GENOVIS PRESENTS

Preparation of Pure Antibody Fab Fragments Using a Robust and Specific Enzyme (IgdE)

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. The newly characterized cysteine protease IgdE (FabALACTICA), cloned from *Streptococcus agalactiae*[1], digests human IgG1 (hIgG1) at one specific site above the hinge without the need for additives or reaction optimization.

The use of general proteases for Fab preparation often requires optimization for individual antibodies in order to avoid overdigestion, loss of activity and a non-homogenous Fab preparation[2]. In this work, the specificities of IgdE, 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 a high yield of pure and intact Fab fragments, IgdE immobilized on agarose resin was used to digest hIgG1 before the Fab and Fc fragments were separated using an Fc-specific resin.

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 preparation of pure Fab 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.

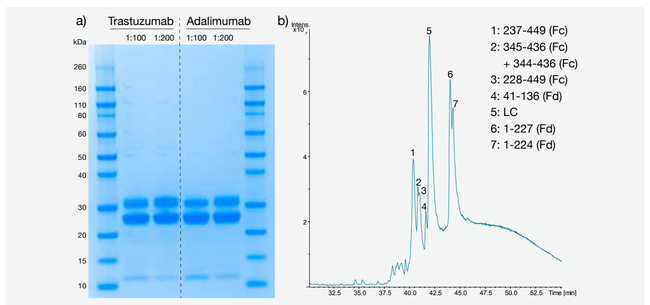

Figure 1. Human IgG1 digested with papain. a) SDS-PAGE (reduced) of trastuzumab and adalimumab digested with papain **b)** MS analysis total Ion Chromatogram (TIC) of trastuzumab digested with papain (1:100). Digestions were performed in PBS, 5 mM L-cysteine, 37°C, overnight (ON), and stopped with inhibitor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

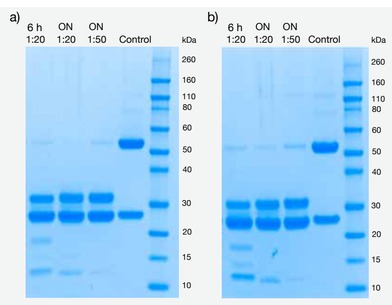

Figure 2. Human IgG1 digested with Lys-C. SDS-PAGE (reduced) of **a)** trastuzumab and **b)** adalimumab digested with Lys-C at different enzyme:substrate ratios. Digestions were performed in 50 mM NaP, 150 mM NaCl, pH 8.0, 37°C and stopped with low pH. ON: overnight.

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*).

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

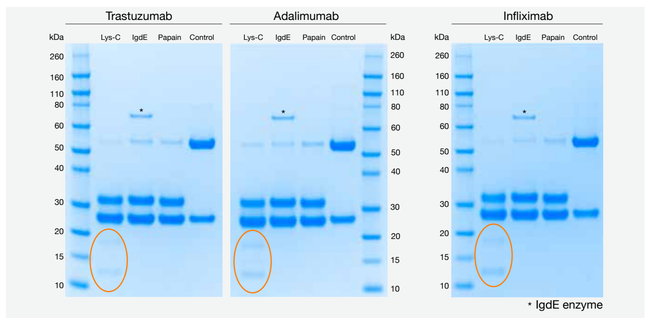

Figure 3. IgdE digestion compared to optimized papain and Lys-C digestions. SDS-PAGE (reduced) of **a)** trastuzumab, **b)** adalimumab and **c)** infliximab digested with Lys-C (1:50, 6 h), IgdE (1 unit/µg hIgG1, ON) and papain (1:100, 5 h). IgdE digestions were performed in 150mM NaP, pH 7.0, 37°C.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

(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 Fab and Fc.

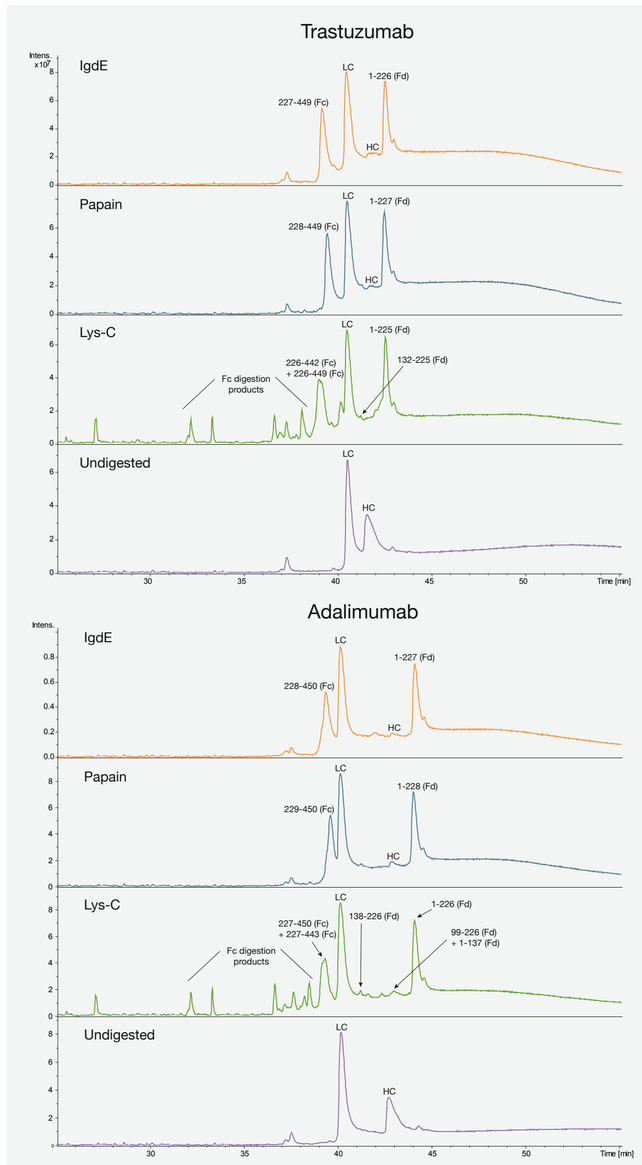

Figure 4. IgdE compared to optimized papain and Lys-C digestions. MS analysis TICs from IgdE (1 unit/µg hIgG1, ON), papain (1:100, 5 h) and Lys-C (1:50, 6 h) digestions of trastuzumab and adalimumab.

Preparation of Pure Fab Fragments Using Immobilized IgdE

To achieve a pure Fab preparation without the presence of enzyme, IgdE was immobilized on agarose beads. Adalimumab, trastuzumab and cetuximab (5 mg/ml) were incubated with immobilized IgdE in spin columns. The Fc was separated from Fab using CaptureSelect™* Fc spin columns by a 30 minute incubation (*Fig. 6*), and a homogenous pool of pure Fab fragments was eluted by a mild centrifugation step (*Fig. 7*). The Fc subunit can be eluted from the Capture Select resin by using a low pH buffer. As displayed in Table 1, pure Fab fragments were obtained in a high yield from all three antibodies.

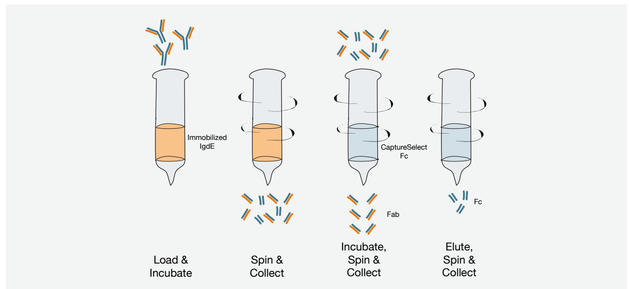

Figure 6. Schematic picture of the preparation and purification of Fab fragments using immobilized IgdE. The hIgG1 is incubated overnight with immobilized IgdE, the Fab is subsequently separated from Fc using an Fc specific resin and the pure Fab is collected by centrifugation.

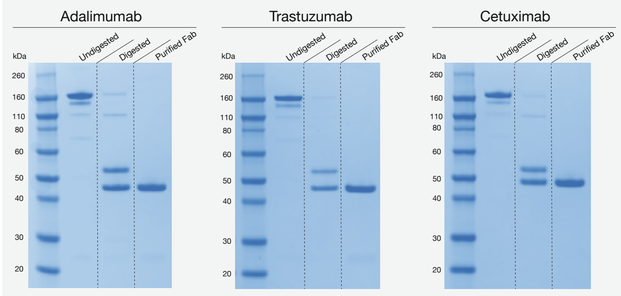

Table 1. Final Yield of Purified Fab

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

Figure 7. Purification of Fab from three hIgG1 digested with IgdE. No-nreduced SDS-PAGE showing starting material, digested hIgG1 and purified Fab. 1µg total protein was applied to each lane. The table shows the final yield of purified Fab when starting from 250 - 500 µg mAb and 100 µl resin.

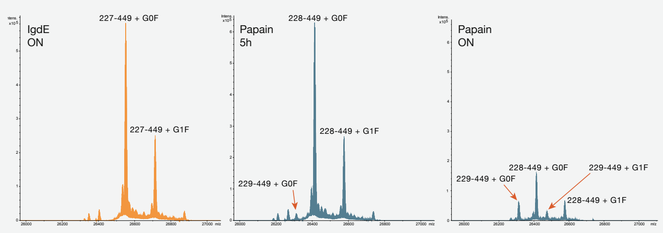

Figure 5. Deconvoluted MS data. Fc fragment spectra generated from IgdE and papain digestion of trastuzumab.

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. 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.
2. 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.