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Journal of Chromatography B, 858 (2007) 254–262
JOURNAL OF CHROMATOGRAPHY B
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Structural effect of a recombinant monoclonal antibody on hinge region peptide bond hydrolysis

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Received 8 June 2007; accepted 30 August 2007
Available online 11 September 2007

Abstract

IgG hinge region peptide bonds are susceptible to degradation by hydrolysis. To study the effect of Fab and Fc on hinge region peptide bond hydrolysis, a recombinant humanized monoclonal IgG1 antibody, its $F(ab')_2$ fragment, and a model peptide with amino acid sequence corresponding to the hinge region were incubated at 40 °C in formulation buffer including complete protease inhibitor and EDTA for 0, 2, 4, 6 and 8 weeks. Two major cleavage sites were identified in the hinge region of the intact recombinant humanized monoclonal antibody and its $F(ab')_2$ fragment, but only one major cleavage site of the model peptide was identified. Hinge region peptide bond hydrolysis of the intact antibody and its $F(ab')_2$ fragment degraded at comparable rates, while the model peptide degraded much faster. It was concluded that Fab region of the IgG, but not Fc portion had significant effect on preventing peptide bond cleavage by direct hydrolysis. Hydrolysis of hinge region peptide bonds was accelerated under both acidic and basic conditions.

Keywords: Recombinant monoclonal antibody; Hydrolysis; Hinge region; Mass spectrometry

1. Introduction

Immunoglobulin Gs (IgGs) are composed of two light chains and two heavy chains. Each light chain has one variable (VL) and one constant (CL) domain and each heavy chain has one variable (VH) and three constant domains (CH1, CH2 and CH3). Each domain has one intrachain disulfide bond. Heavy chains and light chains are connected by interchain disulfide bonds. The region between CH1 and CH2 domains is the so-called hinge, which is the least structured and most susceptible region to enzymatic and non-enzymatic cleavage.

Many enzymes have been shown to cleave IgGs in or around the hinge region. The most common enzyme is papain, which cleaves IgGs in the hinge region to generate Fab and Fc fragments. In addition, cathepsin L, plasmin and lys-C [1–5] can also cleave peptide bonds in the hinge region. Pepsin is another commonly used enzyme, which cleaves IgG to generate $F(ab')_2$ fragment. The cleavage sites of pepsin are located in the CH2 domain of Fc, but close to the hinge region. Metalloproteinases [6] have also been shown to cleave IgGs in CH2 domains, and the cleavage sites are closer to the hinge region than the pepsin cleavage sites.

In addition to enzymatic cleavage, hinge region peptide bonds can also be cleaved non-enzymatically by direct hydrolysis. Jiskoot et al. [7] reported that peptide bond cleavage was observed in a mouse monoclonal IgG1 antibody after storage at 37 °C for 32 days at the pH range of 7.4–10.0. Similar hinge region peptide bond cleavage was observed in a mouse recombinant monoclonal antibody after storage at 5 °C for a year and half [8], a mouse–human chimeric monoclonal antibody stored at 60 °C at pH above 5.0 [9], a mouse–human chimeric monoclonal antibody stored at 60 °C [10], a human monoclonal antibody after incubation at acidic and basic pH in the presence or absence of hydrogen peroxide [11], a fully human recombinant monoclonal antibody after storage at 40 °C for 6 months [12], and a recombinant monoclonal IgG1 antibody after storage at 29 °C for 4 months [13,14]. In the study of hinge region peptide bond cleavage of four humanized recombinant monoclonal antibodies incubated at different temperatures, Cordoba et al. [15] observed that including protease inhibitors and EDTA in

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doi:10.1016/j.jchromb.2007.08.043

the formulation buffers did not have any effect on hinge region peptide bond cleavage, which supports the idea of peptide bond cleavage by direct hydrolysis.

In this report, cleavage of hinge region peptide bonds was studied using a recombinant humanized monoclonal IgG1antibody, its $F(ab')_2$ fragment, and a model peptide with amino acid sequence corresponding to the hinge region. Size-exclusion chromatography (SEC) was used to monitor degradation of the intact antibody and the $F(ab')_2$ fragment. Liquid chromatography mass spectrometry (LC–MS) was used to monitor degradation of the model peptide. Cleavage sites of the intact antibody, its $F(ab')_2$ fragment and the model peptide were identified by LC–MS. Effect of pH on the rate of direct hydrolysis of intact recombinant antibody was also studied.

2. Experimental

The recombinant monoclonal IgG1 antibody was produced by a transfected Chinese hamster ovary (CHO) cell line and purified by several chromatography steps including cation exchange, anion exchange and hydrophobic interaction chromatography at Abbott Bioresearch Center (Worcester, MA). The antibody was formulated in liquid formulation buffer, which includes 5.57 mM sodium phosphate monobasic, 8.69 mM sodium phosphate dibasic, 106.69 mM sodium chloride, 1.07 mM sodium citrate, 6.45 mM citric acid, 66.68 mM mannitol and 0.1% Tween at pH 5.2.

To prepare the $F(ab')_2$ fragment, the recombinant monoclonal antibody was buffer-exchanged to 20 mM sodium acetate, pH 4.5 and then concentrated to 20 mg/mL using Amicon Ultra-15 centrifugal device (Millipore, Billerica, MA) with a molecular weight cut-off of 10 kDa. One milliliter of the antibody solution (20 mg) was mixed 1 mL immobilized pepsin beads (Pierce, Rockford, IL), which had been washed with 20 mM sodium acetate, pH 4.5. The sample was incubated overnight at 37 °C with vigorous shaking. The $F(ab')_2$ fragment was purified using an FPLC system and a Superdex 200 size exclusion column (26 mm × 600 mm) (GE Healthcare, Piscataway, NJ). The column was washed and equilibrated with a mobile phase of 20 mM Tris, 100 mM sodium chloride, 5% glycerol, pH 7.5. After loading the pepsin-digested sample, the column was eluted with the mobile phase at a flow rate of 1 mL/min. Elution was monitored by UV at 214 and 280 nm. The peak with molecular weight corresponding to the predicted $F(ab')_2$ fragment molecular weight was collected. The $F(ab')_2$ fragment was then buffer exchanged into formulation buffer using an Amicon Ultra-15 centrifugal device with a molecular weight cut-off of 10 kDa.

The recombinant monoclonal antibody, its $F(ab')_2$ fragment and a model peptide (New England Peptides Inc., Gardner, MA) with an amino acid sequence corresponding to the hinge region sequence (AADKTHTAA) were diluted to 5 mg/mL using formulation buffer. Complete protease inhibitor (Roche, Indianapolis, IN) and 1 mM EDTA (Sigma, St. Louis, MO) were included in all the sample preparations. Samples were sterilized by filtering through 0.2 μm syringe filter (Gelman Sciences, Ann Arbor, MI) and then incubated at 40 °C. Aliquots were taken at 2, 4, 6, and 8 weeks. Aliquots of each sample were taken before incubation and stored at −80 °C as the zero time point.

In order to determine the pH effect on hinge region peptide bond cleavage, the recombinant monoclonal antibody was diluted to 5 mg/mL using 50 mM citrate (pH 4, 5 and 6), HEPES (pH 7 and 8) and glycine (pH 9 and10) buffers. Complete protease inhibitor and 1 mM EDTA were included in the sample preparations. Samples were incubated at 40 °C for 2 weeks after sterile filtration.

Degradation of intact antibody and $F(ab')_2$ fragment were monitored by SEC using a Shimadzu HPLC and a Superdex 200 column (GE healthcare, 10 mm × 300 mm). Each sample (100 μg) was injected and eluted with a mobile phase of 20 mM sodium phosphate, 150 mM sodium chloride, pH 7.5, at a flow rate of 0.3 mL/min. Elution was monitored by UV 214 and 280 nm. SEC fractions of fragment peaks were collected from intact antibody and $F(ab')_2$ fragment after an 8-week incubation. The collected materials were concentrated using Amicon Ultra-4 centrifugal filter devices with a 5 kDa MW cut-off (Millipore). To facilitate identification, PNGaseF (Prozyme, San leandro, CA) was used to remove the oligosaccharides of peak 1 material from Fig. 3. PNGaseF (1 μL) and *N*-octylglucoside (Roche) were added to the concentrated peak 1 material and incubated at 37 °C over night. The sample was then reduced with 10 mM dithiothreitol (DTT) at 37 °C for 30 min before analysis.

An Agilent HPLC (Agilent, Santa Clara, CA) and a Q star pulsar i LC–MS/MS mass spectrometer (Applied Biosystems, Framingham, MA) were used for molecular weight measurement. For intact recombinant antibody, a protein microtrap (Michrom Bioresources Inc., Auburn, CA) was used to desalt and introduce samples into the mass spectrometer. The sample (5 μg) was loaded at 95% mobile phase A (0.08% formic acid (Sigma) in Milli-Q water) and 5% mobile phase B (0.08% formic acid in acetonitrile (EMD, Gibbstown, NJ)). After eluting for 5 min at 5% mobile phase B, the sample was eluted off the column by increasing mobile phase to 95% B in 0.5 min and running at 95% B for 4.5 min. Mobile phase B was then decreased to 5% in 0.1 min, and the column was equilibrated at 5% mobile B for 4.9 min. The flow rate was set at 50 μL/min. The mass spectrometer was operated in positive mode with a scan range of *m*/*z* from 2000 to 3500. IonSpray voltage was set at 5000 V, and the source temperature was set at 350 °C. For analysis of $F(ab')_2$ and SEC fractions, a protein C4 column (Vydac, 150 mm × 1 mm i.d., 5 μm particle size, 300 Å pore size) was used. Samples were loaded at 5% mobile phase B. After running for 5 min, samples were eluted by increasing mobile phase B to 65% within 35 min. The column was washed by increasing mobile phase B to 95% in 5 min, and equilibrated by decreasing mobile phase B to 5% in 5 min and running at 5% mobile phase B for 10 min before the next injection. The flow rate was set at 50 μL/min. The mass spectrometer scan range was set at a range of *m*/*z* 800–2500. IonSpray voltage was set at 4500 V. The source temperature was set at 350 °C.

For analysis of the model peptide, the Agilent HPLC and Q star mass spectrometer described above were used. A C18 column (Vydac, 150 mm × 1 mm i.d., 5 μm particle size, 300 Å pore size) was used to separate and introduce samples into the

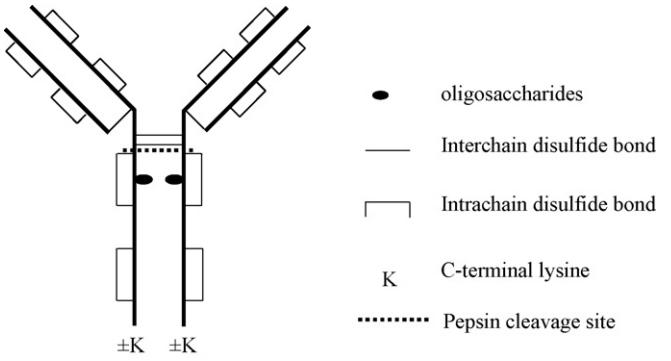

Fig. 1. A typical human IgG1 structure. The major features including disulfide bonds, N-linked oligosaccharides, incomplete processing of C-terminal lysine residues and pepsin cleavage sites are shown in the diagram.

mass spectrometer. Samples were loaded at 2% mobile phase B and 98% mobile phase A at a flow rate of 50 μL/min. Peptides were eluted off the column using an isocratic elution of 2% mobile phase B and 98% mobile phase A for 10 min. The mass spectrometer IonSpray voltage was set at 4200 V. The source temperature was set at 75 °C. The scan range was from *m/z* 250 to 2500.

3. Results

A typical IgG1 structure is shown in Fig. 1. The molecule has two identical light chains and two identical heavy chains. Each light chain has two intrachain disulfide bonds and each heavy chain has four intrachain disulfide bonds. Each light chain is connected to each heavy chain by one interchain disulfide bond, while each heavy chain is connected to each heavy chain by two interchain disulfide bonds. In addition, *N*-glycosylation and C-terminal processing are the two most common post-translational modifications. Oligosaccharides of complex biantennary structures with core fucose and with zero (Gal 0), one (Gal 1) or two (Gal 2) terminal galactose are the major oligosaccharide structures. Incomplete C-terminal lysine processing results in antibody with zero (Lys 0), one (Lys 1) or two (Lys 2) C-terminal lysine residues.

SEC chromatograms of the recombinant monoclonal antibody and its $F(ab')_2$ fragment are shown in Fig. 2A. As expected, the $F(ab')_2$ fragment is smaller and has a longer retention time. Deconvoluted mass spectrum of the $F(ab')_2$ fragment is shown in Fig. 2B. Based on the molecular weights, pepsin cleavage sites and the four major peaks (peaks 1–4) were identified and are summarized in Table 1 and Fig. 2B. There are two possibilities for the peak with molecular weight of 97,374 Da. Two light chains linked through disulfide bonds to either two heavy chains of amino acids 1–238 or one heavy chain with amino acids 1–237 and the other with amino acids 1–239 both result in this molecular weight. Analysis by mass spectrometry after reduction revealed that heavy chain with amino acids 1–238 was the only species contributing to the peak (data not shown). Peak 97,374 was thus assigned as two light chains disulfide bonded to two heavy chains with amino acids 1–238. Several minor peaks in Fig. 2B could be due to pepsin cleavage on other sites

Table 1
Pepsin cleavage sites and identities of peaks as shown in Fig. 2B

	MW(Da)		Identities	Pepsin Cleavage sites
	Observed	Calculated		
1	97262	97263	2L+1HC1-237+1HC1-238	APELLGG
2	97374	97376	2L+2HC1-238	APELLGG
3	97487	97489	2L+1HC1-238+1HC1-239	APELLGG
4	97603	97602	2L+2HC1-239	APELLGG

Arrows indicate the sites of cleavage.

or non-covalent adducts. Deconvoluted mass spectrum of the intact recombinant monoclonal antibody is shown in Fig. 2C. The major peak with a molecular weight of 148,090 Da is in good agreement with the calculated molecular weight (148,081 Da) of intact antibody with oligosaccharide Gal 0 on both heavy chains and without C-terminal lysine residues. Other peaks are formed due to either the addition of galactose on the sugar moiety, the presence of C-terminal lysine(s) or both. The identities of these peaks cannot be assigned without ambiguity at the intact molecular weight level.

SEC chromatograms of the intact antibody after 0, 2, 4, 6, and 8 weeks of incubation at 40 °C are shown in Fig. 3. In addition to the main peak, peaks with shorter and longer retention times were observed. Peaks with shorter retention are higher molecule weight aggregates. Peaks with longer retention times are lower molecular weight fragments. The fragment peaks, labeled peak 1 and 2, were collected from the sample after the 8-week incubation and analyzed by mass spectrometry, shown in Fig. 4. Peak 1 was analyzed after removal of oligosaccharides and DTT reduction for higher sensitivity. In addition to intact light chain and intact heavy chain (not shown in the spectrum), two peaks with molecular weights of 24,937 Da (peak a) and 25,303 Da (peak b) were observed (Fig. 4A). These peaks correspond to heavy chain amino acids 229–450 and 226–450, respectively. Based on the information from mass spectrometry analysis and the elution position of peak 1 on SEC chromatogram, peak 1 was assigned as intact antibody lacking one Fab fragment. The deconvoluted mass spectrum of peak 2 is shown in Fig. 4B. The major peaks (c–f) observed correspond to the Fab region from different hinge region cleavage sites. The corresponding Fc fragments were not observed, which may be due to a lower amount, a lower ionization efficiency, or complete degradation. The cleavage sites and identities of peaks in Fig. 4A and B are summarized in Table 2. It is clear from Table 2, the two major cleavage sites are between amino acids D and K, and H and T in the hinge region sequence DKTHT. The minor cleavage site is between amino acids K and T. The two peaks in between peaks (e) and (f) cannot be assigned.

SEC chromatograms of the $F(ab')_2$ fragment are shown in Fig. 5. Similar to intact antibody, aggregates and fragments were observed in samples after incubation. Fragment peaks from sample after the 8-week incubation were collected and analyzed by mass spectrometry. As shown in Fig. 6, three groups of peaks were observed. The first group of peaks (a–d) was formed due to peptide bond cleavage in the hinge region of both heavy chains.

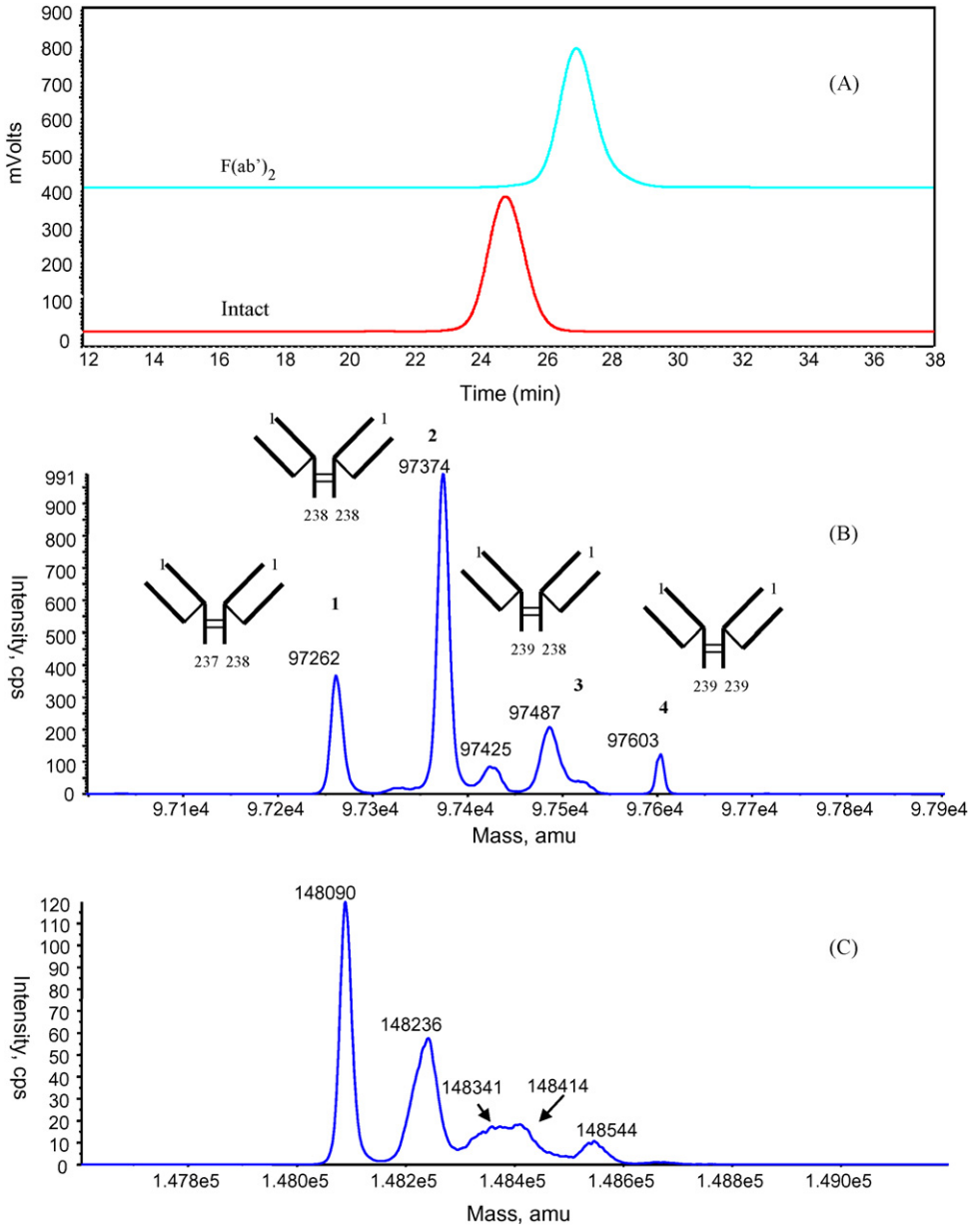

Fig. 2. Analysis of the recombinant monoclonal IgG1 and its F(ab′)$_2$. (A) SEC chromatograms of the intact antibody and F(ab′)$_2$. (B) Deconvoluted mass spectrum of F(ab′)$_2$. Identities of peaks 1–4 are summarized in Table 1 and also shown as insets. Numbers in the inset diagrams indicate the N-terminal and C-terminal amino acids. (C) Deconvoluted mass spectrum of the intact antibody.

Table 2
Cleavage sites and identities of peaks in Fig. 4A and B

Peaks		MW (Da)		Identities	Cleavage sites
SEC	MS spectra	Observed	Calculated		
Peak 1 reduced	a	24937	24935	HC229-450	CDKTHTC
	b	25303	25302	HC226-450	CDKTHTC
Peak 2	c	47297	47297(-18Da)	1L+HC1-225 (-18Da)	CDKTHTC
	d	47315	47315	1L+HC1-225	CDKTHTC
	e	47443	47443	1L+HC1-226	CDKTHTC
	f	47682	47681	1L+HC1-228	CDKTHTC

Arrows indicate the sites of cleavage.

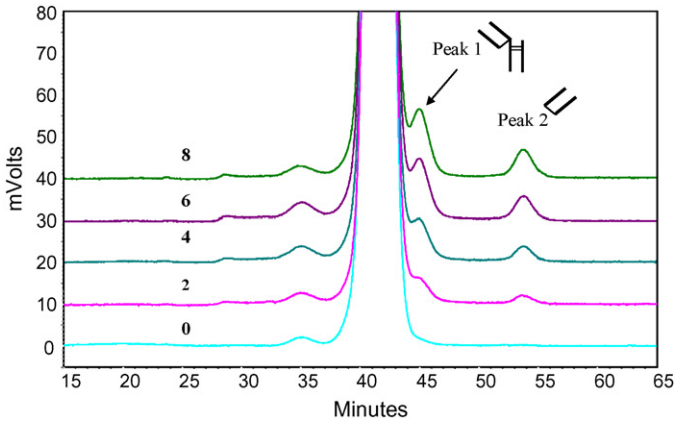

Fig. 3. SEC chromatograms of the intact monoclonal IgG1 after incubation at 40 °C for 0, 2, 4, 6 and 8 weeks as indicated in the figure. Peaks 1 and 2 were collected and further analyzed. The identities of peak 1 and 2 are indicated as insets of this figure.

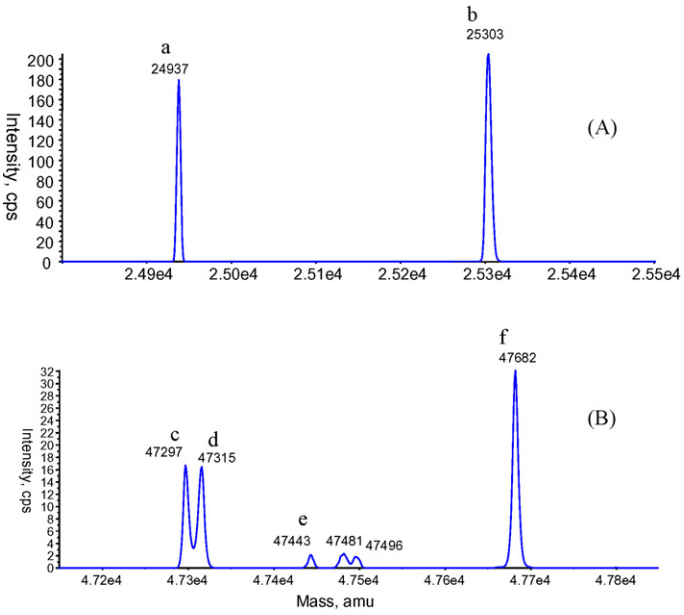

Fig. 4. Mass spectra of peak 1 and peak 2 as shown in Fig. 3. (A) Mass spectrum of peak 1 after removal of oligosaccharides and DTT reduction. (B) Mass spectrum of peak 2. Peaks labeled as (a)–(f) were identified and summarized in Table 2.

The second group of peaks (e–g) was due to reduction of the two inter-heavy-chain disulfide bonds. The third group of peaks (h–k) was due to the cleavage of peptide bonds in the hinge region of one heavy chain. The minor peaks were probably due to cleavage sites of other regions or non-covalent adducts. The cleavage sites and identities of these peaks are summarized in Table 3 and in Fig. 6. Similar to intact antibody, the two major cleavage sites are between amino acids D and K, and H and T of the hinge region amino acids. The minor cleavage site is between amino acids K and T.

Mass spectra of the model peptide without incubation (0 week) and after 8 weeks of incubation at 40 °C are shown in Fig. 7A and B respectively. The model peptide, AADKTHTAA, contains the hinge region amino acid sequence DKTHT. The two alanine residues on both ends is to increase the hydrophobicity of this short peptide and facilitate analysis by reverse-phase chromatography and mass spectrometry. One major peak with a molecular weight (MH+) of 885.4 Da, the calculated molecular weight of the model peptide, was observed in the time zero sample. The peak with a molecular weight of 907.4 Da is probably impurity from the synthesis and it is also present in the sample after 8-week incubation. As shown in Fig. 7B, in addition to the peak with molecular weight corresponding to the intact peptide (885.4 Da), a peak with molecular weight of 628.3 Da was observed. This peak is due to peptide bond cleavage between residues D and K. The peak with molecular weight of 743.4 Da corresponds to a loss of two alanine residues. A lower amount of the 743.4 Da peak was also present in the time zero sample. Therefore, peak 743.4 Da in sample after 8-week incubation is due to degradation of the original sample as well as thermal degradation. The only cleavage site relevant to hinge region

Table 3
Cleavage sites and identities of peaks in Fig. 5

	MW (Da)		Identities	Cleavage sites	
	Observed	Calculated		Hinge	Pepsin cleavage
a	47297	47297(-18Da)	1L+HC1-225 (-18Da)	CDKTHTC	NA
b	47315	47315	1L+HC1-225	CDKTHTC	NA
c	47443	47443	1L+HC1-226	CDKTHTC	NA
d	47682	47681	1L+HC1-228	CDKTHTC	NA
e	48577	48577	1L+HC1-237	NA	APELLGG
f	48689	48690	1L+HC1-238	NA	APELLGG
g	48803	48802	1L+HC1-239	NA	APELLGG
h	49714	49714	1L+HC1-238+HC229-238	CDKTHTC	APELLGG
i	49966	49966	1L+HC1-238+HC229-237	CDKTHTC	APELLGG
J	50080	50080	1L+HC1-238+HC226-238	CDKTHTC	APELLGG
k	50193	50193	1L+HC1-238+HC226-239	CDKTHTC	APELLGG

Arrows indicate the sites of cleavage.

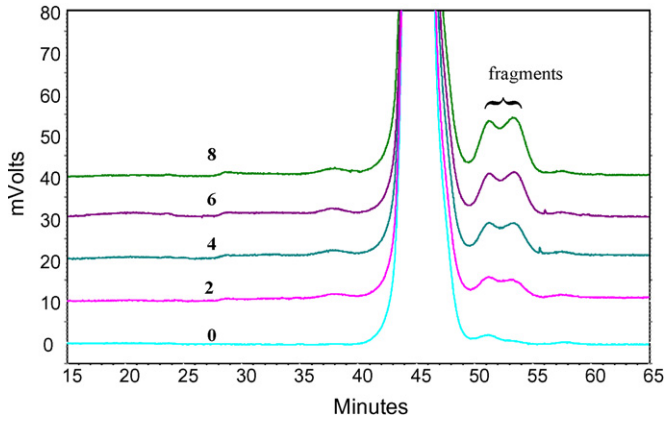

Fig. 5. SEC chromatograms of F(ab′)$_2$ after incubation at 40 °C for 0, 2, 4, 6 and 8 weeks as indicated in the figure. Peaks labeled as fragments were collected and further analyzed.

peptide bond cleavage was between amino acids D and K. The cleavage site between amino acids H and T, which was observed in the degradation of intact antibody and the F(ab′)$_2$ fragment, was not detected in the degradation of this synthesized peptide.

Rates of degradation of the intact recombinant monoclonal antibody, the F(ab′)$_2$ fragment and the model peptide are shown in Fig. 8. The relative percentage of degradants of the intact recombinant monoclonal antibody (peaks 1 and 2 in Fig. 3) and the F(ab′)$_2$ fragment (peaks labeled in Fig. 5) were calculated by integrating the peak areas of aggregate, monomer and degradants. Degradation of the model peptide was calculated by dividing peak intensity of 628.3 Da by the sum of the intensities of peak 628.3 and 885.4 Da. The formation of degradants of the intact antibody and its F(ab′)$_2$ fragment followed almost parallel lines (Fig. 8). Based on Fig. 6, approximately 30% of the F(ab′)$_2$ degradants were formed due to breakage of the inter-heavy-chain disulfide bonds. An analysis omitting the reduction of the disulfide bonds did not change the parallel degradation of the intact antibody and its F(ab′)$_2$ fragment. Therefore, it is concluded that Fc portion of the antibody did not affect hinge region peptide bond cleavage.

The model peptide degraded much faster even though only one cleavage site was present. One may argue that degradation of this peptide only required cleavage of one peptide bond, but as a dimer of this peptide, degradation of the hinge region of both the intact antibody and the F(ab′)$_2$ fragment required cleavage of two peptide bonds. While, even dividing the degradation rate of this peptide by two to reflect the fact that hinge is a dimer of the model peptide, the synthesized peptide still had a much higher degradation rate. In summary, intact antibody and its F(ab′)$_2$ showed a parallel degradation kinetics, while the synthesized peptide had a much faster degradation rate. Therefore, Fab, but not Fc, of this antibody provides significant protection against cleavage in the hinge region.

As mentioned in the experimental section, complete protease inhibitor and EDTA were included in all the sample preparations. Cleavage of the peptide bonds in the hinge region cannot be due to contaminating proteases or metals. Therefore, cleavage was most likely due to hydrolysis, and both acidic and basic conditions should accelerate degradation. To test this hypothesis, intact antibody was incubated in buffers of different pH with complete protease inhibitor and EDTA. The extent of degradation was determined by SEC analysis. As shown in Fig. 9A and B, more degradants were observed in samples incubated in buffers at lower or higher pH than at neutral pH. Hinge region peptide bonds were most stable at pH 6.0. This

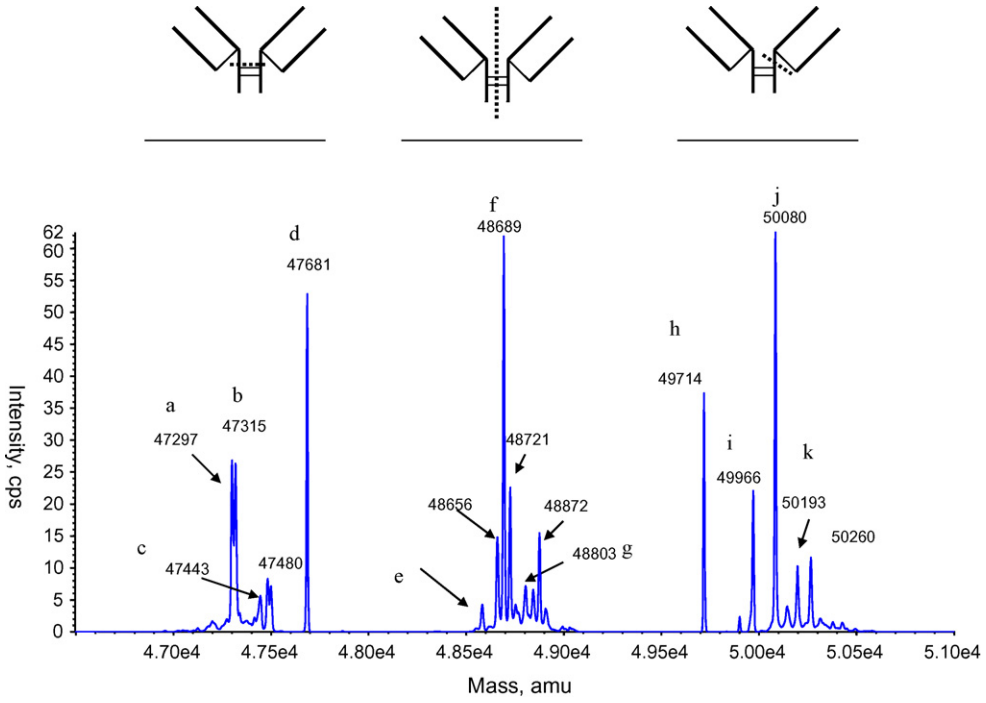

Fig. 6. Mass spectrum of peaks collected as indicated in Fig. 5. The identities of peaks (a)–(k) were summarized in Table 3.

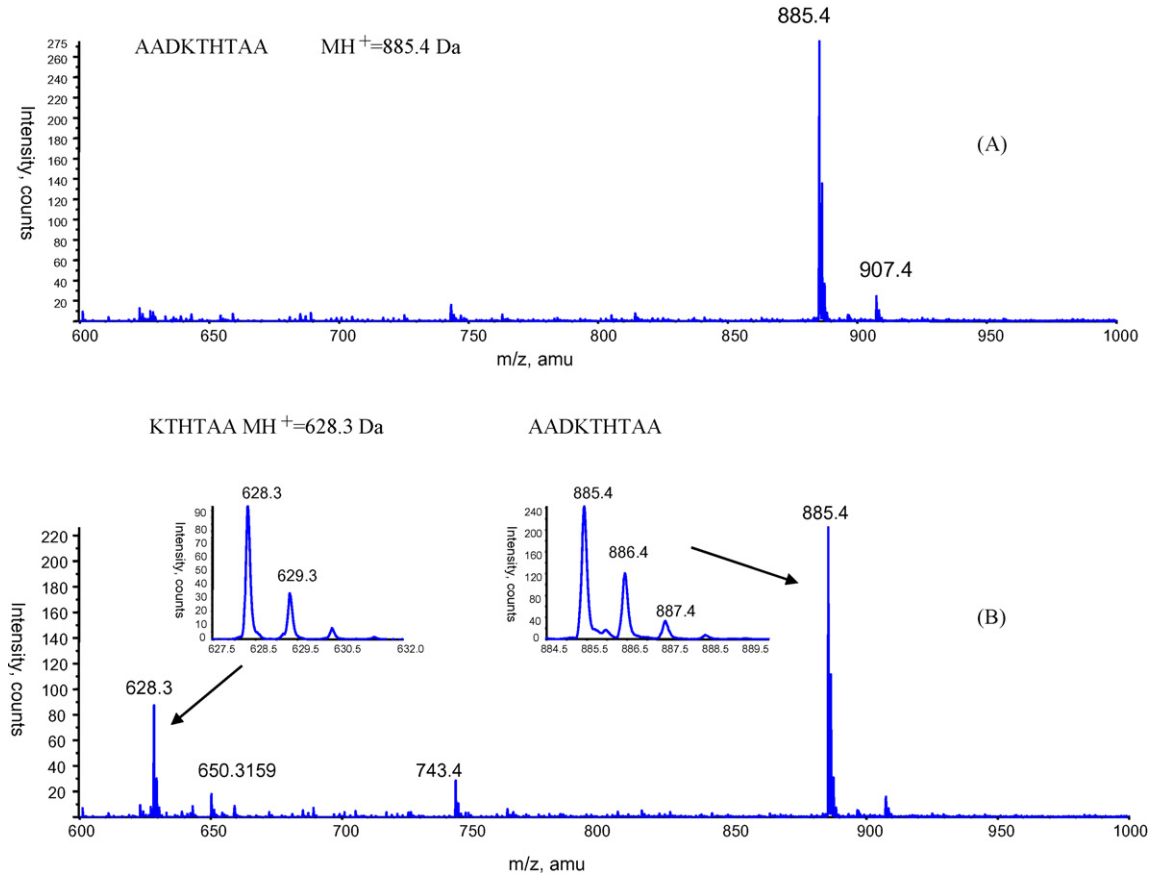

Fig. 7. Mass spectra of the synthesized peptide: (A) without incubation (0 week incubation); (B) after 8 week incubation. The isotopic distributions of peaks 885.4 and 628.3 Da are shown as insets. Also shown as inset is the peptide sequence and the cleavage site.

data supports the hypothesis of acidic and basic catalyzed fragmentation mechanism.

4. Discussion

Hinge region peptide bond cleavage is one of the most common recombinant monoclonal antibody degradation pathways. Several mechanisms may account for the cleavage of hinge region peptide bond including enzymatic, metal catalyzed, and direct hydrolysis.

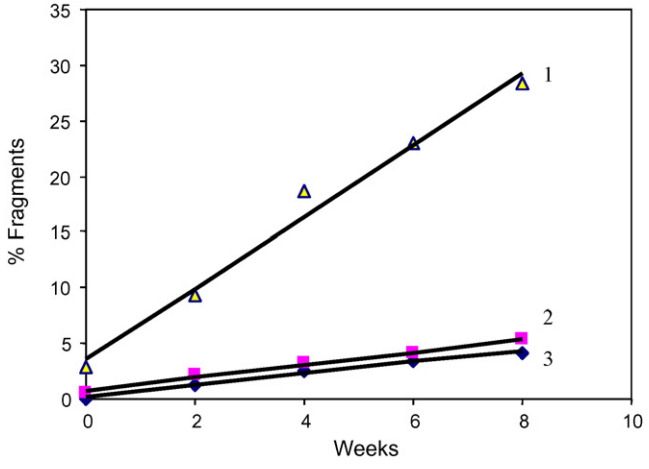

Fig. 8. Degradation of the model peptide (1, triangle), the F(ab′)$_2$ fragments (2, square) and the intact antibody (3, diamond) incubation at 40 °C.

For highly purified recombinant monoclonal antibodies, direct hydrolysis may be the major source of peptide bond cleavage in the hinge region. As it has been reported by Cordoba et al. [15], EDTA and protease inhibitor did not prevent hinge region peptide bond cleavage when recombinant monoclonal antibodies were incubated in liquid formulation buffers at elevated temperature. In agreement with Cordoba et al [15], peptide bond cleavage of an intact recombinant monoclonal antibody, its F(ab′)$_2$, and a model peptide with amino acids corresponding to the hinge region was observed in the presence of complete protease inhibitor and EDTA. The hypothesis of a direct hydrolysis mechanism of the peptide bonds was also supported by the observation that both acidic and basic conditions accelerated hinge region peptide bond cleavage.

The major cleavage sites of the intact monoclonal IgG1 and its F(ab′)$_2$ fragment are between amino acids D and K, and H and T in the hinge region amino acid sequence, DKTHT. The same cleavage sites of recombinant monoclonal antibodies have been reported previously [12,14,15]. Interestingly, only one cleavage site between amino acids D and K was observed in the degradation of the model peptide. However, this observation was not a surprise as peptide bonds involving amino acid D, especially when it is followed by a proline residue, are known to be susceptible to hydrolysis [16,17]. Lacking of cleavage between amino

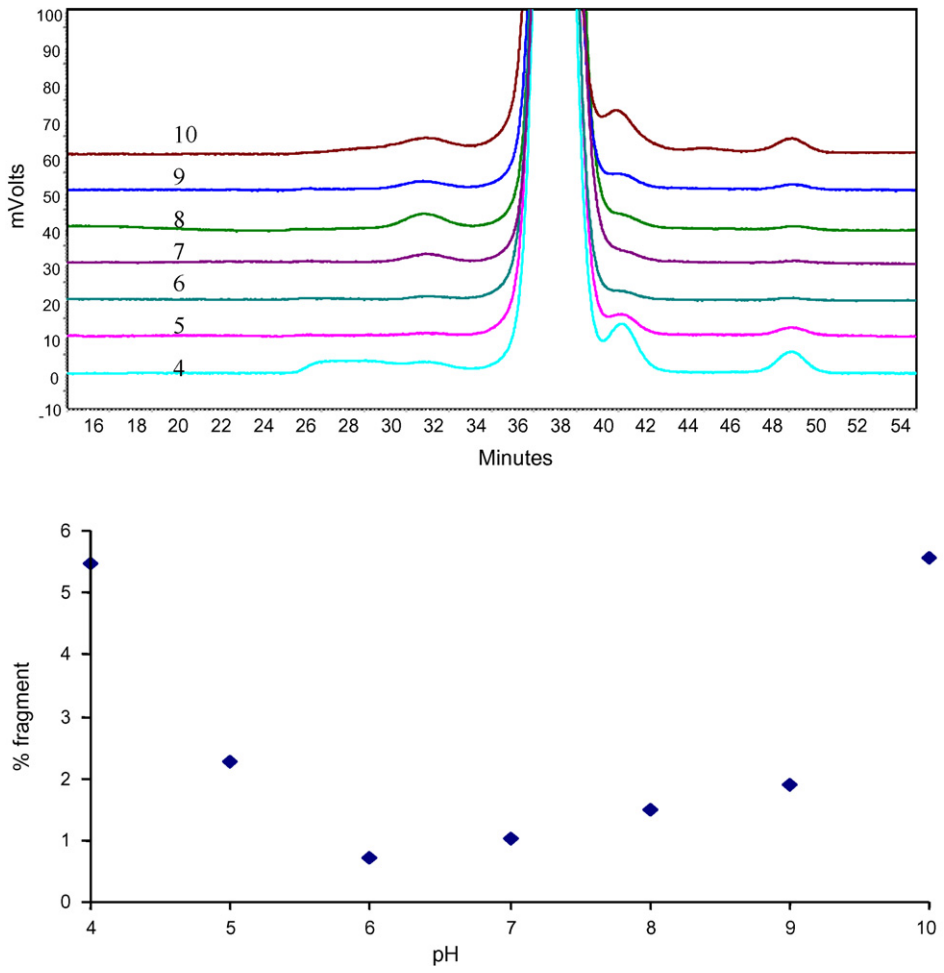

Fig. 9. pH effect on antibody fragmentation. (A) SEC chromatograms of the intact antibody after incubation at 40 °C for 2 weeks in buffers of different pHs as indicated in the chromatograms. (B) Relative % of fragments of the antibody after incubation at 40 °C for 2 weeks in buffers of different pHs as indicated in the chromatograms.

acids H and T in the model peptide suggested that the local structure of the hinge or other regions of the intact antibody and its $F(ab')_2$ fragment play a significant role in defining the susceptibility of the HT peptide bond to hydrolysis. A significant amount of degradation of $F(ab')_2$ fragment, but not the intact antibody, also occurred through the breakage of the inter-heavy-chain disulfide bonds in the hinge region. This observation indicated that Fc may play a role in protecting inter-heavy-chain disulfide bonds in the hinge region. However, the possibility that breakage of the inter-heavy-chain disulfide bonds of intact antibody also occurred but the molecule was still held together by strong non-covalent interactions between the two CH3 domains cannot be excluded. Fragments corresponding to peptide bond cleavage between D and K but with a molecular weight of approximately 18 Da lower were observed as both intact antibody and $F(ab')_2$ fragment thermal degradation products. Similar fragments have been reported previously [12,15]. The 18 Da difference may be due to the presence of succinimide intermediate as cleavage on the C-terminal side of aspartate residues [18–20].

Fragmentation rates of the intact antibody, its $F(ab')_2$ fragment, and the model peptide with the hinge region sequence were compared. Degradation of the intact antibody and $F(ab')_2$ fragment due to peptide bond cleavage in the hinge region progressed with parallel kinetics, which suggests that Fc portion of the IgG1 molecule does not provide much protection against hydrolysis of the hinge region. Degradation of the model peptide, on the other hand, has a much faster rate. Therefore, it is concluded that Fab region, but not Fc region, provides most of the protection from hydrolysis of the hinge region peptide bonds. It has been observed in the crystal structure of a human IgG1 antibody that one Fab is packed on top of Fc, with C_L domain in contact with one CH2 domain [21]. This kind of domain disposition should provide protection to the hinge region.

In summary, peptide bonds between hinge region amino acids D and K, and H and T of the hinge region amino acid sequence DKTHT were susceptible to cleavage by neutral and acid- or base-catalyzed hydrolysis. The rate of hydrolysis was accelerated by an acidic or basic pH. The similar kinetics of hydrolysis of the intact antibody and its $F(ab')_2$ fragments indicated that Fab, but not Fc, provided significant protection of the hinge region peptide bonds.

Acknowledgements

The authors would like to thank Czeslaw H. Radziejewski, Gary J. Welch and Peter Moesta for their support.

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