Size exclusion chromatography analysis of papain-cleaved monoclonal antibody using Superdex 200 Increase columns

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This application note describes a simplified workflow for the production of Fab (fragment, antigen binding). Fab was produced by papain cleavage of a purified monoclonal antibody (MAb) followed by purification on HiTrap™ MabSelect SuRe™ and HiTrap Protein L columns. Intermediates and end products were analyzed with size exclusion chromatography (SEC, also called gel filtration) on two different sized Superdex 200 Increase columns: one 300 mm and one 150 mm in length. The high resolution power of Superdex 200 Increase allows baseline separation between antibody monomer, aggregates, and fragments.

Introduction

MAb fragments are getting increased attention in research because of their small size and lower immunogenicity relative to intact antibodies in *in vivo* experiments.

Fab can be produced either by direct expression in a host, such as *E. coli*, or by papain cleavage of antibodies. Affinity chromatography utilizing protein A and protein L media can, in both cases, be used to obtain a highly pure end product.

In this work, a Fab was produced by papain cleavage of a purified MAb. The generated Fab was purified on HiTrap MabSelect SuRe and HiTrap Protein L columns.

The purity of the Fab was determined by analysis on Superdex 200 Increase (Fig 1). Superdex 200 Increase has the resolution power to separate MAb, Fab and Fc (fragment, crystallizable) from each other as well as MAb and Fab from their respective aggregates.

Fig 1. SEC analysis on Superdex 200 Increase 10/300 GL column was performed on an ÄKTA™ pure chromatography system.

Even though papain treatment of antibodies to produce antibody fragments is not new, the easy workflow for production of pure Fab using HiTrap MabSelect SuRe and HiTrap Protein L, as shown in this work, facilitates the procedure. Analyses on the Superdex 200 Increase columns make it possible to follow the purification procedure in detail.

The chromatography medium in Superdex 200 Increase columns is based on a high-flow agarose base matrix with good pressure/flow properties and a small bead size (average bead size 8.6 µm). In addition, the low nonspecific interaction permits high recovery of biological materials. The small bead size allows for high resolving analytical separations.

Materials and methods

MAb production and papain cleavage

Human monoclonal antibody of the IgG sub class 1, containing kappa 1 light chains, was expressed in a CHO cell line and purified as summarized in Table 1, step 1. Fractions containing purified MAb were collected for SEC analysis and subsequent papain cleavage.

For generation of Fab, purified MAb was incubated with papain (0.1 mg/mg MAb) over night at 37°C. Inhibition of the papain-cleavage reaction was performed by incubation with antipain for 30 min at room temperature.

Purification of Fab

Fab, generated by papain-cleavage of MAb, was separated from uncleaved or partially cleaved MAb and Fc as summarized in Table 1, Step 2, and fractions were collected for SEC analysis.

Fab in the flowthrough was further purified as summarized in Table 1, Step 3 and fractions containing Fab were collected for SEC analysis.

Size exclusion chromatography analyses

All intermediate and end products from the different purification steps were analyzed on two different SEC columns: Superdex 200 Increase 10/300 GL run on ÄKTA pure chromatography system as well as Superdex 200 Increase 5/150 GL run on ÄKTAmicro™ chromatography system. All SEC runs were performed in 0.010 M phosphate buffer, 0.0027 M KCl, 0.14 M NaCl, pH 7.4 (PBS).

Result and discussion

An overview of the Fab purification process is given in Table 1 and molecular weights (M_r) for MAb, Fc, Fab, and papain are summarized in Table 2.

Table 2. Molecular weights of MAb, Fc, Fab, and papain

Protein	M_r	Comment
MAb	146 755*	
Fc	48 540*	Glycosylation will give higher M_r in reality
Fab	47 673*	
Papain	23 406	Information from supplier

* Theoretical values calculated on amino acid sequence.

Table 1. Overview of the Fab generation and purification process

	Step 1	Step 2	Step 3
Sample	MAb in cell culture supernatant	Papain treated MAb	Fab and impurities in flowthrough from Step 2
Sample volume	30.5 ml	17.5 ml	25 ml
Column	HiTrap MabSelect SuRe 5 ml	HiTrap MabSelect SuRe 5 ml	HiTrap Protein L 5 ml
Equilibration	10 CV* of 25 mM sodium phosphate buffer + 500 mM NaCl, pH 7, 10 ml/min	10 CV of 25 mM sodium phosphate buffer + 500 mM NaCl, pH 7, 10 ml/min	10 CV of 25 mM citrate buffer, pH 5.5, 10 ml/min
Binding condition	25 mM sodium phosphate buffer + 500 mM NaCl, pH 7, 2.5 ml/min	25 mM sodium phosphate buffer + 500 mM NaCl, pH 7, 2.5 ml/min	25 mM citrate buffer, pH 5.5, 2.5 ml/min
Wash	5 CV of 25 mM sodium phosphate buffer + 500 mM NaCl, pH 7, 10 ml/min	5 CV of 25 mM sodium phosphate buffer + 500 mM NaCl, pH 7, 10 ml/min	5 CV of 25 mM citrate buffer, pH 5.5, 10 ml/min
Elution	Step, 5 CV of 25 mM citrate buffer, pH 3.5, 5 ml/min	Step, 5 CV of 25 mM citrate buffer, pH 3.5, 5 ml/min	Step, 5 CV of 25 mM citrate buffer, pH 3, 5 ml/min
System	ÄKTA pure	ÄKTA pure	ÄKTA pure
Obtained product			
flowthrough eluate	Impurities MAb	Fab and impurities MAb and Fc	Impurities Fab

* CV = column volumes

Analysis of purified MAb

MAb-containing fractions from purification on the HiTrap MabSelect SuRe column were analyzed by SEC on Superdex 200 Increase 10/300 GL and Superdex 200 Increase 5/150 GL columns, Step 1 in Table 1 (data not shown).

Analysis of Fab-containing flowthrough

Fab-containing flowthrough (Step 2 in Table 1) from purification on HiTrap MabSelect SuRe (Fig 2A) was analyzed by SEC on Superdex 200 Increase (Figs 2B and C). The analysis takes less than a fourth of the time on the shorter 150 mm columns compared to the longer 300 mm column.

SEC analysis of eluate from HiTrap MabSelect SuRe (Fig 2A) confirms that only very small amounts of Fab bind to the column (SEC data not shown).

Analysis of purified Fab

Fab-containing flowthrough (Step 3 in Table 1) from previous separation step was purified on HiTrap Protein L column (Fig 3A). Fractions were collected for purity analysis by SEC (Figs 3B and C).

SEC analysis of flowthrough from HiTrap Protein L column (Fig 3A) confirms that it contains only very small amounts of Fab and Fc (SEC data not shown).

(A)

Column: HiTrap MabSelect SuRe 5 ml
Sample: Papain cleaved MAb, 17.5 ml
System: ÄKTA pure

(B)

Column: Superdex 200 Increase 10/300 GL
Sample: Fab-containing flowthrough, 50 µl
Flow rate: 0.5 ml/min
System: ÄKTA pure

(C)

Column: Superdex 200 Increase 5/150 GL
Sample: Fab-containing flowthrough, 20 µl
Flow rate: 0.3 ml/min
System: ÄKTAmicro

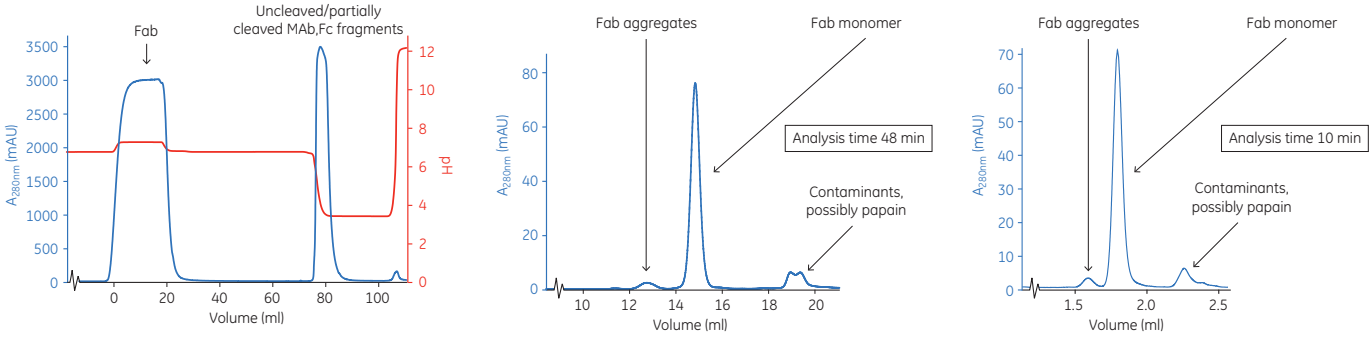

Fig 2. Separation of Fab from uncleaved or partially cleaved Mab and Fc after papain treatment on HiTrap MabSelect SuRe 5 ml column. Flowthrough contains Fab and other Impurities. Eluted fractions contain Fc and uncleaved or partially cleaved MAb (A). SEC analysis of Fab-containing flowthrough using Superdex 200 Increase 10/300 GL (B) and Superdex 200 Increase 5/150 GL (C).

(A)

Column: HiTrap Protein L 5 ml
Sample: Flowthrough from MabSelect SuRe run, 25 ml
System: ÄKTA pure

(B)

Column: Superdex 200 Increase 10/300 GL
Sample: Purified Fab, 50 µl
Flow rate: 0.5 ml/min
System: ÄKTA pure

(C)

Column: Superdex 200 Increase 5/150 GL
Sample: Purified Fab, 20 µl
Flow rate: 0.3 ml/min
System: ÄKTAmicro

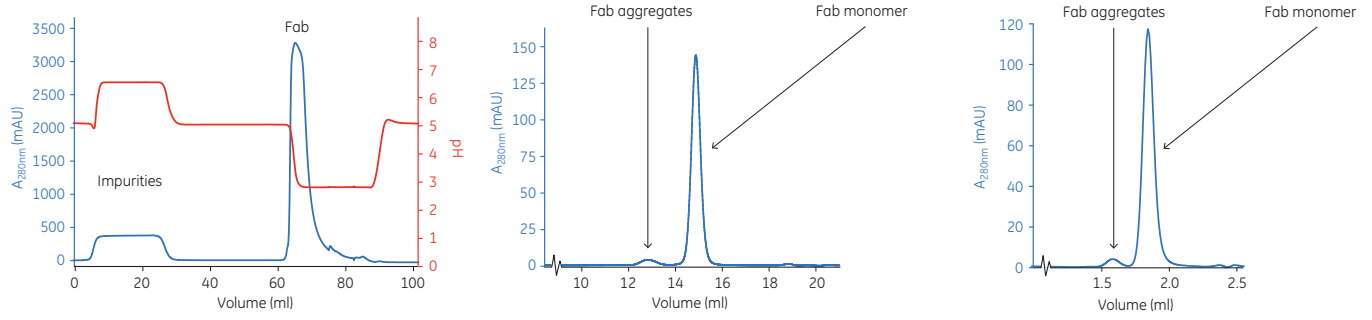

Fig 3. Fab further purified on HiTrap Protein L column. Flowthrough contains impurities. Eluted fraction contains the Fab (A). SEC analysis of Fab using Superdex 200 Increase 10/300 GL (B) and Superdex 200 Increase 5/150 GL (C)..

Overlay of individual analyses of MAb and Fab

Broad separation range (M_r 10 000 to M_r 600 000) of Superdex 200 Increase, with optimized resolution for antibodies (M_r 100 000 to M_r 300 000), allows for separation of a large number of different biomolecules.

The separation efficiency of the Superdex 200 Increase medium is demonstrated here with an overlay of the different SEC analyses of MAb, Fab, and Fc (Fc purification process not described in this application note) (Fig 4).

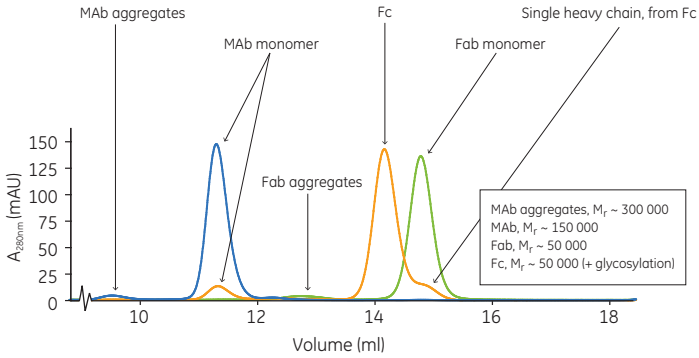

Fig 4. Overlay of separate SEC analyses of purified MAb (blue), Fab (green), and Fc (orange, purification not previously shown) on Superdex 200 Increase 10/300 GL. For comparative purposes, the curves have been normalized against the peak height of each monomer species.

Conclusions

HiTrap MabSelect SuRe and HiTrap Protein L columns enable a simplified workflow to produce pure Fab by papain digestion of MAb.

The high resolution power of Superdex 200 Increase allows baseline separation between antibody monomer, aggregates, and fragments.

Both column formats are well suited as analysis tools and do complement each other:

- Highest resolution is obtained with the 10/300 GL column
- Shortest analysis time is obtained with the 5/150 GL column

Ordering information

Product	Size	Code number
Superdex 200 Increase 10/300 GL	1 × 24 ml	28-9909-44
Superdex 200 Increase 5/150 GL	1 × 3 ml	28-9909-45
HiTrap Protein L	1 × 5 ml	17-5478-15
HiTrap MabSelect SuRe	1 × 5 ml	11-0034-94

To order ÄKTA pure and ÄKTAmicro chromatography systems, please contact your regional sales representative.

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GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

imagination at work

GE Healthcare UK Ltd.
Amersham Place
Little Chalfont
Buckinghamshire, HP7
9NA, UK

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

GE Healthcare Europe, GmbH
Munzinger Strasse 5
D-79111 Freiburg
Germany

GE Healthcare Bio-Sciences Corp.
800 Centennial Avenue, P.O. Box 1327
Piscataway, NJ 08855-1327
USA

GE Healthcare Japan Corporation
Sanken Bldg., 3-25-1, Hyakunincho
Shinjuku-ku, Tokyo 169-0073
Japan

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