

Identification and Characterization of Deamidation Sites in the Conserved Regions of Human Immunoglobulin Gamma Antibodies

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Deamidation of asparagine residues of biological pharmaceuticals is a major cause of chemical degradation if the compounds are not formulated and stored appropriately. The mechanism of this nonenzymatic chemical reaction has been studied in great detail; however, the identification of deamidation sites in a given protein remains a challenge. In this study, we identified and characterized all deamidation sites in the conserved region of a recombinant monoclonal antibody. The conserved region of this antibody is shared by all human IgGs with the exception of minor differences in the hinge region. Our high-performance liquid chromatography method could separate the succinimide, isoaspartic, and aspartic acid isoforms of peptide fragments generated using trypsin. Each of the isoforms was unambiguously identified using tandem mass spectrometry. Deamidation at the identified four sites was slow for the intact, folded antibody at accelerated degradation conditions (pH 7.5 and 37 °C). Deamidation was enhanced after reduction, alkylation, and tryptic digestion, indicating that the three-dimensional structure of the antibody reduced deamidation. Furthermore, after the reduction, alkylation, and tryptic digestion, only 4 of a possible 25 asparagine residues showed deamidation, demonstrating the effect of the primary amino acid sequence, especially the –1 and +1 amino acids flanking the deamidation site. For instance, the amino acid motifs SNG, ENN, LNG, and LNN were found to be more prone to deamidation, whereas the motifs GNT, TNY, YNP, WNS, SNF, CNV, SNT, WNS, FNW, HNA, FNS, SNK, GNV, HNH, SNY, LNW, SNL, NNF, DNA, GNS, and FNR showed no deamidation. Our findings should help predict deamidation sites in proteins and peptides and help develop deamidation-resistant biological therapeutics.

Immunoglobulin Gamma (IgG) antibodies have emerged as a popular modality in protein pharmaceuticals due to their predictable properties, controlled functions, and long circulation life.

Although IgG antibodies are relatively stable molecules, they are subject to a variety of degradation reactions that can occur during manufacturing, formulation, and storage. A number of molecular modifications for antibodies have been reported in the

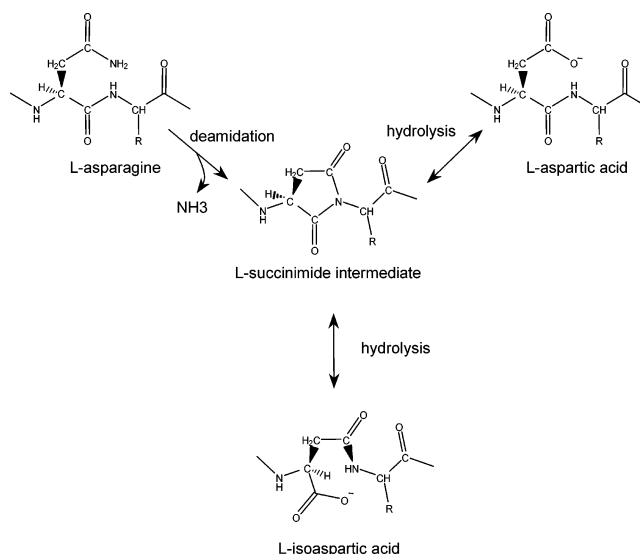


Figure 1. Succinimide-mediated pathway for the nonenzymatic degradation of asparagine and aspartate residues.

literature including aggregation, oxidation, proteolytic cleavage, disulfide bond scrambling, glycosylation, deamidation, and isomerization.^{1–4} These modifications can decrease the activity of protein pharmaceuticals. Deamidation of asparagine residues of biological pharmaceuticals is a major cause of degradation if the pharmaceutical proteins are not formulated and stored appropriately. The mechanism of this nonenzymatic chemical reaction has been studied in great detail. Asparagine residues can form a cyclic imide (succinimide) intermediate (Figure 1), which spontaneously hydrolyzes to a mixture of isoaspartic/aspartic acid at an approximate ratio of 3:1.^{5,6} To complicate matters further, the isomerization is accompanied by partial racemization of the newly formed isoaspartyl and aspartyl residues, which occurs preferentially at the stage of succinimide intermediate.⁷ The effects

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of pH and temperature also have been studied and suggest that high pH and high temperature accelerate the deamidation reaction.^{5,6} The solvent composition and properties (e.g., dielectric constants) seem to affect the rate of deamidation of asparagine. Solutions containing methanol show a decreased rate of deamidation.^{8,9} The rate of deamidation at asparagine residues is markedly reduced in solutions with low dielectric strength.¹⁰ These observations are in line with the theoretical mechanism (Figure 1) in which the deprotonated peptide bond nitrogen anion (the postulated attacking species in succinimide formation) is less stable in solvents with low dielectric constants. The effect of different amino acids at the C-terminal of asparagine for succinimide formation and isomerization has been reported, indicating that glycine, serine, and histidine residues are most favorable for succinimide formation of asparagine residues.¹¹ Other researchers have reported that amino acids at the N-terminal of asparagine do not seem to significantly affect the rate of deamidation.^{12–15}

Effects of the protein and peptide conformation on asparagine deamidation have been investigated. Kosky et al. reported the effect of the α -helical secondary structure on the stability of asparagine residues, showing that deamidation only occurs in nonhelical populations.¹⁶ A local β -turn conformation resulting in structural constraint seems to protect asparagine residue 67 in ribonuclease A from deamidation, and not accessibility, since the residue is exposed on the surface of the protein.¹⁷ The hydrophobic regions of proteins also show conformational constraints that prevent the formation of succinimide.¹⁸

The effect of deamidation on the biological activity of proteins has also been reported. Harris et al. showed that deamidation of asparagine residue 30 in one of the light chains of a commercial recombinant monoclonal antibody reduced its potency to 70%.¹⁹ Cell proliferation assays showed that two recombinant human stem cell factor heterodimeric species, derived from the dimerization between isoaspartyl and other stem cell factor monomers, retained approximately half of the biological activity.²⁰ The homodimer with isoaspartic acid in place of N10 was 50-fold less potent, while the aspartyl homodimer, either isolated during deamidation experiments or recombinantly prepared by site-directed mutagenesis (e.g., N10D and N10D/N11D variants), exhibited higher activity than the standard molecule.²⁰

In this report, we have identified four deamidation sites in the conserved region of a recombinant monoclonal antibody. The conserved region of this antibody is shared by all IgGs with the exception of minor differences in the hinge region. We developed a reversed-phase LC/MS method for peptide mapping, which was capable of separating and identifying the succinimide, isoaspartic, and aspartic acid products.

EXPERIMENTAL SECTION

Materials. The recombinant monoclonal antibody analyzed in this study was expressed in Chinese hamster cells and purified using standard manufacturing process steps. The antibody was prepared in 50 mM sodium acetate, 100 mM NaCl, pH 5.8, at a concentration of 20 mg/mL and stored at -80°C . Buffer exchange was performed using a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) according to the manufacture's protocol.

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Reduction, Alkylation and Tryptic Digestion. The recombinant human antibody was diluted to 2 mg/mL with 6 M guanidine hydrochloride, 0.25 M Tris-HCl, pH 7.5, 1 mM EDTA to a total volume of 500 μL . A 5- μL aliquot of 0.5 M DTT was added, and the reaction mixture was placed at 37°C for 30 min. The sample was cooled to room temperature. A 12- μL aliquot of 0.5 M iodoacetic amide was added, protected from light, and placed at room temperature for 40 min. The reduced and alkylated material was buffer exchanged using a gel filtration NAP-5 column (Amersham BioSciences, Uppsala, Sweden). The column was equilibrated with 10 mL of 0.1 M Tris-HCl pH 7.5 buffer, and the 500- μL volume of sample was loaded on the column. The reduced and alkylated antibody was eluted with a 1-mL aliquot of 0.1M Tris-HCl pH 7.5 buffer to a final protein concentration of ~ 1 mg/mL. Tryptic digestion was performed for 4 h or as specified in the text at 37°C using an enzyme/protein ratio of 1:50 (w/w). The same amount of trypsin (Roche, Indianapolis, IN) was added a second time after 2 h of incubation. The digests were frozen at -20°C and analyzed together.

HPLC Separation of Tryptic Peptides. The tryptic peptides were separated by reversed-phase HPLC using an Agilent 1100 HPLC equipped with a diode-array detector, autosampler, micro-flow cell, and temperature-controlled column compartment (Agilent, Palo Alto, CA). UV absorbance was monitored at 214 and 280 nm. A Polaris Ether column (250 $\times$ 2 mm) packed with a 3- μm nominal diameter, 300- $\text{\AA}$ pore size C_{18} resin (Varian, Torrance, CA) was used for the separations. The solvents were (A) 0.1% trifluoroacetic acid (TFA; Pierce, Rockford, IL) in water, and (B) 0.089% TFA in 90% acetonitrile (Baker, Phillipsburg, NJ) in water. Tryptic peptides were injected onto the RP-HPLC column after the column was equilibrated at 0% solvent B for 30 min. A linear gradient from 0 to 65% B was run over 195 min. The column temperature was maintained at 50°C . A total of 20 μg of protein digest was injected onto the column for mass spectrometry analysis.

Mass Spectrometry Analysis of Tryptic Peptides. The HPLC was directly coupled to a Thermo Finnigan LCQ Deca ion trap mass spectrometer (Thermo Electron, San Jose CA) equipped with an electrospray ionization source. The spray voltage was 4.5 kV, and the capillary temperature was 250°C . The fragmentation mass spectra were obtained using ion trap collision energies of 35%. Each full-scan mass spectrum was followed by a zoom scan

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Table 1. Predicted Peptides from a Trypsin Digestion of the Conserved Region of the Recombinant Monoclonal Antibody Analyzed in This Report^a

Heavy Chain: 124-GPSVFPLAPCSR 136-STSESTAALGCLVK

150-DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVFTVPSNFGTQYTCNVDHKPSNTK

213-VDK 216-TVER 320-K 221-CCVECPPCAPPVAGPSVFLFPPKPK 247-DTLMISR

254-TPEVTCVVVDVSHEDPEVFQFNWYVDGVEVHNAK 287-TKPR 291-EEQFNSTFR

300-VVSVLTVVHQDWLNGK 316-EYK 319-CK 321-VSNK 325-GLPAPIEK 333-TISK

337-TK 339-GQPR 343-EPQVYTLPPSR 354-EEMTK 359-NQVSLTCLVK

369-GFYPSDIAVEWESNGQPENNYK 391-TTPMLDSGDSFFLYSK 408-LTVDK 413-SR

415-WQGNVFSQSVMEALHNHYTQK 438-SLSLSPGK

Light Chain: 109-TVAAPSVFIFPPSDEQLK 127-SGTASVVCLLNIFYPR 143-EAK 146-VQWK

150-VDNALQSGNSQESVTEQDSK 170-DSTYSLSSTLTLSK 184-ADYEK 189-HK

191-VYACEVTHQGLSPVTK 208-SFNR 212-GEC

^a This sequence is shared by all human IgGs. The four deamidation sites are highlighted in boldface type.

and then a data-dependent MS/MS scan of the most intense ion. The dynamic exclusion feature was enabled (repeat counts 2, repeat duration 0.2 min, exclusion duration 2 min, and exclusion mass width 2 Da).

Data Analysis. Peptides were identified automatically by two different computer programs. BioWorks version 3.1 (Thermo Electron, San Jose, CA), which correlated the experimental tandem mass spectra against theoretical tandem mass spectra from a database. The database, containing only one protein (the recombinant monoclonal antibody) was created manually. No enzyme parameter was chosen, and the alkylated cysteines were examined as static modifications. Mass analyzer software developed in-house was also used to correlate the experimental tandem mass spectra against theoretical tandem mass spectra generated from the known antibody amino acid sequence for peptide identification.^{21,22}

The relative abundances of peptides were measured using base peak ion chromatogram data. Standard deviations of peak area measurements were less than 10% for all peptides, which did not show change in concentration. The quantification based on base peak ion chromatograms correlated well with quantification based on UV chromatograms measured by Agilent ChemStation software.

RESULTS AND DISCUSSION

The predicted tryptic peptides of the conserved region of the monoclonal antibody are illustrated in Table 1, the numbering of which is based on the amino acid sequence of the full-length antibody. This conserved region of the antibody is shared by all IgGs with the exception of minor differences in the hinge region.²³ The tryptic peptides containing only one or two amino acids (see Table 1) were not detected, due to the limited retention of small and hydrophilic peptides on the reversed-phase column. However, all peptides containing three or more amino acids could be identified using fragmentation information from the MS/MS spectra and the automated search programs BioWorks 3.1 and mass analyzer. Trypsin digestion of the monoclonal antibody

followed by HPLC separation and mass spectrometry analysis of the peptides resulted in the identification of several deamidation products. Figure 2a–d shows the tandem mass spectra of the four isoforms of the tryptic peptide, 369-GFYPSDIAVEWESNGQPEN-NYK-390 (from the heavy chain), which could be separated using reversed-phase chromatography method and eluted at 121, 122, 123, and 124 min. Figure 2a shows the tandem mass spectrum and the b, and y ion assignments for the native peptide containing asparagine residues at positions 382 and 387. The tandem mass spectra shown in Figure 2b–d resulted in the identification of succinimide, isoaspartic acid, and aspartic acid isoforms, respectively, indicating changes have occurred at amino acid residues 382 and 387 on the heavy chain of the antibody. The assignment for the isoaspartic acid-containing peptide and for the aspartic acid-containing peptide were based on the elution times from the reversed-phase analysis, where aspartic acid peptides elute later than their isoaspartic acid isomers. Succinimide formation at position 382 was determined based on the presence of the ions m/z 1763.4 (b16), 1676.4 (y14), 1577.5 (y13), and 1448.4 (y11) that showed a mass difference of –17 Da compared with the native peptide (Figure 2b). The ions at m/z 764.2 and 950.1 corresponding to the y6 and y8 fragmentation ions, which did not show a mass difference compared with the native peptide, further confirmed the identification and location of the succinimide at position 382, and not at the other two asparagine residues at positions 387 and 388 (Figure 2b). The location of isoaspartic acid formation was unambiguously identified at position 382, based on the presence of the ions at m/z 1782.8 (b16), 1694.8 (y14), 1595.5 (y13), and 1466.2 (y11), with a mass difference of +1 Da compared with the native peptide (Figure 2c). The ions at m/z 764.2 and 949.2, corresponding to the y6 and y8 fragmentation ions, did not differ in mass compared with the native peptide, further confirming the identification and location of isoaspartic acid residue at position 382 (Figure 2c). The formation of aspartic acid was identified at position 387 based on the presence of ions at m/z 668.4 (y5), 765.3 (y6), and 950.4 (y8), with a mass difference of +1 Da compared with the native peptide (Figure 2d).

The identification of four isoforms of tryptic peptide 300-VVSVLTVVHQDWLNGK-315 (from the heavy chain) eluting at 135, 137, 139, and 142 min are shown in Figure 3a–d. Additionally, three isoforms of peptide 127-SGTASVVCLLNIFYPR-142 (from the light chain) could be identified (Figure 4a–c), due to deamidation of amino acid residue 137 in the conserved region of the light chain (C_L). The presence of ions at m/z 696.5 (y6), which does not differ in mass from the native peptide, and the ion at m/z 811.4, which does differ in mass by +1 Da compared with the native peptide, unambiguously identifies that deamidation occurs at position 137 and not 138 (Figure 4b,c).

Since isoaspartic acid and aspartic acid do have the same molecular weight, it is not possible to distinguish between the two amino acids by mass spectrometry. Therefore, we relied on the fact that isoaspartic acid-containing peptides elute earlier as compared with their aspartic acid counterparts when using reversed-phase chromatography.^{13,19,24,25} A previous study has

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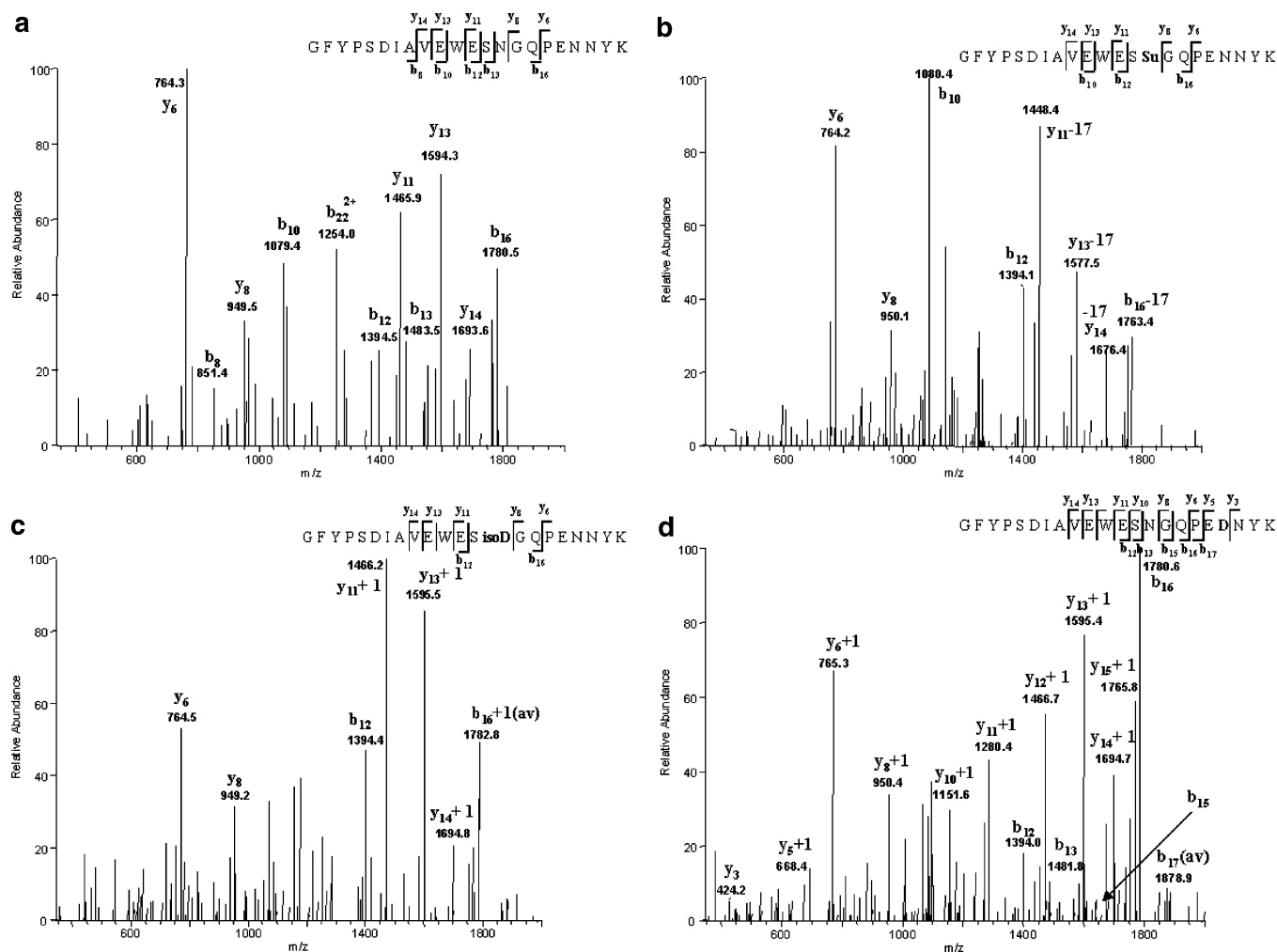


Figure 2. Tandem mass spectra derived by collision-induced dissociation of the $(M + 2H)^{2+}$ precursor ions at m/z 1772.9 (a), 1264.4 (b), and 1273.3 (c,d). The peptides were identified as 369-GFYPSDIAVEWESNGQPENNYK-390 (a) and its three isoforms: Su382 (b), IsoD382 (c), and D387 (d).

shown that b/y ion intensity ratio of complementary b and y ions generated by cleavage of the (D/isoD)–X bond could be used to distinguish between isoaspartic acid and aspartic acid isoforms.²⁶ In our examples, this was not the case, indicating that only a few selected peptides show this phenomenon or that the ion trap produces a different fragmentation pattern compared to the quadrupole mass spectrometry technology used in Lehmann's experiments. Racemization of the cyclic intermediate is another possible reaction involving asparagine deamidation, resulting in the generation of D-isoaspartic acid- and D-aspartic acid-containing peptides.^{7,27} These variants have not been detected in our study.

We further characterized the deamidation reaction to determine whether our sample preparation procedure affected the amount or rate of deamidation. Identification of the succinimide isoform was surprising, since this cyclic intermediate of the deamidation reaction should only be stable at low pH.¹³ To determine whether the deamidation reaction through succinimide formation was caused by the low pH and high temperature on the reversed-phase column, we injected a tryptic digest of the

antibody onto the reversed-phase column and incubated the reaction mixture for 1, 2, 4, and 8 h on the column before elution. The low pH and high temperature of the column did not affect the rate of deamidation for the three peptides studied (data not shown), which excluded the HPLC process as a cause of deamidation. Other possible sources for deamidation are the sample preparation steps including reduction, alkylation, and tryptic digestion, which were performed at pH 7.5 and 37 °C. To determine the rate of deamidation during these steps, we incubated the reduced and alkylated tryptic digestion products for 4, 18, 96, and 168 h at pH 7.5 and 37 °C. As expected, the abundance of deamidation products increased with time under these conditions (Figure 5, Figure 6A). The detection of succinimide isomers after 18 h of peptide incubation for all three peptides at relatively large amounts (~5–10% based on the peak area) was surprising, since the cyclic intermediate is not stable under those conditions.¹³ The effect of the primary amino acid sequence and possible secondary structure of the peptides on the rate of deamidation can be seen in Figure 6. The peptides 300-VVSV-LTVVHQDWLNGK-315 and 127-SGTASVVCLLNFFYPR-142 deamidated at a single amino acid each and formed isoaspartic acid and aspartic acid at a ratio of ~3:1. The intermediate

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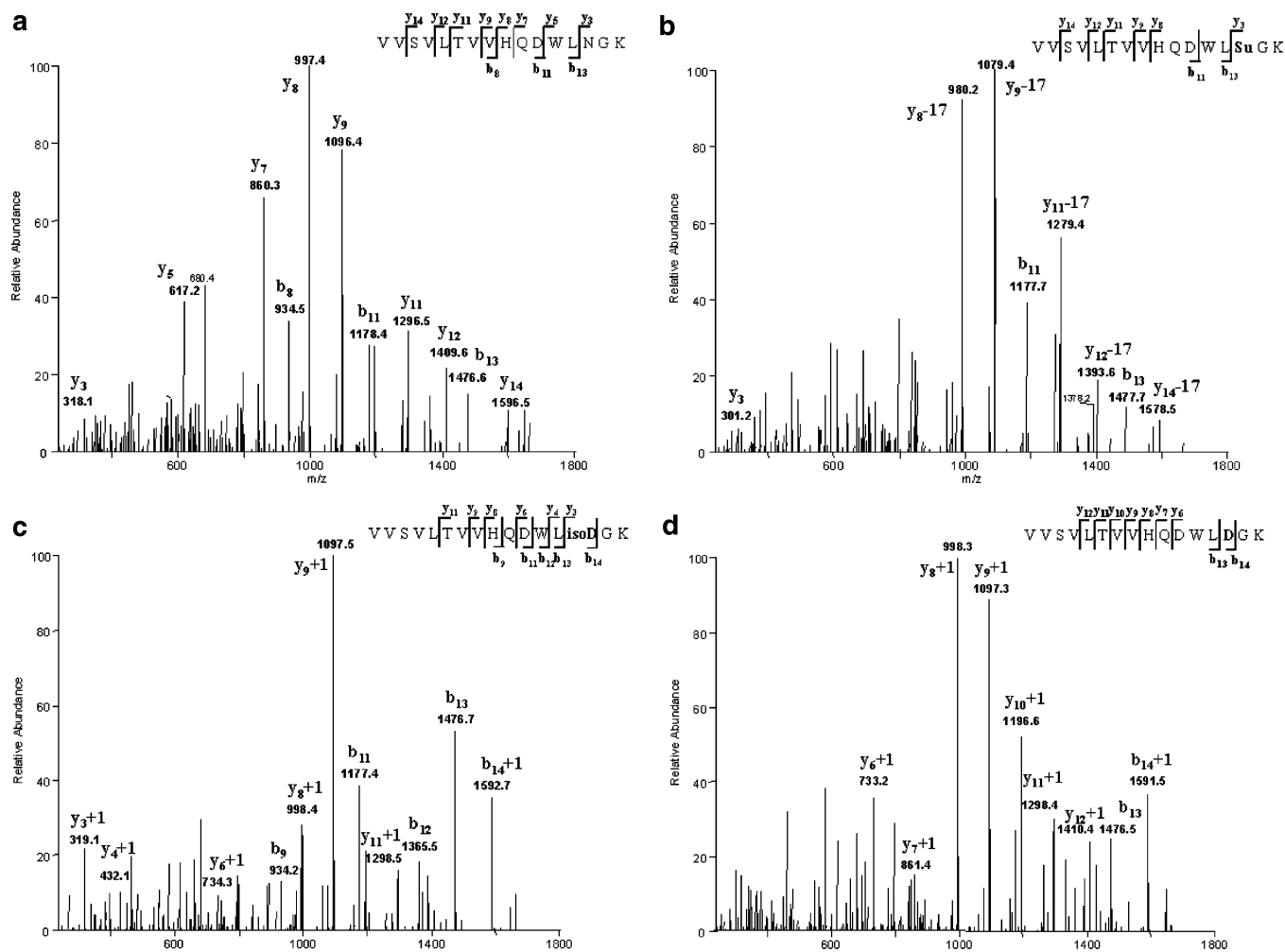


Figure 3. Tandem mass spectra derived by collision-induced dissociation of the $(M + 2H)^{2+}$ precursor ions at m/z 897.7 (a), 889.1 (b), and 898.3 (c,d). The peptides were identified as 300-VVSVLTVVHQDWLNGK-315 (a) and its three isoforms: Su313 (b), IsoD313 (c), and D313 (d).

succinimide was detected for both peptides at a lower amount.

Peptide 369-GFYPSDIAVEWESNGQPENNYK-390 deamidated at two of the three available asparagine residues. The main deamidation reactions were the formation of isoaspartic acid at residue 382 and aspartic acid at residue 387 with minor succinimide formation at residue 382. The ratio of isoaspartic acid to aspartic acid isoforms was still $\sim 3:1$, although the location was at a different residue.

Finally, we determined the rate of deamidation of the intact native antibody as opposed to the deamidation induced by reduction, alkylation, and tryptic digestion. Table 2 compares between the amounts of deamidation from the native antibody samples stored at -80°C (50 mM sodium acetate, 100 mM NaCl, pH 5.8) for 6 months and the amount of degradation from samples stored at pH 7.5 and 37°C for 35 days. The increased amount of deamidation products in the 37°C sample indicated that deamidation does occur at the protein level, but at a much slower rate compared to the deamidation at the peptide level upon storage under increased temperature conditions (Figure 6). To investigate whether the secondary and tertiary structure of the antibody plays a role in deamidation, we determined the rate of deamidation for the reduced and alkylated antibody in Tris buffer at pH 7.5 and 37°C for varying periods of time (Figure 6B). The rate of deamidation after reduction and alkylation was similar to the rate

of deamidation after reduction, alkylation, and tryptic digestion for peptide 369-GFYPSDIAVEWESNGQPENNYK-390 under similar conditions (pH 7.5 and 37°C), indicating that the length of the polypeptide did not affect the rate of deamidation. However, the rate of deamidation after reduction and alkylation was reduced compared with the rate of deamidation after reduction, alkylation, and tryptic digestion for peptides 300-VVSVLTVVHQDWLNGK-315 and 127-SGTASVCLLNFFYPYR-142, indicating that possible secondary structure of the polypeptide did affect the rate of deamidation. The rate of deamidation of the four deamidation sites was significantly affected by the secondary or tertiary structure in the vicinity of the asparagine residues of the intact antibody. To investigate the three-dimensional surroundings of the identified deamidation sites in this study, we looked at the previously solved three-dimensional structure of an IgG1 as a model.²⁸ Although the three-dimensional structure of the antibody studied has not been solved, it appears appropriate that its constant region is very similar to that of a published IgG1 structure. Peptide 369-GFYPSDIAVEWESNGQPENNYK-390 is located in the conserved

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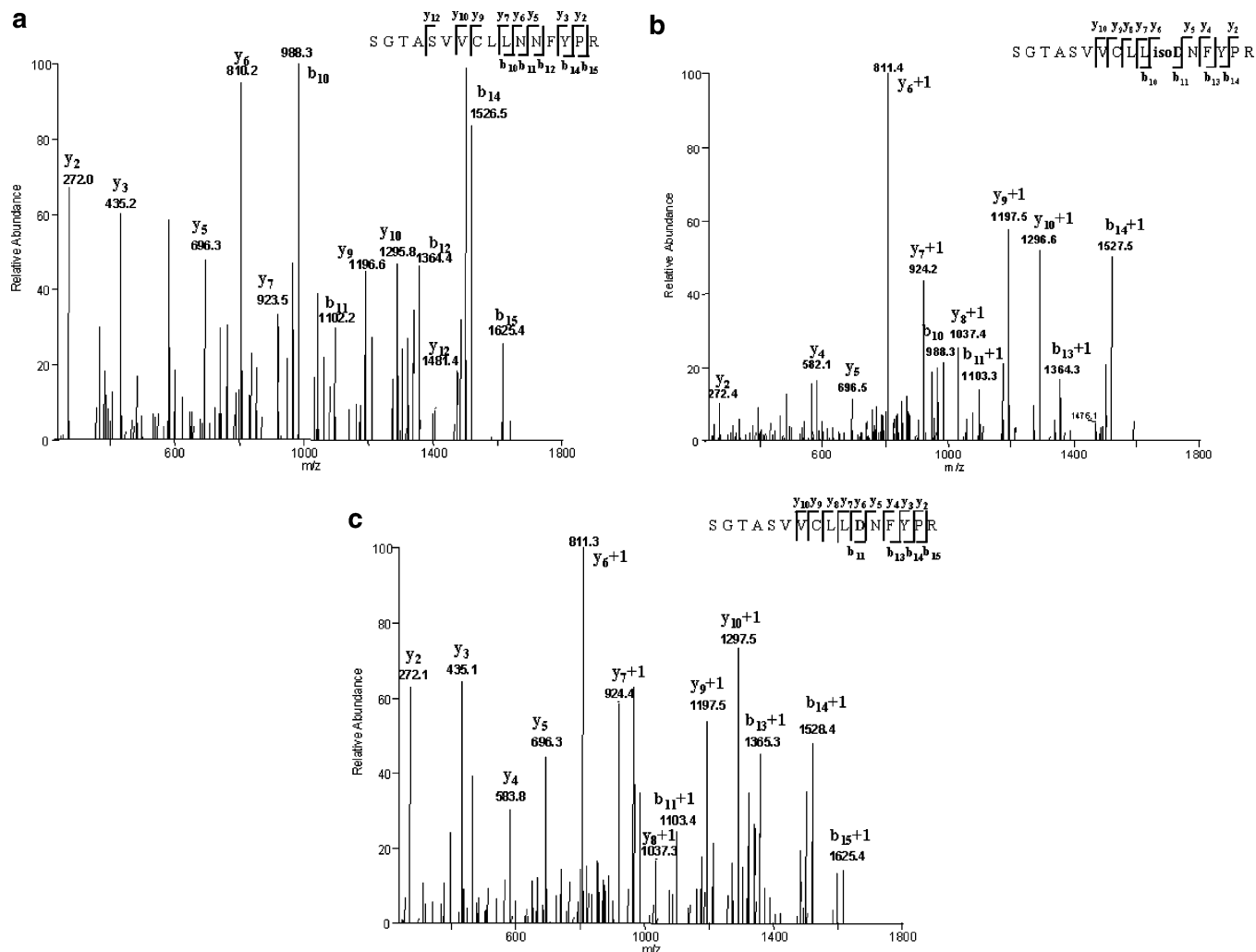


Figure 4. Tandem mass spectra derived by collision-induced dissociation of the $(M + 2H)^{2+}$ precursor ions, m/z 899.7 (a) and 900.3 (b, c). The peptides were identified as 127-SGTASVVCLLNNFYPR-142 (a) and its two isoforms: IsoD137 (b) and D137 (c).

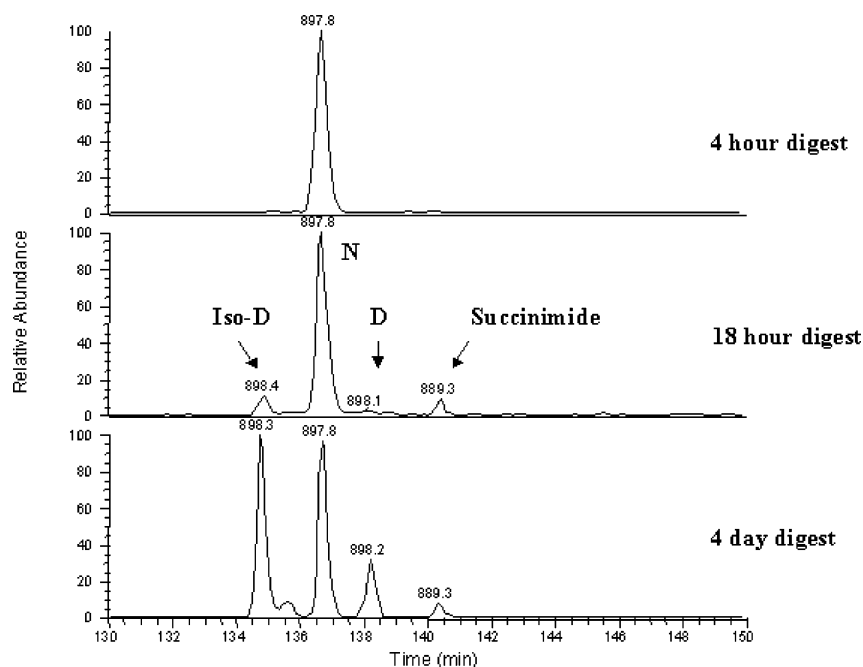


Figure 5. Base peak ion chromatograms of $(M + 2H)^{2+}$ precursor ions at m/z 897.7 (N), 889.1 (Su), and 898.3 (D, isoD) of the four different isoforms of the peptide 300-VVSVLTVHVDWLNGK-315. The peptide was incubated at pH 7.5 and 37 °C in 100 mM Tris buffer for different periods of time. The amount of deamidation products increased with time judging from the peak areas of the base peak ion chromatograms.

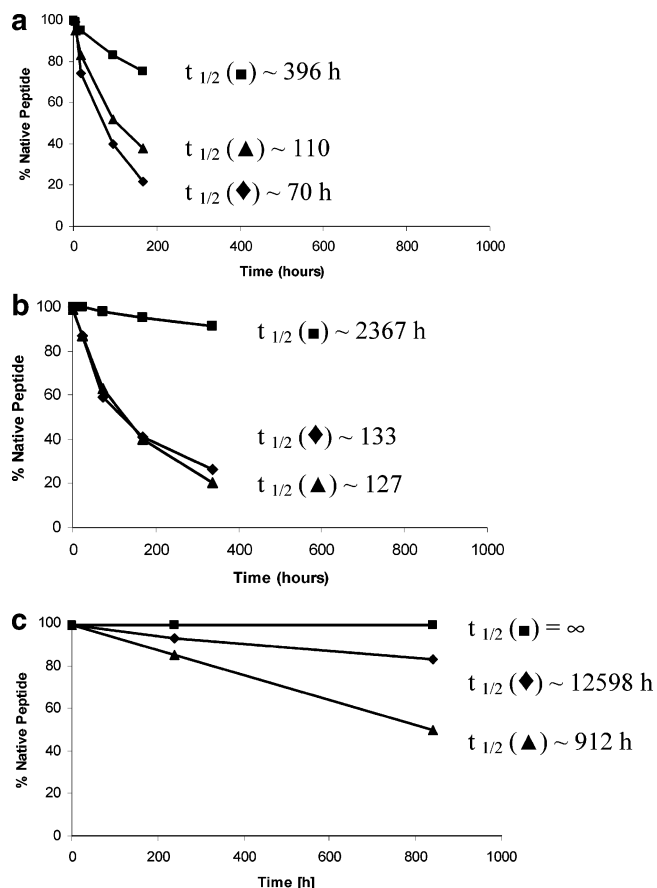


Figure 6. In vitro deamidation of the monoclonal antibody. Abundances of the native forms of three peptides 369-GFYPSDIAVEWESNGQPENNYK-390 (▲), 300-VVSVLTVVHQDWLNGK-315 (◆), and 127-SGTASVCLLNFFYPYR-142 (■) are plotted versus incubation time for the following incubation conditions. The abundance of each isoform was calculated from the peak area of the base peak chromatogram. The half-life ($t_{1/2}$) of deamidation was calculated using SigmaPlot software assuming nonenzymatic deamidation of asparagine residues is a first-order reaction. (A) The recombinant monoclonal antibody was unfolded, reduced, alkylated, and digested using trypsin as described in the Experimental Section. The peptides were incubated for varying periods of time under the digestion conditions of pH 7.5 and 37 °C in 100 mM Tris buffer. (B) The recombinant monoclonal antibody was unfolded, reduced, and alkylated as described in the Experimental Section and incubated for varying periods of time at pH 7.5 and 37 °C in 100 mM Tris buffer before tryptic digestion for 4 h. (C) The recombinant monoclonal antibody was incubated for varying periods of time at pH 7.5 and 37 °C in 100 mM Tris buffer before unfolding, reduction, alkylation, and tryptic digestion for 4 h (see Experimental Section for details).

C_H3 region of the heavy chain. The available structure of an IgG1 shows both deamidation sites are located on the surface of the antibody and are accessible to interactions with water molecules, which are necessary for the deamidation reaction. The slow rate of deamidation could be explained by the reduced flexibility in this region of the protein due to the presence of a loop. Peptide 300-VVSVLTVVHQDWLNGK-315 is located in the conserved C_H2 region of the heavy chain. This asparagine residue is also located at the surface of the antibody, but the flexibility may be reduced due to secondary structure effects in this area of the protein. Finally, peptide 127-SGTASVCLLNFFYPYR-142 is located in the constant C_L region of the light chain. The three-dimensional model of the IgG1 indicates that this asparagine residue is located in a

Table 2. Percentage of Remaining Native Peptide after Unfolding, Reduction, Alkylation, and Tryptic Digestion of the Monoclonal Antibodies^a

peptide	control	degraded
GFYPSDIAVEWESNGQPENNYK	99	50
VVSVLTVVHQDWLNGK	99	83
SGTASVCLLNFFYPYR	99	99

^a Samples were stored in 50 mM sodium acetate, 100 mM NaCl, pH 5.8 at −80 °C (control) and accelerated degradation sample stored in 100 mM Tris buffer at pH 7.5 and 37 °C for 35 days. The abundance of native isoform for each peptide was calculated using their peak areas of the base peak ion chromatograms. The minor loss of native peptide in the control sample was caused by deamidation during unfolding, reduction, alkylation, and tryptic digestion.

β-sheet structure area, where it is not accessible to water. The reduced flexibility in this region of the antibody and the inaccessibility to react with water might explain the reduced rate of deamidation at this position of the intact antibody compared with the peptide after tryptic digestion.

We have shown that tryptic digestion accelerates the rate of deamidation due to enhanced accessibility and flexibility of the deamidation site in the tryptic peptides compared with the intact native protein. A practical conclusion of our study is that overnight digestion at pH 7.5 and 37 °C introduces significant (up to 30%) deamidation at several asparagine residues, similar to the observations for small synthetic peptides.¹¹ We found that a 4-h digestion with a second addition of trypsin after the first 2 h of incubation resulted in completed digestion but did not induce significant amounts of deamidation. The recombinant monoclonal antibody used in this study contains a total of 25 possible deamidation sites. After tryptic digestion and prolonged storage at 37 °C for up to 7 days, only three peptides (Table 2) showed deamidation. The amino acid motifs SNG, ENN, LNG, and LNN resulted in deamidation products with C-terminal glycine residues adjacent to the asparagine exhibiting the fastest rate of deamidation consistent with the literature.⁵ Two of the four deamidation sites were flanked with asparagine residues on the C-terminal side, indicating for the first time that asparagine in addition to the already known amino acids of glycine, serine, and histidine enhances deamidation. We could confirm the findings of others that the ratio of isoasp/asp formed from the deamidation reaction is ~3:1.^{5,6} This ratio does not change over time. However, the amount of succinimide in our experiments seemed larger (5–10% based on the peak area) compared to previously reported studies, especially considering that, under the conditions of pH 7.5 at 37 °C, only trace amounts should have been detected.^{5,6,13} All other potential deamidation sequence motifs found in this antibody (including GNT, TNY, YNP, WNS, SNF, CNV, SNT, WNS, FNW, HNA, FNS, SNK, GNV, HNH, SNY, LNW, SNL, NNF, DNA, GNS, and FNR) did not show deamidation. Surprisingly, C-terminal histidine in one motif and C-terminal serine in four other motifs adjunct to the deamidation site did not show deamidation under accelerated conditions (pH 7.5, 37 °C). We suspect that the N-terminal flanking amino acids in those motifs might play a role in the prevention of deamidation. Secondary structure of the peptides could also play a role for limiting the deamidation reaction.

Deamidation of peptide 369-GFYPSDIAVEWESNGQPENNYK-390 resulted in the formation of succinimide at position 382, but not 387. Additionally, the lack of isoaspartic acid at position 387 and aspartic acid at position 382 indicated different mechanisms for both deamidation reactions.

Glutamine residue can undergo deamidation similar to aspartic acid residues. However, the rate of the deamidation of glutamine residues is at least 100 times slower compared with asparagine residues.¹¹ We did not detect any deamidation of glutamine residues during the time course of our experiments, and we do not believe that deamidation of glutamine residues should be of any concerns for biological pharmaceuticals.

CONCLUSION

Deamidation is a major source of protein degradation, and it is important to monitor in the course of formulation development. In this report, we identified several deamidation sites in the conserved region of a recombinant human monoclonal antibody. The conserved region of this antibody is shared by all IgGs with the exception of minor differences in the hinge region. Deamidation of asparagine residues and the formation of either isoaspartic acid or aspartic acid results in a molecular mass change of only + 1 Da. This 1-Da mass difference cannot be identified with current mass spectrometry technologies for the intact antibody. Therefore, enzymatic digestion (typically trypsin) followed by reversed-phase separation and mass spectrometry analysis is often used to identify such degradation products. Our reversed-phase high-performance liquid chromatography method enabled us to

separate the succinimide, isoaspartic, and aspartic acid degradation products, and these isoforms were unambiguously identified using tandem mass spectrometry. The ion trap technology used in this study was capable of detecting the small differences in mass (1 Da). Additionally, tandem mass spectrometry data were used to identify the location of the deamidation sites in cases where several possible sites in a single peptide existed. We found that deamidation at the four identified sites in this antibody was very slow as long as the antibody was intact even under the conditions of pH 7.5 and 37 °C. The deamidation was greatly enhanced after tryptic digestion, indicating that the three-dimensional structure of the antibody inhibits deamidation. The effect of the primary amino acid sequence, especially the N- and C-terminal amino acids following the deamidation site, was investigated, showing that deamidation preferentially occurs at glycine and asparagine at the C-terminal of asparagine.

ACKNOWLEDGMENT

We thank Gary Pipes and Tom Dillon for the development of the tryptic peptide mapping LC/MS method used in this study. We also thank MaryAnn Foote for proofreading and David Brems and Michael Treuheit for helpful discussions during the preparation of the manuscript.

Received for review April 19, 2005. Accepted July 19, 2005.

AC050672D