

Column Length Effect on Separation and Resolution

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Abstract

Size exclusion chromatography (SEC) is a commonly used technique for separating aggregates from properly folded proteins. As protein yields grow, so does the importance of removing impurities (aggregates). A helpful characteristic of SEC is that column length has an impact on degree of separation and the relationship between the two can be leveraged to improve protein purity and/or yield. Here, we provide proof of principle by coupling two ENrich SEC 650 Columns in a straightforward SEC experiment separating gamma globulin from its dimer.

Introduction

Size exclusion chromatography (SEC) separates molecules based on size via gel filtration. This technique uses spherical beads that contain pores of a specific size distribution. Smaller molecules will diffuse into the pores to varying depths according to their size. Large molecules, however, cannot enter the pores and are instead eluted into the void volume of the column. As a result, molecules separate as they flow through a column and will elute in order of decreasing molecular weight (MW).

Due to the nature of SEC separations, a target protein should not interact with the chosen resin matrix. If a protein of interest elutes at a smaller-than-expected size, this is indicative of an unwanted interaction with the resin matrix and can result in a lower purity and/or yield. It is possible that a different resin matrix may be required, depending on the target protein's properties.

To make sure high target purity is achieved, complete resolution between chromatogram peaks is essential. Occasionally, this degree of resolution is not met and the separation parameters need to be optimized. There are several techniques that can help to improve separation, such as optimizing buffer conditions, flow rates, or internal system volume (flush volume). It has also been shown that peak resolution improves as column length increases. Herein, we show that increasing the length of a Bio-Rad ENrich SEC 650 Column by running two columns in tandem significantly improves peak resolution and, therefore, target purity.

Materials and Methods

The Bio-Rad Gel Filtration Standard (catalog #1511901) was rehydrated with 0.5 ml of deionized H₂O. A 0.125 ml aliquot of the standard was diluted to 500 µl with 1x phosphate buffered saline (PBS, #1610780). A Bio-Rad ENrich SEC 650 Column (#7801650) was equilibrated with 1x PBS and the gel filtration standard was injected using a 500 µl injection loop at a flow rate of 1 ml/min using a Bio-Rad NGC Quest 10 Plus System with column switching valve (CSV, #7880003). The standards were eluted with 30 ml of 1x PBS at 1 ml/min for each individual column. The two columns were then coupled using a PEEK Column Coupler, Male/Male – ½ inch (IDEX, #U-288) and equilibrated with 1x PBS at a flow rate of 1 ml/min. A 500 µl standard was injected onto the coupled columns and eluted with 60 ml of 1x PBS.

Results and Discussion

A significant improvement in peak resolution was seen with the increase in column length (Figure 1). The shoulders seen on the first main peak (thyroglobulin) are clearly resolved in the coupled column run. Resolution factor, a critical indicator of success in this experiment, is defined as the difference between peak retention times divided by average peak width:

$$R = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(w_1 + w_2)}$$

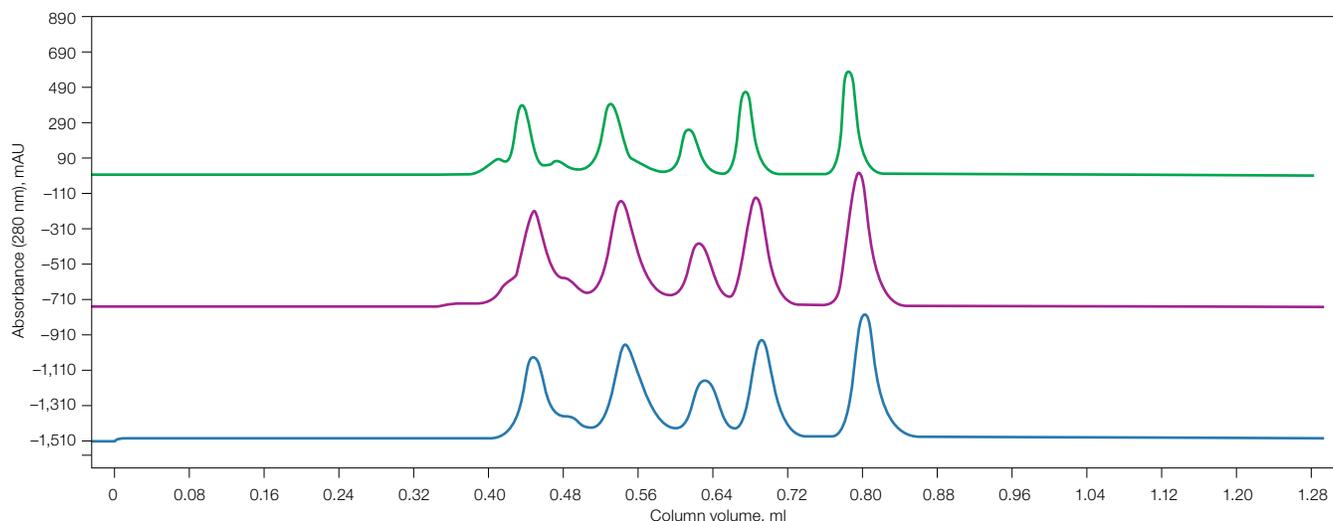


Fig. 1. Chromatogram overlay showing full resolution of the first peak in the coupled column run. The tandem SEC columns (—, top) show a clear increase in resolution on the initial peak shoulders compared to the individual column runs (—, middle; —, bottom), which display poor resolution between the peak shoulders. Chromatograms are normalized to column volume.

Table 1. Recorded performance data from experimental runs.

	SEC Column 1	SEC Column 2	SEC Column 1 + 2
Gamma Globulin			
Retention time, min	12.92	12.80	25.06
Peak width, ml	2.13	2.07	4.18
Gamma Globulin Dimer			
Retention time, min	11.42	11.37	22.33
Peak width, ml	1.24	1.20	1.61
Resolution factor	0.89	0.87	0.94

While the individual columns demonstrated a resolution factor between gamma globulin and its dimer of 0.89 and 0.87, when the columns were coupled the resolution factor showed an increase to 0.94 (Table 1). Attaining this improvement did not require extensive method optimization, but rather resulted from a simple and straightforward solution.

Conclusion

SEC continues to be a popular method for protein purification as well as size determination. It is a common step in antibody purification as elution from Protein A requires low pH and often produces protein aggregates. Aggregates need to be removed prior to downstream processes to ensure the highest purity of the target protein. Therefore, SEC is a standard last step to separate properly folded protein from high-MW aggregates, as well as to ensure protein is in the appropriate conditions for future analysis.

By coupling two high-resolution SEC columns, we were able to improve chromatogram peak resolution, which resulted in higher purity without drastically increasing method time or reducing the flow rate. This simple procedure can be used when an increase in resolution of two similar peaks is desired and alternative method optimizations would prove to be too time consuming.

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