

Structural Characterization and Comparison of Temperature and Pressure Stress on a Protein Library Across pH and Concentration Using Microfluidic Modulation Spectroscopy

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Abstract

Thermal stress is the most common stress condition utilized for gauging protein stability, however, melt curves from a protein under different conditions can appear very different but still produce the same T_m from a derivative plot. Microfluidic Modulation Spectroscopy (MMS) is an automated mid-IR technique capable of measuring protein secondary structure at very high resolution across a broad range of concentrations and buffer conditions. MMS can be used to measure and monitor the structural changes that are occurring, leading to protein unfolding, and then thermally induced aggregation. With MMS we can monitor the loss of native protein secondary structure elements as the protein is being subjected to thermal stress. We looked at thermal unfolding for lysozyme across a range of pHs and found the T_m to be pH dependent. We looked at an IgG across a range of concentrations and found the T_m to be modestly concentration independent. Additionally, we used MMS to compare temperature and pressure stress applied to ovalbumin and found that each stress unfolds ovalbumin differently.

Introduction

The melting temperature (T_m) of biologic drugs is critical to the characterization as it sheds light on the stability, functionality, and manufacturability. T_m refers to the temperature at which the molecule unfolds and is specifically the temperature at which half the molecules are unfolded and half are folded. Therefore, the higher the T_m , the more stable the biologic drug. Historically, instruments that measure T_m provide just the T_m with no other information or context on the structure.¹ However, here we present a way to determine T_m using MMS, giving full secondary structure information at each temperature along the ramp so we can observe which structures are changing and use 2D heat maps to compare the different ways proteins unfold.

MMS interrogates the amide I band of the IR spectrum to sensitively probe protein structure while modulating against the reference buffer for accurate, real-time background subtraction in aqueous-based samples. As it is so sensitive, this technique is particularly useful for quality control and is compatible with many different formulation buffers. The second-generation MMS system, Aurora Tx, was used for this study and is equipped with a high-power Quantum Cascade Laser that is significantly more intense than traditional FTIR light sources. The combination of more light and modulating background subtraction makes MMS about 30 times more sensitive than FTIR and 5 times more sensitive than CD to small changes in structure.²

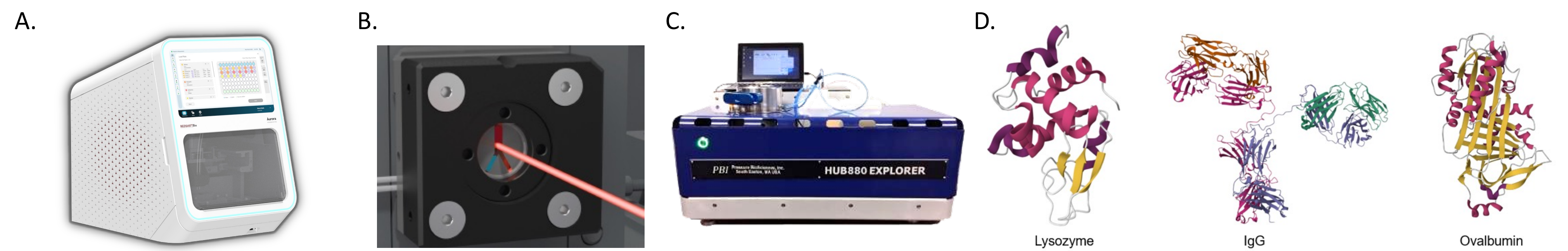


Figure 1A. MMS second generation Aurora Tx (RedShiftBio) used for secondary structure analysis. **(B)** MMS flow cell. **(C)** HUB 880 Explorer (Pressure BioSciences) used for pressure stressing up to 95 kpsi. **(D)** Crystal structures of lysozyme, IgG, and ovalbumin, which were the proteins used in this work.

Results

MMS thermal ramp: Lysozyme at different pHs

Formulation development is the process for determining the optimum buffer and excipients for a biologic drug to be the most stable, soluble, and active. One of the most important features in determining biologic stability is the pH. This can be determined using T_m to see which pH leads to the highest T_m value, therefore, the most resistant to temperature stress. Figure 2A shows the melting curve of lysozyme at pH 3 focusing on just the signal at 1656 cm^{-1} . The first derivative plot depicts how the T_m can be calculated from the curve. Figure 2B shows the comparison of 4 different pH values and how pH 4 and 5 are very similar in T_m , but pH 4 is slightly higher, which is similar to other reports.³

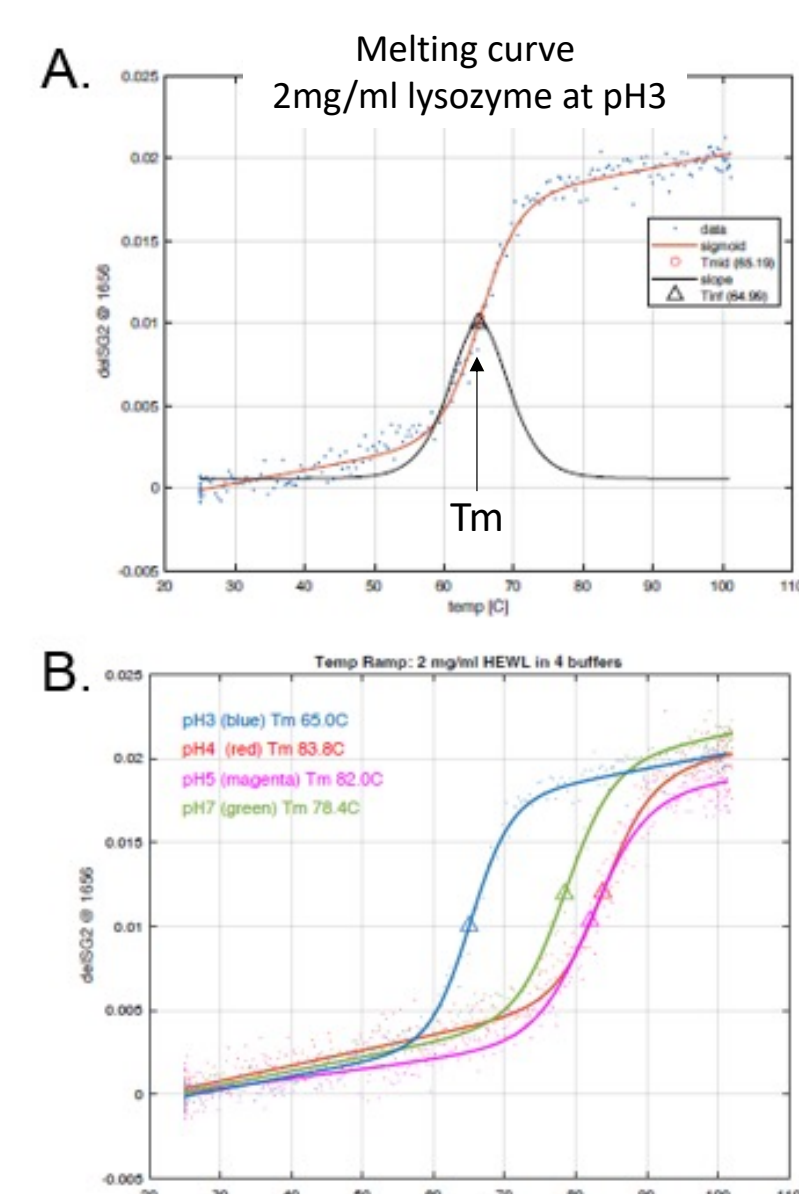


Figure 2A. Melt curve for 2 mg/mL lysozyme at pH 3. The first derivative plot indicates the T_m . **(B)** Overlay of the melt curves for pH 3, 4, 5, and 7.

2D MMS thermal ramp: Lysozyme

Unlike traditional techniques used for determining T_m , MMS provides structural information across the entire melt curve. These results are depicted in Figure 3A for lysozyme (pH 3), where the y-axis is temperature, the x-axis is wavenumber, yellow regions represent loss in structure, and dark blue represents increase in structure. Figure 3B shows the higher order structure (HOS) at 3 of the temperatures. This shows the conversion of alpha-helix to turn structure due to the thermal stress.

2D MMS thermal ramp: IgG

T_m s were calculated for 0.5, 1, and 2 mg/mL and were all approximately 81°C . Shown in Figure 3C is the 2D plot for 0.5 mg/mL and it is clear that the unfolding of IgG occurs at a much higher temperature compared to lysozyme. The major components that we see are native beta-sheet converting to intermolecular beta-sheet (beta-).

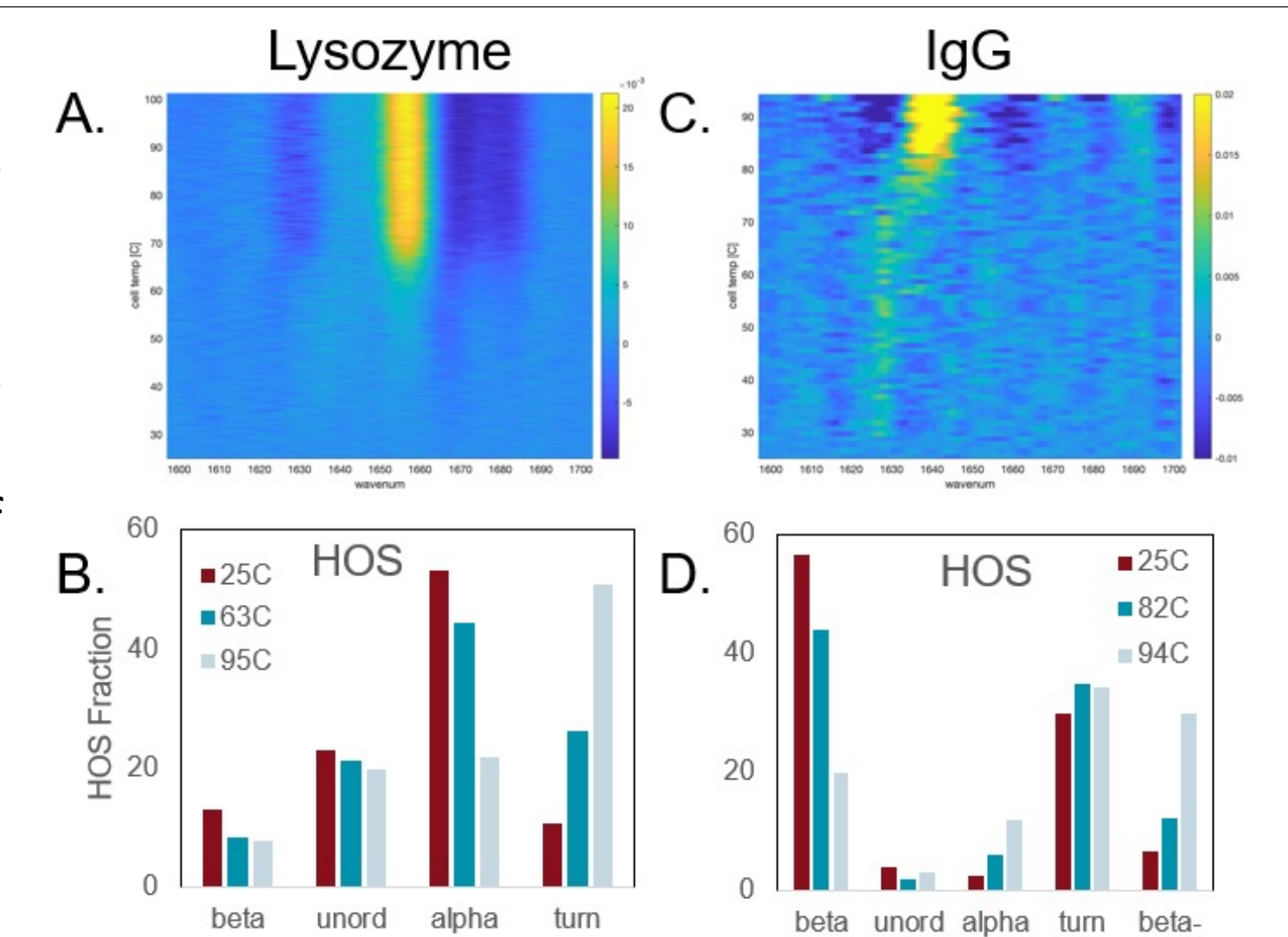


Figure 3A. MMS thermal melt plot as an inverted heat map. **(B)** The higher order structure (HOS) bar graph for lysozyme shows the loss in alpha-helix and increase in turn structure caused by temperature. **(C)** 2D MMS melt plot for IgG and **(D)** the structure changes observed for IgG, specifically loss in native beta-sheet and gain in intermolecular beta (beta-).

Traditional vs MMS thermal ramp: Ovalbumin

Figure 4A shows the first derivative melt curve of native ovalbumin with two transition temperatures. By pre-stressing the sample with temperature or pressure, the melt curve could appear very different, but yield the same T_m (Figure 4B). We tested ovalbumin in MMS to tease out which structures were affected by the thermal stress. Figure 4C shows the stability plot and how each secondary structure is affected by 5 mins incubation at each temperature and below in Figure 4D is the MMS thermal ramp corroborating the loss in native beta-sheet structure and gain in intermolecular beta-sheet structure. Both T_m s are visible and now we can see T_{m1} corresponds to a loss in alpha-helix and T_{m2} corresponds to a loss in native beta-sheet structure.

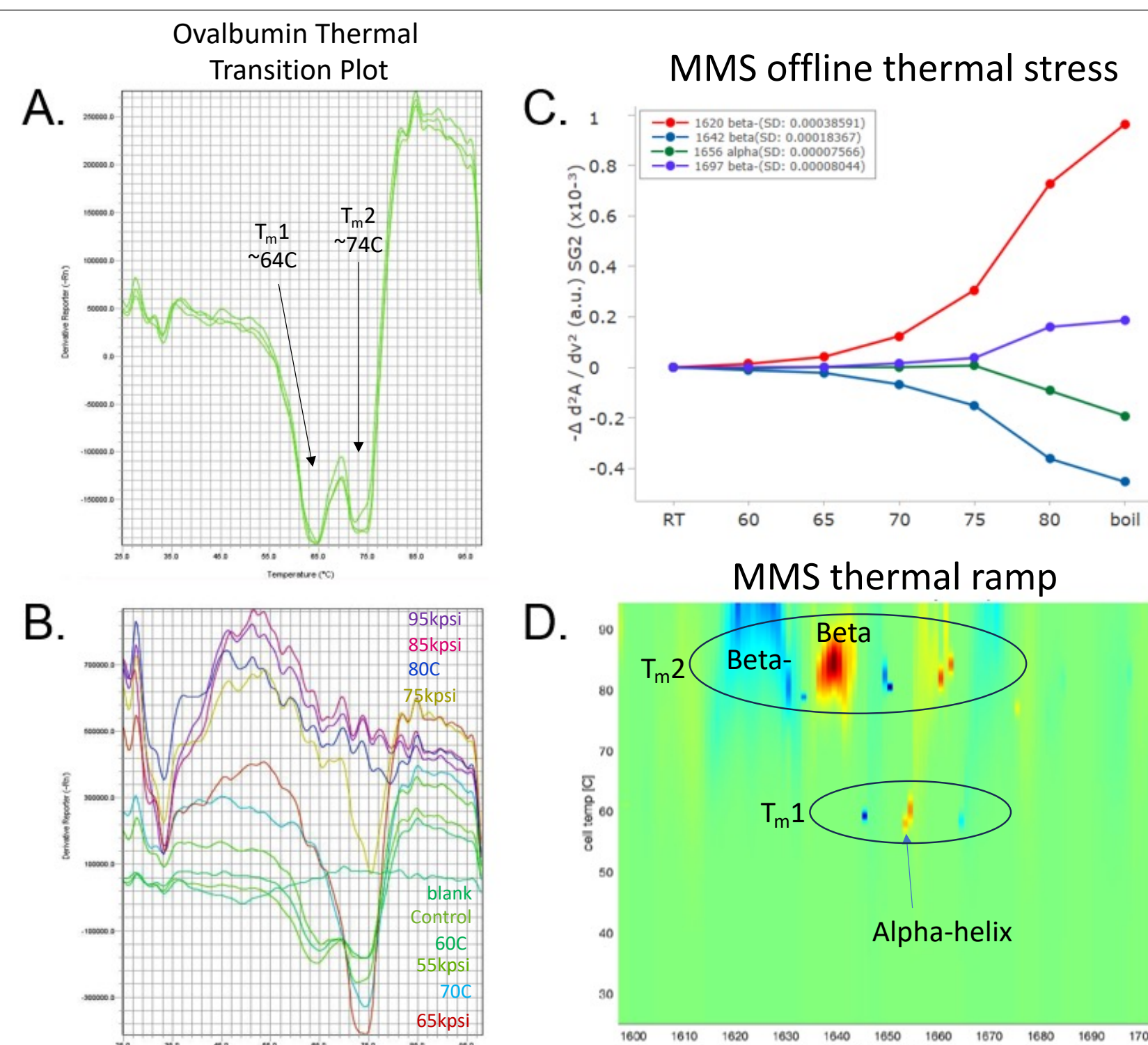


Figure 4A. Melt curve for ovalbumin using a Thermo 7500 instrument with Sypro orange dye to visualize protein unfolding. **(B)** Pre-stressing the ovalbumin for 5 mins at various temps, or 10 mins using Pressure BioScience's HUB 880 Explorer led to different looking curves, but all had the same T_m . **(C)** MMS data can show which secondary structure is affected by the temp applied. **(D)** MMS thermal ramp shows both T_{m1} s and loss in structure is shown as yellow/red, gain in structure as blue/dark blue.

MMS thermal stress vs pressure stress: Ovalbumin

Figure 5A and B show the temperature stress results for ovalbumin, where the largest change occurs at 1620 cm^{-1} , causing a loss in native beta-sheet and increase in aggregated beta-sheet (beta-). The pressure results shown in Figure 5C and D show similar structure changes, but there is less aggregated beta-sheet formed than the temp stress and more unordered structure caused by pressure stress. Notice the discrepancies in the pressure delta plot compared to the temp delta plot, specifically at 1620 cm^{-1} where the pressure plot shows two much smaller peaks than the temp stress. This is the cause of less beta- in pressure stress. The large increase at 1650 cm^{-1} in the pressure samples is the cause of higher unordered structure.

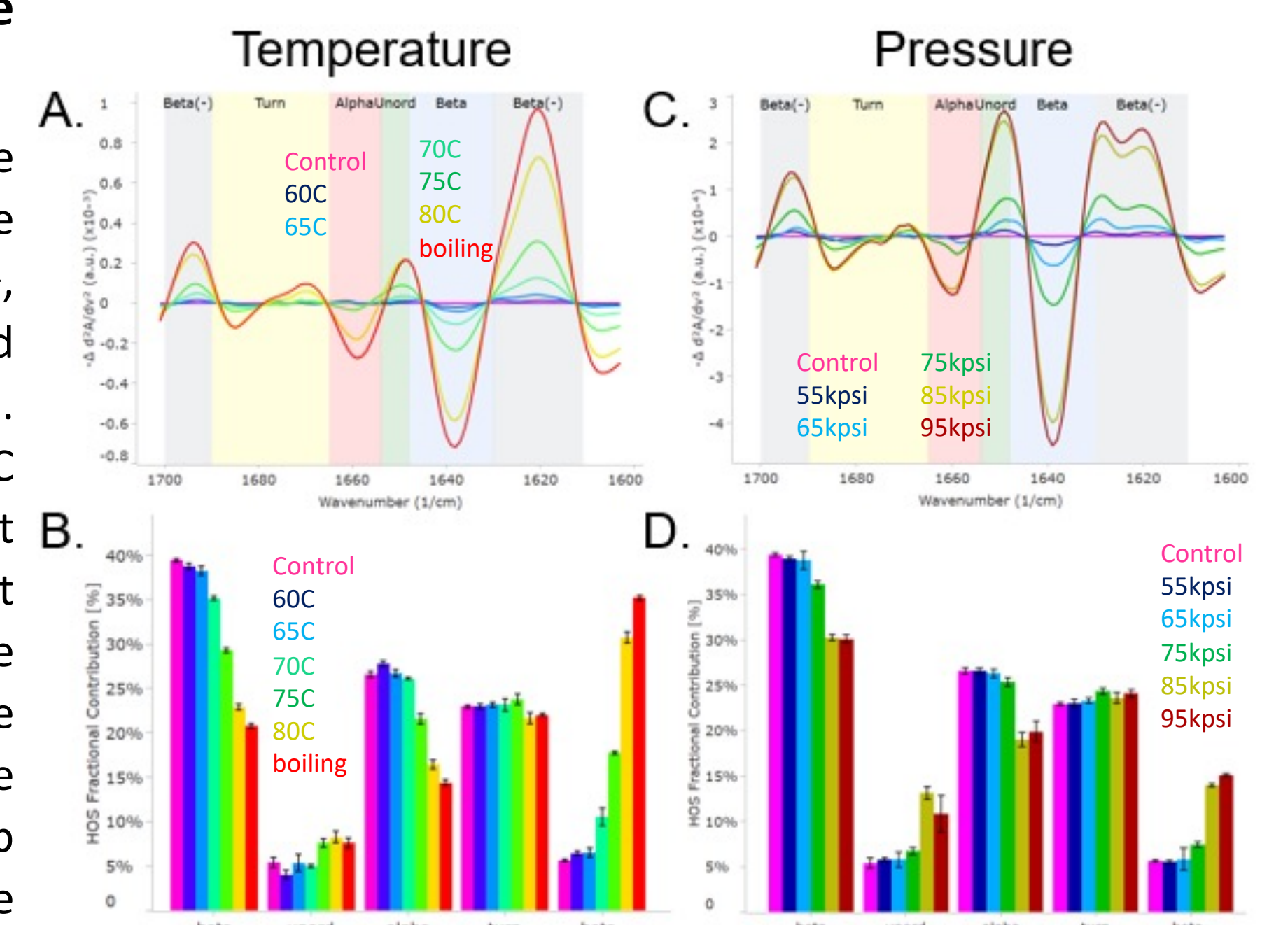


Figure 5. Ovalbumin was stressed from 25-100C for 5 mins at each temperature, cooled to room temp, and run on MMS. The delta plot **(A)** and HOS bar chart **(B)** show the structure changes of ovalbumin caused by temperature stress. Separate samples were pressure stressed from 55 kpsi-95 kpsi for 10 mins in the Pressure BioScience's HUB 880 Explorer, equilibrated to atm pressure, then run on MMS to study structural differences. The delta plot **(C)** and HOS bar chart **(D)** show distinct structural changes compared to temperature stress.

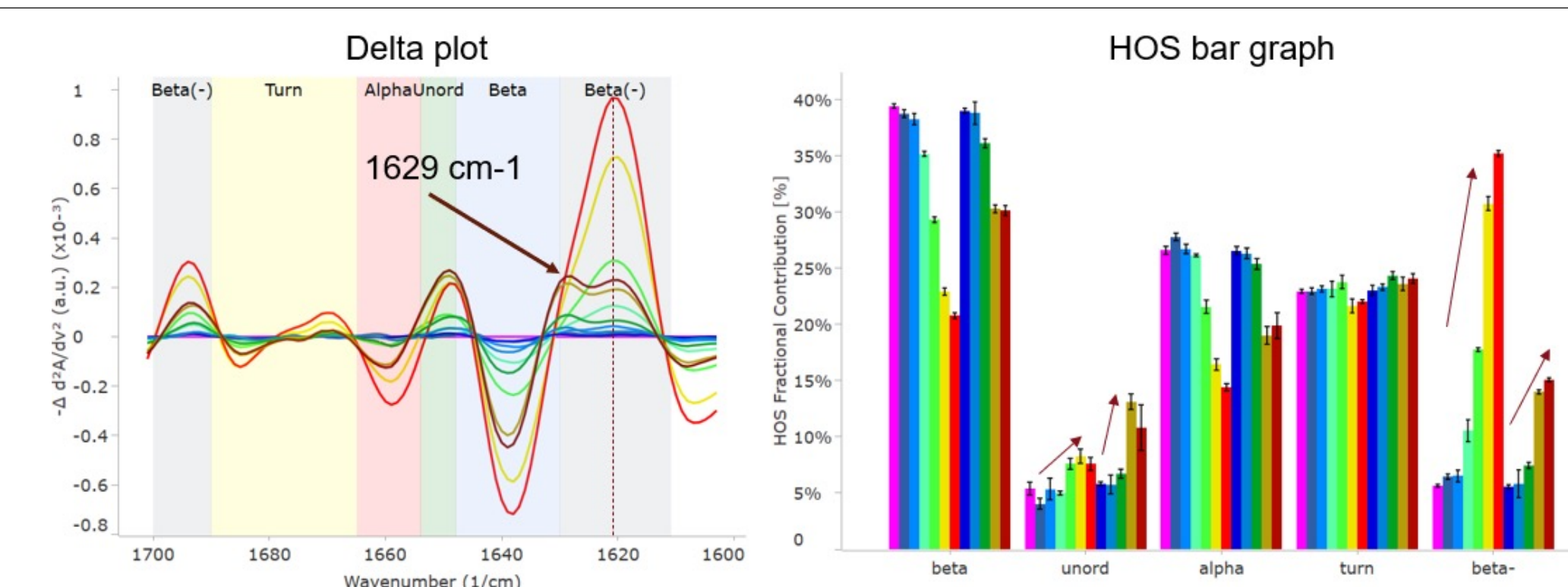


Figure 6. Overlay of the temperature and pressure data for ovalbumin, highlighting the difference the two stressors cause in the ovalbumin structure. Temp stress causes one large peak at 1620 cm^{-1} , whereas pressure causes two much smaller peaks, one at 1620 , the other at 1629 cm^{-1} . The darker shades are the pressure-treated samples.

Conclusions

- MMS can be used for structural characterization and for thermal melting, correlating structure and stability.
- Lysozyme at pH 4 is the most thermally stable of the pHs that were tested, and lysozyme has a much lower T_m than IgG.
- Temperature stress unfolds lysozyme by converting alpha-helical structure to turn structure, whereas the same temperature stress causes IgG native beta-sheet to form intermolecular beta-sheets.
- Pre-stressed ovalbumin melt curves using traditional methods result in the same T_m but appear very different.
- MMS thermal ramp data can distinguish both T_{m1} s and correlate which secondary structures are involved.
- MMS structure characterization of ovalbumin can detect structural differences between temperature stress and pressure stress.
- Specifically, pressure appears to unfold the ovalbumin, whereas temperature seems to aggregate it.
- This could be helpful in testing aggregation intermediates or aggregation pathways in proteins that are quick to aggregate.

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