Analysis method of Aggregates and Additives in Antibody Drugs

1. Introduction

Antibody drugs are important potent therapeutic drugs. Because of their highly specific targeting capacity to the cancer cells, it can cause less side effects. However, it is known that they may aggregate to form dimers and other larger aggregates during manufacturing process and/or during storage period. The larger aggregates have concerns of producing antibodies or becoming immunogenic conjugates that have properties of eliciting cell immune responses inside the body. Therefore, monitoring of such aggregates are important for quality control (QC) purposes. Generally, silica-based aqueous size exclusion chromatography columns are used for the separation of aggregates. Shodex™ PROTEIN LW-803 is one of them and demonstrates high separation capability for the purpose. This application shows the effectiveness of the LW-803 by comparing it to other columns. IgG (molecular weight (MW) about 150,000) and Bovine serum albumin (BSA, MW 66,500) were analyzed for the comparison. The effects of injection volume for the resolution of polyclonal IgG was also tested.

During the production of antibody drugs, small amounts of surfactants may be added to solubilize or stabilize IgG, knowing the concentration of such surfactants are important QC factors. Quantification of surfactants can be difficult as they exist as complex matrixes and have low UV absorption. Therefore, by using a polymer-based column, Shodex™ ODP2 HP-2D, we developed a rapid LC/MS method for quantifying polysorbate (Tween) in the presence of IgG and NaCl as matrixes, without the need of sample pretreatment.

2. Separation performance of LW-803

2-1. Comparison of four columns for analysis of IgG

Table 1 shows the analytical conditions used. Test samples were prepared by dissolving polyclonal IgG from human serum or monoclonal IgG from recombinant CHO cell in the eluent. Fig. 1 shows the chromatograms of polyclonal IgG by injecting 5 µL of 10 mg/mL sample. Monomer as well as dimer and even larger aggregates were detected.

Table 1. Analytical conditions

Columns	Shodex™ PROTEIN LW-803 (8.0 mm I.D. x 300 mm, 3 µm) Shodex™ PROTEIN KW-803 (8.0 mm I.D. x 300 mm, 5 µm) Column A from other company (7.8 mm I.D. x 300 mm, 4 µm) Column B from other company (7.8 mm I.D. x 300 mm, 2.7 µm)
Eluent	50 mM Sodium phosphate buffer (pH 7.0) + 0.3 M NaCl
Flow Rate	1.0 mL/min
Detector	UV (280 nm)
Column Temp.	Ambient

LW-803 demonstrated a superior separation efficiency between monomer and dimer compared to KW-803 and columns from other company recommended for the analysis of antibodies.

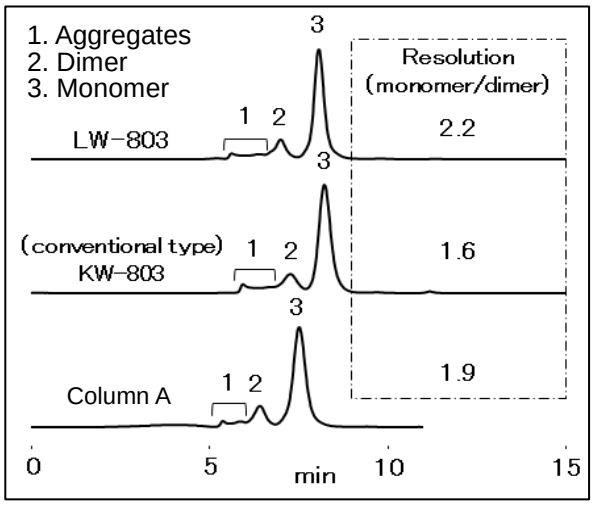

Fig. 1 Chromatograms of polyclonal IgG

Next, monoclonal IgG was analyzed by injecting 20 µL of 1 mg/mL sample (Fig. 2). Similar to polyclonal IgG analysis, the monomer, as well as the dimer, trimer, and larger aggregates were detected. Table 2 summarizes the resolution between monomer and dimer and the resolution between dimer and trimer. Again, LW-803's separation capability for both aggregate pairs was proved to be superior.

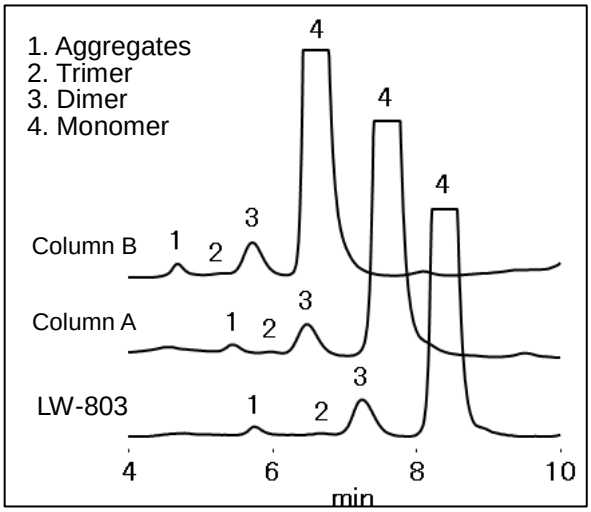

Fig. 2 Chromatograms of monoclonal IgG

Table 2. Resolutions between each peak

	Monomer / Dimer	Dimer / Trimer
Column B	2.1	2.6
Column A	2.5	2.4
LW-803	2.6	3.4

2-2. Comparison of three columns for analysis of BSA

Table 3 shows the analytical conditions used. Fig. 3 shows the chromatograms of BSA by injecting 5 µL of 2 mg/mL sample. The monomer, dimer and trimer were detected.

Table 3. Analytical conditions

Columns	Shodex™ PROTEIN LW-803 (8.0 mm I.D. x 300 mm, 3 µm) Column C from other company (7.8 mm I.D. x 300 mm, 3 µm) Column D from other company (7.8 mm I.D. x 300 mm, 5 µm)
Eluent	50 mM Sodium phosphate buffer (pH 7.0) + 0.3 M NaCl
Flow Rate	1.0 mL/min
Detector	UV (280 nm)
Column Temp.	25 ℃

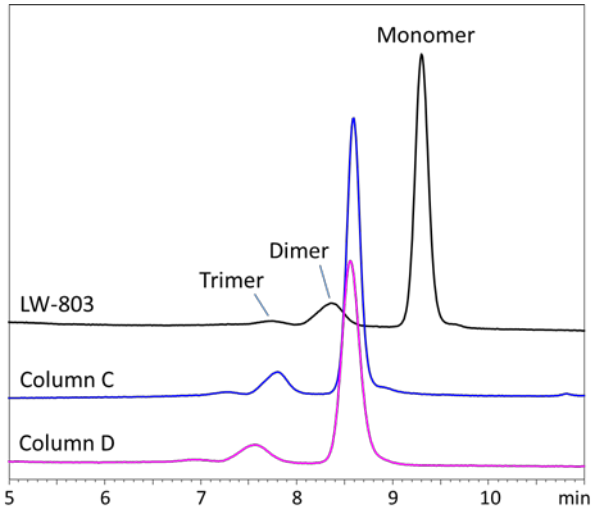

Fig. 3 Chromatograms of BSA

Fig 4 depicts a superimposed chromatogram of the above three analysis by aligning the BSA monomer peak of each.

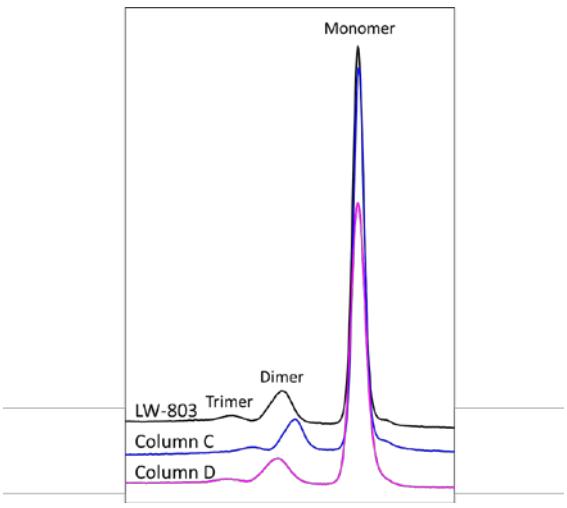

Fig. 4 Superimposed chromatograms of BSA

Theoretical plate numbers (TPNs) and resolutions between monomer and dimer for each column are summarized in Table 4. LW-803 had the highest resolution over other columns.

Table 4. TPNs and resolutions (monomer/dimer) for the analysis of BSA

	RT (min)	TPN	Rs (M/D)
LW-803	9.30	17478	2.33
Column C	8.59	14986	2.07
Column D	8.56	8594	2.07

2-3. Effects of injection volume for the analysis of IgG

Between 1 and 100 µL of 10 mg/mL polyclonal IgG was injected to LW-803. The chromatograms and the graph showing the relationships between the injection volume vs. TPNs and resolutions between monomer and dimer are presented in Fig. 5.

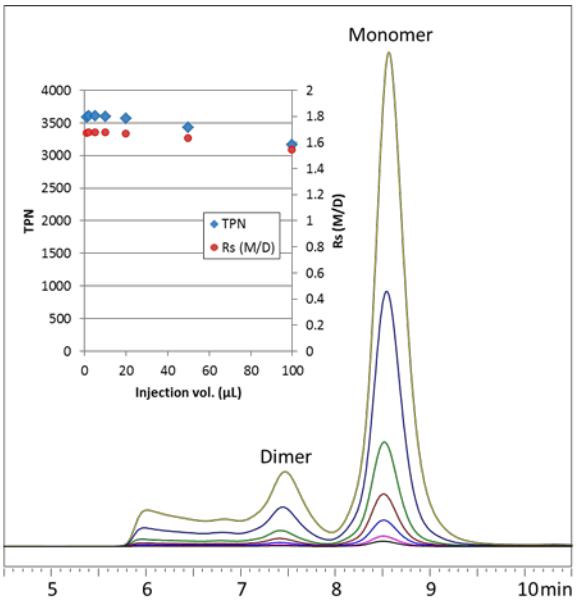

Fig.5 The effects of sample injection volume

The separation efficiency was consistent among all injection volumes tested in the range of 1-20 µL. It also demonstrated the method's capability for handling larger injection volume (100 µL, absolute sample loading of 1 mg) as there was no significant decrease in its separation efficiency (resolution larger than 1.5). The resolution obtained in this experiment (2-3) was lower than the data obtained in 2-1. This is due to the differences in the analytical systems being used. It is worth noting that the system's dead volume may affect the resolution.

3. LC/MS analysis of surfactant in antibody drugs

The analytical conditions used can be found in Table 5. Shodex ODP2 HP-2D is packed with polyhydroxy methacrylate gels. Unlike regular ODS columns, ODP2 HP is highly hydrophilic and prevents proteins to be retained inside the column. Hydrophobic interaction which is typically the cause of protein retention can be suppressed almost fully by using alkaline eluent. Also, ODP2 HP's small pore size (40Å) adds size exclusion effect that helps eluting proteins at V_0.

Table 5. Analytical conditions

Column	Shodex™ ODP2 HP-2D (2.0 mm I.D. x 150 mm, 5 µm)
Eluent	(A) 0.1% Ammonia aq. (B) CH_3CN
Gradient Program	(B%) 20% (0 to 5 min), 20 to 90% (5 to 19 min), 90% (19 to 24 min), 90 to 20% (24 to 25 min)
Flow Rate	0.2mL/min
Detectors	UV (280 nm) ESI-MS (SIM Positive)
Column Temp.	40 ℃

Using those features, ODP2 HP can completely separate IgG from surfactants that are retained by reverse phase mode. In order to prevent IgG from entering the MS, a flow-switching valve was placed after the UV detector. The column eluate obtained between 0 and 5 minutes was collected in a waste bottle and the eluate obtained after 5 minutes was injected into the MS.

Tween was selected as a test surfactant in this application as it is often added in antibody drugs. Tween consists a sorbitan backbone, polymers of ethylene oxide (EO), and long chain fatty acids connected by ester bonding (Fig. 6).

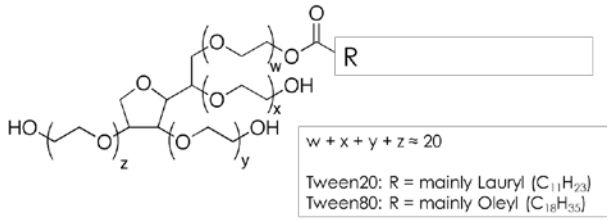

Fig.6 Structure of Tween

Tween does not exist as a single compound, but exists in several different forms. Therefore, the most abundant component was selected by pre-scanning. Quantification curve was prepared using the result obtained by MS (SIM mode) measurement of the selected ion. Among available Tween standards, Tween 20 and Tween 80 were used for this analysis. As a test sample, a mixture containing 100 mg/L Tween 20 or Tween 80, 1 g/L polyclonal IgG, and 0.3 mol/L NaCl was prepared. Injection volume of 2 µL was used throughout the experiment.

3-1. Analysis of Tween 20

Fig 7 (blue) represents the SIM chromatogram of Tween 20. Ions at m/z 735.7 was monitored. This ion is assumed to be a divalent ion consisting a sorbitan backbone, 20 or more EO units, glycerol diesters (MW 1435) of C14 and C12 with either one having a double bond, and two ammonium ions. Two chromatograms, one includes IgG and NaCl (Fig. 7-a) and the other that does not include IgG and NaCl (Fig. 7-b) demonstrated very similar results, showing that there was minimum ion-suppression effect. Good linearity was obtained for the quantification curve of the peak of 11.5 min derived from Tween 20 (Fig. 8).

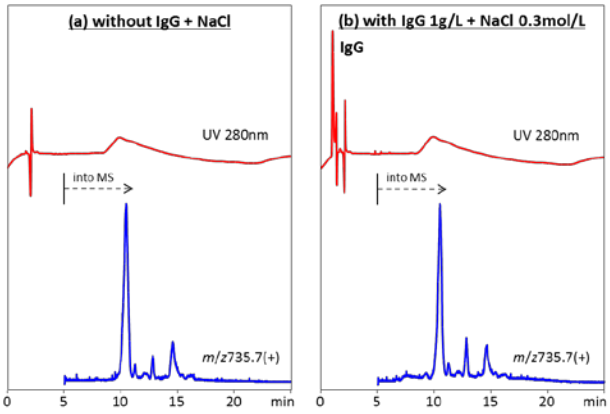

Fig. 7 SIM chromatogram of Tween 20: (a) with and (b) without 1 g/L IgG and 0.3 mol/L NaCl.

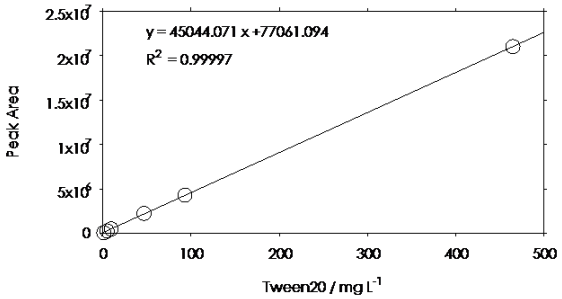

Fig. 8 Calibration curve of Tween 20

3-2. Analysis of Tween 80

Tween 80 was analyzed using the same method as for Tween 20. Ion at m/z 848.8 was monitored (Fig. 9). This ion is assumed to be a divalent ion consisting a sorbitan backbone, 28 or more EO units, glycerol monoester (MW 1661) of C18 with a double bond, and two ammonium ions. Consistent with Tween 20's analysis result, the chromatograms of samples containing with and without IgG and NaCl were very similar, showing the minimum ion suppression. Good linearity was obtained for the quantification curve of the peak of 13.1 min derived from Tween 80 (Fig. 10).

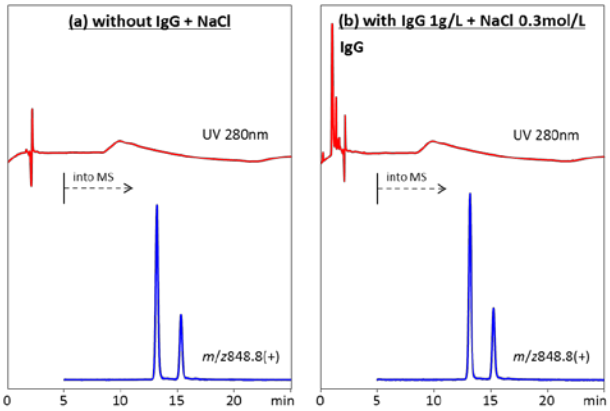

Fig. 9 SIM chromatogram of Tween 80: (a) with and (b) without 1 g/L IgG and 0.3 mol/L

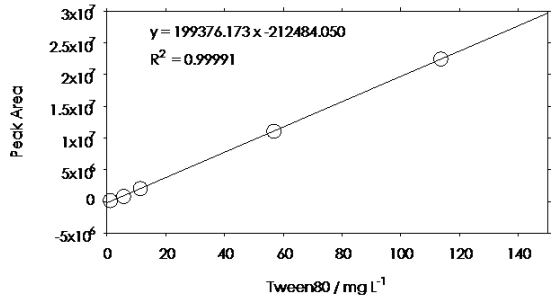

Fig.10 Calibration curve of Tween 80

4. Conclusions

Shodex™ PROTEIN LW-803 demonstrated its superior separation capability for IgG monomers and its aggregates. It also has high separation capability including lower molecular weight ranges. This allows the analysis of anti-body metabolites and other proteins. Moreover, the column is capable of handling high loading volumes. Therefore, LW-803 has a wide usability for the separation of not only antibody aggregates but for other proteins.

On the other hand, Shodex™ ODP2 HP-2D proved its separation capability of analyzing non-ionic surfactant, Tween, in the presence of IgG and NaCl, without the necessity of deproteinization nor desalting sample pretreatment. Here, alkaline eluent is effective for eluting IgG at V_0, which can be only done by polymer-based reverse phase columns such as ODP2 HP that are durable against alkaline conditions. The method developed in this application is fast and high-sensitive for the analysis of Tween which has potentials in antibody drug QC analysis. Even faster analysis is expected by using a shorter column (50 mm), ODP2 HP-2B, coupled with MS.