Detection of protein heterogeneity by HPLC

Atis Chakrabarti

Tosoh Bioscience LLC, King of Prussia, PA 19406

Introduction

Protein heterogeneity is generated by…

- Post-translational modification

  phosphorylation（dephosphorylation）, glycosylation, lipidation, methylation, acetylation, protein splicing, …

- Decomposition

  proteolysis, deamidation, oxydation, …

- Others

  chemical modification（e.g. PEGylation), denaturation, aggregation, ...

Introduction

Therapeutic proteins

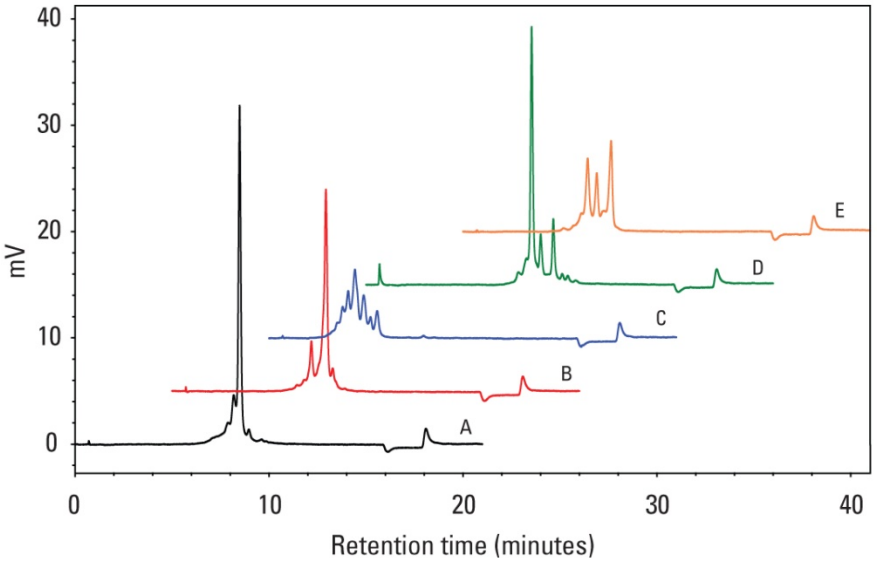
- Therapeutic antibodies and recombinant proteins are now widely used for therapeutic treatment.
- Heterogeneity evaluation is essential during development, stability testing, and in the quality control of the final product.
- Analysis of the aggregates and denaturated proteins is also important because they might increase the risk of anaphylaxis or immunoreaction.

Contents

1. Ion Exchange Chromatography (IEC)
2. Size Exclusion Chromatography (SEC)
3. Hydrophobic Interaction Chromatography (HIC)
4. Reversed Phase Chromatography (RPC)

1. Ion Exchange Chromatography

Therapeutic antibody analysis (1) using a TSKgel CM-STAT column

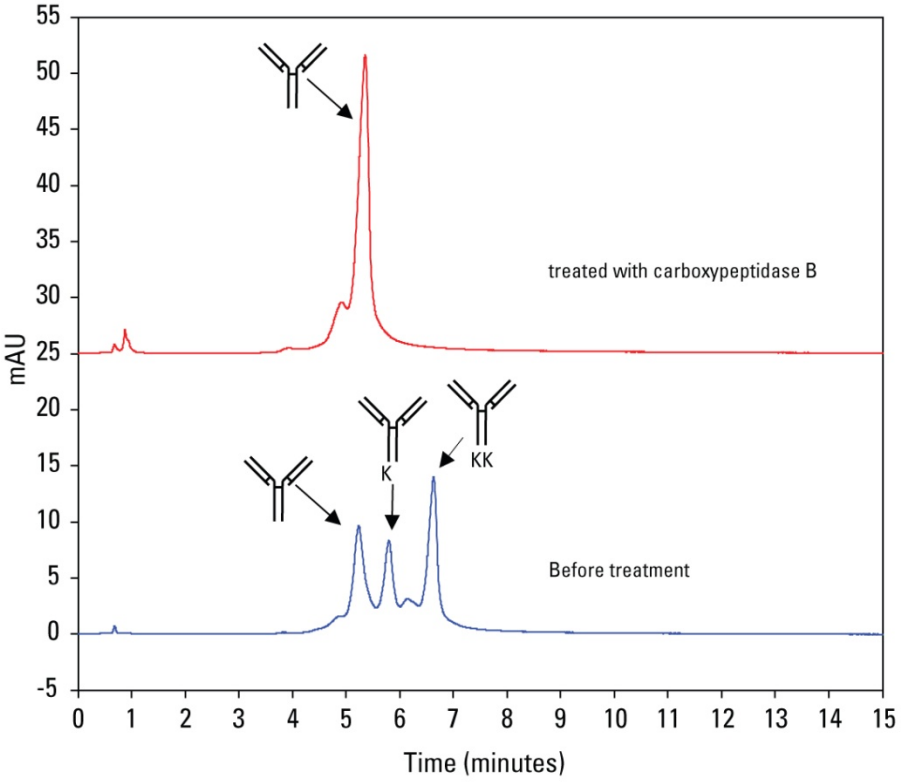


Column:	TSKgel CM-STAT, 4.6 mm ID x 10 cm
Mobile phase A:	20 mmol/L MES, pH 6.0
Mobile phase B:	20 mmol/L MES + 0.5 mol/L NaCl, pH 6.0
Gradient:	10% B (0 min), 30% B (15 min), 100% B (15.1 min), 100% B (18 min), 10% B (18.1 min), 10% B (23 min)
Flow rate:	1.0 mL/min
Detection:	UV@280 nm
Temperature:	25°C
Injection vol.:	20 µL
Concentration:	0.5 g/L
Sample:	A: humanized, IgG_1
	B: humanized, IgG_1
	C: chimera, IgG_1
	D: chimera, IgG_1
	E: chimera, IgG1

MES: 2-morpholinoethanesulfonic acid

High resolution analysis profiles of five antibodies were obtained on a TSKgel CM-STAT column. Each antibody shows multiple variants.

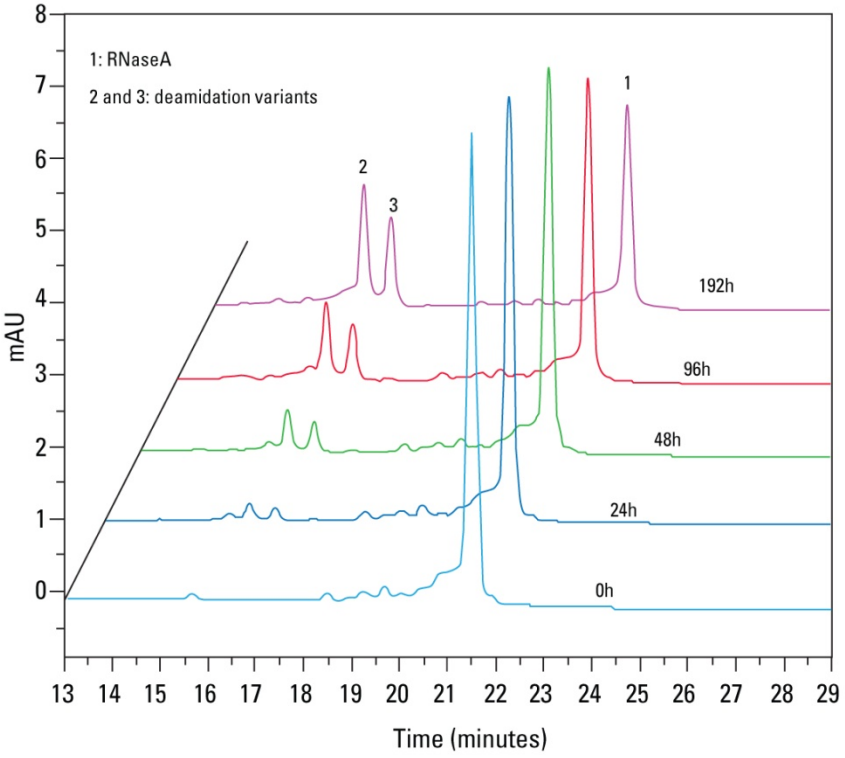
Therapeutic antibody analysis (2) using a TSKgel CM-STAT column

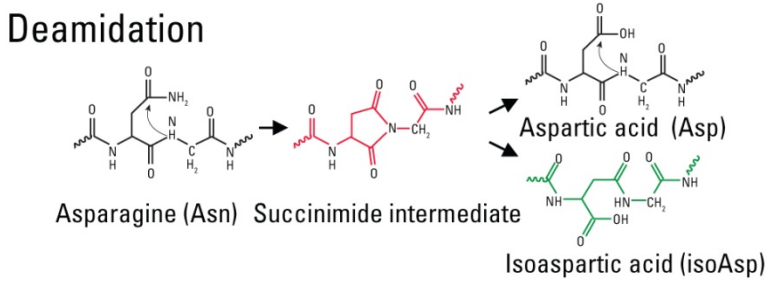


Column:	TSKgel CM-STAT, 4.6 mm ID x 10 cm
Mobile phase A:	20 mmol/L MES, pH 6.0
Mobile phase B:	20 mmol/L MES + 0.5 mol/L NaCl, pH 6.0
Gradient:	10% B (0 min), 30% B (15 min) 100% B (15.1 min), 100% B (18 min), 10% B (18.1 min), 10% B (23 min)
Flow rate:	1.0 mL/min
Detection:	UV@280 nn
Temperature:	25°C
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.

The TSKgel CM-STAT column can detect even one amino residue difference.

Deamidation of RNaseA using a TSKgel CM-STAT column





Column:	TSKgel CM-STAT, 4.6 mm ID x 10 cm
Mobile phase A:	20 mmol/L MES, pH 6.0
Mobile phase B:	20 mmol/L MES +1 mol/L NaCl, pH 6.0
Gradient:	5% B (0 min), 25% B (30 min), 100% B (30 min), 100% B (34 min), 5% B (34 min), 5% B (40 min)
Flow rate:	1.0 mL/min
Detection:	UV@280 nm
Temperature:	25°C
Injection vol.:	20 µL
Concentration:	0.25 g/L

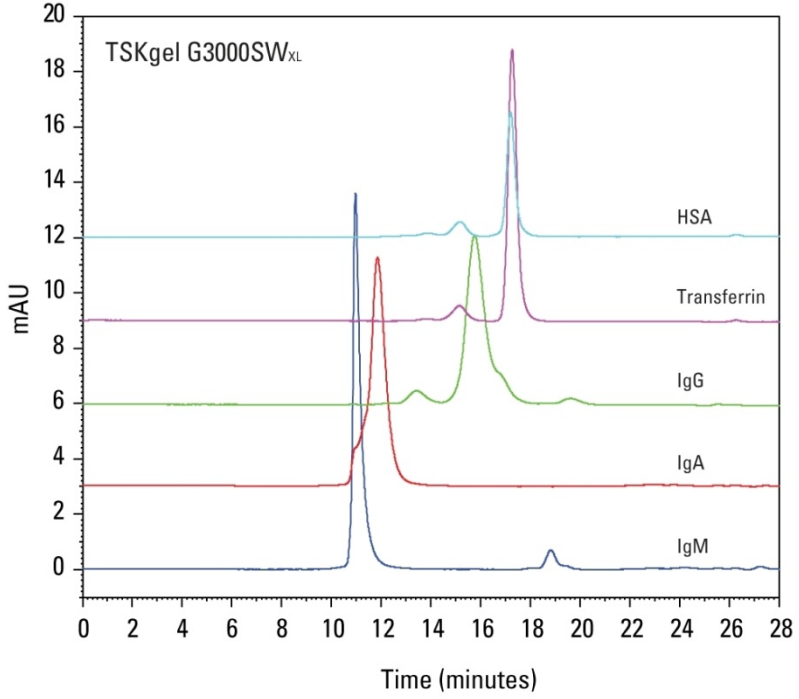
Procedure: RNaseA was dissolved at a concentration of 5 g/L in 1% ammonium carbonate buffer at pH 8.2 and incubated at 37°C for 0 to 192 hours. After incubation, diluted with 20 mmol/L MES, pH 6.0, at a concentration of 0.25 g/L and analyzed.

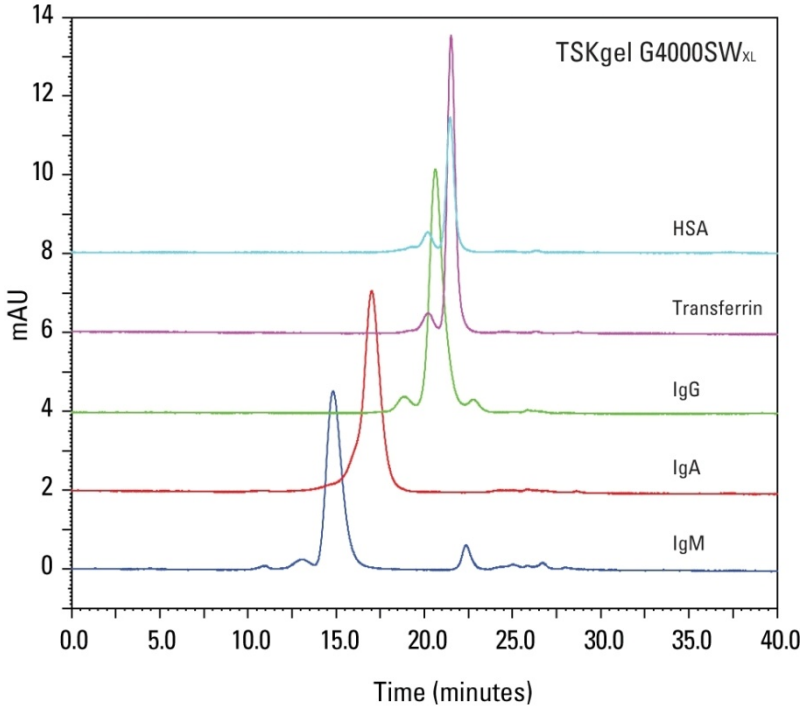
Note: Position 67 asparagine of the Ribonuclease A is well known of in the mild alkaline condition and generates 2 kinds of variants (Asp67 and isoAsp67).

RNaseA and the two variants were completely separated using the TSKgel CM-STAT column.

2. Size Exclusion Chromatography

Separation of immunoglobulins using TSKgel G3000SWXL and G4000SWXL columns





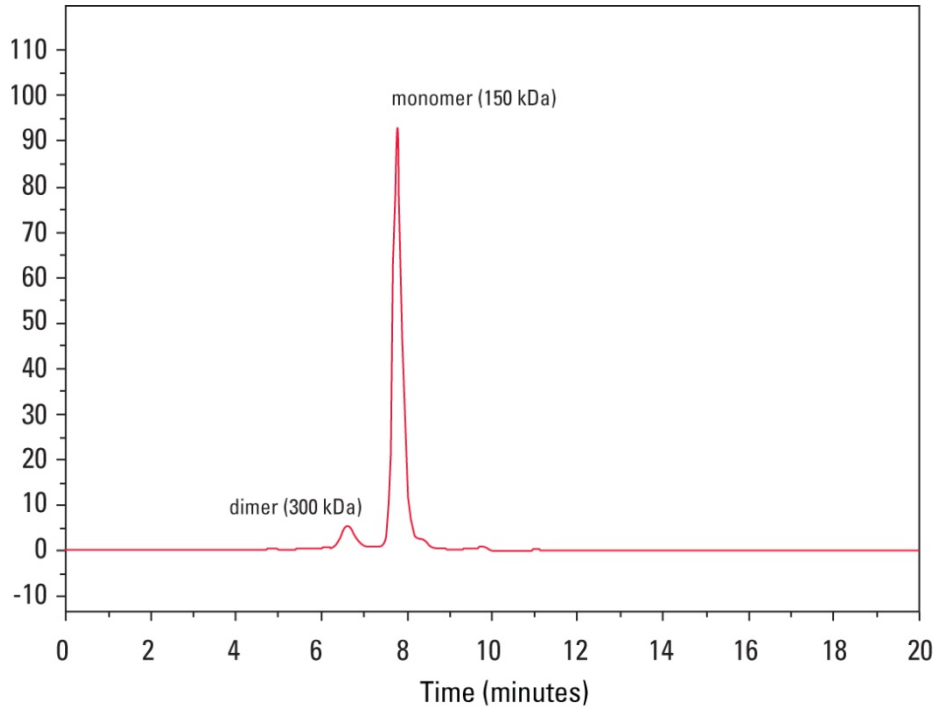
Column:	TSKgel G3000SWXL, 7.8 mm ID x 30 cm TSKgel G4000SWXL, 7.8 mm ID x 30 cm
Mobile phase:	20 mmol/L phosphate buffer + 0.3 mol/L NaCl, pH 7.0
Flow rate:	0.5 mL/min
Detection:	UV@280 nm
Temperature:	25°C
Injection vol.:	15 µL
Concentration:	0.17 g/L

Samples:

1. human IgM (from myeloma, MW: 970 kDa)
2. human IgA (colostrum, MW: 390 kDa)
3. human IgG (MW: 150 kDa)
4. transferrin (MW: 80 kDa)
5. human serum albumin (MW: 66 kDa)

The separation of immunoglobulins and serum-related proteins was successfully analyzed using TSKgel SWXL columns.

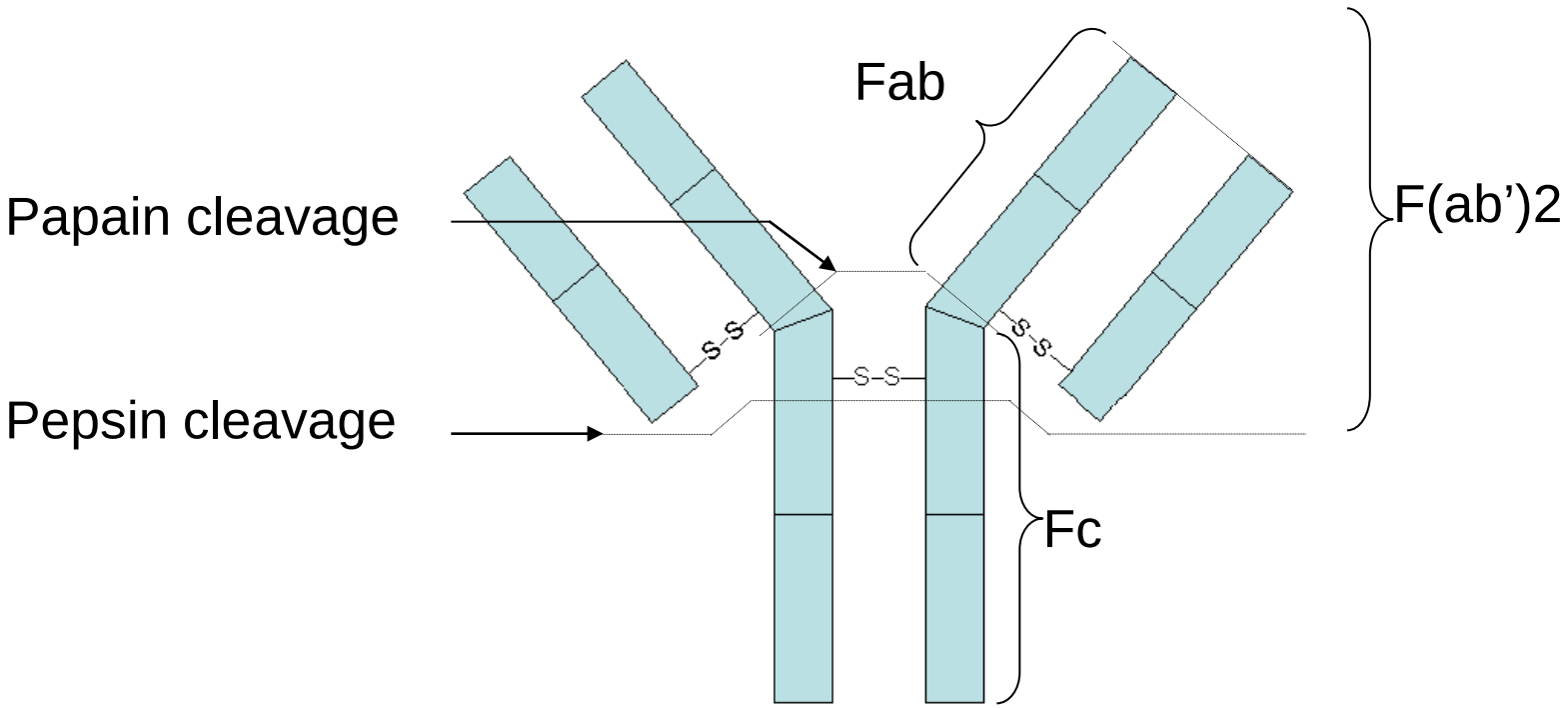
Aggregation analysis of an antibody using a TSKgel SuperSW3000 column



Column:	TSKgel SuperSW3000, 4.6 mm ID x 30 cm
Mobile phase:	20 mmol/L phosphate buffer + 0.3 mol/L NaCl, pH 7.0
Flow rate:	0.35 mL/min
Detection:	UV@280 nm
Temperature:	room temperature
Injection vol.:	5 µL
Sample:	human monoclonal antibody (4.6 g/L, IgG_1)

The monomer antibody (approximate molecular size of 150 kDa) and the dimer (molecular size of 300 kDa) are separated with the TSKgel SuperSW3000 column.

Structure of IgG_1



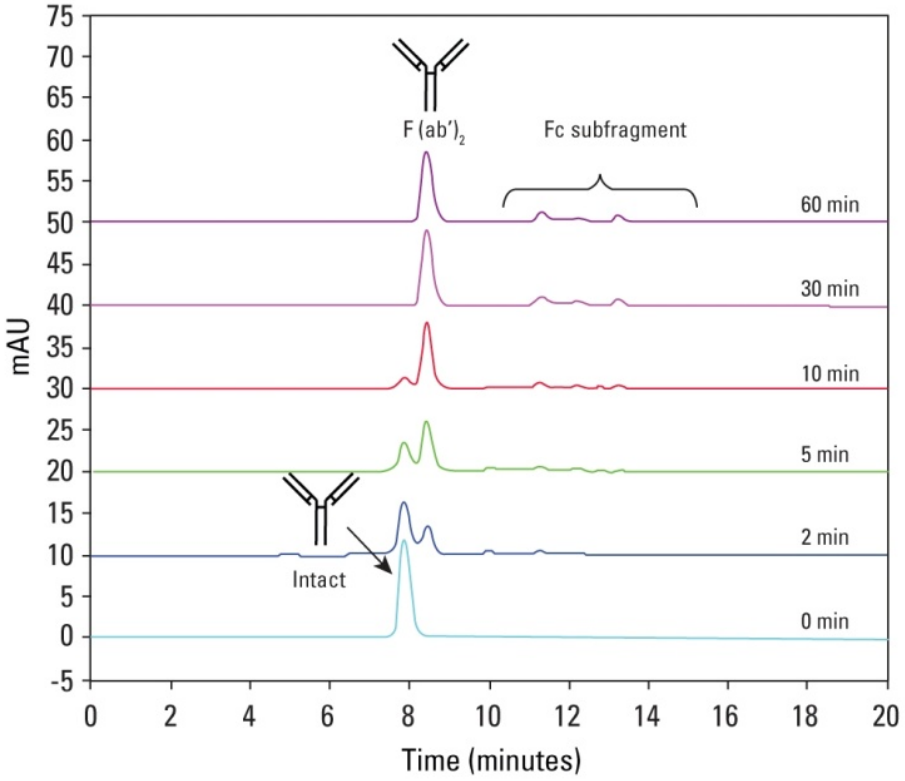
IgG is a relatively large molecule (approx. 150 kDa) and in order to improve the penetration to the tissue , fragmentation is carried out. Digestion with papain or pepsin is commonly applied to obtain antibody fragments without the loss of activity.
When papain is used for the antibody digestion, 2 Fab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

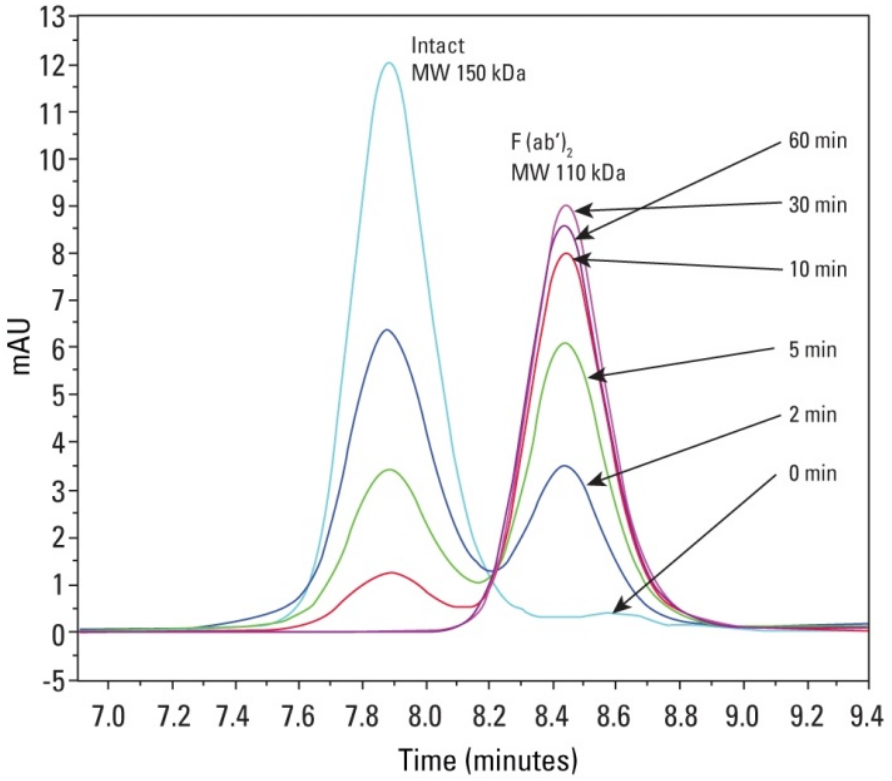
Pepsin digestion

Mouse IgG_1
(5 g/L, 0.1 mol/L citrate buffer, pH 3.7)
↓
Addition of Pepsin 40 U/mg-IgG
(1 g/L, 0.1 mol/L citrate buffer, pH 3.7)
↓
40°C
↓
Sampling 40 µL
↓
Addition of 10 µL 3 mol/L Tris
↓
Addition of 990 µL 20 mmol/L phosphate buffer + 0.3 mol/L NaCl, pH 7.0
↓
Analysis

Pepsin: from porcine gastric mucosa,
Sigma P7012, 2540 units/mg

Pepsin digestion using a TSKgel G3000SWXL column





Column:	TSKgel G3000SWXL, 7.8 mm ID x 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

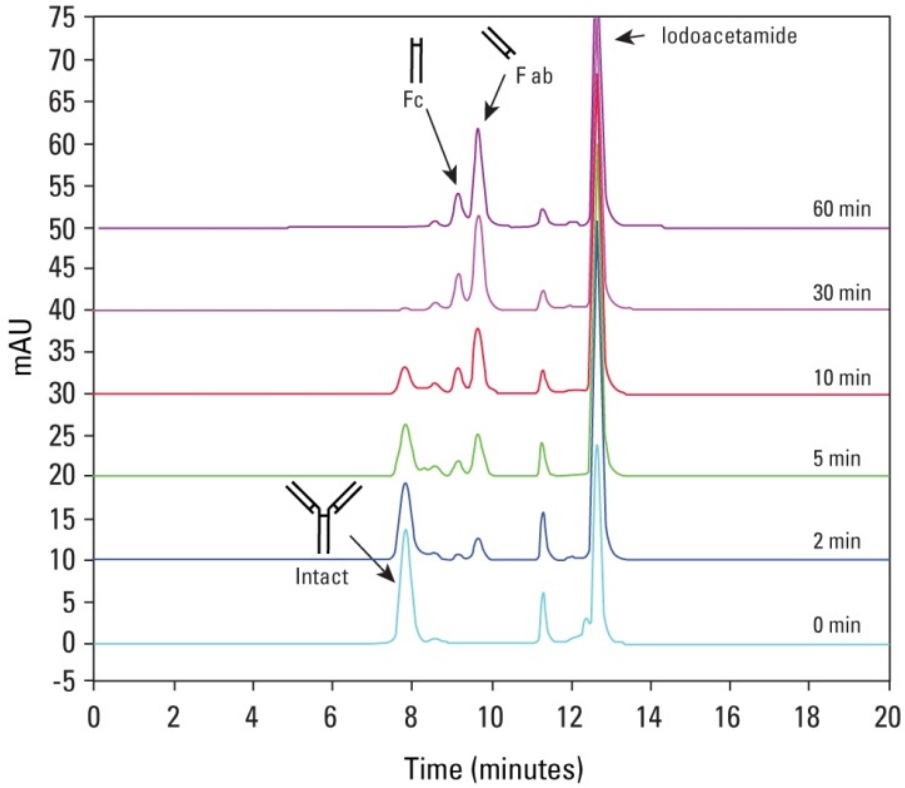
As the reaction proceeds, the Fc fragment is further digested to a small subfragment so its peak is not seen, as is expected from its molecular size.

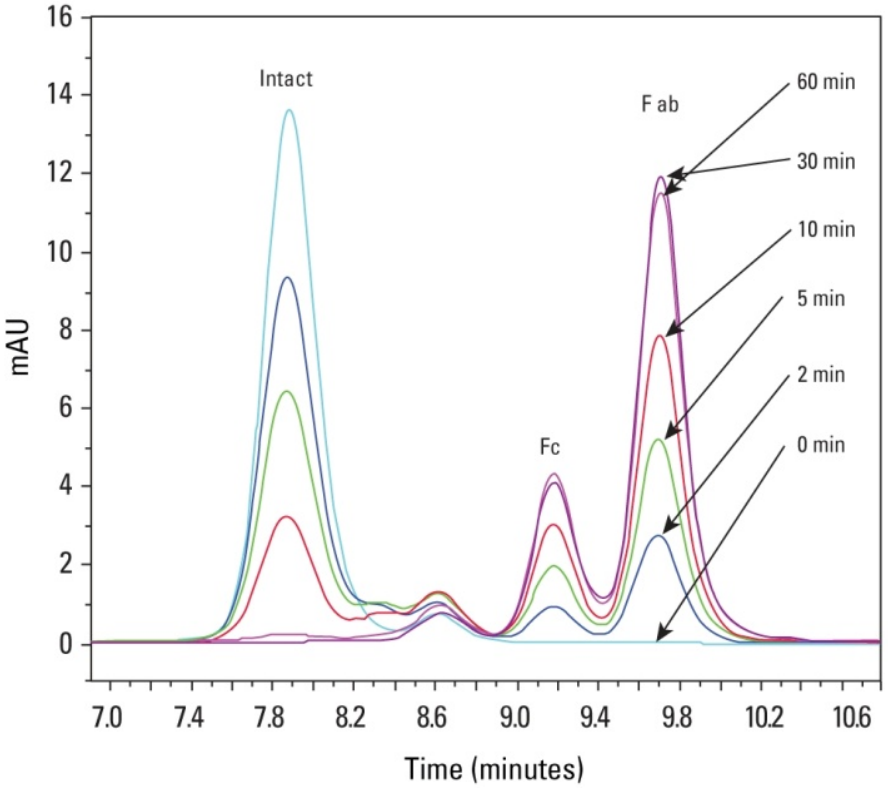
Papain digestion

Mouse IgG_1
(5 g/L, 10 mmol/L phosphate buffer, pH 7.3 + 0.15 mol/L NaCl
+ 1 mmol/L EDTA • 2Na + 25 mmol/L β-mercaptoethanol)
↓
Addition of Papain solution,10 vol%
(1 g/L, 10 mmol/L phosphate buffer, pH 7.3 + 0.15 mol/L NaCl
+ 1 mmol/L EDTA • 2Na + 25 mmol/L β-mercaptoethanol)
↓
37°C
↓
Sampling 48.5 µL
↓
Addition of 1.5 µL 1 mol/L iodoacetamide
↓
40°C, 15 min
↓
Addition of 950 µL 20 mmol/L phosphate buffer + 0.3 mol/L NaCl, pH 7.0
↓
Analysis by SEC

Papain: from papaya latex
Sigma P4762, 14 units/mg protein

Papain digestion using a TSKgel G3000SWXL column





Column:	TSKgel G3000SWXL, 7.8 mm ID x 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

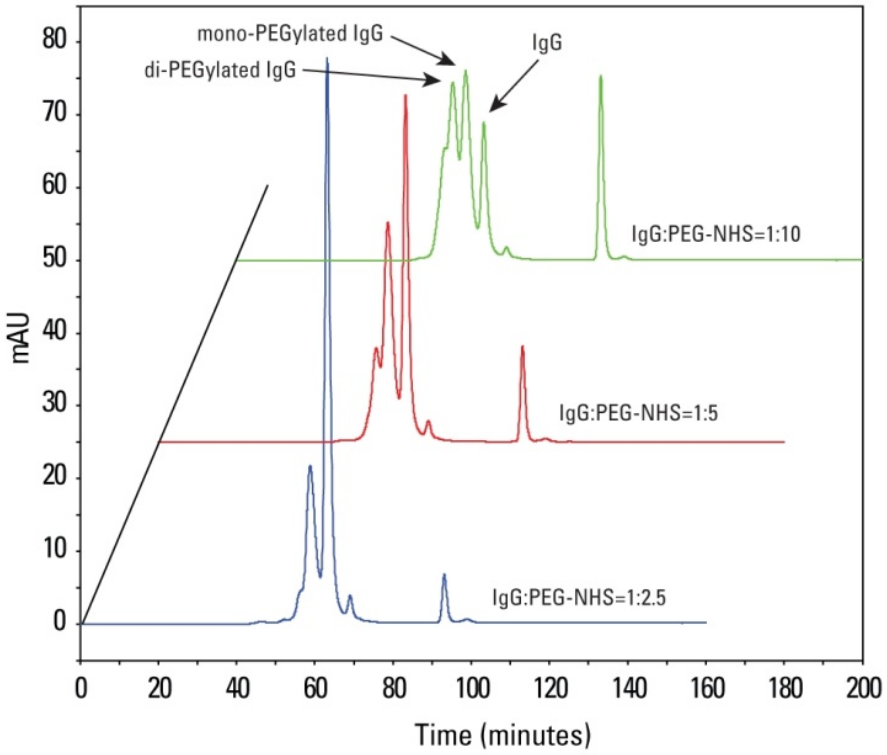
As the reaction proceeds, the intact antibody reduces and Fab and Fc peaks become larger.

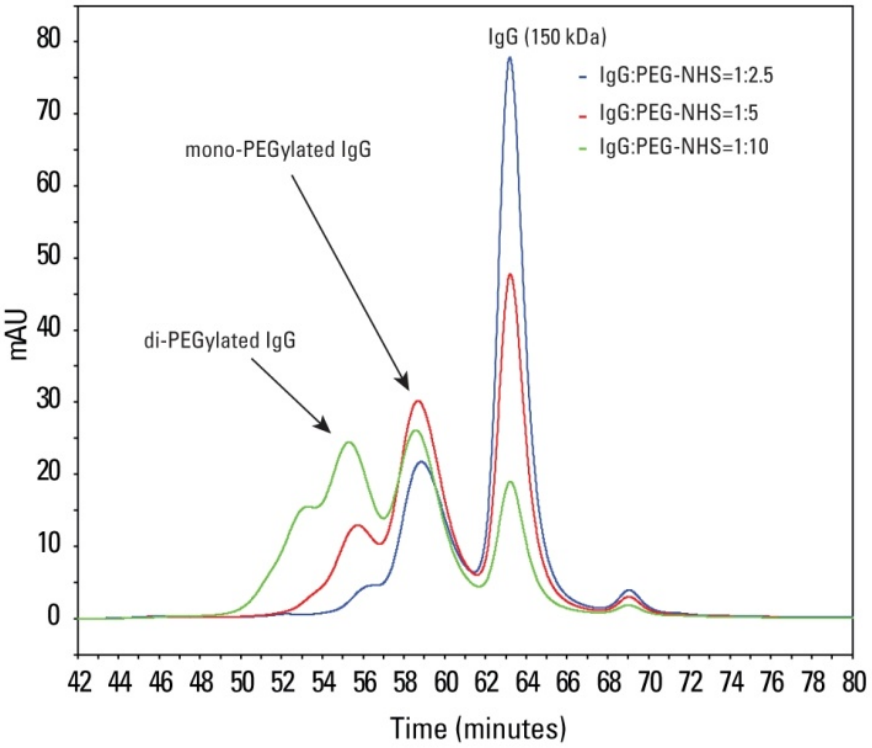
Separation of PEGylated protein (PEG:5000)

Mouse IgG_1
(5 g/L, 20 mmol/L phosphate buffer + 0.3 mol/L NaCl, pH 7.0)
↓
Addition of 2.5, 5, 10 equivalent molar of PEG-NHS/DMF
↓
4°C, 17 h
↓
To a 100 µL of sample solution add 900 µL 20 mmol/L of phosphate buffer + 0.3 mol/L NaCl, pH 7.0
↓
Analysis by SEC

PEG-NHS: PEG, N-hydroxysuccinimidyl ester
SUNBRIGHT ME-050CS, MW: 5000, NOF CORPORATION, JAPAN

Separation of PEGylated protein (PEG:5000) using a TSKgel G3000SWXL column



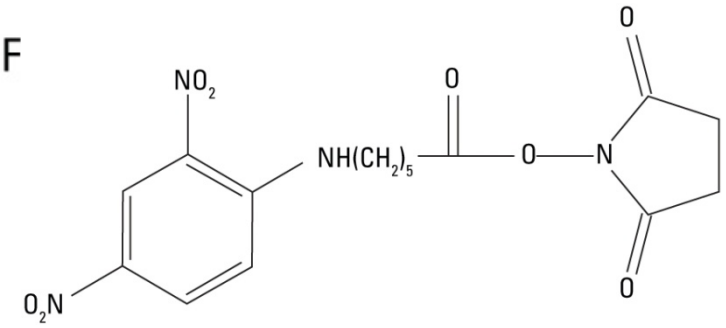


Column:	TSKgel G3000SWXL, 7.8 mm ID x 30 cm, 2 columns
Mobile phase:	20 mmol/L phosphate buffer + 0.3 mol/L NaCl, pH 7.0
Flow rate:	0.25 mL/min
Detection:	UV@280 nm
Temperature:	25°C
Injection vol.:	20 µL
Concentration:	0.5 g/L

From this analysis we can get information not only on the average number of PEG that were introduced to the antibody, but also the distribution of the PEG.

Analysis of hapten-conjugated protein

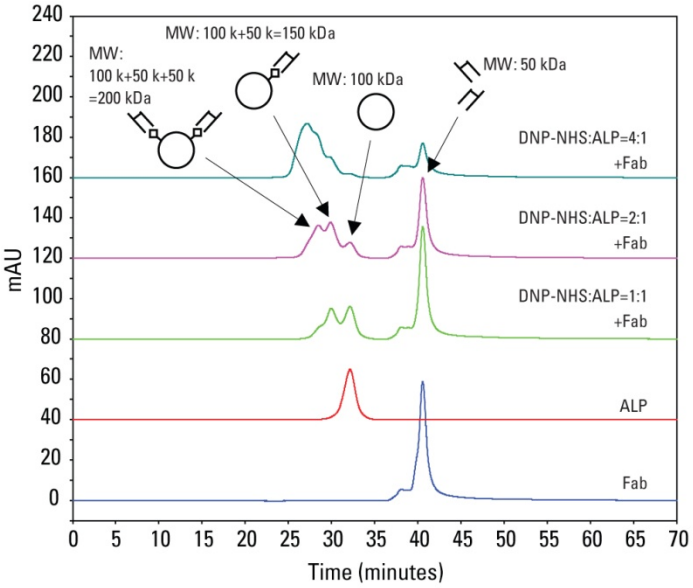
Alkaline Phosphatase (ALP)
(5 g/L)
↓
Addition of 1, 2, 4 equivalent molar of DNP-X-NHS/DMF
↓
4°C, 17 h
↓
Purified by SEC to remove unreacted DNP
↓
Addition of anti-DNP antibody (Fab)
↓
Analysis by SEC

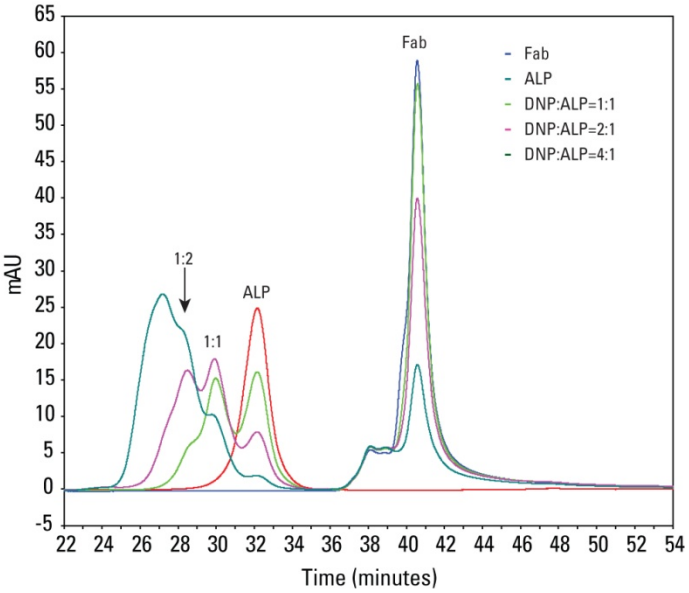


DNP-X-N-hydroxysuccinimidyl ester
MW: 394.34

DNP-X-NHS: AnaSpec, Cat# 81228
Anti-DNP MAb: Cosmo Bio, Cat# LO-DNP-61
Antibody was papain digested and Fab was purified by SEC before use

Analysis of hapten-conjugated protein using a TSKgel G3000SWXL column





Column:	TSKgel G3000SWXL, 7.8 mm ID x 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%

Because the molecular weight of hapten is only about 300 Da compared to the 100 kDa weight of alkaline phosphatase, molecular size doesn't change enough to change the retention time of alkaline phosphatase when the hapten has conjugated to the protein.

By adding anti-hapten Fab, in this case anti-DNP Fab, Fab binds to the hapten of the alkaline phosphatase surface. As a result, it shows a large molecular size increase, allowing hapten-conjugated and non-conjugated alkaline phosphatase to be separated by SEC.

3. Hydrophobic Interaction Chromatography

Analysis of therapeutic antibody (Papain digested)

Therapeutic antibody

↓

Fractionation by CIEC (TSKgel BioAssist S)

↓

Adjust to 2 mg/mL
(0.1 mol/L Tris-HCl, 20 mmol/L Cysteine, 4 mmol/L EDTA, pH 7.2)

↓

Addition of Papain solution, 2 vol%
(1 g/L, 0.1 mol/L Tris-HCl, 20 mmol/L Cysteine, 4 mmol/L EDTA, pH 7.2)

↓

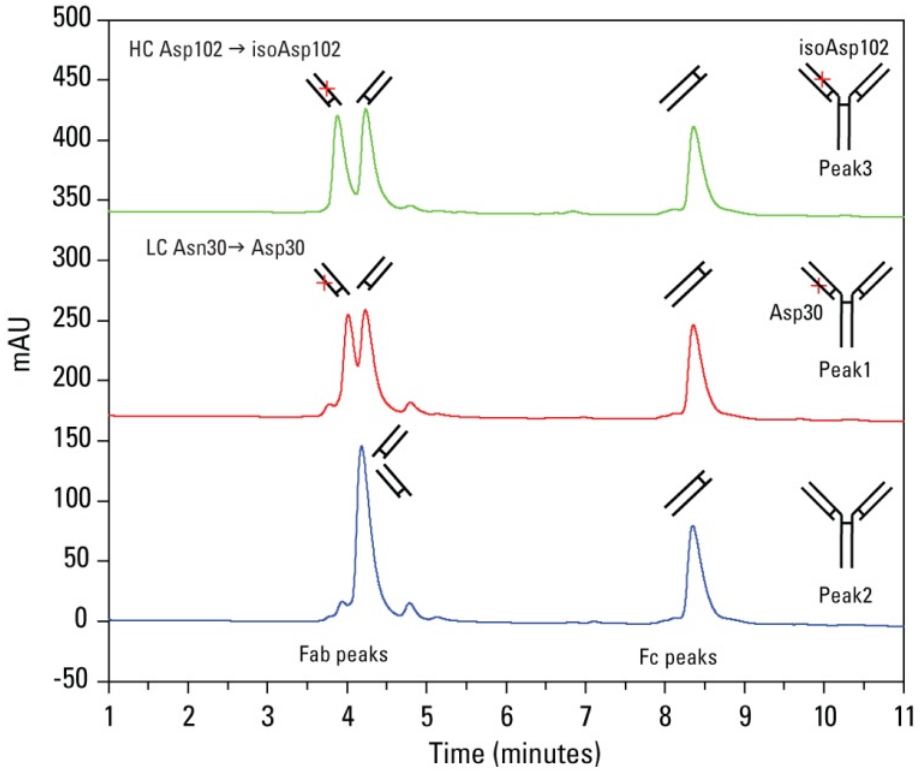
37°C, 2 h

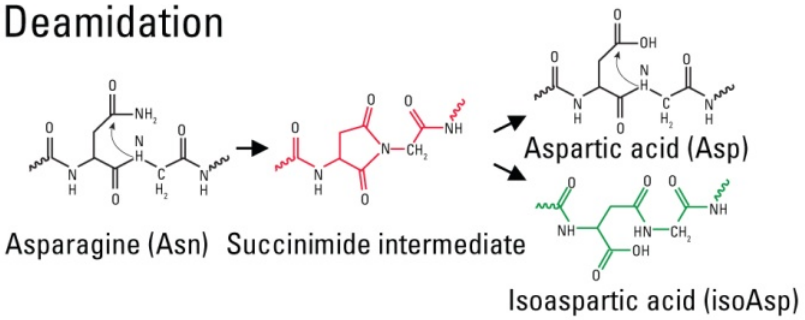
↓

Analysis by TSKgel Butyl-NPR

Papain: from papaya latex
Sigma P4762, 14 units/mg protein

Analysis of therapeutic antibody (Papain digested) using a TSKgel Butyl-NPR column





Column:	TSKgel Butyl-NPR, 4.6 mm ID x 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

TSKgel Butyl-NPR is also a superior tool for detecting even one residue difference of the proteins.

Analysis of therapeutic antibody (Papain digested)

Therapeutic antibody

↓

Adjust to 1 mg/mL
(5 mmol/L NaOAc, 150 mmol/L NaCl, 0.01% Tween20, pH 5.0)

↓

Addition of t-BHP
(70% t-BHP solution was x10 diluted with PW before use, 1% or 0.1%vol of diluted t-BHP were added)

↓

25°C, 20 h

↓

Dialysis
(0.1 mol/L Tris-HCl, pH 7.2)

↓

Adjust to 2 mg/mL
(0.1 mol/L Tris-HCl, 20 mmol/L Cysteine, 4 mmol/L EDTA, pH 7.2)

↓

Addition of Papain solution, 2 vol%
(1 g/L, 0.1 mol/L Tris-HCl, 20 mmol/L Cysteine, 4 mmol/L EDTA, pH 7.2)

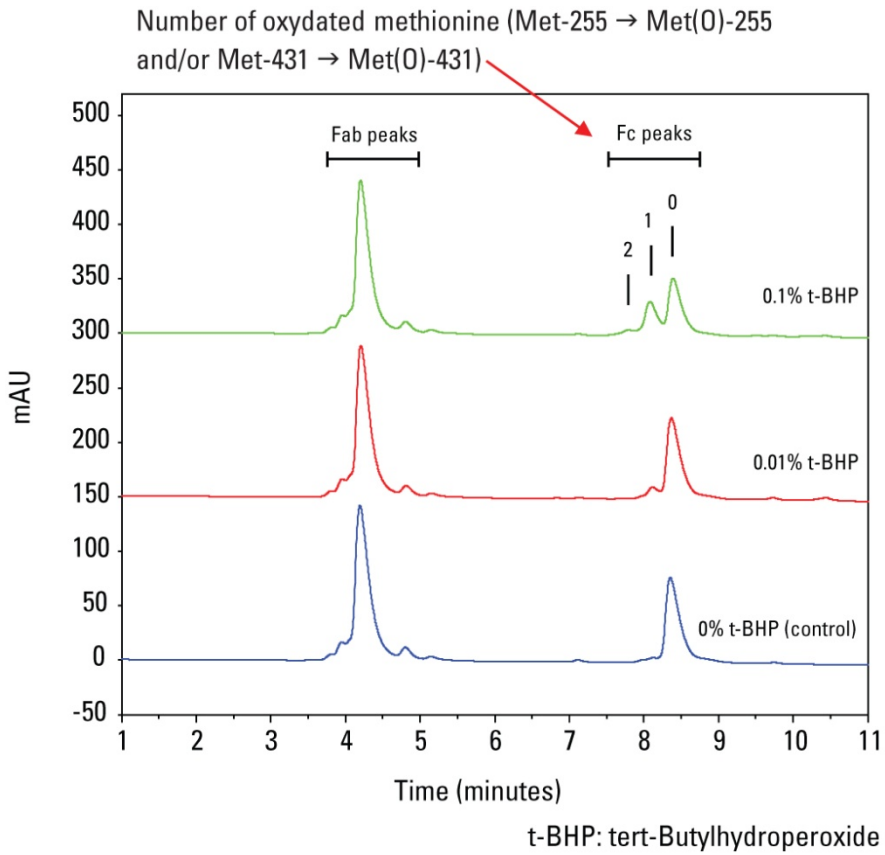
↓

37°C, 2 h

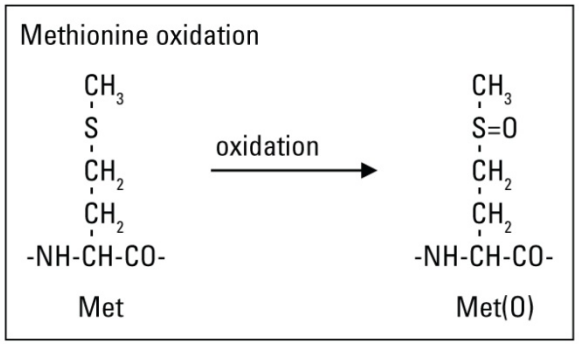
↓

Analysis by TSKgel Butyl-NPR

Analysis of therapeutic antibody (Papain digested) using a TSKgel Butyl-NPR column



t-BHP: tert-Butylhydroperoxide



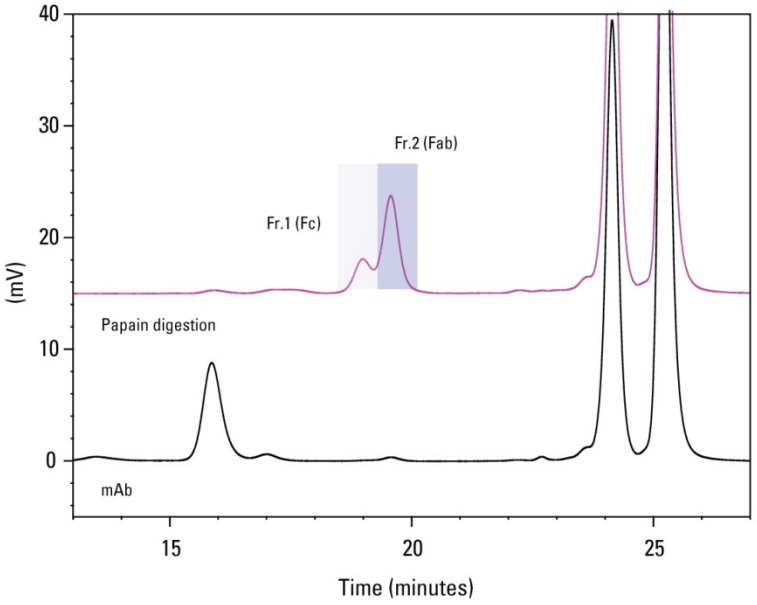
Column:	TSKgel Butyl-NPR, 4.6 mm ID x 3.5 cm
Mobile phase A:	20 mmol/L phosphate buffer containing 2 mol/L $(NH_4)_2SO_4$, pH 7.0
Mobile phase B:	20 mmol/L phosphate buffer, pH 7.0
Gradient:	10 min linear gradient from 25 to 60% B
Flow rate:	1.0 mL/min
Detection:	UV@214 nm
Temperature:	25°C
Injection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

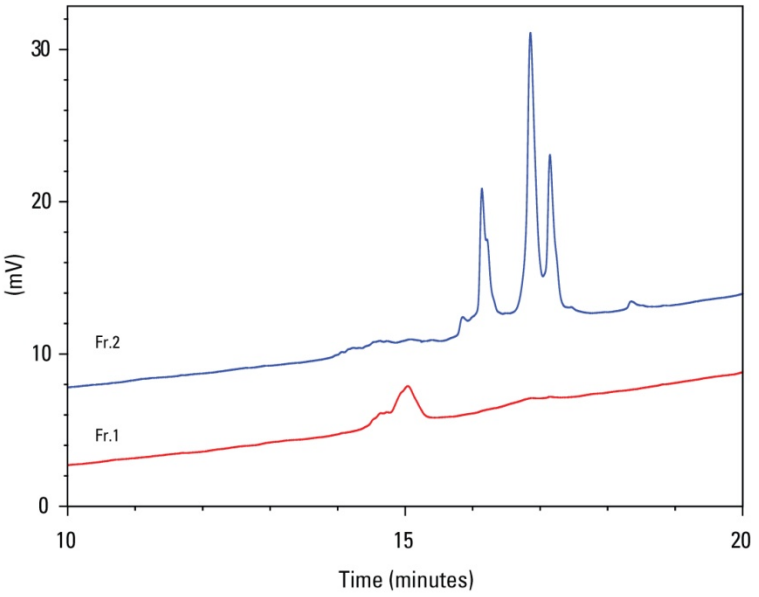
Therapeutic antibody was oxidated by incubating the antibody solution with t-BHP and analyzed with TSKgel Butyl-NPR after papain digestion.

Methione residue of Fc was oxidated, and as a result multiple peaks were observed. Whereas no change was observed with Fab peaks.

4. Reversed Phase Chromatography

Analysis of antibody fragment using a TSKgel Protein C_4-300 column





Conditions for SEC

Column:	TSKgel G3000SW$_{XL}$, 7.8 mm ID x 30 cm x 2
Mobile phase:	20 mmol/L phosphate buffer, pH 7.0 + 0.3 mol/L NaCl
Flow rate:	1.0 mL/min
Temperature:	25°C
Sample:	monoclonal antibody (human IgG$_1$)

Conditions for RPC

Column:	TSKgel Protein C_4-300, 4.6 mm ID x 15 cm
Mobile phase A:	0.05% TFA in water
Mobile phase B:	0.05% TFA in acetonitrile
Gradient:	5% B (0 min), 50% B (20 min)
Flow rate:	1.0 mL/min
Temperature:	70°C
Sample:	monoclonal antibody (human IgG$_1$)

Human antibody was papain digested and separated with SEC. Two fractions were obtained and each fraction was analyzed with the TSKgel Protein C_4-300 column. Three peaks were observed with the analysis of Fab.

Conclusions

- Therapeutic antibodies and recombinant proteins are now widely used for therapeutic treatment and evaluation of their heterogeneity is essential for the development, stability testing, and in the quality control of the final product.
- High resolution TSKgel nonporous resin columns for Ion Exchange Chromatography and Hydrophobic Interaction Chromatography can detect even one residue difference of the proteins.
- Size exclusion chromatography is suitable for detecting aggregates, fragments, and PEGylated proteins.
- The reversed phase chromatography column, TSKgel Protein C4-300, which has a large pore size of 300 Å, is applicable for the protein analysis.

Generous support was received from Dr. Hitoshi Kakidani, Sagami Chemical Research Institute.