Separation of Fab and Fc Fragments from Monoclonal Antibody Papain Digest on Ceramic Hydroxyapatite and Ceramic Fluoroapatite

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Abstract

Antibody fragments have a wide variety of uses in analytical and diagnostic applications. They are also becoming a major biotherapeutic agent for treatment of human diseases. Protein A and Protein G affinity separations, ion exchange, and gel filtration chromatography are techniques commonly used to separate Fab and Fc antibody fragments.

This study describes an alternative technique for separating Fab and Fc antibody fragments using CHT™ ceramic hydroxyapatite Type I and Type II and CFT™ ceramic fluoroapatite Type II.

Materials and Methods

Purified monoclonal antibody (Mab-G) was digested with immobilized papain for 6 and 17 hr. The digestion mixtures were analyzed using a Bio-Sil® SEC 250-5 size based separation column. A small quantity of undigested antibody remained.

Separations of Fab and Fc fragments were evaluated on CHT ceramic hydroxyapatite and CFT ceramic fluoroapatite using a sodium phosphate gradient at two different pH values, 6.5 and 7.0. Crude digested antibody was loaded onto the column with 0.005 M sodium phosphate as the loading buffer at 311 cm/hr (2 ml/min) using a BioLogic DuoFlow™ chromatography system. Fab and Fc fragments were eluted off the column from 0–0.25 M sodium phosphate buffer for 40 column volumes. Fractions collected were analyzed by SDS-PAGE under nonreducing conditions with Precision Plus Protein™ molecular weight standards and Fab standard. Fab standard was obtained by purifying the crude digested material in CHT support equilibrated with 25 mM HEPES plus 2.5 mM CaCl2, pH 7.0 (Gagnon et al. 2009). The results of separations of Fab into Fc and residual antibody (Ab) fragments on both apatite media were analyzed by nonreducing SDS-PAGE. Equal volumes of pooled fractions were loaded into the gel except the original material (undigested) and digested monoclonal antibody where differing volumes were used.

Results

The separations of Fab from Fc and intact IgG from the mixture that was digested 6 hr were evaluated on CHT Type I, 20 and 40 µm particle sizes, and CFT Type II, 40 µm particle size, with the following results (Figure 1):

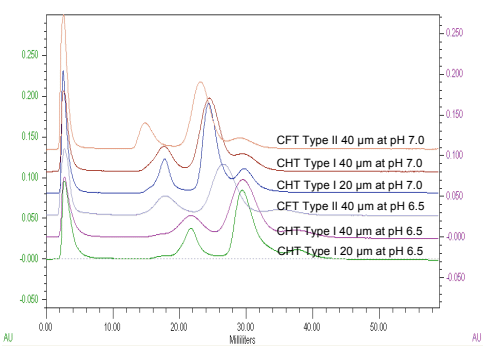

Figure 1. Separations of Fab to Fc and intact IgG on CHT and CFT media on a sodium phosphate gradient at pH 6.5 and 7.0.

Chromatographic Conditions:

Sample:	Crude digested antibody (digested for 6 hr)
Columns:	CHT Type I, 20 and 40 µm CFT Type II, 40 µm
Bed volume:	1 ml
Buffer A:	0.005 M sodium phosphate, pH 6.5 and 7.0
Buffer B:	0.250 M sodium phosphate, pH 6.5 and 7.0
Buffer B2:	0.500 M sodium phosphate, pH 6.5 and 7.0
Flow rate:	2 ml/min (311 cm/hr)
Gradient profile:	100% A for 7 CV; 0–100% B1 for 40 CV; 100% B1 for 5 CV; 100% B2 for 5 CV

Chromatograms presented in Figure 1 have the same general elution profile. CHT Type I, 40 µm, at pH 6.5 and 7.0 has broader peaks than CHT Type I, 20 µm, at pH 6.5 and 7.0 because of its larger particle size. The antibody fragments and intact IgG eluted earlier on CFT support than on CHT support, which indicates lower affinity to the media.

The first major peak on Figure 1 was caused by the difference in buffer composition; this was determined later in this study. The second major peak on Figure 1 represents the Fc fragment; the third peak represents the Fab fragment and intact IgG was eluted at the end. Eluted peaks at pH 6.5 were more strongly retained than those at pH 7.0. This exhibits strong affinity at lower pH value.

Separations of antibody fragments were performed also using low-phosphate buffer with a high concentration of sodium chloride. There were no separations of Fab to Fc and intact IgG observed; hence all components eluted as one peak (data not shown).

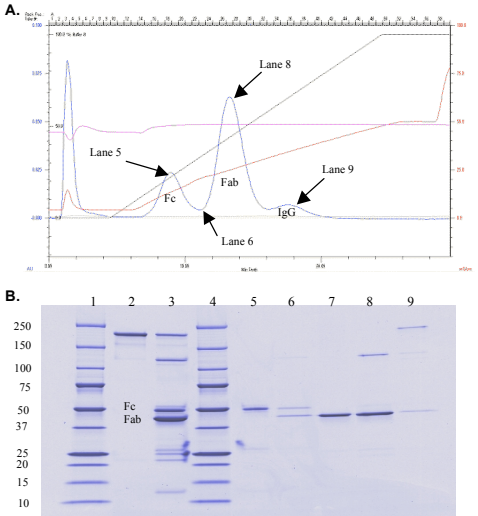

Figure 2. A) Fab, Fc, and intact IgG chromatographic elutions on CFT Type II 40 µm; B) Nonreduced SDS-PAGE of Fab, Fc, and intact IgG; lanes 1 and 4, Precision Plus Protein molecular weight standards; lane 2, undigested Ab; lane 3, digested Ab; lane 5, Fc fragment; lane 6, Fc and Fab fragments; lane 7, Fab standard; lane 8, Fab fragment; lane 9, intact IgG.

All chromatographic data were analyzed by nonreducing SDS-PAGE. Electropherograms showed the same results on all chromatography experiments (data not shown).

Figure 2A represents the CFT Type II, 40 µm, chromatogram from Figure 1. Figure 2B is representative of pooled fractions analyzed by SDS-PAGE on all experimental runs performed on CHT and CFT media.

Fab and Fc fragments were converted into smaller fragments after 6 hr of digestion. Digestion yielded low molecular weight fragments of Fc and Fab shown in Figure 2B, lane 3, which were identified by LC-MS (liquid chromatography and mass spectrometry) analysis (courtesy of Dr Tim Wehr).

The conditions used for separations of Fab to Fc and intact IgG after 17 hr digestion, evaluated on CHT and CFT media were as follows:

Sample:	Crude digested antibody (digested for 17 hr)
Columns:	CHT Type I, 20 and 40 µm CHT Type II, 40 µm CFT Type II, 40 µm
Bed volume:	1 ml
Buffer A:	0.005 M sodium phosphate, pH 7.0
Buffer B1:	0.250 M sodium phosphate, pH 7.0
Buffer B2:	0.500 M sodium phosphate, pH 7.0
Flow rate:	2 ml/min (311 cm/hr)
Gradient profile:	100% A for 7 CV; 0–100% B1 for 40 CV; 100% B1 for 5 CV; 100% B2 for 5 CV

The results obtained for these experiments were shown in Figure 3. Chromatographic profiles of all the experimental runs were the same except for the retention time. Antibody fragments were retained less on CHT Type II than in the rest of the experimental runs, which exhibited weak binding to the media.

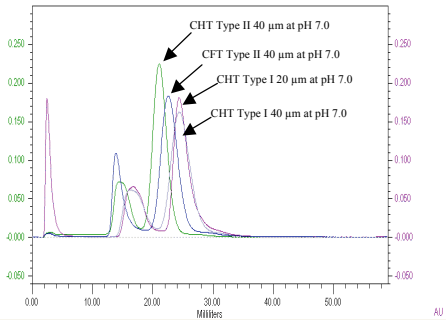

Figure 3. Separations of Fab to Fc and intact IgG on CHT Type I and II, 20 and 40 µm, and CFT Type II, 40 µm, at pH 7.0.

The first major peak on CHT Type I, 20 µm, was an artifact peak due to the difference in buffer composition. This was determined when the digested monoclonal antibody sample was buffer exchanged to low sodium phosphate buffer before loading to the column (see Figure 3 for the experimental runs). With or without buffer exchange of the digested antibody sample, selectivity was not affected on CHT and CFT media.

More of the intact IgG was converted to Fab and Fc fragments after 17 hr of digestion than was converted after 6 hr of digestion. Residual amounts of intact IgG were still present (see Figure 4, lane 4).

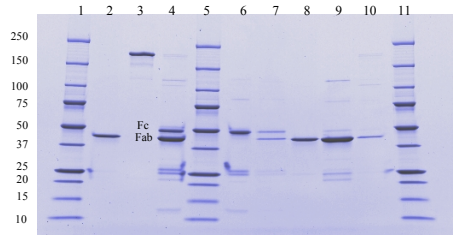

Figure 4. Nonreduced SDS-PAGE of Fab to Fc separations on CFT Type II, 40 µm, at pH 7.0. Lanes 1, 5, and 11, Precision Plus Protein molecular weight standards; lane 2, Fab standard; lane 3, undigested Ab; lane 4, digested Ab; lane 6, Fc fragments; lane 7, Fc and Fab fragments; lane 8, Fab standard; lane 9, Fab fragments; lane 10, intact IgG.

Increased amounts of low molecular weight Fc and Fab antibody fragments were seen throughout the gel in Figure 4. This might be due to overdigestion of the monoclonal antibody sample. It was apparent from the SDS-PAGE that prolonged digestion time resulted in further cleavage of the antibody fragments into smaller fragments.

Conclusions

Separations of Fab to Fc and intact IgG can be performed on CHT and CFT media using a sodium phosphate gradient system alone. This technique is very simple and can be implemented in a large-scale manufacturing process.

Overdigestion or prolonged digestion time of monoclonal antibody in papain generated increased amounts of low molecular weight fragments of Fc and Fab antibody fragments.

Acronyms

Fab	- Fragment having the antigen binding site
Fc	- Fragment that crystallizes
SEC	- Size exclusion chromatography
IgG	- Immunoglobulin G
CV	- Column volume
SDS-PAGE	- Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Reference

Gagnon et al. 2009, Reverse calcium affinity purification of Fab with calcium derivatized hydroxyapatite, J. Immnunol. Methods 342, 115-118.

Acknowledgement

Thanks to Dr Tim Wehr for performing LC-MS identification of Fab and Fc fragments on CHT and CFT fractions.

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