Detection of protein heterogeneity by HPLC

Atis Chakrabarti

Tosoh Bioscience LLC, King of Prussia, PA 19406

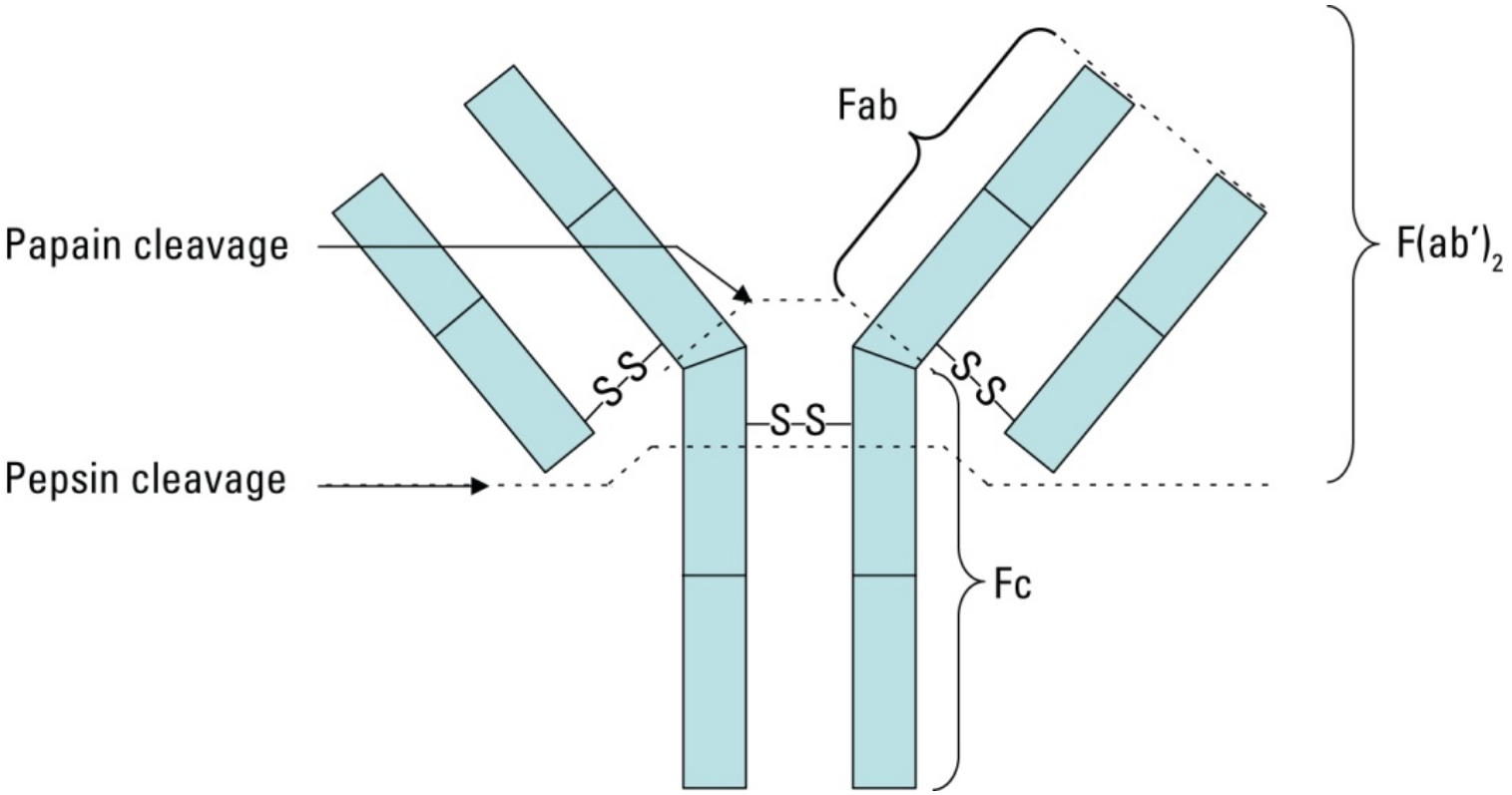
Introduction

- Proteins are not homogeneous.
- A protein isoform is any of several different forms of the same protein.
- Protein heterogeneity is generated by post-translational modifications, decomposition, or chemical modification, etc.
- Isoforms differ in modifications of individual amino acid side chains, or the N- or C-terminus.
- Typical modifications are deamidation, phosphorylation, acetylation, methylation, oxidation, or glycosylation.
- Isoforms may differ in biological activity and stability.
- Antibodies and recombinant proteins are now widely used for therapeutic treatment. Separation of the monomer (150 kDa) of a monoclonal antibody from its dimer (300 kDa) with a very good baseline resolution is very important for quality control. The heterogenic moieties increase the risk of anaphylaxis or immunoreaction if not separated. Detection of even 1 residue difference of the proteins may be challenging.

Introduction continued

- Enzymatic digestion products of monoclonal antibodies need to be resolved to separate digested fragments from the intact protein to baseline resolution.
- Among several disulfide bonds present in IgG, the hinge-region is most accessible and susceptible to reduction leading to half-IgG. Selective or complete reductions are carried out frequently for conjugation, antibody immobilization, or enzyme labeling, etc. Selective or complete reduction requires the separation of a number of the fragments and impurities.
- Similarly, analysis of PEGylated proteins and hapten-conjugated proteins are also important. Fast separation of monoclonal antibody isoforms is important for profiling and mass spectrometric determination.
- The evaluation of the heterogeneity of the therapeutic antibody is essential during development, stability testing, and in the quality control of the final product.
- Here we report the detection and separation of protein heterogeneity by HPLC using four different modes of chromatography: SEC, IEX, RPC and HIC.

Schematic diagram of IgG



- **IgG is a relatively large molecule (approx. 150 kDa) and in order to improve the penetration to the tissue , fragmentation is carried out. Digestion with papain or pepsin is commonly applied to obtain antibody fragments without the loss of activity.**
- **When papain is used for the antibody digestion, 2 Fab and 1 Fc are obtained from 1 antibody. When pepsin is used, a $F(ab')_2$ is obtained.**

Material and methods

Size Exclusion Chromatography columns

- TSKgel® SuperSW mAb HTP, 4.6 mm ID × 15 cm, 4 µm particle*
- TSKgel SuperSW mAb HR, 7.8 mm ID × 30 cm, 4 µm particle*
- TSKgel UltraSW Aggregate, 7.8 mm ID × 30 cm, 3 µm particle*
- TSKgel G3000SWXL, 7.8 mm ID × 30 cm, 5 µm particle
- TSKgel G2000SWXL, 7.8mm ID × 30 cm; 5 µm particle

Reversed Phase Chromatography Column

- TSKgel Protein C4-300, 4.6 mm ID × 15 cm, 3 µm particle

Ion Exchange Chromatography Column

- TSKgel CM-STAT, 4.6 mm ID × 10 cm, nonporous

Hydrophobic Interaction Chromatography Column

- TSKgel Butyl-NPR, 4.6 mm ID × 3.5 cm, 2.5 µm particle

All of the TSKgel columns were manufactured by Tosoh (Tokyo, Japan).

*prototype columns

Material and methods continued

Instrumentation

Tosoh liquid chromatograph equipped with pump (DP-8020), column oven (CO-8020), UV detector (UV-8020), and data processor (LC-8020 model II).

Agilent 1200 (Chemstation - Rev B.04.02)

Samples

monoclonal antibodies:

- Kaketsuken (Kumamoto, Japan) (*figures 4-7*)
- Monoclonal antibody: BI-mAb-2 from Boehringer-Ingelheim (gift from Tosoh Bioscience GmbH); concentration: 4.5 g/L in glycine/Na phosphate, pH 6.0
- BI-mAb-01 from Boehringer-Ingelheim (gift from Tosoh Bioscience GmbH); in 0.1 mol/L citrate buffer, pH 6.0; concentration: 28 g/L
- Human IgG (Sigma I8640-10MG; Tech grade >80% SDS-PAGE)
- Mouse IgG (Tech grade from serum, Sigma I8765-10MG Lot #95H8845)

Aggregate formation by heat denaturation was carried out by adjusting the pH of the antibody solution from pH 6.0 to 5.5 using dilute phosphoric acid followed by incubation at 60 °C over time.

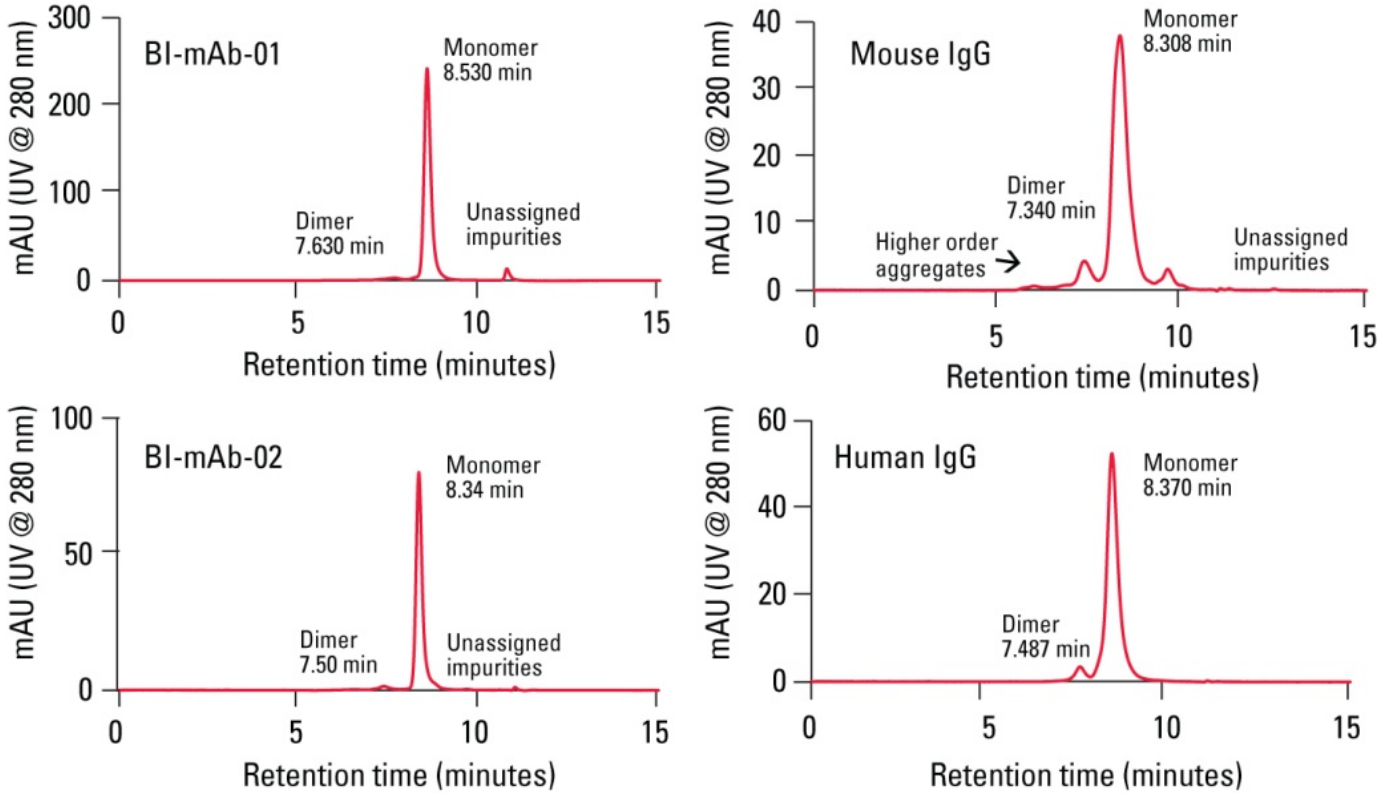
Chromatographic conditions

Mobile Phase:	100 mmol/L potassium phosphate buffer, 100 mmol/L sodium sulfate, pH 6.7 + 0.05% NaN_3; unless mentioned otherwise
Flow rate:	1.0 mL/min (0.35 mL/min for 15 cm column)
Detection:	UV @ 280 nm
Temperature:	ambient/25 °C except during heat denaturation study
Injection vol.:	10 µL unless mentioned otherwise

High purity HPLC grade Sigma Aldrich chemicals were used in this study.

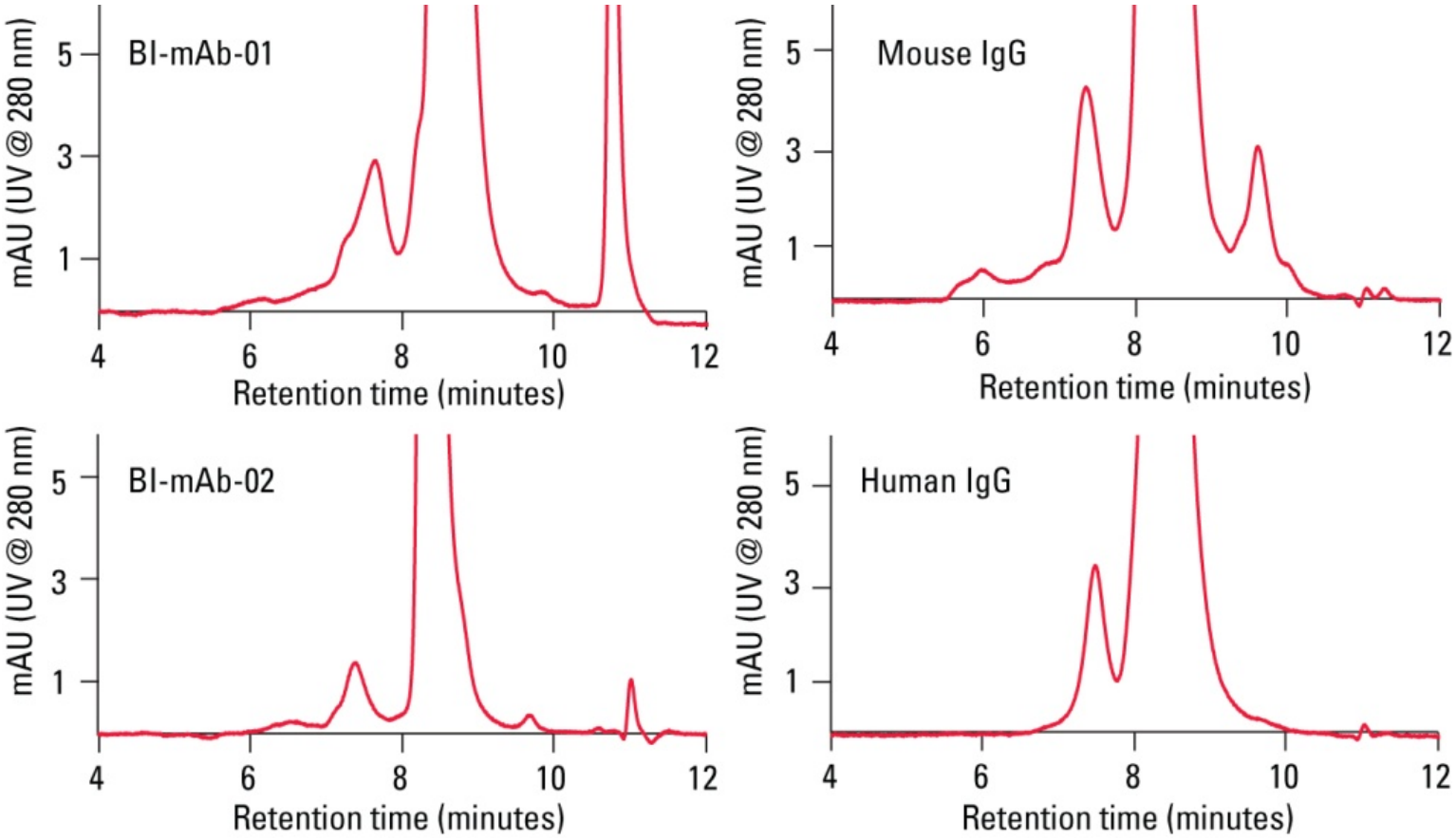
High purity 18.2 m.Ohm-cm quality water was used to make buffer and samples.

Figure 1: Size exclusion chromatographic separation of IgG monomer and dimer using a prototype TSKgel UltraSW Aggregate column



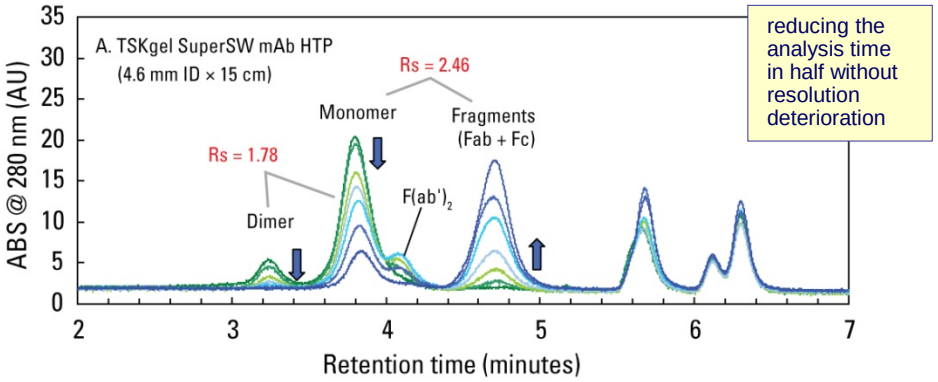
- **Size exclusion chromatography (SEC) was used to separate the monomer (150 kDa) and the dimer (300 kDa) of a human monoclonal antibody to very good baseline resolution.**
- **Heterogeneous impurities are separated from the monomer peaks of four different kinds of monoclonal antibodies under native conditions using SEC.**
- **Different types of monoclonal antibodies have different kinds and amounts of impurities such as aggregates, fragments, etc. which can be separated by SEC.**

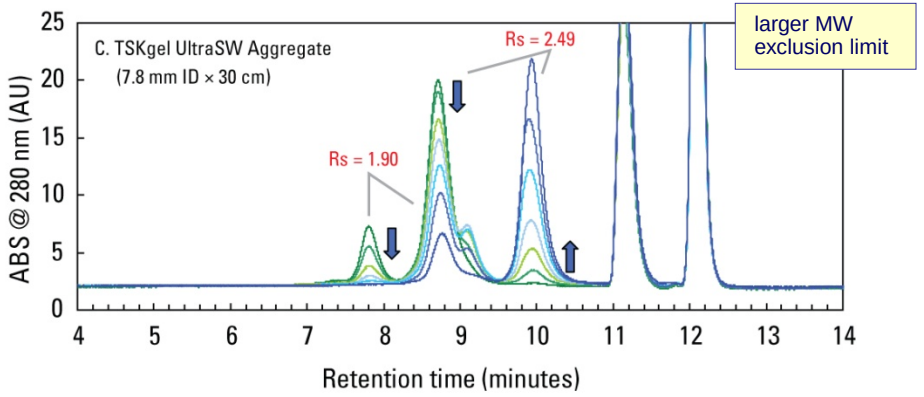
Figure 2: Size exclusion chromatographic separation of IgG monomer and dimer using a prototype TSKgel UltraSW Aggregate column (contd.)

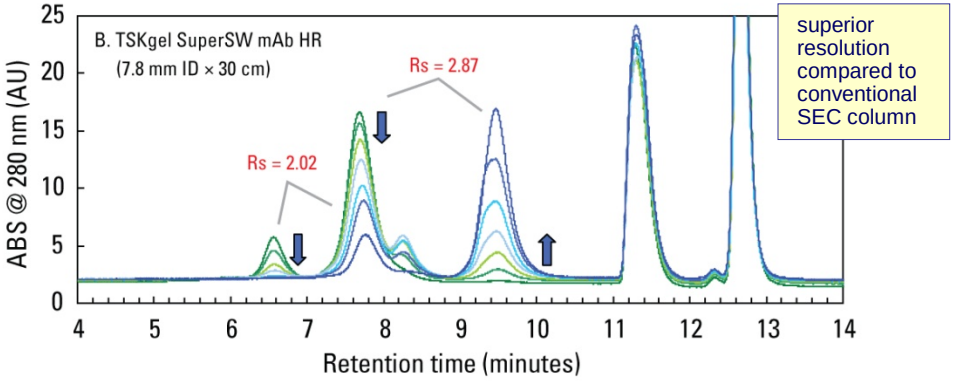


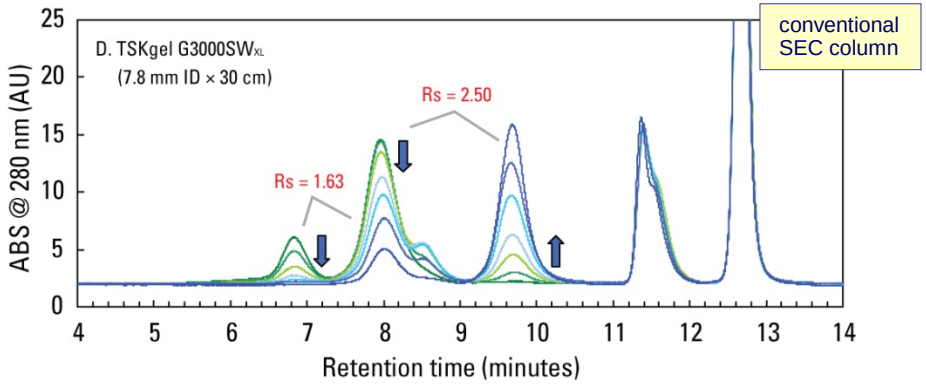
Heterogeneous impurities separated from the monomer peaks of four different kinds of monoclonal antibodies under native conditions using size exclusion chromatography are clearly seen in this zoomed in figure.

Figure 3: Size exclusion chromatographic separation of IgG monomer, dimer, and fragments from papain digested IgG by 3 novel prototype SEC columns: TSKgel SuperSW mAb HTP, SuperSW mAb HR, UltraSW Aggregate









Mobile phase:	200 mmol/L phosphate buffer + 0.05% NaN_3, pH 6.7
Flow rate:	A 0.35 mL/min; B-D 1.0 mL/min
Injection volume:	A 5 µL; B-D 10 µL
Temperature:	25°C,
Detection:	UV @ 280 nm
Samples:	10 g/L IgG digested with papain for 0-24 hr

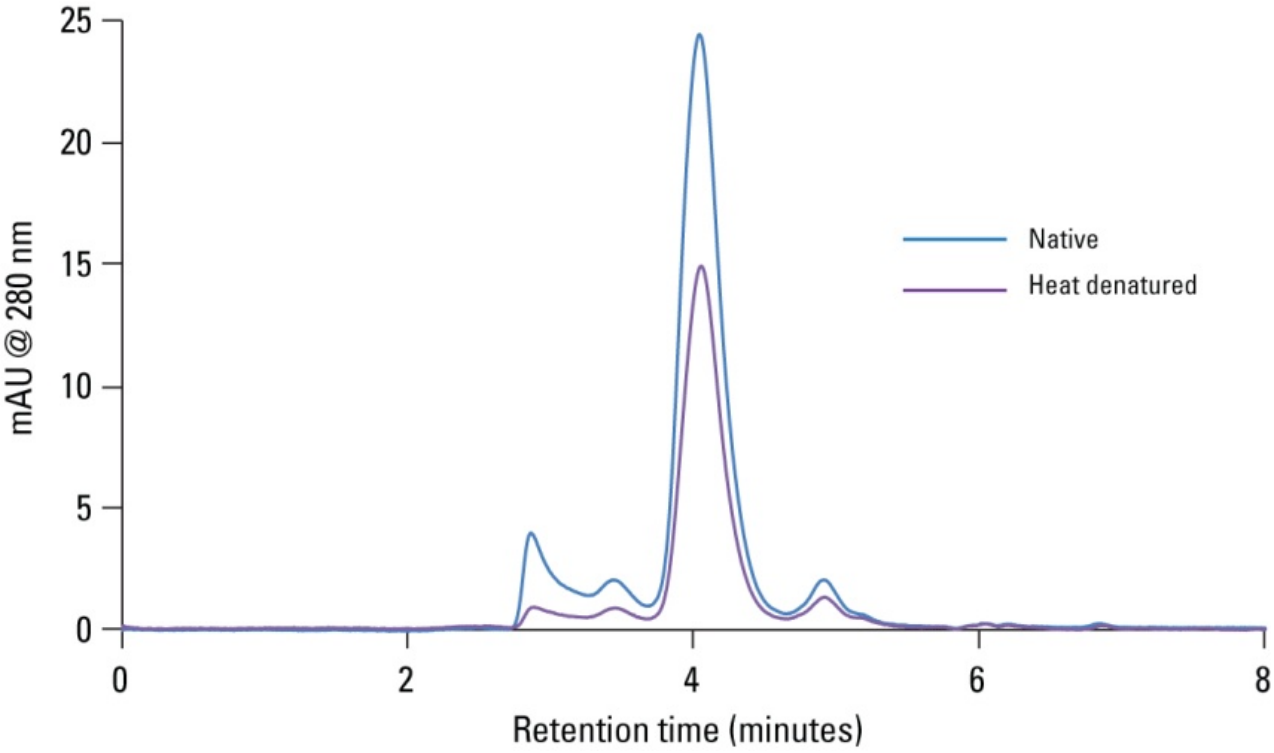
Columns:
A. TSKgel SuperSW mAb HTP, 4.6 mm ID × 15 cm*
B. TSKgel SuperSW mAb HR, 7.8 mm ID × 30 cm*
C. TSKgel UltraSW Aggregate, 7.8 mm ID × 30 cm*
D. TSKgel G3000SWXL, 7.8 mm ID × 30 cm

*prototype columns

Figure 3: conclusions

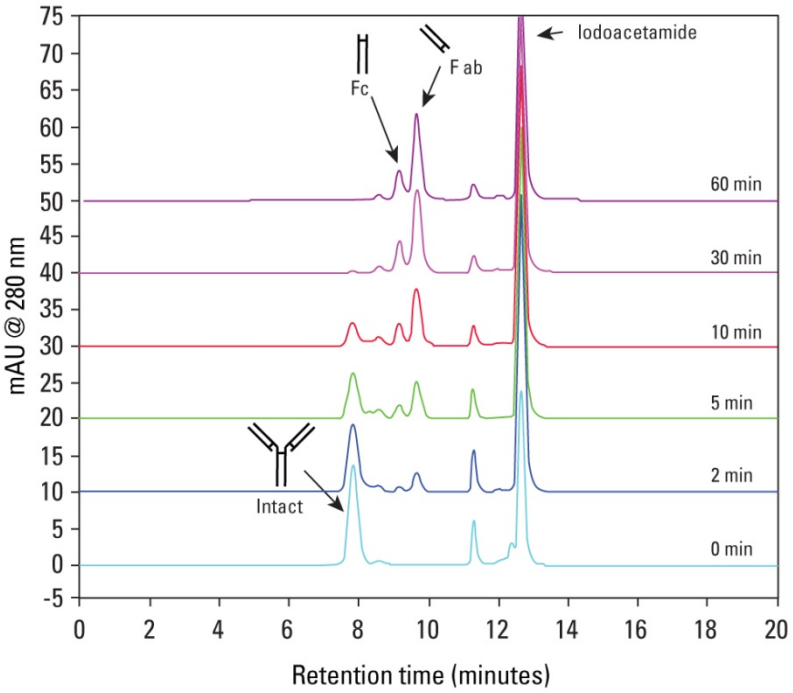
- 3 prototype novel columns especially designed for the analysis of antibody were used for the separation of heterogeneous impurities such as dimer, higher order aggregates, fragments, etc. during the analysis of a papain-digested antibody.
- TSKgel SuperSW mAb HTP: a semi-micro 15 cm column packed with 4 micron particles separated dimer, monomer, and fragments at the same resolving power but in half the analysis time compared to the conventional column
- TSKgel SuperSW mAb HR: a standard 30 cm column packed with the same particles as the TSKgel SuperSW mAb HTP column provided higher resolution than the conventional column in the same analysis time
- TSKgel UltraSW Aggregate column, packed with a particle size of 3 micron and a pore size of 30 nm, is designed for the separation of aggregates and has a larger molecular weight exclusion limit than the other 3 columns. It possesses a wider separation window for IgG oligomers and aggregates with high molecular weight ($5x10^5$ - $5x10^6$ Da) than the TSKgel G3000SW$_{XL}$ column.

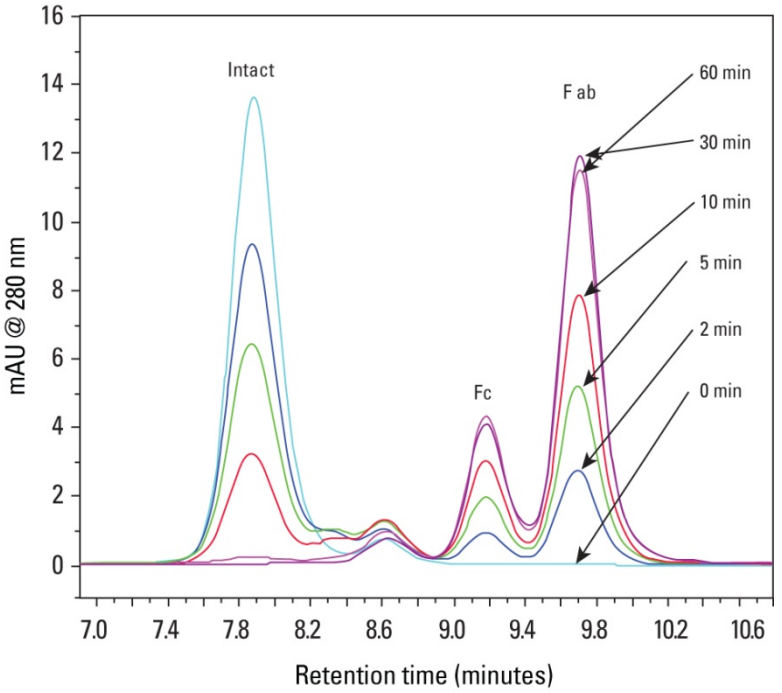
Figure 4: Size exclusion chromatographic separation of aggregates and impurities from heat denatured monoclonal antibody (mouse IgG) using a prototype TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm column (0231S4)



- **Stability studies or studies using forced degradation need to be analyzed for heterogeneities arising out of decomposition, degradation, and fragmentation.**
- **The increased amount of dimer and higher order of aggregates obtained from heat denaturation were separated.**
- **Similarly, heterogeneous impurities were separated from the monomer peaks of four different kinds of monoclonal antibodies under native and denatured conditions (data not shown here).**

Figure 5: Size exclusion chromatographic separation of intact monoclonal antibody from its fragments from papain digestion

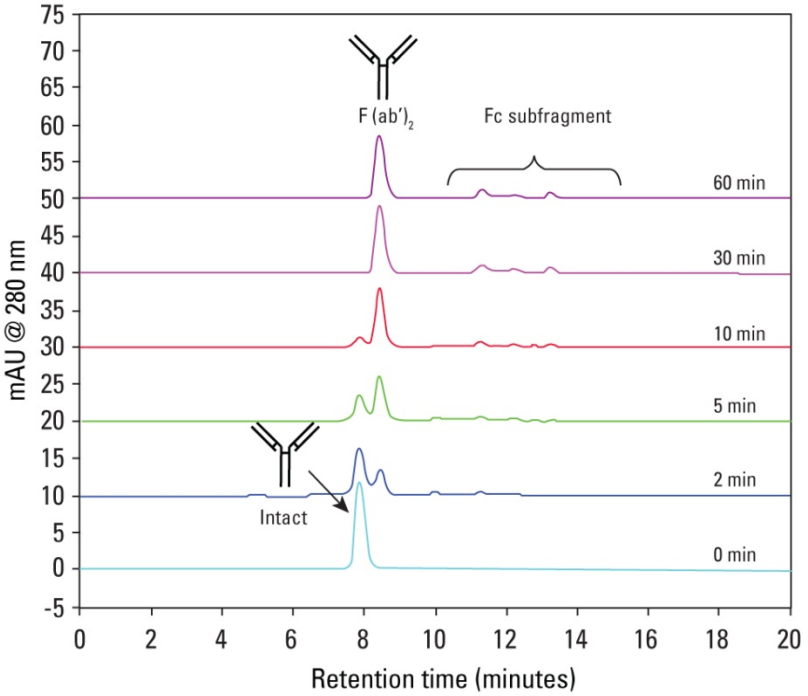


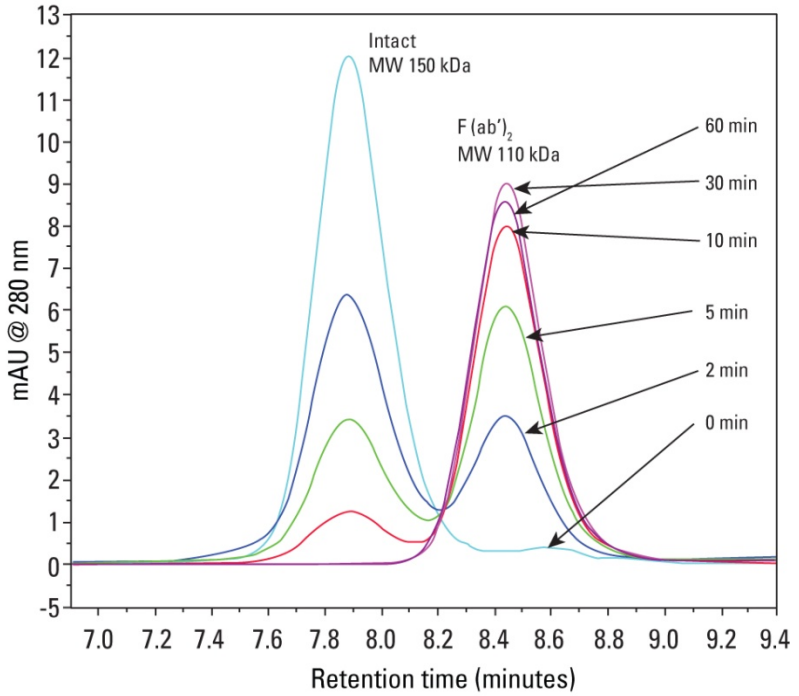


Column:	TSKgel G30000SW$_{XL}$, 7.8 mm ID × 30 cm
Mobile phase:	20 mmol/L phosphate buffer + 0.3 mol/L NaCl, pH 7.0
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	room temperature
Injection vol.:	10 µL
Concentration:	0.24 g/L

The time course of papain digestion shows that as the reaction proceeds, the intact antibody peak intensity decreases and Fab and Fc peak intensity increases.

Figure 6: Size exclusion chromatographic separation of intact monoclonal antibody from its fragments from pepsin digestion

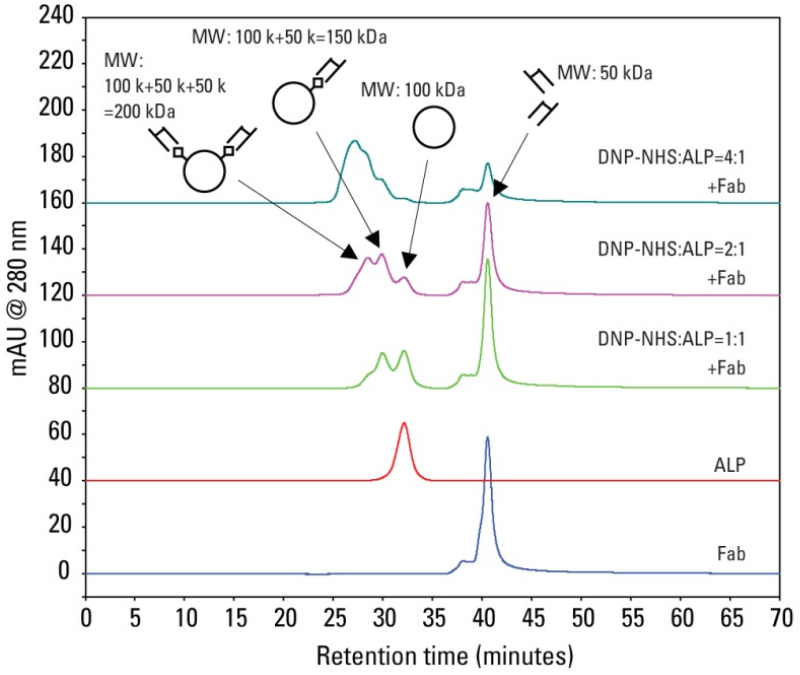


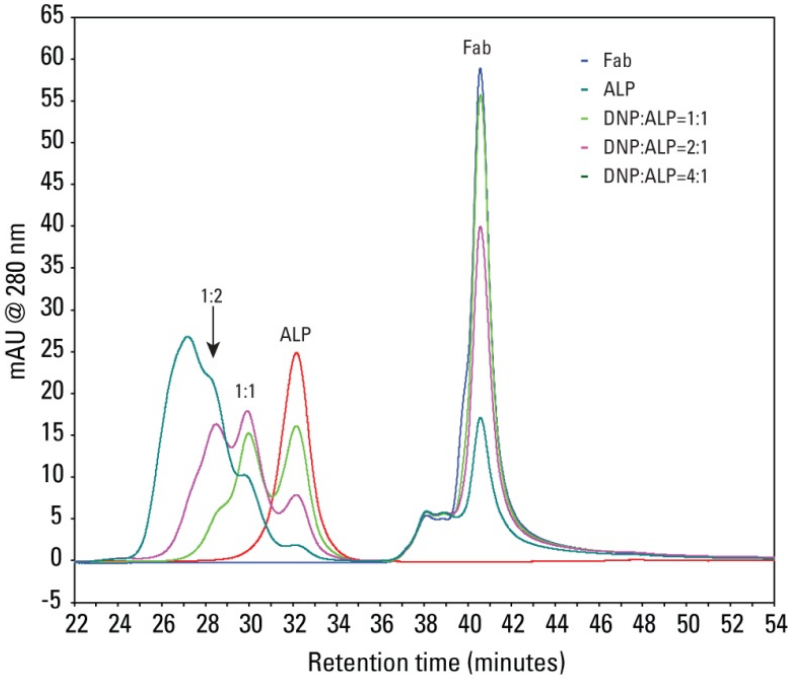


Column:	TSKgel G3000SWXL, 7.8 mm ID × 30 cm
Mobile phase:	20 mmol/L phosphate buffer + 0.3 mol/L NaCl, pH 7.0
Flow rate	1.0 mL/min
Detection	UV @ 280 nm
Temperature:	room temperature
Injection vol.:	10 µL
Concentration:	0.19 g/L

The time course of pepsin digestion shows that as the reaction proceeds, the intact antibody peak intensity decreases and F(ab)'$_2$ peak intensity increases. The Fc fragment is further digested to a small subfragment, so the peak cannot be seen as is expected from the molecular size.

Figure 7: Size exclusion chromatographic separation of hapten-conjugated monoclonal antibody from its impurities





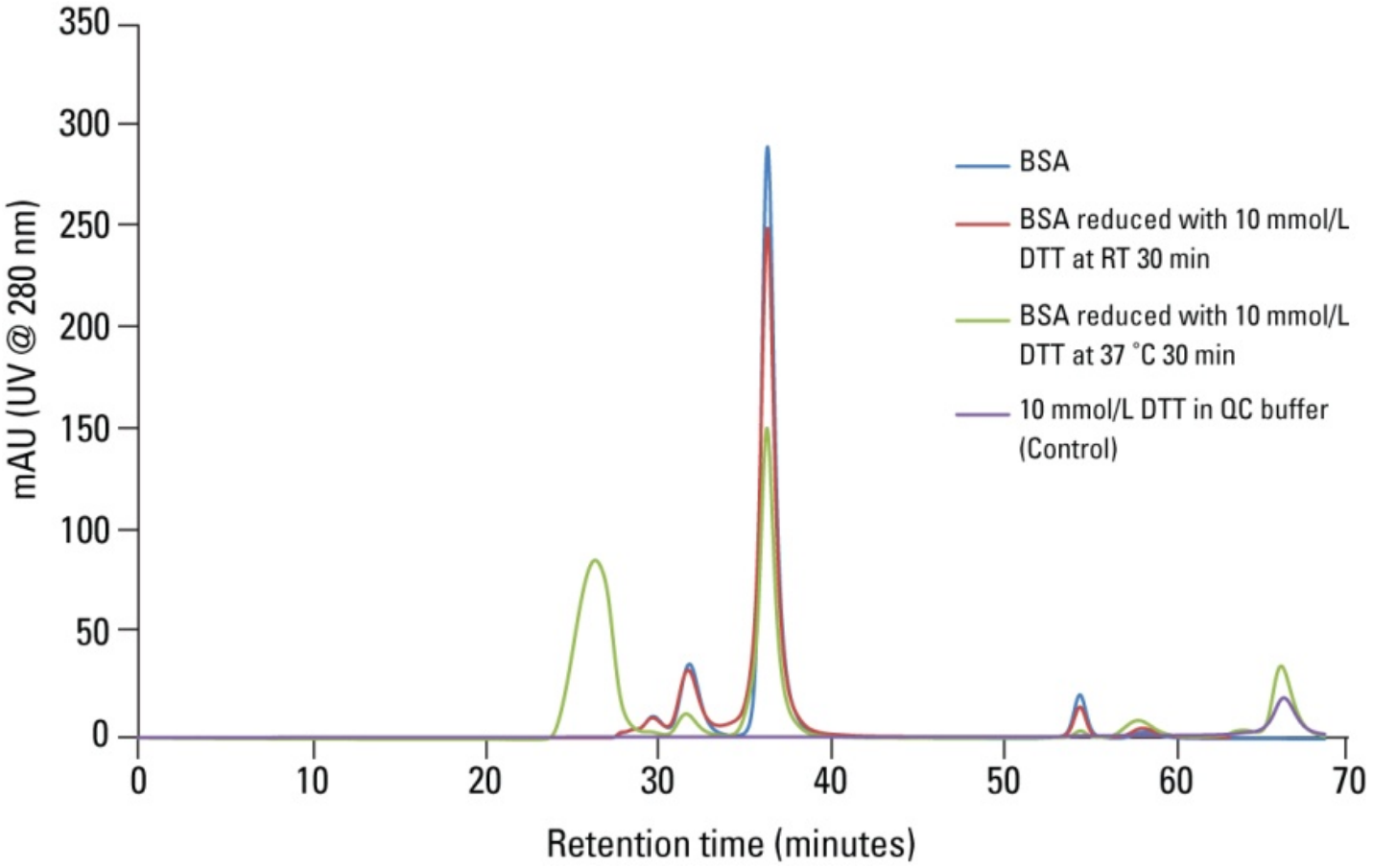
Column:	TSKgel G3000SW$_{XL}$, 7.8 mm ID × 30 cm
Mobile phase:	20 mmol/L phosphate buffer + 0.3 mol/L NaCl, pH 7.0
Flow rate:	0.25 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	20 µL
Concentration:	DNP-ALP: 0.67 g/L, anti-DNP Fab: 1 g/L

Mixed ratio of DNP-NHS:ALP	Ratio (%)			
	DNP introduced (number)			
	0	1	2	≥3
1:1	61%	32%	7%	0%
2:1	30%	40%	22%	8%
4:1	7%	19%	30%	44%

Figure 7: conclusions

- Small chemical compounds (haptens) are generally not good immunogens until they are attached to macromolecules as carriers, such as proteins and antibodies.
- Once conjugated, the hapten-conjugated antibodies or proteins need to be purified from their impurities.
- The TSKgel G3000SW$_{XL}$ column was successfully used for the separation of hapten-conjugated proteins from its impurities.

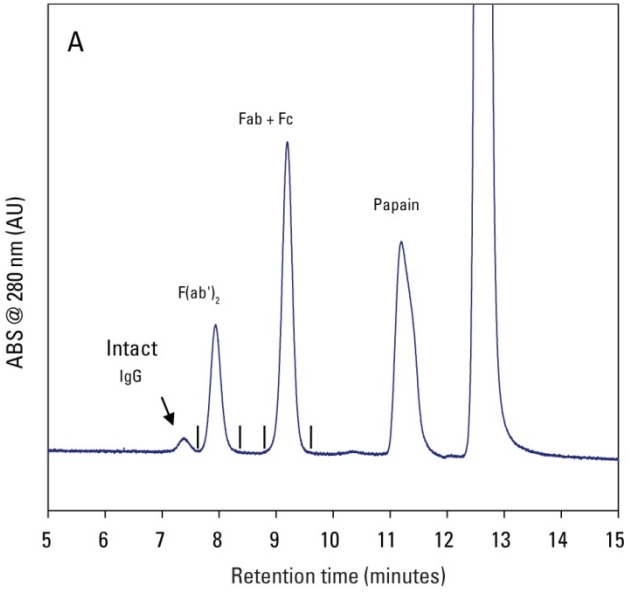
Figure 8: Size exclusion chromatographic separation of reduced monoclonal antibody in the separation of BSA using a TSKgel G2000SWXL, 5 µm, 7.8 mm ID × 30 cm column



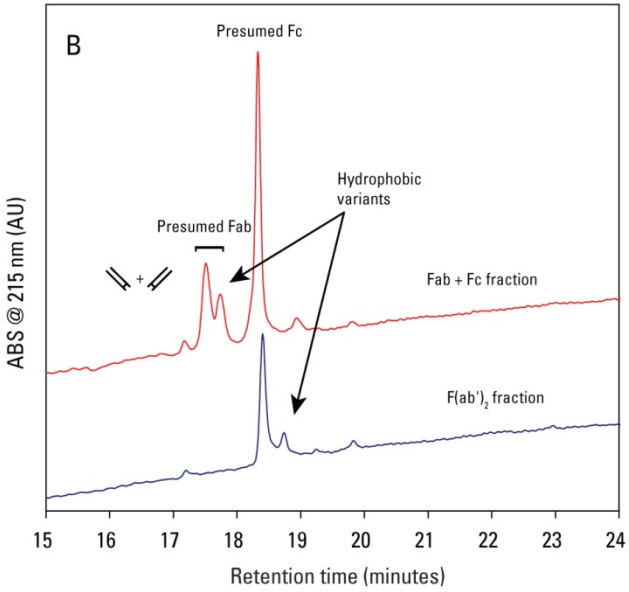
The increased amount of aggregate formed upon the reduction of the mAb using BSA was successfully separated to the baseline from the dimer and monomer peak. This shows that the column can be used for the separation of selectively or completely reduced antibodies targeted for conjugation, immobilization, or enzyme labeling.

Figure 9: Reversed phase chromatographic separation of hydrophobic variants of papain-digested IgG fragments using a TSKgel Protein C4-300, 3 µm, 4.6 mm ID × 15 cm column

A: Separation of papain-digested IgG by SEC



B: Chromatograms of F(ab')$_2$ fraction and Fab+Fc fraction collected from SEC obtained with TSKgel Protein C4-300



Conditions for SEC

Column:	TSKgel SuperSW3000, 4 µm, 4.6 mm ID × 30 cm
Mobile phase:	200 mmol/L phosphate buffer + 0.05% NaN_3, pH 6.7
Flow rate:	0.35 mL/min
Temperature:	25 °C
Detection:	UV @ 280 nm
Injection vol.:	100 µL
Sample:	papain digest of mouse monoclonal IgG

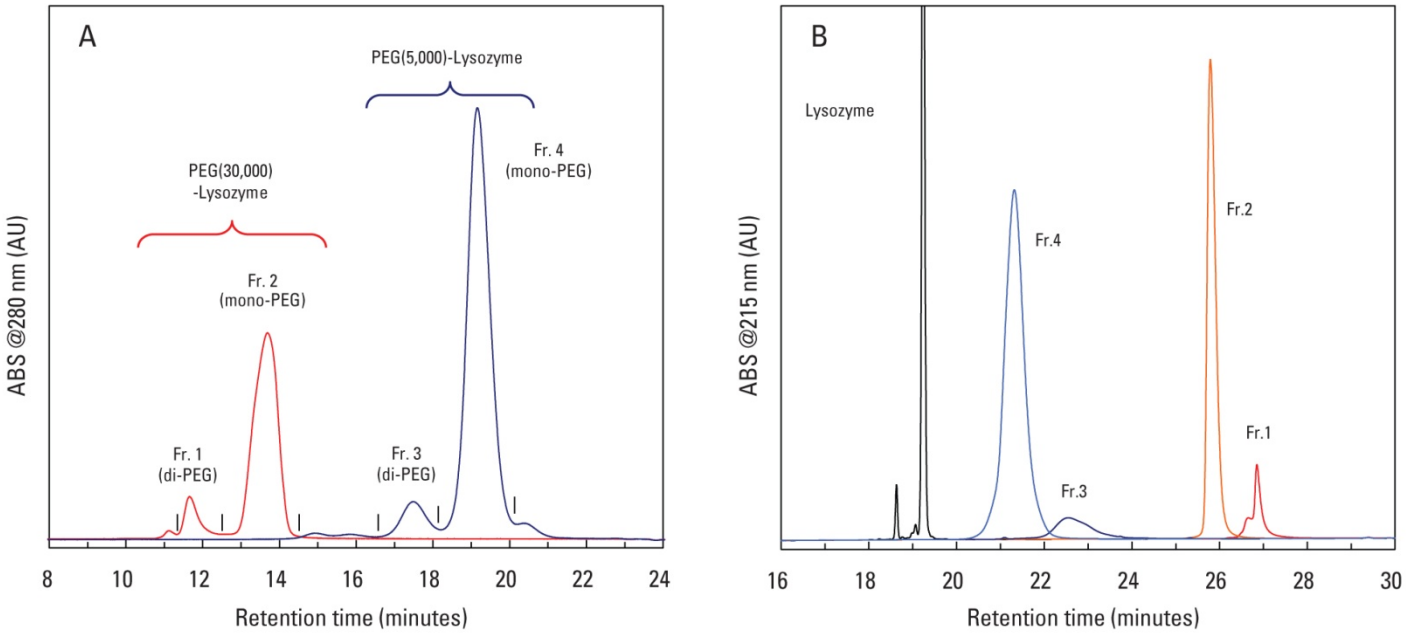
Conditions for RPC

Column:	TSKgel Protein C4-300, 3µm, 4.6 mm ID × 15 cm
Mobile phase A:	H_2O/acetonitrile/TFA = 90/10/0.05 (v/v/v)
Mobile phase B:	H_2O/acetonitrile/TFA = 20/80/0.05 (v/v/v)
Gradient:	0% → 100% B in 45 min
Temperature:	50 °C
Detection:	UV @ 215 nm
Injection vol.:	100 µL
Samples:	SEC fractions of mouse IgG fragments

Figure 9: conclusions

- Intact mAb, the partially digested fragment, and completely digested fragments were separated on the basis of the molecular sizes.
- Each fraction of antibody fragments eluting as a single peak was collected and then applied to a RPC column, the TSKgel Protein C_4-300, to show the presence of heterogeneity in terms of hydrophobicity.

Figure 10: Reversed phase chromatographic separation of hydrophobic variants of the separation of a PEGylated protein from intact protein using a TSKgel Protein C4-300 column



Conditions for SEC

Column:	TSKgel SuperSW3000, 4.6 mm ID × 30 cm × 2
Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	0.35 mL/min
Temperature:	25 °C
Detection:	UV @ 280 nm
Injection vol.:	50 µL
Samples:	5 g/L PEG(MW 5,000)-Lysozyme, 5 g/L PEG(MW 30,000)-Lysozyme

Conditions for RPC

Column:	TSKgel Protein C_4-300, 4.6 mm ID × 15 cm
Mobile phase A:	H_2O/acetonitrile/TFA = 90/10/0.05 (v/v/v)
Mobile phase B:	H_2O/acetonitrile/TFA = 20/80/0.05 (v/v/v)
Gradient:	0%→ 100% B in 45 min
Temperature:	40 °C
Detection:	UV @ 215 nm
Injection vol.	100 µL
Samples:	0.1 g/L lysozyme, SEC fractions 1~4 shown in the left figure.

Figure 10: conclusions

- PEG has been predominantly used to reduce the immunogenicity and to increase the circulating half-lives of antibodies.
- The use of a covalent attachment of poly(ethylene glycol) to various antibodies and antibody fragments lead to heterogeneity, which need to be separated.
- PEGylated lysozyme was separated by SEC, depending on the molecular weight of PEG, or the number of PEG chains.
- Each SEC fraction was applied to the RPC column to separate on the basis of hydrophobicity.
- The PEG-conjugated species were more strongly retained by RPC, than the intact species.

Figure 11: Lot-to-lot variations in reversed phase chromatographic TSKgel Protein C_4-300, 3 µm, 4.6 mm ID × 5 cm and 2.0 mm ID × 10 cm columns

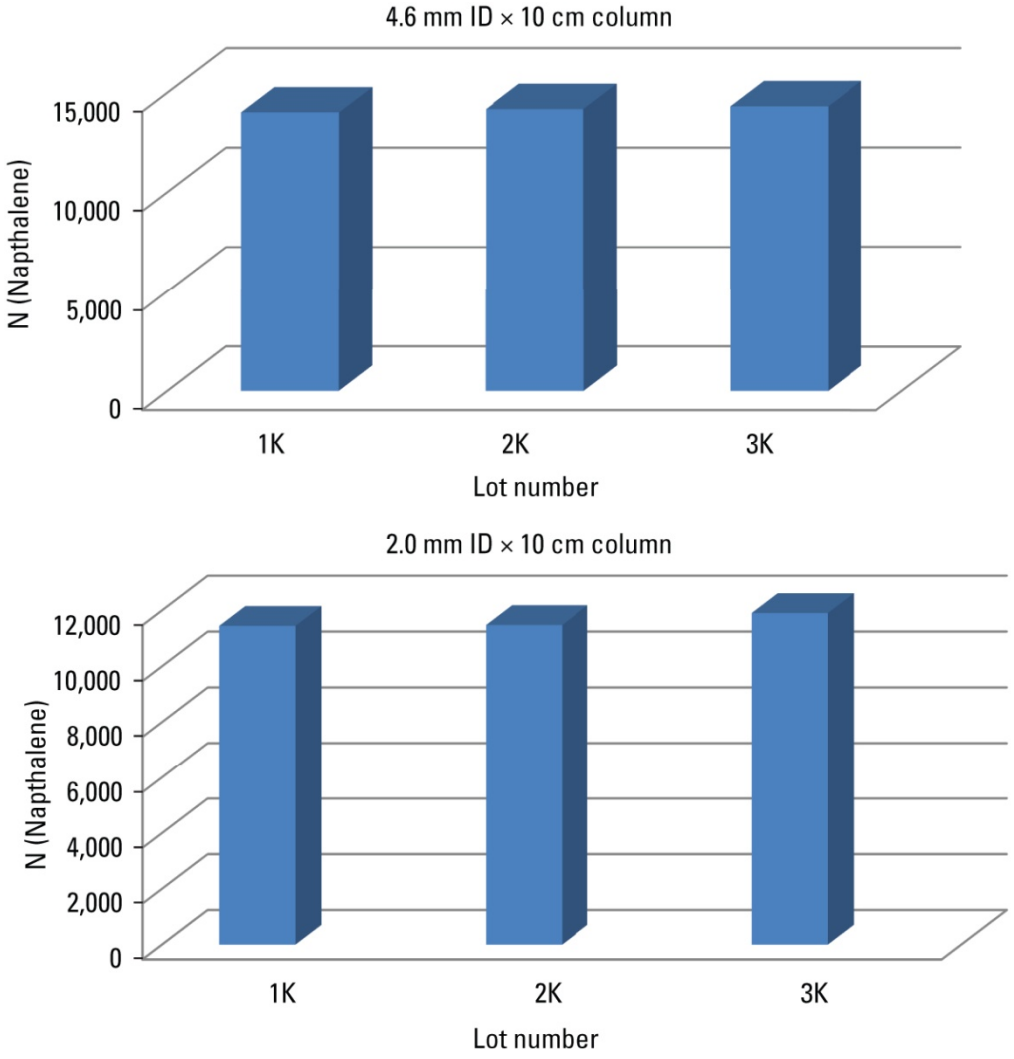


Figure 12: Lot-to-lot variations in reversed phase chromatographic TSKgel Protein C_4-300, 3 µm, 4.6 mm ID × 5 cm and 2.0 mm ID × 10 cm columns

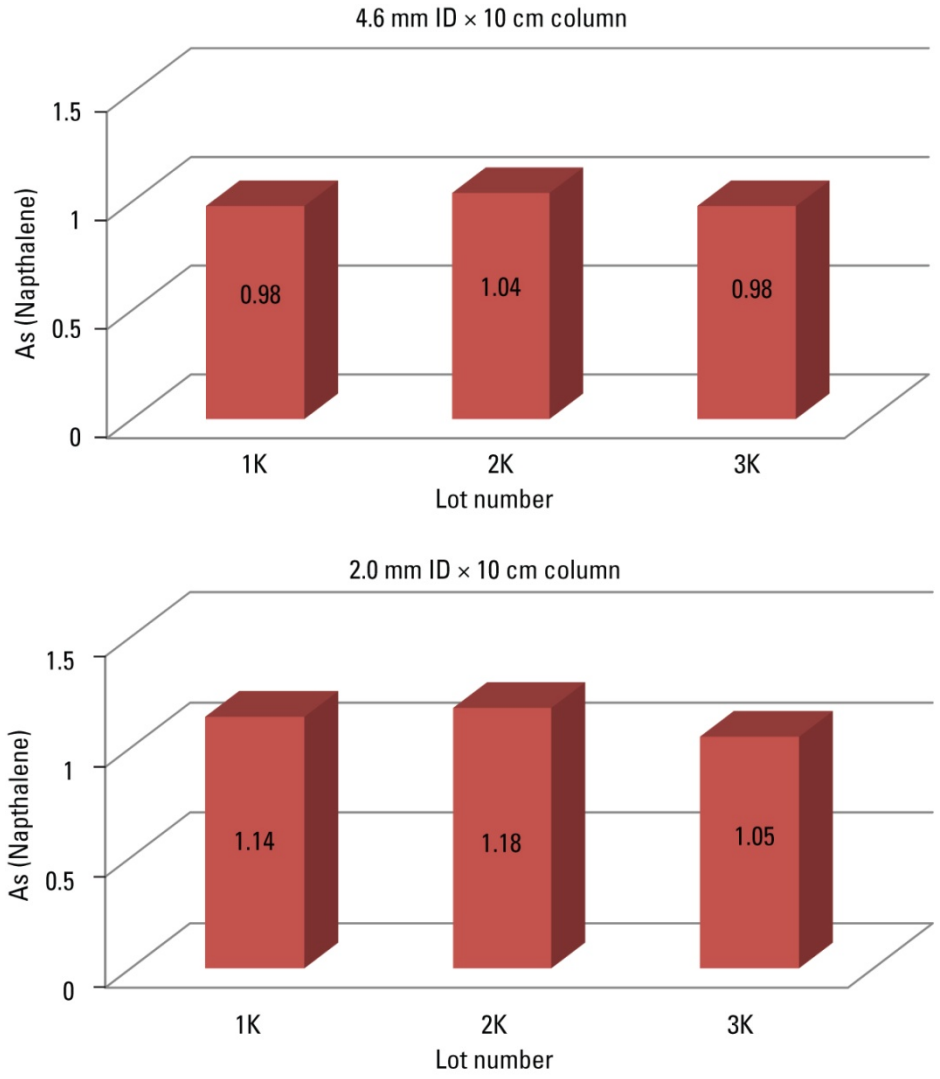
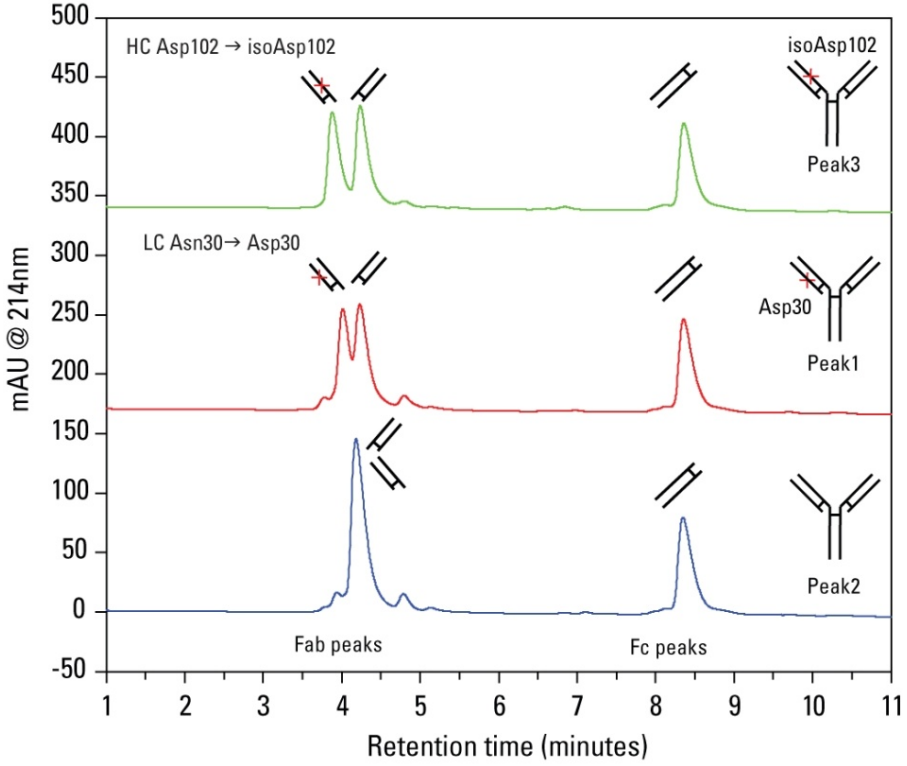
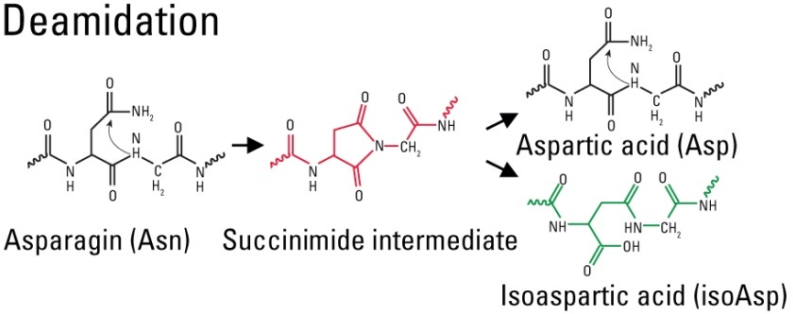


Figure 13: Hydrophobic interaction chromatographic separation of deamidation variants of mAbs using a TSKgel Butyl-NPR, 4.6 mm ID x 3.5 cm column





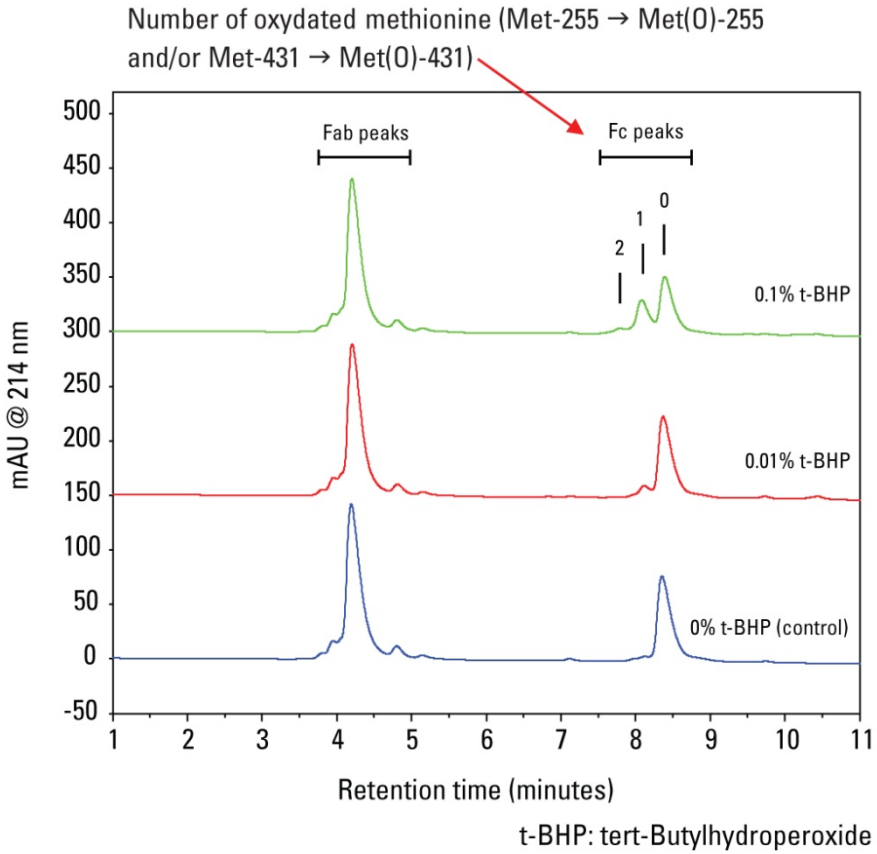
Column:	TSKgel Butyl-NPR, 4.6 mm ID × 3.5 cm
Mobile phase A:	20 mmol/L phosphate buffer containing 2 mol/L $(NH_4)_2SO_4$, pH 7.0
Mobile phase B:	20 mmol/L phosphate buffer, pH 7.0
Gradient:	10 min linear gradient from 25 to 60% B
Flow rate:	1.0 mL/min
Detection:	UV @ 214 nm
Temperature:	25 °C
Injection vol.:	2 µL (4 µg)

Peak	Structural difference	at LC Asn30	at HC Asn55	at HC Asp102
1	Deamidated (to Asp) at Asn30 of one light chain	Asn/Asp	Asn/Asn	Asp/Asp
2	Main peak form	Asn/Asn	Asn/Asn	Asp/Asp
3	Isomerized (to isoAsp) at Asp102 of one heavy chain	Asn/Asn	Asn/Asn	Asp/isoAsp

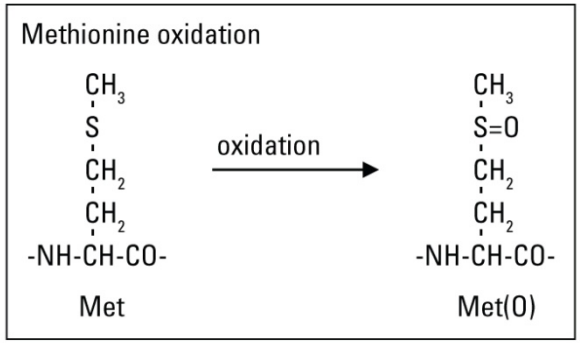
Figure 13: conclusions

- Three variants of monoclonal antibody were digested with papain and then separated using a TSKgel Butyl-NPR column.
- TSKgel Butyl-NPR is a HIC column packed with 2.5 micron nonporous particles.
- The antibody without deamidation was separated into one Fab peak and one Fc peak.
- In contrast, antibodies with deamidated residue at positions 30 or 102 were separated into two Fab peaks and one Fc peak.

Figure 14: Hydrophobic interaction chromatographic separation of methionine-oxidized variants of mAbs using a TSKgel Butyl-NPR, 4.6 mm ID x 3.5 cm column



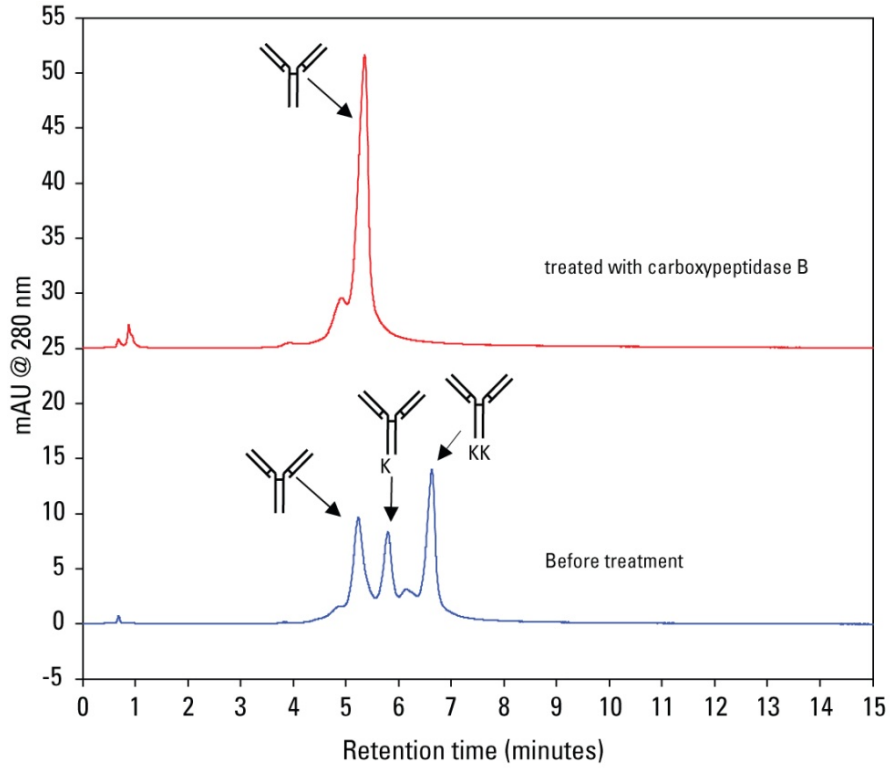
t-BHP: tert-Butylhydroperoxide



Column:	TSKgel Butyl-NPR, 4.6 mm ID × 3.5 cm
Mobile phase A:	20 mmol/L phosphate buffer containing 2 mol/L $(NH_4)_2SO_4$, pH 7.0
Mobile phase B:	20 mmol/L phosphate buffer, pH 7.0
Gradient:	10 min linear gradient from 25 to 60% B
Flow rate:	1.0 mL/min
Detection:	UV @ 214 nm
Temperature:	25 °C
Injection vol.:	2 µL (4 µg)

- **Therapeutic antibody was oxidated by incubating the antibody solution with t-BHP and analyzed with TSKgel Butyl-NPR after papain digestion.**
- **Methione residue of Fc was oxidated, and as a result multiple peaks were observed. Whereas no change was observed with Fab peaks.**

Figure 15: Ion exchange chromatographic separation of C-terminal lysine variants of IgG using a TSKgel CM-STAT, 4.6 mm ID × 10 cm column



Column:	TSKgel CM-STAT, 4.6 mm ID × 10 cm
Mobile phase A:	20 mmol/L MES, pH 6.0
Mobile phase B:	20 mmol/L MES + 0.5 mol/L NaCl, pH 6.0
Gradient:	10% B (0 min), 30% B (15 min) 100% B (15.1 min), 100% B (18 min), 10% B (18.1 min), 10% B (23 min)
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nn
Temperature:	25 °C
Injection vol.:	20 µL
Concentration:	0.5 g/L
Sample:	therapeutic antibody treated and untreated with carboxypeptidase B
Procedure:	To a 35 µL of therapeutic antibody (10 g/L), 1 µL of carboxypeptidase B (Sigma C9584, 140 U/mg protein, 5 g/L in PBS) was added and incubated for 3 hours at 37 °C. After adding 664 µL of 20 mmol/L MES, pH 6.0, to dilute the antibody concentration of 0.5 g/L, 20 µL of the diluted sample was injected.

Figure 15: conclusions

- Lysine is a positively charged amino acid, so that the lysine variants are separated by IEC, depending on the number of lysine residues.
- An antibody was digested with carboxypeptidase B to cleave C-terminal lysine residues, followed by analysis using a TSKgel CM-STAT column.
- The 3 peaks observed before digestion converged into a single peak. The results indicate that C-terminal lysine variants were separated by the TSKgel CM-STAT column.

Conclusions

- Detection of protein heterogeneity is needed not just for academic research purposes, but also to separate the impurities to get the best quality biotherapeutics agents with the highest potency and free from toxicity. And just one mode cannot achieve the highest purity.
- Tosoh offers a variety of columns from different chromatographic modes for the detection of protein heterogeneity.