L445P Mutation on Heavy Chain Stabilizes IgG4 Under Acidic Conditions

ABSTRACT#

Changai Xu, Matthew R. Wallace, Stephen Farrand, Vijay Chhajlani, and Kristin Krukenberg

Jounce Therapeutics, Inc., Cambridge, MA USA

ABSTRACT

IgG4, a common type of therapeutic antibody, is less stable during manufacturing processes compared with IgG1. Aggregation and fragmentation are the two main challenges. In this study, we report instability of the heavy chain (HC) C-terminal region under acidic conditions, which leads to cleavage and aggregation. Leu445, at the C-terminal region of the HC in IgG4, plays a critical role in its acid-induced fragmentation and subsequent aggregation. We found that mutating HC C-terminal Leu445 to Pro (the corresponding residue in IgG1) in IgG4_CDR-X significantly reduces fragmentation and aggregation, while mutating Pro445 to Leu in IgG1_CDR-X promotes fragmentation and aggregation. HC C-terminal Gly446 cleavage was observed in low pH citrate buffer and resulted in further fragmentation and aggregation, whereas, glycine buffer can completely inhibit the cleavage and aggregation. It is proposed that cleavages occur through acid-induced hydrolysis under acidic conditions and glycine stabilizes IgG4 via two main mechanisms: 1) product feedback inhibition of the hydrolysis reaction, and 2) stabilization of protein conformation by direct interaction with the peptide backbone and charged side chains. Experiments using IgG4 molecules IgG4_CDR-Y and IgG4_CDR-Z with the same CH domains as IgG4_CDR-X, but different complementarity-determining regions (CDRs), indicate that the stability of the HC C-terminal region is also closely related to the sequence of the CDRs. The stability of IgG4_CDR-X is significantly improved when binding to its target. Both observations suggest that there are potential interactions between Fab and CH2-CH3 domains, which could be the key factor affecting the stability of IgG antibodies.

Purpose and Rational

A 57 Da cleavage from the HC C-terminal of one IgG4 molecule was observed during purification. It was suspected to be caused by the low pH hold step, resulting in fragmentation, subsequent aggregation, and low yield.

To address the issue the following approaches were applied.

- Comparison with and without low pH hold step
- Screening elution buffer types and concentration
- HC C-terminal point mutation, L445P (as indicated in Fig. 1)
- CDR mutations and target binding check

Protein was produced in CHO cells and purified via ProA column. Aggregation was checked by SEC-HPLC, cleavage and fragmentation by Mass Spec, and binding affinity by SPR.

CH3 (EU 341 to 447)

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IgG4: GQPREPQVYT LPPSQEEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS
IgG1: GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS

IgG4: DGSFFLYSRL TVDKSRWQEG NVFSCSVMHE ALHNHYTQKS LSLSLGK
IgG1: DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK
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Figure 1: Sequence alignment of CH3 in IgG1 and IgG4. Conserved residues are shown in black. Similar residues are shown in green. Residues with different properties are shown in red. The numbering of the amino acids is based on the EU numbering system.

RESULTS

Acid-induced fragmentation and aggregation of IgG4_CDR-X

Table 1: MS analysis of HC of IgG4_CDR-X purified by different procedures. A 57 Da mass loss was observed consistently during process.

Samples	Theoretical mass (Da) (PTM)	Determined mass (Da)	Mass difference (Da)
Sample_1[a]	50245.1 (pyro Q–Lys + G0F)	50188.0	−57.1
	50407.2 (pyro Q–Lys + G1F)	50350.0	−57.2
	50569.3 (pyro Q–Lys + G2F)	50512.5	−56.8
	48800.9 (pyro Q–Lys + 1 Da) (deglycosylated)[c]	48743.5	−57.0
	23775.7 (Fc/2 by IdeS)[d]	23718.5	−57.2
Sample_2[b]	50245.1 (pyro Q–Lys + G0F)	50245.0	−0.1
	50407.2 (pyro Q–Lys + G1F)	50407.0	−0.2
	50569.3 (pyro Q–Lys + G2F)	N/D	N/A
	48800.9 (pyro Q–Lys + 1 Da) (deglycosylated)[c]	48801.0	0.1
	23775.7 (Fc/2 by IdeS)[d]	23775.5	0.2

[a]Eluted from ProA column with 50 mM acetate buffer and held in the same buffer for 1 hr before neutralization.
[b]Eluted from ProA column with 100 mM acetate buffer and immediately neutralized.
[c]De-*N*-glycosylation was done with PNGase F treatment, which results in conversion of asparagine to aspartic acid, a 1 Da mass increase.
[d]Fc/2 was generated by IdeS digestion.

Glycine inhibits and citrate promotes acid-induced C-terminal fragmentation and subsequent aggregation

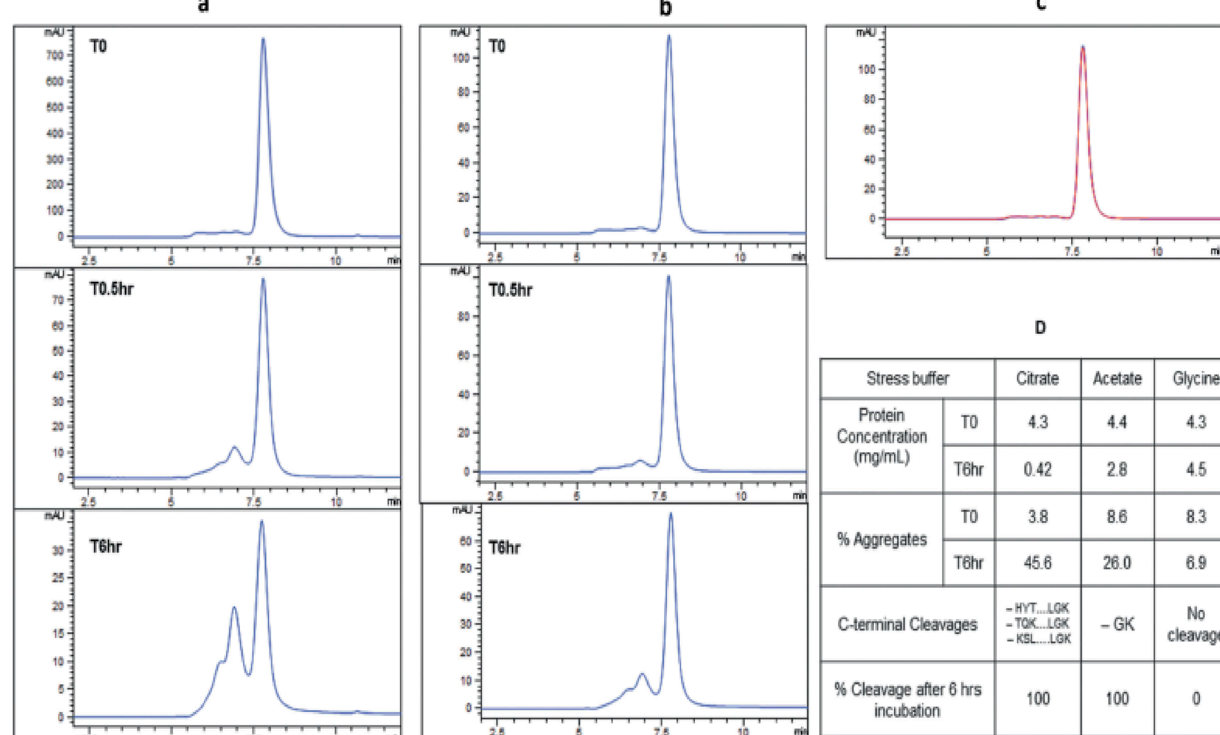

Figure 2: SE-HPLC and MS analysis of IgG4_CDR-X under acidic stress in different buffers. A) Stacked SE-HPLC chromatograms in sodium citrate buffer; B) Stacked SE-HPLC chromatograms in sodium acetate buffer; C) Overlaid SE-HPLC chromatograms in L-glycine buffer; D) Summary of protein loss determined by A280, aggregation determined by SE-HPLC, and C-terminal cleavages detected in mass analysis for samples after stress.

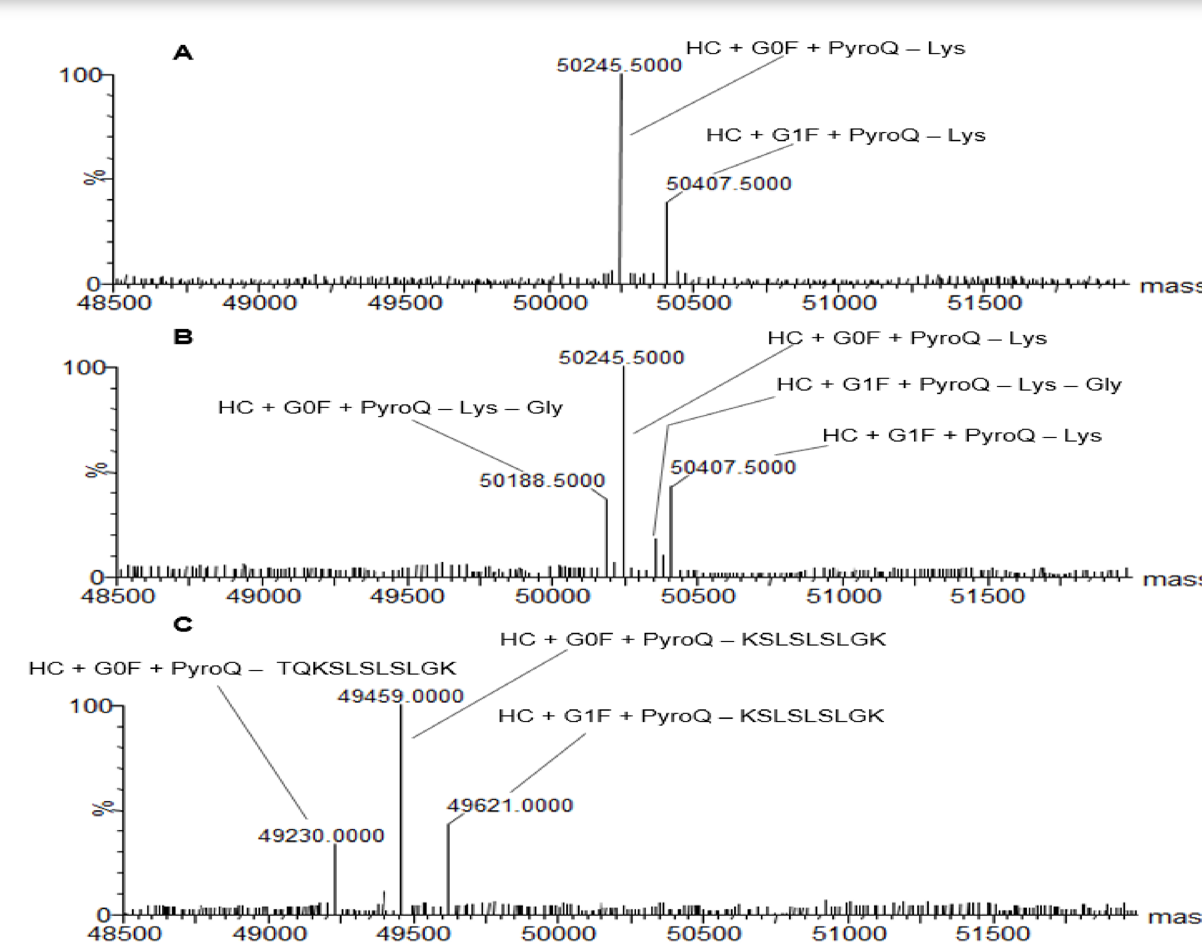

Figure 3: Deconvoluted heavy chain mass spectra showing pH-dependent C-terminal cleavages of IgG4_CDR-X. A) Neutral pH, protein was eluted from ProA column with 100 mM citrate buffer, pH 3.5, and immediately neutralized; B) pH 4.0, protein was eluted from ProA column with 100 mM citrate buffer, pH 4.0, followed by hold in the same buffer for 1 hr before neutralization; C) pH 3.5, protein was eluted from ProA column with 100 mM citrate buffer, pH 3.5, followed by hold in the same buffer for 1 hr before neutralization.

Point mutation L445P on IgG4_CDR-X heavy chain significantly reduces HC C-terminal truncation and antibody aggregation with no changes of Tm and affinity

Table 2: Heavy chain C-terminal cleavage of IgG4_CDR-X, IgG1_CDR-X and their mutants IgG4_CDR-X(L445P), IgG1_CDR-X (P445L) after acidic stress. Approximately 40% of the IgG4 L445P CDR-X molecules experienced no cleavage (except for C-terminal Lys).

Samples		Theoretical Mass (Da)	Observed Mass (Da)	HC modifications and cleavages determined by MS analysis
IgG_4_CDR-X	T0	48944.9	50245.5	HC + G0F + PyroQ – Lys
			50407.5	HC + G1F + PyroQ – Lys
			50571.5	HC + G2F + PyroQ – Lys
	T24hr supernatant		48923.5	HC + G0F + PyroQ – HYTQ…LGK
			49228.5	HC + G1F + PyroQ – TQKS…LGK
			49458.5	HC + G2F + PyroQ – KSLS….LGK
	T24hr Pellet		48815.0	HC + G0F + PyroQ – NHYT….LGK
			48928.0	HC + G0F + PyroQ – HYTQ….LGK
			49229.5	HC + G1F + PyroQ – TQKS….LGK
			49453.0	HC + G2F + PyroQ – KSLS…. LGK
IgG_4_CDR-X ($L^{465}P$)	T0	48928.9	50229.0	HC + G0F + PyroQ – Lys
			50353.5	HC + G0F + PyroQ
			50387.5	HC + G1F + PyroQ – Lys
	T24hr supernatant		49330.0	HC + G0F + PyroQ – QKSL….PGK
			50353.5	HC + G0F + PyroQ
	T24hr pellet		49330.0	HC + G0F + PyroQ – QKSL….PGK
			50179.5	HC + G0F + PyroQ – Gly - Lys
			50353.5	HC + G0F + PyroQ*
IgG_1_CDR-X	T0	49132.3	50431.5	HC + G0F + PyroQ – Lys
			50593.5	HC + G1F + PyroQ – Lys
	T24hr supernatant		50592.0	HC + G1F + PyroQ – Lys
			50431.0	HC + G0F + PyroQ – Lys
	T24hr pellet		50432.0	HC + G0F + PyroQ – Lys
			50594.0	HC + G1F + PyroQ – Lys
IgG_1_CDR-X ($P^{465}L$)	T0	49148.3	50450.5	HC + G0F + PyroQ – Lys
			50609.0	HC + G1F + PyroQ – Lys (<1%)
	T24hr supernatant		49864.5	HC + G0F + PyroQ – LSLSLGK
	T24hr pellet		49864.5	HC + G0F + PyroQ – LSLSLGK

*Percentage of HC + G0F + PyroQ was ~40% based on MS data.

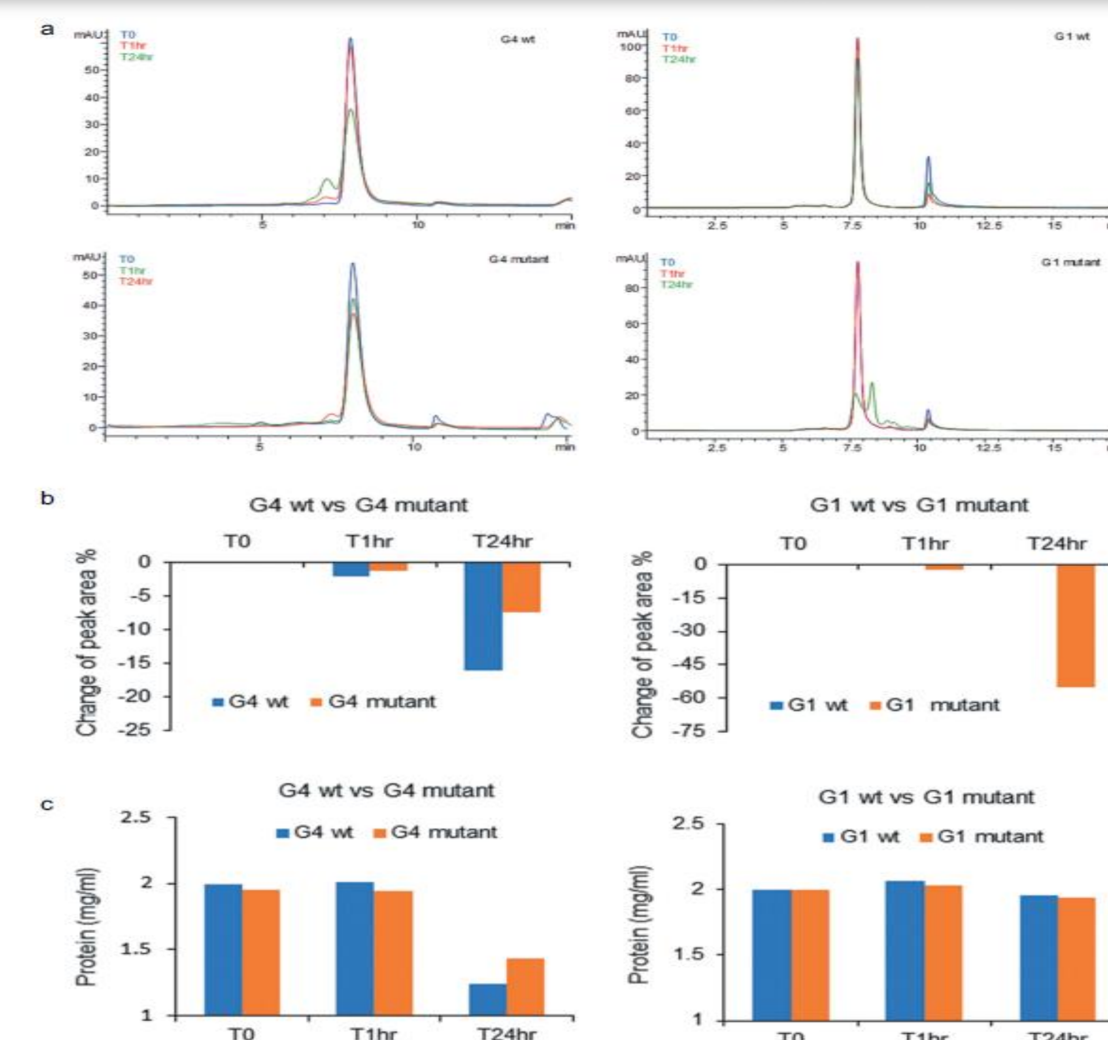

Figure 4: SE-HPLC monomer peak change and protein loss of IgG4_CDR-X, IgG1_CDR-X and their mutants IgG4_CDR-X(L445P), IgG1_CDR-X(P445L) after acidic stress. A) Overlaid SE-HPLC chromatograms of T0, T1 hr and T24 hr; B) SE-HPLC monomer peak area change; C) Protein loss measured by A280.

Table 3: Affinity and Tm comparison of wild type antibodies IgG4_CDR-X, IgG1_CDR-X, and their mutants IgG4_CDR-X (L445P), IgG1_CDR-X(P445L) by SPR and VP-DSC. No changes were observed between wild type and mutants.

Samples	k_a [1/(M·s)]	k_d [1/s]	K_D (M)	Fab T_m (°C)
IgG_4_CDR-X	6.6×10^4	2.3×10^{-4}	3.4×10^{-9}	69.8
IgG_4_CDR-X($L^{445}P$)	5.9×10^4	2.1×10^{-4}	3.6×10^{-9}	70.2
IgG_1_CDR-X	6.1×10^4	2.2×10^{-4}	3.5×10^{-9}	82.0
IgG_1_CDR-X($P^{445}L$)	6.2×10^4	2.2×10^{-4}	3.5×10^{-9}	82.8

IgG4 Fc stability is influenced by possible interaction between Fab and Fc

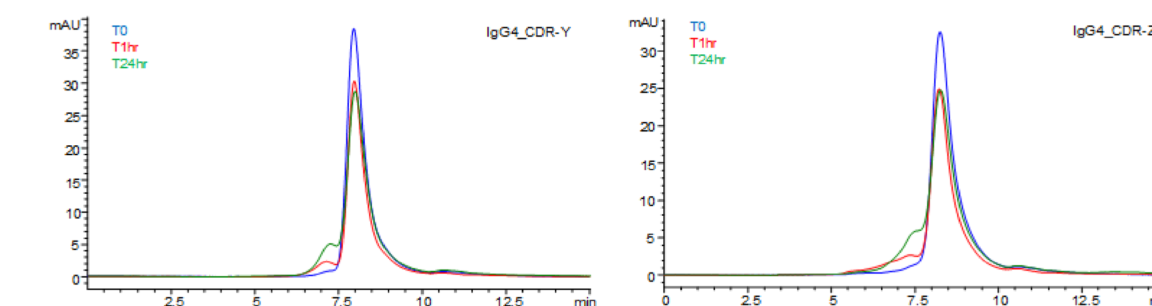

Figure 5: Overlaid SE-HPLC chromatograms of IgG4_CDR-Y and IgG4_CDR-Z after acidic stress in citrate buffer. IgG4_CDR-Y and IgG4_CDR-Z target the same ligand as IgG4_CDR-X but with different CDRs. Soluble aggregate was comparable after acidic stress with all three antibodies.

Table 4: Soluble aggregation and precipitation of IgG4_ CDR-Y and IgG4_CDR-Z compared with IgG4_CDR-X after acidic stress. 1). Soluble aggregation as determined by SE-HPLC. 2). Precipitation as determined by A280.

Incubation time	IgG_4_CDR-X		IgG_4_CDR-Y		IgG_4_CDR-Z	
	% HMW[1]	% protein loss[2]	% HMW[1]	% protein loss[2]	% HMW[1]	% protein loss[2]
T0	0	n/a	0	n/a	0.0	n/a
T1hr	2.2	0	2.3	3.5	2.9	3
T24hr	16.1	37.4	11.2	2	12.7	2

Table 5: Comparison of acid-induced soluble aggregation, precipitation and heavy chain C-terminal cleavages of IgG4_ CDR-X in antigen-complexed and uncompleted form. 1) Soluble aggregation as determined by SE-HPLC. 2) Precipitation as determined by A280. 3) Cleavages by MS analysis

Incubation time	Aggregates IgG_4_CDR-X complex		HC cleavage IgG_4_CDR-X complex		Aggregates IgG_4_CDR-X		HC cleavage IgG_4_CDR-X	
	% HMW[1]	% protein loss[2]	%	Cleaved peptides[3]	% HMW[1]	% protein loss[2]	%	Cleaved peptides[3]
T0	0.0	n/a	0.0	n/a	0	n/a	0.0	n/a
T1hr	1.8	0.0	45	SLSLSLGK	2.2	0	100	NHYTQKSLSLSLGK; HYTQKSLSLSLGK; TQKSLSLSLGK; KSLSLSLGK
T24hr	28.7	0.0	88	SLSLSLGK and KSLSLSLGK	16.1	37.4	100	

CONCLUSION

In conclusion, heavy chain C-terminal Gly 446 cleavage was found in IgG4_CDR-X at low pH and is likely through an acidic-induced hydrolysis. Gly 446 cleavage triggers further C-terminal cleavages. Its aggregation is induced by HC C-terminal cleavage. Two mitigating approaches are found to improve IgG4 stability. Glycine buffer prevents cleavage of Gly446, while the L445P mutation provides greater stability to the HC C-terminus. Furthermore, IgG4 has a unique spatial orientation, with the Fab located near the Fc region. Certain mutations in the CDR and antigen binding can also help to stabilize the CH2-CH3 domains which suggests presence of interaction between the Fab and CH2-CH3 domains. These key factors are important to be considered when IgG4 is exposed to manufacturing conditions.

REFERENCE

1 Skamris T, Tian X, Thorolfsson M, Karkov HS, Rasmussen HB, Langkilde AE, Vestergaard B. Monoclonal antibodies follow distinct aggregation pathways during production-relevant acidic incubation and neutralization. Pharm Res. 2016; 33(3): 716-28.

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