High Resolution Analysis of Monoclonal Antibody Heterogeneity by Size Exclusion Chromatography and Hydrophilic Interaction Chromatography

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Introduction

- Monoclonal antibodies (mAbs) bind specifically to target molecules and therefore they are expected to be more effective and less harmful in disease treatments compared to low molecular weight drugs.
- mAbs may undergo structural modifications and become heterogeneous during production, storage, and transportation.
- The heterogeneity of mAbs results from various processes: aggregation, fragmentation, truncation of C-terminal lysine residues, deamidation of asparagine residues, oxidation of methionine residues, and formation of glycosylation variants. These variants may impact therapeutic effect or safety.
- Thus, the determination of heterogeneity in therapeutic mAbs is essential for pharmaceutical development and quality control.
- mAb heterogeneity can be examined in detail by HPLC.
- Here we report :
    - the separation of mAb monomer from impurities with different molecular sizes by the use of size exclusion chromatography (SEC) columns with high resolving power
    - the separation of oligosaccharides released from the polypeptide chains of mAbs by the use of hydrophilic interaction chromatography (HILIC) columns, so that the heterogeneity in the glycosylation pattern of mAb was evaluated.

Experimental

Experimental

Materials

- Disodium hydrogenphsophate 12-water, potassium dihydrogen phosphate, sodium azide, p-aminobenzoic acid, acetic acid, triethylamine, and HPLC grade acetonitrile were obtained from Wako Pure Chemical Industries (Osaka, Japan).
- Thyroglobulin, γ-globulin, ovalbumin, and ribonuclease A were obtained from Sigma-Aldrich (St. Louis, MO, USA).
- PA-glucose oligomer (3-22 mer) was obtained from TaKaRa Bio (Shiga, Japan).
- Water was purified with the Milli-Q® system (Merck Millipore, Darmstadt, Germany).
- mAb-1: mouse monoclonal antibody, ca. 20 g/L
- mAb-2: human monoclonal antibody, ca. 5 g/L

Instrumentation

- Tosoh liquid chromatograph equipped with pump (DP-8020), column oven (CO-8020-C), UV detector (UV-8020), and data processor (LC-8020 model II)
- Fluorescence was detected with Waters 2475 multi λ fluorescence detector (Waters, Milford, MA, USA)

Experimental

Release of *N*-linked oligosaccharides with PNGase F

- Each of the SEC-fractionated mAb monomers was concentrated and buffer-exchanged into digestion buffer (100 mmol/L ammonium bicarbonate, pH 8.6) with Amicon® Ultra-0.5 100 K device (Merck Millipore) using the procedure described by the manufacturer, to yield ca. 5 g/L of mAb monomer solution.
- 40 µL of mAb monomer solution (ca. 200 µg) was added with 20 µL of water and 2 µL (10 mU) of PNGase F, and incubated at 37 ºC for 24 hr to release *N*-linked oligosaccharides from polypeptide chains.
- Added 50 µL of 100 mmol/L ammonium acetate, pH 4.0, and incubated at 37 ºC for 60 min to liberate ammonia from glycosylamines, to yield 112 µL of reaction mixture.
- From 50 µL of the reaction solution, the released oligosaccharides were purified with a TaKaRa cellulose cartridge glycan preparation kit (TaKaRa Bio) using the procedure described by the manufacturer.
- Dried overnight *in vacuo* at room temperature.

Experimental

Fluorescence labeling of oligosaccharides

- The dried oligosaccharides were fluorescence-labeled with 2-aminopyridine following the protocol in the TaKaRa pyridylamination manual kit (TaKaRa Bio).
- The labeled oligosaccharides were purified with a TaKaRa cellulose cartridge glycan preparation kit using the procedure described by the manufacturer.
- Dried overnight *in vacuo* at room temperature.
- Dissolved in 100 µL of water to yield a sample solution.
- The sample solution was diluted 100-fold with 80% acetonitrile and analyzed by HILIC-FL.

Experimental

Size exclusion chromatography (SEC)

- Column: TSKgel® SuperSW mAb HR, 4 µm, 7.8 mm ID × 30 cm (Tosoh)
- Mobile phase: 200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
- Flow rate: 1.0 mL/min
- Detection: UV @ 280 nm
- Temperature: 25 ^oC

Hydrophilic interaction chromatography (HILIC)

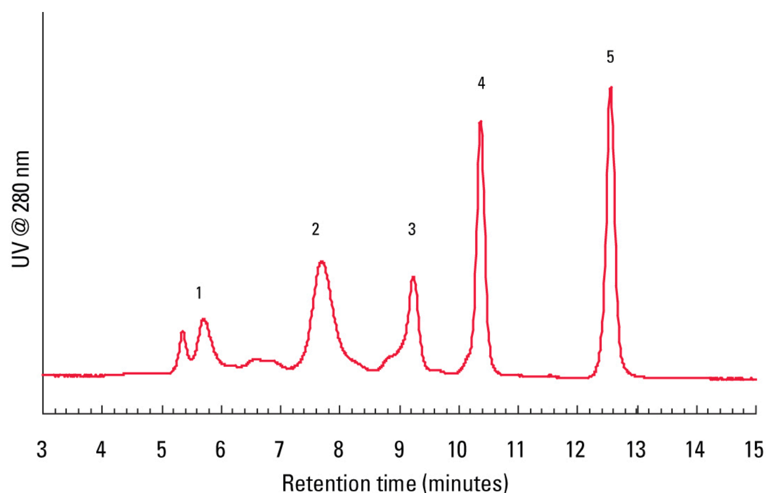
- Column: TSKgel Amide-80, 3 µm, 2.0 mm ID × 15 cm (Tosoh)
- Mobile phase: A: 200 mmol/L acetic acid-triethylamine, pH 7.3
  B: acetonitrile
- Gradient: 75% B (0-5 min), 75-50% B (5-80 min, linear)
- Flow rate: 0.5 mL/min
- Detection: Fluorescence (ex. 315 nm, em. 380 nm)
- Temperature: 40 ^oC

Characteristics of the SEC Columns

Table 1: Basic Properties of the SEC Columns

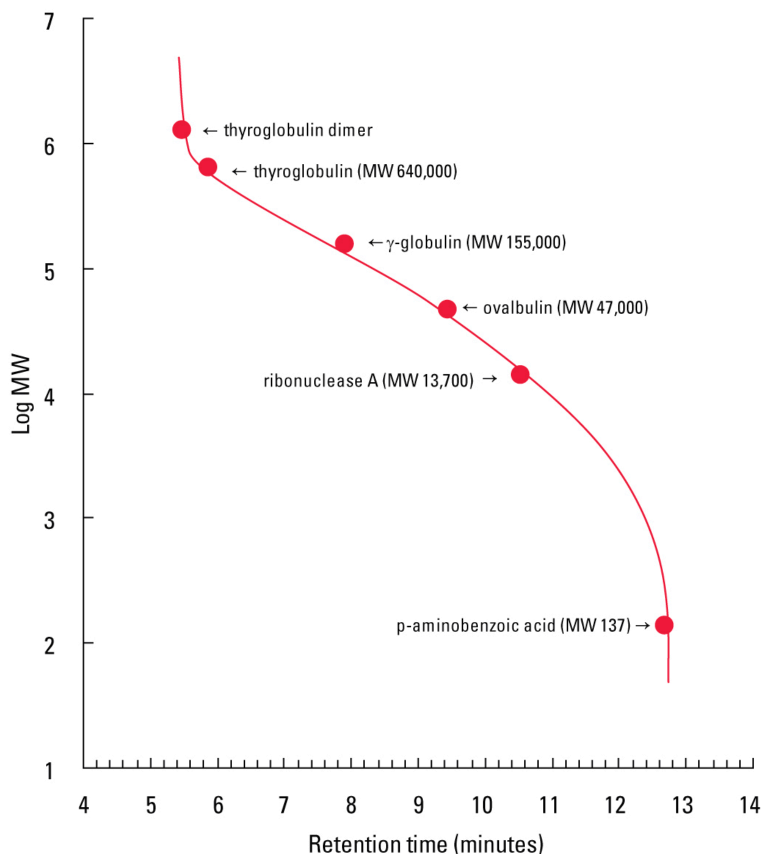
TSKgel SuperSW mAb HR	
Dimensions	7.8 mm ID × 30 cm
Base material	Silica gel
Functional group	Diol
Particle size	4 μm
Pore size	25 nm
Separation range	10,000-500,000 Da (for globular proteins)
Features	Superior resolving power compared to Tosoh's conventional SEC column, TSKgel G3000SWXL; Compatible with conventional LC systems.
Applications	Separation of mAb dimer/monomer/fragments with high resolution

Figure 1: Chromatograms of Standard Proteins



Column: TSKgel SuperSW mAb HR, 7.8 mm ID × 30 cm
Mobile phase: 200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate: 1.0 mL/min
Detection: UV @ 280 nm
Temperature: 25 °C
Injection vol.: 10 μL
Samples:
1. 0.5 g/L thyroglobulin (MW 640,000)
2. 1.0 g/L γ-globulin (MW 155,000)
3. 1.0 g/L ovalbumin (MW 47,000)
4. 1.5 g/L ribonuclease A (MW 13,700)
5. 0.01 g/L p-aminobenzoic acid (MW 137)

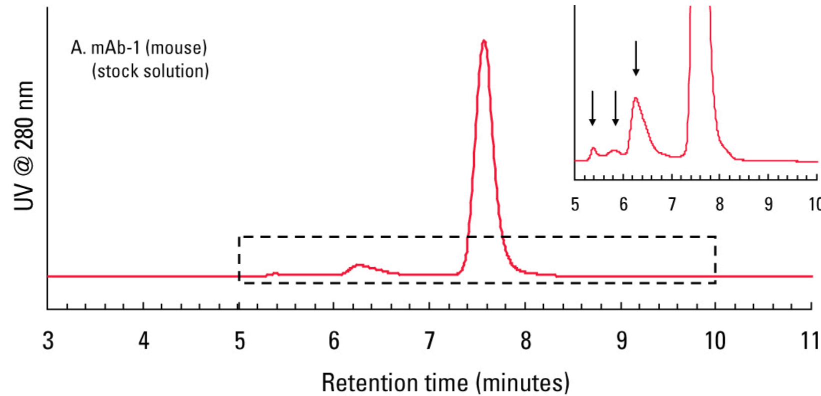
Figure 2: Calibration Curve of TSKgel SuperSW mAb HR

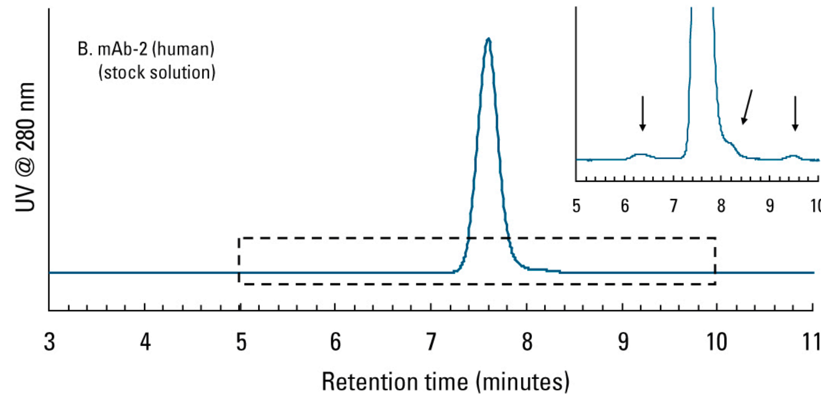


TSKgel SuperSW mAb HR has a calibration curve for globular proteins with a shallow slope around γ-globulin. This is suitable for the separation of mAb monomer from its aggregates or degradation products.

Results

Figure 3: Analysis of mAb purity by SEC



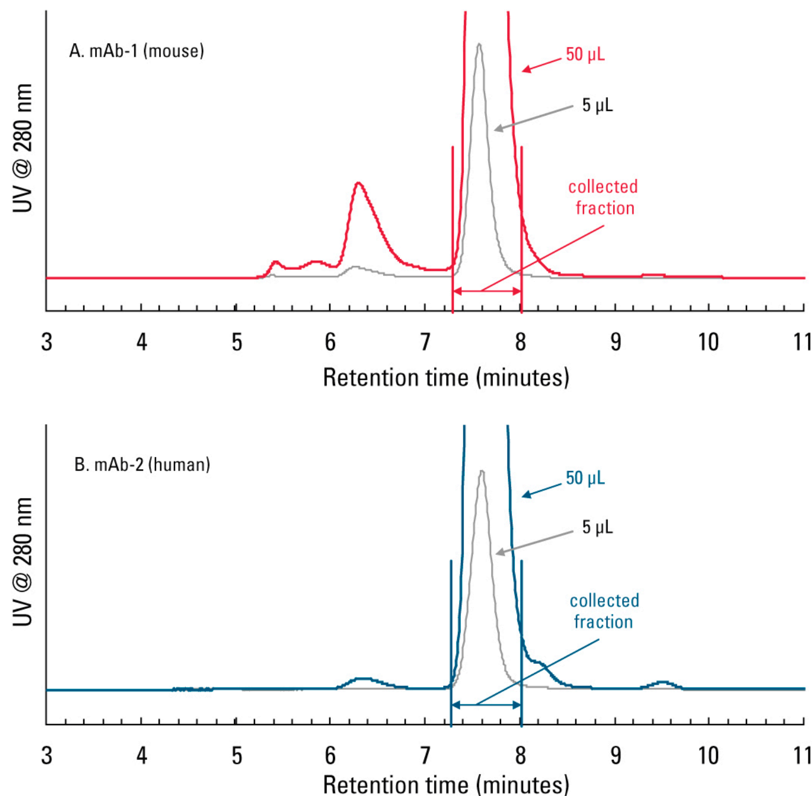


Column:	TSKgel SuperSW mAb HR, 7.8 mm ID × 30 cm
Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	5 µL
Samples:	A. mAb-1 (mouse monoclonal, 20 g/L) B. mAb-2 (human monoclonal, 5 g/L)

SEC analysis revealed that original stock solution of mAb-1 contained higher molecular size impurities (indicated with arrows), presumed to be aggregates of the antibody.

Similarly, mAb-2 solution proved to contain higher and lower molecular size impurities, the latter was presumed to be degradation products of the antibody.

Figure 4: Fractionation of mAb Monomer

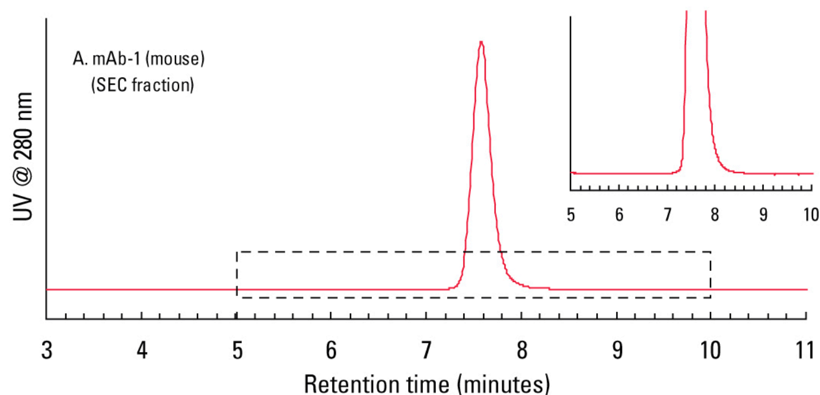


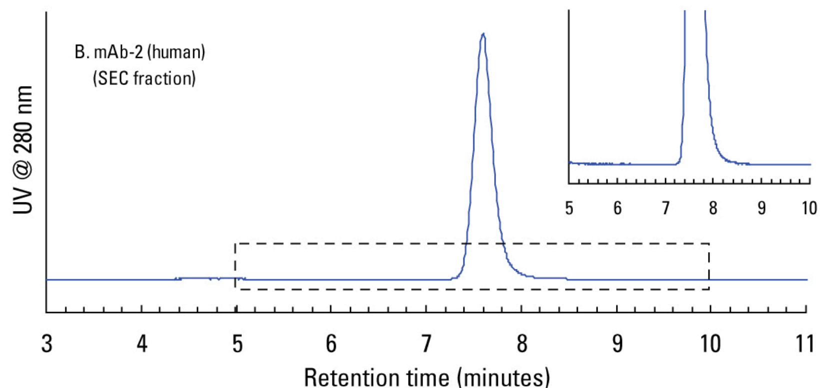
Column:	TSKgel SuperSW mAb HR, 7.8 mm ID × 30 cm
Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	50 µL
Samples:	A. mAb-1 (mouse monoclonal, 20 g/L) B. mAb-2 (human monoclonal, 5 g/L

50 µL of mAb stock solution was injected into the SEC column and 7.3-8.0 min elution fraction was collected to yield 700 µL of purified mAb monomer solution.

Figure 4 shows the comparison between the chromatograms obtained with different sample injection volumes. A 50 µL injection (*red* or *blue*) gave acceptable separation of mAb monomer from impurities, despite the broader peak compared to a 5 µL injection (*gray*).

Figure 5: Confirmation of the Removal of Impurities from mAb Monomer Solution



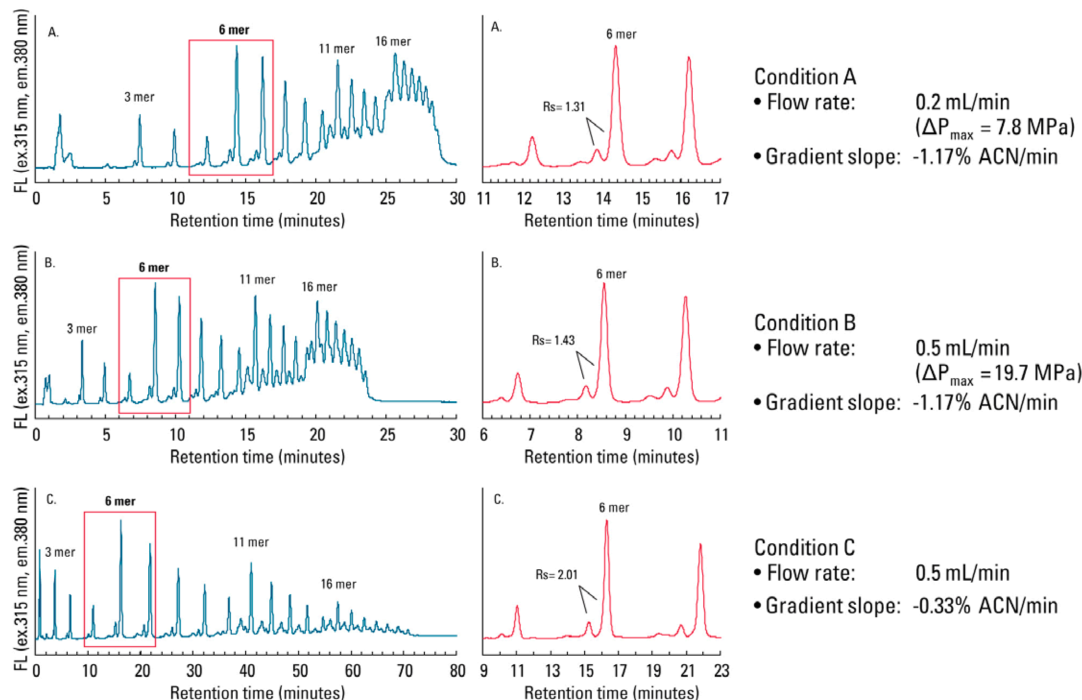


Column:	TSKgel SuperSW mAb HR, 7.8 mm ID × 30 cm
Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	20 µL
Samples:	A. SEC section of mAb-1 monomer B. SEC section of mAb-2 monomer

SEC analysis revealed that each SEC-fractioned mAb monomer solution contained no higher or lower molecular size impurities.

N-linked oligosaccharides were released from these purified mAb monomers, derivatized with fluorescence labeling reagent (2-aminopyridine), and separated with HILIC, as described in "Experimental" section.

Figure 6: Effect of Chromatographic Conditions on the Separation of PA-Glucose Oligomers by HILIC

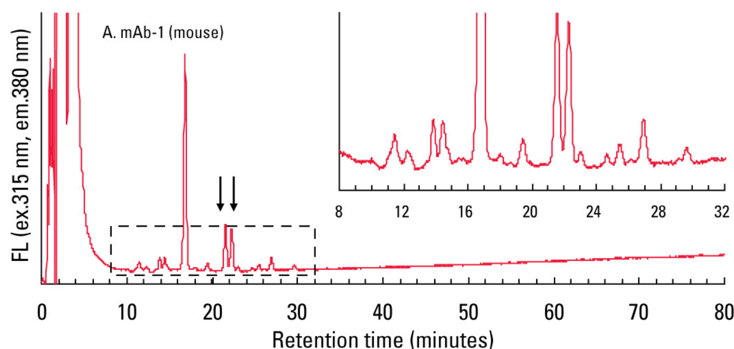


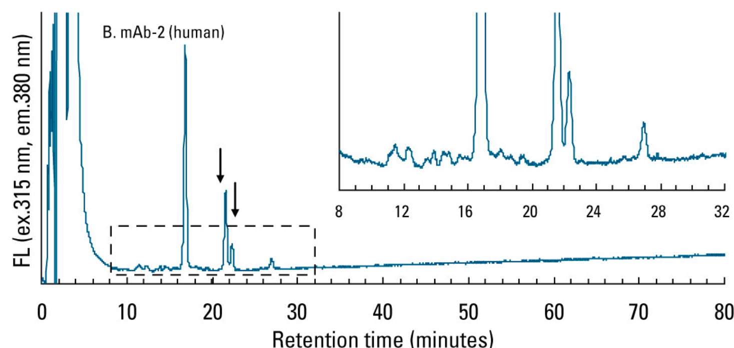
Column:	TSKgel Amide-80, 3 µm, 2.0 mm ID × 15 cm
Mobile phase:	A: 200 mmol/L acetic acid + triethylamine, pH 7.3 B: acetonitrile
Gradient:	A,B: 0-30 min, linear (75-50%B) C: 0-5 min (75%B), 5-80 min, linear (75-50%B)
Flow rate:	A: 0.2 mL/min, B,C: 0.5 mL/min
Detection:	Fluorescence (ex. 315 nm, em. 380 nm)
Temperature:	40 °C
Injection vol.:	50 µL
Samples:	PA-glucose oligomer (3-22 mer) (TaKaRa Bio)

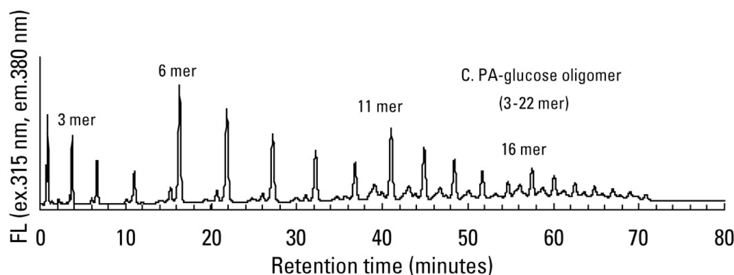
Figure 6: Conclusions

- Figure 6 shows the entire chromatogram of fluorescence-labeled standard sugar chains (*left*) and the enlargement of glucose hexamer peaks (*right*) obtained under different chromatographic conditions.
- These chromatograms revealed that with the separation of oligosaccharides by HILIC, higher flow rate and shallower gradient slope gave higher resolving power.
- On the basis of these results, condition C was adopted as the analytical condition for oligosaccharides released from mAbs.

Figure 7: Analysis of *N*-linked Oligosaccharides Released from mAb







Column:	TSKgel Amide-80, 3 µm, 2.0 mm ID × 15 cm
Mobile phase:	A: 200 mmol/L acetic acid + triethylamine, pH 7.3 B: acetonitrile
Gradient:	0-5 min (75% B), 5-80 min, linear (75-50% B)
Flow rate:	0.5 mL/min
Detection:	Fluorescence (ex. 315 nm, em. 380 nm)
Temperature:	40 °C
Injection vol.:	50 µL
Samples:	A. pyridylaminated oligosaccharides released from mAb-1 B. pyridylaminated oligosaccharides released from mAb-2 C. PA-glucose oligomer (3-22 mer) (TaKaRa Bio)

Several oligosaccharides were detected at around 5-8 glucose units (GU) from both samples.

The comparison of the elution profiles between mAb-1 and mAb-2 revealed that:

(1) The peak area ratio of the two peaks that eluted at ~7GU (indicated with arrows) was obviously different between mAb-1 and mAb-2.

(2) The sample from mAb-1 included a larger variety of oligosaccharides than that from mAb-2. In other words, mAb-1 had a more heterogeneous glycosylation pattern than mAb-2.

Conclusions

- In this study, the heterogeneity of monoclonal antibodies (mAbs) in the glycosylation pattern was evaluated by the use of size exclusion chromatography (SEC) and hydrophilic interaction chromatography (HILIC).
- SEC analysis using TSKgel SuperSW mAb HR revealed that the stock solutions of mAb contained higher or lower molecular size impurities. They were presumed to be aggregation and degradation products of the antibodies produced during storage, respectively. These impurities were successfully removed by SEC fractionation to yield pure mAb monomer solutions.
- *N*-linked oligosaccharides released from the polypeptide chains of the mAb monomer were analyzed by the HILIC column, TSKgel Amide-80, 3 µm. Prior to the analysis of real samples, analytical conditions for oligosaccharides were examined, and high resolution separation was achieved by high flow rate and shallow gradient slope. In this analytical condition, oligosaccharides released from mouse mAb and human mAb were satisfactorily separated, and the difference in the glycosylation pattern between the two was observed. The difference was estimated to be caused by the difference in the production processes of the mAbs, including cell line and cell culture conditions.
- The high resolving power of the SEC column and the HILIC column mentioned in this study is suitable for the evaluation of mAb heterogeneity.