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# Non-enzymatic hinge region fragmentation of antibodies in solution

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**Abstract**

Liquid formulations of monoclonal antibodies (MAbs) typically undergo fragmentation near the papain cleavage site in the hinge region, resulting in Fab and Fab + Fc forms. The purpose of this study was to investigate whether this fragmentation is due to proteases. Four closely-related MAbs were exchanged into a pH 5.2 acetate buffer with NaCl and stored at −20 °C, 5 °C, 30 °C, or 40 °C for 1 month. Fragmentation generated size-exclusion chromatography (SEC) peak fractions that were analyzed by electrospray mass spectrometry to identify the cleavage sites. The effects of protein inhibitors or host cell proteins on fragmentation were also studied. The extent of fragmentation was equivalent for all four antibodies, occurring in the heavy chain hinge region Ser–Cys–Asp–Lys–Thr–His–Thr sequence. The fragment due to cleavage of the Asp–Lys bond showed two forms that differ by 18 Da. A synthetic peptide with the hinge region sequence terminating with Asp did not show fragmentation or the loss of 18 Da after incubation. Protease inhibitors did not affect rates of cleavage or modify sites of fragmentation. Degradation was not affected by host cell protein content. Fragmentation appears to be a kinetic process that is not caused by low levels of host cell proteases.


*Keywords:* Hinge region fragmentation; Protease inhibitors; Monoclonal antibodies; Size-exclusion chromatography; Stability

## 1. Introduction

Monoclonal antibodies (MAbs) typically undergo fragmentation over time in the liquid state. This phenomenon is usually observed by size-exclusion chromatography (SEC). Fragmentation occurs in one heavy chain hinge region, near the papain cleavage site, resulting in Fab and Fab + Fc forms. Others have reported on this phenomenom. Jiskoot et al. [1] identified Fab and Fab + Fc fragments as products of degradation for two monoclonal antibodies after incubation at different pH values and temperatures. Alexander and Hughes [2] monitored the degradation of a chimeric monoclonal antibody under high temperature conditions using MALDI-TOF MS, observing ions corresponding in mass to loss of one light chain from the antibody, loss of a Fab arm to yield a Fab + Fc fragment, and separated HC and LC moieties. $Cu^{++}$ mediated cleavage of the molecule within the hinge region to generate Fab, Fc, and Fab + Fc fragments has also been reported [3].

Cleavage of immunoglobulins by enzymes (e.g. papain, pepsin) is facilitated by the accessibility of the hinge region (Fig. 1). Flexibility of the IgG in solution potentially favors kinetic cleavage at its weakest link (the hinge region). X-ray crystallographic studies have shown that the hinge region is mobile or completely disordered or very fluid and flexible [4–7]. Roux et al. [8] defined the different modes of flexibility. These include hinge-like folding or bending, conical wagging, rotation about the long axis, and translation (in-and-out motion). In another study using high-resolution nuclear

*Abbreviations:* MAb, monoclonal antibody; SEC, size-exclusion chromatography; HC, heavy chain; LC, light chain; CHOP, Chinese hamster ovary cell protein; LC-ESI/MS, liquid chromatography-electrospray mass spectrometry; HPLC, high performance liquid chromatography; Da, Dalton; a.m.u, atomic mass units

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magnetic resonance (NMR), Endo and Arata [9], concluded that in solution a hinge segment (upper hinge) undergoes an extensive degree of internal motion. Their results indicated that in solution the hinge region contributes significantly to the internal flexibility of IgGs.

By determining cleavage rates and clip sites of MAbs during storage that have been purified under different process conditions, a better understanding of the nature of the fragmentation at the hinge region could be obtained. We investigated whether host cell proteins could enhance fragmentation, and whether protease inhibitors could reduce fragmentation. Four recombinant IgG1 MAbs were exchanged into a pH 5.2 acetate buffer with NaCl and stored at different temperatures (ranging from −20 °C to 40 °C) for 1 month. The extents of cleavage of the four MAbs were measured by SEC. The cleavage products were assigned by electrospray mass spectrometry.

## 2. Experimental

Four humanized IgG1 monoclonal antibodies (MAb A, MAb B, MAb C, and MAb D) produced by Genentech Inc. were reconstituted with sterile water for injection (SWFI). A 100 mg sample of each MAb was transferred into dialysis tubing (Spectra/Por, 6000–8000 MWCO, from Spectrum Laboratories Inc., CA, USA), then exchanged into 10 mM sodium acetate, 140 mM NaCl, 0.05% sodium azide, pH 5.2. Dialyzed samples were transferred to Falcon tubes and buffer was added to each sample to make the final protein concentration 10 mg/ml. The samples were sterile-filtered with 0.22 μm membrane filters (Lida Manufacturing Corp., PRO-X filter unit) and transferred into 2 ml autoclaved vials. Duplicate 1 ml aliquots of each MAb sample were stored at −20 °C, 5 °C, 30 °C, or 40 °C for 1 month.

Similarly, samples of MAb B at different stages of the recovery process (initial, intermediate and final pools) were also prepared following the procedure described above using the same buffer, and stored in duplicates at −20 °C, 5 °C, 30 °C, or 40 °C for 1 month. An ELISA for Chinese hamster ovary cell protein [10] (CHOP) performed at Analytical Operations Department at Genentech, was used to quantify the levels of CHOP in the pool samples.

In a parallel experiment, MAb D samples were incubated in a different buffer (5 mM histidine, 85 mM sucrose, 0.01% Polysorbate 20, pH 6.0) for 1 month at 40 °C with and without a mixture of protease inhibitors containing E64, leupeptin, benzamidine, E-amino caproic acid, pepstatin A, and EDTA (Table 1). All protease inhibitors were obtained from Calbiochem (San Diego, CA, USA).

Samples were analyzed by SEC using a HP1090 HPLC system (Hewlett-Packard, Santa Clara, CA, USA). A TOSO HAAS TSK 3000SWXL (Tosoh Biosep LLC, Montogomeryville, PA, USA) 7.8 mm × 30 cm, 5 μm column was used at ambient temperature. The mobile phase was 100 mM $K_2HPO_4$ (J.T. Baker), the pH was adjusted to 6.8 with 85% phosphoric acid (Mallinckrodt), and the flow rate was 0.5 ml/min. Peak fractions from SEC were collected manually, then exchanged into 100 mM Tris, pH 6.8, and concentrated to 0.1–2 mg/ml using Centricon YM-10 centrifugal filter devices (Millipore Corporation, Bedford, MA, USA).

Table 1
Protease inhibitors added to MAb D cocktail sample

| Inhibitor | Type | Concentration |
|---|---|---|
| E64 | Irreversible inhibitor of cysteine proteinases | 25 μM |
| Leupeptin | Inhibits serine and cysteine proteases | 20 μM |
| Benzamidine | Inhibits trypsin-like serine proteinases | 4 mM |
| E-amino caproic acid | Inhibits carboxypeptidase B | 2 mM |
| Pepstatin A | Inhibitor of various aspartic proteinases | 10 μM |
| EDTA | Metalloproteinase inhibitor | 7.5 mM |

Peak fractions were analyzed by electrospray mass spectrometry (LC-ESI/MS). Samples were reduced with 25 mM dithiothreitol (DTT, Boehringer Mannheim, Indianapolis, IN, USA) at 37 °C for 20 min, then desalted by injecting onto a perfusion chromatography reversed phase column, 10 μm, 0.33 mm × 20 cm, packed with POROS R2 resin (PerSeptive Biosystems, Framingham, MA, USA) using an HP1090 HPLC. The column was held at 100% solvent A (0.1% aqueous formic acid) for 5 min, and the samples were eluted with a linear gradient from 20% to 70% solvent B (0.08% formic acid in acetonitrile) over 16 min. The column effluent was directly introduced into the electrospray source of a PE Sciex API3000 triple quadrupole mass spectrometer (Foster City, CA, USA).

The mass-to-charge (*m*/*z*) range 50–3000 of Q1 and Q3 were tuned by using polypropylene glycol standard (PPG's) and polypropylene glycol 3000 standard (PPG's 3000) solution to give an approximate resolution of >2000 (50% valley definition) across the mass-to-charge range between 1000 and 3000. After using myoglobin for calibrating and optimizing the spray condition, these samples were analyzed with the described instrument parameters. For the reduced fragments, spectra were recorded by scanning the *m*/*z* range of the first quadruple from 1100 to 2400 in 0.1 a.m.u steps with a total scan time of 13.0 s. For intact antibodies, the scanned *m*/*z* range was changed to 1800–3000 in 0.1 a.m.u steps with a total scan time of 12.0 s to accommodate the ionization window. The resulting background was subtracted from the spectra and then deconvoluted to the true mass using the manufacturer's software (Bioanalyst, version 1.3).

Five milligrams of a synthetic peptide KKVEPKSCD were prepared by the Bio-Organic Chemistry Department at Genentech Inc. The peptide was reconstituted with 10 mM sodium acetate (trihydrate), 140 mM NaCl, 0.05% sodium azide, pH 5.2 at a concentration of 1 mg/ml. Under aseptic conditions, the peptide was transferred into 2 ml depyrogenated vials; 1 ml duplicates were stored at −20 °C and

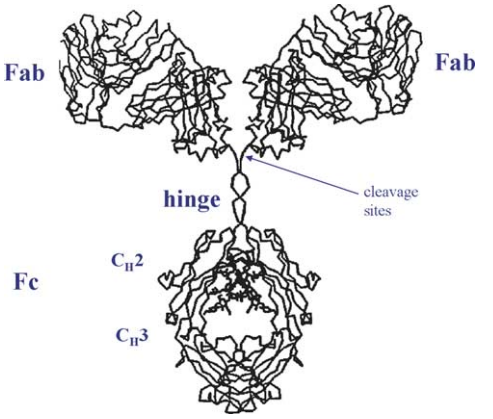


Fig. 1. Computer generated model of human IgG [4].

40 °C for 1 month. The peptide samples were analyzed by LC–ESI/MS (as described above).

## 3. Results

The SEC profiles of the samples after 1-month storage were very similar for the different MAbs. All 30 °C and 40 °C samples showed two additional peaks eluting after the intact form, the first one as a shoulder of the monomer and the second one eluting about 3.6 min later (peaks 2 and 3, respectively). The profiles for MAb D at 40 °C are shown in Fig. 2.

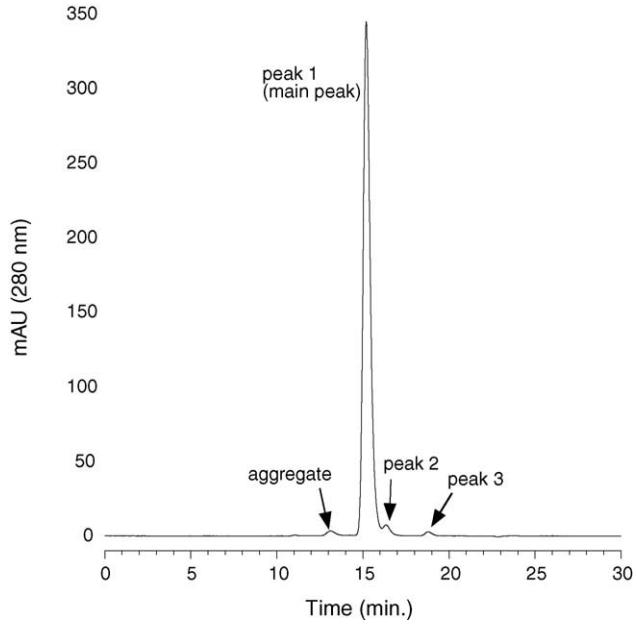


Fig. 2. Size-exclusion chromatography of MAb D after 1-month incubation at 40 °C.

Structures were assigned for the collected peak fractions by comparison of the observed masses to the known heavy and light chain structures, including Fc glycosylation. Peak 2 was assigned as the Fab + Fc (one Fab arm plus the Fc portion

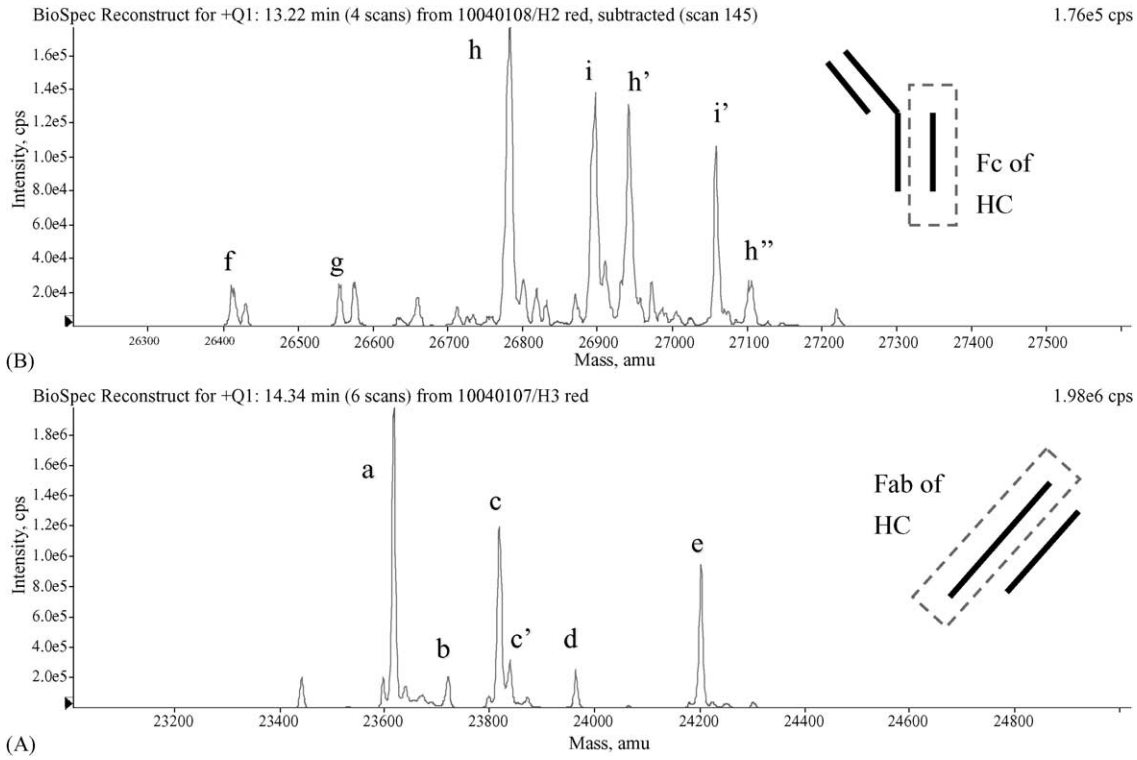


Fig. 3. LC/ESI-MS spectra of reduced MAb B fragments collected from size-exclusion chromatography. LC mass (23,442 Da) and HC mass (50,602 Da) do not appear in these region of the spectra. Panel A represents the Fab part of the heavy chain of peak 3 (Fab fragment). Panel B represents the Fc part of the heavy chain of peak 2 (Fab + Fc fragment). Identities of the peaks are shown in Table 2.

Table 2
LC/ESI-MS fragments of MAb B observed in Fab and Fab + Fc forms collected from SEC

| Peak | Residues[a] | Expected mass | Observed mass | Cleavage site |
|---|---|---|---|---|
| a | HC:1–222 | 23619.5 | 23617.3 | Ser–Cys |
| b | HC:1–223 | 23722.6 | 23721.0 | Cys–Asp |
| c | HC:1–224 (−18 Da) | 23837.7 | 23818.1 | Asp–Lys |
| c′ | HC:1–224 | 23837.7 | 23836.5 | Asp–Lys |
| d | HC:1–225 | 23965.9 | 23964.6 | Lys–Thr |
| e | HC:1–227 | 24204.1 | 24203.1 | His–Thr |
| f | HC:228–449 w/G0 | 26415.7 | 26414.6 | His–Thr |
| g | HC:227–449 w/G0 | 26552.9 | 26556.5 | Thr–His |
| h | HC:225–449 w/G0 | 26782.2 | 26782.0 | Asp–Lys |
| h′ | HC:225–449 w/G1 | 26944.2 | 26942.1 | Asp–Lys |
| h″ | HC:225–449 w/G2 | 27106.2 | 27105.9 | Asp–Lys |
| i | HC:224–449 w/G0 | 26897.2 | 26897.0 | Cys–Asp |
| I′ | HC:224–449 w/G1 | 27059.3 | 27059.2 | Cys–Asp |

[a] G0, G1, and G2 refer to Fc oligosaccharides with 0, 1, or 2 galactose residues, respectively.

of the IgG, formed after loss of one Fab arm) fragment, and peak 3 contained the Fab fragment. Fig. 3 shows the spectra obtained for MAb B peaks 2 and 3. The derived structures of each form are shown in Table 2. These structures indicate the presence of multiple cleavage sites in the heavy chain hinge region. No cleavage of light chains was observed in any peak fraction. Peak 1 gave the expected intact heavy chain and light chain masses for all of the MAbs (data not shown).

Fragments containing the heavy chain component of the Fab fragment with Asp C-termini appear with two forms. The main form is 18 Da lower than expected (Table 2, Fig. 3). A synthetic peptide with the sequence KKVEPKSCD did not exhibit the −18 Da form, even after incubation at 40 °C for 1 month in the acetate–NaCl buffer used to incubate the four antibodies, eliminating the possibility that the 18 Da mass difference observed for the forms with a C-terminal Asp was due to sample preparation or was an electrospray mass spectrometry artifact. The synthetic peptide presented no fragmentation after 1-month incubation at 40 °C. The complementary Fc fragments of the antibodies resulting from cleavage at the Asp–Lys bond had the expected masses and did not exhibit any +18 Da forms, eliminating the possibility of an alternate

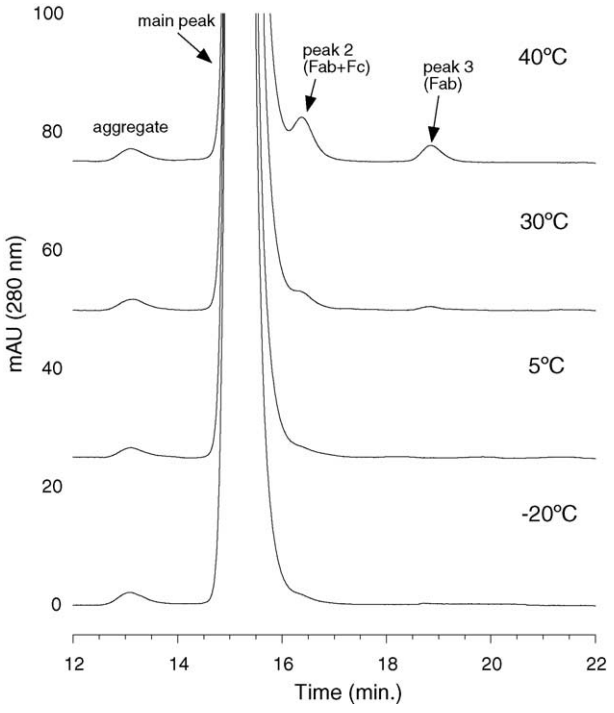


Fig. 4. MAb D after 1-month incubation at different temperatures.

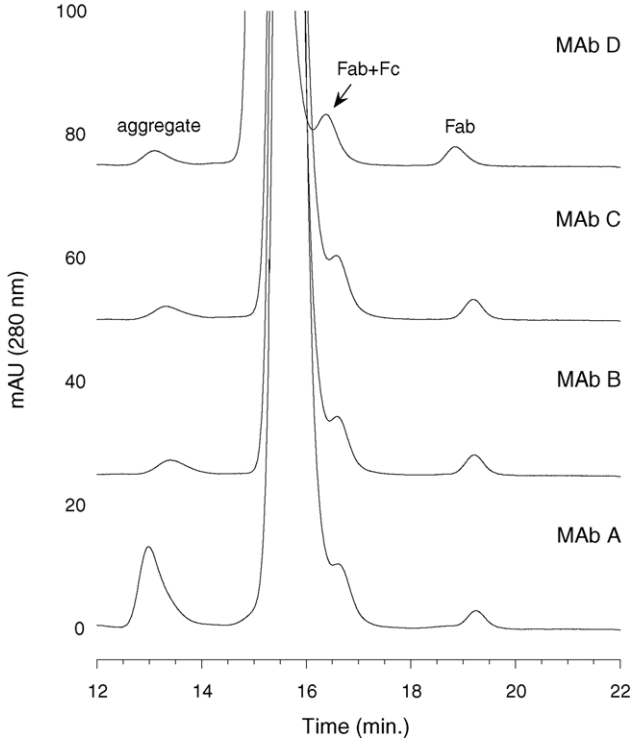


Fig. 5. Size-exclusion chromatography of MAbs A, B, C, and D after 1-month incubation at 40 °C.

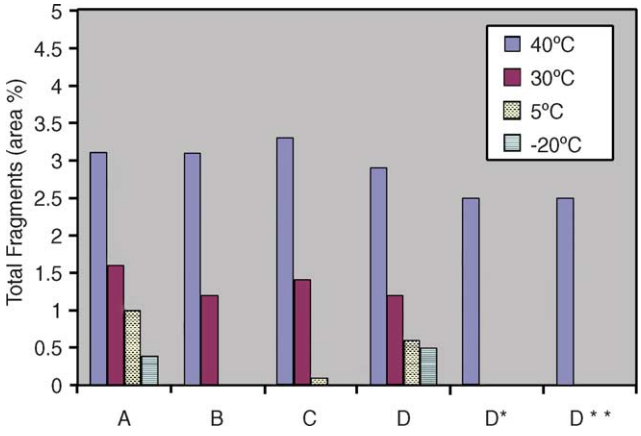


Fig. 6. Total fragments (Fab + Fc + Fab) area % of MAbs after 1-month incubation at different temperatures. Samples A, B, C and D were incubated in acetate–NaCl buffer, pH 5.2. Samples D* (MAb D control) and D** (MAb D cocktail) were incubated in histidine–sucrose buffer, pH 6.0.

cleavage site or rearrangement. Asp-containing proteins are susceptible to an intramolecular reaction by the nitrogen of the amino acid on the C-terminal side of the Asp residue onto the aspartyl chain. This reaction results in a cyclic imide intermediate, which has shown to be stable under certain conditions [11,12]. Though the formation of a succinimide at the Asp site would cause a loss of 18 Da, cleavage of the succinimide ring would restore the expected aspartic mass (i.e. not a loss of 18 Da). The lack of −18 Da forms for the other heavy chain Fab fragments suggests that this Asp residue does not readily form the succinimide. The loss of 18 Da from the C-terminal Asp forms, presumably by a loss of water to form the anhydride, appears to occur post-cleavage by an unknown mechanism.

The area of the fragments increased proportionally with temperature. When stored at 5 °C or lower, fragmentation was low or not observed in all samples by SEC. Profiles of MAb D at the different temperatures are shown in Fig. 4. Similar profiles at different temperatures were observed for the other MAbs (data not shown). A detailed view of the chromatogram section showing the fragments of the four MAbs incubated at 40 °C is shown in Fig. 5. MAb A has higher levels of aggregate than the other three MAbs at all time points (data not shown). Fig. 6 shows the total fragment area percent value for the four MAbs at the different incubation temperatures. The extents of cleavage at elevated temperatures were equivalent between MAbs.

A summary of the clip sites for all the MAbs is shown in Fig. 7. It is observed how the cleavage takes place within the same heavy chain hinge region Ser–Cys–Asp–Lys–Thr–His–Thr sequence for all the MAbs. Complementary fragments are generally observed for all sites except for

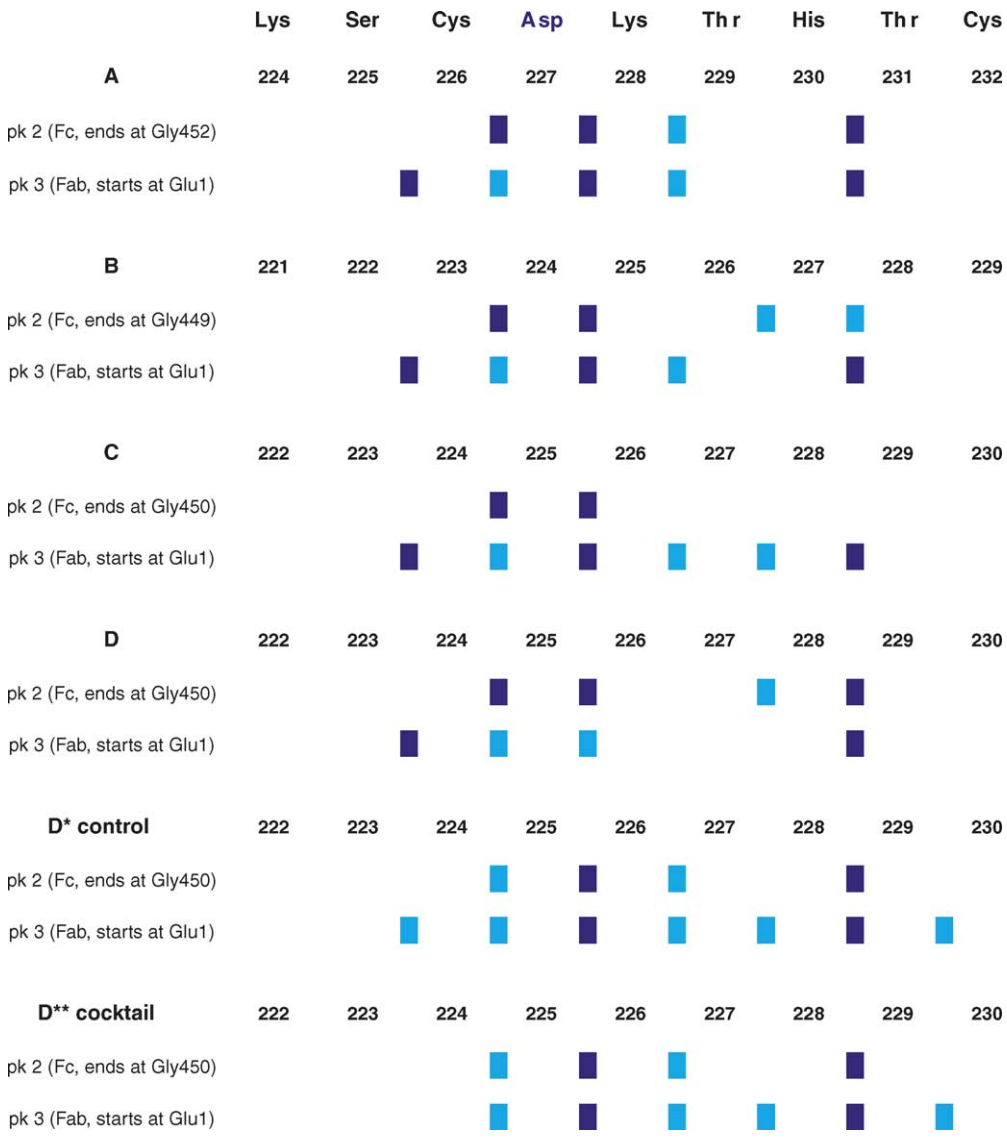


Fig. 7. Summary of clip sites for MAbs incubated for 1 month at 40 °C. Peak 2 is the shoulder peak (Fab + Fc) after the main SEC peak. Peak 3 is the Fab peak that elutes later in the SEC profile. Light blue indicates a minor form. LC-MS data shows a loss of 18 Da when the C-terminal residue is Asp. Samples A, B, C and D were incubated in acetate–NaCl buffer, pH 5.2. Samples D control and D cocktail were incubated in histidine–sucrose buffer, pH 6.0.

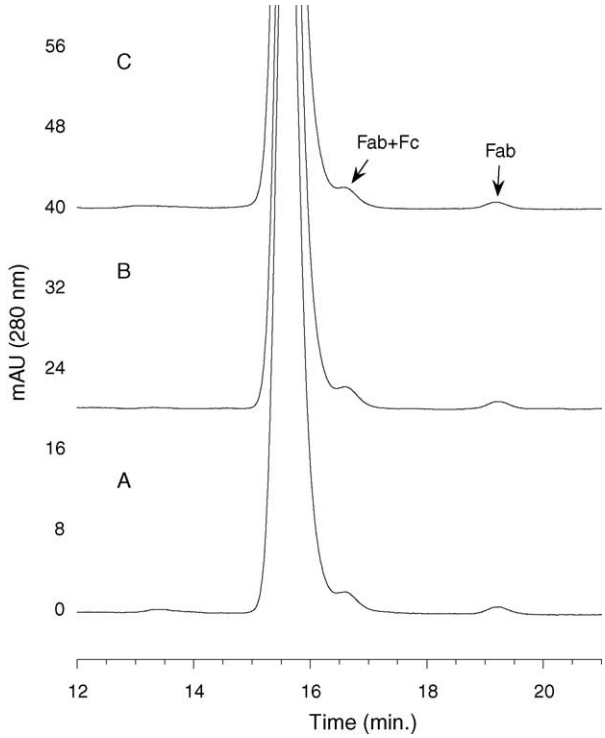


Fig. 8. MAb B process recovery pools after 1-month incubation at 40 °C. (A) Final pool (<1 ng CHOP/mg MAb B). (B) Intermediate pool (15 ng CHOP/mg MAb B). (C) Initial pool (160 ng CHOP/mg MAb B).

the Ser–Cys bond, where the Fab fragment with serine C-terminus is observed, but the corresponding Fc fragment with an N-terminal cysteine is not observed.

The use of protease inhibitors did not modify sites of fragmentation. The main clip sites are between Asp–Lys and His–Thr (the papain clip site) of the hinge region (Fig. 7). The extent of cleavage was not diminished (Fig. 6) and no cleavage site was eliminated in the presence of the inhibitors (Fig. 7). The extent of cleavage at 40 °C of the samples incubated in the pH 6.0 buffer is somewhat lower than the pH 5.2 buffer samples, but the addition of protease inhibitors had no effect on sample degradation when compared to its control sample (Fig. 6).

After 1-month incubation, the SEC profiles of samples from different recovery pools of MAb B look very similar (Fig. 8). No new peaks were observed in the profiles. The extents of cleavage between pools were equivalent, despite increasing levels of host cell proteins (0, 15, and 160 ng CHOP/mg MAb). An increased level of cleavage in the less pure pools would have suggested the presence of proteases, but we did not observe any such increase.

## 4. Discussion

Recombinant antibodies have a tendency to produce low levels of Fab and Fab + Fc fragments upon extended incubation in the liquid state. The fragments can be resolved by size-exclusion chromatography, allowing the extent and sites of cleavage to be assigned. We found in this study that four closely-related humanized IgG1 antibodies undergo fragmentation to the same extent and at similar sites, all in the hinge region of one heavy chain. This cleavage was not increased by the presence of host cell proteins, nor was the cleavage rate decreased by co-incubation with protease inhibitors. Cleavage only occurred at peptide bonds. Our initial assumption had been that residual CHO-derived proteases were present in these preparations, but the experiments described in this study indicate that non-enzymatic processes must be responsible.

The overall IgG1 structure contains highly compact, relatively inflexible Fab and Fc domains that are connected by an extended, protease-susceptible heavy chain hinge region, akin to dense modules that are connected by a weak link. Several studies have demonstrated that the hinge region is highly flexible [4]. This local flexibility may lower the activation energy needed for hydrolytic cleavage, allowing the cleavage of one heavy chain to produce the observed Fab and Fab + Fc forms. The predominant cleavage sites are adjacent to Asp and His residues, which may make the local environment more acidic or basic, accelerating hydrolysis at these sites. It should be feasible to control the rate of cleavage by manipulation of pH and/or excipients; we did not examine this aspect. Lowering the storage temperature can minimize hinge-region fragmentation. Understanding that the fragmentation of recombinant antibodies is not due to proteases should simplify the challenges of developing purification processes, while adding an important aspect that should be investigated during the development of stable liquid formulations.

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