Monoclonal Antibody Fragment Separation and Characterization Using Size Exclusion Chromatography Coupled with Mass Spectrometry

Application Note ZM-1008

Protein Separation

Authors

Haiying Chen
Katherine McLaughlin
Sepax Technologies, Inc.
5 Innovation Way
Newark, DE 19711
USA

Abstract

Monoclonal antibodies have increasingly become a major part of protein therapeutics. Monoclonal antibody fragments (such as Fab and F(ab')$_2$) offer advantages over using intact MAbs such as reducing nonspecific antigen binding from Fc. Size exclusion chromatography has been widely used in protein analysis. Aggregates, monomers and degradation products of monoclonal antibodies can be separated on size exclusion columns based on their molecular weights under native conditions. In general, protein native buffer conditions such as salts at neutral pH are not mass spectrometry friendly. In this study, we investigated antibody fragments such as heavy and light chains, Fab/Fc, and F(ab')$_2$ using SEC separation. Heavy and light chains were also analyzed by SEC coupled with mass spectrometry with volatile mobile phases. The effect of different percentages of TFA, formic acid and acetonitrile in mobile phases on the antibody fragments separation was also explored.

Introduction

Size exclusion chromatography (SEC) has been applied successfully to separate different sizes of proteins under native conditions. It has been routinely used for the characterization and quality control of monoclonal antibody therapeutics in the pharmaceutical industry (1). However, with normal SEC salt containing mobile phases, direct mass spectrometry analysis of antibody fragments is not feasible. Here we investigated salt and volatile mobile phase effects on the separation of monoclonal antibody fragments analyzed by Zenix™-SEC. Figure 1 shows the schematic illustrations on the generation of monoclonal fragments. Monoclonal antibodies were reduced to heavy and light chains using DTT. Fab and Fc fragments were generated by papain digestion while the $F(ab')_2$ fragment was obtained by pepsin digestion. With an optimized volatile mobile phase, the direct molecular weight analysis of a monoclonal antibody was achieved using online SEC with mass spectrometry.

Sepax Zenix™ SEC columns are based on uniform, hydrophilic, and neutral nanometer thick films chemically bonded on high purity and mechanically stabilized silica. Zenix™ SEC-300 is specifically designed for protein and large peptide separations with a molecular exclusion limit of 1,250,000 Da. In this application note, we applied Zenix™ SEC-300 for the separation of heavy light chains, Fab and Fc, and $F(ab')_2$ for the characterization of a monoclonal antibody. Mobile phases including salts and different concentrations of TFA, formic acid and acetonitrile were evaluated for the fragment separations.

Experimental

HPLC system

Agilent 1200 HPLC with binary pump

Q-TOF mass spectrometer

Waters Q-Tof Ultima. The scan range is from 350 to 3000 m/z. The instrument settings are: source temperature = 80°C, desolvation temperature = 150°C, capillary voltage = 4.44 kV

SEC column and LC method

Zenix™ SEC-300 (3 µm, 300 Å, 7.8 x 300 mm and 4.6 x 300 mm) was used for intact MAb and MAb fragment separations. Mobile phases include different percentages of TFA, formic acid and acetonitrile, the regular SEC mobile phase is 150 mM sodium phosphate buffer, pH 7.0. Flow rates were 0.5 mL/min and 0.2 mL/min for 7.8 x 300 mm and 4.6 x 300 mm columns, respectively.

SEC-MS

The Shimadzu HPLC with Sepax's Zenix™ SEC-300 7.8x300 mm column was used to separate the heavy and light chains. Online Waters Q-TOF mass spectrometer was used to analyze the heavy and light chains. The mobile phase was 0.1% TFA, 0.1% formic acid and 20% acetonitrile. The flow rate was 0.2 mL/min. 5 µg of reduced MAb 321 was injected for analysis.

Chemicals and Reagents

Recombinant monoclonal antibody (MAb 321) was produced by transfected Chinese Hamster ovary (CHO) and purified by a local biotechnology company.

Papain from papaya latex and pepsin from porcine gastric mucosa were purchased from Sigma Aldrich.

4-12% Bis-Tris gels and reagents were purchased from Invitrogen.

Sample preparation

Reduction

DTT reduction was carried out on MAb 321. MAbs were diluted to 1mg/mL with 150 mM phosphate buffer, pH 7.0. Antibodies were reduced with a final concentration of 20 mM DTT at 65°C for 15 minutes. Various amounts of reduced MAbs were injected. SEC runs were monitored using 280 nm UV absorbance.

Papain digestion

Papain digestion was modified from the procedure reported previously (2). The digestion was carried out by incubating MAb 321 (1 mg/mL) in 100 mM Tris-HCl, pH 7.6, 2 mM EDTA and 5 mM Cysteine. The digestion was started by adding 1 mg/mL papain. The papain/MAb ratio was at 1:100. The digestion

mixture was incubated for 2, 3, 3.5 and 4 hours at 37°C.

Pepsin digestion

Pepsin digestion was performed with a method modified from the previously reported (3,4). MAb 321 was incubated at a final concentration of 1 mg/mL in 20 mM sodium acetate, pH 4.0 with a pepsin to MAb 321 ratio of 1:40. The digestion was carried out at 37 °C for 15.5 hours. The reaction was stopped by adding 2 M TRIS to increase the pH to 8.0.

Papain digestion and Pepsin digestion mixtures were stored at -20°C until use. Freshly reduced MAb 321 was prepared each time before use. All samples were stored in the chilled autosampler for sequenced HPLC runs.

Results

Monoclonal antibody fragment separations on Zenix SEC-300 with 0.1% TFA, 0.1% formic acid and 20% acetonitrile

Reduced MAb

Separations of heavy chain and light chain were achieved using both 4.6 x 300 mm and 7.8 x 300 mm Zenix™ SEC-300 columns. HPLC profiles in figure 2 and 3 indicate that the elution times of heavy and light chains were later than that of intact MAb. Heavy and light chains were separated based on their molecular elution order. 4-12% Bis-Tris gel image shows the correct molecular weight for each collected fraction containing heavy (50 kD) and light (25kD) chains. There was a small fraction of the dimerized fragments in each peak according to the gel bands with the dimer molecular weights.

With a 7.8 x 300 mm column at a 0.2 mL/min flow rate and directly coupled to online mass spectrometry, identities of the heavy and light chains were confirmed. Heavy chain and light chain were separated. Sample were directly introduced to the mass spectrometer without a flow split (LC profile not shown, similar to figure 3 with later retention time due to slower flow rate). Figure 4 represents the deconvoluted mass spectra of heavy and light chain peaks. Heavy chain has a major fragment with a mass of 50,598 Da. Another prominent peak shown in the spectra was 50,760 Da with an additional mass of 162 Da. The possible difference between these two peaks is the mass of galactose due to the different glycosylation on the MAb. Light chain fragment has a mass of 23,441 Da.

Papain digestion – Fab/Fc separation

Papain digestion generated Fab and Fc fragments from the intact MAb. A time course of papain digestion at 37 °C yielded optimum 3.5 hours of incubation time. Time point material was taken and subjected to SEC analysis similar to figure 5 (3.5 hour time point). At 3 hours of incubation, based on peak integration from a 7.8 x 300 mm run with 20 µg digested MAb injection, there was a 0.28% intact MAb and 0.5% Fab/Fc dimer present. Intact MAb, incomplete digestion fragments, Fab and Fc were all well separated on Zenix™ SEC-300 (data not shown here). Figure 5 shows an overlay profile of individually injected intact MAb 321, papain digested MAb 321 and papain blank on a 4.6 x 300 mm column. Fab and fc maintained the baseline separation with 5 µg injection. The gel image of the collected peaks indicates the correct molecular weight order elution.

Pepsin digestion – $F(ab')_2$

Pepsin digestion generated the $F(ab')_2$ fragments and many small fc fragments. Figure 6 panel A shows the digested MAb separation on Zenix™ SEC-300 4.6 x 300 mm. $F(ab')_2$ eluted as a single peak with a small shoulder due to a smaller fragment which is indicated by the gel image shown in panel B. This smaller fragment had an estimated molecular weight of 70 kD. There was also a small fraction of undigested intact MAb 321, indicated by a tiny visible peak before the $F(ab')_2$ peak and a faint gel band at 160 kD in the gel image.

Comparison of mobile phases with different TFA concentrations in volatile buffer 0.1% formic acid with 20% acetonitrile and salt mobile phase

Figures 7, 8 show chromatogram overlays for heavy/light chains, Fab/Fc and $F(ab')_2$ separations with 0.02%, 0.05% and 0.1% TFA in 0.1% formic acid and 20 % acetonitrile on a 4.6 x 300 mm column, respectively. The results indicate that when TFA concentration is reduced to 0.05%, all fragments still can be baseline separated with slightly broader peak width in comparison to 0.1% TFA. At 0.02%, all fragments in each sample could not be separated and were merged into the buffer peaks.

When the mobile phase was switched to a 150 mM sodium phosphate buffer, only the papain digested sample was analyzed; Fab/Fc fragments were eluted as one peak with a delayed retention time as shown in figure 9.

Effect of formic acid concentration on monoclonal antibody fragment separations

With 0.02% TFA, 0.1% formic acid and 20% acetonitrile, there is no separation of heavy/light, Fab/ Fc and $F(ab')_2$. When the concentration of formic acid was increased to 1% in 0.02% TFA and 20% acetonitrile, all fragments were separated again on the same column. Figure 10 and 11 show the heavy/light, Fab/Fc, and $F(ab')_2$ separations under three different mobile phases (A. 0.02% TFA in 1% formic acid and 20% acetonitrile; B. 1% formic acid in 20% acetonitrile; C. 0.1% TFA in 0.1% formic acid and 20% acetonitrile). When 0.02% TFA was omitted from the mobile phase, heavy and chains were baseline separated while $F(ab')_2$ exhibited a much broader peak width, indicating a stronger retention on the column. Both Fc and Fab peaks were eluted earlier and there was a slight overlap between the two peaks without the 0.02% TFA as shown in figure 11. Table 1 summarizes the column performances using the three different mobile phases. 0.02% TFA in 1% formic acid and 20% acetonitrile has similar effect on the separation of Fab and Fc as 0.1% TFA in 0.1% formic acid and 20% acetonitrile.

Effect of acetonitrile concentration on Fab/Fc separations

When the percentage of acetonitrile was changed to 50% from 20% in 0.02% TFA and 1% formic acid, both heavy/light and $F(ab')_2$ separation maintain a similar separation as with 20% acetonitrile. All fragment peaks were eluted with earlier retention times (data not shown). However with Fab/Fc separation as shown in figure 12, 50% acetonitrile can't separate Fab and Fc fragments.

Sample loading test on Zenix™ SEC-300 4.6 x 300 mm

Increasingly low sample loadings are required in the characterization and QC process of a monoclonal antibody and its fragments. When the injection of papain digested MAb 321 was reduced to 0.1 μg, Fab and Fc remained baseline separated with 0.02% TFA, 1% formic acid and 20% acetonitrile, as shown in figure 13. The other fragment peak, next to the Fab peak, was visible at 0.1 μg.

Conclusion

Zenix™ SEC-300 4.6 x 300 mm can successfully separate MAb fragments including heavy/light chains, Fab/Fc and $F(ab')_2$ from their reaction mixtures, respectively. Volatile mobile phases were optimized for the separation of MAb fragments. To minimize the TFA MS signal suppression effect, TFA concentrations can be reduced to lower than 0.05%. At 0.02% TFA, 1% formic acid and 20% acetonitrile, all fragments can be separated from their reaction mixtures with similar separation efficiency as 0.1% TFA. Even without TFA, all MAb fragments can be separated with some reduced column performance. Both mobile phases are suitable for the MAb fragment separation and SEC-MS online analysis. With low sample loading and mass spec friendly mobile phases, the on-line SEC-MS method can be applied to general protein separation and mass detection.

For more information on Zenix™- SEC products, please visit Sepax website: http://www.sepax-tech.com/Zenix.php or contact us at 1-877-SEPAX-US

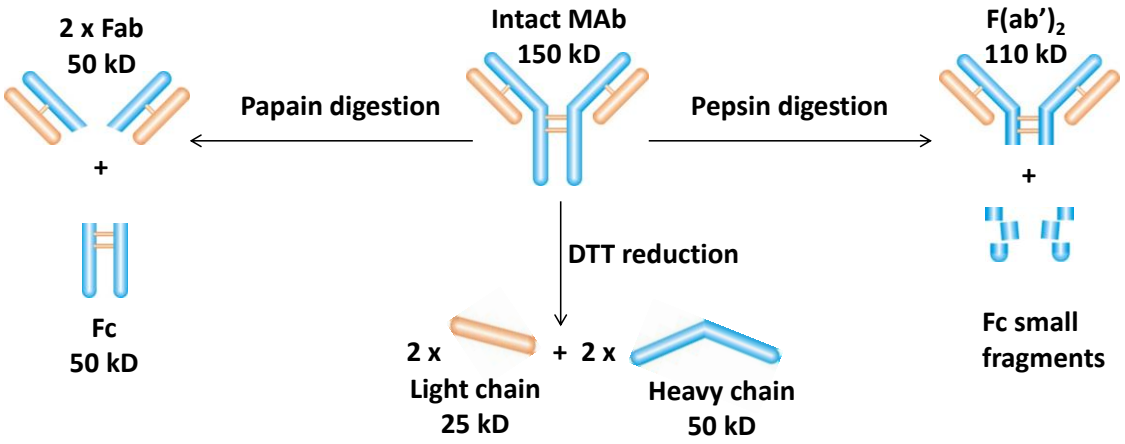

Figure 1. Schematic illustrations of monoclonal antibody fragment generation.

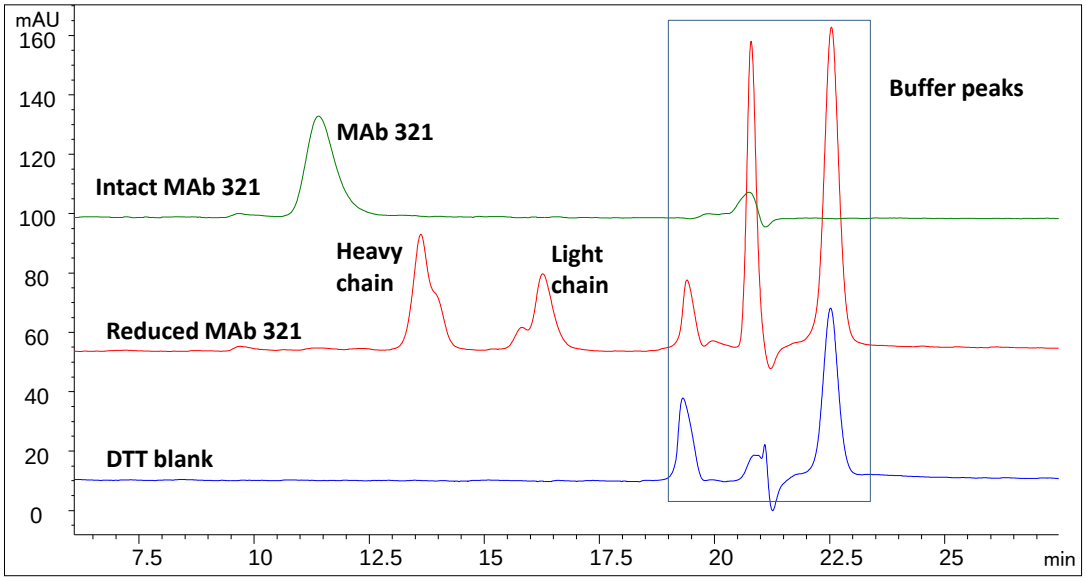

Figure 2. Reduced MAb 321 separation on Zenix™ SEC-300, 4.6 x 300 mm. Mobile phase was 0.1% TFA, 0.1% formic acid with 20% acetonitrile. Flow rate was at 0.2 mL/min. UV detection was set at 280 nm. 5 µg of intact MAb 321 and 20 µg of reduced MAb 321 were injected.

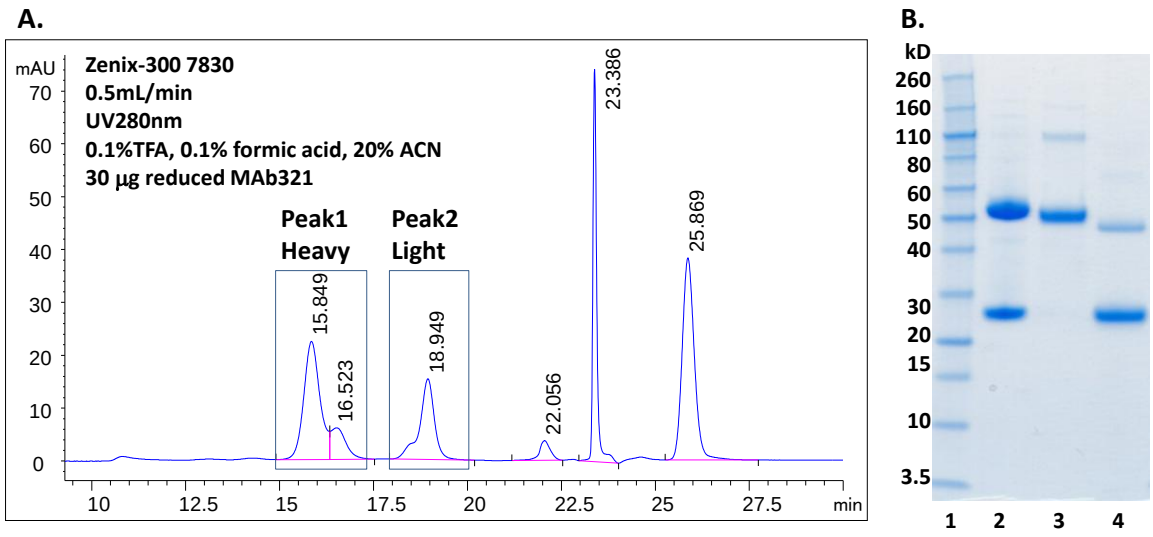

Figure 3. Panel A shows a separation profile of reduced MAb 321 on Zenix™ SEC-300, 7.8 x 300 mm. Peak 1 and Peak 2 were each collected and speed-vac dried. Dried samples were then dissolved in Invitrogen LDS sample buffer. Panel B. shows the 4-12% Bis-Tris gel image of reduced MAb sample and Peak 1, 2 fractions. Lane 1 protein marker; lane 2 reduced MAb sample mixture; lane 3 peak 1 heavy chain; lane 4 peak 2 light chain.

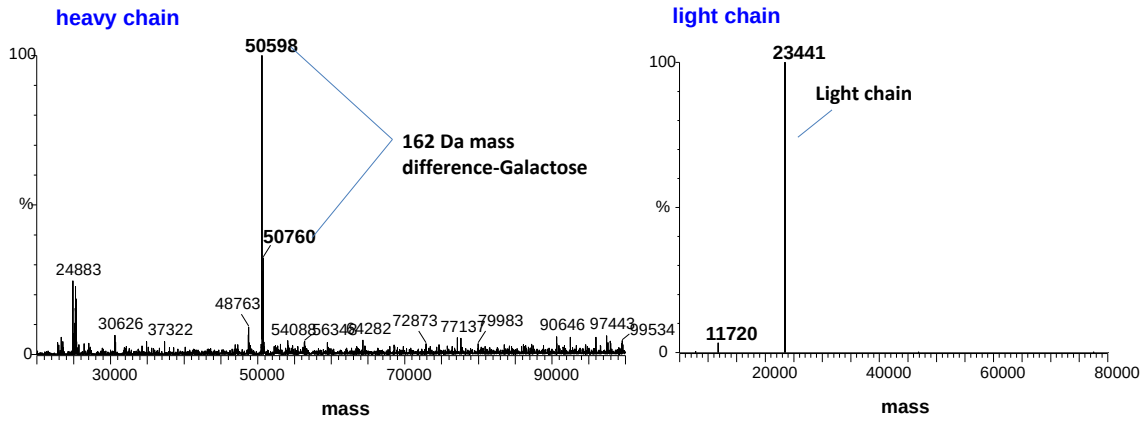

Figure 4. Online MS analysis of heavy and light chain from SEC separation of reduced MAb 321. A 7.8 x 300 mm Zenix™ SEC-300 column was used to separate the heavy and light chains. Flow rate was 0.2 mL/min with 0.1% TFA, 0.1% formic acid and 20% acetonitrile as the mobile phase. Samples were directly introduced to online Q-TOF after SEC. Deconvoluted mass spectra of peaks were shown as in heavy chain and light chain.

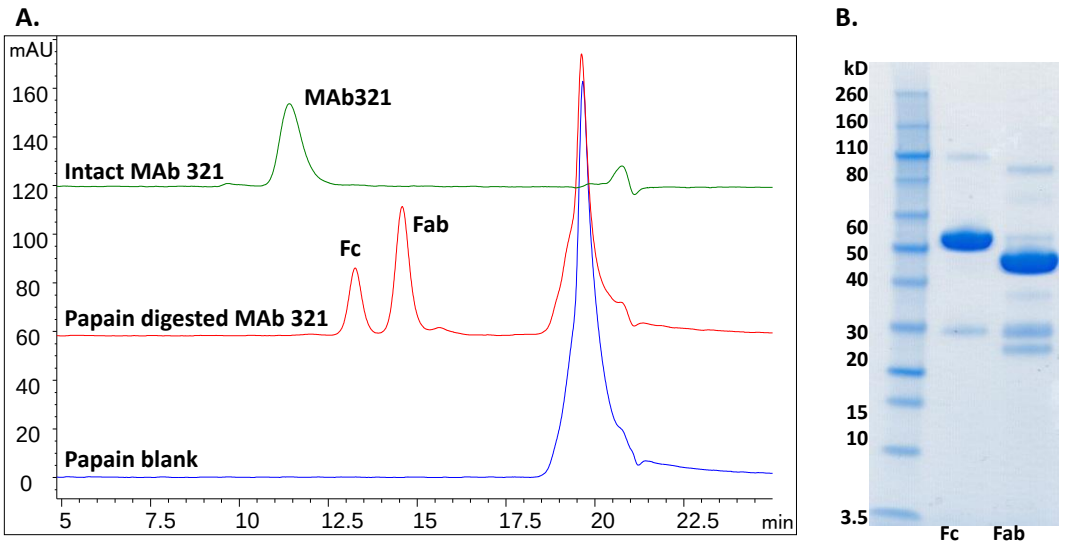

Figure 5. Panel A shows the papain digested MAb 321 (3.5 hour incubation time) -Fab/Fc separation on Zenix™ SEC-300, 4.6 x 300 mm. Mobile phase was 0.1% TFA, 0.1% formic acid with 20% acetonitrile. Flow rate was 0.2 mL/min. UV detection was set at 280 nm. 5 µg of intact MAb 321 and 5 µg of papain digested MAb 321 were injected. Panel B shows the 4-12% Bis-Tris gel image of collected Fc and Fab fractions separated on a Zenix™ SEC-300, 7.8 x 300 mm column at 0.5 mL/min (LC profile not shown).

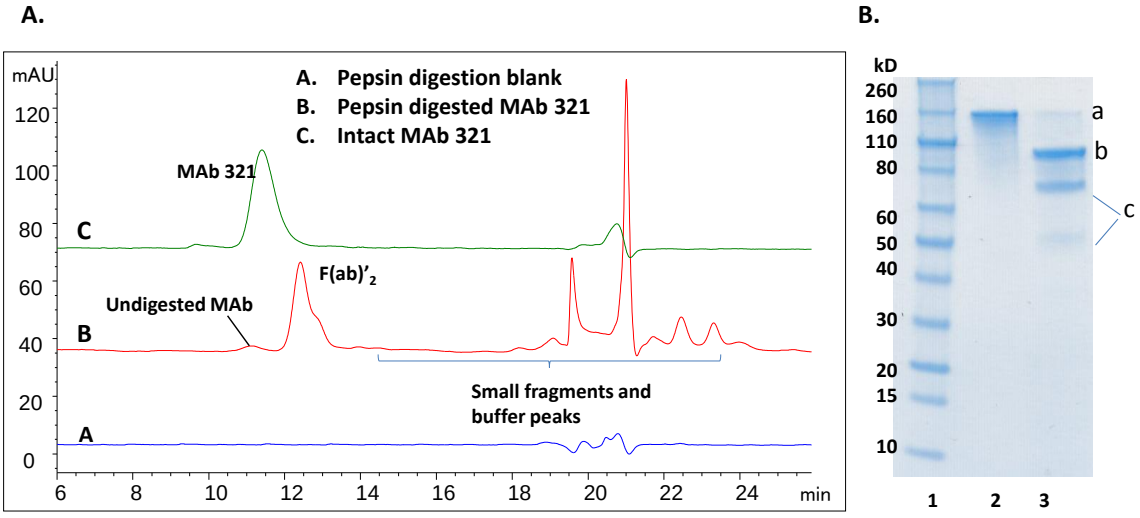

Figure 6. Panel A showed the pepsin digested MAb 321-F(ab')$_2$ separation on Zenix™ SEC-300, 4.6 x 300 mm. Mobile phase was 0.1% TFA, 0.1% formic acid with 20% acetonitrile. Flow rate was 0.2 mL/min. UV detection was set at 280 nm. 5 µg of intact MAb 321 and 15 µg of papain digested MAb 321 were injected. Panel B shows 4-12% Bis-Tris gel image. 5 µg of each sample were loaded. Lane 1 protein markers; lane 2 undigested MAb 321; lane 3 pepsin digested MAb 321. Band (a) is undigested MAb, band (b) is F(ab')$_2$, and bands (c) are smaller fragments from the digestion.

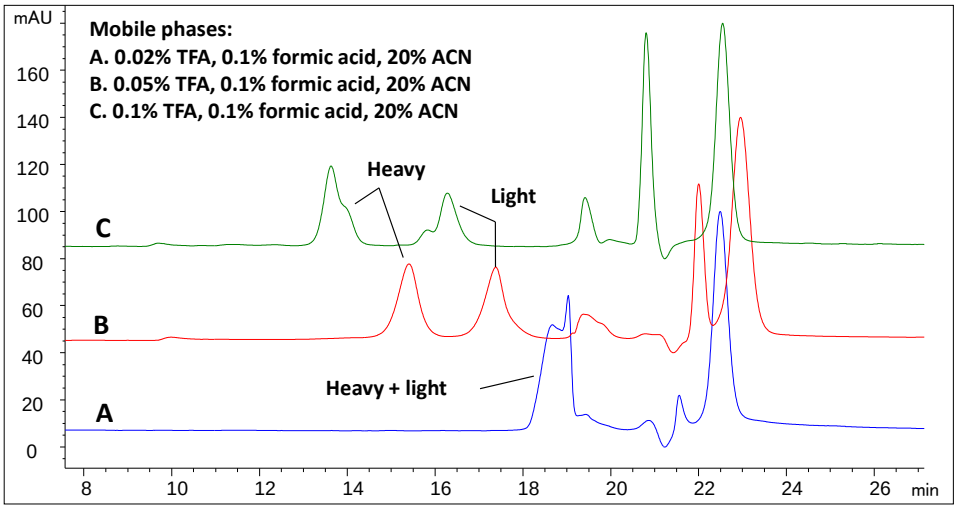

Figure 7. Effect of different TFA concentrations on the separation of heavy and light chains on a Zenix™ SEC-300, 4.6 x 300 mm column. Flow rate was 0.2 mL/min. UV detection was set at 280 nm. 20 µg of reduced MAb 321 was injected. At 0.05% TFA, heavy and light chains can be baseline separated. However, at 0.02% TFA, heavy and light chains are not separated.

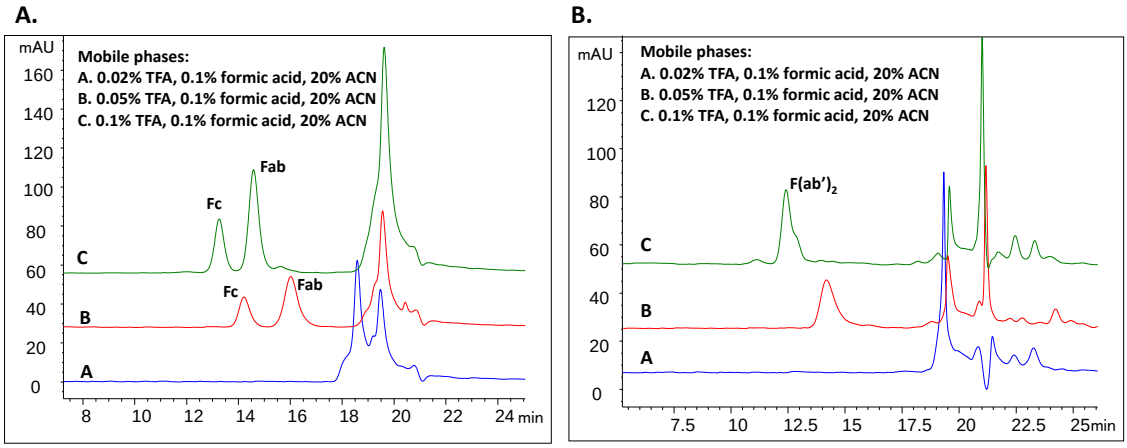

Figure 8. Effect of different TFA concentrations in 0.1% formic acid and 20% acetonitrile on the separation of Fab/ Fc and $F(ab')_2$ on a Zenix™ SEC-300, 4.6 x 300 mm column. Flow rate was 0.2 mL/min. UV detection was set at 280 nm. Panel A shows the chromatogram overlays of 5 µg of papain digested MAb 321 with the mobile phases indicated. Panel B shows the chromatogram overlays of 15 µg pepsin digested MAb 321.

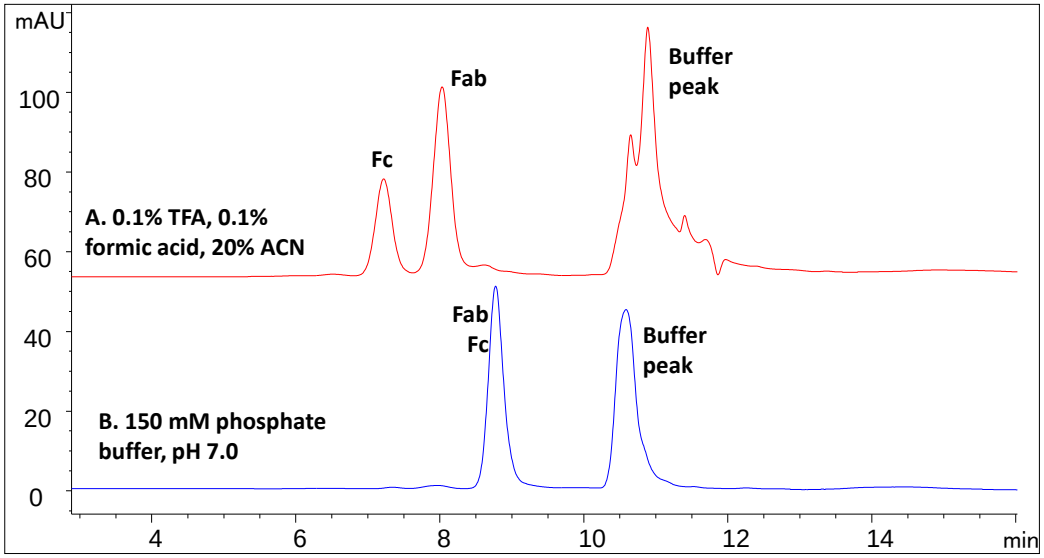

Figure 9. Organic mobile phase vs. salt mobile phase for Fab/Fc separations on Zenix™ SEC-300, 4.6 x 300 mm. Flow rate was 0.35 mL/min, 5 µg of papain digested MAb 321 was injected for both runs. LC profile A was obtained with 0.1% TFA, 0.1% formic acid in 20% acetonitrile, while profile B was generated with 150 mM sodium phosphate buffer, pH 7.0.

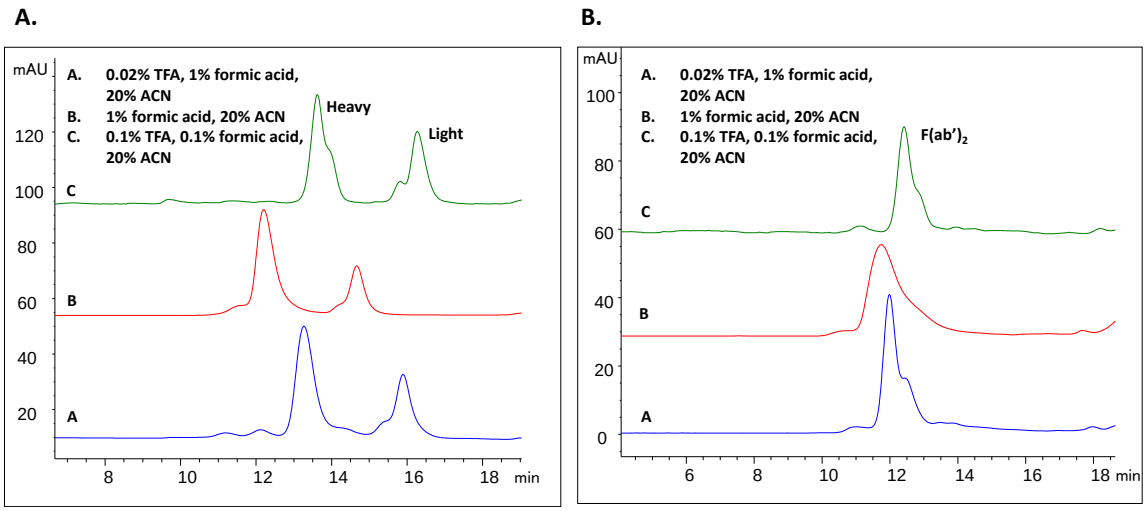

Figure 10. Heavy/light chains, $F(ab')_2$ separations on Zenix™ SEC-300, 4.6 x 300 mm. Flow rate was 0.2 mL/min with UV 280 nm detection. Panel A shows the chromatogram overlays of reduced MAb 321 separation (heavy and light chains). Panel B shows the chromatogram overlays of pepsin digested MAb 321 separation ($F(ab')_2$). Mobile phase A was 0.02% TFA, 1% formic acid, 20% acetonitrile. Mobile phase B was 1% formic acid and 20% acetonitrile. Mobile phase C was 0.1% TFA, 0.1% formic acid and 20% acetonitrile.

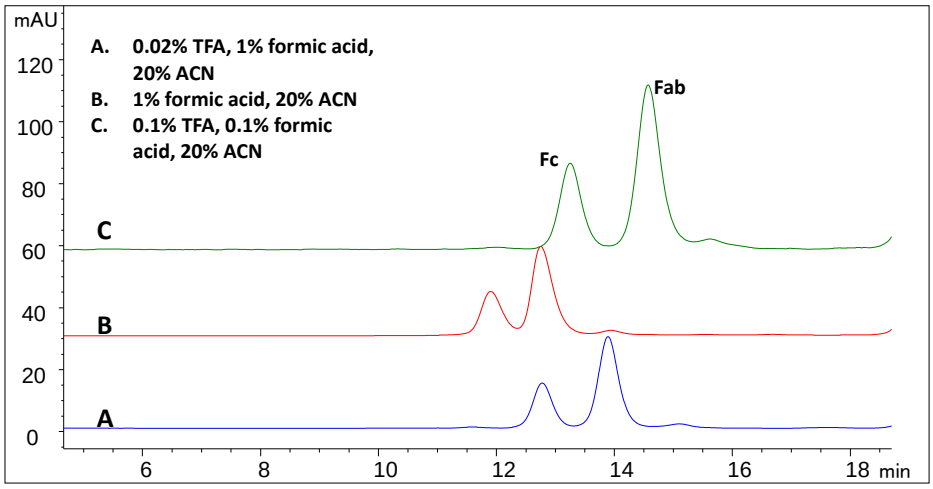

Figure 11. Fab and Fc separations on Zenix™ SEC-300, 4.6 x 300 mm with different mobile phases. Flow rate was 0.2 mL/min, 5 µg of papain digested MAb 321 was injected for all three runs.

Table 1. Zenix™ SEC-300 4.6x300mm column performance dependence on mobile phase compositions for Fab/Fc separations.

Peak	Mobile phase	RT (min)	Plate	Tailing	Resolution
Fc	0.02% TFA, 1% formic acid, 20% ACN	12.78	6824	1.09	
	1% formic acid, 20% ACN	11.91	4909	1.05	
	0.1% TFA, 0.1% formic acid, 20% ACN	13.25	5884	1.08	
Fab	0.02% TFA, 1% formic acid, 20% ACN	13.89	7551	1.09	1.77
	1% formic acid, 20% ACN	12.75	5824	1.55	1.26
	0.1% TFA, 0.1% formic acid, 20% ACN	14.58	6322	1.19	1.85

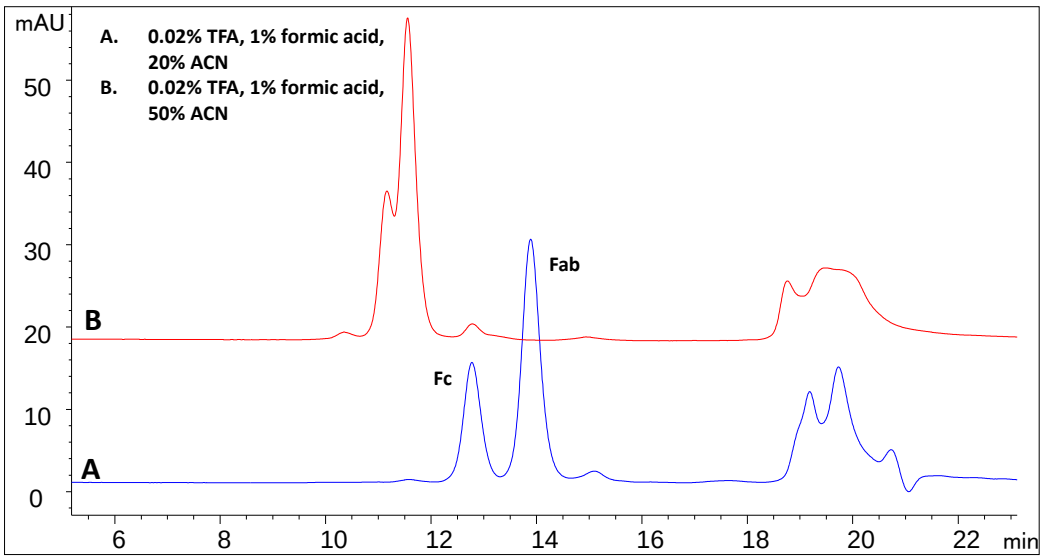

Figure 12. Acetonitrile concentration effect on Fab/Fc separations on Zenix™ SEC-300, 4.6 x 300 mm. Flow rate was 0.2 mL/min, 5 µg of papain digested MAb 321 was injected for both runs. LC profile A was obtained with 20% acetonitrile, while profile B was generated with 50% acetonitrile.

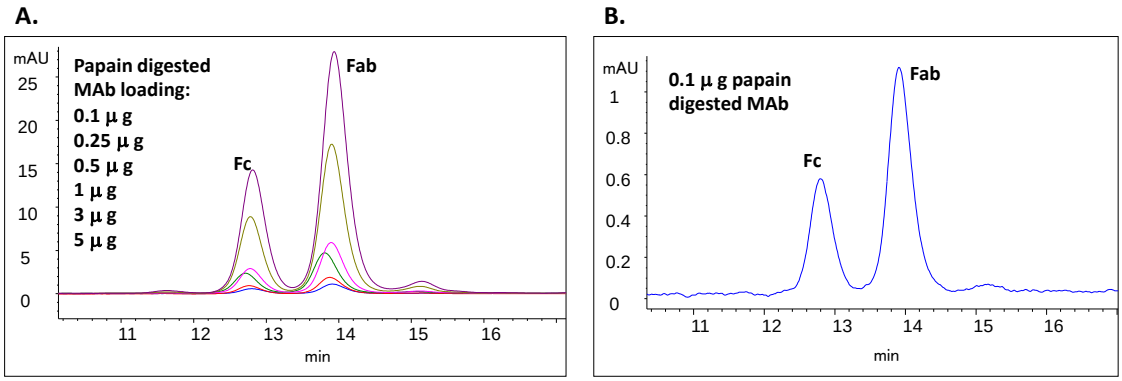

Figure 13. Panel A shows the chromatogram overlays of different sample loadings of papain digested MAb from 0.1 µg to 5 µg on Zenix™ SEC-300, 4.6 x 300 mm. HPLC mobile phase was 0.02% TFA in 1% formic acid and 20% acetonitrile. Flow rate was 0.2 mL/min with UV 280 nm detection. Panel B shows the separation profile with 0.1 µg loading. Fab and Fc maintained a baseline separation at 0.1 µg loading.

Reference:

1. Analysis of Reduced Monoclonal Antibodies Using Size Exclusion Chromatography Coupled with Mass Spectrometry, J. Am. Soc. Mass Spectrom. 20, 2258-2264 (2009)
2. Analysis of Post-translational Modifications in Recombinant Monoclonal Antibody IgG1 by Reversed-phased Liquid Chromatography/mass Spectrometry. Journal of Chromatography A, 1164, 153-161 (2007)
3. Papain Digestion of Different Mouse IgG Subclasses as Studied by Electrospray as Mass Spectrometry. Journal of Immunological Methods, 237, 95-104 (2000)
4. Enzyme Digestion of Monoclonal Antibodies from the Protein Protocols Handbook, 2nd edition by Sarah M. Andrew.

Order information

Part Number	Particle Size	Pore Size	ID×Length
213300-2105 [1]	3 µm	300 Å	2.1×50mm
213300-2130	3 µm	300 Å	2.1×300mm
213300-4605 [1]	3 µm	300 Å	4.6×50mm
213300P-4605 [1] [3]	3 µm	300 Å	4.6×50mm
213300-4615	3 µm	300 Å	4.6×150mm
213300-4625	3 µm	300 Å	4.6×250mm
213300-4630	3 µm	300 Å	4.6×300mm
213300P-4630 [3]	3 µm	300 Å	4.6×300mm
213300-7805 [1]	3 µm	300 Å	7.8×50mm
213300-7815	3 µm	300 Å	7.8×150mm
213300-7820	3 µm	300 Å	7.8×200mm
213300-7825	3 µm	300 Å	7.8×250mm
213300-7830	3 µm	300 Å	7.8×300mm
213300-10005 [1]	3 µm	300 Å	10.0×50mm
213300-10010	3 µm	300 Å	10.0×100mm
213300-10015	3 µm	300 Å	10.0×150mm
213300-10025	3 µm	300 Å	10.0×250mm
213300-10030	3 µm	300 Å	10.0×300mm
213300-21205 [1]	3 µm	300 Å	21.2×50mm
213300-21225	3 µm	300 Å	21.2×250mm
213300-21230	3 µm	300 Å	21.2×300mm

[1] Guard column
[2] Column packed with PEEK tubing