TECH NOTE

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Thermal Stability Analysis of Tryptophan-Free Proteins Using SUPR-DSF

Overview

Intrinsic differential scanning fluorimetry (intrinsic DSF) is most commonly associated with proteins containing tryptophan residues, whose strong fluorescence signal provides excellent sensitivity for monitoring thermal unfolding. However, because tryptophan is the least abundant amino acid found in proteins, a substantial fraction of naturally occurring and engineered proteins contain few or no tryptophan residues, making sensitive detection of tyrosine fluorescence essential for broad applicability of the technique.

This technical note demonstrates the ability of SUPR-DSF™ to characterize challenging proteins that contain tyrosine as the sole intrinsic fluorescent amino acid. Using four commercially available proteins spanning a wide range of molecular weights, tyrosine contents, and thermal stabilities, we evaluate both the practical sensitivity limits of the technique and the influence of protein concentration on measured thermal stability. The results demonstrate that full-spectrum fluorescence acquisition combined with BCM analysis enables robust thermal unfolding measurements at protein concentrations as low as 0.1 mg/mL, even for proteins containing only a single tyrosine residue.

Introduction

SUPR-DSF measures protein conformational stability by monitoring changes in intrinsic protein fluorescence during a controlled thermal ramp. As proteins unfold, buried aromatic residues become exposed to the solvent environment, producing characteristic changes in fluorescence intensity and spectral shape that can be used to determine unfolding transitions. Unlike traditional differential scanning fluorimetry methods that rely on extrinsic fluorescent dyes (e.g., SYPRO Orange), SUPR-DSF directly measures intrinsic fluorescence from native protein samples without added probes, minimizing the potential for dye-induced perturbations or non-specific interactions with formulation components. The technique supports high-throughput analysis in standard 384-well plates, with a typical thermal unfolding experiment only requiring approximately 90 minutes while simultaneously monitoring hundreds of samples. Full-spectrum fluorescence detection enables resolution of multiple independent unfolding events (T_m) within a single protein.

Intrinsic protein fluorescence arises primarily from tryptophan and tyrosine residues; however, tryptophan-containing proteins are generally easier to characterize because tryptophan exhibits substantially greater fluorescence intensity due to its higher molar absorptivity ($\varepsilon_{280} \approx 5540\ M^{-1}\ cm^{-1}$ vs. $1480\ M^{-1}\ cm^{-1}$ for tyrosine), fluorescence quantum yield ($\Phi \approx 0.20–0.25$ vs. ~0.14 for tyrosine), and lower susceptibility to quenching (e.g., via hydrogen bonding). As a result, tryptophan fluorescence in proteins is often observed to be approximately an order of magnitude (~10-20×) greater than that of tyrosine, making proteins lacking tryptophan residues a more challenging measurement scenario for intrinsic DSF.[1,2]

Residue abundance further complicates this situation. Tryptophan is the least abundant amino acid found in proteins, comprising only ~1.2% of residues on average, and proteins lacking tryptophan residues are therefore relatively common, particularly among smaller proteins and engineered constructs. In contrast, tyrosine residues occur at a higher frequency (~3% abundance), making proteins completely lacking tyrosine substantially less common.[3,4] Based on a simple probabilistic analysis of amino acid abundance statistics, approximately 30% of proteins 100 amino acids in length would be expected to lack tryptophan residues, compared to only 4–5% lacking tyrosine. For proteins of average size (~300 amino acids, 33 kDa), these values decrease to approximately 2–3% and 0.008%, respectively. Consequently, the ability to sensitively detect tyrosine fluorescence is critical to the broad applicability of intrinsic DSF for protein stability characterization.

Table 1: *Commercially available proteins examined in this study along with their molecular weights, fluorescent amino acid content, and approximate expected melting temperatures.*[5-9]

Protein (Species)	MW (kDa)	# of W Residues	# of Y Residues	Y Residues per kDa	Reported T_m (°C)
Protein A (*S. aureus*)	42	0	4	0.10	~70-80 (broad/multiple transitions)
Calmodulin (*H. sapiens*)	16.8	0	2	0.12	T_{m1} = 92 T_{m2} = 110 (holo)
Ubiquitin (*H. sapiens*)	8.6	0	1	0.12	92
Ribonuclease A (*B. taurus*)	13.7	0	6	0.44	63

This technical note highlights how the SUPR-DSF's full-spectrum fluorescence detection enables robust and highly sensitive thermal stability measurements across a diverse set of challenging proteins that lack tryptophan residues. Four commercially available proteins containing at least one tyrosine residue but no tryptophan residues were selected for this study. Relevant characteristics, including molecular weight (MW), number of tyrosine residues (both absolute and normalized to protein molecular weight), and approximate melting temperatures reported from various biophysical techniques, including both intrinsic and extrinsic DSF, differential scanning calorimetry (DSC), or circular dichroism (CD) measurements, are summarized in Table 1.

- **Protein A (ProtA; from Staphylococcus aureus)** is a bacterial cell wall protein widely used in antibody purification and immunoassay applications due to its multiple homologous high-affinity binding domains targeting the Fc region of IgG immunoglobulins. The commonly used recombinant constructs of Protein A, including the 42 kDa construct used in this study, contain no tryptophan residues and 4 tyrosine residues, making Protein A a useful model for evaluating tyrosine-only intrinsic fluorescence measurements. Protein A-derived domains are generally highly thermostable, with reported melting temperatures for closely related bacterial IgG-binding domains typically falling within the ~70–80°C range depending on construct design and solution conditions.[5]
- **Human calmodulin (CaM)** is a highly conserved calcium-binding regulatory protein involved in numerous intracellular signaling pathways through modulation of calcium-dependent enzymes and target proteins. Calmodulin consists of N- and C-terminal lobes, each containing two calcium-binding sites; however, both tyrosine residues are located exclusively within the C-terminal lobe, and the protein contains no tryptophan residues. In its calcium-free (apo) state, calmodulin exhibits relatively low thermal stability and unfolds through two broad domain-specific transitions occurring between approximately 45–60°C. In contrast, the fully calcium-bound (holo) form adopts a substantially more stabilized conformation, with reported DSC melting transitions near 92°C and 110°C, which have been proposed to correspond to unfolding of the N-terminal domain (faster calcium binding kinetics) and C-terminal domain (higher calcium affinity), respectively.[6]
- **Human ubiquitin (Ub)** is a small regulatory protein responsible for post-translational protein ubiquitination, a process central to protein degradation, trafficking, and cellular signaling. Human ubiquitin contains 1 tyrosine residue and no tryptophan residues. Ubiquitin is exceptionally thermally stable, with a single reported melting temperature of approximately 92°C depending on the precise solution condition and method.[7]
- **Bovine ribonuclease A (RNase A)** is a well-characterized pancreatic endoribonuclease commonly used as a model protein in folding and stability studies due to its robust structure and extensive biophysical characterization. Bovine RNase A contains 6 tyrosine residues and no tryptophan residues. Reported melting temperatures are generally centered around a single transition at 63°C under near-neutral aqueous buffer conditions.[7-9]

Results & Discussion

Intrinsic DSF Measurements of Tyrosine-Containing Proteins

In this study, we focus exclusively on four proteins in which tyrosine serves as the sole intrinsic fluorescent amino acid, in the complete absence of tryptophan residues (Table 1). Figure 1a shows a representative set of thermal unfolding fluorescence isotherms for Protein A. As expected for tyrosine fluorescence, the emission maximum is observed at approximately 315 nm. Figure 1b displays the same data after normalization to the folded and unfolded baselines, revealing a pronounced redshift in fluorescence emission toward ~350 nm upon unfolding. The SUPR-DSF records the full fluorescence emission spectrum from 310–420 nm, enabling sensitive characterization of proteins exhibiting a wide range of intrinsic fluorescence behaviors. Correspondingly, SUPR-Suite can generate thermal melt curves using fluorescence intensity at a selected wavelength, fluorescence ratios, or the barycentric mean (BCM). The BCM method, which calculates the spectral center of mass of the fluorescence waveform (i.e., the point on the curve where the areas on either side are equal), is particularly sensitive to unfolding-induced changes in tyrosine fluorescence because it captures subtle shifts in the overall emission distribution across the full recorded spectrum.

Thermal Stability Analysis of Four Tyrosine-Containing Proteins

All four proteins listed in Table 1 were analyzed across a concentration series to assess both the feasibility and sensitivity of measuring tyrosine-derived intrinsic fluorescence using the SUPR-DSF, as well as to investigate potential concentration-dependent effects on thermal stability. All samples were measured using quadruplicate wells, providing high precision and reproducibility. Application of the BCM analysis method generated high-resolution thermal unfolding curves from the acquired fluorescence spectra (data not shown). The resulting first-derivative dBCM traces were fitted with Gaussian functions using the SUPR-Suite's proprietary autofitting algorithm (Table 5, Appendix) to quantitatively determine melting transition temperatures (T_m). The results, summarized in Table 2, include both tabulated T_m values and overlays of the corresponding dBCM traces for each protein concentration series.

- **Protein A** was evaluated across a concentration range of 0.1–10 mg/mL. Robust fluorescence signals were obtained throughout the series, with appreciable noise observed only at the lowest concentration (0.1 mg/mL, blue series). The resulting melt profile exhibited a broad, asymmetric unfolding transition peaking between 76-78°C and spanning approximately 30°C, characterized by a progressive redshift in fluorescence and consistent with overlapping denaturation of Protein A's multiple homologous Fc-binding domains.

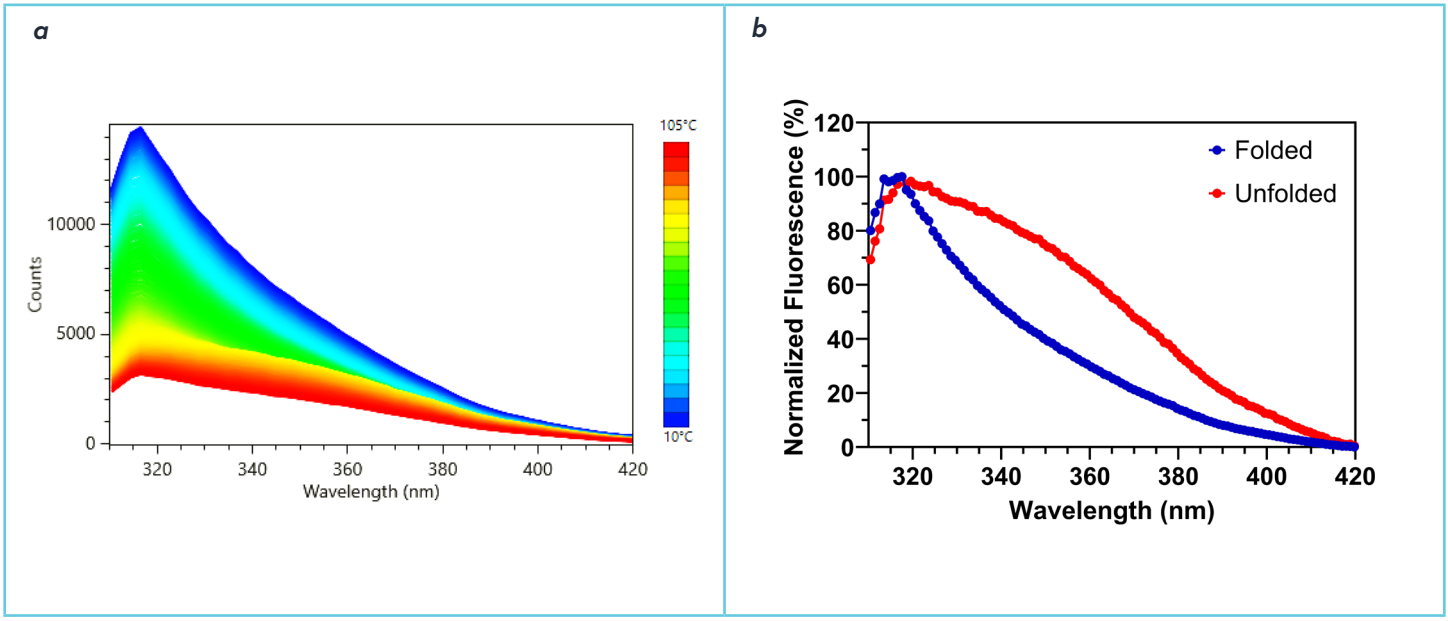
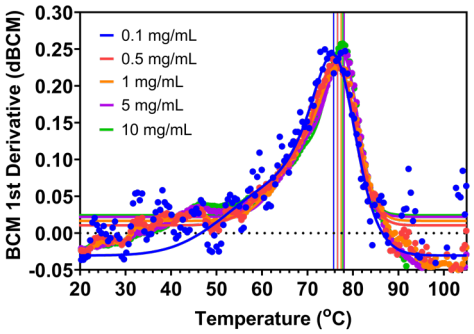
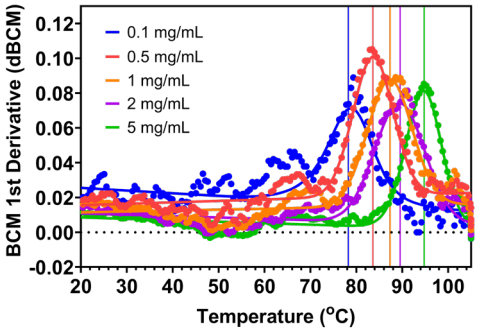
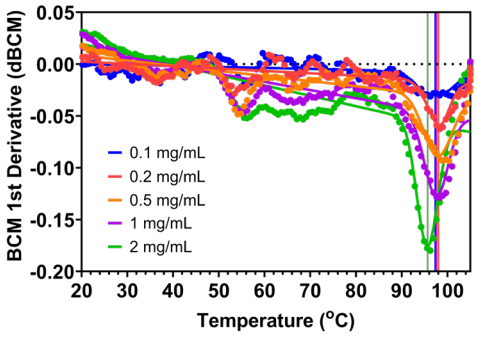
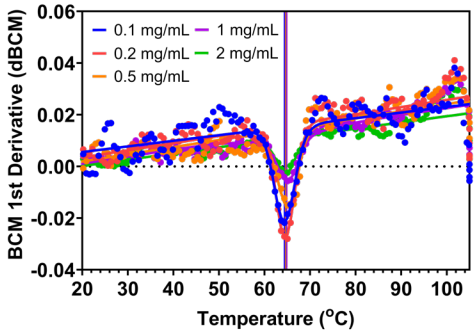


Figure 1: *Tyrosine unfolding spectra from Protein A. a) Tyrosine fluorescence isotherms plotted as raw fluorescence counts vs. wavelength. b) Normalized Protein A fluorescence spectra visualizing both the initial folded (blue) and final unfolded (red) states.*

Table 2: *Tabulation of proteins analyzed in this study, including tested concentrations and observed melting transitions (mean ± SD). Only the major transition (T_{m2}) is reported for Protein A fits. The far right column shows an overlay of dBCM (derivative) traces for the various tested concentrations; dots are averaged raw data, and solid lines are the corresponding Gaussian fit traces. Vertical lines mark the respective T_m values.*

Protein	Concentration (mg/mL)	T_m (°C) (Mean ± SD)	dBCM Overlays
Protein A	0.1	75.76 ± 0.79	
	0.5	76.63 ± 0.27	
	1	77.41 ± 0.24	
	5	78.08 ± 0.06	
	10	77.79 ± 0.10	
Calmodulin	0.1	78.26 ± 0.99	
	0.5	83.60 ± 0.20	
	1	87.33 ± 0.16	
	2	89.54 ± 0.47	
	5	94.77 ± 0.18	
Ubiquitin	0.1	97.28 ± 1.86	
	0.2	98.03 ± 0.45	
	0.5	97.86 ± 0.13	
	1	97.54 ± 0.26	
	2	95.66 ± 0.14	
Ribonuclease A	0.1	64.35 ± 0.42	
	0.2	64.60 ± 0.37	
	0.5	64.89 ± 0.73	
	1	64.90 ± 0.51	
	2	64.79 ± 0.42	

Although the data were best fit using a two-transition model, only the dominant transition (T_{m2}) is reported here for ease of comparison. A modest concentration-dependent stabilization of approximately 2°C was observed with increasing protein concentrations.

- **Calmodulin** was evaluated across a concentration range of 0.1–5 mg/mL. All samples were prepared in PBS supplemented with 2 mM $CaCl_2$ to promote formation of the fully calcium-loaded (holo) state. Red-shifted unfolding transitions were successfully fit across the entire concentration series, although the lowest concentration tested (0.1 mg/mL) exhibited appreciable noise.

  Notably, calmodulin displayed a remarkably broad range of apparent melting temperatures, spanning nearly 20°C from 75.8°C (0.1 mg/mL, blue series) to 94.8°C (5 mg/mL, green series). Unlike the modest concentration-dependent effects observed for the other proteins in this study, the large T_m shift observed for calmodulin is more likely attributable to differences in calcium occupancy resulting from the varying protein-to-calcium ratios across the concentration series, as the $CaCl_2$ concentration remained constant while protein concentration varied. Furthermore, calcium binding has been reported to increase calmodulin fluorescence intensity by approximately 2–3-fold, which may also influence the observed thermal transitions.[6] These results highlight the need to take into account the effects of protein concentration, buffer composition and pH, and calcium loading state on the measured thermal stability of calmodulin.

- **Ubiquitin** was evaluated across a concentration range of 0.1–2 mg/mL and, despite containing only a single tyrosine residue, yielded a measurable thermal transition even at the lowest concentration tested (0.1 mg/mL, blue series). The protein exhibited an exceptionally high melting temperature (~95–98°C) accompanied by an unusual blueshift in fluorescence following unfolding, likely indicative of rapid aggregation subsequent to the denaturation event.[10,11] In contrast to Protein A, ubiquitin displayed a modest concentration-dependent destabilization, with T_m decreasing by approximately 2°C at higher protein concentrations.

- **Ribonuclease A** was evaluated across a concentration range of 0.1–2 mg/mL. Consistent with literature reports obtained using a variety of biophysical techniques, bovine RNase A exhibited a highly reproducible melting transition between 64–65°C and provided excellent signal-to-noise ratios even at the lowest concentration tested (0.1 mg/mL). Notably, published intrinsic fluorescence thermal unfolding measurements of RNase A using NanoDSF were unable to be performed below a protein concentration of approximately 0.5 mg/mL despite the 6 tyrosine residues (0.44 per kDa), highlighting the strong sensitivity achieved here at five-fold lower concentration using the SUPR-DSF.[7]

  Similar to ubiquitin, RNase A displayed a blue-shifted fluorescence response upon unfolding. Interestingly, the magnitude of the dBCM response decreased with increasing protein concentration, suggesting a progressive shift toward a red-shifted fluorescence signature at sufficiently high concentrations. One possible explanation is the onset of concentration-dependent intermolecular interactions or aggregation following unfolding, which may alter the local environment of tyrosine residues and consequently affect the quenching interactions and therefore the observed spectral response. However, the precise origin of this behavior remains unclear and warrants further investigation.

Collectively, these results demonstrate that the SUPR-DSF is capable of generating high-quality thermal unfolding data from tyrosine fluorescence at protein concentrations as low as 0.1 mg/mL, even for proteins containing only one tyrosine residue. Because protein concentrations in DSF experiments are typically reported on a mass basis (mg/mL), the number of fluorescent residues per unit molecular weight provides a useful predictor of intrinsic fluorescence signal intensity. Protein A, calmodulin, and ubiquitin possess similar tyrosine densities (~0.10–0.12 Tyr/kDa), whereas RNase A contains approximately four-fold more tyrosine residues relative to its molecular weight (~0.44 Tyr/kDa; Tables 1 & 3).

Fluorescence intensities were measured at 315 nm near each protein's T_m. The proteins with similar tyrosine densities (Protein A and calmodulin) produced broadly comparable fluorescence signals of 2,401 and 2,876 counts, respectively. Ubiquitin exhibited somewhat lower fluorescence intensity of 1,017 counts, likely due to differences in the local tyrosine environment and associated quenching effects. In contrast, ribonuclease A, which contains approximately four-fold more tyrosine residues per unit mass, exhibited a substantially greater fluorescence intensity of 4,815 counts.

Despite these differences in signal intensity, all four proteins yielded readily analyzable unfolding transitions at 0.1 mg/mL. To further quantify the sensitivity of these measurements, lower limits of detection were estimated from concentration-dependent fluorescence calibration curves.

Table 3: *Comparison of tyrosine density and intrinsic fluorescence intensity for the four proteins evaluated in this study. Tyrosine density is reported as the number of tyrosine residues normalized to protein molecular weight (Tyr/kDa). Fluorescence intensities correspond to average emission counts measured at 315 nm near the melting transition temperature (T_m) for each protein.*

Protein	Tyrosine Residues per kDa	Average Fluorescence Counts (315 nm)
Protein A	0.10	2401
Calmodulin	0.12	2876
Ubiquitin	0.12	1017
Ribonuclease A	0.44	4815

Limit of Detection (LOD)

Visual inspection of the melt curve overlays in Table 2 demonstrates that DSF melt curves can be successfully fit using the SUPR-Suite autofitting algorithm at concentrations down to 0.1 mg/mL, allowing corresponding T_m values to be determined. To provide a more quantitative assessment of sensitivity, concentration series data from all four proteins were used to calculate theoretical lower limits of detection (LOD), summarized in Table 4. LOD values were calculated according to:

$$LOD = \frac{3.3\sigma}{S}$$

where σ is the residual standard error obtained from linear regression analysis in Excel and S is the slope of the calibration curve. As shown in Table 4, calculated LOD values ranged from approximately 0.1–0.3 mg/mL across all proteins examined.

These values compare favorably with internal observations for proteins containing only one to two tryptophan residues, where sensitivity, or lowest practical concentration for analysis, is often approximately one order of magnitude greater (LOD typically ~0.01–0.05 mg/mL). Together, the visual and quantitative analyses indicate that proteins lacking tryptophan residues and containing merely one tyrosine residue can still generate sufficient intrinsic fluorescence signal to support reliable thermal melt analysis at concentrations as low as a few tenths of a mg/mL.

It is important to note, however, that while thermal melt measurements can be performed at very low protein concentrations, concentration itself can influence the apparent thermal stability (T_m) of certain proteins. Protein-protein interactions, self-association, and other intermolecular effects may manifest as concentration-dependent shifts in T_m values, leading to apparent changes in stability independent of signal sensitivity. Therefore, although the results presented here demonstrate that reliable measurements can be obtained at concentrations as low as ~0.1 mg/mL, maintaining similar sample concentrations across experiments is best practice to ensure meaningful comparison of thermal stability data.

Table 4: *Calculated lower limits of detection (LOD) for each protein concentration series. For each set of sample replicates, the mean fluorescence intensity at 315 nm during the thermal transition midpoint (T_m) was tabulated; 315 nm was selected as it approximates the fluorescence emission maximum of tyrosine. Mean fluorescence intensity values were plotted against protein concentration over a range of 0.1–1 mg/mL to generate calibration curves for each protein. LOD values were calculated using* $LOD = 3.3 * \sigma / S$ *where* σ *is the residual standard error obtained from linear regression analysis in Excel, and S is the slope of the calibration curve.*

	Protein A	Calmodulin	Ubiquitin	Ribonuclease A
LOD (mg/mL)	0.32	0.24	0.07	0.13

Conclusion

The results presented here demonstrate that the SUPR-DSF is capable of performing sensitive intrinsic DSF measurements on proteins lacking tryptophan residues and relying exclusively on tyrosine fluorescence for signal generation. Across four structurally and functionally diverse model proteins, high-quality thermal unfolding data were obtained at concentrations as low as 0.1 mg/mL, including for ubiquitin, which contains only a single tyrosine residue. Quantitative lower limits of detection ranged from approximately 0.1–0.3 mg/mL, confirming that useful thermal stability measurements can be achieved even for challenging low-fluorescence samples.

In addition to demonstrating sensitivity, these experiments also highlight the importance of considering concentration-dependent effects on apparent thermal stability. While some proteins exhibited minimal variation in T_m across the tested concentration range, others displayed measurable shifts that may arise from intermolecular interactions, aggregation, ligand occupancy, or other solution-dependent phenomena. Consequently, maintaining consistent protein concentrations remains important when comparing thermal stability measurements across samples or experimental conditions.

These findings highlight the exceptional sensitivity of the SUPR-DSF for label-free characterization of proteins lacking tryptophan residues and suggest that useful thermal stability data can be obtained from samples available only in limited quantities and at concentrations substantially below values typically reported for conventional intrinsic fluorescence DSF approaches.

Materials & Methods

Biological Sample Information

The following commercially available samples were used for the experimental procedures conducted:

- Protein A from Staphylococcus aureus (Sigma, P6031)
- Recombinant Human Calmodulin (Santa Cruz, sc-471287)
- Recombinant Human Ubiquitin (R&D Systems, U-100H)
- Ribonuclease A from Bovine Pancreas (Sigma, R6513)

Sample Preparation

1. **Samples:** All proteins were reconstituted in PBS (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) according to the manufacturers' instructions.
    - Protein A was formulated in PBS at a concentration of 20 mg/mL. All running concentrations were prepared by diluting this stock protein in PBS.
    - Calmodulin was formulated as a 10 mg/mL PBS stock that also contained 2 mM $CaCl_2$. This stock was serially diluted into PBS containing 2 mM $CaCl_2$ to the final running concentrations.
    - Ubiquitin and Ribonuclease A were both reconstituted in PBS at 10 mg/mL. This stock was diluted into HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) to make a 2 mg/mL intermediate stock, followed by direct dilution into HBS to the final running concentrations.
2. **Plate Loading & Replicates:** All samples (10 µL aliquots per well) were loaded into a standard black 384-well PCR microplate (FrameStar™ 384-Well Skirted PCR Plate; Azenta Life Sciences, 4ti-0386), with measurements performed in quadruplicate for each condition. Matching buffer blanks were included for each protein series to control for background signal contributions from the formulation buffer.
3. **Sealing:** The plate was briefly centrifuged to remove air bubbles and then sealed with an optically clear, pressure-activated adhesive film (qPCR Adhesive Seal; Azenta Life Sciences, 4ti-0560) to prevent sample evaporation and concentration changes during the thermal ramp.
4. **Data Acquisition:** The SUPR-DSF instrument was configured for the standard DSF thermal ramp experiment type using the parameters listed below.

Differential Scanning Fluorimetry (DSF)

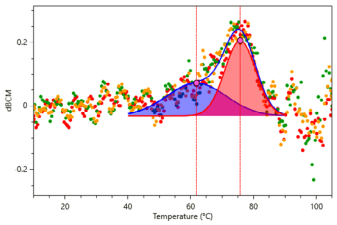
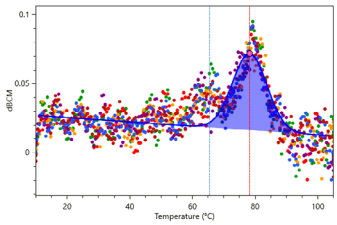
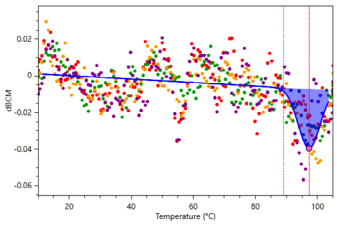
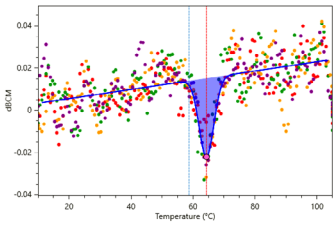
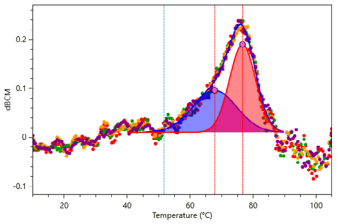
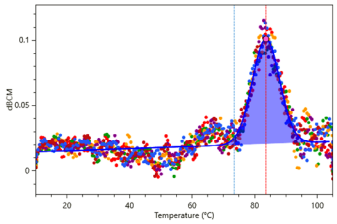
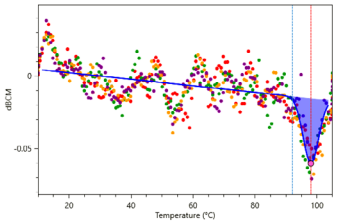
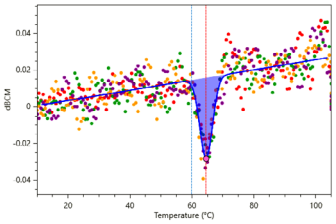
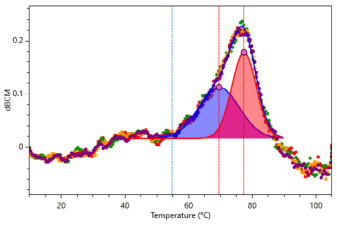
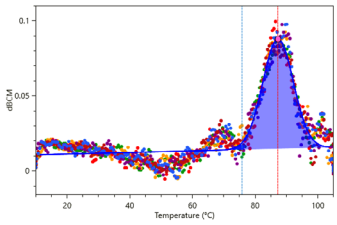
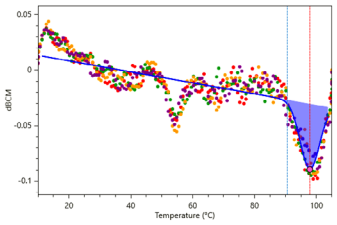
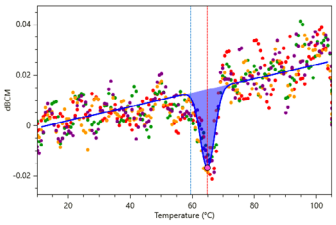
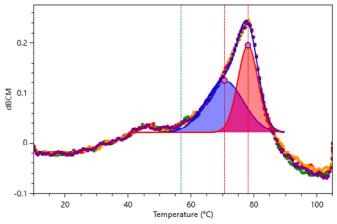
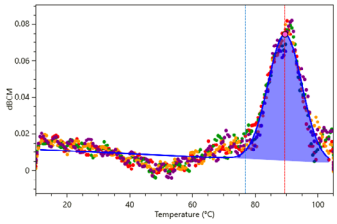
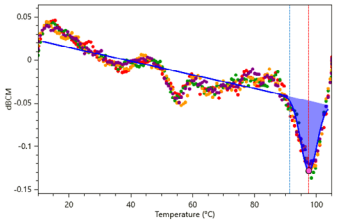
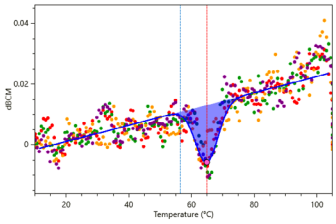
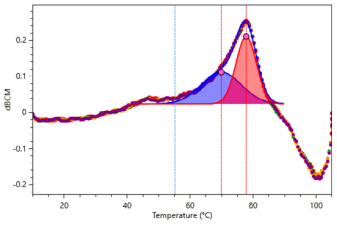
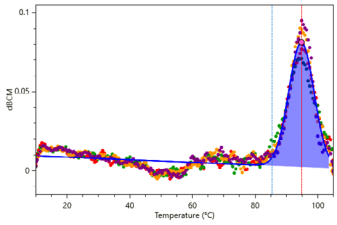
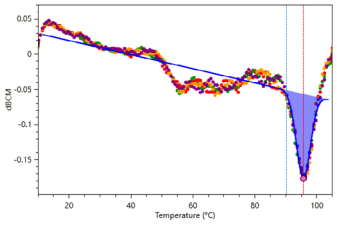
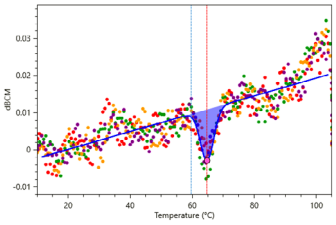
Thermal unfolding (T_{onset} & T_m) was directly measured in a conventional DSF experiment using a linear thermal temperature gradient.

- Temperature range: 10°C to 105°C
- Ramp rate: 1°C per minute
- Excitation: 280 nm
- Scan interval = 0.5°C (194 total spectra were obtained from each sample)
- Integration time: 35 ms
- Detection: Full spectrum fluorescence emission (310-420 nm)
- Total experiment time: 100 minutes

SUPR-Suite software (v2.1.11.0) was used for data acquisition and analysis. Spectral data were processed using the Barycentric Mean (BCM) method from 310–360 nm, and melting temperatures (T_m) were determined from the corresponding first-derivative (dBCM; Gaussian) fits using the SUPR-Suite's proprietary autofitting algorithm.

Appendix

Table 5: *Individual dBCM (derivative) plots with corresponding Gaussian fits for each protein at every tested concentration. Fits were calculated using the proprietary SUPR-Suite autofitting algorithm. T_{onset} denoted by vertical blue line and T_m transition(s) denoted by vertical red line(s); T_{m1} transition (blue shaded area) and T_{m2} transition (red region; Protein A) highlighted for clarity.*

Protein A	Calmodulin	Ubiquitin	Ribonuclease A
 0.1 mg/mL	 0.1 mg/mL	 0.1 mg/mL	 0.1 mg/mL
 0.5 mg/mL	 0.5 mg/mL	 0.2 mg/mL	 0.2 mg/mL
 1 mg/mL	 1 mg/mL	 0.5 mg/mL	 0.5 mg/mL
 5 mg/mL	 2 mg/mL	 1 mg/mL	 1 mg/mL
 10 mg/mL	 5 mg/mL	 2 mg/mL	 2 mg/mL

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