GENOVIS PRESENTS

Characterization of a Novel Protease (IgdE) for Above Hinge Antibody Subunit Generation

AUTHORS

P. Onigman[1], M. W. Sandberg[1], S. Björk[1], M. Nordgren[1], M. Mejàre[1], J. Bunkenborg[2], E. Mørtz[2] and F. Olsson[1]

[1)] Genovis AB, Lund, Sweden; [2)] Alphalyse, Odense, Denmark

INTRODUCTION

Antibody subunit analysis using IdeS enzymatic digestion and LC/MS has become a widely accepted analytical strategy for rapid characterization of therapeutic antibodies and related products. The IdeS enzyme digests IgG just below the hinge, generating F(ab')2 and Fc fragments. However, for applications involving intact Fab or Fc fragments, digestion above hinge is desirable. In this study, we characterized the IgG proteolytic activity of a novel cysteine protease, IgdE from *Streptococcus agalactiae*. IgdE was found to digest human IgG1 at one specific site just above the hinge cysteines, generating a homogenous pool of intact Fab and Fc fragments. The recombinant enzyme was found active in a broad range of pH and salt concentrations, without the need of any co-factors or reducing reagents. These features make the enzyme suitable for subunit LC/MS analysis under reducing or non-reducing conditions. The specificity of IgdE was demonstrated by paired glycan analysis of intact and homogenous Fc fragments of trastuzumab. IgdE may be utilized for applications including characterization of bi- or multispecific antibodies, monovalent binding studies, crystallization and NMR studies of higher order structure of antibodies.

SUMMARY

- IgdE protease shows high specificity for digestion above the hinge of hIgG1
- One single digestion site as verified by MS; KSCDKT / HTCPPCP
- No buffer additives or reducing agent required for activity, active in a broad pH range
- Masses of intact Fab and Fc fragments of trastuzumab were determined
- Paired glycan analysis of the intact Fc fragments is possible
- Active in immobilized form for preparation of pure Fab and Fc fragments

RESULTS

Enzyme Characterization

Characterization of IgdE confirmed that its natural substrate is hIgG1. IgdE was neither active on any other subclass of human IgG nor on IgG from any other tested species (data not shown). Optimal digestion conditions were obtained in 150 mM NaP at 37°C, pH 6.5-8 (Fig. 1b) in 0-150 mM NaCl. Digestion yield after incubation overnight was typically 90-95%, as determined by SDS-PAGE.

The monoclonal hIgG1 antibodies adalimumab, trastuzumab and cetuximab were digested with a purified IgdE protease preparation with an E:S ratio of 1:10 at 37°C overnight in 150 mM NaP, pH 7.0. The digestions were analyzed by non-reduced SDS-PAGE (Fig. 1c) and RP-HPLC (Fig. 1d). HPLC was performed on Agilent 1260 using Waters Acquity UPLC® BEH300 C4, 1.7 µm, 2.1x100 mm column in an acetonitrile/isopropanol gradient at 60°C.

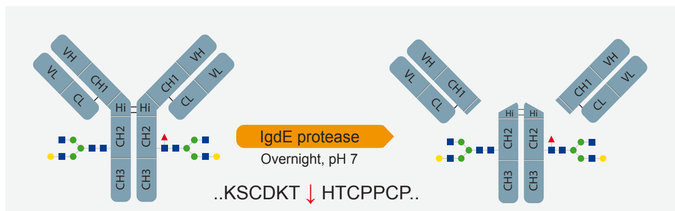

Fig 1a.

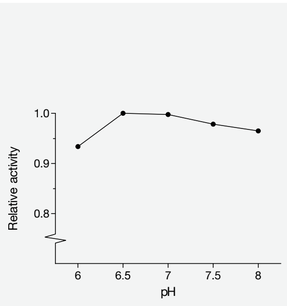

Fig 1b.

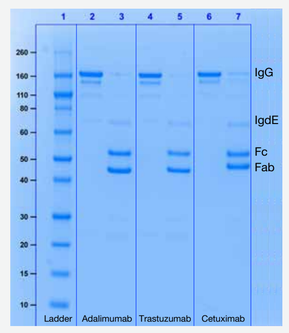

Fig 1c.

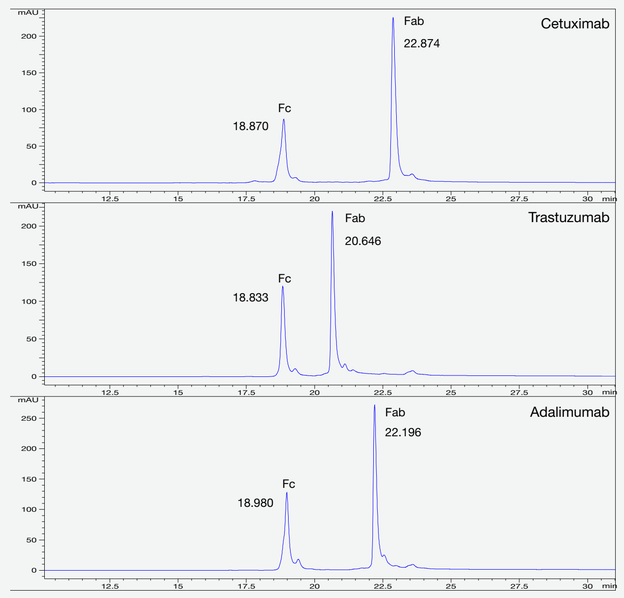

Fig 1d.

Subunit Analysis of Trastuzumab Digested with IgdE Enzyme

Non-reduced

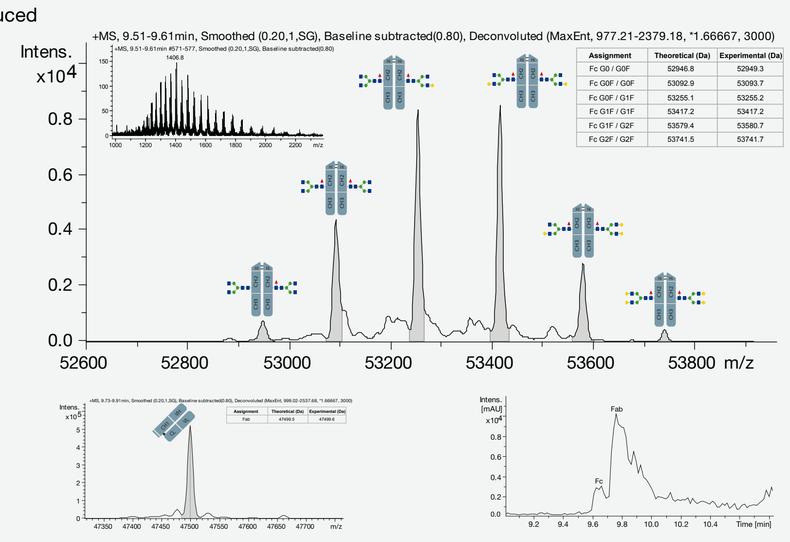

Fig 2a.

Reduced

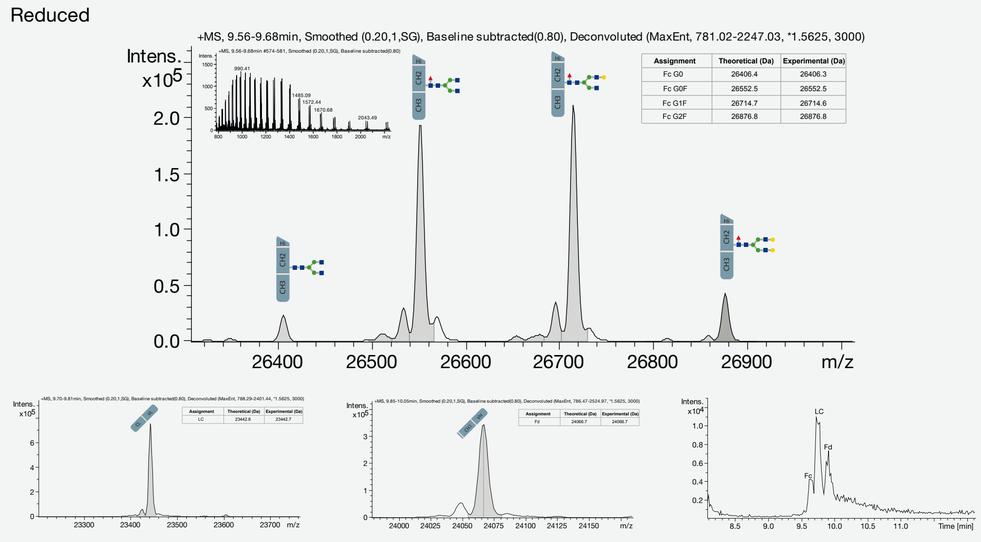

Fig 2b.

Fab and Fc fragments were generated by digestion of trastuzumab with the novel cysteine protease IgdE. No reducing agent is required for the enzyme to be active, thus intact fragments are generated, enabling intact Fc glycan profiling. The Fc glycan profile of IgdE digested trastuzumab was determined with LC/MS. The IgdE protease digestion site was determined to be …KSCDKT / HTCPPCP…

Sample preparation and LC/MS: Trastuzumab was digested with an E:S ratio of 1:20 in PBS, pH 7.4 at 37°C, overnight. The reduced sample was lyophilized and re-dissolved in 6 M GndHCl, 50 mM ABC pH 8.5. The reduction was achieved by adding 1M DTT to a final concentration of 50 mM and incubated at 65°C for 30 min. LC/MS was run both non-reduced and reduced samples. The digested sample was separated by reversed phase HPLC (Agilent 1200 system) using a 2.1x7.5 mm Poroshell 300SB-C8 column (Agilent) coupled online to a Q-TOF mass spectrometer (Bruker Maxis Impact). The obtained raw spectra were smoothed, baseline corrected and deconvoluted using the MaxEnt algorithm to determine the masses.

Fab Preparation and Purification under Native Conditions

Adalimumab was incubated with immobilized IgdE in 50 mM NaP, 150 mM NaCl pH 6.5 at room temperature overnight and the fragments were purified using a CaptureSelect™* Fc spin column (Fig. 3a). The pure Fab was isolated by centrifugation and the Fc was subsequently eluted using 0.1 M Glycine pH 2.8. The fractions were analysed by non-reduced SDS-PAGE (Fig. 3b).

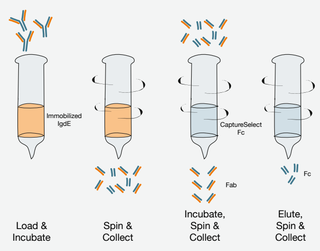

Fig 3a.

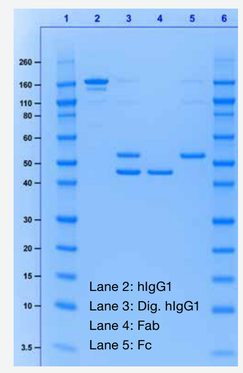

Fig 3b.

* Thermo Scientific™ CaptureSelect™ resin from Thermo Fisher Scientific Inc. and its subsidiaries.
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REFERENCES

1. Spoerry C, Hessle P, Lewis MJ, Paton L,Woof JM, von Pawel-Rammingen U (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