X-ray Scattering coupled Chromatography: a New Tool for Studying protein interactions at Deep Ocean Pressures

Cody Cummings[1], Qingqiu Huang[2], Durgesh Kumar Rai[2], and Richard Gillilan[2]

1 School of Chemistry and Materials Science, Rochester Institute of Technology, Rochester, New York
2 Cornell High Energy Synchrotron Source (CHESS), Cornell University, Ithaca, New York

Abstract

There are countless bacterial/archaean species thriving in the deep oceans and the subsurface world, but how pressure affects biomolecular structure is still obscure to us surfacers (mesophiles). Small-angle x-ray scattering (SAXS) combined with high-performance liquid chromatography is a powerful tool for unlocking biophysical phenomena at room pressure. For the first time, we have extended this technique to deep ocean pressures to get ideas of how conformationally "pressurized" structures help organisms to survive in the deep sea. The Cornell High Energy Synchrotron Source (CHESS) is a large underground particle accelerator devoted to producing ultra-bright X-rays for research use. We have used several protein standards to study how well size-exclusion and anion-exchange type chromatographies work in. combination with X-rays under extreme pressure. Scattered protein standards under high pressure without a column were also reported. We observed that applied high-pressure (100 MPa) changes oligomeric states of some protein standards but the analysis of conformational changes in those proteins is still in progress. We also found some protein standards were only slightly changed or resistant from being "pressurized" (no significant changes at 100 MPa). The most interesting is that the structure of the unfolded l-lactate dehydrogenase was probably altered at 100 MPa. Kratky plots were produced to prove that protein standards were folded during the experiments. Finally, Guinier plots were effective to monitor that protein standards weren't severely damaged by x-ray radiation. In the future, we still need to confirm shifts in peaks seen in AEX. Although the anion protein standards were slightly shifted at between room pressure and 25 MPa but we believe these shifts will show how protein surface electrostatic field changes with pressure.

Introduction

The biophysical phenomena of proteins in the deep oceans are extraordinary but still recondite to us. The most attentive parts of those oceanic proteins are their structures and dynamics which makes them to be thermodynamically stabilized under extremely high pressure. The deepest trench of the oceans called the Challenger Deep in the Mariana Trench which is located in the western Pacific Ocean. The depth of the Mariana Trench reaches to more than 10,000 meters [1]. In other words, the pressure of the Mariana Trench is estimated 1100 bar or 110 million pascals [1]. The average temperature surrounding hydrothermal vents are 350^{o}C [1]! High-performance liquid chromatography (HPLC) coupled with a pressure generator is used to create an artificial high-pressure environment as an equivalent to the deep ocean and can separate proteins using high-pressure-resistant columns [2]. High pressure column hardware is available, but not packed with the type of chromatography media that we needed. So our experiments required that we pack our own columns with several different types of material [2]. Small-angle x-ray scattering (SAXS) is a powerful technique for analyzing tertiary/quaternary conformations of proteins which can be achieved with ultra-bright x-ray radiation [3]. For producing x-rays, the particle accelerator of the Cornell High Energy Synchrotron Source (CHESS) is utilized for this research project [3]. With those instruments and certain analytical software, the conformational changes and thermodynamic

stability under high pressure can be studied with well-known protein standards. This research report gives an overview of our hardware, software, biological materials, and data.

Hardware of the BIO-SAXS Beamline

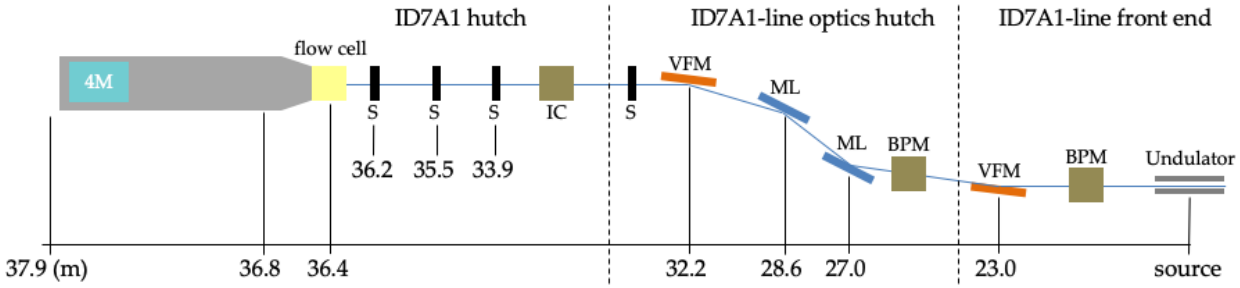


Figure 1. Simplified Diagram of the BIO-SAXS Beamline inside of the 1D7A1 hutches

The moveable x-ray components residing in the 1D7A1-line front end and 1D7A1-line optics hutch are controlled using a program called SPEC. For 1D7A1 hutch, the upstream components of the beamline are arranged as seen in Figure 2.

Figure 3

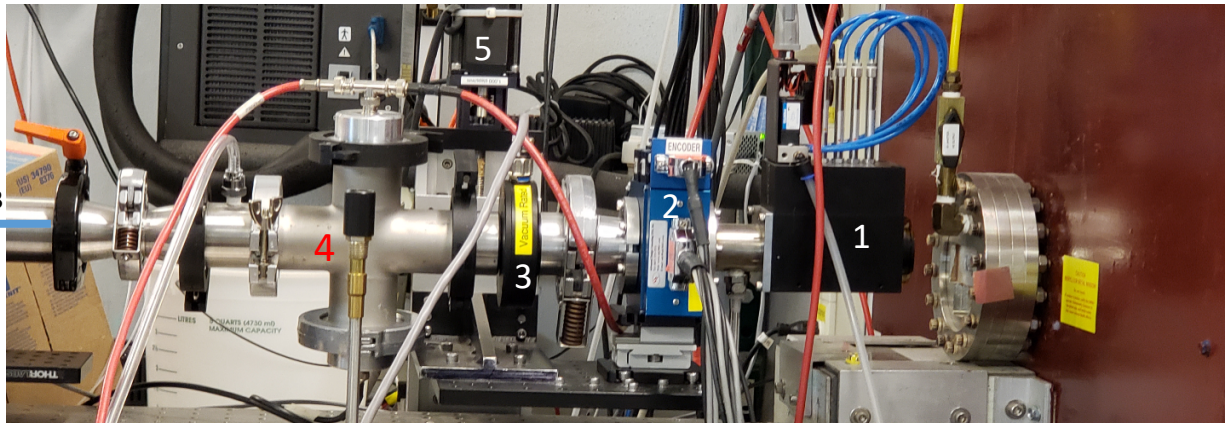


Figure 2. The leftmost selection of the front 1D7A1 beamline (next to G-Cave x-ray micro-window.) The section #1 is an attenuation; #2 is a controllable S4 slit (and is also movable using #5, an upstream motor); #3 is a vacuum rated shutter; and #4 is an ion-chamber (I_4), a measurement of x-ray intensity.

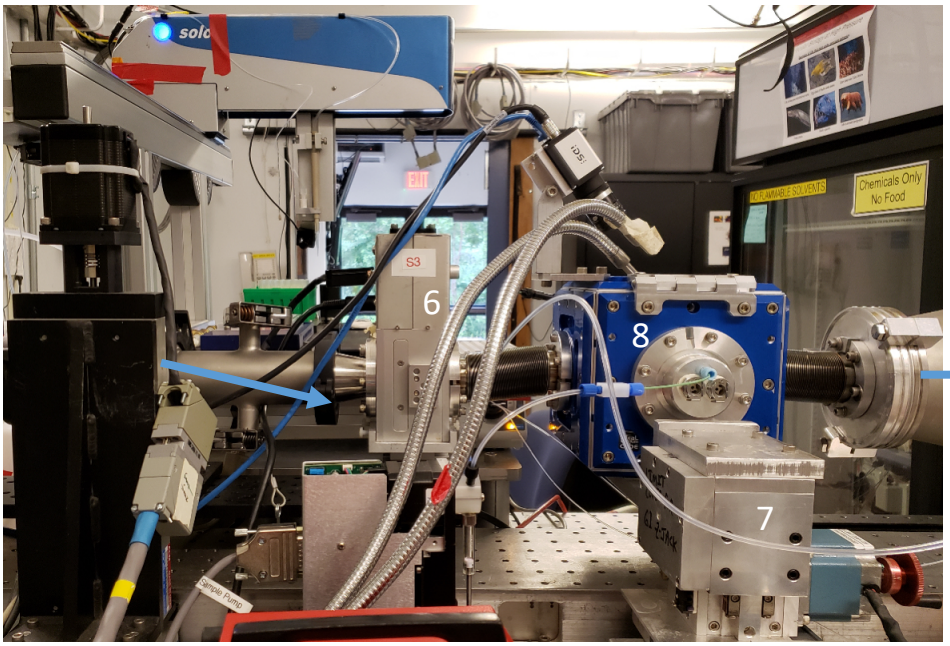


Figure 3. The middle selection of the behind 1D7A1 beamline. #6 is a guard slit S_3 (to clean up "parasite" scatter"); #7 is a backside of X-Z stage to support and move #8, a blue cube (detailed in Figure 10.)

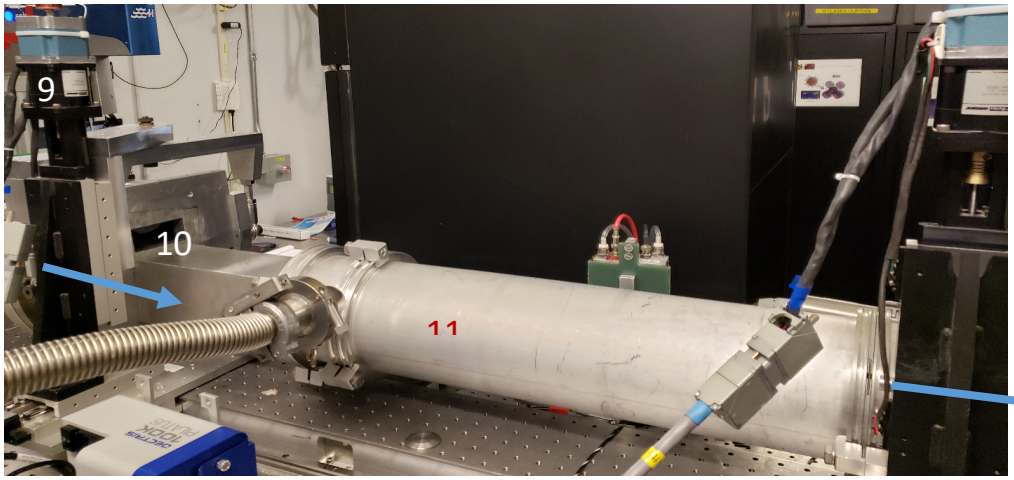


Figure 4. The middle-rightmost selection of the behind 1D7A1 beamline; #9 is Fl1x/Fl1z motor, to move the upstream end of the pipe; #10 is a vacuum window (must be sealed with plastic paper and epoxy-based glue); and #11 is a flight path (pipe).

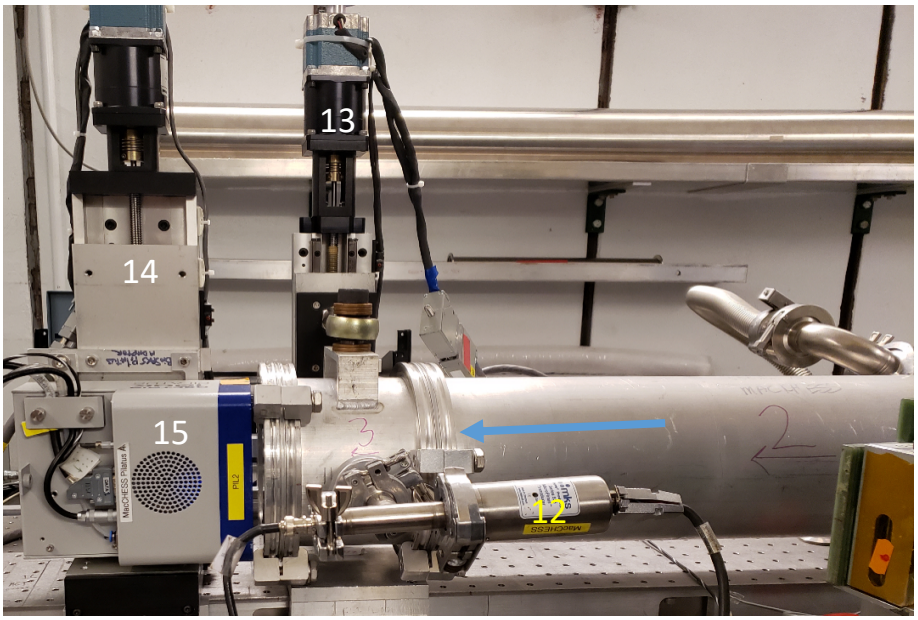


Figure 5. The rightmost selection of the front 1D7A1 beamline; #12 is a MicroPirani Transducer connecting to a vacuum gauge (not visible in Figure 5); #13 is a bsx/bsz motor of beamstop; #14 is a ccdx/ccdz motor of #15, Pilatus 100K detector, to detect scatters of x-ray beams. Vacuum of the flight path must be equal to 10 mTorr or lower to get the best quality of scattering.

Hardware of the Biological Experiments

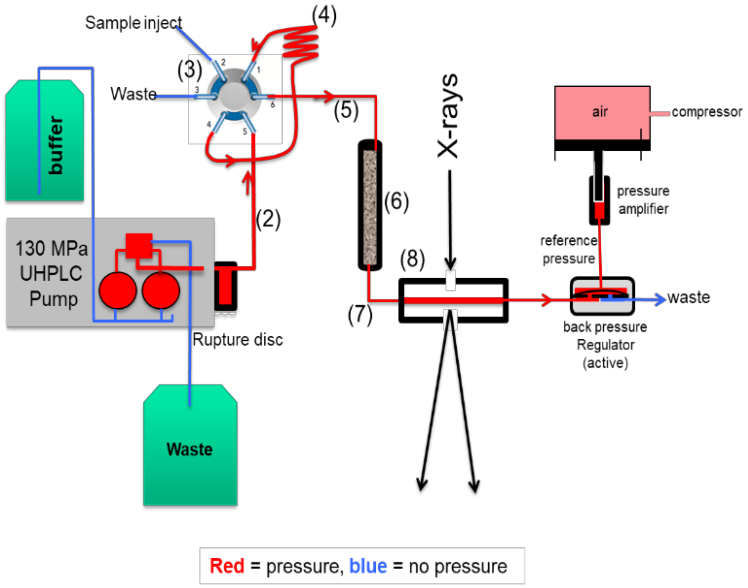


Figure 6. Simplified Diagram of the Sample and Buffer Flow under Pressure. 2# Metal tube; 3# IDEX injection value; 4# 100 microliter loop; 5# metal tube; 6# high-pressure column; 7# metal tube; and 8# sapphire capillary (inside of the blue cube).

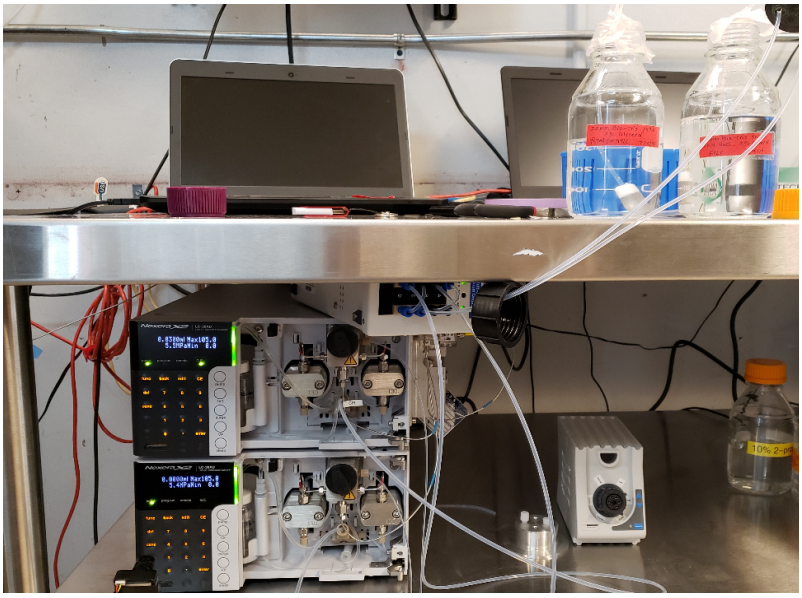


Figure 7. Nexera X2 – LC-30AD pumps setup (two pumps were required for AEC experiments - more details at bottom)

Nexera X2 LC-30AD pumps should be connected to the injection system (in Figure 8) using high-pressure steel tubing (fittings must be tight) while a plastic tube was used to feed buffer to the pumps.

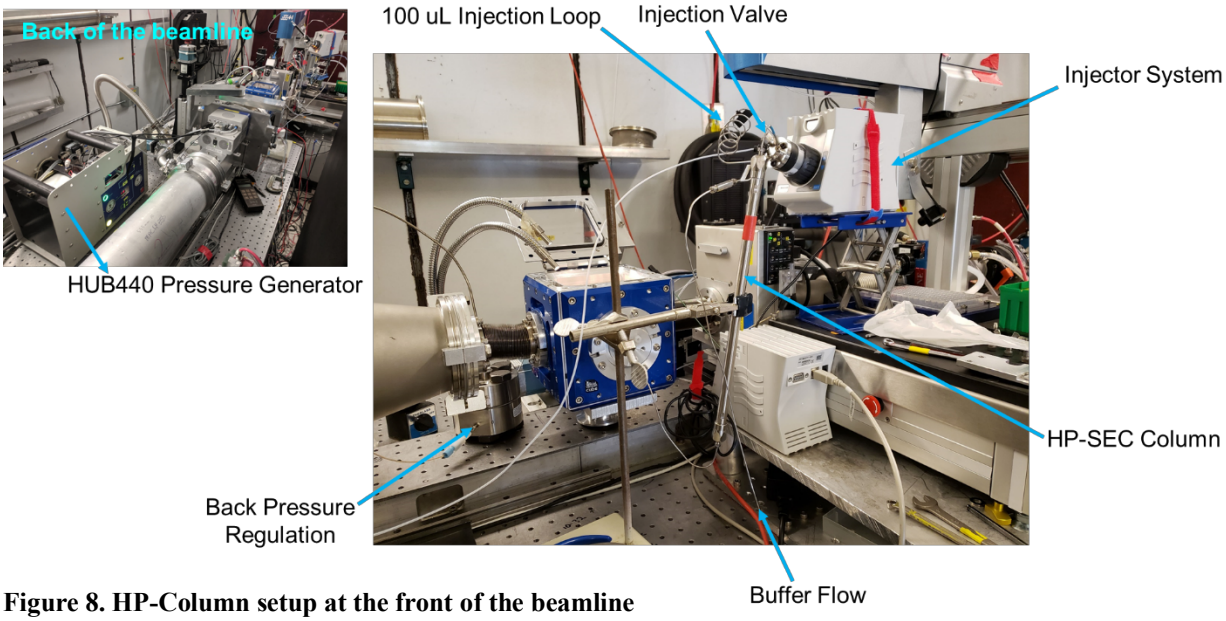


Figure 8. HP-Column setup at the front of the beamline

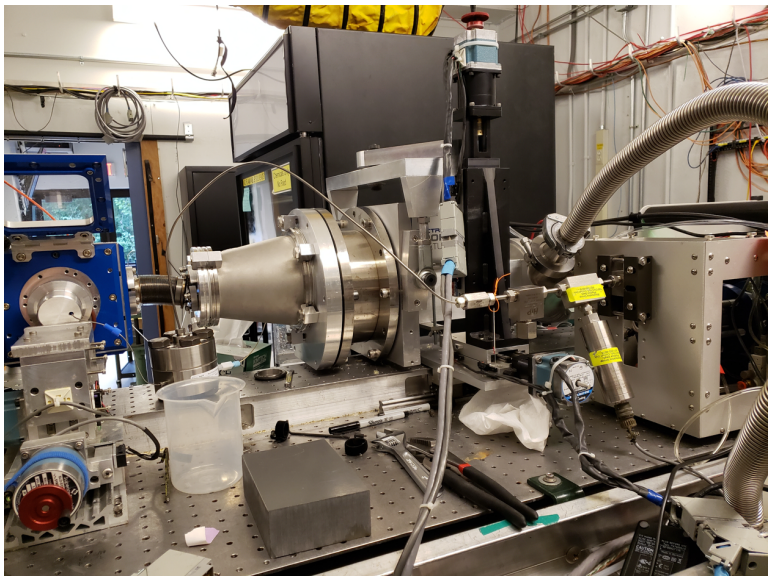


Figure 9. HP-Column and Pressure Generator set-up at the behind of the beamline

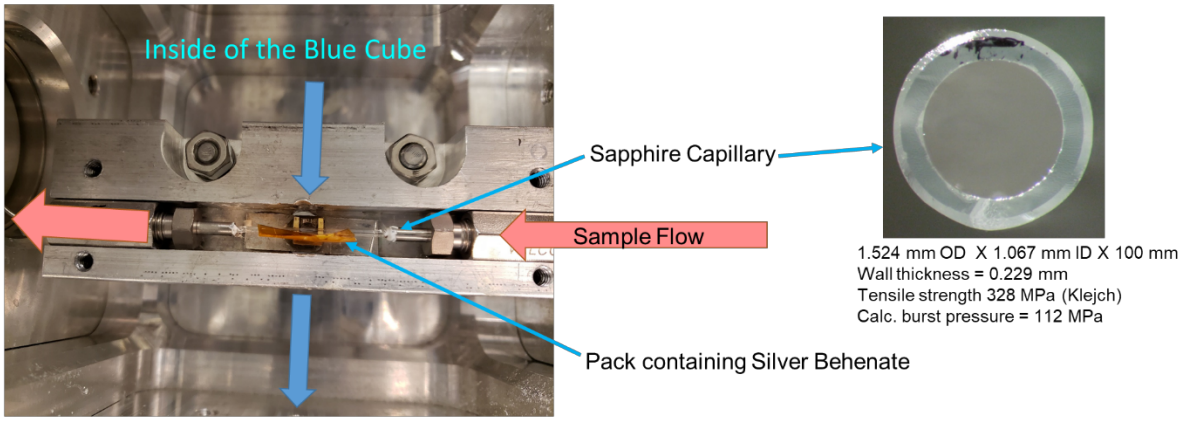


Figure 10. Inside of the blue cube and sapphire capillary setup

Software Requirements

- **Rheodyne Titan Value Control Software** for flow controls such as injecting sample into a capillary.
- **Pressure Bioscience software** for HUB pressure generator (to operate the pressure inside of the sample/buffer flows)
- **Avasoft 8.9 software** for Avantes UV spectrometer
- **BIO-SAXS RAW** for manipulating and analysis the data including series plots, Guinier plots, and Kratky plots

Packing High-Pressure Column

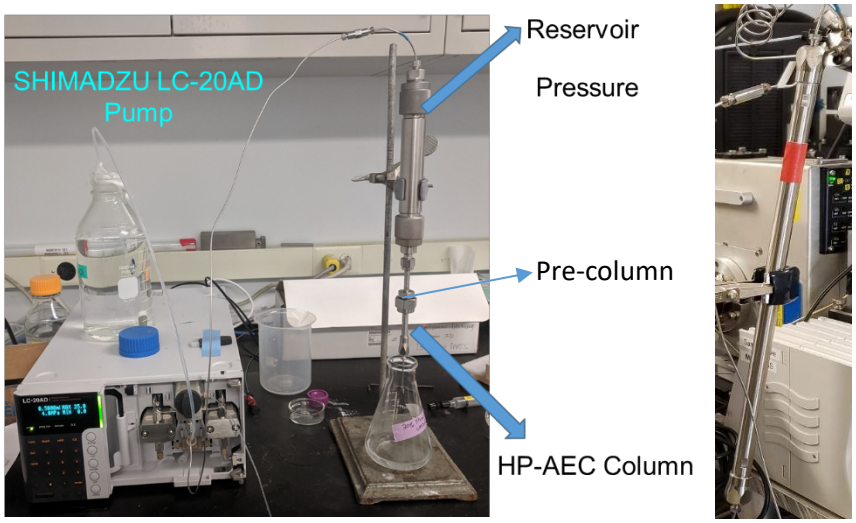


Figure 11. Packing the High-Pressure set-up (left) and HP-SEC column (right)

High pressure column can be packed with a reservoir, a SHIMADZU LC-20AD pump, and a chromatographic resin as shown in Figure 11; there were two HP-columns were prepared - one for size-exclusion chromatography (SEC) and another one for anion-exchange chromatography (AEC) for this research project. A chromatographic resin was removed from a used column by forcefully rinsing it with 20% ethanol (in this case - YMC-pack Diol-120 silica resin for SEC and Mono QTM 5/50 GL resin for AEC), placed it into a 50mL centrifuge tube, and centrifuged it at high speed. The total volume of an empty HP-column and pre-column, and resin volume must be measured prior to packing a column. A reservoir tube and a pre-column (connecting to a metal column) was filled with appropriate volume of centrifuged resins in 20% ethanol, sealed it, and connected to the LC-20AD pump. LC-20AD pump was turned on to push resins slowly (between 2.0 mL/min to 0.1 mL/min) into a metal column until the pressure becomes constant (for more than five minutes.) HP-column can be removed and sealed until it can be used for experiments. (The HP-column must be rinsed with deionized water to remove 20% ethanol before equilibrating it with a buffer.)

Biological Materials

Protein	Buffer Used	Molecular Weight (Da)	Column Used?
L-Lactate Dehydrogenase	100mM Bis-TRIS[1] + 10 mM DDT[2] + 1 mM EDTA[3]	140,000	HP-SEC
Glucose Isomerase	100mM Bis-TRIS + 10 mM DDT + 1 mM EDTA	170,000	HP-SEC
Glyceraldehyde-3-Phopshate Dehydrogenase	100mM Bis-TRIS + 10 mM DDT + 1 mM EDTA	36,000-37,000	HP-SEC
Bovine Serum Albumin	100mM Bis-TRIS + 10 mM DDT + 1 mM EDTA	66,500	HP-SEC
Beta-Lactoglobulin	20 mM Bis_TRIS + 100 mM NaCl	18,000	No column used
Myoglobin*	20 mM TRIS-HCl + 1.0 M NaCl	16,700	HP-AEX
Conalbumin*	20 mM TRIS-HCl + 1.0 M NaCl	76,000	HP-AEX
Ovalbumin*	20 mM TRIS-HCl + 1.0 M NaCl	42,700	HP-AEX
Soybean Trypsin Inhibitor*	20 mM TRIS-HCl + 1.0 M NaCl	21,000	HP-AEX

1. Bis-TRIS Methane; 2. Dichlorodiphenyltrichloroethane; 3.Ethylenediaminetetraacetic acid. *RAD-BIO protein exchange standard

- **L-Lactate Dehydrogenase (hog muscle)** - 10mg/mL - Roche - Lot# 10107085001
- **Glyceraldehyde-3-phosphate dehydrogenase** - Sigma - Lot#
- **Bovine Serum Albumin Fraction V** - 10mg/mL - Sigma - Lot# A3059-10G
- **Beta-lactoglobulin** – Sigma L7880 (Lot # 051M7002V
- **BIO-RAD Protein Exchange Standard** - conc. - BIO-RAD - Lot # 1250561

<u>Preliminary Results (Note: most of our results are not shown here because they are being prepared for peer-reviewed publication. An updated version of this report will be available once our results are published).</u>

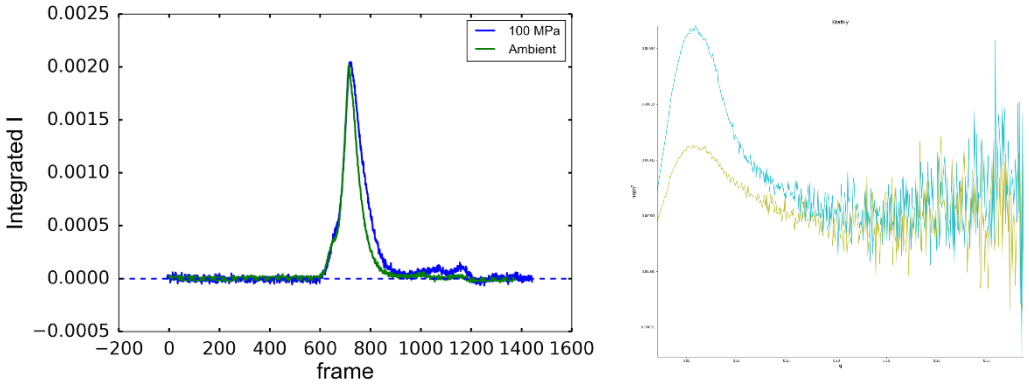
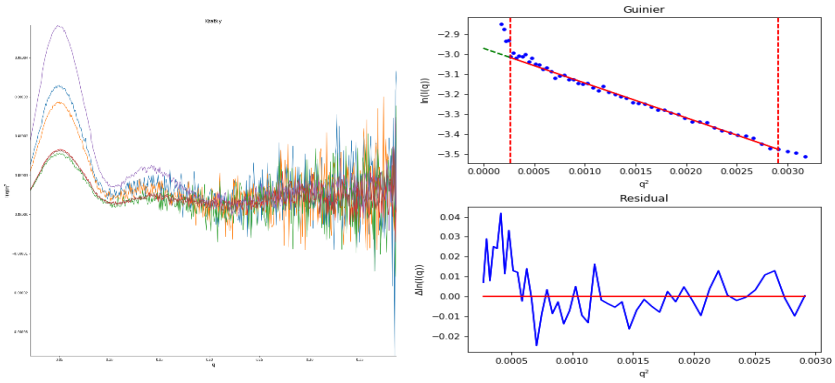


Figure 14. SAXS Plots of Bovine Serum Albumin under various pressure. Left plot was the series plot (Integral I vs Frame #) - Green line was pressure room (5 Mpa); Blue line was 100 MPa. Right plot was Kratky plot ($I(q)q^2$ vs q) – Yellow-Green line was room pressure and Cyan line was 100 MPa.



Discussion and Conclusion

Although bovine serum albumin was probably resistant from being significantly changed under high-pressure (up to 100MPa); it appears there is some broadening of the peak under pressure (Figure 14). We believe this is related to column packing and not the protein itself. The Kratky plot shown in the figure is important evidence that the protein is folded. The Guinier plot shows some minor aggregates or damage at smallest angles, but otherwise is linear. Preliminary anion exchange experiments were successful in separating protein standards under pressure. Data to be published later.

SAXS coupled chromatography technique and BIO-SAXS RAW software are effective to accurately analyze the changes in proteins under high pressure. Oligomeric-state changes in some proteins under high pressure are indicated by the peak (data still being analyzed for publication). Kratky plots and Guinier plots were analyzed to check the quality of the data and to monitor possible structure and/or radiation damages. In the future, the shifts in peaks of the Bio-Rad protein anion exchange standard at 100 MPa will be examined to see if they relate to how protein surface electrostatics change with pressure. Furthermore, the cation exchange chromatography and cationic protein standards at high pressure are also interested to be examined in the future.

Acknowledgment

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