Application Note

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Aggregate Analysis of a Recombinant L-Asparaginase from *E. coli*

Authors

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Abstract

Quantification of the aggregation of biotherapeutic proteins is an essential part of ensuring quality and safety. Recombinant proteins encounter a range of conditions that can contribute to aggregation, including changes in pH, concentration, temperature, and exposure to surface and shear forces. The enzyme L-asparaginase poses a challenge for quantifying aggregation because its structure makes it prone to producing unusual high molecular weight aggregates.

This application note explores the use of two types of size exclusion column (SEC) to quantify aggregation: a silica-diol-type stationary phase (Agilent ProSEC 300S) and a hydrophilic polymer-coated silica phase (Agilent AdvanceBio SEC). The study also verified the robustness of the method by comparing performance in prolonged testing.

Introduction

The anticancer properties of the enzyme L-asparaginase were identified many years ago.[1,2] The enzyme converts asparagine into aspartate and ammonia. Certain cancer cells lack asparagine synthetase, so they cannot convert aspartate into asparagine. When such cells have no supply of asparagine, they suffer cell apoptosis. L-Asparaginase therefore became an important biotherapeutic target, resulting in several recombinant enzyme products.

As with many recombinant biotherapeutics, aggregation of L-asparaginase is a critical quality attribute and must be quantified during manufacture and quality control. However, asparaginase in its active form is a 148 kDa enzyme that contains four identical 37 kDa subunits. Therefore, possible aggregates include not only low molecular weight species but also octamers and higher-order species.

The challenges of quantifying aggregation of recombinant asparaginase have been reported previously.[3] Variability in quantification of higher-order aggregates has been noted for various size exclusion columns from different vendors, including lot-to-lot variation.

Experimental

Reagents and chemicals

- All reagents and chemicals were HPLC grade or higher.
- L-Asparaginase from *E. coli* was purchased from Sigma-Aldrich.

Instrumentation

Analyses were performed on an Agilent 1260 Infinity II Bio-inert LC system, comprising the following modules:

- 1260 Infinity II bio-inert quaternary pump (G5654A)
- 1260 Infinity II bio-inert multisampler (G5668A)
- 1290 Infinity II Multicolumn Thermostat (G7116B), and
- 1260 Infinity II Multiple Wavelength Detector with bio-inert flow cell (G7165A).

Data were acquired with Agilent OpenLab CDS 2.6 software.

Light scattering analysis was done with an Agilent 1260 Infinity Bio-SEC multidetector system (G7805A) added to the setup.

Analysis of the light scattering data was performed using Agilent GPC/SEC software.

Sample preparation

Stock solutions of bovine serum albumin (BSA), 20.0 mg/mL, and myoglobin (MYO), 10.0 mg/mL, were prepared in mobile phase. Equal volumes were mixed to create a BSA–MYO system suitability standard.

L-Asparaginase was prepared by dissolving the contents of a vial with 1.0 mL of mobile phase.

A BioRad gel filtration standard (GFS) protein standard was prepared by dissolving the contents of a vial in 5.0 mL of mobile phase.

Mobile phase preparation

Mobile phase of 100 mM sodium phosphate and 100 mM NaCl was prepared by combining NaH_2PO_4 and Na_2HPO_4 solutions with NaCl and adjusting the pH to 7.2. The solution was then passed through a 0.2 µm membrane filter.

For light scattering analysis, the mobile phase was passed three times through a 0.1 µm membrane filter.

Table 1. Method conditions.

	HPLC Conditions
Column	Agilent ProSEC 300S 5 µm, 300 Å, 7.5 × 300 mm (p/n PL1147-6501), or Agilent AdvanceBio SEC 2.7 µm, 300 Å, 7.8 × 300 mm (p/n PL1180-5301)
Mobile Phase	100 mM sodium phosphate + 100 mM NaCl, pH 7.2
Flow Rate	0.5 mL/min
Column Temperature	25 °C
Injection Volume	20 µL
Total Run Time	30.0 minutes
Detection	UV, 280 nm (unless stated otherwise)
Sequence	Ramp phase (including flush) 0.1 mL/min, 5 min 0.2 mL/min, 5 min 0.3 mL/min, 5 min 0.4 mL/min, 5 min 0.5 mL/min, 120 min (flush) Analysis phase 0.5 mL/min, 30 min

Results and discussion

Each column was subjected to the same sequence of steps, as identified in the previous section.

The initial phase involved ramping the flow rate in increments of 0.1 mL/min until the desired operating flow rate of 0.5 mL/min was reached. Each column was then flushed for 120 minutes (approximately 8 to 9 column volumes). The injection sequence included blanks (mobile phase), the BioRad GFS protein standard, and the BSA–MYO system suitability standard. Injections of recombinant asparaginase were followed by further injections of the BSA–MYO and BioRad GFS standards.

Figures 1A and 1B compare the chromatograms from the two columns investigated. The dimensions of the ProSEC 300S column are 7.5 × 300 mm, giving a column volume of 13.3 mL. The dimensions of the AdvanceBio SEC column are 7.8 × 300 mm, giving a column volume of 14.3 mL.

At a flow rate of 0.5 mL/min, retention times could be up to 2.2 minutes later for the larger AdvanceBio SEC column. However, Figures 1A and 1B clearly show that the larger proteins elute earlier in the AdvanceBio SEC column. This result indicates that the AdvanceBio SEC column has a much larger pore volume (6.4 mL versus 5.0 mL), with around 30% greater separation capability.

The chromatograms show the separation of the BioRad GFS protein standard and the BSA–MYO system suitability standard on both column types. The plots are normalized to the largest peak in the chromatogram. Peak 7, myoglobin, is common to both samples and shows excellent reproducibility of retention time. Minor peaks, corresponding to dimers of several of the proteins present in the samples, are well resolved and identified in the figure legend.

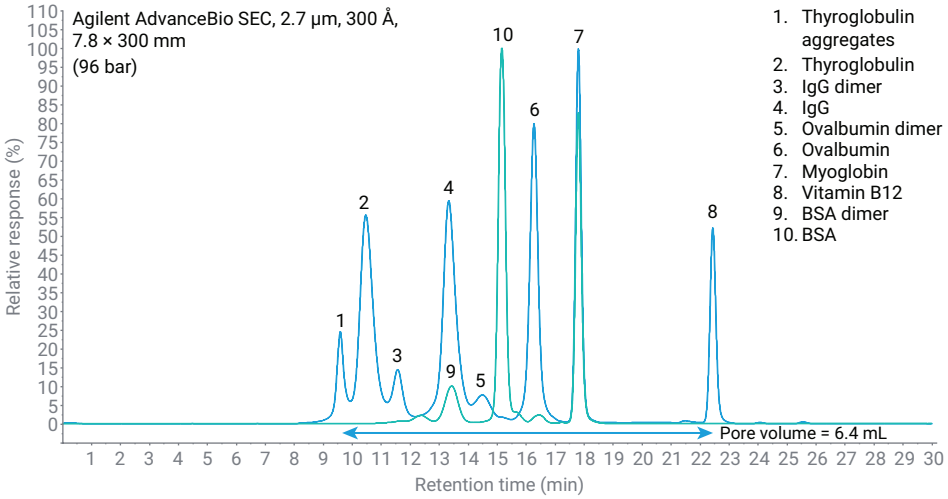

Figure 1A. Overlay of chromatograms from the Agilent AdvanceBio SEC column for BioRad GFS protein standard (peaks 1 to 8) and BSA–MYO system suitability standard (peaks 9 and 10).

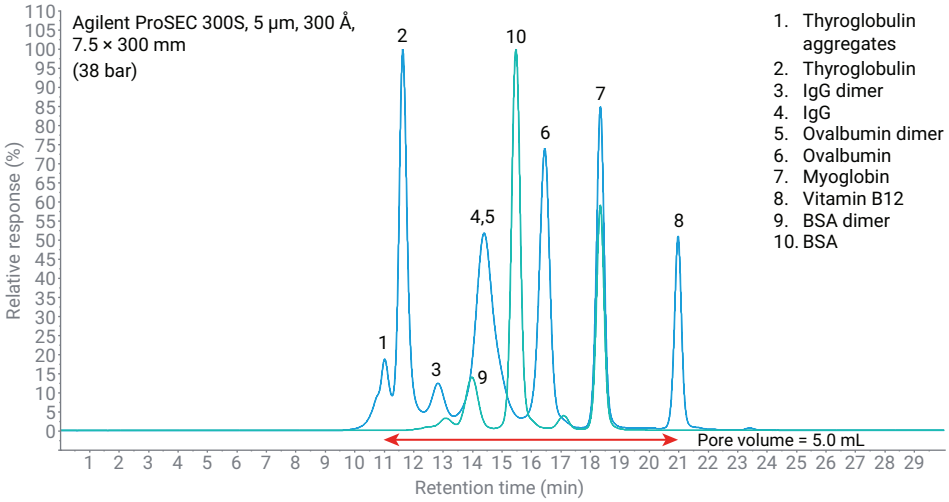

Figure 1B. Overlay of chromatograms from the Agilent ProSEC 300S column for BioRad GFS protein standard (peaks 1 to 8) and BSA–MYO system suitability standard (peaks 9 and 10).

Table 2 contains the resolution values for the system suitability mixture, between the BSA dimer and monomer peaks and between the BSA monomer and myoglobin monomer peaks.

A combination of higher pore volume and smaller particle size (resulting in higher column efficiency) leads to a significant increase in resolution for the AdvanceBio SEC column compared to the ProSEC 300S column.

Figure 2 shows the calibration curve created by plotting the logarithm of the molecular weight on the Y-axis versus retention time on the X-axis. The curve clearly demonstrates the difference in pore volume. Further tests were therefore performed.

Using a second AdvanceBio SEC column from a different stationary phase lot, the performance over time was monitored. A sequence was devised consisting of 25 consecutive injections, including:

- Blank (mp)
- BioRad GFS protein standard
- BSA–MYO standard.

This sequence was repeated 12 times for a total of 300 injections over 6 days, and the performance of the column was compared across sequences.

Figure 3 shows the consistency of the resolution for BSA-MYO over the duration of the 300 injection experiment.

Table 2. Comparison of resolution values for BSA-MYO.

	Agilent AdvanceBio SEC (2.7 µm, 300 Å, 7.8 × 300 mm)	Agilent ProSEC 300S (5 µm, 300 Å, 7.5 × 300 mm)
BSA Dimer – BSA Resolution	2.64	2.16
BSA–MYO Resolution	6.28	5.49

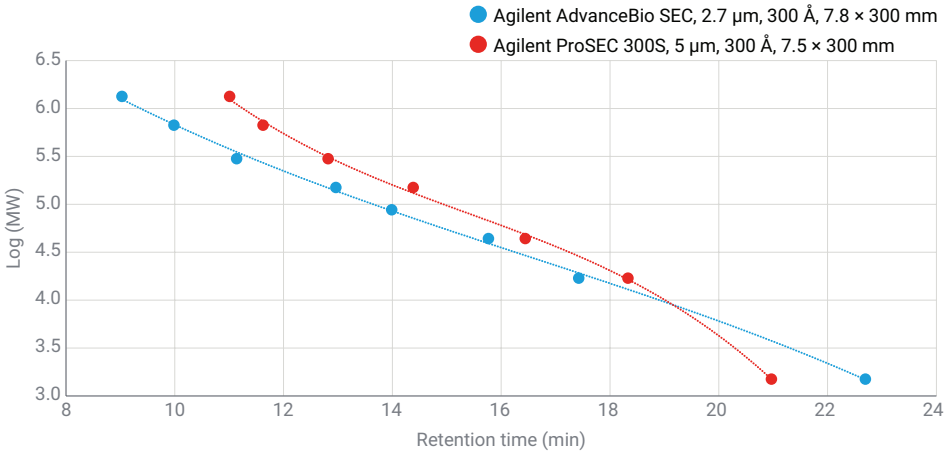

Figure 2. Calibration curves based on protein retention times.

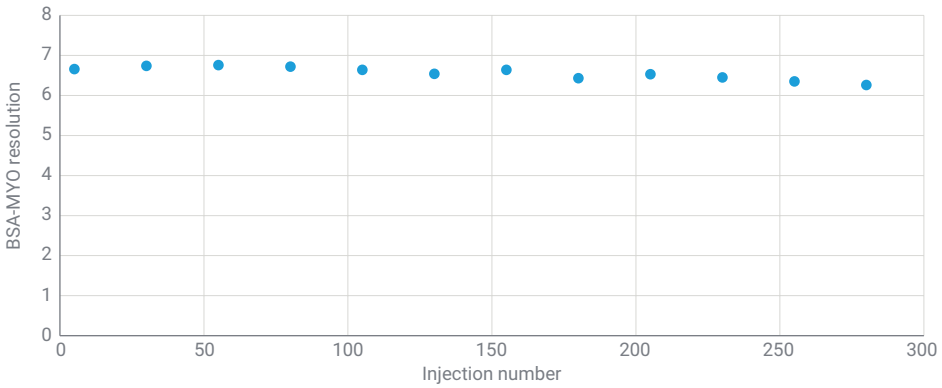

Figure 3. BSA-MYO resolution during a 300 injection sequence.

Figures 4A and 4B show chromatograms taken from the start and end of the sequence, demonstrating the robustness of the analysis.

Results are also comparable to those from the first AdvanceBio SEC column, showing good lot-to-lot consistency.

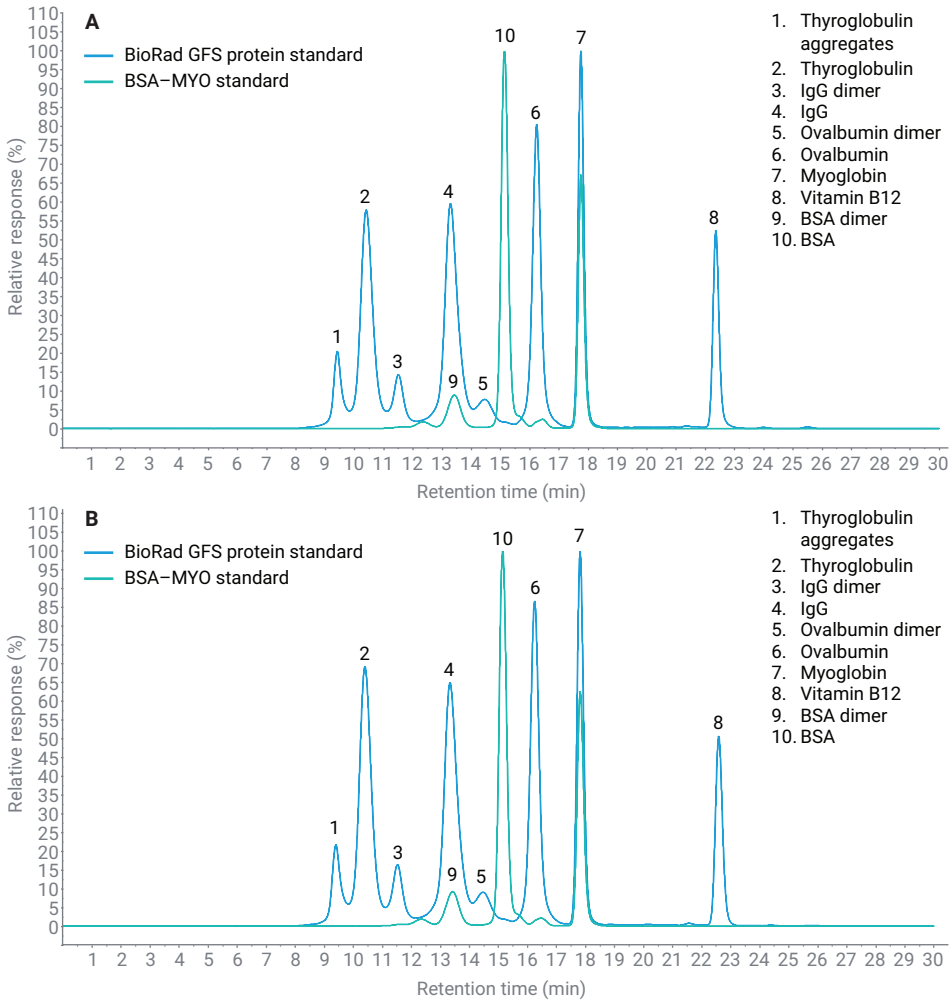

Figure 4. Overlay of chromatograms from the Agilent AdvanceBio SEC column for BioRad GFS protein standard (peaks 1 to 8) and BSA–MYO system suitability standard (peaks 9 and 10) at the start (A) and end (B) of a prolonged 300-injection sequence.

Figures 5A and 5B show the chromatogram of asparaginase, which has an expected profile. The main peak, 2, corresponds to the tetrameric form of the ASNase enzyme. Peak 4 is expected to be the monomeric form, Peak 1 is expected to be aggregated material, and Peak 3 is unknown, possibly a dimeric form.

To confirm these assumptions, these data points were plotted onto the calibration curve previously obtained from the protein standards (Figure 6).

Unexpectedly, the asparaginase peaks elute later than expected (i.e., the molecule is behaving as if smaller in hydrodynamic radius) compared to other globular proteins contained in the standards tested. It was therefore decided to use a light scattering detector as well as a UV detector for further analysis.

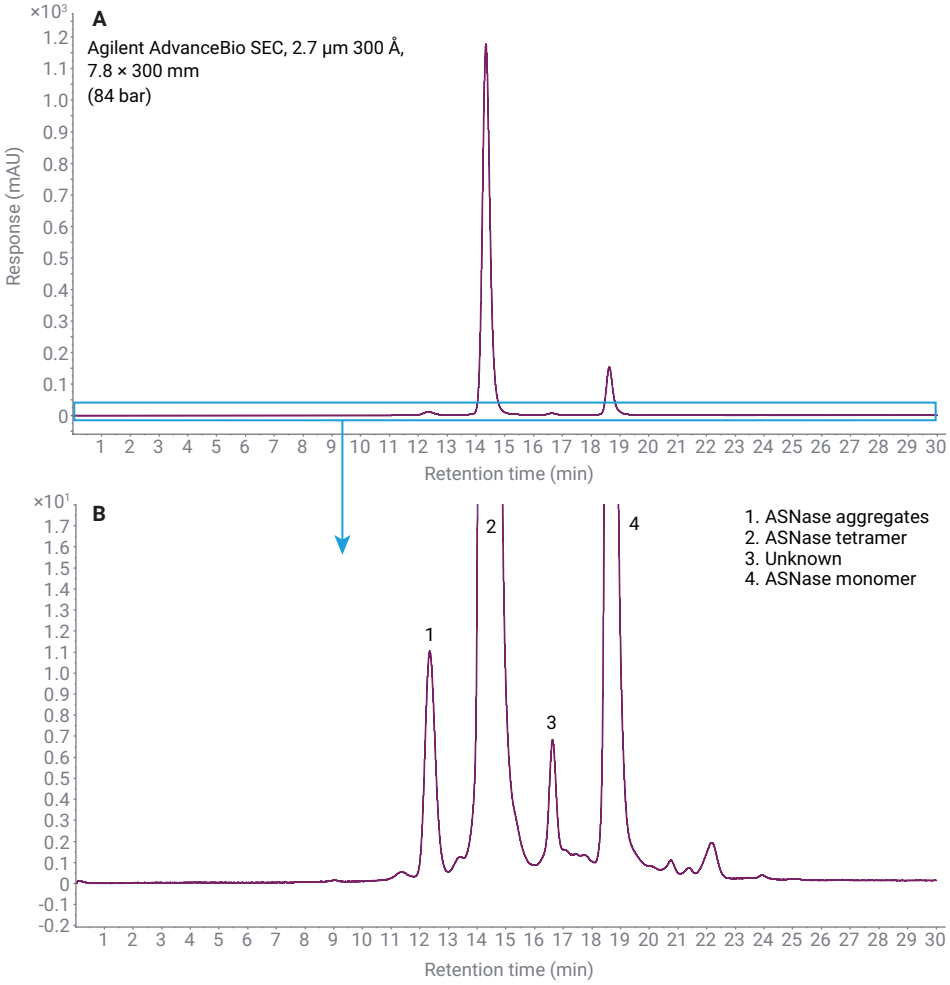

Figure 5. (A) Chromatogram of asparaginase on an Agilent AdvanceBio SEC column. (B) Enlarged region.

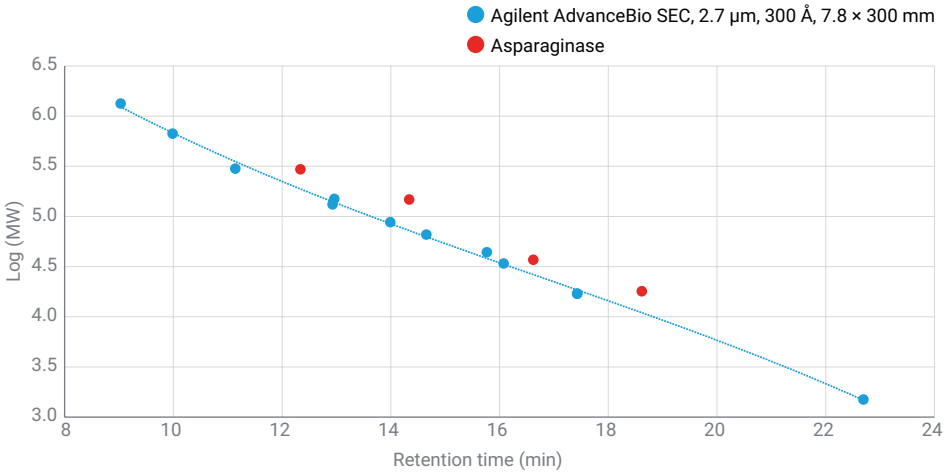

Figure 6. Calibration curve based on protein standards (same as Figure 2) with asparaginase data points overlaid.

Figure 7 shows the outcome of the light scattering analysis, comparing the AdvanceBio SEC column with the ProSEC 300S column.

The response from the AdvanceBio SEC column enabled molecular weight analysis using the light scattering detector signal in conjunction with the UV detector signal. Unfortunately, the light scattering signal from the ProSEC 300S was too noisy to enable a comparable analysis.

Using the GPC/SEC software, the molecular weight of the main peak was determined to be 139 kDa. This calculation was made with a specific refractive index increment (dn/dc) value of 0.186 and a theoretical UV extinction coefficient ($\varepsilon^{0.1\%}$ 280 nm) of 0.64. This molecular weight is in excellent agreement with the expected value for a molecule containing four 37 kDa subunits.

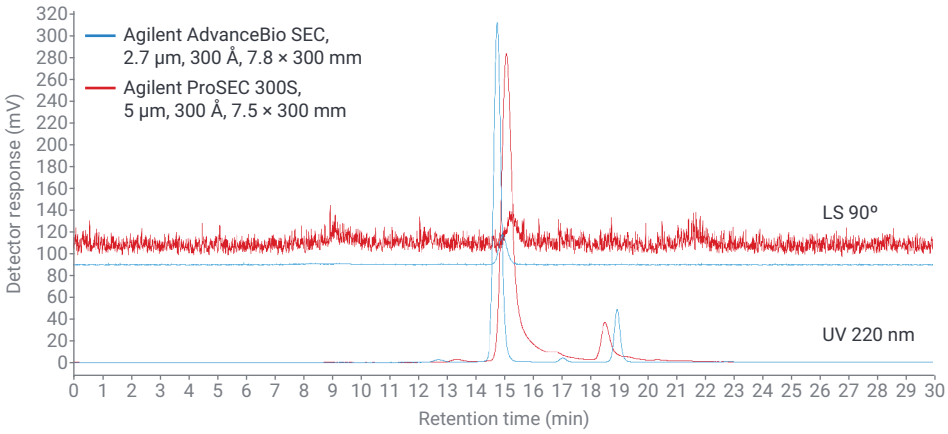

Figure 7. Light scattering (LS) detector analysis of asparaginase on an Agilent AdvanceBio SEC column.

Conclusion

In this application note, the suitability of the Agilent AdvanceBio SEC column as an effective and improved alternative to the Agilent ProSEC 300S column for the aggregate analysis of L-asparaginase, a biotherapeutic enzyme, has been demonstrated.

The combination of smaller particle size and increased pore volume leads to a significant increase in resolution with only a modest increase in column operating pressure. Furthermore, excellent and consistent performance over a period of prolonged use (300 injections over 6 days) as well as improved performance in conjunction with light scattering detection was demonstrated.

References

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