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Identification and characterization of oxidation and deamidation sites in monoclonal rat/mouse hybrid antibodies

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ABSTRACT

Oxidation of methionine residues and deamidation of asparagine residues are the major causes of chemical degradation of biological pharmaceuticals. The mechanism of these non-enzymatic chemical reactions has been studied in great detail. However, the identification and quantification of oxidation and deamidation sites in a given protein still remains a challenge. In this study, we identified and characterized several oxidation and deamidation sites in a rat/mouse hybrid antibody. We evaluated the effects of the sample preparation on oxidation and deamidation levels and optimized the peptide mapping method to minimize oxidation and deamidation artifacts. Out of a total number of 18 methionine residues, we identified six methionine residues most susceptible to oxidation. We determined the oxidation rate of the six methionine residues using 0.05% H_2O_2 at different temperatures. Methionine residue 256 of the mouse heavy chain showed the fastest rate of oxidation under those conditions with a half life of approximately 200 min at 4 °C and 27 min at 37 °C. We identified five asparagine residues prone to deamidation under accelerated conditions of pH 8.6 at 37 °C. Kinetic characterization of the deamidation sites showed that asparagine residue 218 of the rat heavy chain exhibited the fastest rate of deamidation with a half live of 1.5 days at pH 8.6 and 37 °C. Analysis of antibody isoforms using free flow electrophoresis showed that deamidation is the major cause of the charged variants of this rat/mouse hybrid antibody.

1. Introduction

Triomab® monoclonal antibodies are an innovative antibody format with the potential to revolutionize cancer therapy. The unique structure of Triomab® antibodies, comprising of a rat and mouse half antibody (rat/mouse hybrid), allows the targeting of a tumor specific antigen on a cancer cell, the simultaneous binding to a target on a T-cell, and attracting macrophages through the Fc receptor mediated pathway [1–6]. A cartoon of the structure and proposed mechanism of action is shown in Fig. 1. Recently, the European Medicines Agency (EMEA) approved the first Triomab® antibody Removab® for the treatment of malignant ascites.

Like other antibodies, rat/mouse hybrid antibodies are relatively stable molecules, but they are still subject to a variety of degradation reactions that can occur during manufacturing, formulation and storage. Oxidation and deamidation are among the most common modifications of biopharmaceuticals.

Oxidation of methionine residues occurs naturally and is even considered as an internal antioxidant, protecting proteins from oxidation damage [7]. Other amino acids like cysteine, tryptophane, tyrosine and histidine can oxidize as well, but typically at a much slower rate compared to methionine residues. Methionine oxidation results in the formation of methionine sulfoxide and, under extreme conditions, sulfones. Peroxides have been widely used for studying the kinetics and mechanisms of methionine oxidation in proteins [8–10]. Peroxides react with metal ions to form free radicals that can initiate oxidation of proteins [11,12]. Methionine can also be photooxidized by a free radical pathway [13] or via singlet oxygen intermediate formation [14]. Oxidation of protein drug products can be influenced by many factors, including the buffer and its concentration, excipients, and formulation pH [15].

The mechanism of the deamidation reaction has been studied in great detail [16]. Asparagine residues can form a cyclic imide (succinimide) intermediate, which spontaneously hydrolyzes to a mixture of isoaspartic/aspartic acid at an approximate ratio of 3:1 [17,18]. High pH and high temperatures accelerate the deamidation reaction [17,18]. An effect of different amino acids at the C-terminus of asparagine on succinimide formation and isomerization has been reported, indicating that glycine, serine and histidine residues are most favorable for succinimide formation of asparagine residues [19]. Others have reported that amino acids at the N-terminus of

Abbreviations: HPLC, high performance liquid chromatography; LC/MS, liquid chromatography mass spectrometry; ESI, electrospray ionization; MWCO, molecular weight cutoff; FFE, free flow electrophoresis; IEF, isoelectric focusing.

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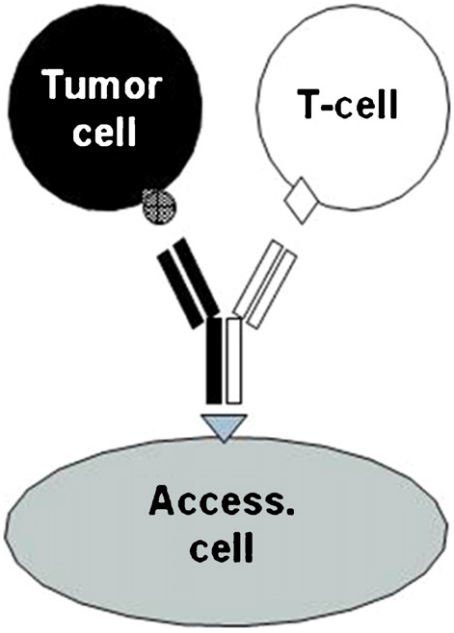

Fig. 1. Structure and mechanism of action of Triomab® antibodies.

asparagine do not seem to significantly affect the rate of deamidation [20–23]. Effects of the protein and peptide conformation on asparagine deamidation have also been investigated. Kosky et al. reported the effect of the alpha-helical secondary structure on the stability of asparagine residues, and showed that deamidation only occurs in non-helical populations [24]. 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 [25]. The hydrophobic regions of proteins also show conformational constraints that prevent the formation of succinimide [26]. The effect of deamidation on the biological activity of proteins has also been reported. Harris et al. [27] showed that deamidation of asparagine residue 102 and the formation of isoaspartic acid in one heavy chain of a commercial recombinant monoclonal antibody reduced its potency by more than 3-fold.

We developed a reversed-phase LC/MS peptide mapping method for the identification and quantification of oxidation and deamidation sites. We identified and characterized several oxidation and deamidation sites in a rat/mouse hybrid antibody, and we used free flow electrophoresis fractionation of charge-isoforms for the identification of the source of the charge heterogeneity.

2. Materials and methods

2.1. Material

The monoclonal antibody analyzed in this study was expressed using quadroma production technology and purified using standard manufacturing processing steps (protein A and ion exchange chromatography). The antibody was prepared in 100 mM sodium citrate buffer pH 5.6; 0.02% Tween-80 at a concentration of 0.1 mg/mL and stored at −80 °C.

All chemicals were analytical grade purchased from Sigma (St. Louis, MO) unless stated otherwise in the text.

2.2. Generation of stressed material

For pH-stress, the sample buffer was exchanged to 100 mM sodium citrate pH 8.6 by centrifugation through a 10 kDa MWCO membrane (Millipore, Billerica, MA). The samples were then incubated at 4 °C or 37 °C for the time specified in the text.

Peroxide stress samples were generated by the addition of hydrogen peroxide to a final concentration of 0.05% and incubation at 4 °C and 37 °C for the time specified in the text. Hydrogen peroxide was removed by buffer exchange (see below).

2.3. Reduction, alkylation and enzymatic digestion

Samples (1 mL, 0.1 mg/mL) were concentrated by a factor of 5 (0.2 mL, 0.5 mg/mL) by filtration through 10 kDa MWCO membranes (Millipore, Billerica, MA). During this procedure, the buffer was changed to 4 M urea in 100 mM tris(hydroxymethyl)aminomethane/hydrochloric acid pH 8.4. After addition of hydroxylamine hydrochloride to a final concentration of 10 mM (to prevent carbamylation) and endoproteinase Lys-C (Wako, Osaka, Japan) at a ratio of 1:20 enzyme to substrate, samples were incubated at 37 °C overnight. Dithiothreitol (2 μL, 0.2 M) was then added and the mixture was incubated at 37 °C for 45 min. Afterwards, the samples were alkylated by adding 2 μL iodoaceteamide (0.5 M) and incubation at room temperature for 45 min in the dark. The samples were analyzed immediately after the alkylation procedure or stored at −20 °C waiting analysis.

Prior to the reduction, alkylation and enzymatic digestion procedure, the FFE fractions were incubated at 65 °C for 30 min and the cellulose was removed by centrifugation for a few seconds.

2.4. Optimized reduction, alkylation and enzymatic digestion procedure

The optimized method included an acetone precipitation step after the concentration step, by adding four volumes of ice-cold acetone to the sample, incubation at −20 °C for 1 h, centrifugation at 4000 × *g* for 10 min, followed by resuspension of the protein pellet in 4 M urea in 100 mM Tris pH 8.4.

2.5. HPLC separation of peptides generated by the Lys-C digest

The peptides were separated on a reversed-phase HPLC using an Agilent 1200 HPLC system equipped with a diode-array detector, an auto-sampler, and a temperature controlled column compartment (Agilent, Waldbronn, Germany). The eluents were A: 0.1% TFA in water (Merck, Darmstadt, Germany) and B: 0.1% TFA in acetonitrile (Merck, Darmstadt, Germany). Separation was performed on a Jupiter C5 column packed with 5 μm particles, 300 Å pore size (Phenomenex, Torrance, CA). The column was heated at 50 °C to enhance separation and the flow rate was 200 μL/min. The column was initially equilibrated at 2% solvent B. Three minutes after sample injection, the concentration of mobile phase B was increased to 50% over 127 min. The column was re-equilibrated by ramping up mobile phase B to 100% over 5 min, holding for 5 min at 100% B, dropping down to 2% B over 5 min followed by 15 min at 2% B. UV absorption was monitored at 214 and 280 nm.

2.6. Mass spectrometry analysis of the separated peptides

The HPLC was directly coupled to an Agilent 6330 ESI-ion trap (ESI-IT) mass spectrometer or an Agilent 6220 ESI-TOF mass spectrometer (Agilent, Santa Clara, CA). The ESI voltage for the ion trap and TOF mass spectrometer were set at 3500 V and 4000 V respectively in positive ion mode. The capillary temperature was 250 °C for both instruments. The fragmentation in positive mode was obtained using ion trap collision energies of 1 V. Each full-scan mass spectrum in the ESI-IT analysis was followed by two data-dependent MS/MS scans of the most intense ions. Both

instruments were calibrated and tuned by infusion of calibration solution.

2.7. Data analysis

Peptides were identified manually by comparing the observed tandem mass spectra with the theoretical mass spectra generated from the known amino acid sequence, or automatically by Mascot software. Large peptides were identified manually by comparing the observed mass with the theoretical mass of the peptides using accurate mass information from ESI-TOF experiments.

The relative abundances of the peptides were measured using the extracted ion chromatogram of the most abundant ions of the peptides or the isotope ratios as described in the text in more detail. The quantification based on the extracted ion chromatograms correlated well with quantification based on UV chromatograms measured by Agilent ChemStation software.

Kinetic parameters were calculated using the one site decay function of GraphPad Prism® software (GraphPad Software Inc., La Jolla, CA).

2.8. Free flow electrophoresis

The isolation of the antibody isoforms was performed by free flow electrophoresis unit (Becton Dickinson GmbH, Munich, Germany). The sample was concentrated to 1–1.2 mg/mL and the buffer was changed to 10 mM tris(hydroxymethyl)aminomethane/hydrochloric acid pH 4.5 by ultracentrifugation. Hydroxypropylmethyl cellulose (HPMC) and glycerol were added to a concentration corresponding to the separation medium (see below). The concentrated antibody was separated according to the isoelectric points of the isoforms in a total of six FFE-runs, collected in two microtiter plates. The separation medium was 25% glycerol, 0.2% HPMC, 1% Servalyt pH range 6–8. After loading of 150 μL sample, fractionation was achieved at 1200 V, 30 mA, 10 °C over approximately 50 min. Immediately before sample application, a p*I* marker test with six p*I* markers was performed to verify the pH gradient in the separation chamber. After FFE equilibration and p*I* marker test, the samples were infused into the chamber for FFE. 96 sample-fractions (approximately 300 μL per analysis) were collected. The instrument was maintained under the same conditions for all FFE-runs. The same media solutions were used in all of the separation experiments. Consequently, all separation parameters remained constant throughout the separation of the sample providing reproducible separation conditions.

2.9. Isoelectric focusing

Isoelectric focusing of stressed samples was performed using a horizontal electrophoresis unit (Serva, Heidelberg, Germany) with agarose IEF plates pH 3–10 (Cambrex, East Rutherford, NJ) according to the manufacturer's protocol. The electrophoresis unit was programmed as follows: pre-focus for 10 min at 2000 V, 150 mA, 1 W, and main-focus for 75 min at 1500 V, 50 mA, and 25 W. After fixation, the gel was stained with 100 mL Brilliant Blue G-Colloidal solution (prepared from concentrate according to the manufacturer's instructions) for 2 h. After destaining, the gel was scanned and analyzed using Pharmacia Biotech Image Master Gel Analysis Software 1D Elite version 3.0.

Isoelectric focusing of FFE fractions was performed using the ZOOM IPGRunner® system (Invitrogen Carlsbad, CA) with Novex® IEF gels pH 3–10 (Invitrogen, Carlsbad, CA). The electrophoresis unit was programmed as follows: 1 h at 100 V, 1 h at 200 V, and 30 min at 500 V. The gels were stained using the SilverQuest® kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. FFE fractions containing one prominent band with considerably lower amounts of other isoform bands were selected for further analysis.

3. Results

3.1. Identification of oxidation and deamidation sites

Lys-C digestion of control and stressed antibody preparations followed by HPLC separation and mass spectrometry analysis of the peptides resulted in the identification of several oxidation and deamidation products. The results are summarized in Table 1. Only methionine residues were found to be susceptible to oxidation using 0.05% H_2O_2 stress conditions and only asparagine residues were found to be susceptible to deamidation using pH 8.6 stress conditions.

Oxidation sites were identified by comparing the peptide map UV chromatogram (see Fig. 2) of a control sample with the UV chromatogram of peroxide stressed sample (0.05% H_2O_2, 37 °C, 4 h). Additionally, oxidation sites were identified by comparing the base peak ion chromatograms of all methionine containing peptides and their hypothetical oxidized form (+16 Da) of the control and stressed sample. The oxidation levels of all methionine residues susceptible to oxidation were calculated based on the peak areas of the extracted ion chromatogram of the native and the oxidized peptides. Fig. 3 shows the extracted ion chromatogram of peptide 345-GSVRAPQVYVLPPPEEEMTK-364 (from the mouse heavy chain), and its oxidized form as an example. The identified oxidized peptides were confirmed by tandem mass spectrometry. The oxidized methionine containing fragmentation ions showed an increase of 16 Da compared to the fragmentation ions of the native peptide.

Similar to the identification of oxidation sites, deamidation sites were identified by comparing the peptide map UV chromatogram of a control sample with the UV chromatogram of pH-stressed sample (pH 8.6, 37 °C, 7 days). Fig. 4 shows the detection of two new peaks eluting at 53.2 and 54.2 min in the peptide map of a degraded sample in comparison with a control sample. Additionally, deamidation sites were identified by comparing the isotope pattern of all asparagine containing peptides of the control and stressed sample. The extracted ion chromatogram could not be used for the calculation of the deamidation levels, since separation of the native and deamidated species was not always possible. Instead, the deamidation levels of asparagine residues susceptible to deamidation were calculated based on the isotope profile. Fig. 5 shows the theoretical and the observed isotope profile of peptide 213-KVERRNGGIGHK-224 (from the rat heavy chain). The amount of deamidation was calculated as followed: Since Asn and Asp (deamidation: Asn → Asp) containing peptides differ by 1 Da, the natural isotope distribution of the Asn peptides partially overlapped in the mass spectrum with the Asp peptide. The first isotopes peak (Fig. 5) represents the first isotope peak of the Asn peptide. The theoretical intensity of the Asn peptide was calculated by using the intensity of the first peak for normalization (see Table 2). The theoretical intensities for each isotope peak of the Asn peptide is also shown in the red line in Fig. 5. All other isotope peaks shown (black line in Fig. 5) are contributed by both Asn and Asp peptides. The second isotope peak for examples is the sum of the first isotope peak of the Asp peptides plus the second isotope peak of the Asn peptide. The Asp isotope peak intensity can be calculated by subtracting the normalized theoretical intensity of each Asn peptide isotope peak from the intensity of the measure isotope peak (in black line in Fig. 5). The percentage of deamidation can be calculated using the calculated Asp peptide peak intensities and the normalized Asn peptide peak intensities by dividing the intensity of the first Asp peptide isotope with the sum of first Asn and Asp peptide isotope. The second

Table 1
Monoclonal antibody peptides generated by Lys-C proteolysis containing all methionine residues prone to oxidation (A) and all asparagine residues prone to deamidation (B) shown in underline bold. All masses are given as monoisotopic masses of neutrals. Cysteine residues are calculated as S-carbamidomethylcysteine.

Peptide	Amino acid sequence	RT	*z*	*m/z*	Observed mass	Theoretical mass
(A)						
MH15[a]	253-DVL**M**ISLSPIVTCVVVDVSEDDPDVQI SWFVNNVEVHTAQTQTHREDYNSTL RVVSALPIQHQDWMSGK-321	114.8	4	2323.83	9291.32	9291.39
MH21	345-GSVRAPQVYVLPPPEEE**M**TK-364	67.4	3	743.03	2226.09	2226.14
MH23	366-QVTLTCMVTDF**M**PEDIYVEWTNNGK-390	99.7	3	997.82	2990.46	2990.38
RH6[a,b]	77-*XXXXXX*M*XXXXXXXXXXXXXXXXXXXXXX*X *XXXXXXX***M***XXXXX*AQTTAPSVYPLAPGCG DTTSSTVTLGCLVK-149	94.8	4	2056.48	8221.92	8221.87
RH15	264-VTCVVVDVSEEEPDVQFSWFVNNVEV HTAQTQPREEQYNSTFRVVSALPIQHQDW**M**SGK-322	91.0	4	2080.98	8319.92	8319.82
RH25	398-NTEPV**M**DSDGSFFMYSK-414	69.4	2	977.94	1953.88	1953.81
(B)						
ML11	156-IDGSERQ**N**GVLNSWTDQDSK-175	55.2	3	750.33	2247.99	2248.04
MH23	366-QVTLTCMVTDFMPEDIYVEWTN**N**GK-390	99.7	3	997.82	2990.46	2990.38
MH28	420-NWVER**N**SYSCSVVHEGLHNHHTTK-443	53.9	3	964.47	2890.41	2890.36
RL8	154-A**N**GAPISQGVDTANPTK-170	42.1	2	820.91	1639.82	1639.82
RH9_10	213-KVERR**N**GGIGHK-224	28.7	2	675.86	1349.72	1349.76

[a] Oxidation site M256 of the mouse heavy chain and M114 of the rat heavy chain was confirmed by additional Asp-N digestion, separating the methionine residues in separate peptides (data not shown). All other oxidation and deamidation sites were confirmed by MS/MS data.
[b] Methionine residues M83 and M114 of the rat heavy chain are located in the Fab framework of the antibody. The amino acids of constant region and the location of the methionine residues are shown; all others amino acids are marked with an *X*.

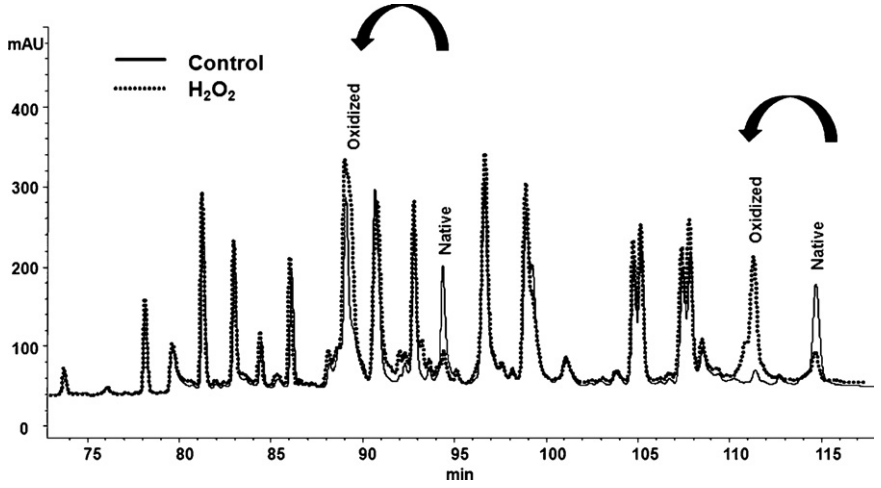

Fig. 2. Reverse phase chromatograms with UV absorbance at 214 nm of the Lys-C peptides of the monoclonal antibody stressed with 0.05% H_2O_2 at 37 °C for 4 h (dotted line) and the unstressed control (solid line). A new peak eluting at 112 min was detected and identified as the oxidized form of peptide MH15 that eluted at 115 min. Further analysis using Asp-N digestion showed that methionine residue 256 was oxidized and not methionine 318.

isotope peaks of the Asp and Asn peptides could be used for the calculation of the amount of deamidation as well to confirm the results (Table 2).

The identified deamidated peptides were also confirmed by tandem mass spectrometry. The deamidated asparagine containing fragmentation ions showed an increase of 1 Da compared to the fragmentation ions of the native peptide. Differentiation of the iso-Asp and Asp containing peptide was not possible with our method, however, the elution order and the iso-Asp to Asp ratio of approximately 3:1 allowed a putative assignment.

The deamidated form of peptide 213-KVERRNGGIGHK-224 (from the rat heavy chain) coeluted with its native isoform at 28.7 min, making the conformation of the deamidation site by tandem mass spectrometry tricky. However, using the isotope pattern of the fragmentation ions, it was still possible to confirm deamidation of asparagines residue 218 (data not shown).

Table 2
Calculation of the deamidation percentage of rat heavy chain peptide 213-KVERR**N**GGIGHK-224 using the theoretical and measured isotope intensities (see Fig. 5).

Isotopes of the RH9_10 peptide	1st	2nd	3rd
Measured peak intensities	396	762	469
Theoretical peak intensities[a]	100	71	28
Normalized theoretical peak intensities[b]	396	281	111
Difference of measured to normalized[c]	0	481	358
Percentage of Asp containing peptide	–	55	56

[a] The theoretical isotope distribution was calculated using MassHunter software.
[b] Represents the intensities of the native (Asn containing) peptide.
[c] Represents the intensities of the deamidated (Asp containing) peptide.

3.2. Effect of sample preparation on oxidation and deamidation

Lys-C peptide mapping procedure required exposure of the analyte to denaturing conditions (4 M urea), high temperature, and basic conditions. We investigated the effect of these conditions to the oxidation and deamidation analysis. The effect of the pH during the digestion was evaluated by comparing the Lys-C peptide maps at 5 different pH values (pH 7.0, 7.5, 8.0, 8.4, and 9.1). Digestion

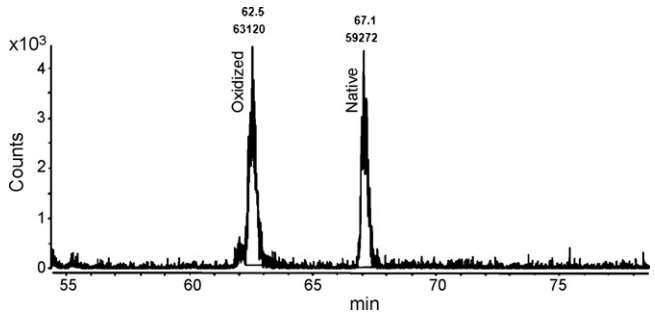

Fig. 3. Extracted ion chromatogram of ions correlating to the native and oxidized forms of the peptide GSVRAPQVYVLPPPEEEMTK. The sample was incubated with 0.05% H_2O_2 prior to the peptide mapping analysis.

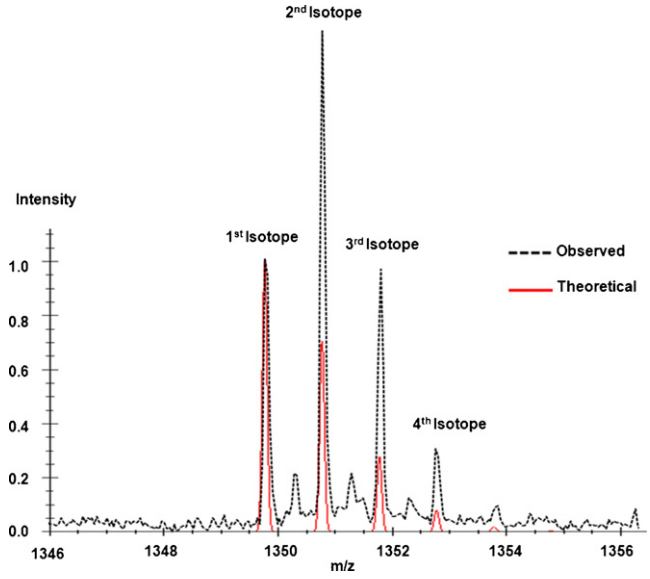

Fig. 5. The mass spectrum quantifying deamidation of peptide RH9_10 eluting at 28.7 min was obtained by a high resolution ESI-TOF. The spectrum shows the isotope peak intensity distribution for the singly charged peptide ions. Peaks plotted by red line represent the theoretical native isotope distribution and peaks in solid black line represent the observed isotope peak intensity distribution. The isotope peak intensity of the deamidated peptide was calculated by subtracting theoretical native isotope intensity from the observed isotope peak intensity. For example, the intensity of the monoisotopic peak (1st isotope) of the deamidated peptide equals the intensity of the second observed isotope peak (black line) minus the second isotope peak of the normalized theoretical peak (2nd isotope peak: red line).

at pH of 7.0 and 7.5 resulted in the presence of incomplete digestion products, whereas digestion conditions of pH 8.0, 8.4 and 9.1 did not seem to differ based on the UV chromatogram (data not shown). However, small differences in the levels of oxidation or deamidation are difficult or even impossible to evaluate based on a visual comparison of the UV chromatograms. Therefore, the levels of oxidation and deamidation were evaluated using the mass spectrometry data. The results of the oxidation and deamidation levels at different pH value digestion conditions and different digestion times are summarized in Fig. 6. The different pH conditions during the digestion did not seem to affect the oxidation levels, however a clear difference in the deamidation levels was detected. As expected, digestion at higher pH values resulted in an increase in deamidation. We concluded that the optimal pH range for the Lys-C digestion was pH 8.4. At lower pH values, the digestion was incomplete and at higher pH values the deamidation increased.

We also evaluated the effect of the digestion time on oxidation and deamidation. We digested the antibody for 2, 4, 6, 16, and 24 h and compared the resulting peptide maps. Again, a visual inspection of the UV chromatograms clearly showed increased levels of undigested material at digestion condition times of 2, 4, and 6 h. The addition of larger amounts of Lys-C did not solve this problem (data not shown). The levels of oxidation and deamidation for the different digestion times are shown in Fig. 6. Clearly, shorter digestion times did result in less deamidation. However, a shorter digestion time resulted in incomplete digestion products. We concluded that an overnight digestion was the method of choice.

Some antibody formulations contain polysorbates like Tween for stabilization, which could not be removed during the buffer exchange procedure, but influenced the mass spectrometric analysis. We evaluated different methods for the removal of the polysorbate (acetone, heat, and trichloroacetic acid precipitation) and found that precipitation of the protein using 80% acetone at −20 °C for 1 h, followed by resuspension in digestion buffer (4 M urea, 100 mM Tris pH 8.4) removed the Tween almost completely. A comparison of the mass spectra with and without the acetone precipitation is shown in Fig. 7, demonstrating the advantages of the acetone precipitation step. Deamidation levels were not affected by the acetone precipitation step, but the oxidation levels were slightly increased compared to the standard method (data not shown). However, the advantages of removing Tween for increased sensitivity and avoiding contamination of the instruments outweighed

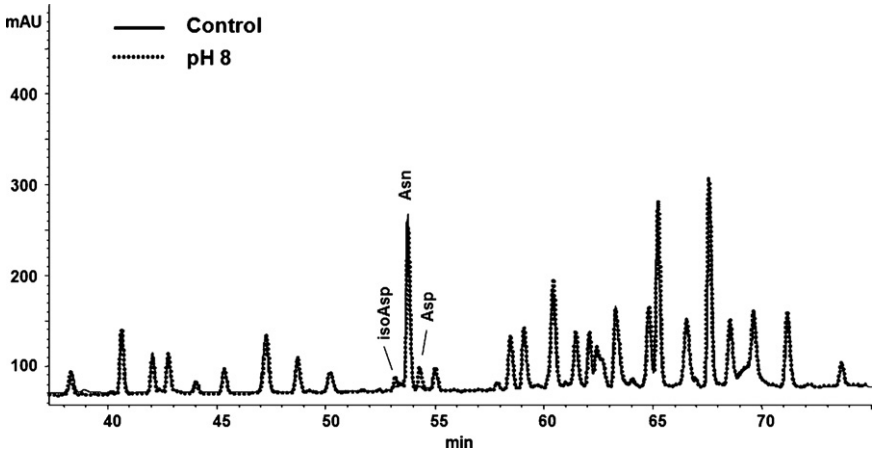

Fig. 4. Reverse phase chromatograms with UV absorbance at 214 nm of the Lys-C peptides of the monoclonal antibody stressed at pH 8.6 at 37 °C for 7 days (dotted back line) and the unstressed control (black line). New peaks eluting at 53.2 and 54.2 min were detected and identified as the isoform of peptide NWVERNSYSCSVVHEGLHNHHTTK of the mouse heavy chain that eluted at 53.9 min. Mass spectrometry analysis of these isoforms showed that they had iso-Asp and Asp at residue 425 (see details in the text).

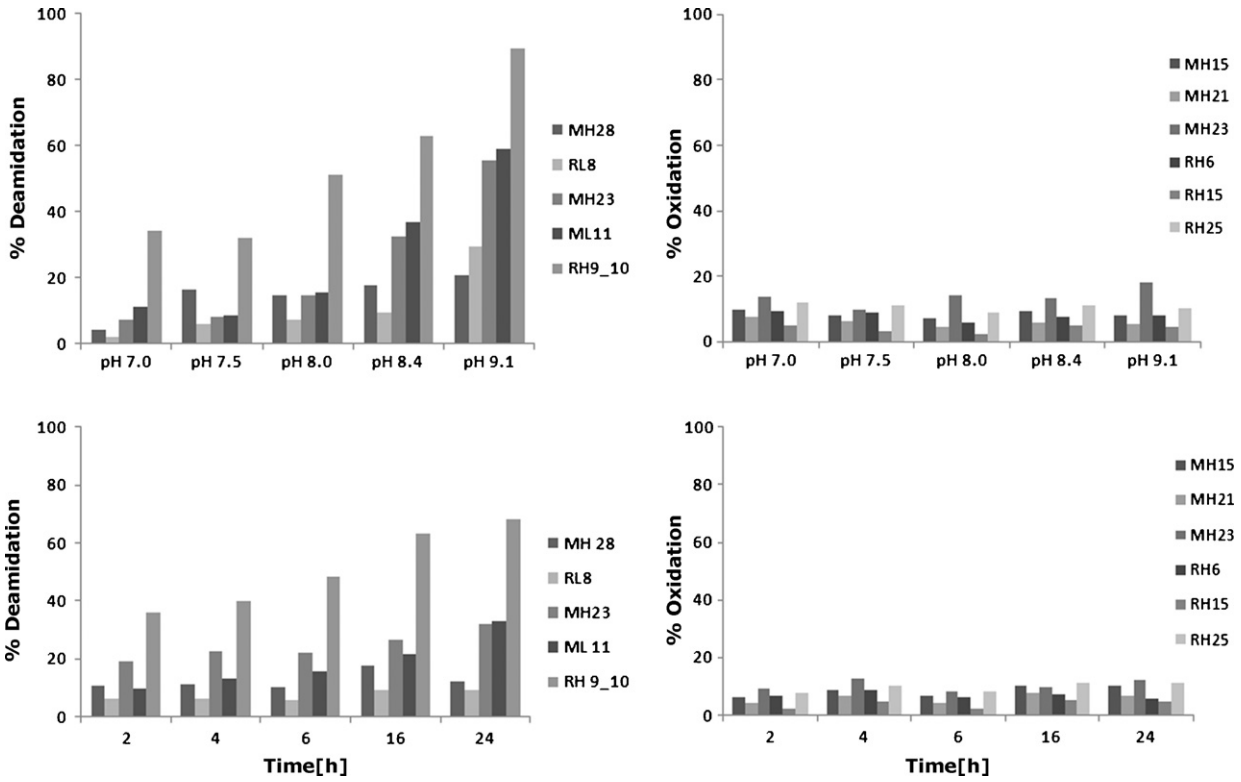

Fig. 6. Effect of different Lys-C digestion conditions on oxidation and deamidation. Samples were incubated with Lys-C at different pH values and different times. The amount of oxidation and deamidation for a selected number of peptides was calculated as described in Section 2.

the disadvantages of a slight increase of the oxidation level. All the following experiments were performed using the acetone precipitation method.

3.3. Oxidation and deamidation kinetics of the intact antibody

After optimizing the Lys-C peptide mapping method for the quantification of oxidation and deamidation, we further characterized the oxidation and deamidation susceptibility of the rat/mouse hybrid antibody. The kinetics of the oxidation reactions of the identified methionine residues were determined using 0.05% H_2O_2 at 4 °C and 37 °C. Samples were analyzed at the following time points: 0, 30, 60, 120, 240, 480, 1440, 2880, and 4320 min. The results are summarized in Table 3. Methionine 256 of the mouse heavy chain showed the fastest oxidation rate with a half life of approximately 200 min at 4 °C and 27 min at 37 °C.

Table 3
In vitro oxidation kinetic parameters of different methionine residues oxidized using 0.05% H_2O_2 stress conditions at 4 °C and 37 °C.

Peptide	Methionine	4 °C		37 °C	
		K [min^{-1}]	$t_{1/2}$ [min]	K [min^{-1}]	$t_{1/2}$ [min]
RH6	114	0.0019	369.5	0.016	44.6
RH15	319	0.0023	301.2	0.011	65.1
RH25	403	0.0024	289.9	0.024	29.3
MH15	256	0.0035	199.6	0.026	26.6
MH21	362	0.0016	424.0	0.012	58.0
MH23	372	0.002	345.0	0.018	38.6

Kinetic parameters were calculated using the one site decay function of GraphPad software. R^2 values were between 0.981 and 1.000.

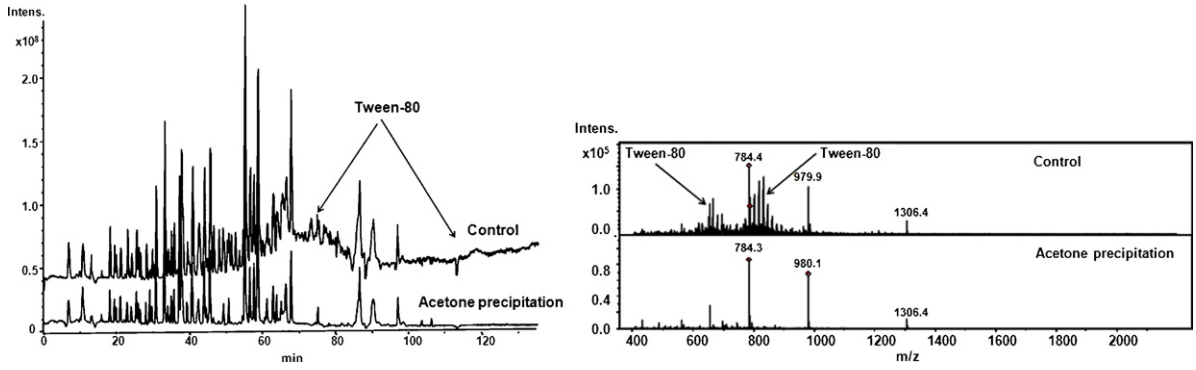

Fig. 7. Effect of acetone precipitation before Lys-C peptide mapping on MS data. Samples were submitted to peptide mapping as described in Section 2, either with or without prior acetone precipitation. The total ion chromatogram as well as the mass spectrum at 75 min showed Tween-80 signals in the control samples, which could be removed by acetone precipitation.

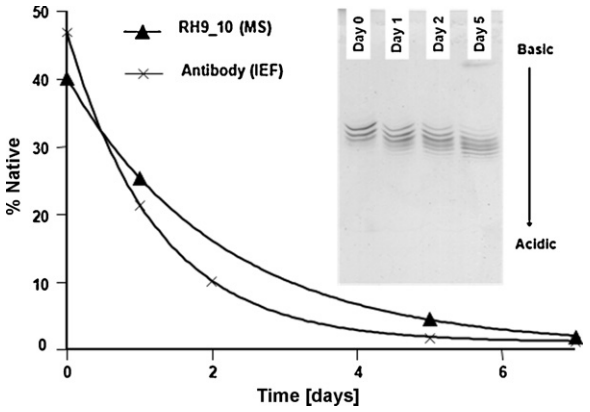

Fig. 8. Comparison of deamidation of peptide RH9_10 measured using mass spectrometry of the Lys-C digested antibody (▲) and degradation of isoform 1 (most basic antibody isoform) measured using IEF of the intact antibody (×). The antibody was incubated at pH 8.6, 37 °C and analyzed by peptide mapping and IEF as described in Section 2. The IEF of the sample at 7 days was analyzed on a different gel (not shown).

The kinetics of the deamidation reactions of the asparagine residues identified as susceptible to deamidation were determined using pH 8.6 conditions at 4 °C and 37 °C. The samples were analyzed at the following time-points: 0, 1, 2, 5, 7, 11, and 14 days. Asparagine 218 of the rat heavy chain showed the fastest deamidation rate with a half live of approximately 1.5 days and a K value of 0.46 days^{-1} at 37 °C (R^2 = 1.0) followed by asparagine residue 388 of the mouse heavy chain with a half live of approximately 3.6 days and a K value of 0.19 days^{-1} at 37 °C (R^2 = 0.982). The kinetic parameters of the other asparagine residues susceptible to deamidation could not be calculated because the reaction was to slow (less than 10% change in 14 days). No deamidation increase was observed at incubation temperatures of 4 °C at pH 8.6 for up to 14 days.

3.4. Characterization of charged variants

The rat/mouse hybrid antibody could be separated into three major isoforms using isoelectric focusing techniques. Fig. 8 shows the IEF gel of the control and the pH 8.6 stressed samples. Using the IEF information, the kinetics of the degradation of the basic isoform could be calculated. Comparing the rate of degradation with the rate of deamidation of peptide 213-KVERRNGGIGHK-224 (from the rat heavy chain), it was concluded that deamidation of asparagine 218 of the rat heavy chain is the major cause of the changing IEF profile. Deamidation of other asparagine residues, especially N388 of the mouse heavy chain contribute to the changing IEF profile as well

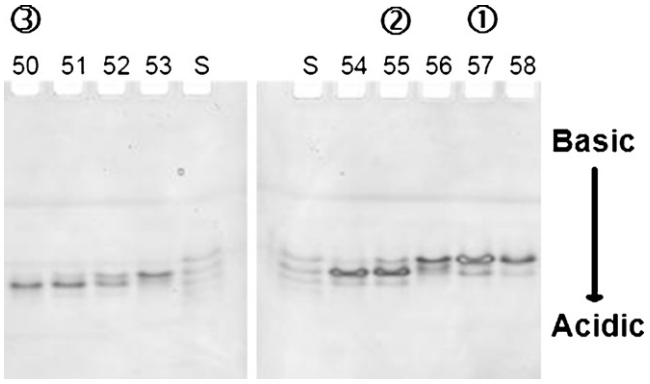

Fig. 9. IEF analysis of antibody fractions separated by FFE fractionation. Fractions of interest ((1) 57, (2) 55, and (3) 50) were further analyzed by peptide mapping as described in Section 2.

Table 4
Deamidation percentage of peptides after unfolding, Lys-C digestion, reduction, and alkylation of the monoclonal antibody isoforms isolated by FFE and a control sample. The amount of deamidation for each peptide was calculated using their observed and theoretical isotope profile.

Peptide	% Deamidation			
	FFE 1	FFE 2	FFE 3	Control
RL8	−1[a]	1	9	9
RH9_10	50	75	100	63
MH28	1	11	29	18
ML11	8	15	65	22
MH23	n.d.	n.d.	n.d.	26

n.d., not detected.
[a] Since negative values are not possible, the value is an error of the quantification method and represents no deamidation.

and explained the differences in the faster degradation rate of the basic isoform (half life of 0.9 days; K = 0.81 days^{-1}) compared to the deamidation rate of peptide 213-KVERRNGGIGHK-224 (half life of 1.5 days; K = 0.46 days^{-1}).

To identify the differences in the three major isoforms of the rat/mouse hybrid antibody, we used free flow electrophoresis for preparative fractionation of the major isoforms. Fractions were collected into 96-well plates and the protein concentration of the 96 samples was measured. Samples containing protein were analyzed by IEF and the results are shown in Fig. 9. The samples (1–3) containing the three antibody isoforms of interest, were analyzed by Lys-C peptide mapping as described in Section 2. Prior to concentration and buffer exchange, cellulose (which is present in the FFE buffer) needed to be removed which was achieved by precipitation of the cellulose at 65 °C for 30 min followed by centrifugation. The results of the levels of deamidation in the three isoforms are shown in Table 4. Again, deamidation of peptide 213-KVERRNGGIGHK-224 (from the rat heavy chain) was the major difference between the three isoforms. No other possible difference between the three FFE fractions (like glycosylation, N-terminal pyroglutamate formation, C-terminal lysine variants, or oxidation) was detected.

4. Discussion

We have optimized a Lys-C peptide mapping method for the identification and quantification of oxidation and deamidation of antibodies. Lys-C digestion has the advantage of improved sequence coverage compared to the more common tryptic digestion procedure for peptide mapping. In this study, we achieved 99% sequence coverage of the rat/mouse hybrid antibody. Another advantage of a Lys-C compared to trypsin digestion is the possibility of digesting the non-reduced antibody, giving information about the disulfide structure. Our experiments showed that significant deamidation occurs during the peptide mapping procedure and the levels of deamidation determined using this technology are not the levels of deamidation present in the protein sample before analysis. This observation was reported for trypsin digestions as well, and efforts to reduce the method induced artifact have been described [28–30]. We have identified several peroxide- or pH-stress induced oxidation and deamidation sites in a rat/mouse hybrid antibody. Some oxidation and deamidation sites identified in this study are in the conserved regions of the antibody and are shared by other rat and mouse antibodies; some are even shared by human antibodies as well. Methionine residue 256 of the mouse heavy chain was found to be most susceptible for oxidation using peroxide stress conditions. Methionine residue 256 is located in the conserved region of the antibody and although the mouse sequence shows high sequence homology with the rat sequence, the rat heavy chain does not contain this particular methionine residue. Human IgG contains this methionine residue and it has

been reported to be prone to oxidation in human IgG antibodies as well [31,32]. The identified asparagine residues susceptible to deamidation contain either a C-terminal glycine or a serine residue confirming, that asparagine residues with small amino acids like glycine or serine at the C-terminus are most susceptible to deamidation [19]. Asparagine residue 218 of the rat heavy chain was identified as most susceptible to deamidation. This amino acid is also located in the conserved region of the antibody, but it is not shared by mouse or human IgG antibodies. Deamidation of human antibodies was studied by others, showing that N382 and N387 of the heavy chain were most susceptible to deamidation [28,33]. The N382 residue is shared by mouse and rat antibodies and N387 is not. We identified N388 of the mouse heavy chain, which corresponds to N382 in human antibodies, as the second fastest deamidation site. No deamidation of the corresponding rat asparagine residue could be detected. It is possible, that our method was not capable of detecting this deamidation site, since it is located on a large peptide of 5426 Da, making the quantitative analysis of the isotopes difficult.

Deamidation of asparagine to aspartic acid, changes a neutral to an acidic site chain. The deamidation degradation has the potential to change the charge of the whole antibody to a more acidic isoform. Comparison of the kinetic data of asparagine residue 218 deamidation analyzed by mass spectrometry with the kinetic degradation of isoform 1 analyzed by isoelectric focusing indicated, that deamidation of asparagine 218 of the rat heavy chain is the major cause of the changing IEF profile during stress studies. Separation of the isoforms in the rat/mouse hybrid antibody preparation by FFE and analysis of the fraction by mass spectrometry revealed, that deamidation is also the major cause of the charge variants of the antibody. The observed amounts of antibody isoforms in a control sample on IEF gels are: isoform 1 = 63%, isoform 2 = 27%, and isoform 3 = 10% (numbering from the most basic to the most acidic). The data matched well with theoretical calculations based on the amount of deamidation measured for the different asparagine residues by mass spectrometry. The Lys-C digestion at pH 7.0 showed the lowest levels of deamidation and therefore the lowest levels of method induced artificial deamidation (34%, 11%, 7%, 4% and 2% for the peptide RH9_10, ML11, MH23, M28, and RL8 respectively). Assuming that deamidation reactions are independent events, it can be estimated that statistically, 51% of the antibody should not be deamidated, 40% of the antibody should have one deamidation site, 8% two deamidation sites, and 1% three deamidation sites. It is not surprising that the mass spectrometry data overestimated the amounts of deamidation (only 51% native) compared to the IEF data (63% native), due to artificial deamidation during the Lys-C digestion. Taking all the information into account, we concluded, that the most basic isoform (isoform 1) of the rat/mouse hybrid antibody does not contain any deamidation sites, isoform 2 contains one deamidation site, isoform 3 contains two deamidation sites, and so on. Since deamidation reaction is favored at high pH and high temperatures, we suspect, that deamidation occurs during the manufacturing process of the antibody, especially during fermentation (pH 7.2 at 37 °C).

After the identification and characterization of oxidation and deamidation sites in rat/mouse hybrid antibodies, the information was used to investigate oxidation and deamidation during real storage conditions and to evaluate different formulations. The effect of the pH and excipients in different formulations was studied to minimize oxidation and deamidation during storage. We found that formulations at pH 5.6 did not result in significant deamidation at storage conditions of 2–8 °C for up to 2 years. The levels of oxidation did not seem to increase significantly under these conditions as well, indicating that excipients to prevent oxidation are not required [15]. Additionally, the levels of oxidation in polysorbate containing formulations to stabilize the protein did not seem to be different compared to polysorbate-free formulations [34].

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