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Research Paper

Fragmentation of a Recombinant Monoclonal Antibody at Various pH

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Purpose. To determine the relative importance of direct hydrolysis and β-elimination, two common mechanisms of antibody hinge region fragmentation, and the impact of the conserved N-linked oligosaccharides in affecting antibody fragmentation under various pH.
Methods. A recombinant monoclonal antibody was incubated in buffers of various pH at 40°C for 5 weeks. The level of fragmentation was measured using size-exclusion-chromatography (SEC). The specific sites of fragmentation were determined by analyzing SEC fractions using liquid chromatography mass spectrometry (LC-MS).
Results. Direct hydrolysis was accelerated by acidic and basic pH, while β-elimination contributed to hinge region fragmentation at pH 7 and above. In addition, a shift of the major peptide bond hydrolysis sites in the hinge region towards the C-terminal direction with the decrease of sample pH from 9 to 5 was observed. At pH 4, the major cleavage site shifted outside the hinge region and was localized in the CH2 domain. Oligosaccharides did not affect hinge region fragmentation in the pH range of 5–9, however, at pH 4 oligosaccharides slowed down fragmentation in the CH2 domain.
Conclusions. Antibody fragmentation level, sites and mechanisms were affected by pH. Oligosaccharides only affected the rate of fragmentation at pH 4.

KEY WORDS: fragmentation; hinge region; mass spectrometry; recombinant monoclonal antibody.

INTRODUCTION

IgG antibodies are composed of four regions: two Fab regions, one Fc region and one hinge region. The hinge region connects the two Fab regions to the Fc region, and it is the least structured and the most flexible region of antibodies. Consequently, the hinge region is susceptible to enzymatic and non-enzymatic attack, ultimately leading to fragmentation of the antibody.

Many enzymes have been used to cleave IgG antibodies in the vicinity of the hinge region. For example, papain and pepsin are the two most commonly used enzymes to generate antibody fragments, which cleaves peptide bonds in the hinge region or in the hinge proximal region of the CH2 domain. Metalloproteinases (1) have also been shown to cleave IgGs in the CH2 domain and the cleavage sites are even closer to the hinge region than the pepsin cleavage sites. In addition, cathepsin L, plasmin and Lys-C have been demonstrated to cleave antibodies in the hinge region (2–6).

Antibody fragmentation also often occurs spontaneously within the hinge region through non-enzymatic cleavage. As demonstrated by Cordoba *et al.* (7), protease inhibitors and EDTA did not prevent cleavage of the peptide bonds in the hinge region, which supports non-enzymatic cleavage in this region. Fragmentation of antibodies within the hinge region has been studied at various pH and temperatures. Cleavage of peptide bonds in the hinge region was identified in several monoclonal antibodies after long-term storage at 5°C (8) and short-term storage at elevated temperatures at various pH (9–15). Major cleavage sites within the hinge region were identified to be between amino acids S/C, C/D, D/K, and H/T in the sequence SCDKTHTC of four IgG1 antibodies after incubation at 40°C at pH 5.2 for one month (7).

Two different mechanisms that are involved in antibody fragmentation are direct hydrolysis and hydrolysis through a β-elimination mechanism. Direct hydrolysis is the mechanism that is responsible for most of the fragmentation that have been observed. Tao *et al* (20) identified cleavage sites between amino acids D/K and H/T in the hinge region sequence of SCDKTHTC of a fully human monoclonal IgG1 antibody after incubation at 40°C for 8 weeks at pH 5.2. Cleavage between these amino acids was accelerated when the antibody was exposed to pH above and below 6, which was attributed to a direct hydrolysis mechanism. More recently, Cohen *et al.* (16) reported cleavage of the peptide bond between S/C in the hinge region through a β-elimination mechanism. β-elimination of a disulfide bond leads to the formation of a dehydroalanine residue, which can be hydrolyzed. Hydrolysis of dehydroalanine results in a N-terminal fragment with an amide group and a C-terminal fragment with a pyruvyl group, which is different from a hydroxyl group and an amine group found in the N-terminal and C-terminal fragments respectively from direct hydrolysis. β-elimination of disulfide bonds followed by the formation of dehydroalanine is known to occur more readily under basic conditions (17–19).

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This explains why antibody fragmentation through this mechanism was observed only at basic pH (16), and not at acidic pH (7,20).

Previous studies focused on the mechanism of pH dependence of hinge region fragmentation, however it is unclear whether susceptibility and levels of cleavage of peptide bonds between specific amino acids in the hinge region changes when exposed to different pH. This information could potentially give more insight into the importance of antibody structure and stability. Therefore, in the current study, we focused on monitoring both the levels and sites of hinge region fragmentation after the antibody was exposed to various pH at 40°C. It is also known that N-linked oligosaccharides attached to the conserved asparagine residue in the CH2 domain plays a significant role in maintaining antibody stability. Therefore, we also monitored the effects of oligosaccharides on antibody fragmentation.

MATERIAL AND METHODS

Materials

The 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). N-octylglucoside was purchased from Roche (Indianapolis, IN). Acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ) and PNGaseF was purchased from Prozyme (San Leandro, CA).

Deglycosylation of the Recombinant Monoclonal Antibody

The antibody (71 mg/mL) in formulation buffer (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-20) at pH 5.2, was diluted to 10 mg/mL with Milli-Q water, and then digested with PNGaseF (1 μL enzyme: 500 μg antibody) at 37°C for 18 h. N-octylglucoside at a final concentration of 1% was included in the sample preparation in order to achieve complete digestion. A sample prepared the same way as the sample for PNGaseF digestion but without the addition of PNGaseF was also incubated at 37°C for 18 h and was used as a control.

Reversed-Phase High Performance Liquid Chromatography and Mass Spectrometry

An Agilent HPLC (Santa Clara, CA) and a Qstar mass spectrometer (Applied Biosystems, Framingham, MA) were used to analyze the reduced antibody and fractions collected from size exclusion chromatography (SEC). The samples were first diluted to 0.5 mg/mL using PBS and then reduced with 10 mM DTT at 37°C for 30 min. Five μg of each sample was loaded separately onto a protein C4 column (Vydac, 150×1 mm i.d., 5 μm particle size, 300 A pore size) at 95% mobile phase A (0.08% FA in Milli-Q water) and 5% mobile phase B (0.08% FA in acetonitrile). After 5 min at 5% mobile phase B, the light chain and heavy chain of the reduced antibodies were eluted off the column by increasing mobile phase B to 65% within 35 min. The column was washed with 95% mobile phase B for 5 min and then equilibrated at 5% mobile phase B for 10 min before the next injection. The flow rate was set at 50 μL/minute and the column oven was set at 60°C. The mass spectrometer was operated at positive mode with a scan range of m/z 800 to 2500. IonSpray voltage was set at 4500 volts and the source temperature was set at 350°C.

Thermal Stress of the Antibody with and without Oligosaccharides

PNGaseF digested antibody (deglycosylated) and the control (glycosylated) were concentrated and buffer exchanged to Milli-Q water using Amicon-ultra centrifugal filter devices with a molecular weight cut-off of 30 kDa (Millipore, Billerica, MA). Antibody concentration was determined by UV absorption at 280 nm and the extinction coefficient calculated from the known amino acid sequence of this antibody. The samples (>100 mg/mL) were diluted to 5 mg/mL using 10 mM citric acid and 10 mM sodium phosphate at various pH of 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. The final pH of each sample was measured to be 4.0, 5.0, 6.0, 6.9, 7.9 and 8.8 respectively and the pH did not change over the 5-week incubation period. The buffer pH was used to refer to the samples in the following sections. The samples were then sterilized by filtering through 0.2 μm syringe filters (Gelman Sciences, Ann Arbor, MI) and incubated at 40°C for 5 weeks. Aliquots of the sample in pH 4 buffer were taken at earlier time points of 4, 6, 8, 10 and 14 h for analyses. Samples stored at −80°C were used as the time zero point.

Size Exclusion Chromatography

A Shimadzu HPLC and a Superdex 200 column (10× 300 mm, GE healthcare, Piscataway, NJ) were used to separate aggregates, monomer and fragments. Each sample (50 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. Samples incubated for 5 weeks at pH 5, 6, 7, 8 and 9 were used to collect fractions of peaks 1 and 2 (Fig. 2). Fractions corresponding to peak 2 were collected from samples incubated at pH 4 for 4, 6, 8, 10 and 14 h. The fractions were concentrated using Amicon-ultra centrifugal filter devices with a molecular weight cut-off of 5 kDa (Millipore). The fractions from pH 5, 6, 7, 8 and 9 were analyzed by LC-MS after reduction with DTT, while fractions from the pH 4 samples were analyzed before and after reduction.

RESULTS

Deglycosylation of the Recombinant Monoclonal Antibody

Glycosylation of the conserved asparagine residue in the CH2 domain with N-linked oligosaccharides and incomplete processing of the C-terminal lysine (Lys) residues are the two major post-translational modifications of this recombinant monoclonal antibody. The major oligosaccharide species are

core fucosylated complex structure with either zero (Gal 0) or one terminal galactose (Gal 1). While incomplete C-terminal processing results in antibody heavy chain with zero (Lys 0) or one Lys (Lys 1).

N-linked oligosaccharides were removed from the antibody with PNGaseF and complete digestion was confirmed by mass spectrometry. In the mass spectrum of the native antibody heavy chain, three major peaks were observed with molecular weights of 50634 Da, 50758 Da, and 50796 Da (Fig. 1A), which corresponded to the heavy chain without C-terminal Lys with Gal 0 (50636 Da), with C-terminal Lys with Gal 0 (50764 Da), and without C-terminal Lys with Gal 1 (50798 Da) respectively. After deglycosylation with PNGaseF, only two peaks were observed with molecular weights of 49189 Da and 49314 Da (Fig. 1B), which corresponded to the heavy chain without oligosaccharides, without the C-terminal Lys (49191 Da) and with the C-terminal Lys (49319 Da) respectively. The later result confirmed the removal of oligosaccharides.

Degradation of the Antibody at the pH Range from 5 to 9

Degradation of the antibody with and without oligosaccharides was monitored by size exclusion chromatography. Representative chromatograms of the antibody with or without oligosaccharides after incubation at various pH for 5 weeks are shown in Fig. 2. The peak that eluted in the time window of approximately 40–45 min corresponded to the antibody monomer. Aggregates eluted in the time window of approximately 27–39 min. The chromatographic profile between 34–39 min (dimer) was similar between the native and deglycosylated samples, however, it was different between 27–34 min (large aggregates). It was thus possible that the absence of oligosaccharides had an effect on the formation of large aggregates. Fragments eluted later than the monomer peak between the retention time windows of 45–48 minutes and 52–56 min and referred to as peak 2 and peak 1 respectively. There were minor peaks that eluted after peak 1 that were not included for further analysis. This study focused on the analysis of the fragments that were generated.

1. **Effect of pH and oligosaccharides on the level of antibody fragmentation**

Fragmentation of the antibody with and without oligosaccharides at various pH was analyzed by integrating the peak areas of aggregates, monomer and fragments and comparing them after incubation at 40°C for 5 weeks. Peaks 1 and 2 were summed together and referred to as fragments. As shown in Fig. 3, there was no significant difference in the percentage of fragments that was detected between the native and the deglycosylated antibody when they were incubated at the same pH. This result suggested that the presence or absence of oligosaccharides did not affect the level of antibody fragmentation. On the other hand, pH had a significant

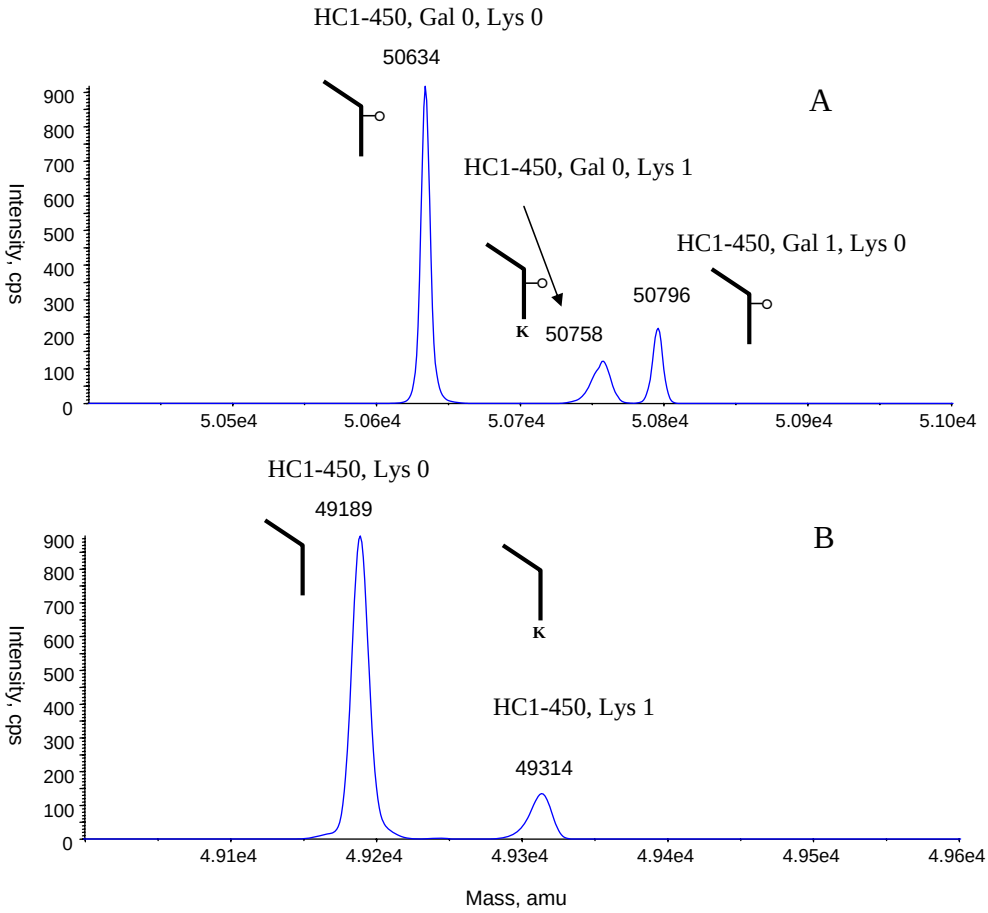

Fig. 1. Mass spectra of the antibody before (A) and after deglycosylation (B). The spectra were acquired after DTT reduction. Oligosaccharides are indicated as –O. C-terminal lysine is indicated as *K*.

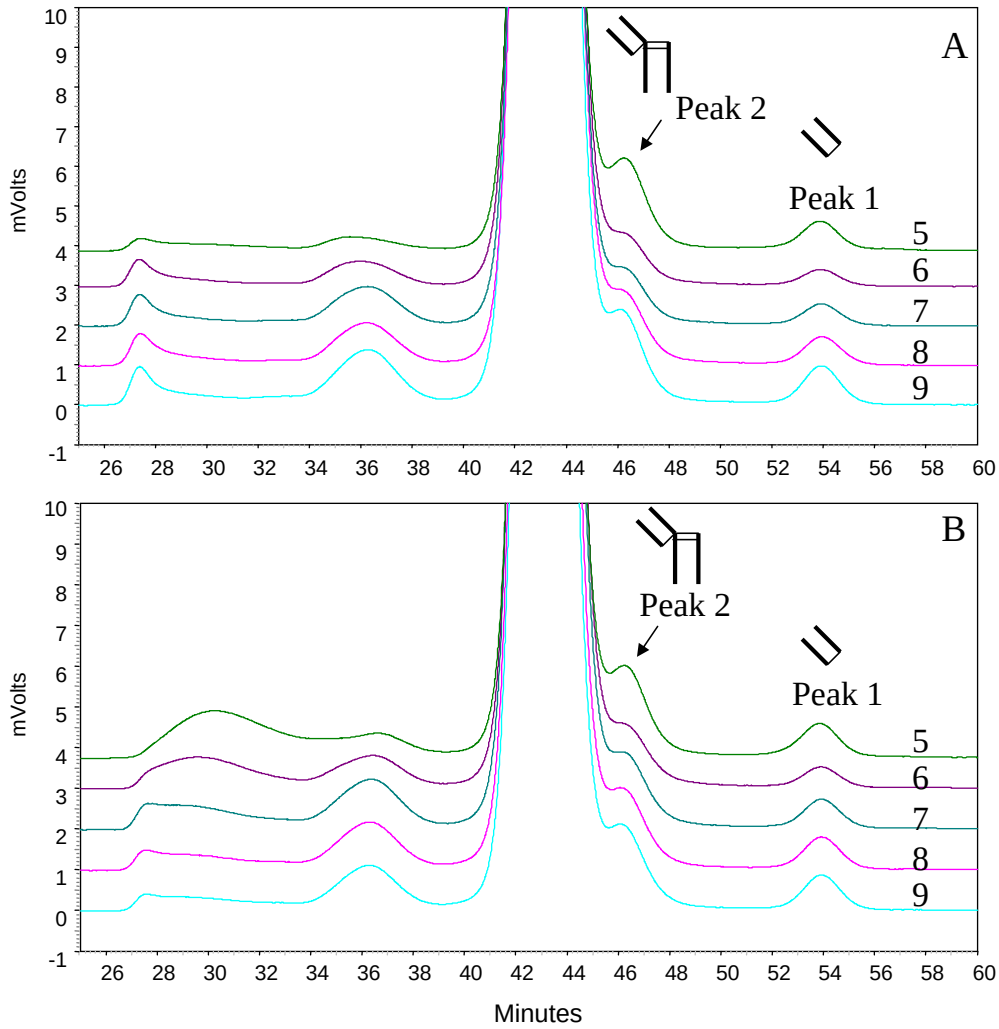

Fig. 2. Representative chromatograms of the antibody before (A) and after deglycosylation (B). The chromatograms are of samples that were incubated at 40°C for 5 weeks at pH 5 (5), 6 (6), 7 (7), 8 (8) or 9 (9). Peak 1 includes fragments of Fab and peak 2 includes fragments of antibody missing one Fab arm as indicated in the figure.

impact on the level of antibody fragmentation. In agreement with a previous study (20), higher levels of fragments were observed in the samples incubated at a pH either lower or higher than 6.

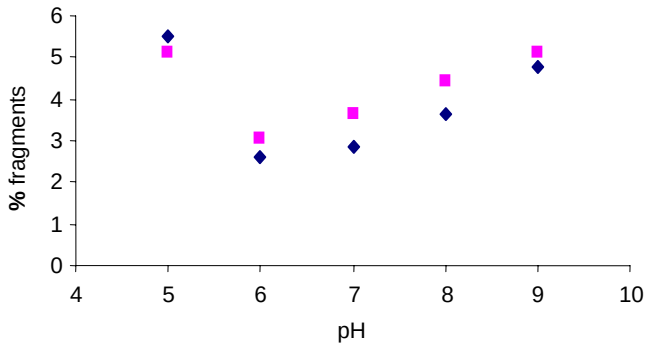

Fig. 3. Relative percentage of fragments of the antibody before (*diamond*) and after deglycosylation (*square*) after incubation at 40°C for 5 weeks at pH 5, 6, 7, 8 or 9.

2. **Analysis of peak 1**

Peak 1 material from the antibody with and without oligosaccharides after incubation at various pH for 5 weeks was collected and analyzed by LC-MS. All of the fragments observed were related to the Fab region. The mass spectra are shown in Fig. 4 and peak identities are summarized in Table I. No significant cleavage pattern difference between the samples with or without oligosaccharides was observed, which suggested that oligosaccharides did not play a significant role in affecting the peptide bond hydrolysis pattern in the hinge region. However, the cleavage pattern of the peptides in the hinge region of both the deglycosylated antibody and the native antibody was significantly affected by pH. There was a shift of peptide bond hydrolysis towards the C-terminal direction with decreasing pH (Fig. 4). The major hydrolysis sites of the samples incubated at pH 5 and 6 were between amino acids D/K and H/T. Cleavage also occurred between K/T at pH 6. The major cleavage sites of the samples incubated at pH 7 were between C/D, K/T, and H/T, with lower levels of cleavage between S/C, D/K and T/H.

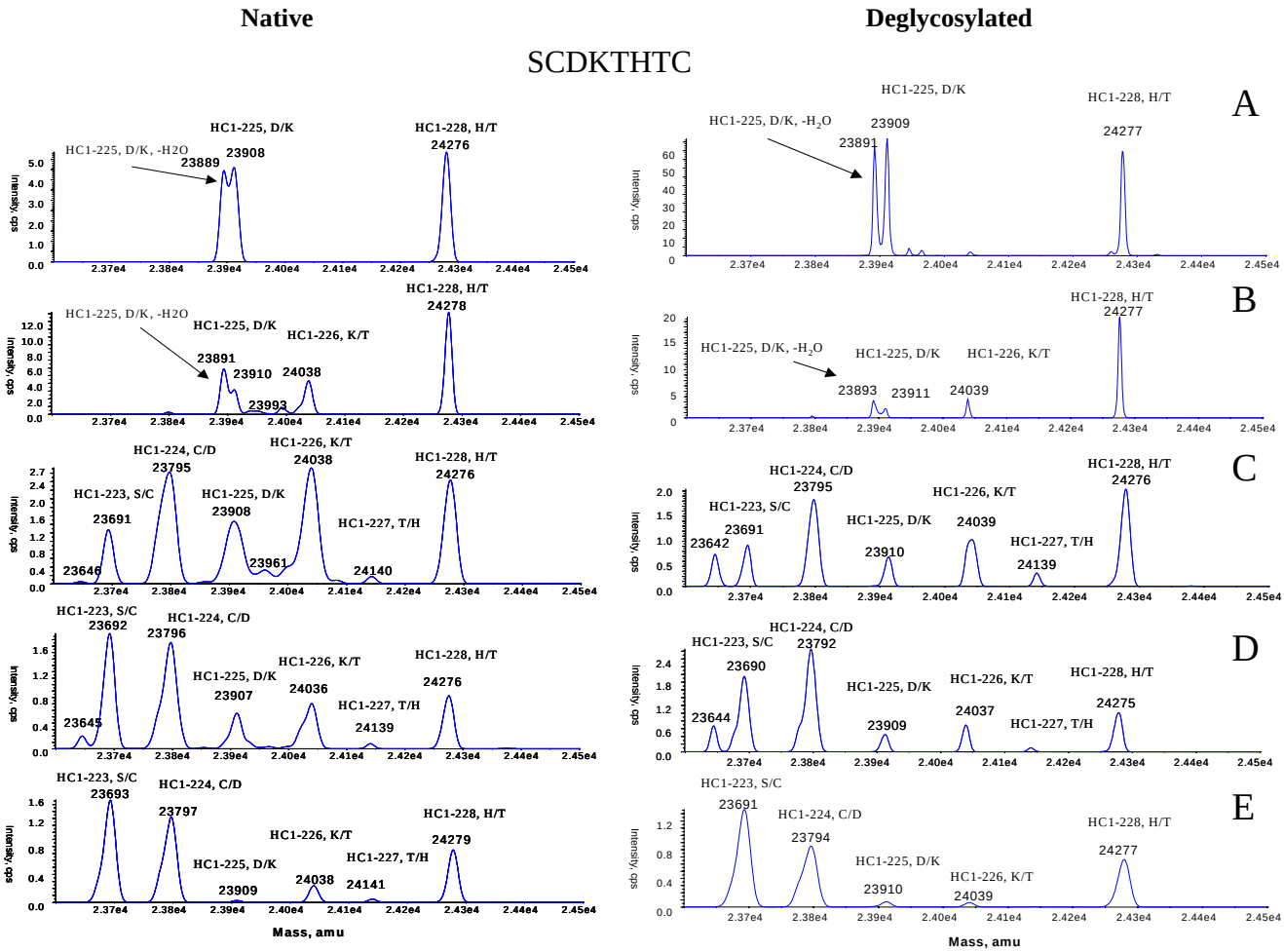

Fig. 4. Mass spectra of peak 1 (Fig. 2) collected from the native and deglycosylated samples after incubation at 40°C for 5 weeks at pH 5 (A), 6 (B), 7 (C), 8 (D) and 9 (E). The peaks in the figure are labeled with (a) HC followed by the position of the amino acids of the fragment sequence and (b) the single letter code of the amino acids immediately before and after the cleavage site.

The cleavage sites for the samples incubated at pH 8 and 9 were similar to the sites observed at pH 7 but with the major sites of cleavage between S/C and C/D.

Direct hydrolysis of the S/C bond and hydrolysis of dehydroalanine formed from β-elimination of the disulfide bond results in a N-terminal fragment with the same number of amino acids but with different C-terminal end groups. Direct hydrolysis results in a hydroxyl group, while hydrolysis of dehydroalanine results in an amide group. The 1 Da difference between the amide and hydroxyl groups cannot be differentiated at this level by mass spectrometry. Therefore the mechanism of cleavage between S/C at pH 7, 8 or 9 could not be determined at this point based on Fab fragments alone.

Table I. Identities of Peak 1 Fragments as Shown in Fig. 4

Calculated MW (Da)	Amino acids	Cleavage sites
23691	HC1-223	↓SCDKTHTC
23794	HC1-224	SCD↓KTHTC
23891	HC1-225 (-18 Da)	SCDK↓THTC
23909	HC1-225	SCDK↓THTC
24037	HC1-226	SCDKTHT↓C
24275	HC1-228	SCDKTHT↓C

A peak with a molecular weight of 18 Da lower than that of the calculated molecular weight of amino acids 1–225 was observed in samples incubated at pH 5 and 6 (Fig. 4A and B). The loss of 18 Da was observed previously (7,13,20) and was attributed to the loss of water, which was due to succinimide formation most likely by the mechanism reported by Geiger and Clark (21), in which a C-terminal succinimide was formed by the attack of the β-amide nitrogen on the peptide bond carbonyl.

3. **Analysis of peak 2**

Analysis of the Fc portion of the fragments would confirm the cleavage sites observed in peak 1 by providing information on the C-terminal portion of the fragments. Fc fragments

were expected to elute slightly earlier or at least co-elute with the Fab fragments (in peak 1) when analyzed by SEC. However, there was no obvious peak that eluted immediately before peak 1 and the Fc portion was not detected in the peak 1 fraction when analyzed by mass spectrometry. The absence of a Fc peak when analyzed by SEC can be attributed to low abundance and/or instability of the Fc portion. There was still a possibility that the Fc portion co-eluted with the Fab fragment in peak 1 even if it was not detected by mass spectrometry. The Fc portion contains heterogeneity that is caused by different types of N-linked oligosaccharides and C-terminal Lys variation, which would contribute to lowering the signal of any one particular fragment. Therefore, to gain insight of the Fc related cleavage products, peak 2, the shoulder on the descending side of the monomer peak, was collected and analyzed. Peak 2 consisted of antibody that was missing one Fab arm.

The mass spectra acquired from the analyses of the Fab portions collected from antibody with and without oligosaccharides showed no significant difference in the cleavage site or levels of fragmentation at various pH as discussed in the previous section (Fig. 4). Therefore, we focused on the spectra obtained from the antibody with oligosaccharides. The peak 2 mass spectra acquired from the antibody with oligosaccharides after incubation for 5 weeks at various pH are shown in Fig. 5 and the identities of the peaks are summarized in Table II. The C-terminal fragments observed confirmed the sites of cleavage observed in peak 1. At pH 5 and 6 (Fig. 5A–B), the major cleavage sites were between amino acids H/T, D/K and C/D. A minor cleavage between K/T was observed in the pH 6 sample. The major cleavage sites of the sample incubated at pH 7 were between amino acids H/T and C/D, with lower levels between K/T and D/K. AT pH 8 and 9, the major cleavage site was between C/D, with lower levels of cleavage between H/T, K/T and D/K. The cleavage sites shifted to the C-terminal direction with the decrease of pH, which is in agreement with the results obtained for peak 1.

The relative intensities of a specific fragment with either Gal 0 or Gal 1 compared well with the relative intensities of the original non-fragmented heavy chain with Gal 0 or Gal 1 (Fig. 1). For example, the relative intensities of the peak with a molecular weight of 26746 (HC226–450, Gal 0) and the peak with a molecular weight of 26908 (HC226–450, Gal 1) (Fig. 5A) were similar to the relative intensities of the peak with a molecular weight of 50634 (HC1–450, Gal 0) and the peak with a molecular weight of 50796 Da (HC1–450, Gal 1), shown in Fig. 1. This was observed in all of the samples incubated at various pH which indicated that antibodies with different types of oligosaccharides (at least Gal 0 and Gal 1) did not differ in their impact on fragmentation.

It is interesting to note that a peak with a molecular weight of approximately 26930 Da was observed in the samples incubated at pH 8 and 9 (Fig. 5, D and E). This molecular weight was 70 Da higher than expected from cleavage of the C/D bond through direct hydrolysis. The 70 Da difference can be attributed to the presence of a pyruvyl group, which was formed from hydrolysis of the dehydroalanine residue as a result of β-elimination of the interchain disulfide bond as reported by Cohen *et al.* (16). The presence of this peak was only observed at basic pH.

Degradation of the Antibody at pH 4

Degradation of the antibody with and without oligosaccharides at pH 4 occurred much more rapidly than at other pH. Approximately 80% and 25% fragments were detected in the deglycosylated and native antibody samples respectively after incubation at 40°C for 14 h (Fig. 6). This result suggested that oligosaccharides played a major role in slowing down fragmentation as the native antibody degraded much slower.

Peak 2 material was collected and analyzed from the deglycosylated and native antibody after incubation at 40°C for 6, 8, 10, and 14 h. The mass spectra of the sample incubated for 14 h are shown in Fig. 7A and B. After reduction, two peaks were observed. The peak with a molecular weight of 23411 Da corresponded to the intact light chain, while the peak with a molecular weight of 25458 Da corresponded to the N-terminal portion of the heavy chain from amino acids 1 to 240. This result indicated that hydrolysis occurred between the two glycine residues in the CH2 domain. The molecular weight of 97713 Da acquired before reduction (Fig. 7B) corresponded to an antibody fragment with two intact light chains and two heavy chains consisting of amino acids 1–240, which confirmed cleavage between the two glycine residues. Therefore, the only detectable cleavage site was between amino acids G240 and G241 in the CH2 domain after the samples were incubated at 40°C at pH 4.0. This was in contrast to all of the samples incubated at the pH range of 5–9.

DISCUSSION

Hinge region peptide bond cleavage is one of the major degradation pathways of monoclonal antibodies in liquid formulation. Two different mechanisms, direct hydrolysis and hydrolysis of dehydroalanine resulting from β-elimination are responsible for most of the antibody cleavage in the hinge region. The levels of hydrolysis of hinge region peptide bond between particular pairs of amino acids at different pH and the impact of oligosaccharides were investigated further.

The results from this study confirmed earlier studies that direct hydrolysis and β-elimination contributed to hinge region fragmentation. Analysis by mass spectrometry revealed that direct hydrolysis was the major hinge region peptide bond cleavage mechanism and was accelerated by pH either lower or higher than 6. In addition, hydrolysis of dehydroalanine after β-elimination of the inter light chain and heavy chain disulfide bond contributed to antibody

Fig. 5. Mass spectra of peak 2 (Fig. 2) collected from the antibody after incubation at 40°C for 5 weeks at pH 5 (A), 6 (B), 7 (C), 8 (D) and 9 (E). The peaks in the figure are labeled with (a) HC followed by the position of the amino acids of the fragment sequence and (b) the single letter code of the amino acids immediately before and after the cleavage site. Gal 1 indicates fragments with one terminal galactose on the oligosaccharide. Lys 1 indicates the presence of a C-terminal Lys. Two minor peaks with molecular weights of 97,764 Da and 97,821 Da were not identified.

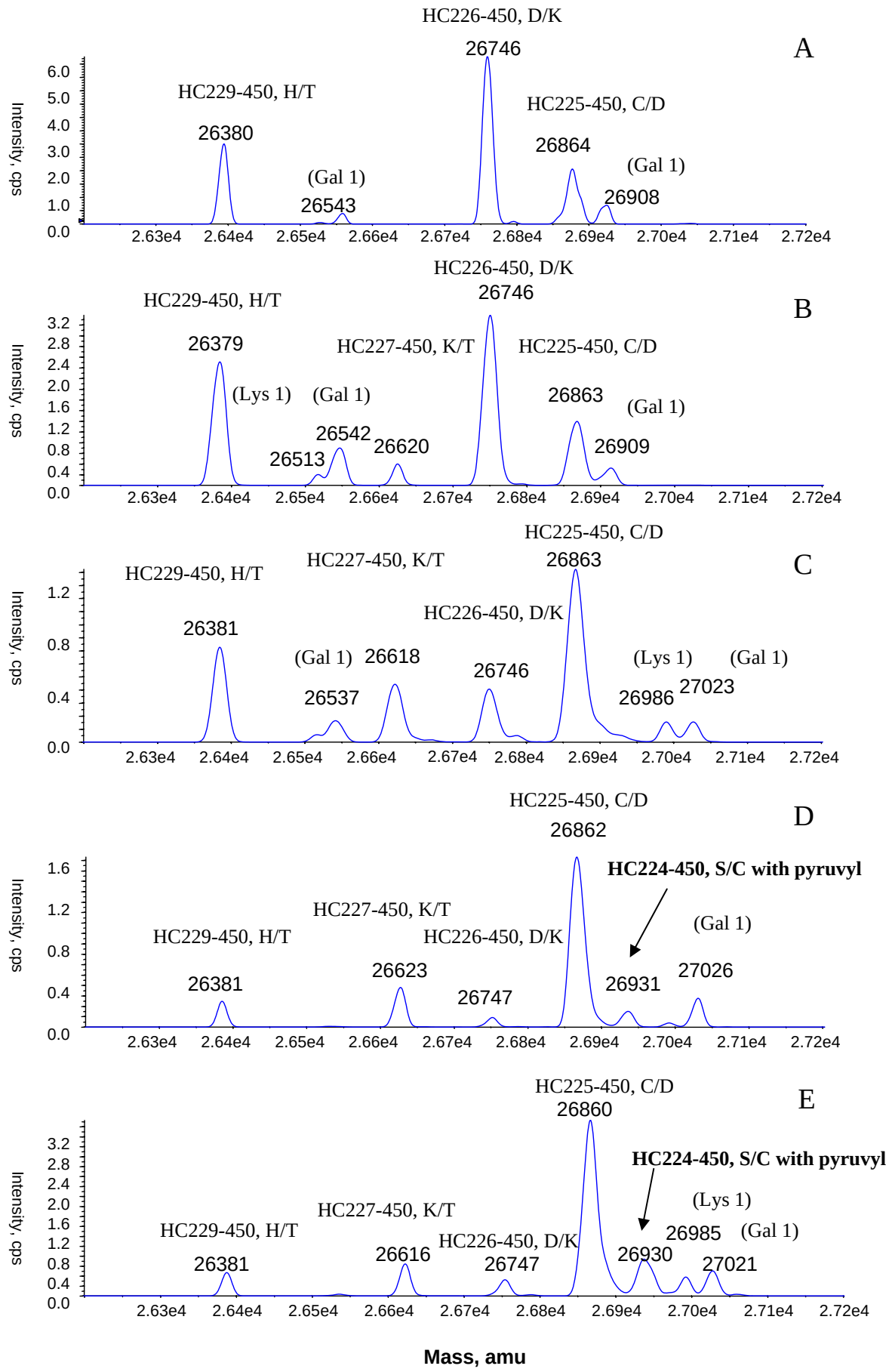
A
HC226-450, D/K
26746
HC229-450, H/T
26380
(Gal 1)
26543
HC225-450, C/D
26864
(Gal 1)
26908
Intensity, cps
6.0
5.0
4.0
3.0
2.0
1.0
0.0
2.63e4 2.64e4 2.65e4 2.66e4 2.67e4 2.68e4 2.69e4 2.70e4 2.71e4 2.72e4
B
HC226-450, D/K
26746
HC229-450, H/T
26379
(Lys 1)
(Gal 1)
26542
26513
26620
HC227-450, K/T
HC225-450, C/D
26863
(Gal 1)
26909
Intensity, cps
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
2.63e4 2.64e4 2.65e4 2.66e4 2.67e4 2.68e4 2.69e4 2.70e4 2.71e4 2.72e4
C
HC225-450, C/D
26863
HC227-450, K/T
HC229-450, H/T
26381
HC226-450, D/K
(Gal 1)
26618
26746
26537
(Lys 1)
(Gal 1)
26986
27023
Intensity, cps
1.2
0.8
0.4
0.0
2.63e4 2.64e4 2.65e4 2.66e4 2.67e4 2.68e4 2.69e4 2.70e4 2.71e4 2.72e4
D
HC225-450, C/D
26862
HC224-450, S/C with pyruvyl
HC227-450, K/T
(Gal 1)
HC229-450, H/T
HC226-450, D/K
26623
26381
26747
26931
27026
Intensity, cps
1.6
1.2
0.8
0.4
0.0
2.63e4 2.64e4 2.65e4 2.66e4 2.67e4 2.68e4 2.69e4 2.70e4 2.71e4 2.72e4
E
HC225-450, C/D
26860
HC224-450, S/C with pyruvyl
(Lys 1)
HC227-450, K/T
HC229-450, H/T
26985
(Gal 1)
HC226-450, D/K
26616
26930
26381
26747
27021
Intensity, cps
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
2.63e4 2.64e4 2.65e4 2.66e4 2.67e4 2.68e4 2.69e4 2.70e4 2.71e4 2.72e4
Mass, amu

Table II. Identities of peak 2 fragments as shown in Fig. 5

Calculated MW (Da)	Amino acids	Cleavage sites
26379	HC229-450 (Gal 0,Lys 0)	SCDKTHTC
26507	HC229-450 (Gal 0,Lys 1)	SCDKTHTC
26541	HC229-450 (Gal1,Lys 0)	SCDKTHTC
26618	HC227-450 (Gal 0,Lys 0)	SCDKTHTC
26746	HC226-450 (Gal 0,Lys 0)	SCDKTHTC
26860	HC225-450 (Gal 0,Lys 0)	SCDKTHTC
26930	HC225-450 (Gal 0, Lys 0, with pyruvoyl group)	SCDKTHTC
26988	HC225-450 (Gal 0, Lys 1)	SCDKTHTC
27022	HC225-450 (Gal 1, Lys 0)	SCDKTHTC

fragmentation only at a pH higher than 7. This observation is in agreement with the study reported by Cohen *et al.* (16), where they observed significant amount of fragmentation caused by β-elimination mechanism under basic pH conditions.

Information on the cleavage sites was obtained from the analysis of peak 1 and peak 2 by mass spectrometry. Peak 1 contained Fab fragments, and peak 2 contained intact antibody lacking one Fab arm, which together provided complementary confirmation of the cleavage sites. The peak intensities of corresponding C-terminal and N-terminal fragments from the analysis of the same cleavage site from peak 1 and peak 2 were in agreement with one exception. As shown in Fig. 4D and E, the N-terminal fragment corresponding to amino acids 1–223 resulting from the cleavage of a peptide bond between S/C was one of the major peaks. Based on this result, the corresponding C-terminal fragment, amino acids 224–450 without C-terminal Lys and with Gal 0 was also expected to be a major peak, but a peak corresponding to this molecular weight (26839 Da) was not found (Fig. 5D and E). Only a C-terminal fragment containing a pyruvyl group with a molecular weight of 26931 Da was observed. This suggested that cleavage between S/C was due solely to the β-elimination mechanism. However, there was a discrepancy between the intensity difference between the N-terminal fragment H1–223 (Fig. 4C, D, and E) and the corresponding C-terminal fragment H224–450 with a pyruvyl group. There are several possible reasons for the lower intensity of the C-terminal fragment. First, higher levels of heterogeneity associated with this fragment caused by different types of oligosaccharides and the presence or absence of the C-terminal Lys could lower its intensity. Second, further hydrolysis of the fragment with the pyruvyl group can lower the abundance of this fragment. And last, it is also possible that the C-terminal fragment with the pyruvyl group had a lower ionization efficiency than its N-terminal counterpart.

The peptide bond cleavage patterns were different at different pH within the hinge region sequence SCDKTHTC, which was one of the interesting findings from this study. Overall, the major cleavage sites shifted towards the C-terminus with the decrease of pH. At pH 4, the sole cleavage site shifted entirely out of the hinge region and into the CH2 domain between amino acids G240 and G241. These results suggest that the Fab regions moved closer towards the hinge with decreasing pH, resulting in greater protection of the N-terminus of the hinge region. At pH 4, a structural rearrangement of the antibody provided complete protection of the hinge region. Other studies have shown antibody structural rearrangements that were pH dependent. Kats *et al.* (22) showed, using micellar electrokinetic capillary chromatography that a monoclonal chimeric antibody existed in one of five different isoforms depending on pH, due to subtle differences in antibody domain disposition. It has also been reported that acidic treatment of rabbit IgG resulted in increased interaction between the CH1 and CH2 domains (23–25). Our observation of the shift in peptide bond cleavage patterns provides further support that the domain organization of the IgG molecules varied with pH, which affects the susceptibility of different regions of the antibody to hydrolysis.

Based on this study, oligosaccharides did not have a significant affect on hinge region peptide bond hydrolysis when the antibody was incubated at the pH range of 5–9 at 40°C for 5 weeks. The extent and pattern of the hinge region peptide bond hydrolysis of the native as well as the deglycosylated antibody was similar for samples incubated at the same pH. In addition, different types of oligosaccharides did not differ in their impact on fragmentation. However, it was shown that oligosaccharides played a significant role in slowing down the cleavage between G240 and G241 in the CH2 domain at pH 4. This finding is supported by other studies, which showed that deglycosylation of an antibody resulted in structural changes and decreased stability of the CH2 domain (26–31). For example, local conformational differences around the conserved asparagine residue between glycosylated and aglycosylated IgGs were detected by NMR (27,28). Tao and Morrison (26) reported increased protease susceptibility of aglycosylated chimeric mouse-human IgG1 and IgG3 compared to the native IgG molecules. Decreased thermal stability (29,30) and increased protease susceptibility (31) of antibodies upon the removal of oligosaccharides has also been reported. It is also possible that the mere presence of the oligosaccharides near the CH2 domain provided direct protection to the peptide bond

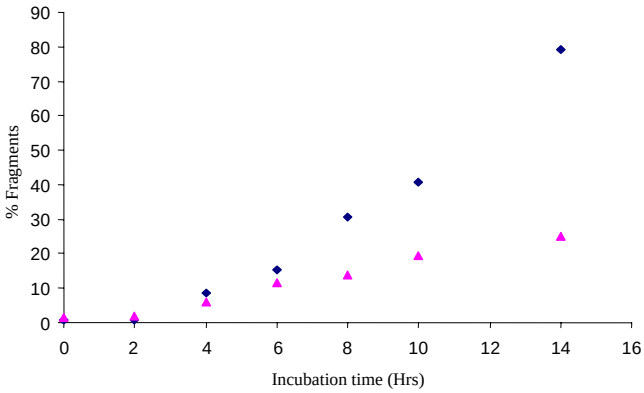

Fig. 6. Relative percentage of fragments of the antibody before (*triangle*) and after (*diamond*) deglycosylation after incubation at pH 4 for various time points.

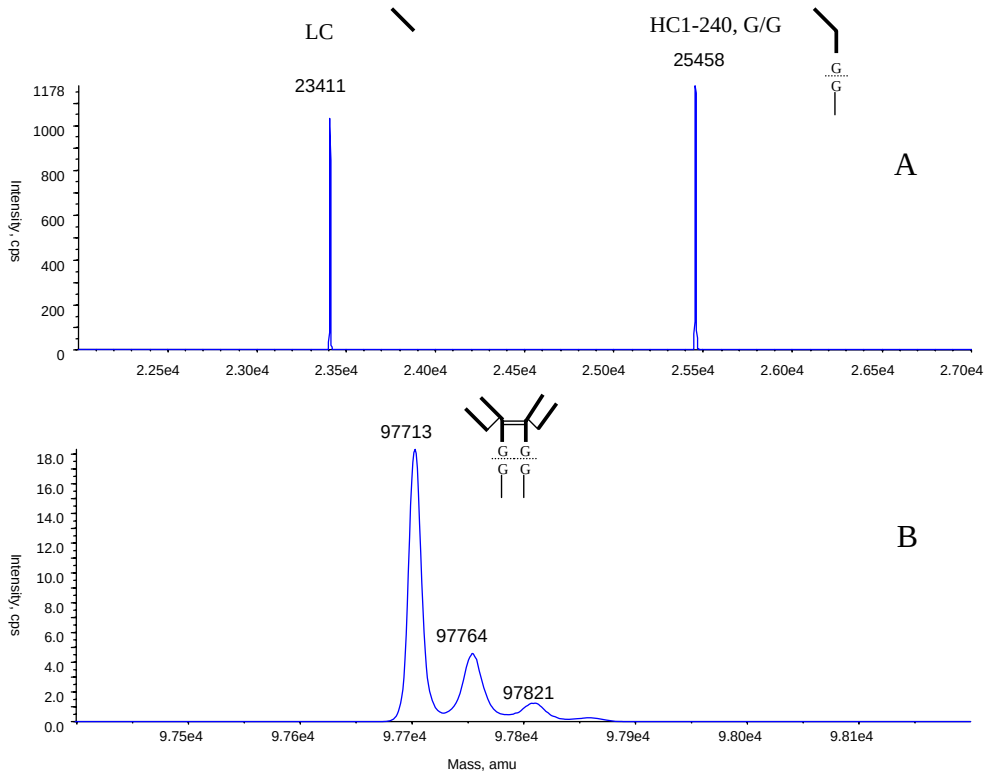

Fig. 7. A representative deconvoluted mass spectrum of the antibody after incubation at pH 4 for 14 h at 40°C. The spectrum was acquired from the antibody before (B) and after reduction (A).

between G240 and G241. Degradation of the antibody with or without oligosaccharides at pH 4 occurred more rapidly than at any other pH. The rapid degradation was not observed in our previous study (20), where the same batch of antibody was used but with a buffer consisting of 50 mM citrate. Based on additional repetitive experiments (n=4) in our laboratory, it was confirmed that antibody fragmentation, at least for this particular antibody, was highly pH dependent. A pH difference of 0.1 unit resulted in a significant change in the fragmentation rate at 40°C. This suggests that subtle difference in buffer pH or composition under acidic conditions may lead to dramatic conformational changes that may alter the rate and site of peptide bond hydrolysis.

In summary, we demonstrated that although the levels of antibody hinge region fragments can be easily quantified by simple analytical techniques such as SEC, a detailed analysis by mass spectrometry was necessary to understand the impact of pH on the sites of peptide bond cleavage by the two different mechanisms of hydrolysis. The results provided evidence of pH dependent antibody structural rearrangements, which impacted the levels, and sites of peptide bond cleavage. It suggests that a pH near 6 would be optimal for the stability of this antibody in liquid formulation, at least from the hinge region fragmentation point of view. Properties of antibody molecules such as the presence or absence of the N-linked oligosaccharides as well as conditions such as pH and temperature can all affect antibody stability. Although it is reasonable to predict that the level of antibody fragmentation will be significantly lower at 2–8°C, the combined effects of pH and temperature may make it difficult to predict the fragmentation pattern, which is modulated by antibody conformation. Understanding antibody fragmentation mechanisms and the role of buffer pH could provide invaluable information for proper formulation of antibodies, which is an integral part for the continuous and successful development of monoclonal antibody therapeutics.

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