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Assessment of antibody fragmentation by reversed-phase liquid chromatography and mass spectrometry

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ABSTRACT

Antibody fragmentation in the hinge region and other regions, and the impact of pH on the level and pattern of antibody fragmentation were investigated by reversed-phase (RP) liquid chromatography and mass spectrometry (LC–MS). Extensive fragmentation was observed in the hinge and in regions other than the hinge of a recombinant monoclonal antibody that was incubated in buffers of various pH at 40 °C for 10 weeks. Peptide bonds that were susceptible to hydrolysis were located mainly around the domain–domain interfaces close to or in the loop structures. The sites as well as the level of peptide bond hydrolysis were affected by the buffer pH. In agreement with previous findings when only the hinge region fragmentation was monitored, pH 6 was optimal for slowing down antibody fragmentation in regions other than the hinge. It also demonstrated that analysis by RPLC–MS provided a better assessment of the susceptible regions of recombinant monoclonal antibodies than size-exclusion chromatography (SEC) followed by fraction collection and mass spectrometry identification.

1. Introduction

Antibody fragmentation is one of the major degradation pathways of protein therapeutics including recombinant monoclonal antibodies in liquid formulation [1,2]. As a common practice, antibodies are routinely incubated at stressed conditions such as high temperature to generate fragments within a short period of time. Although it may be questionable to predict long-term stability at low temperature by using information obtained from high temperature, information on the regions that are susceptible to fragmentation could provide invaluable information for the selection of appropriate pH in formulations.

It is well documented that peptide bonds in the hinge region of antibodies are susceptible to hydrolysis, generating fragments corresponding to the Fab region, Fc region, and antibody lacking one Fab arm (one-armed antibody) [3–6]. The fragments are readily separated by size-exclusion chromatography (SEC) and identified by mass spectrometry. Incubation of a chimeric monoclonal antibody at pH 8.5 at 60 °C led to a progressive loss of the intact antibody over time, partially due to fragmentation in the hinge region [7]. Analysis of fragments of four different humanized IgG1 monoclonal antibodies after incubation at 40 °C for 1 month at pH 5.2 suggested that peptide bonds in the upper hinge region were susceptible to hydrolysis [4]. Such fragmentation in the hinge region was accelerated by acidic and basic conditions [5], which confirmed an acid or base catalyzed mechanism leading to hinge region peptide bond cleavage. Other modes of fragmentation that occurred in close proximity to the hinge region have also been identified. Cohen et al. [8] reported that β-elimination of the disulfide bond between the light chain and heavy chain can lead to the formation of dehydroalanine, which can be hydrolyzed resulting in peptide bond cleavage. Recently, it has been reported that the pattern of antibody fragmentation in the hinge region was dependent on the pH, possibly by modulating antibody structure around the hinge region [9].

Hydrolysis can also occur in regions other than the hinge. For example, multiple fragments from hydrolysis of peptide bonds in the CH2 domain were detected in an accelerated stability sample of a recombinant fully human monoclonal antibody after incubation at 40 °C for 6 months [10]. A fragment corresponding to the heavy chain variable domain (V_H) region was also observed in an IgG1 monoclonal antibody after storage at 29 °C for 4 months [6]. It has been demonstrated that at pH 4 or lower, one peptide bond in the CH2 domain was susceptible to hydrolysis and the presence of the conserved N-linked oligosaccharides slowed down the rate of hydrolysis significantly [9]. Overall, however, compared to the wealth of information on peptide bond hydrolysis in the hinge region, the information on peptide bond hydrolysis in other regions is sparse.

In the current study, fragmentation of a recombinant fully human monoclonal antibody was investigated after incubation

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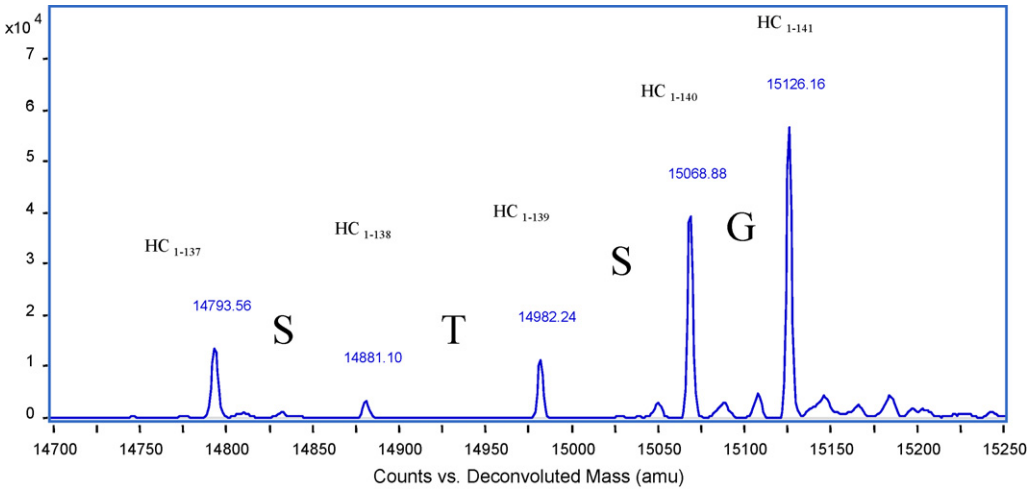

Fig. 1. Deconvoluted mass spectra of antibody fragments from the sample incubated at pH 9. Multiple peaks corresponding to hydrolysis of peptide bonds between amino acids 137–142 were observed. S, T and G are amino acids serine, threonine and glycine.

at 40 °C for 10 weeks at pH of 5, 6, 7, 8 and 9. Antibody fragmentation in the hinge region has been commonly monitored by size-exclusion chromatography [4,5,8,9]. However, SEC cannot differentiate fragments from peptide bond hydrolysis in other regions from intact antibody. Such fragments are most likely held together by non-covalent interactions within domains and between domains of antibodies therefore making them indistinguishable from intact antibodies by SEC. On the other hand, the organic solvents employed for reversed-phase chromatography can efficiently disrupt these non-covalent interactions and various fragments can thus be separated based on their hydrophobicities. It has been demonstrated that reversed-phase chromatography followed by mass spectrometry (MS) analysis, can identify fragmentation by-products of antibodies in regions other than the hinge [10]. Therefore, RP-HPLC was employed in the current study, which was followed by mass spectrometry analysis. Analysis of these fragments by RP-HPLC and MS provided a better understanding of antibody fragmentation due to peptide bond hydrolysis in both the hinge and in domain–domain interfaces close to or in the loop structure.

2. Experimental

2.1. Materials

A recombinant, fully human monoclonal IgG1 antibody was produced by a transfected Chinese hamster ovary (CHO) cell line and purified by multiple chromatography steps (Abbott Bioresearch Center, Worcester, MA). Dithiothreitol (DTT) was purchased from Sigma (St. Louis, MO). Formic acid (FA) was purchased from EMD (Madison, WI). Trifluoroacetic acid (TFA) and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ).

2.2. Incubation of samples at 40 °C

The recombinant monoclonal antibody at 70 mg/mL was diluted to 5 mg/mL using 20 mM citric acid, 20 mM glycine and 20 mM sodium phosphate. The pH of the samples after dilution was adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0 using hydrochloric acid or sodium hydroxide. The samples were then filtered through 0.2 μm syringe filters (Gelman Sciences, Ann Arbor, MI) and incubated at

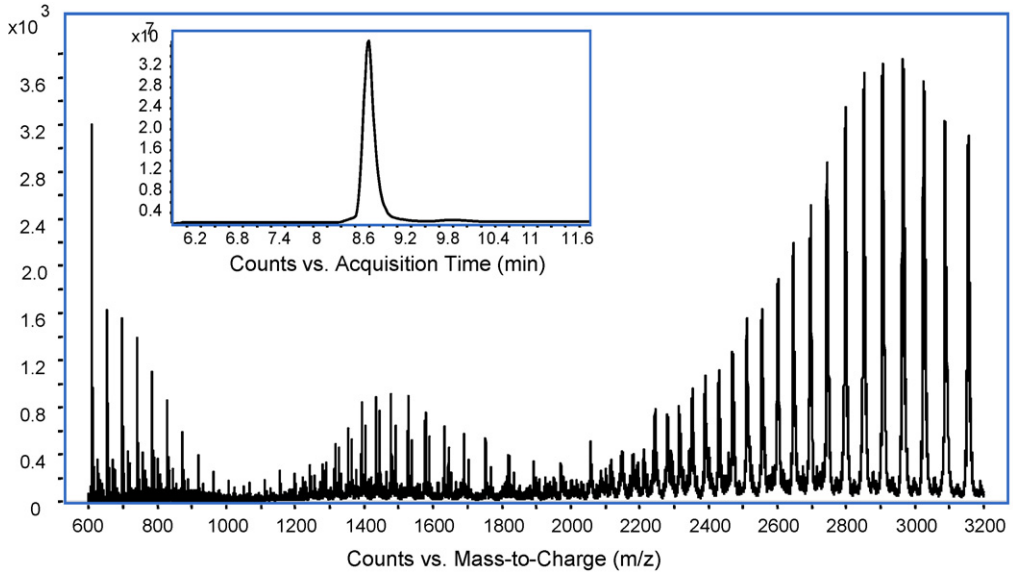

Fig. 2. A typical mass spectrum to show the *m*/*z* envelops. The spectrum was acquired using the sample incubated at pH 5 at 40 °C for 10 weeks. Inset shows the total ion chromatogram of this sample.

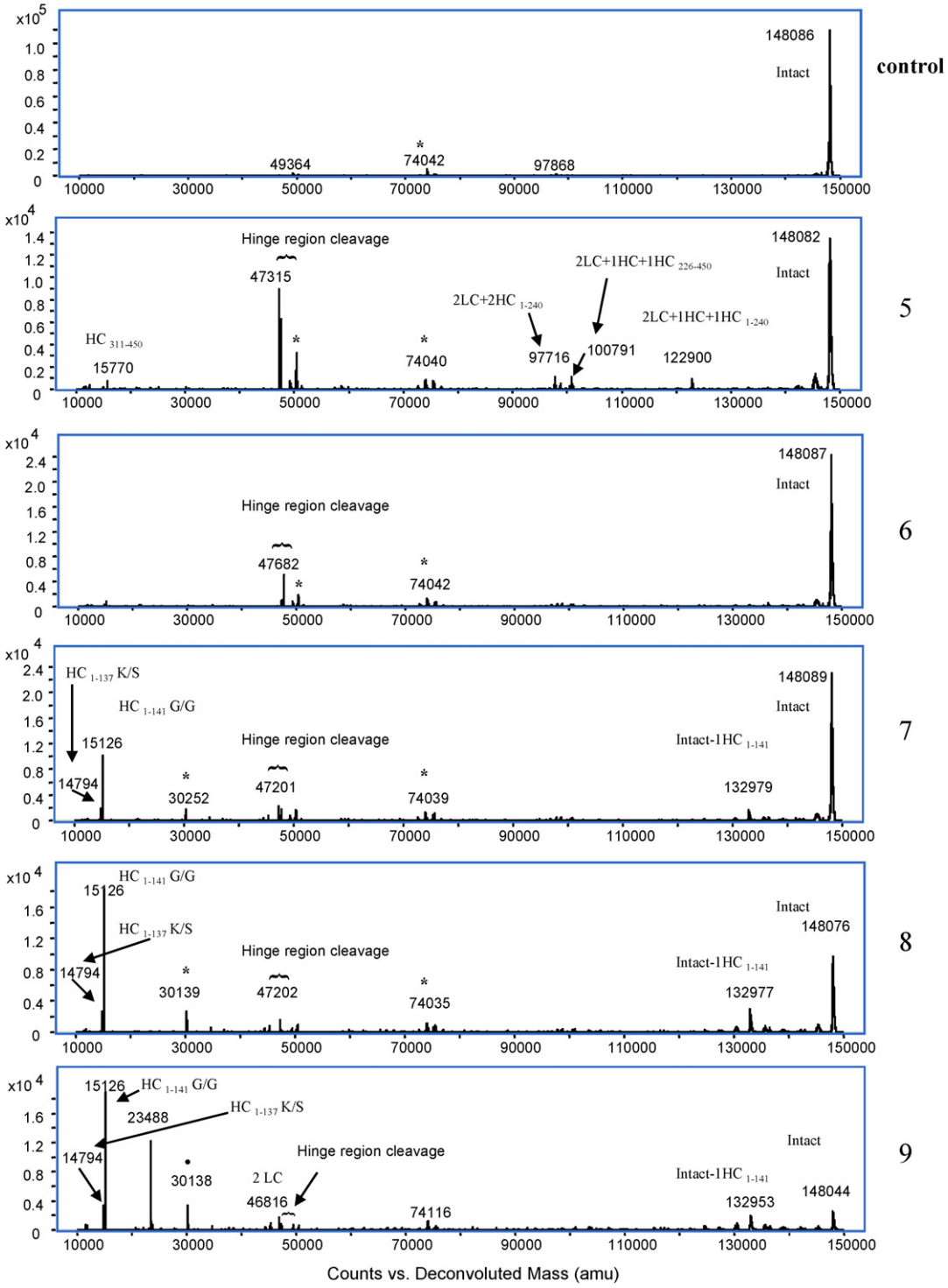

Fig. 3. Deconvolution mass spectra of the control and the samples incubated at 40 °C for 10 weeks at pH 5 (5), 6 (6), 7 (7), 8 (8) and 9 (9). Fragments corresponding to the peaks are labeled in the Figures. Peaks under the bracket are expanded and shown in Fig. 4. Peaks labeled with * are deconvolution artifacts.

40 °C for 10 weeks. A sample stored at −80 °C was used as a control.

2.2.1. LC–MS analysis using a protein microtrap

An Agilent HPLC and a Q-TOF 6510 LC/MS system (Agilent, Santa Clara, CA) were used to determine the molecular weights of the antibody and its various fragments. Approximately 2 μg of each sample was loaded on to a protein microtrap (Michrom Bioresources, Auburn, CA) at 95% mobile phase A (0.02% TFA and 0.08% FA in Milli-Q water) and 5% mobile phase B (0.02% TFA and 0.08% FA in acetonitrile). After running at 5% mobile phase B for 5 min, the mobile phase B was increased to 95% within 1 min, maintained at 95% mobile phase B for 4 min and then decreased to 5% mobile phase B in 1 min. The protein microtrap was equilibrated using 5% mobile phase B for 4 min before the next injection. The flow-rate was set at 50 μL/min with the column oven set at 60 °C.

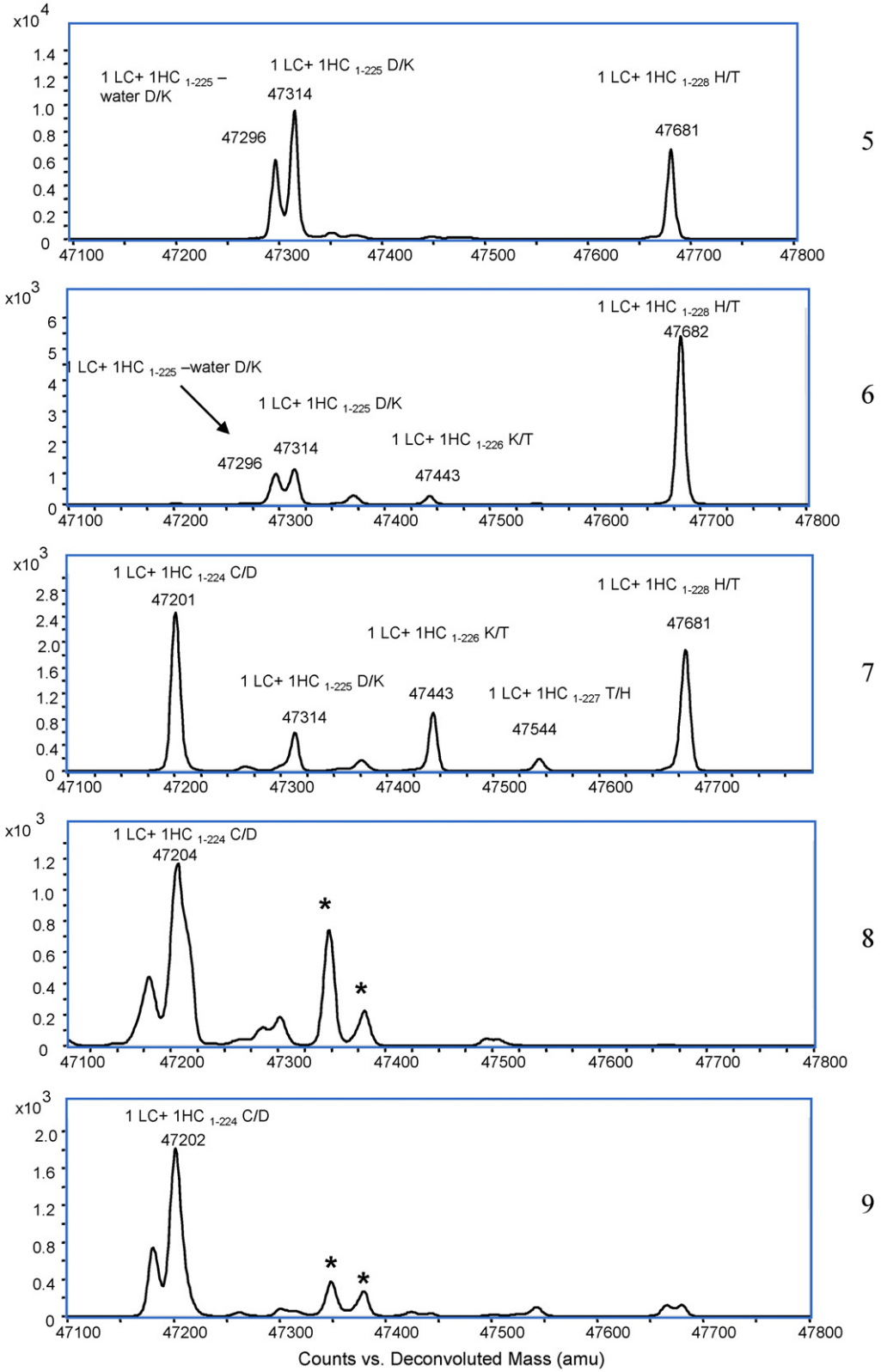

Fig. 4. Expanded view of the peaks corresponding to antibody fragmentation in the hinge region from Fig. 3. The spectra were from the samples incubated at pH 5 (5), 6 (6), 7 (7), 8 (8), and 9 (9). Identities of the peaks are labeled in the Figure. Peaks labeled with * are deconvolution artifacts.

The mass spectrometer was set at an m/z range of 600–3200, source gas temperature of 350 °C and Vcap voltage of 4750 V.

2.2.2. LC–MS analysis using a protein C4 reversed-phase column

The samples were also analyzed using the Agilent HPLC and mass spectrometer in combination with a protein C4 column (Vydac, 150 mm × 1 mm i.d., 5 μm particle size, 300 A pore size). For analyzing the samples before reduction, 2 μg of each sample was loaded at 5% mobile phase B. Mobile phase B was increased to 25% within 5 min, and the sample was eluted by increasing mobile phase B from 25% to 60% in 35 min. The column was washed using 95% mobile phase B for 5 min and equilibrated at 5% mobile phase B for 6 min before the next injection. For analyzing the samples after reduction, 50 μL of each sample diluted with PBS to 0.2 mg/mL was reduced with 10 mM DTT at 37 °C for 30 min and 2 μg of each sample was loaded into the column at 5% mobile phase B. After increasing the mobile phase B to 20% in 5 min, the column was eluted by increasing the percent of mobile phase B from 20% to 45% in 25 min. The column was washed with 95% mobile phase B for 5 min and then equilibrated at 5% mobile phase B for 6 min before the next injection. The flow-rate was set at 50 μL/min with the column oven set at 60 °C. The mass spectrometer parameters were the same as for analysis with the protein microtrap.

3. Results

3.1. Strategy for fragment identification

Identification of the fragments was first based on the agreement of the observed molecular weights with the calculated molecular weights of the fragments. If a match was found, additional information such as the known post-translational modifications, peak clusters, the likelihood of such fragment, and other modifications such as oxidation of methionine residues were used to further confirm the assignment.

The recombinant monoclonal antibody used for this study possesses two major post-translational modifications, which are incomplete C-terminal lysine (Lys) processing and N-linked glycosylation of the conserved asparagine (Asn) in the CH2 domain. The additional Lys results in a molecular weight increase of 128 Da. The major oligosaccharide is composed of core-fucosylated biantennary complex structures with either (Gal 0), one (Gal 1) or two terminal galactose (Gal 2) residues. The attachment of Gal 0 increases the molecular weight by 1445 Da and the addition of each terminal galactose increases the molecular weight by an additional 162 Da. Information on the C-terminal Lys and the presence of oligosaccharides especially with the addition of galactose was invaluable in determining the identities of the fragments.

The presence of a cluster of peaks around the main peak of each fragment provided further evidence for the assignment. This point is clearly demonstrated in Fig. 1. A cluster of peaks corresponding to heavy chain fragments from amino acids 1–137, 1–138, 1–139, 1–140 and 1–141 was observed. The molecular weight difference between the neighboring peaks matched the molecular weight of the individual amino acid in the sequence, providing further evidence for a positive identification.

The likelihood of generating such fragments was also an important consideration. Compared to fragments with either the original N-terminus or the original C-terminus of the intact antibody, fragments with newly generated N and C-termini were less likely to be present. It is also possible that several fragments were associated with the same major site of hydrolysis.

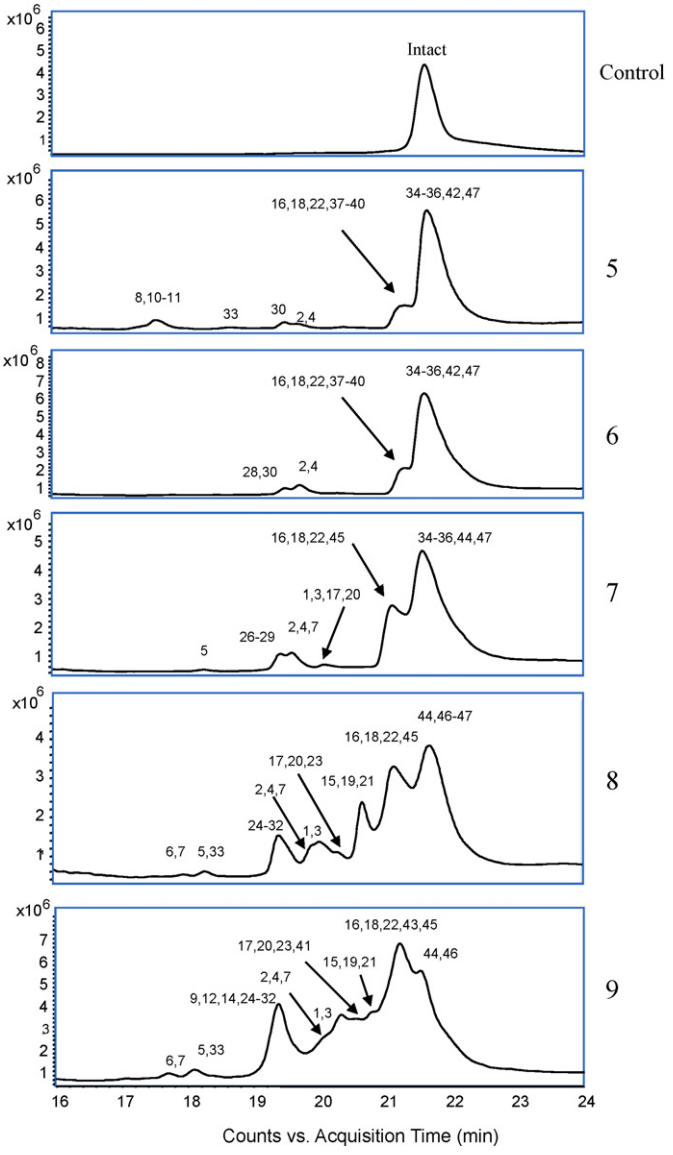

Fig. 5. Chromatograms of the control and the samples incubated at 40 °C for 10 weeks. The chromatograms were acquired using the control (control), the samples incubated at pH 5 (5), 6 (6), 7 (7), 8 (8) and 9 (9). Peak identities are summarized in Table 1. Numbers in the Figure correspond to fragment numbers in Table 1.

In addition to the known post-translational modifications, other chemical modifications that occurred during the incubation of the samples or generated during sample preparation were very helpful for fragment assignment. For example, peaks with an increase of 16 Da were observed in many fragments containing Met432 of this antibody, which is known to be susceptible to oxidation [11–13]. The other common chemical modification is desulfurization, which occurred mainly at basic pH [14], and can lead to cross-linking by thioether formation cleavage of peptide bond and the conversion of the original cysteine residue to a dehydroalanine residue [8,15].

3.2. Analysis of the samples before reduction

3.2.1. Analysis using a protein microtrap column

Samples after incubation at 40 °C for 10 weeks were analyzed by LC–MS before reduction. A sample stored at −80 °C was used as a control. The samples were first analyzed using a short gradient and

Table 1
Identification of antibody fragments in the samples incubated at various pH without reduction.

Fragment	Molecular weight (Da)			pH				
	Observed	Calculated	Identities	5	6	7	8	9
1	11,279	11,279	HC1–102 ($-H_2O$)			+	+	+
2	11,297	11,297	HC1–102	+	+	+	+	+
3	11,538	11,538	HC1–105 ($-H_2O$)			+	+	+
4	11,556	11,556	HC1–105	+	+	+	+	+
5	11,875	11,875	HC346–450 ($-H_2O$)			+	+	+
6	11,949	11,948	HC32–141			+	+	
7	12,003	12,003	LC106–214				+	+
8	12,275	12,275	HC342–450	+				
9	12,574	12,576	HC339–450				+	
10	12,704	12,704	HC338–450	+				
11	13,524	13,524	HC330–450	+				
12	13,747	13,748	LC1–126					+
13	13,835	13,835	LC1–127			+	+	+
14	13,892	13,892	LC1–128					+
15	14,776	14,776	HC1–137 ($-H_2O$)				+	+
16	14,793	14,794	HC1–137	+	+	+	+	+
17	14,881	14,881	HC1–138			+	+	+
18	14,981	14,982	HC1–139	+	+	+	+	+
19	15,051	15,051	HC1–140 ($-H_2O$)				+	+
20	15,069	15,069	HC1–140			+	+	+
21	15,109	15,109	HC1–141 ($-H_2O$)			+	+	
22	15,126	15,126	HC1–141		+	+	+	+
23	15,183	15,183	HC1–142				+	+
24	22,015	22,015	LC1–201				+	+
25	22,102	22,102	LC1–202				+	+
26	22,963	22,963	LC1–210			+	+	+
27	23,176	23,176	LC1–212			+	+	+
28	23,304	23,305	LC1–213		+	+	+	+
29	23,374	23,374	LC1–213 (+dehydroalanine)			+	+	+
30	23,408	23,408	LC (1 free SH)	+	+	+	+	+
31	23,440	23,440	LC (+a sulfur group)				+	+
32	23,488	23,488	HC20–240				+	+
33	25,200	25,200	HC241–450	+			+	+
34	47,297	47,297	1LC + HC1–225 ($-H_2O$)	+	+	+		
35	47,314	47,315	1LC + HC1–225	+	+	+		
36	47,682	47,681	1LC + HC1228	+	+	+		
37	100,419	100,418	1LC + HC + HC229–450	+	+			
38	100,579	100,580	1LC + HC + HC229–450 (Gal)	+	+			
39	100,786	100,785	1LC + HC + HC226–450	+	+			
40	100,948	100,947	1LC + HC + HC226–450 (Gal)	+	+			
41	117,861	117,865	Intact-2HC1–141					+
42	122,899	125,900	2LC + 1HC + 1HC1–240	+	+			
43	132,962	132,955	Intact-1HC1–141 ($-H_2O$)					+
44	132,977	132,973	Intact-1HC1–141			+	+	+
45	133,123	133,117	Intact-2HC1–141 (Gal, $-H_2O$)			+	+	+
46	148,060	148,063	Intact ($-H_2O$)				+	+
47	148,080	148,081	Intact	+	+	+	+	

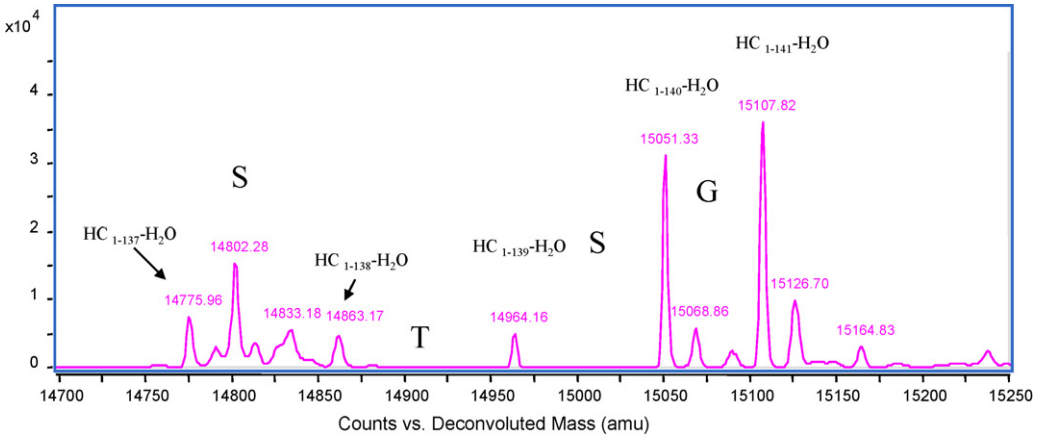

Fig. 6. Deconvoluted mass spectra of antibody fragments from the sample incubated at pH 9. Multiple peaks corresponding to hydrolysis of peptide bonds between amino acids 137–142 were observed as shown in Fig. 1 but with molecular weight decrease of 18 Da. S, T and G are amino acids serine, threonine and glycine.

a protein microtrap column to elute antibody and its fragments as one peak. Deconvolution of the single peak with a wide mass range provided an overall picture of the antibody fragments.

A typical mass spectrum and the corresponding total ion chromatogram (TIC) from the sample incubated at pH 5 using the protein microtrap column is shown in Fig. 2. There was no difference between the TIC peaks of different samples and all eluted off the protein microtrap column as a single peak. Multiple charge envelops in the mass spectrum indicated that there were multiple components in the sample. Deconvolution spectra of the samples incubated at different pH are shown in Fig. 3. The peaks are identified in the figure. In addition to the fragment peaks, peaks corresponding to the molecular weights of the intact antibody with various post-translational modifications were observed. Fragments corresponding to the Fab region from hydrolysis of peptide bonds in the hinge region were observed in the samples that were incubated at all pH levels in the study. Incubation at pH 6 led to the generation of the fewest fragments, whereas fragments increased at pH either lower or higher than 6. Fragments from hydrolysis of peptide bonds in the Fc region such as $HC_{311-450}$, intact-$2HC_{1-240}$ and intact-HC_{1-240} were observed in the sample incubated at pH 5. On the other hand, fragments from the hydrolysis of peptide bonds in the Fab region such as HC_{1-137} and HC_{1-141} were observed in the samples incubated at pH 7, 8 and 9. The intensity of the peak corresponding to the intact antibody was comparable at pH 6 and 7, but decreased at pH 5, 8 and 9, which suggested that the intact antibody degraded faster at pH 5, 8 and 9. The molecular weight of the intact antibody also decreased in the samples incubated at pH 8 and 9, which as demonstrated later was likely due to dehydration and desulfurization.

The regions of the deconvoluted mass spectra corresponding to peptide bond hydrolysis in the hinge region were expanded and shown in Fig. 4. One or more fragments of the Fab region from the hydrolysis of peptide bonds between amino acids of CDKTHT (224–229) of the entire upper hinge region were observed in the samples incubated at all pH levels studied. In agreement with a previous study, a shift of the hydrolysis sites towards the C-terminus with decreasing pH was observed [9]. The peak corresponding to 18 Da lower than the molecular weight of Fab fragment 1LC + HC_{1-225} was also observed previously [4,5,10], and was attributed to a loss of water from the formation of a C-terminal succinimide [16].

3.2.2. Analysis using a protein C4 chromatography column

The same set of samples was further analyzed using a protein C4 column. As shown in Fig. 5, one peak was observed in the control with an observed molecular weight of 148,081 Da, which was in agreement with the calculated molecular weight of 148,081 Da. However, multiple additional peaks and shoulders, indicating degradation, were observed in the samples after incubation at pH 5 through 9. In agreement with the previous observation, the antibody was most stable at pH 6, where the fewest additional peaks and shoulders were observed (Fig. 5). Additional peaks or peaks with increased intensities were observed in the samples incubated at pH 5, 7, 8 and 9.

The fragments identified in the peaks in Fig. 5 are summarized in Table 1. More fragments were detected in the samples incubated at pH 7, 8 and 9 than at pH 5 or 6. Fragments detected in the samples incubated at pH 6 were generally similar to fragments detected in the sample incubated at pH 5. Whereas, fragments in the samples incubated at pH 7 and 8 corresponded to fragments in the sample incubated at pH 9.

It is clear from Table 1 that peptide bonds in several regions are susceptible to hydrolysis. At pH 5 and 6, the major susceptible regions were around heavy chain amino acids 225–229 with less

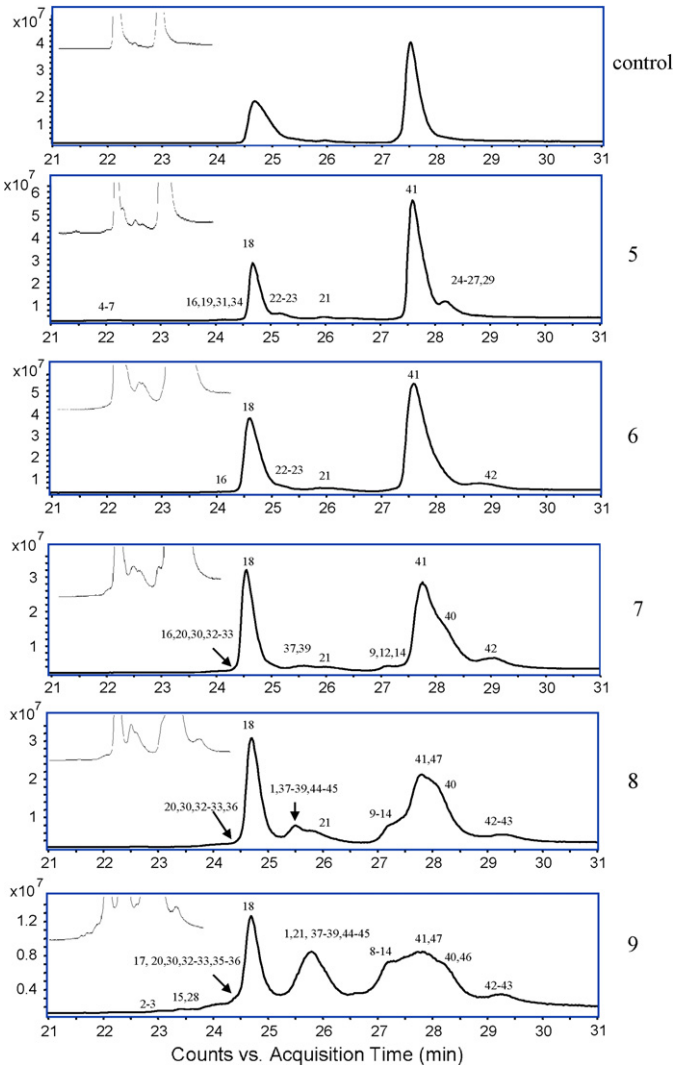

Fig. 7. Chromatograms of the control and the samples incubated at 40 °C for 10 weeks after reduction. The chromatograms were acquired using the control (control), the samples incubated at pH 5 (5), 6 (6), 7 (7), 8 (8) and 9 (9). Peak identities are summarized in Table 2. Numbers in the Figure correspond to fragment numbers in Table 2. Insets are expanded views of regions of the corresponding chromatograms to show peaks with low intensities.

fragmentation at 102–105, 137–142, and 330–342. At pH 9, hydrolysis of the light chain around amino acids 126–128 and 201–213 in addition to the susceptible regions in the pH 5 sample were also observed.

Several modifications of various antibody fragments were also observed. First, a fragment corresponding to the Fab region consisting of light chain and heavy chain amino acids 1–225 but with 18 Da lower was observed in the samples incubated at pH 5, 6 and 7. This was observed previously and was attributed to the loss of water. Second, several peaks with molecular weights corresponding to the loss of water were observed when the samples were incubated at pH 7, 8 and 9 (Table 1). As discussed earlier, a cluster of peaks corresponding to heavy chain N-terminal fragments consisting of amino acids 1–137, 1–138, 1–139, 1–140, and 1–141 was observed (Fig. 1). A cluster of peaks corresponding to the same series of fragments but with a decrease of 18 Da was also observed when the samples were incubated at basic pH (Fig. 6). This suggested that β-elimination caused a loss of water from residues serine or threonine [17]. Third, peaks with various modifications such as the formation

Table 2
Identification of antibody fragments in the samples incubated at various pH after reduction.

Fragment	Molecular weight (Da)			pH				
	Observed	Calculated	Identities	5	6	7	8	9
1	11,539	11,538	HC1–105 ($-H_2O$)				+	+
2	11,949	11,947	HC345–450 (2 free SH)					+
3	12,004	12,004	LC106–214					+
4	12,275	12,275	HC342–450					+
5	12,704	12,704	HC338–450	+				
6	13,524	13,524	HC330–450	+				
7	13,639	13,638	HC329–450	+				
8	14,776	14,776	HC1–137 ($-H_2O$)					+
9	14,794	14,794	HC1–137			+	+	+
10	14,963	14,963	HC1–139 ($-H_2O$)				+	+
11	15,051	15,051	HC1–140 ($-H_2O$)				+	+
12	15,069	15,069	HC1–140			+	+	+
13	15,108	15,108	HC1–141 ($-H_2O$)				+	+
14	15,126	15,126	HC1–141			+	+	+
15	16,777	16,774	HC302–450 (3 free SH)					+
16	21,480	21,478	HC275–450 (2 free SH)	+	+	+		
17	22,102	22,102	LC1–202					+
18	23,408	23,407	LC (1 free SH)	+	+	+	+	+
19	23,424	23,424	LC (oxidation)	+				
20	23,426	23,424	LC (oxidized, 3 free SH)			+	+	+
21	23,524	23,524	LC (modified)	+	+	+	+	+
22	23,564	23,564	LC (modified)	+	+			
23	23,582	23,582	LC (modified)	+	+			
24	23,890	23,890	HC1–225 ($-H_2O$)	+				
25	23,909	23,908	HC1–225 (1 free SH)	+				
26	24,275	24,274	HC1–228 (1 free SH)	+				
27	25,200	25,200	HC241–450	+				
28	25,202	25,200	HC241–450 (2 free SH)					+
29	25,455	25,452	HC1–240 (3 free SH)	+				
30	26,620	26,616	HC227–450 (4 free SH)			+	+	+
31	26,746	26,744	HC226–450 (2 free SH)	+				
32	26,749	26,744	HC227–450 (+K, 4 free SH)			+	+	+
33	26,863	26,859	HC225–450 (4 free SH)			+	+	+
34	26,908	26,906	HC226–450 (Gal, 2 free SH)	+				
35	26,934	26,929	HC225–450 (pyruvyl, 4 free SH)					+
36	27,024	27,021	HC225–450 (Gal, 4 free SH)				+	+
37	35,531	35,526	HC 142–450 (5 free SH)			+	+	+
38	35,589	35,583	HC141–450 (5 free SH)				+	+
39	35,694	35,688	HC 142–450 (Gal, 6 free SH)			+	+	+
40	50,621	50,615	HC ($-H_2O$, 6 free SH)			+	+	+
41	50,637	50,634	HC (3 free SH)	+	+	+	+	+
42	50,639	50,634	HC (5 free SH)			+	+	+
43	50,641	50,634	HC (7 free SH)				+	+
44	58,905	58,901	LC + HC142–450 by thioether (4 free SH)			+	+	+
45	58,964	58,958	LC + HC141–450 by thioether (6 free SH)				+	+
46	73,996	73,990	LC + HC by thioether ($-H_2O$, 6 free SH)					+
47	74,015	74,008	LC + HC by thioether (7 free SH)				+	+

of a dehydroalanine group and modification of the light chain with a free sulfhydryl were observed in samples incubated at basic pH (Table 1).

3.3. Analysis of the samples after reduction

The samples were also analyzed using a protein C4 column after reduction. As shown in Fig. 7, only two peaks were observed in the control at retention times of approximately 24.5–25 and 27–28 min, which corresponded to antibody light chain and heavy chain, respectively. Several small peaks and shoulders off the light chain and heavy chain peaks were observed in the sample incubated at pH 5. Two small peaks and a shoulder off the heavy chain peak were observed in the sample incubated at pH 6, whereas more peaks and shoulders with higher intensity were detected with the increase in pH from 7 to 9. The heavy chain degraded more with the increase in pH, while the light chain did not degrade significantly at any pH tested.

Identities of the fragments are summarized in Table 2. More fragmentation was observed at pH 5, 7, 8 and 9 than at pH 6. As in the case where the samples were analyzed before reduction using the protein C4 column, fragments in the samples incubated at pH 7 and 8 were, in general, similar to fragments in the pH 9 sample, and fragments in the sample incubated at pH 6 were similar to fragments observed in the pH 5 incubation sample.

In addition to the modifications discussed in the previous section, several fragments consisting of the light chain and heavy chain linked by a thioether were observed in the samples incubated at basic pH. These were not detected previously, perhaps due to their co-elution with other fragments. The observed molecular weights were close to the calculated molecular weights with cysteine residues in the free sulfhydryl state. This suggested that the disulfide bonds associated with these fragments were reduced. The number of free cysteine residues was estimated using the molecular weight difference. There were two peaks that were detected in the samples incubated at pH 5 and 6 with molecular weights of 23,564 and 23,582 Da (Table 2), which were observed previously [10]. In addition, a peak with molecular weight of 23,524 Da was detected in the samples of all pH. These fragments were modifications on the light chain (MW, 23,407 Da) and

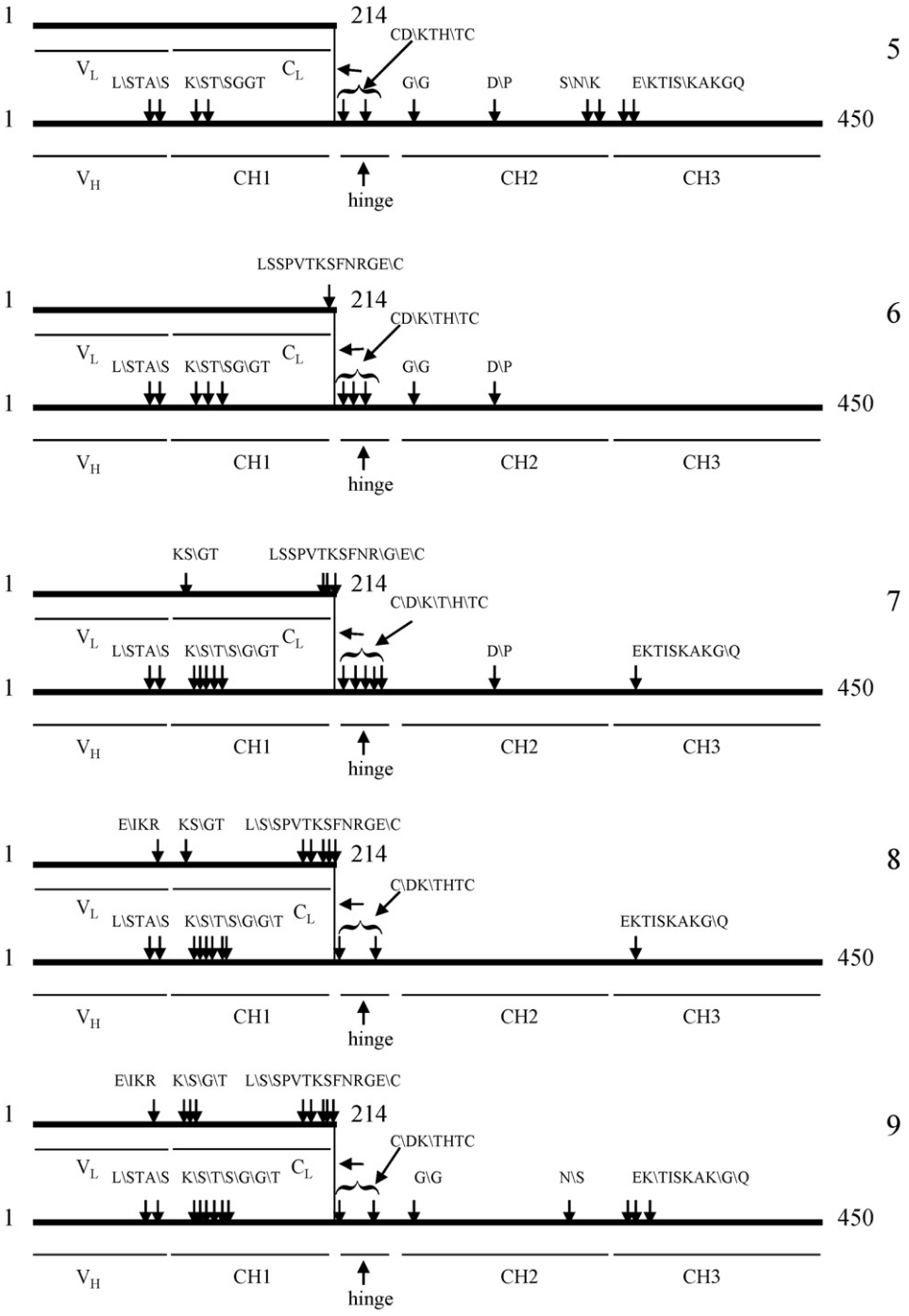

Fig. 8. Fragmentation of the antibody after incubation at various pH. Only one light chain and one heavy chain are shown in the diagram to represent an antibody structure. Amino acids in the susceptible regions are shown in the diagram with the hydrolysis sites indicated by "\". Sites of hydrolysis are also labeled using arrows.

the specific nature of the modification will be further investigated.

3.4. Summary of the fragments

Combined fragments shown in Fig. 3 and listed in Tables 1 and 2, reveal specific regions of the antibody in which peptide bonds are susceptible to hydrolysis. This is displayed graphically in Fig. 8. The light chain was stable when the sample was incubated at pH 5. Only one peptide bond between amino acids 213/214 of the light chain was hydrolyzed in the sample incubated at pH 6. Peptide bonds between amino acids 127/128 and several peptide bonds between amino acids 210–214 of the light chain were hydrolyzed in the sample incubated at pH 7. Peptide bonds in the regions of amino acids 105–108, 126–129, and 210–214 of the light chain were hydrolyzed when the samples were incubated at pH 8 and 9. Clearly, more peptide bonds of the light chain were hydrolyzed with the increase in pH.

The susceptible peptides of the heavy chain were also limited to specific regions, which included amino acids 102–106, 137–142, 337–346 and amino acids 225–229 of the hinge region. Hydrolysis of peptide bonds between amino acids 240/241 only occurred in

the samples incubated at pH 5, 6 and 9. The peptide bond between amino acids 274/275 was hydrolyzed only in the samples that were at pH 5 or 6. Hydrolysis of peptide bonds in the region of amino acids 328–330 was only observed in the sample incubated at pH 5. Hydrolysis of the peptide bond between amino acids 301/302 was observed in the sample incubated at pH 9.

There is also evidence that hydrolysis of peptide bonds occurred on the antibody fragments after they were formed. For example, fragment HC_{1-141} was observed in samples stored at pH 5 through 9, and fragment HC_{32-141} was observed in samples stored at pH 8 and 9. This indicates that the 141/142 bond is less stable under the storage conditions, implying that it was first hydrolyzed and the resulting fragment was then hydrolyzed at the 31/32 bond. Similarly, HC_{20-240} was probably formed from hydrolysis of the peptide bonds between amino acids 19/20 of fragment HC_{1-240}. Fragments from further hydrolysis of the original fragments were not included in Fig. 7.

4. Discussion

The impact of pH on antibody fragmentation was investigated in this study. The use of reversed-phase chromatography coupled with mass spectrometry allowed detection of antibody fragments from hydrolysis of peptide bonds not only in the hinge region, but also in other regions of the molecule. These results provide a better understanding of peptide bond susceptibility to hydrolysis in different regions of the antibody.

A pH of 6 was demonstrated to be optimal for slowing down antibody fragmentation in the hinge region which is in agreement with previous findings [5,9]. This is also true for fragmentation in other regions of the antibody. Therefore, for recombinant monoclonal antibodies in liquid formulation, pH 6 should be optimal to slow down antibody fragmentation through hydrolysis in the hinge as well as other regions.

The light chain was more stable than the heavy chain. No fragments from the light chain were observed in the sample incubated at pH 5, while multiple fragments from the heavy chain were observed. Fragments from hydrolysis of a single peptide bond of the light chain in the sample incubated at pH 6 and several peptide bonds in the samples incubated at pH 7, 8 and 9 were observed. However, more fragments were observed in the heavy chain in samples incubated at the same pH. The stability of the light chain compared to the heavy chain was also observed in the TIC chromatograms of the reduced samples (Fig. 7). The light chain peaks did not change significantly, whereas the heavy chain peaks changed dramatically, particularly in the samples incubated at pH 8 and 9.

Peptide bonds in regions within the IgG domain structures were more stable than regions around the domain–domain interfaces. In addition to the peptide bonds in the hinge region, peptide bonds in the regions of 105–108, 126–129 and 201–214 of the light chain and 102–106, 137–142 and 328–346 of the heavy chain are located close to the domain–domain interfaces, either at the edges of β-sheets or in the loops that connect the β-sheets [18,19]. The lack of structural protection and flexibility are the likely reasons for susceptibility of peptide bond to hydrolysis in these regions. The peptide bond between G240/G241 was susceptible to hydrolysis at pH 5 and at pH 9. At pH 6, 7 and 8, no significant cleavage was observed at this site. G240 and G241 are located in the CH2 domain in close proximity to the hinge but they are not in the β-sheet structure [18]. Thus, the flexibility of the peptide bond between the two glycine residues and the lack of structural constraints increased the susceptibility of this peptide bond to hydrolysis. Peptide bond between D274/P275, located in a loop structure [18], was also susceptible at neutral and acidic pH. In addition, it is well-known that the peptide bond between aspartic acid and proline is susceptible to low pH [20,21]. At pH 9, a cleavage between amino acid N301, which is the conserved residue for N-glycosylation, and S302 was observed. These residues are in the DE-loop and are highly exposed [18].

There were several chemical modifications in addition to hydrolysis of peptide bonds. First, several peptides with molecular weights that were lower by 18 Da than the calculated molecular weights were observed. As discussed earlier, the loss of water under the acidic condition was probably due to the formation of a C-terminal succinimide from aspartic acid [16]. On the other hand, the loss of water of several fragments observed in the samples that were incubated at basic pH was probably due to a β-elimination that caused a loss of water from serine or threonine residues [17]. It was also possible that the loss of water was due to the accumulation of succinimide as an aspartate isomerization and asparagine deamidation intermediate [22,23]. Second, several modifications were related to the instability of the inter-light chain and heavy chain disulfide bond. It is known that desulfurization is one of the major protein degradation pathways under basic conditions [14,24,25], and was observed in this study. Multiple products from such a reaction were detected, including the conversion of a cysteine residue to dehydroalanine (Table 1), the addition of a pyruvyl group (Table 2) and cross-linking of the light chain and the heavy chain through a thioether linkage (Table 2).

In summary, antibody fragmentation through peptide bond hydrolysis was investigated in this study using a combination of reversed-phase liquid chromatography and mass spectrometry. Fragmentation was observed not only in the hinge region, but also in other specific regions of the antibody. Identification and better understanding of the hydrolytically susceptible regions of the antibody was achieved. As expected, peptide bonds within the IgG domain structures were more stable than peptide bonds around the domain–domain interfaces. In agreement with the data obtained by monitoring peptide bond hydrolysis in the hinge region, pH 6 was also demonstrated to be optimal to retard fragmentation in all regions of the antibody, including the hinge.

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