Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 47 (2008) 23–30
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

# Identification and quantification of degradations in the Asp–Asp motifs of a recombinant monoclonal antibody

Gang Xiao, Pavel V. Bondarenko *

*Amgen, Department of Pharmaceutics, One Amgen Center Drive, Thousand Oaks, CA 91320, United States*

Received 12 September 2007; received in revised form 20 November 2007; accepted 28 November 2007
Available online 14 December 2007

**Abstract**

Two degradations of aspartate residues located in Asp–Asp motifs in the CDR3 region of a recombinant monoclonal antibody were identified and quantified after the antibody was aged in a mildly acidic buffer at elevated temperatures. The degraded sample aged at 25 °C for 1 month generated 1.8% antibody molecules that had isomerization in the aspartate residues, while the degraded sample after aging at 45 °C for 1 month contained 7% isomerization. Peptide bond cleavages at the aspartate residues were also detected and characterized. The percentage of clipped antibody molecules after 1 month of storage was 1% at 25 °C and 4.4% at 45 °C. The generated cleaved polypeptides were noncovalently attached to the intact antibody molecule and were not involved in the aggregation formation. They were not detected by native size-exclusion chromatography because of their strong non-covalent association to the rest of the antibody molecules. On the other hand, the cleaved polypeptides were dissociated and detected as fragments under denaturing conditions of reversed-phase HPLC, denaturing size-exclusion chromatography and MALDI-TOF mass spectrometry. It was demonstrated that the cleavages at the above aspartate residue sites occurred due to the aging of the sample at elevated temperatures and were not method-induced by the reversed-phase HPLC and other methods used in this study.
© 2007 Elsevier B.V. All rights reserved.

*Keywords:* Isoaspartate; Immunoglobulin gamma antibody; Peptide bond hydrolysis; Reversed-phase liquid chromatography; Mass spectrometry

## 1. Introduction

Proteins are subject to degradation via both chemical and physical pathways, presenting challenges to the pharmaceutical industry during the process of protein purification, formulation, storage and delivery. Mildly acidic buffers have been widely used for formulation of proteins including antibodies, because physical changes in proteins, including dimer formation and aggregation and covalent modifications, such as deamidation, enzymatic cleavages and oxidation, tend to be minimized at pH 4–6 [1,2]. However, under these conditions, aspartate (Asp) residues can undergo degradations through the following two main pathways. One is isomerization of Asp residues [3–10]. Asp residues can form a cyclic imide intermediate (succinimide, Suc), which then hydrolyzes into Asp and isoaspartate (isoAsp) residues typically at a ratio of 1:3 [4] in short peptides (Fig. 1). It has been reported in great detail that low pH and high temperature accelerate Suc formation [4–7,11]. The effect of different amino acids C-terminal to Asp residue has been studied, indicating that C-terminal glycine (Gly) and serine (Ser) facilitate aspartate isomerization and Suc formation in peptides and proteins [12,13] including antibodies [14–18]. Histidine (His) was identified as particularly labile among several tested residues N-terminal to Asp [19].

The other main Asp degradation pathway is Asp cleavage. It was well documented that acid hydrolysis occurred at the C-terminal side of Asp residue that was followed by proline (Pro) [20], glycine (Gly) [6] and alanine (Ala) [21] residues. Several mechanisms of the aspartyl peptide bond cleavage were proposed [6,19,22].

Complementarity determining regions (CDRs) of antibodies are flexible and solvent exposed loops of antibodies responsible for binding to therapeutic target. The lack of secondary structure and exposure to solvent make aspartate residues in CDRs susceptible to degradations, which can lead to reduction in binding and biological activity [14]. Therefore, it is very important to identify and quantify Asp degradations particularly in the variable regions for therapeutic proteins. However, an accurate

* Corresponding author. Tel.: +1 805 447 7215; fax: +1 805 447 3259.
*E-mail address:* pavel.bondarenko@amgen.com (P.V. Bondarenko).

0731-7085/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2007.11.050

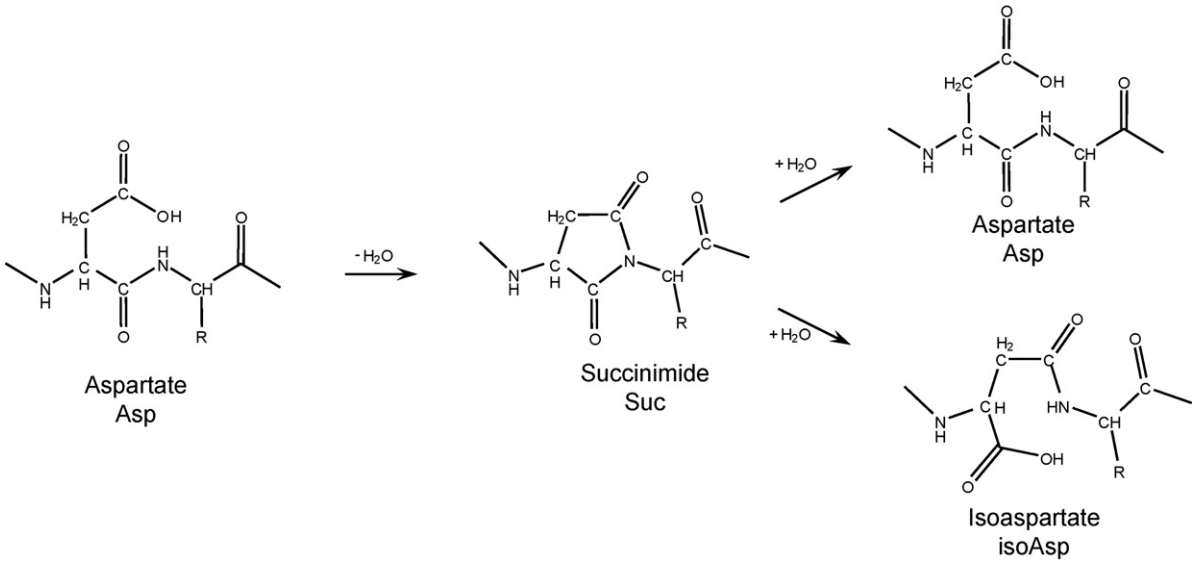


Fig. 1. Isomerization of the Asp through formation of the Suc that then hydrolyzes into Asp and isoAsp typically at a ratio of 1:3.

analysis of aspartate degradation products brings up an analytical challenge since the analysis itself may cause cleavage and isomerization. For example, the reversed-phase chromatography can be complicated by the on-column acid hydrolysis at the low pH and elevated temperatures. In addition, aspartate isomerization does change neither charge nor mass, which makes this modification difficult for detection by mass spectrometry. To complicate things further, therapeutic antibodies may contain multiple Asp residues in close vicinity. Ion exchange [14] and hydrophobic interaction [18] chromatography methods were utilized to monitor isoAsp, but these techniques will not identify location of isomerization.

In the present study, different analytical methods were employed to identify and quantify Asp degradations in a monoclonal recombinant antibody that had two pairs of Asp residues separated apart by two other residues in the heavy chain variable region.

## 2. Experimental

### 2.1. Materials

The recombinant monoclonal IgG2 antibody analyzed in this study was expressed at Amgen and purified by standard manufacturing procedures. It has been stored in mildly acidic (pH 5.2) buffers at 4, 25 and 45 °C for 1 month prior to analysis. The control sample has been stored frozen at −70 °C in the same buffer.

### 2.2. RP HPLC/MS of intact antibodies

Reversed-phase separation was carried out on an Agilent 1100 series CapLC equipped with an Agilent Zorbax SB 300 C8, 50 mm × 1 mm column (part #HP865630-902) according to the previously reported methods [23,24]. A protein sample of 5 μg was typically injected, and elution was achieved with a linear gradient from 21 to 27% of solvent B over 22 min for the intact protein. Solvent A was 0.1% aqueous TFA and solvent B contained 90:9.9:0.1 of *n*-propanol:water:TFA. The flow-rate and temperature were maintained at 50 μl/min and 75 °C throughout the run, respectively.

Mass spectrometric analysis was carried out on a Waters LCT Premier orthogonal-TOF mass spectrometer equipped with an electrospray ionization (ESI) source. The analysis was carried out in positive ion V mode. The capillary and cone voltages were set at 3000 and 100 V, respectively. Ion guide voltage was at 125 V. The dissolvation and source temperatures were set at 400 and 100 °C, respectively. The ESI mass spectra of intact antibodies were deconvoluted using Waters Masslynx MaxEnt 1 software to obtain deconvoluted masses [25].

### 2.3. Reduction, alkylation and tryptic digestion

The original protein sample (20–30 mg/ml) was diluted to 2 mg/ml in a 0.5 ml buffer containing 7.5 M guanidine HCl (GndHCl), 0.1 M Tris–HCl, and 1 mM EDTA at pH 7.5. Five microliters of 0.5 M DTT solution was added and the solution was incubated at 37 °C for 30 min. It was then cooled down to room temperature, a 13 μl aliquot of 0.5 M IAA solution was added, and the sample was placed at room temperature for 40 min in the dark. The sample was then buffer exchanged to 1 ml of 0.1 M Tris–HCl at pH 7.5 by NAP-5 column (Amersham BioSciences, Uppsala, Sweden) following the manufacturer's protocol. Tryptic digestion was performed for 2 h at 37 °C using an enzyme: protein weight ratio of 1:50. Then, the same amount of trypsin was added and protein was digested for another 2 h.

### 2.4. Peptide mapping

The antibody digestion products were separated using a Polaris C18-Ether 3 μm particle, 250 mm × 2 mm column (Varian, part number 2021-250X020) maintained at 50 °C. The

mobile phase consisted of 0.1% aqueous TFA as solvent A and 90:9.015:0.085% of acetonitrile:water:TFA as solvent B. The linear gradient from 0 to 50% B over 195 min was performed at a flow rate of 0.2 ml/min. The HPLC system was directly coupled to a Finnigan LCQ Deca ion trap mass spectrometer (Thermo Electron, San Jose, CA, USA) equipped with an ESI source. The electrospray voltage was 4.5 kV and the capillary temperature was 250 °C. The mass spectra were obtained using a triple play method with a full survey scan followed by zoom scan and MS/MS scan of the most intensive peak from the full scan. Collision energy of 35% was used to obtain fragmentation spectra. A Dynamic Exclusion feature was enabled with a repeat count of 2, repeat duration of 0.5 min, and exclusion duration of 1.0 min. Instrument was tuned using a synthetic peptide with *m/z* of 786. A Mass Analyzer software developed in-house [26] was used for peptide identification by correlating the sequence of the antibody to fragmentation mass spectra. Manual identification was required for several peptides containing sugars and for some non-specific or missed cleavages. Thermo Finnigan BioWorks 3.1 software was also used for peptide identification.

### 2.5. Size-exclusion chromatography under non-denaturing (SEC) and denaturing (dSEC) conditions

SEC was performed isocratically on an Agilent HP1100 HPLC system equipped with a TSK SWXL 3000 column. It was maintained at 37 °C and eluted by a mobile phase consisting of 0.1 M sodium phosphate (pH 7.0), 0.5 M sodium chloride, and 5% of ethanol at a flow rate of 0.2 ml/min. dSEC was carried out on the same column but using a mobile phase consisting of 0.1 M sodium phosphate (pH 6.5) and 2.5 M guanidine HCl. The sample was diluted to 1 μg/μl either using water for SEC or using a buffer consisting of 0.1M Tris and 7.5 M GndHCl at pH 7.5 for dSEC. Typically, a protein sample of 20 μg was loaded onto the column.

### 2.6. MALDI-TOF analysis of the intact antibody samples

MALDI-TOF analysis was performed using a Voyager-Pro (Applied Biosystems, Foster City, CA, USA) in a positive ion linear mode with an accelerating voltage of 25 kV and extraction delay time of 750 ns. Each spectrum was acquired with 100 laser shots. A saturated sinapic acid (SA, Sigma) solution was prepared using a solvent containing 0.3% TFA, 50% acetonitrile and 50% water. The SA solution was sonicated for 5 min and then allowed to stand 10 min at room temperature prior to use. A 1 μl aliquot of the intact antibody sample and 1 μl aliquot of SA matrix supernatant was mixed in a 0.5 ml Eppendorff tube and spotted onto a MALDI gold plate (Applied Biosystems).

## 3. Results and discussion

### 3.1. Isomerization of Asp

To determine whether any Asp residues were isomerized during aging, tryptic peptide mapping has been performed on frozen (−70 °C) and degraded samples (aged at 4, 25 and 45 °C for 1 month). Fig. 1 shows the RP chromatograms of the samples after storage at 4 and 25 °C. All peaks of the peptide map chromatograms were well aligned with the exception of the two peaks eluting at 144 and 146 min. The peak eluting at 146 min slightly decreased and was identified by ion trap tandem mass spectrometry as a peptide of heavy chain containing the CDR3 region with the four closely located Asp residues (Fig. 2, peptide H11). In addition, a new peptide with the same mass eluted at 144 min (isoH11; Fig. 2). This isopeptide (isoH11) eluted 2 min earlier and had the same mass and sequence, according to the tandem mass spectra, as peptide H11, indicating this isopeptide was due to Asp isomerization (Fig. 3). It was reported that peptides with isoaspartyl residues possess the same mass, but typically elute earlier than with aspartyl residues [4]. In previous studies, succinimide was accumulated under the mildly acidic conditions [15,16] and even survived several hours at neutral and alkaline pH and elevated temperatures, and appeared on peptide maps [4,18,27]. In this study, Suc peak was not detected in the peptide map (Figs. 2 and 3), indicating that there was no or little accumulation of Suc in the peptide. By the peptide mapping analysis, 0.4, 1.8 and 7.4% of Asp isomerization have been detected after aging at 4, 25 and 45 °C for 1 month, respectively. Since there were four aspartic acid residues in the peptide, the site of isomerization could not be determined by this peptide mapping method. Edman sequencing was previously used to establish exact location of isoAsp in peptides. However, because isoH11 was a very long peptide and the four Asp residues were close to C-terminus, the Edman sequencing would be difficult considering the poor yield for the amino acid residues that are far away from the N-terminus of the peptide. None of the other Asp residues of this recombinant monoclonal antibody, including heavy chain Asp280Gly281, Asp399Ser400, Asp401Gly402 and light chain Asp167Ser168, Asp171Ser172 (Edelman numbering), underwent isomerization, probably because those residues were engaged in hydrogen bonding creating secondary structure and were not exposed to the solvent like the residues in the flexible loops of the CDR [28].

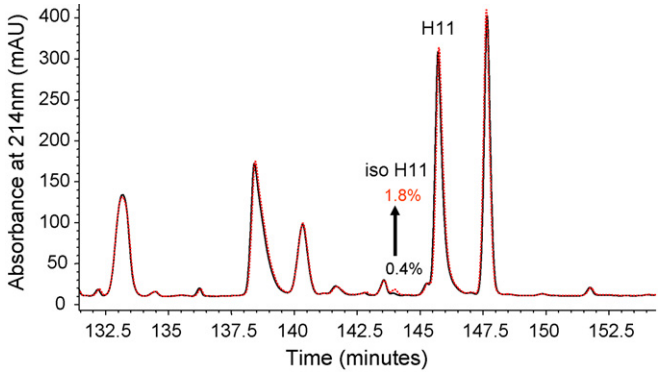


Fig. 2. A section of tryptic peptide maps of the antibody samples aged in a mildly acidic buffer (pH 5.2) at 4 °C (solid line) and 25 °C (dotted line) for 1 month showed an increase for the isomerized tryptic peptide number 11 of heavy chain (isoH11) in the 25 °C sample.

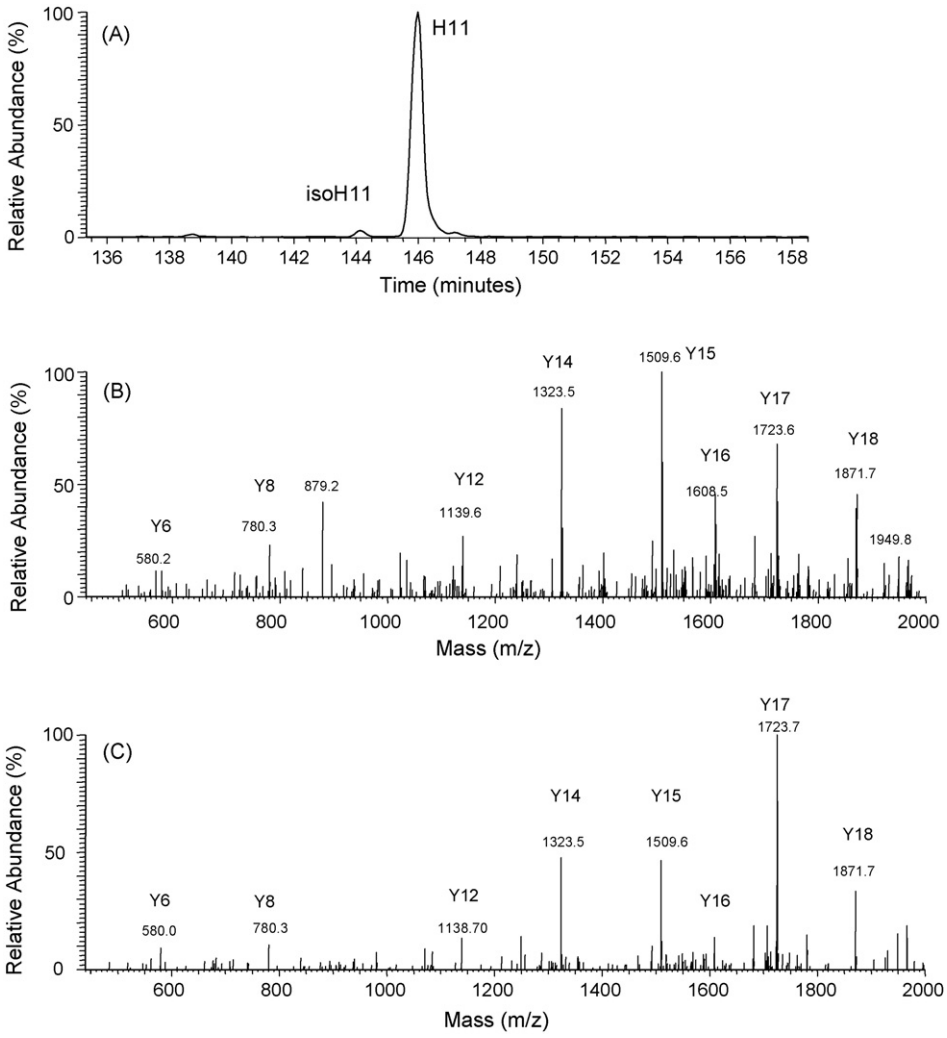


Fig. 3. (A) Reconstructed ion chromatogram for the heavy chain tryptic peptide number 11(H11) containing two Asp–Asp motifs in the close vicinity in the primary amino acid sequence of CDR3, and its isopeptide. (B and C) Fragmentation mass spectra of peptides H11 eluting at 146 min and isoH11 eluting at 144 min, respectively.

### 3.2. Detection and identification of the Asp cleavages

In addition to isomerization, cleavages at the C-terminal side of Asp residues were detected by RP HPLC/MS analysis of intact antibodies. The generated N-terminal fragments of the heavy chain have been detected for degraded samples as earlier eluting peaks containing N-terminal fragments of heavy chain (Fig. 4). The fragments were increasing at 8.5 min in the degraded samples. For the samples after storage at 4, 25 and 45 °C for 1 month, there was 0.8, 1.0 and 4.4% of the clipped molecules detected, respectively (Fig. 4). Only 0.7% of the clips have been detected for the frozen sample that was used as a control. The masses of these clips corresponded to N-terminal fragments of heavy chains generated by the cleavage at the C-terminal sides of the Asp residues in the CDR3 region (Fig. 5). Because the remaining counterpart (the high molecular weight fragment) of the clipped antibody molecule was not chromatographically resolved from the main peak, the percentages of clipped antibody molecules in Fig. 4 were calculated using peak areas of the low molecular weight (LMW) fragments, considering the molecular weight (MW) ratio of the LMW fragments (11 kDa) and the intact antibody molecules (147 kDa). The percentage of the clipped antibody molecules equals to the peak area percentage of the LMW fragments on the chromatogram multiplied by the ratio of 147 kDa/11 kDa (Fig. 4). Shown in Fig. 5 is the deconvoluted ESI mass spectrum that contains four peak groups corresponding to the masses of the four N-terminal fragments (named 1–4). This was further confirmed by tryptic peptide mapping of the LMW fragments, collected at 8 min elution time (Fig. 4), that identified the C-terminal amino acid of each fragment (data not shown). The intact antibody was partially separated on three peaks eluting between 29 and 40 min (Fig. 4). The measured MW values for these peaks were the same within the error margin of the mass spectrometer (±2 Da). This polymorphism was previously attributed to disulfide heterogeneity found in IgG2 antibodies by reversed-phase HPLC [23] and will not be discussed here.

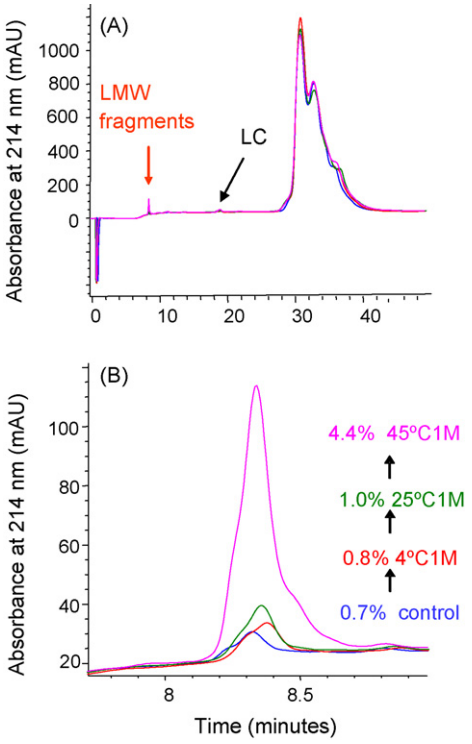


Fig. 4. Reversed-phase chromatograms (A) and sections of the chromatograms around 8.5 min containing low molecular weight fragments (B) for the antibody samples aged at elevated temperatures. A frozen sample (at −70 °C), containing 0.7% of low molecular weight fragments, was used as a control.

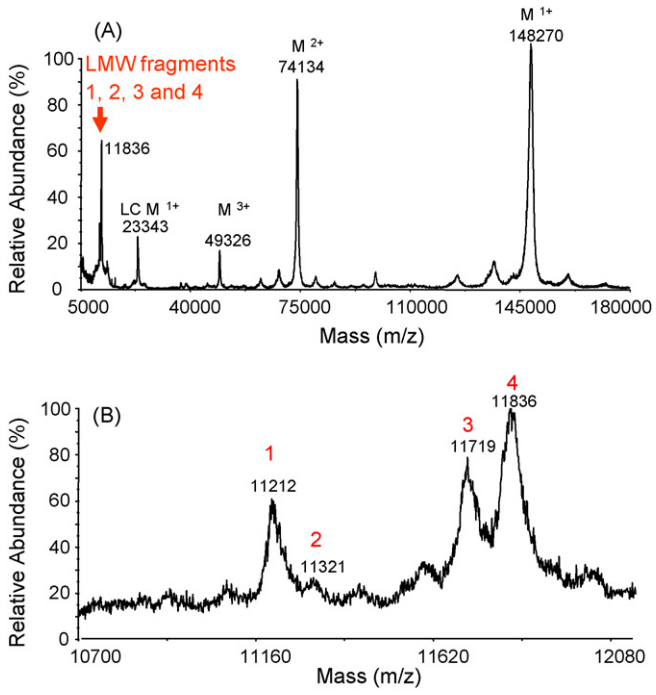


Fig. 6. (A) MALDI-TOF mass spectrum of the intact antibody sample aged at elevated temperature (45 °C for 1 month) containing singly ($M^{1+}$), doubly ($M^{2+}$) and triply ($M^{3+}$) charged intact antibody ions and clipped fragment ions. (B) A section of mass spectrum with the N-terminal cleavage fragments of the heavy chain, which was also detected by RP-HPLC/MS using the ESI orthogonal-TOF MS in Figs. 3 and 4. The calculated MW values were 148,510 Da for intact antibody, 50823 Da for the heavy chain and 23,432 Da for the light chain.

### 3.3. The Asp cleavages were due to the sample aging in a mildly acidic buffer at elevated temperatures

Under the high-temperature, low-pH conditions of the RP HPLC method used in this study, Asp270Pro271 peptide bond (in the heavy chain of the Fc region, Edelman numbering) cleavage was detected (data not shown). To verify that the clipped fragments from Asp cleavage were due to the aging condition and not induced by the RP column conditions, further analyses by other methods were carried out. MALDI-TOF MS analysis of the frozen and aged samples clearly showed the increase of the fragments in the aged samples, similar to the fragment increased observed by RP-HPLC/MS using the ESI orthogonal-TOF MS in Figs. 3 and 4. The MALDI-TOF mass spectrum of the sample aged at 45 °C for 1 month contained singly, doubly and triply charged intact antibody ions and the N-terminal LMW fragments 1–4 (Fig. 6). The mass spectrum of the frozen control sample contained only the multiply changed antibody ions and no fragments (data not shown). MALDI-TOF analysis confirmed that the fragments observed in Figs. 4 and 5 are real and not related to the low-pH column conditions.

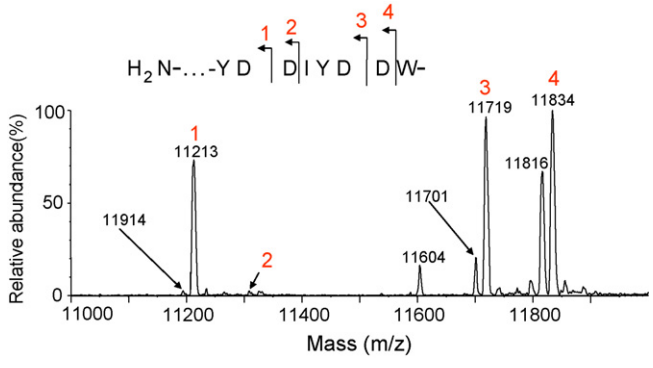


Fig. 5. Deconvoluted ESI mass spectrum of the fragments that eluted at 8.5 min in the chromatograms in Fig. 3. Four groups of peaks were identified as the N-terminal low molecular weight fragments from cleavage at the C-terminal sides of each Asp residue in the CDR3 of the heavy chain. The C-terminus amino acids have been confirmed to be Asp by tryptic peptide mapping. The loss of water ($-H_2O$) was through the gas-phase dissociation in the atmosphere vacuum interface.

SEC of the recombinant monoclonal samples stored frozen at −70 °C and aged at 45 °C for 1 month showed an increase in the dimer and aggregate peaks (Fig. 7A), but did not detect any LMW fragment species. It was initially expected that the heavy chain fragments will elute in the LMW region. In contrast to SEC, dSEC of the same samples allowed detection of LMW species (Fig. 7B). The RP HPLC/MS analysis has been performed for the collected later eluting species and main peak fractions from dSEC (Fig. 8). RP HPLC/MS analysis of these species has identified them as the light chain (LC) and the N-terminal LMW fragments also observed in Figs. 4 and 5.

In addition, the dSEC main peak fractions were collected from the control and degraded samples and were further analyzed by RP HPLC/MS (Fig. 8C). No fragments were observed for these fractions, further proving that the N-terminal fragments

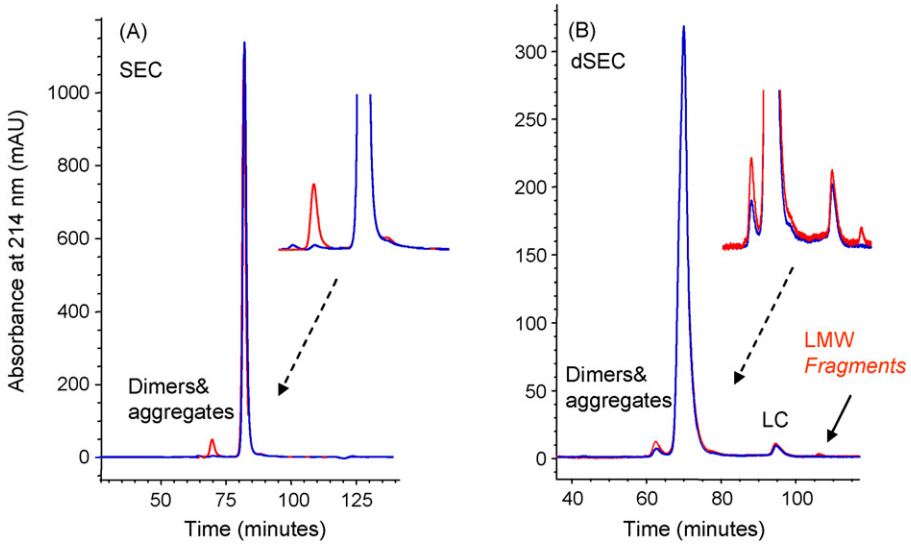


Fig. 7. (A) Size-exclusion chromatography (SEC) of the recombinant monoclonal antibody samples frozen at −70 °C (blue line) and aged at elevated temperature (45 °C for 1 month, red line) in the mildly acidic buffers. (B) Denaturing size-exclusion chromatography (dSEC) of the same samples.

completely dissociated under dSEC conditions and the Asp and isoAsp containing peptide bonds at the above sites are not susceptible to on-column hydrolysis under the RP HPLC/MS conditions used in this study.

### *3.4. The cleaved N-terminal polypeptides were attached to the intact antibody molecules and were not involved in the dimer and aggregate formation measured by SEC*

The fact that the cleaved N-terminal heavy chain polypeptides were not observed by SEC (Fig. 7A), but were detected by denaturing SEC (Fig. 7B) indicated that these cleaved polypeptides were strongly noncovalently bound to the rest of the antibody molecules, which prevented their dissociation and detection by native SEC. The N-terminal heavy chain polypeptides generated by cleavage at the Asp residues included the entire variable domain of the heavy chain (VH). This domain is packed against the variable domain of light chain (VL) via strong hydrophobic interactions [29]. After cleavage at the C-terminal side of the Asp residues, the N-terminal polypeptides were still attached to the antibody molecules via the strong interaction between the variable

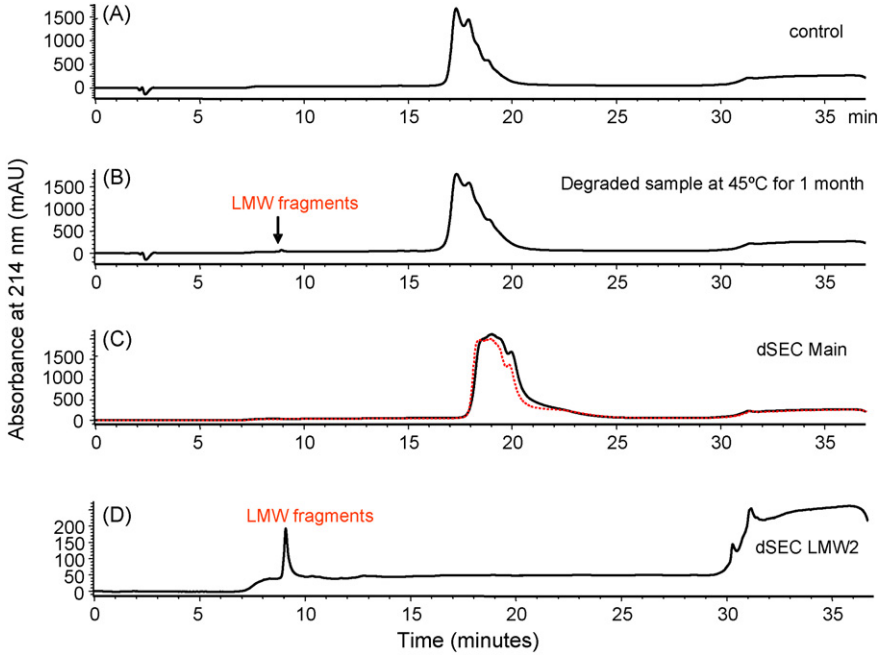


Fig. 8. Reversed-phase chromatograms for (A) frozen control sample, (B) the sample aged in a mildly acidic buffer at 45 °C for 1 month, (C) collected fractions of dSEC main peak from the frozen control sample (solid) and aged in a mildly acidic buffer at 45 °C for 1 month (dotted) and (D) LMW fragments from the aged sample shown in Fig. 7B.

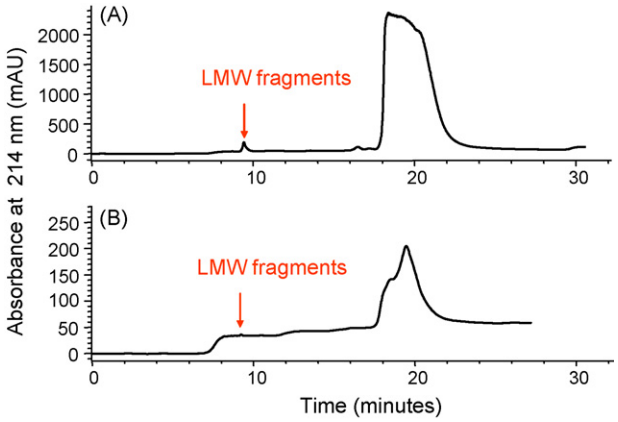


Fig. 9. Reversed-phase chromatograms of the collected SEC fractions from the sample aged in the mildly acidic buffer at 45 °C for 1 month: main peak fraction (A) and dimer and aggregate fraction (B). The size-exclusion chromatography utilized to separate and collect these fractions is shown in Fig. 7B.

domains unless this interaction was disrupted under denaturing conditions.

The fragments (dissociated cleaved polypeptides) were thought to initiate aggregation, which resulted in appearance of the dimmer and aggregate species. In order to investigate this possibility, SEC main peak and dimer and aggregate fractions were collected and analyzed by RP HPLC/MS (Fig. 9). Contrary to the expectations, the abundance of the fragments was not elevated in the dimer and aggregate fraction. The experiment indicated that the formation of the dimers and aggregates was not seeded by the cleaved polypeptides, but rather due to other reasons. The cleaved polypeptides remained bound to the remaining monomer antibody molecules and were not involved in formation of dimer and aggregate species.

Although several mechanisms have been proposed previously for Asp–Xxx [6,22] and Xxx–Asp [19] cleavage, they did not directly address the Asp–Asp motif. In this study, one of those mechanisms was adopted for Asp–Asp using the following rationale (Fig. 10). Joshi et al. [22] reported that cleavage rates for several Asp–Xxx sites of glucagon in acidic buffers (pH 2–5) were correlated with their p$K_a$ values measured by NMR. The authors found that the Asp residues in Asp–Tyr and Asp–Ser motifs with lowest apparent p$K_a$ had highest cleavage rate. The proposed cleavage pathway involved acid catalyzed intramolecular nucleophilic attack of the aspartyl carboxylic acid side chain on the peptide backbone carbonyl center (C=O), resulting in the formation of a cyclic anhydride intermediate [22]. The ionized form of the Asp side chain should be more reactive because it is a better nucleophile. Applying this mechanism to the case of the Asp–Asp motif, we suggest that two neighboring Asp can lower each other p$K_a$ values by forming a hydrogen bond with side chain carboxyl acid protons (Fig. 10A). This facilitates ionization of the carboxyl group, followed by the nucleophilic attack and cleavage (Fig. 10B). Another hypothesis (added by one of the reviewers of this manuscript) is that the Asp–Asp cleavage could be a result of electrostatic repulsion between the neighboring residues when both are ionized. It would be interesting to test this hypothesis using peptide models.

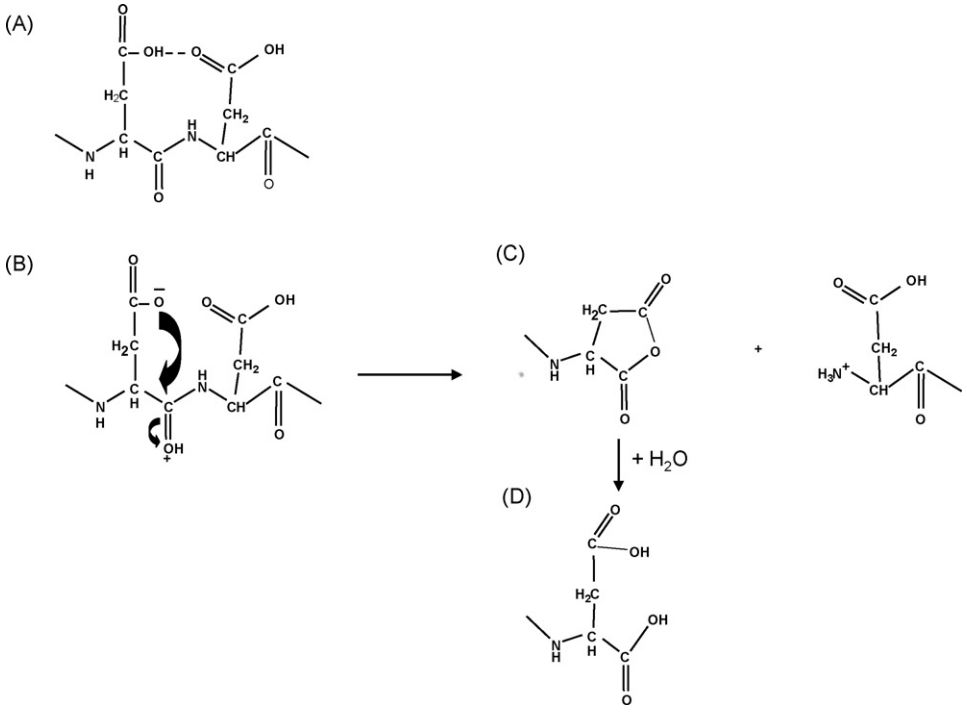


Fig. 10. Proposed mechanism for the cleavage at the Asp–Asp motifs in a CDR region of the recombinant monoclonal antibody under the mildly acidic conditions (pH 4–5). (A) The side chain carboxyl acid protons are hydrogen bonded to the oxygen of the carboxyl groups of their neighboring Asp residue, lowering p$K_a$ and facilitating ionization of the side chain carboxyl. (B) The ionized side chain attacks the peptide backbone carbonyl to cleave the peptide bond at the C-terminus of aspartic acid and form an anhydride intermediate (C). (D) The anhydride intermediate is hydrolyzed after reaction with water leading to mass increase by 18 Da.

## 4. Conclusions

The degradations of two Asp–Asp motifs closely positioned in a complementarity determining region (CDR) in heavy chain of a recombinant monoclonal IgG2 antibody were studied after aging in mildly acidic buffers at elevated temperatures. Asp isomerization and cleavage were the two main chemical degradation pathways in these motifs. It was demonstrated that these degradations occurred during aging, and were not induced by the high-temperature, low-pH environment of the RP HPLC column. Several N-terminal polypeptides from the Asp cleavages included entire variable domain of the heavy chain (VH) and were strongly noncovalently attached to the rest of the antibody molecules. The cleaved polypeptides were dissociated, separated and detected only under the denaturing conditions of dSEC, RP HPLC/MS and MALDI, but not by native SEC.

## Acknowledgements

We thank Lejla Karamujic and Deirdre Piedmonte for providing samples for this study. We would like to acknowledge Mike Treuheit for valuable discussions and suggestions. We are grateful to Alexis Lueras for carefully reading the manuscript.

## References

[1] A.C. Herman, T.C. Boone, H.S. Lu, in: R. Pearlman, Y.J. Wang (Eds.), Formulation, Characterization, and Stability of Protein Drugs. Case Histories, Plenum Press, New York, 1996, pp. 303–328.
[2] X.M. Lam, J.Y. Yang, J.L. Cleland, J. Pharm. Sci. 86 (1997) 1250–1255.
[3] M.C. Manning, K. Patel, R.T. Borchardt, Pharm. Res. 6 (1989) 903–918.
[4] T. Geiger, S. Clarke, J. Biol. Chem. 262 (1987) 785–794.
[5] C. Oliyai, R.T. Borchardt, in: J.L. Cleland, R. Langer (Eds.), Formulation and Delivery for Proteins and Peptides, American Chemical Society, Washington, DC, 1994, pp. 47–58.
[6] C. Oliyai, R.T. Borchardt, Pharm. Res. 10 (1993) 95–102.
[7] C. Oliyai, R.T. Borchardt, Pharm. Res. 11 (1994) 751–758.
[8] T. Abern, M.C. Manning, Stability of Protein Pharmaceutics, Plenum, New York, 1992.
[9] M.F. Powell, in: R. Pearlman, Y.J. Wang (Eds.), Formulation, Characterization and Stability of Protein Drugs: Case History, vol. 9, Plenum Press, New York, 2006, pp. 1–140.
[10] J.L. Cleland, M.F. Powell, S.J. Shire, Crit. Rev. Ther. Drug Carrier Syst. 10 (1993) 307–377.
[11] S. Clarke, Int. J. Pept. Protein Res. 30 (1987) 808–821.
[12] B.A. Johnson, J.M. Shirokawa, W.S. Hancock, M.W. Spellman, L.J. Basa, D.W. Aswad, J. Biol. Chem. 264 (1989) 14262–14271.
[13] D.W. Aswad, M.V. Paranandi, B.T. Schurter, J. Pharm. Biomed. Anal. 21 (2000) 1129–1136.
[14] R.J. Harris, B. Kabakoff, F.D. Macchi, F.J. Shen, M.Y. Kwong, J.D. Andya, S.J. Shire, N. Bjork, K. Totpal, A.B. Chen, J. Chromatogr. B Biomed. Sci. Appl. 752 (2001) 233–245.
[15] G.C. Chu, D. Chelius, G. Xiao, H.K. Khor, S. Coulibaly, P.V. Bondarenko, J. Pharm. Res. 24 (2007) 1145–1156.
[16] G. Xiao, P.V. Bondarenko, J. Jacob, G.C. Chu, D. Chelius, Anal. Chem. 79 (2007) 2714–2721.
[17] A.A. Wakankar, J. Liu, D. Vandervelde, Y.J. Wang, S.J. Shire, R.T. Borchardt, J. Pept. Sci. 96 (2007) 1708–1718.
[18] A.A. Wakankar, R.T. Borchardt, C. Eigenbrot, S. Shia, Y.J. Wang, S.J. Shire, J.L. Liu, Biochemistry 46 (2007) 1534–1544.
[19] T.V. Brennan, S. Clarke, Int. J. Pept. Protein Res. 45 (1995) 547–553.
[20] R. Lamed, R. Kenig, E. Morag, S. Yaron, Y. Shoham, E.A. Bayer, Appl. Biochem. Biotechnol. 90 (2001) 67–73.
[21] P.J. Groenen, P.R. van den Ijssel, C.E. Voorter, H. Bloemendal, W.W. de Jong, FEBS Lett. 269 (1990) 109–112.
[22] A.B. Joshi, M. Sawai, W.R. Kearney, L.E. Kirsch, J. Pharm. Sci. 94 (2005) 1912–1927.
[23] T.M. Dillon, P.V. Bondarenko, D.S. Rehder, G.D. Pipes, G.R. Kleemann, M.S. Ricci, J. Chromatogr. A 1120 (2006) 112–120.
[24] D.S. Rehder, T.M. Dillon, G.D. Pipes, P.V. Bondarenko, J. Chromatogr. A 1102 (2006) 164–175.
[25] H.S. Gadgil, G.D. Pipes, T.M. Dillon, M.J. Treuheit, P.V. Bondarenko, J. Am. Soc. Mass Spectrom. 17 (2006) 867–872.
[26] Z. Zhang, Anal. Chem. 76 (2004) 3908–3922.
[27] D. Chelius, D.S. Rehder, P.V. Bondarenko, Anal. Chem. 77 (2005) 6004–6011.
[28] A. Honegger, A. Pluckthun, J. Mol. Biol. 309 (2001) 657–670.
[29] C. Branden, J. Tooze, in: C. Branden, J. Tooze (Eds.), Introduction to Protein Structure, Garland Publishing Inc., New York, 1999, pp. 299–324.