GENOVIS PRESENTS

Enzymatic Strategies for Generation of Intact Fab and Fc Fragments – IgdE Results in Strict Specific Digestion Compared to Lys-C and Papain

AUTHORS

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INTRODUCTION

Antibody subunit preparation using IdeS (FabRICATOR®) enzymatic digestion has become a widely accepted strategy for characterization of therapeutic antibodies due to its specificity and robustness. The IdeS enzyme digests IgG just below the hinge, generating F(ab')2 and Fc fragments. For applications involving intact Fab or Fc fragments, digestion above the hinge is desirable. Preparation of Fab and Fc using general proteases often requires optimization[1] for individual antibodies to avoid overdigestion, loss of activity and a non-homogenous Fab preparation. The newly characterized cysteine protease IgdE, cloned from *Streptococcus agalactiae*[2], digests human IgG1 (hIgG1) at one specific site above the hinge without the need of reducing agents or optimization of digestion.

In this work, the specificities of IgdE (FabALACTICA™), rLys-C (from *Pseudomonas aeruginosa*) and papain (from papaya latex) were evaluated and compared with respect to generation of Fab and Fc fragments from clinically approved human IgG1 monoclonal antibodies. To achieve high yields of pure and intact Fab and Fc fragments, IgdE immobilized on agarose resin was used to digest hIgG1 before the Fab and Fc fragments were isolated using separate affinity resins.

SUMMARY

- Aim: Comparison of Fab and Fc fragments preparation using IgdE, Lys-C and papain
- IgdE has high specificity with a single digestion site (DKT/HTC) above the hinge of human IgG1
- Lys-C and papain digestion result in additional digestion sites in the Fc and Fab fragments
- The IgdE digestion protocol is robust and can be applied as a platform method
- Lys-C and papain digestion protocols require optimization
- IgdE is active in immobilized form for digestion and preparation of pure Fab and Fc in a high yield

RESULTS

IgdE, Papain and Lys-C for Fab Generation

Since papain and Lys-C have multiple digestion sites on the IgG molecule, the protocols for Fab generation using these enzymes need optimization. The digestion protocols for papain and Lys-C were evaluated for human IgG1 digestion using an enzyme to substrate ratio of 1:20 to 1:200, and incubation times ranging from 2 to 18 h. Non-optimized conditions led to overdigestion (*Fig. 1* and *2*). For example, when a non-optimized papain digestion protocol was used to digest trastuzumab, the LC-MS analysis showed that digestion occured both at the primary digestion site (H227/T228) on the HC and at other sites (such as D224/K225, A40/P41 and K136/S137 in the Fd and E236/L237 in the Fc; Fig. 1b). For Lys-C digestion, it was difficult to find an optimum for achieving a high yield of Fab fragments and minimizing overdigestion (*Fig. 2*). However, based on the SDS-PAGE results, optimized protocols were developed for both papain and Lys-C, and digestions using these enzymes were compared to IgdE digestion.

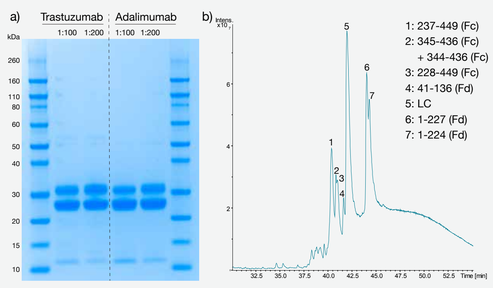

Figure 1. Human IgG1 digested with papain. a) SDS-PAGE (reduced) of trastuzumab and adalimumab digested with papain **b)** MS analysis total Ion Chromatogram (TIC) of trastuzumab digested with papain (1:100). Digestions were performed in PBS, 5 mM L-cysteine, 37°C, overnight (ON), and stopped with inhibitor E-64.

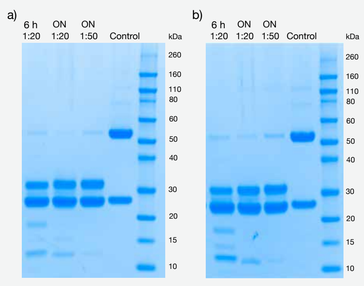

Figure 2. Human IgG1 digested with Lys-C. SDS-PAGE (reduced) of **a)** trastuzumab and **b)** adalimumab digested with Lys-C at different enzyme:substrate ratios. Digestions were performed in 50 mM NaP, 150 mM NaCl, pH 8.0, 37°C and stopped with low pH. ON: overnight.

Three human IgG1 antibodies (trastuzumab, adalimumab and infliximab) were digested using both IgdE and the optimized protocols for papain and Lys-C. No overdigestion was observed using IgdE or papain, however, samples digested with Lys-C showed additional degradation products for all antibodies (*Fig. 3*).

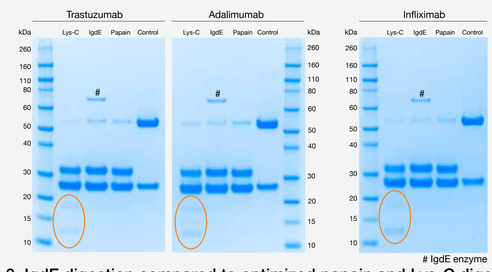

Figure 3. IgdE digestion compared to optimized papain and Lys-C digestions. SDS-PAGE (reduced) of **a)** trastuzumab, **b)** adalimumab and **c)** infliximab digested with Lys-C (1:50, 6 h), IgdE (1 unit/µg hIgG1, ON) and papain (1:100, 5 h). IgdE digestions were performed in 150mM NaP, pH 7.0, 37°C. Additional degradation products are highlighted by orange circles.

LC-MS analysis of IgdE-digested trastuzumab and adalimumab showed three peaks each with masses matching the theoretical masses of LC, Fc and Fd, indicating a single digestion site at T226/H227 of the HC (*Fig. 4*). The papain MS data also showed three peaks. However, the deconvoluted Fc peak spectrum of digested trastuzumab showed a minor degree of digestion at an additional site located one amino acid C-terminally from papain's primary digestion site (H227/T228). The amount of this additional fragment increased by overnight incubation (*Fig. 5*). The primary digestion site of Lys-C on adalimumab is K226/T227. Several additional digestion sites were observed C-terminally of lysine, most of which were found in the Fc region. Some were also found in the Fd region, such as K137/S138 and K98/V99 (*Fig. 4*). IgdE digestion was robust and reliable, and generated homogenous fragments of both Fab and Fc.

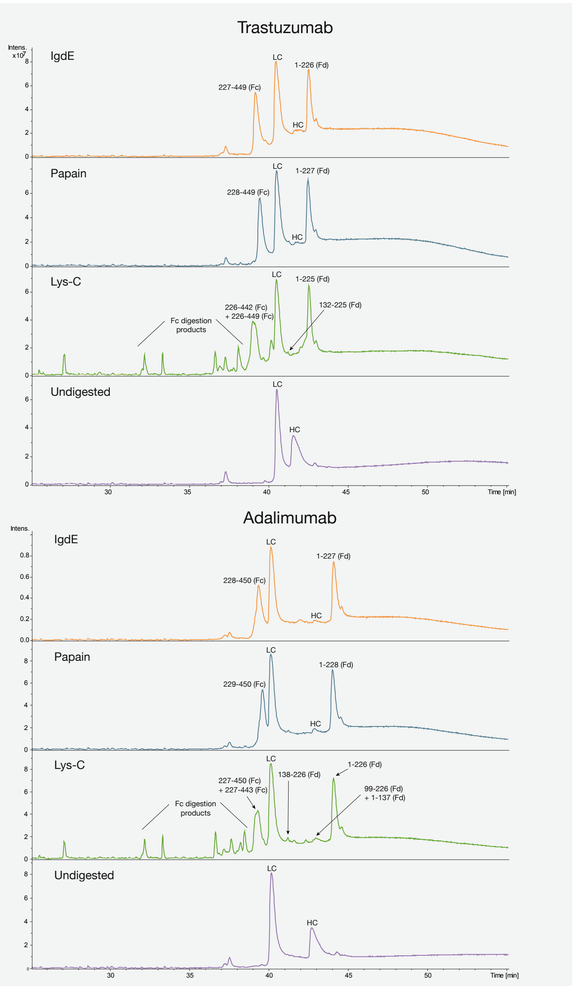

Figure 4. IgdE compared to optimized papain and Lys-C digestions. MS analysis TICs from IgdE (1 unit/µg hIgG1, ON), papain (1:100, 5 h) and Lys-C (1:50, 6 h) digestions of trastuzumab and adalimumab.

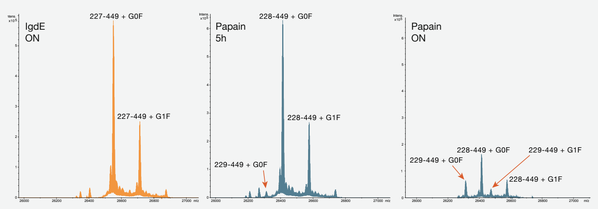

Figure 5. Deconvoluted MS data. Fc fragment spectra generated from IgdE and papain digestion of trastuzumab.

Preparation of Pure Fab and Fc Fragments Using Immobilized IgdE

To achieve Fab and Fc fragments without the presence of enzyme, IgdE was immobilized on agarose beads. A selection of monoclonal antibodies was incubated with immobilized IgdE in spin columns. Fab fragments were isolated using CaptureSelect™* Fc spin columns by a 30 minute incubation, and a homogenous pool of pure Fab fragments was eluted by a mild centrifugation step (*Fig. 6a*). Pure Fc fragments were obtained using the same procedure with Capture Select CH1™* as the affinity resin (*Fig. 6b*). As displayed in Table 1 and 2, pure Fab and Fc fragments were obtained in a high yield from all tested hIgG1 antibodies.

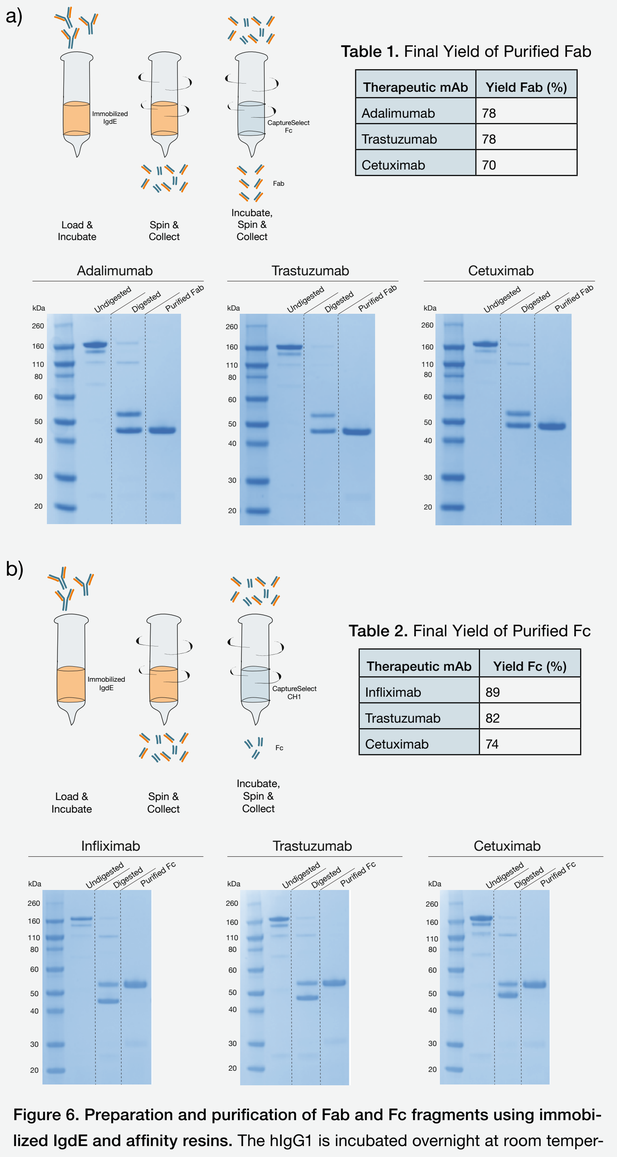

Table 1. Final Yield of Purified Fab

Therapeutic mAb	Yield Fab (%)
Adalimumab	78
Trastuzumab	78
Cetuximab	70

Table 2. Final Yield of Purified Fc

Therapeutic mAb	Yield Fc (%)
Infliximab	89
Trastuzumab	82
Cetuximab	74

Figure 6. Preparation and purification of Fab and Fc fragments using immobilized IgdE and affinity resins. The hIgG1 is incubated overnight at room temperature on an immobilized IgdE column (100 µl). Subsequently, the IgdE digest is incubated with affinity resins specific for Fc or Fab for efficient separation of the fragments. The purified fragments are then collected in flow-through by a mild centrifugation step. The pure Fab is separated from Fc using an Fc specific resin (a) and the pure Fc is separated from Fab using a Fab specific resin (b).
The gel pictures show non-reduced SDS-PAGE of antibody material as undigested, digested and purified Fab (a) and Fc (b) respectively. 1 µg total protein was applied to each lane. The final yields of Fab (Table 1) and Fc (Table 2) were obtained when processing 250-500 µg hIgG1.

* Thermo Scientific™ CaptureSelect™ resin from Thermo Fisher Scientific Inc. and its subsidiaries. Thermo Scientific and CaptureSelect are trademarks of Thermo Scientific Inc. and its subsidiaries.

LC-MS method. The RP-LC separation was performed on a BEH300 C4 column from Waters on denatured and reduced samples and were desalted in-line prior to ESI-Q-TOF MS (Bruker Impact II). The obtained raw spectra were deconvoluted using the MaxEnt algorithm to determine masses.

REFERENCES

1. Moelleken J., et al. 2017. GingisKHAN Protease Cleavage allows a High-throughput Antibody to Fab Conversion enabling Direct Functional Assessment during Lead Identification of Human Monoclonal and Bispecific IgG1 Antibodies. mAbs. doi: 10.1080/19420862.2017.1364325.
2. Spoerry C., et al. 2016. Novel IgG-Degrading Enzymes of the IgdE Protease Family Link Substrate Specificity to Host Tropism of Streptococcus Species. PLoS ONE 11(10): e0164809. doi:10.1371/journal.pone.0164809.

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