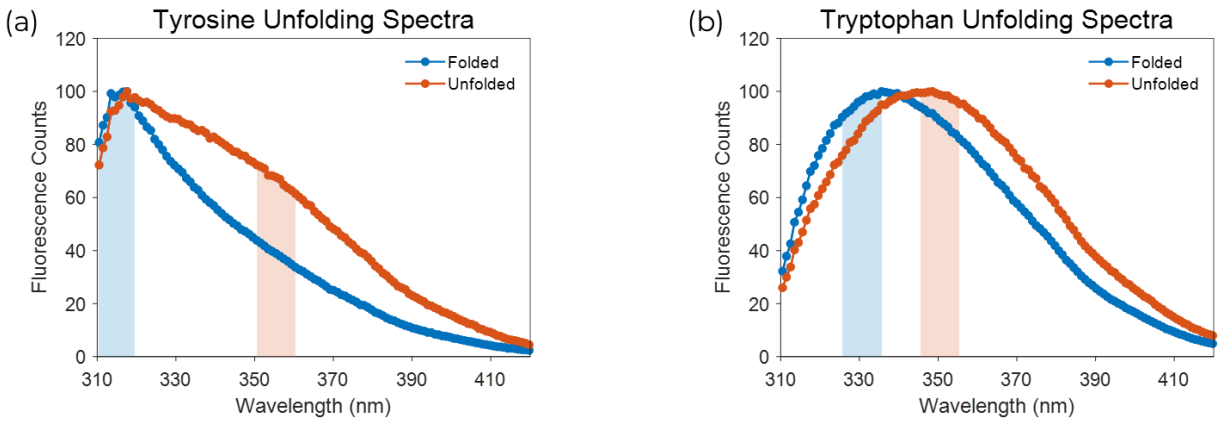


Figure 1 *– Normalized protein unfolding fluorescence spectra generated with the SUPR-DSF for (a) Protein A containing only tyrosine residues and (b) Lysozyme containing tryptophan residues.*

Analysis Methods

The SUPR-DSF acquires the full emission spectrum allowing melt curves to be computed with three analysis methods: ratio, barycentric mean (BCM), and intensity. The ratio measurement computes wavelength shifts by measuring two bands of the spectrum: one band in the folded region and another in the unfolded region. The BCM measures wavelength shifts by finding the center of mass of the fluorescence waveform, which is the point on the curve where the areas on either side are equal. Lastly, the intensity calculation generates melt curves by monitoring the intensity change at a specific wavelength. Typically, a wavelength in the unfolded region generates the best intensity melt curves. Each method has its unique advantages and having all three methods allows one to characterize a wider selection of samples. In this example, both the ratio and BCM methods proved to be the best and were used to generate the melt curves for Protein A.

Ratio

The ratio measurement computes melt curves by comparing the protein fluorescence signal in the unfolded region to the signal in the folded region, allowing wavelength shifts to be tracked during a thermal melt. To generate the best melt curves, it is essential to select the optimal folded and unfolded wavelengths. In this example, Protein A has a peak folded band at 315 nm, and an unfolded band at 355 nm as shown in ***Figure 1a***. The SUPR-DSF allows you to select these optimal wavelengths with its full emission spectrum. Other instruments are limited to 330 nm and 350 nm filter sets, which is optimized for tryptophan fluorescence (***Figure 1b***).

The ratio unfolding curve for Protein A is shown in ***Figure 2a*** for five different concentrations. The figure illustrates a red-shifted melt curve, which means the protein fluorescence shifted to longer wavelengths during unfolding. The red shift is evident by observing a higher ratio value at 90°C in the unfolded state of the protein compared to the folded state at 30°C. A red shift melt curve is very common among proteins and is caused by the buried tryptophan or tyrosine residues becoming exposed to the aqueous environment as the protein unfolds. Another indicator of a red-shifted melt curve can be shown by looking at the first derivative of the melt curve (***Figure 2b***). A positive peak implies a red-shifted melt curve while a negative peak indicates a blue shift.

A single melting temperature is observed in ***Figure 2b*** indicated by one peak. All concentrations tested down to 0.1 mg/mL showed this behavior. The melt curves look excellent for concentrations down to 0.5 mg/mL, but there is a higher level of noise appearing in the 0.1 mg/mL curve implying that the limit of quantification is being reached. Fitting the first derivative to a Gaussian resulted in a melting temperature of 78°C for the higher concentrations with a slight decrease in the melting temperature at the lower concentrations. A decrease in the melting temperature at lower concentrations may imply that Protein A is more prone to aggregation at these concentrations, or that intermolecular interactions are important for the stability of Protein A. See ***Table 1*** for results.

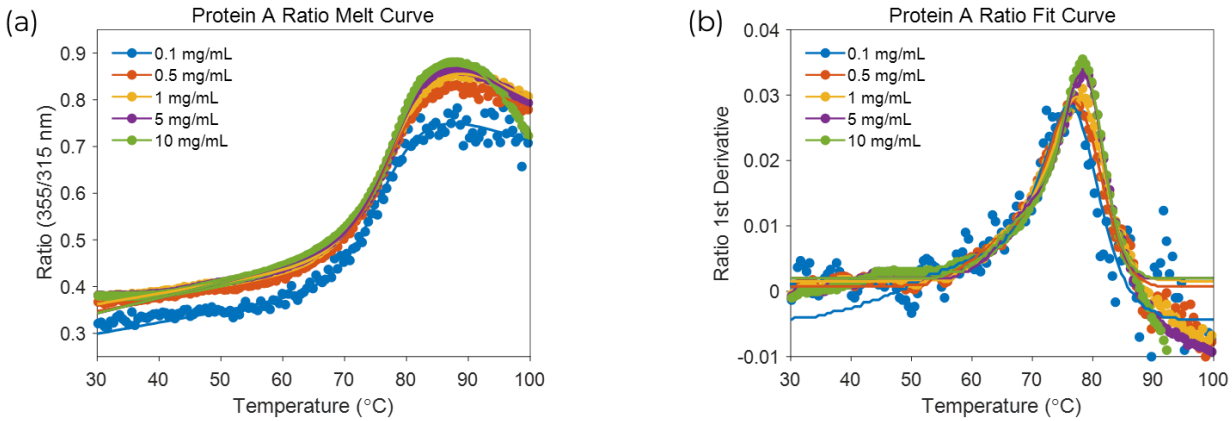


Figure 2 – *Protein A ratio melt curve. (b) Protein A ratio first derivative fit curve.*

Barycentric Mean

In addition to the ratio, the full spectrum capability of the SUPR-DSF enables the calculation of the barycentric mean (BCM). The BCM computes the center of mass of the fluorescence spectrum, enabling the calculation of wavelength shifts for either tryptophan or tyrosine residues. The BCM unfolding curve for Protein A is shown in ***Figure 3a*** for the same sample concentrations illustrated in ***Figure 2a***. As with the ratio melt curve, the BCM melt curve shows the fluorescence spectrum shifting to longer wavelengths, indicating a red-shifted spectrum. In general, the ratio and BCM melt curves will produce very similar results because each method is tracking wavelength shifts. One advantage of the BCM method over the ratio method is that it can produce higher quality melt curves by using the entire fluorescence spectrum in its calculation versus only two wavelength bands with the ratio calculation.

Fitting the BCM first derivative melt curve results in a melting temperature of 78°C, matching the ratio results. Each method will produce slightly different values, but they are typically within ±1°C. The melting temperatures for both methods are shown in ***Table 1*** for all concentrations tested with Protein A. The BCM melting temperatures displayed the same behavior with the melting temperature of Protein A slightly decreasing at lower protein concentrations.

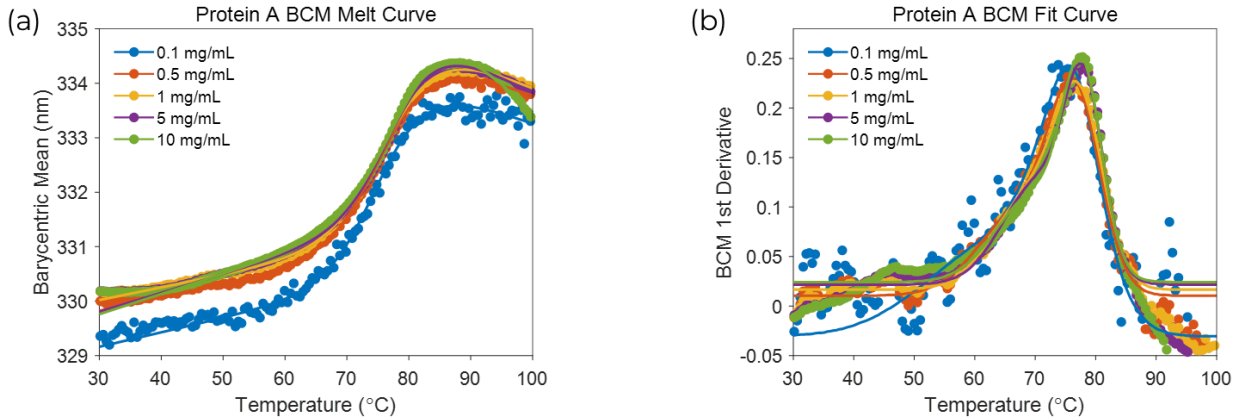


Figure 3 *– Protein A BCM melt curve. (b) Protein A BCM first derivative fit curve.*

Conclusion

The SUPR-DSF full spectrum capability enabled the use of both the ratio and BCM calculations to generate melt curves for Protein A, which lacks tryptophan residues. The melting temperatures for the 5.0 mg/mL and 10.0 mg/mL samples were found to be 78.0°C for both analysis methods with the melting temperatures slightly decreasing at the lower concentrations. This decrease in the melting temperature at lower concentrations may suggest that Protein A is more prone to aggregation at these concentrations, or that intermolecular interactions are important for the stability of Protein A. These results show how the SUPR-DSF facilitates easy thermal melt analysis of non-tryptophan proteins by utilizing the entire fluorescence spectrum to compute wavelength shifts to determine melting temperatures using either a ratio or BCM calculation.

Table 1 *– Ratio and BCM first derivative fitted melting temperatures with errors (4 replicates) for the dilution series. One replicate was excluded in the 0.1 mg/mL sample.*

Protein A Concentration	Ratio T_m (°C)	SD (°C)	BCM T_m (°C)	SD (°C)
0.1 mg/mL	76.4	0.64	75.8	0.79
0.5 mg/mL	77.3	0.21	76.6	0.27
1.0 mg/mL	78.1	0.28	77.4	0.24
5.0 mg/mL	78.8	0.08	78.1	0.06
10.0 mg/mL	78.5	0.08	77.8	0.10

Methodology

Protein A (Sigma, P-6031) was formulated in 0.01 M PBS (pH 7.4, Sigma, P-3813) at a concentration of 20 mg/mL. All concentrations were made by diluting the stock protein in PBS. Samples were dispensed with a Dragonfly® liquid handler in quadruplicate into a 384-well plate (Azenta Life Sciences, 4ti-0386) at a 10 µL well volume. The sample can be dispensed with manual pipettes if liquid handlers are unavailable. After dispensing, the plate was sealed with an optically clear adhesive film (Azenta Life Sciences, 4ti-0560), spun at 1000 x g for 30 seconds to remove air bubbles, and inserted into the SUPR-DSF for measurement.

The SUPR-DSF was configured to measure the fluorescence spectra of Protein A from 10°C to 105°C with a 1°C per minute ramp rate. The spectral shift, which indicates protein denaturation, was quantified using the ratio calculation with a 10 nm band at wavelengths of 315 nm and 355 nm. The BCM calculation was performed over a wavelength range of 310 nm to 360 nm. Melting temperatures were found by fitting the first derivative of the ratio and BCM melt curves to a Gaussian function.

References

[1] – Mach H, Middaugh CR, Lewis RV. Statistical Determination of the Average Values of the Extinction Coefficients of Tryptophan and Tyrosine in Native Proteins. Analytical Biochemistry. 1992, Vol 200, p. 74-80.

[2] – Goding JW. Use of Staphylococcal Protein A as an Immunological Reagent. Journal of Immunological Methods. 1978, Vol 20, p. 241-253.

Protein Stable

21 Mole Business Park
Leatherhead
Surrey
KT22 7BA
United Kingdom