Application Note
JUN 2022
AN-850-0125

Measuring Buffer-Induced Structural Changes in a Beta-sheet Rich Protein using MMS

- ○ Biosimilars
- ○ mAbs
- ○ ADCs
- ○ AAVs
- ○ Ligand Binding
- ✓ Protein/Peptide Analysis
- ○ VLPs
- ○ Nucleic Acid
- ○ Fusion Proteins
- ○ Enzyme Analysis

- ⬢ Aggregation
- ⬢ Quantitiation
- ✓ Structure
- ⬢ Stability
- ✓ Similarity

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Introduction

The critical relationship between structure and function has been well studied[1], and the ability to detect structural changes with ultra-precision and ultra-sensitivity is important to ensure the safety and efficacy of a biopharmaceutical product. Whether a folded protein is primarily comprised of alpha-helix or beta-sheet structures, the buffer in which a protein is prepared can affect its higher order structure and thus, its function[2]. It is not advantageous to forgo this critical quality attribute because current technology presents many limitations that make gathering this information difficult and subjective[3]. Now, there is a solution to obtaining important structural information across the development process in a user-friendly manner.

Microfluidic Modulation Spectroscopy (MMS), a novel mid-IR spectroscopy technology developed by RedShift BioAnalytics, Inc., fills the structural characterization gap by offering ultra-sensitive protein secondary structure measurements directly in formulation conditions with little to no interference by aqueous excipients and common stabilizing additives. MMS generates reproducible measurements that are readily achieved through the combination of a high-power quantum cascade laser (QCL) coupled with a microfluidic flow cell to enable real-time modulation between sample and reference. This results in fully automated, background-subtracted spectra and minimizes human error. Breaking with traditional techniques, MMS demonstrates much greater sensitivity and repeatability than Fourier-transform Infrared spectroscopy (FTIR) and Circular Dichroism (CD)[3] and therefore, it succeeds in providing important structural information and quantifying changes in structure on a reliable, user-friendly, and automated platform.

In this study the structural differences of alpha-chymotrpysin, a beta-sheet rich protein, were characterized in water and three common formulation buffers using MMS. Alpha-chymotrypsin was prepared in HPLC-grade water as a reference, as well as in Phosphate Buffer (PB), Phosphate Buffered Saline (PBS), and Tris buffer. Absorption spectra in the Amide I region were automatically collected and processed to monitor critical structural components including the beta-sheet content, HOS motif percentages, and to calculate the overall structural similarities between samples in all prepared conditions. The resulting analysis and comparison across all buffer conditions shows that alpha-chymotrypsin undergoes buffer-induced structural changes.

Methods

10 mg/mL Alpha-chymotrypsin (Sigma #C4129) was prepared in HPLC-grade water and three buffers: 100 mM PB pH 7, 1x PBS pH 7.4, and 100 mM Tris buffer pH 8. All samples were analyzed in triplicate at room temperature using the AQS^{3}pro first generation MMS platform at a modulation rate of 1 Hz and a backing pressure of 5 psi. The secondary structure components of the prepared protein solutions were determined using delta Data Analysis software. All spectra were normalized for concentration and cell path length prior to generation of Absolute Absorbance, Second Derivative, Delta, and HOS results.

Results

I. Absolute Absorbance, Second Derivative, and Delta

By overlaying the Absolute Absorbance spectra from alpha-chymotrypsin prepared in water and the three buffers, differences are noticeable at the peak of the overlay as shown in Figure 1 (A). These differences are magnified in the overlaid second derivative spectra shown in Figure 1 (B). Here, the most significant buffer-induced difference is observed in the beta-sheet region (1618-1643 cm^{-1}) and where the characteristic signals in this region differ relative to alpha-chymotrypsin in water in increasing difference of PBS < PB < Tris.

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Results, continued

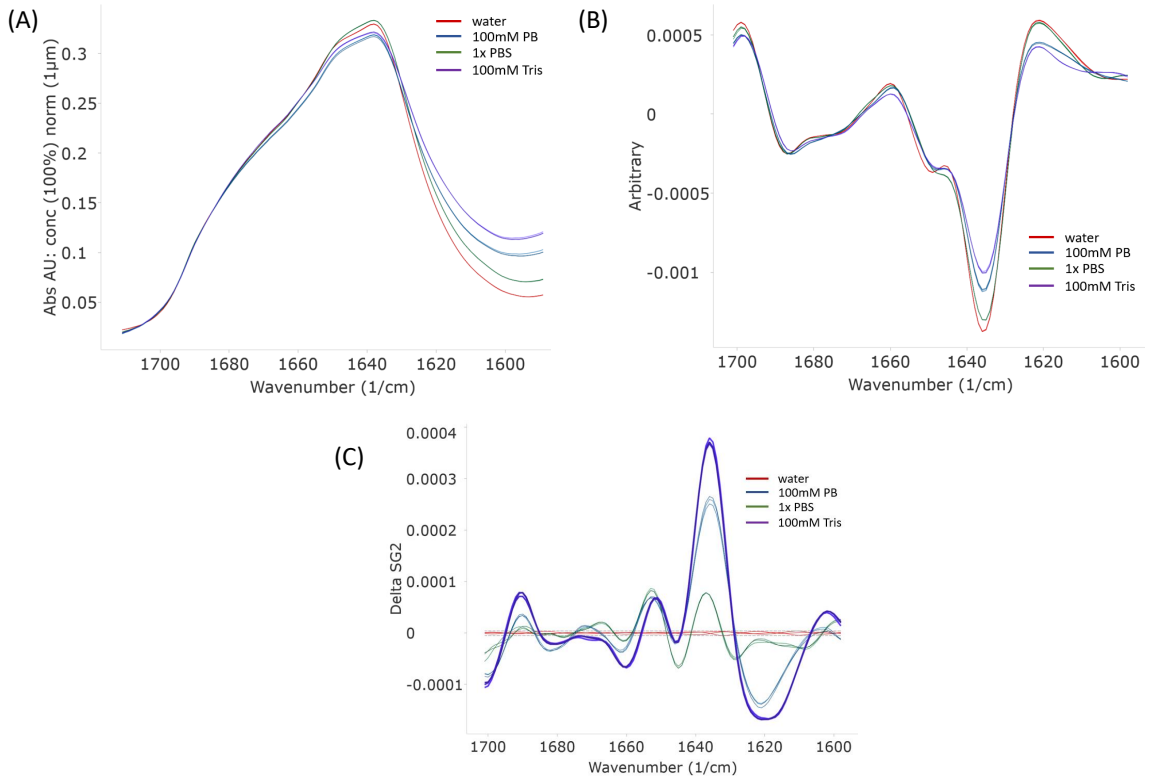

Figure 1. (A) Overlay of Absolute Absorbance spectra (B) Overlay of Second Derivative of the Absolute Absorbance spectra, and (C) Delta plot comparing second derivative spectra for 10 mg/mL alpha-chymotrypsin in water (reference) to PB, PBS, and Tris buffers.

The Delta plot shown in Figure 1 (C) gives another representation of the differences in secondary structure relative to alpha-chymotrypsin in water. The Y axis indicates the differences in second derivatives compared to the average of alpha-chymotrypsin in water that is represented by the red lines bounded by dashed lines at the zero value on the Y axis. This plot further highlights that the greatest difference in protein folding was observed in Tris buffer relative to alpha-chymotrypsin in water, followed by PB, and then PBS.

II. Similarity by AO and WSD: The overall structural similarity of alpha-chymotrypsin in water and the three buffer systems was calculated using two common methods: Area of Overlap (AO) and Weighted Spectral Difference (WSD). Figure 2 (A) shows the similarity plots by AO and Figure 2 (B) by WSD for alpha-chymotrypsin in water overlaid with the protein in the three buffers.

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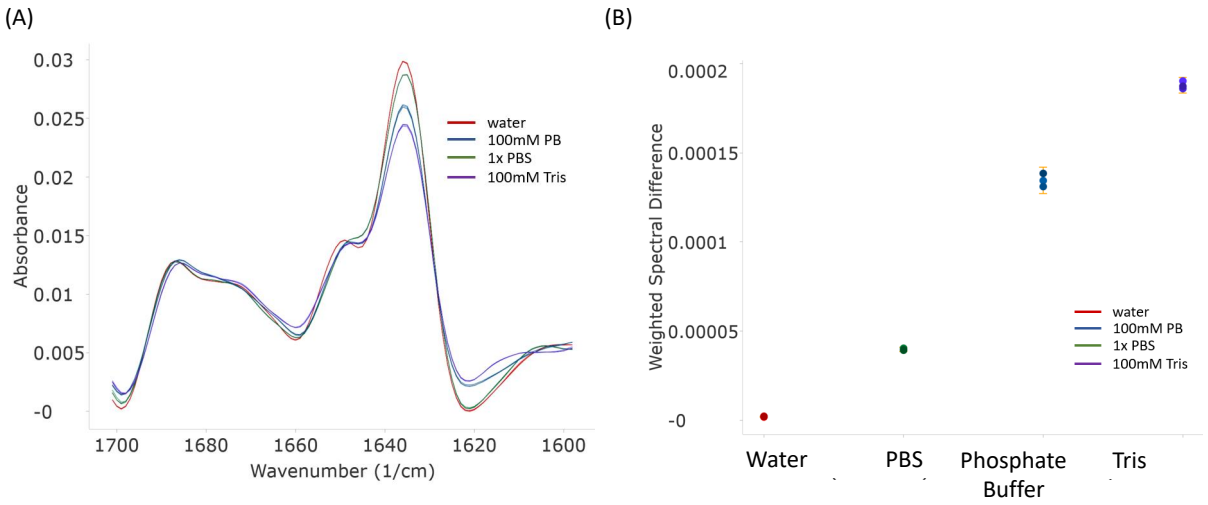

Figure 2: (A) Similarity plot comparing Area of Overlap (AO) and (B) Weighted Spectral Difference (WSD) for alpha-chymotrypsin in water and three buffers.

As detailed further in Table 1, replicate-to-replicate measurements show a repeatability of greater than 99.8% as prepared in all buffers and water, and the overall structure of alpha-chymotrypsin changed 2-6% in the buffers tested versus alpha-chymotrypsin in water when compared by AO. When evaluated using WSD, a similar trend is seen with the overall structure changing the most in Tris, and the least in PBS as compared to water.

Table 1. Percent Similarity by Area of Overlap (AO) and Weighted Spectral Difference (WSD) for 10 mg/mL alpha-chymotrypsin in water, Tris, PB, and PBS.

10 mg/mL alpha-chymotrypsin prepared in	Percent Similarity by AO		Similarity by WSD	
	Among Replicates	vs alpha-chymo in water	Among Replicates ($x10^{-6}$)	vs alpha-chymo in water ($x10^{-6}$)
Water	99.85 ± 0.01	100	2.47 ± 0.57	0
Tris, 100 mM pH 8	99.82 ± 0.06	93.91 ± 0.06	2.90 ± 1.08	188 ± 2.2
PB, 100 mM pH 7	99.85 ± 0.03	95.66 ± 0.12	3.30 ± 0.96	135 ± 3.7
PBS, 1x pH 7.4	99.82 ± 0.07	98.04 ± 0.03	2.47 ± 0.57	39.7 ± 0.6

III. Higher Order Structure: For alpha-chymotrypsin analyzed in water and three buffers, the percent HOS motif composition was determined using Gaussian curve fitting from the AO similarity plot shown previously in Figure 2 (A). The resulting bar graph is presented in Figure 3 relative to the control of alpha-chymotrypsin in water.

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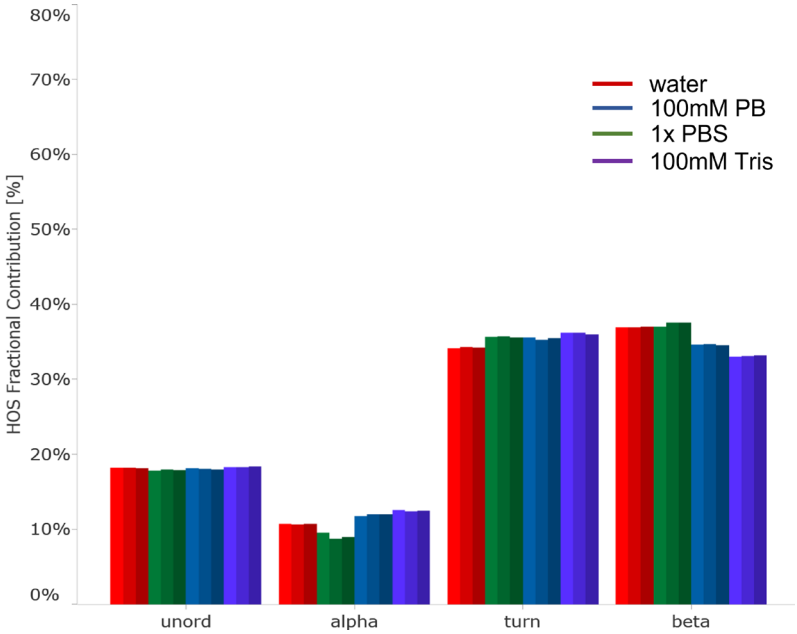

Figure 3: Higher Order Structure (HOS) plot of alpha-chymotrypsin in different buffers.

The HOS plot shows a decrease in beta-sheet structures relative to the control in water, with the protein in Tris showing the greatest change in structure, followed by PB and then PBS.

Table 2 below gives the percentage breakdown of secondary structure motifs in each buffer condition. The amount of characteristic beta-sheet composition decreases in the order of water ≈ PBS > PB > Tris. This correlates well with the results shown in earlier sections, and the overall HOS quantification compares well to the X-ray crystallography data cited in literature[4,5]. Note, the conditions used for the X-ray crystallography data were 0.2 M Citrate buffer, pH 4.2.

Table 2. Percent Similarity by Area of Overlap (AO) and Weighted Spectral Difference (WSD) for 10 mg/mL alpha-chymotrypsin in water, Tris, PB, and PBS.

As measured in or measured by	Percent HOS motif			
	turn	alpha	unordered	beta
Water	34 ± 0.06	11 ± 0.07	18 ± 0.02	37 ± 0.03
Tris, 100 mM pH 8	36 ± 0.1	12 ± 0.07	18 ± 0.06	33 ± 0.06
PB, 100 mM pH 7	35 ± 0.1	12 ± 0.1	18 ± 0.05	35 ± 0.09
PBS, 1x pH 7.4	36 ± 0.07	9 ± 0.4	18 ± 0.4	37 ± 0.3
X-ray cryst.	34	12	19	34

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Conclusions

To characterize important buffer-induced changes in structure, a reliable and sensitive technique must be employed. For alpha-chymotrypsin prepared in water and three common buffers, MMS was used to generate mid-IR absorption spectra to monitor the HOS motifs in each buffer and to rank the changes in structure relative to the protein in water.

In this study, both Tris and Phosphate Buffer (PB) reduced the formation of beta-sheet structures while PBS did not induce significant change relative to the protein in water. This information is important when considering the ideal conditions for formulating this protein and the associated activity that could be affected in each buffer.

Compared to traditional FTIR and CD, MMS measurements have been shown to be more sensitive with outstanding system repeatability to enable very small changes to be detected in higher order structure with accuracy and confidence. MMS also permits direct analysis in the formulation of interest with no need for dilution or buffer exchange due to the real-time buffer-subtraction capability of the instrument and the ability to subtract interference from excipients or common additives. As a novel tool that accurately monitors important critical quality attributes, MMS enables the detection of subtle differences in protein structure under different buffer conditions, therefore informing decisions about formulation and protein stability.

References

1. Lee, D., Redfern, O. & Orengo, C. Predicting protein function from sequence and structure. Nature Reviews Molecular Cell Biology 8, 995–1005 (2007).
2. Gupta, B. S., Taha, M. & Lee, M.-J. Superactivity of α-chymotrypsin with biological buffers, TRIS, TES, TAPS, and TAPSO in aqueous solutions. RSC Advances 4, 51111–51116 (2014).
3. Kendrick, B. S., Gabrielson, J. P., Solsberg, C. W., Ma, E. & Wang, L. Determining Spectroscopic Quantitation Limits for Misfolded Structures. Journal of Pharmaceutical Sciences 109, 933–936 (2020).
4. Tsukada, H. & Blow, D. M. Structure of a-Chymotrypsin Refined at 1433 A Resolution. J. Mol. Biol vol. 184 (1985).
5. pdb4CHA

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