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Application News

High Performance Liquid Chromatograph
Prominence™ Inert HPLC System, LH-40

Seamless Process from Protein Purification to Evaluation

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User Benefits

- By choosing target peaks after fractionation, appropriate fractions can be automatically reinjected for analysis.
- Equipped column-switching valve allows automatic column switching between purification and analysis.
- It is also convenient for comparing several samples to optimize culture conditions.

■ Introduction

Size exclusion chromatography (SEC) is one technique used to separate molecules based on size, and it is one of the main methods used for verification of protein multimer formation. However, this technique can not distinguish proteins from ones with similar molecular sizes. Because smaller molecules can enter pores of materials packed in an SEC column, but larger molecules cannot. Therefore, larger molecules elute earlier than smaller molecules. In the case of target proteins in serum or culture supernatant, the samples have to be purified before SEC analysis. This article describes a seamless process of target proteins for purification, fractionation, and re-injection utilizing a liquid handler.

■ Seamless Analysis with Liquid Handler

The liquid handler (LH-40) serves as an autosampler as well as a fraction collector for the LC system. That means samples fractionated during the first run can be injected directly for the second run without transferring them from a fraction collector to an autosampler. For example, with this system (Fig. 1 and 2), the target protein is purified by an affinity column and fractionated as the first step, and then the fractionated protein elution is re-injected for SEC analysis as the second step. These two steps can be performed simply by specifying the method and fraction.

In this example, we evaluated IgG in human plasma using an LH-40 liquid handler installed in a Prominence inert LC system.

■ Analytical Conditions and Samples

5 mL of commercial human plasma EDTA-2Na was placed in a 15 mL tube, diluted 5-fold using a 10 mmol/L (sodium) phosphate buffer (pH 6.9, mobile phase A), and placed in the liquid handler rack. Then the sample was purified by affinity chromatography using an IgG purification column, based on the conditions in Table 1. The fractions were collected in a 96-well deep well plate placed in the liquid handler. In this case, 100 µL of the fraction around the peak top was analyzed by SEC based on the conditions in Table 2.

Table 1 Analytical Conditions for Affinity Purification

Column	: HiTrap™ rProtein A FF (1 mL, Cytiva)
Mobile Phase A	: 10 mmol/L (sodium) phosphate buffer (pH 6.9)
Mobile Phase B	: 100 mmol/L (sodium) citrate buffer (pH 4.0)
Time Program (B. Conc.)	: 0% (0 – 10 min) → 100% (10.01 - 20 min) → → 0% (20.01 – 35 min)
Flowrate	: 1.0 mL/min
Column Temp.	: 15 ˚C
Injection Volume	: 5 mL
Detection	: 280 nm (SPD-20A)
Flow Cell	: Inert flow cell

Table 2 Analytical Conditions for SEC

Column	: Shim-Pack™ Bio Diol-300*1 (300 mm × 4.6 mm I.D., 5 µm)
Guard Column	: Shim-Pack Bio Diol-300 (G)*2 (30 mm × 8.0 mm I.D., 5 µm)
Mobile Phase A	: 10 mmol/L (sodium) phosphate buffer (pH 6.9)
Flowrate	: 0.5 mL/min
Column Temp.	: 15 ˚C
Injection Volume	: 100 µL
Detection	: 280 nm (SPD-20A)
Flow Cell	: Inert flow cell

*1: P/N 227-31010-04, *2: P/N 227-31010-06

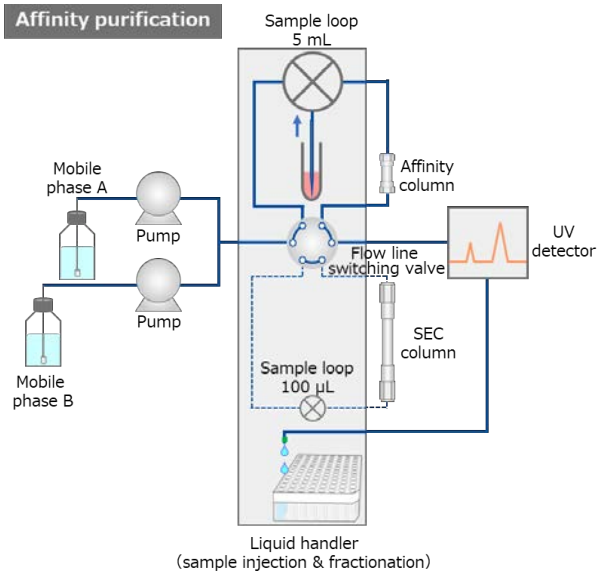

Fig. 1 Flow Path Diagram of Affinity Purification

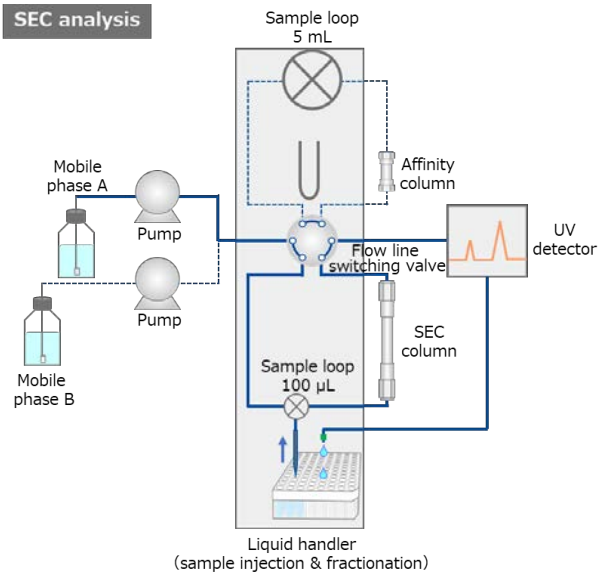

Fig. 2 Flow Path Diagram of SEC Analysis

■ Results of Affinity Purification

5 mL of the diluted human plasma sample was injected into the IgG purification column after being equilibrated with mobile phase A. Then the non-specific adsorbates were washed out with mobile phase A (Fig. 3). Finally, IgG was eluted by mobile phase B (Fig. 4).

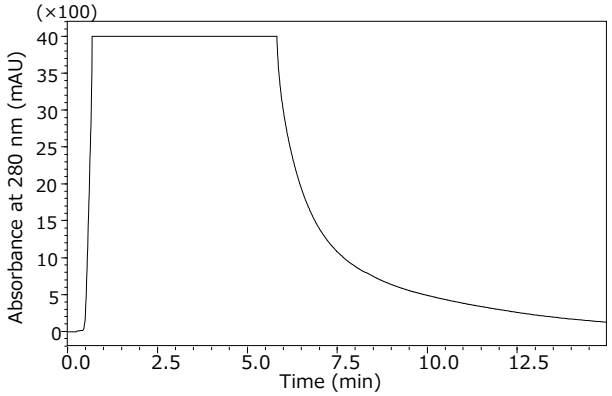

Fig. 3 Chromatogram of Binding Section during Affinity Purification

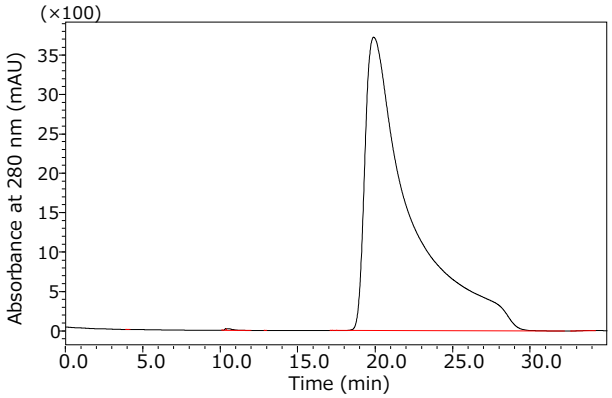

Fig. 4 Chromatogram of Elution during Affinity Purification

■ Results of SEC Analysis

100 µL of the fraction around the peak top eluted by the affinity purification as the first step was re-injected and evaluated by the SEC analysis as the second step. The results are shown in Fig. 5. In the SEC analysis, a main peak (Peak 2) and a broad peak (Peak 1) before Peak 2 were detected. To briefly estimate the molecular weight of the protein from the peaks obtained by SEC analysis, the mixture of thyroglobulin (669 kDa), aldolase (158 kDa), and ovalbumin (44 kDa) and the mixture of ferritin (440 kDa) and conalbumin (75 kDa) were evaluated as standard proteins by SEC (Fig. 6). The elution times of the protein standard solutions indicate that almost all the purified IgG from human plasma existed as monomers.

■ SDS-PAGE Analysis

All fractions from affinity purification and SEC analysis were collected in a 96-well deep well plate. The peak-top fractions were evaluated by SDS-PAGE analysis (non-reducing and reducing). The peak-top fractions collected from both affinity purification and SEC analysis showed bands at the same position as standard human IgG (Fig. 7). Based on the results, both peaks 1 and 2 in SEC were IgG. Structurally similar 4 sub-classes of IgG in human blood could give broadened peak shapes due to the small differences in molecular size and/or structure.

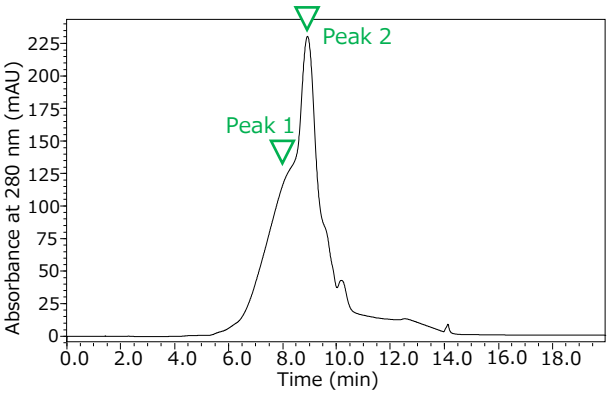

Fig. 5 Chromatogram from SEC Analysis

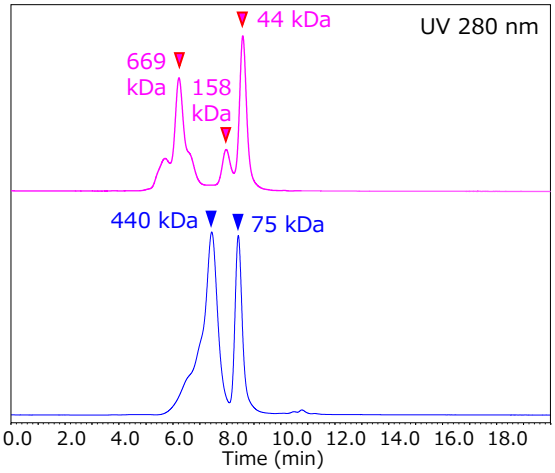

Fig. 6 SEC Chromatograms of Standard Proteins

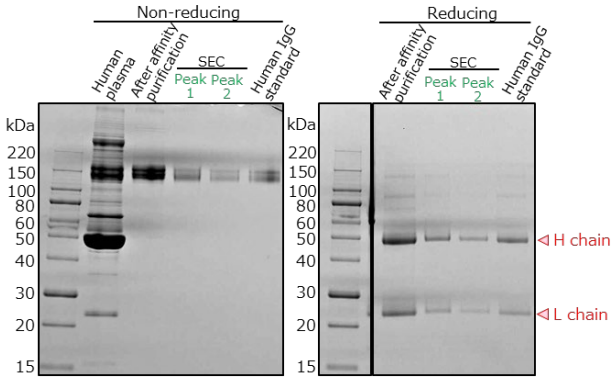

Fig. 7 SDS-PAGE Analysis

■ Conclusion

The liquid handler (LH-40) installed in the LC system provided a seamless process from purification to analysis and required simply placing the sample in the liquid handler. For routine work with prespecified targets, it can be used to analyze only the target fractions. By adding one more column switching valve and increasing the number of columns, the system can also be used to screen purification parameters or purify samples in multiple steps. After fractions are collected in a 96-well plate, they can be used directly for SDS-PAGE, ELISA, or various other analytical methods.

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