Characterization of an IgG-Cleaving Protease from *Streptococcus equi* with Improved Activity Against Mouse IgGs

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1. Introduction

IdeS Protease is an immunoglobulin degrading enzyme isolated from *Streptococcus pyogenes* and it has become a valuable tool for characterization of therapeutic antibodies, Fc-fusion proteins and antibody-drug conjugates. The reasons for its value are:

- Rapid digestion (~30 minutes) of human IgGs exclusively at a single site below the hinge region yielding F(ab')2 and Fc fragments.
- Reduction of the digestion products produces three fragments of ~25kDa that are readily analyzed by LC-MS and allow for:
  - Improved chromatographic separation of variants
  - Improved detection of modifications using high resolution MS.

Nevertheless, Ides protease shows poor activity against mouse IgGs.

IdeZ Protease was originally identified in *S. equi* subsp, *zooepidemicus*[1]. Here we have expressed and purified a modified recombinant IdeZ, and show that it has significantly improved activity against mouse IgG2a and IgG3 subclasses when compared to IdeS. We also demonstrate the use of IdeZ in LC-MS workflows for human and mouse IgG characterization.

[1]Lannergard & Guss, *FEMS Microbiol Lett* (2006).

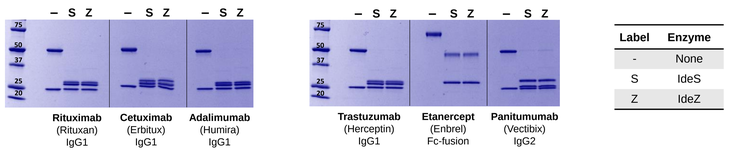
Table 1. Core and lower hinge sequence of human and mouse IgG subclasses. IdeS activity against each subclass is indicated. Cleavage of human IgGs by IdeS has been previously shown to occur between two glycine residues highlighted in red.

	Subclass	Hinge/CH2 Sequence	IdeS Activity	IdeZ Activity
Human	IgG1	CPPCPAPELL**GG**PSVF	+++++	+++++
	IgG2	CPPCPAPP_VAGPSVF		
	IgG3	CPRCPAPELL**GG**PSVF		
	IgG4	AHHAQAPEFL**GG**PSVF		
Mouse	IgG1	PCICTVPEV___SSVF	-	-
	IgG2a[a]	CPPCAAPNLLGGPSVF	+	++++
	IgG2b	CHKCPAPNLEGGPSVF	-	-
	IgG3	GSSCPAGNILGGPSVF	+	++++

2. Comparison of IdeS and IdeZ Activity

IdeZ & IdeS have similar performance against human and chimeric IgGs and Fc fusion proteins.

A. Therapeutic IgG Panel



Label	Enzyme
-	None
S	IdeS
Z	IdeZ

Figure 1. Digestion of panel of therapeutic IgGs and Fc-fusion proteins. 50µg of therapeutic IgG or fusion protein were digested with 50 units of IdeS or IdeZ (Promega) for 30 min at 37°C in a final volume of 25µl in pH 6.6 buffer. Undigested controls and samples were analyzed by SDS-PAGE under reducing conditions.

> 95% Digestion of mouse IgG2a in 2 hours with IdeZ.

B. Mouse IgG2a Digestion

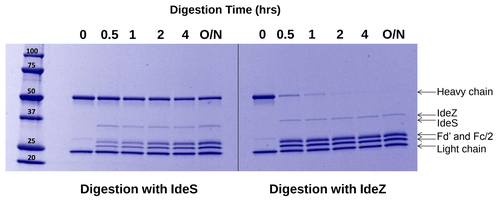
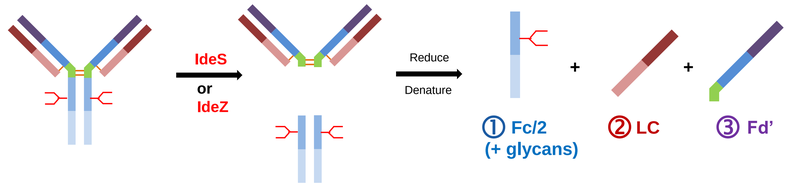


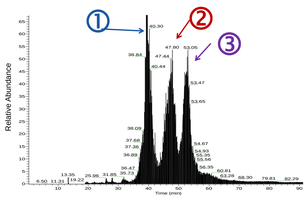
Figure 2. Digestion of recombinant mouse IgG2a with IdeS and IdeZ. 50µg of recombinant mIgG2a anti-hCD20 (Invivogen) were digested with 50 units of IdeS or IdeZ (Promega) for various time points at 37°C in a final volume of 25µl in pH 6.6 buffer. Samples were analyzed by SDS-PAGE under reducing conditions. IgG2a is completely digested in 2 hours with IdeZ whereas is only partially digested with IdeS even after O/N digestion.

3. IdeS and IdeZ Cleave Rituximab at the Same Site

IdeS and IdeZ rapidly digest IgGs below the hinge resulting in three ~25 kDa fragments after reduction.

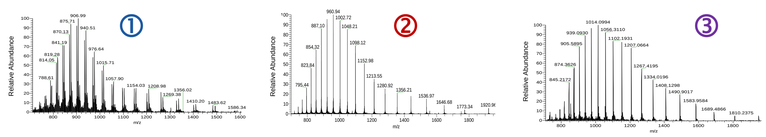


A. TIC Chromatogram of IdeS-digested Rituximab



B. Full MS Spectra

Digestion with IdeS



Digestion with IdeZ

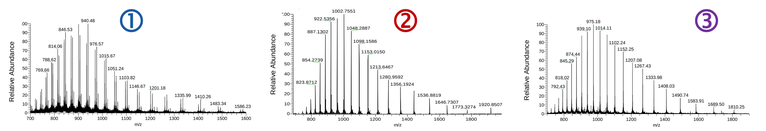


Figure 3. Middle up MS-Analysis of rituximab digested with IdeS or IdeZ.
A) Total ion chromatogram of IdeS-digested rituximab. B) Full MS spectra of rituximab fragments after digestion with IdeS (left) or IdeZ (right). 100µg of rituximab were digested with 100 units of IdeS or IdeZ (Promega) for 30min at 37°C. Fragments were incubated with 10mM TCEP and 6M GuHCl at 60°C for 60 min. Samples were then buffer exchanged into 0.1% formic acid 3x with a 10kDa MWCO filter, reducing the volume to 50µl after each dilution. The final solution was loaded on a 150µm PLRP-S trap column maintained at 75°C over 30 minutes. Fragments were then resolved with a 75µm PLRP-S Analytical column operated at 300nl/ min with a 120 min gradient.

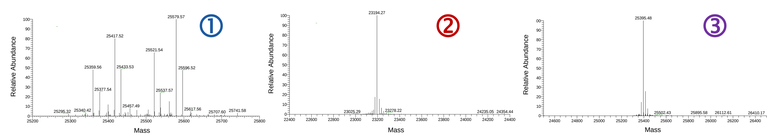
Table 2. Deconvoluted Rituximab fragment masses after digestion with IdeS or IdeZ proteases. Monoisotopic intact masses (Daltons) were determined using Xtract (Thermo Fisher) with a S:N cutoff of 3, manually correcting for off-by-N errors. Fragment masses are the same whether digested with IdeS or IdeZ.

		IdeS Digestion		IdeZ Digestion	
Fragment	Theoretical Mass	Measured Mass	Mass Error (ppm)	Measured Mass	Mass Error (ppm)
Fc-G0F-K	25188.51	25188.52	-0.5	25188.59	-3.2
Fc-G1F-K	25350.56	25350.46	3.9	25350.62	-2.4
Fc-G2F-K	25512.62	25512.49	4.9	25512.64	-0.8
pLC	23025.32	23025.19	5.5	23025.40	-3.4
pFd'	25312.36	25312.27	3.5	25312.43	-2.8

4. MS Characterization of Mouse IgG2a Antibodies After Digestion with IdeZ

IdeZ protease efficiently cleaves mouse IgG2a at the Gly-Gly bond just below the hinge as shown by MS analysis.

A. mIgG2a Anti-hCD20



B. mIgG2a Anti-hTNFα

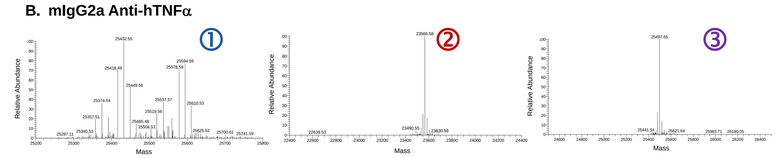


Figure 4. Deconvoluted MS spectra of two mouse IgG2a antibodies digested with IdeZ Protease. A) mIgG2a anti-hCD20. B) mIgG2a anti-TNFα. Recombinant mouse IgG2a antibodies were obtained from Invivogen's anti-hCD20 (Rituximab) and anti-hTNFα (Adalimumab) isotype collections. 100µg of each antibody were digested with 100 units of IdeZ (Promega) overnight at 37°C. Samples were analyzed by LC/MS using the same method as for Rituximab.

Table 3. Deconvoluted monoisotopic IgG fragment masses after digestion with IdeZ protease. Masses were calculated using Xtract (Thermo Fisher) and corrected for off-by-N errors. Masses are reported in Daltons. Theoretical masses are reported assuming IdeZ cleaves between the Gly-Gly bond just below the hinge.

	mIgG2a anti-hCD20			mIgG2a anti-hTNFα		
Fragment	Theoretical Mass*	Measured Mass*	Mass Error (ppm)	Theoretical Mass	Measured Mass	Mass Error (ppm)
Fc-G0F-K -G,Pam	25358.47	25358.56	-3.5	25358.47	25358.51	-1.6
Fc-G0F-K	25416.47	25416.52	-2.0	25416.47	25416.46	0.4
Fc-G0F-K ox	25432.46	25432.53	-2.8	25432.46	25432.55	-3.5
Fc-G0F-K 2-ox	25448.45	-	nd	25448.45	25448.56	-4.3
Fc-G1F-K, -G,Pam	25520.53	25520.54	-0.4	25520.53	25520.56	-1.2
Fc-G1F-K	25578.53	25578.57	-1.6	25578.53	25578.59	-2.3
Fc-G1F-K ox	25594.52	25594.52	0.0	25594.52	25594.59	-2.7
Fc-G1F-K 2ox	25610.51	-	nd	25610.51	25610.53	-0.8
pLC	n/a	23194.27	nd	n/a	23566.58	nd
pFd'	n/a	25395.48	nd	n/a	25497.65	nd

n/a – not available; nd – not determined.

Glossary of PTMS
-K = missing C-terminal lysine
-G = missing C-terminal lysine and glycine
Pam = amidated C-terminal proline
Ox = oxidation; 2-ox = double oxidation
p = N-terminal pyroglutamic acid

5. Conclusions

- **IdeZ is a highly specific protease that cleaves IgGs at the same site as IdeS yielding three ~25kDa fragments after reduction.**
- **IdeZ & IdeS have similar performance against human & chimeric IgGs as well as Fc-fusion proteins.**
- **IdeZ has significantly improved activity against mouse IgG2a and IgG3 compared to IdeS.**
- **IdeZ is a useful alternative to IdeS for antibody characterization.**

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