*Pharmaceutical Research, Vol. 24, No. 6, June 2007 (© 2007)*
DOI: 10.1007/s11095-007-9241-4

*Research Paper*

# Accumulation of Succinimide in a Recombinant Monoclonal Antibody in Mildly Acidic Buffers Under Elevated Temperatures

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*Received October 24, 2006; accepted January 9, 2007; published online March 24, 2007*

***Purpose.*** The purpose of this paper was to identify the location of a succinimide and determine the rate of its formation and hydrolysis in a recombinant human monoclonal IgG2 antibody aged in mildly acidic buffers at elevated temperatures.
***Materials and Methods.*** Cation exchange (CEX) HPLC separated multiple Main Peaks and high levels (up to 50%) of basic variants, the identification of which was an analytical challenge and required several complementary techniques. The relative abundance of the CEX basic variants was used to quantify the percentage of succinimide and to study the rates of its formation and hydrolysis.
***Results.*** Mass decrease by approximately 18 Da for intact antibodies from the CEX basic fractions suggested succinimide formation from aspartic acid as the major modification. Reversed-phase HPLC/MS of the reduced and trypsin-digested samples detected an isoaspartate 30 (isoD30) in the light chain peptide A25-R37. Direct evidence that isoD30 was from succinimide was obtained by performing succinimide hydrolysis in $H_2{}^{18}O$ followed by tryptic digestion in $H_2{}^{16}O$.
***Conclusions.*** Succinimide formation increased as pH became more acidic, whereas its hydrolysis was faster as pH became neutral and alkaline. Succinimide hydrolysis in a denatured sample was estimated to have completed in less than 2 h, but approximately three days for a similar pH but without denaturant. These observations suggest that protein conformation affects succinimide hydrolysis.

**KEY WORDS:** antibody; hydrolysis; ion exchange; mass spectrometry; succinimide.

## INTRODUCTION

In many cases, covalent modifications and physical changes in proteins, including dimer formation and aggregation, tend to be minimized at mildly acidic pH (1,2). However, one reaction that occurs at this pH is succinimide (cyclic-imide, Su) formation from an aspartic acid residue (3). At neutral pH, the formation of a succinimide intermediate is followed by its hydrolysis that results in isoaspartate and aspartate (4). The formation of isoaspartate may lead to the loss of biological activity (5,6) and even to a number of immunologic responses (7–9). Therefore it is important to monitor the formation of succinimide and isoaspartate in protein therapeutics. Aspartic acid is susceptible to succinimide formation because of the close proximity of its side-chain carbonyl group to the peptide-bond nitrogen atom of the $n$+1 residue. In mildly acidic solutions, the isomerization reaction includes a nucleophilic attack of the carbon atom of the carboxyl group of the aspartic acid residue by the $n$+1 nitrogen atom, leading to the formation of the succinimide intermediate (4). The succinimide ring is susceptible to hydrolysis at either of the two C-N bonds to produce α- or β-carboxyl groups, which generates isoaspartate and aspartate, respectively, at a ratio of 3:1 in short, unstructured peptides (4,10). Once succinimide is formed, its hydrolysis is typically quite rapid at neutral and alkaline pH. In mildly acidic solutions, the rate of cyclic imide formation is higher than the rate of its hydrolysis. Consequently the succinimide intermediate can accumulate (11–13).

Succinimide has been detected in intact proteins such as human growth hormone (14), aging crystallins (15), glial cell line-derived neurotrophic factors (16), lysozyme (17,18), and tubulin (19). The formation of succinimide and isoaspartate readily occurs at an asparagine followed by a glycine or serine (20,21), or at an aspartic acid followed by a glycine (20). Two published reports on succinimide in monoclonal antibodies have focused on the identification of the aspartic acid residue involved in cyclization and isomerization (5,6). Aspartic acid D32 of the light chain CDR1 followed by a glycine in an anti-IgE antibody converted to a succinimide intermediate and isoaspartate (5). Aspartic acid D102 followed by glycine G103 in the heavy chain of the monoclonal IgG1 antibody Herceptin converted to isoaspartic acid and a minor fraction of succinimide intermediate in a pH 6 buffer (6). The above reports did not address in details the kinetics of succinimide formation and hydrolysis in the intact folded protein. Previously, the rate of succinimide formation was obtained primarily through peptide models (12,13,22). In this study, we used several novel and improved analytical methods including cation-exchange (CEX) HPLC with a mildly-acidic mobile phase, reversed-phase (RP) HPLC/MS (23) of intact CEX fractions, peptide mapping and hydrolysis in the presence of $H_2{}^{18}O$ (24,25) to characterize the rare accumu-

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lation of the succinimide intermediate in an intact human monoclonal IgG2 antibody. We also examined the kinetics of succinimide formation and hydrolysis in a natively folded intact protein at mildly acidic, neutral, and alkaline pH.

## MATERIALS AND METHODS

### Materials

The recombinant human monoclonal IgG2 antibody analyzed in this study was expressed at Amgen and purified using standard manufacturing procedures. An aging study that examined the effects of pH, in the range of 4.1–6.2, and temperature, in the range of 4–45°C, on this antibody was performed and compared with a control sample that was stored frozen at −70°C.

### Cation Exchange HPLC and Fraction Collection

Weak cation exchange (CEX) HPLC separation of the intact IgG2 was performed using a Dionex WCX Propac 10 column (Dionex Corp., Sunnyvale, CA, USA) and an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA). Protein elution was achieved with a mobile phase gradient from 0 to 100 mM NaCl in 10 mM sodium acetate, pH 5.7, at 25°C. Fractions of the resolved charged variants were collected manually, using the same CEX analytical method. The solvent of the collected CEX antibody fractions was exchanged with the original mildly acidic buffer, using Amicon Ultra centrifugal filter devices with a molecular weight cut-off (MWCO) of 30 kDa (Millipore Corp., Bedford, MA, USA). Concomitantly the fractions were concentrated to 3–18 mg/ml. The purity of each fraction was verified by CEX HPLC. Fractions were stored at 4°C until use.

### Reversed-phase HPLC of Intact Antibody in the CEX Fractions

RP HPLC of the CEX fractions was performed with an Agilent 1100 Capillary HPLC system, using an Agilent Zorbax stable-bond SB300 C8, 50×1 mm, column with 3.5 μm particle size and 300 Å pore size (Micro-Tech Scientific Inc, Vista, CA, USA) according to (26). The mobile phase was water with 0.1% (v/v) trifluoroacetic acid (TFA, J.T. Baker, Phillipsburg, NJ, USA) in solvent A and 90% (v/v) N-propanol (Burdick & Jackson, Muskegon, MI, USA), 9.9% (v/v) water, and 0.1% (v/v) TFA in solvent B. The elution gradient was from 21 to 27% B, with the column at 80°C and a flow rate of 50 μl/min. The column eluate was analyzed by the UV detector and then directed to the on-line electrospray ionization (ESI) time of flight (ESI-TOF) mass spectrometer.

### Reduction and Alkylation of the Antibody

Reduction and alkylation was performed on the antibody under denaturing conditions to produce free heavy and light chains for further analytical characterization. The antibody was diluted to a volume of 0.5 ml and a concentration of 2 mg/ml, using a pH 7.5 buffer comprised of 7.5 M guanidine hydrochloride (GndHCl, Mallinckrodt, Phillipsburg, NJ, USA), 0.1 M Tris-HCl (Sigma, St. Louis, MO, USA), and 1 mM ethylenediaminetetraacetic acid (EDTA, Sigma). An aliquot of a 0.5 M stock solution of the reducing agent dithiothreitol (DTT, Sigma) was added to the protein solution to obtain a final concentration of 5 mM DTT. Subsequently, the overall reaction mixture was incubated at 37°C for 60 min, and then cooled to room temperature. Alkylation of the reduced antibody was done by adding an aliquot of a 0.5 M iodoacetic acid (IAA, Sigma) stock solution, making the final concentration of the IAA in the protein solution 13 mM. The protein solution was then stored at room temperature in the dark for an additional 40 min. A second addition of DTT (7 mM) was used to quench any unused IAA. To remove the excess DTT and IAA, the protein solution was buffer exchanged with 10 mM sodium acetate, pH 5, using a NAP-5 gel filtration column packed with Sephadex G-25 resin (Amersham Pharmacia Biotech, Orsay, France) following the procedure described by the manufacturer. The concentration of the final reduced and alkylated protein solution was 1 mg/ml.

### Reversed-phase HPLC of the Reduced and Alkylated Antibody

The light and heavy chains from the reduced and alkylated IgG sample were separated and analyzed by RP HPLC/MS on an Agilent 1100 Capillary HPLC system similar to the protocol described in (27). The mobile phase included water with 0.1% TFA in solvent A and 80% (v/v) N-propanol, 10% (v/v) acetonitrile (ACN; J.T. Baker, Phillipsburg, NJ, USA), 9.9% (v/v) water, and 0.1% (v/v) TFA in solvent B. Similar to that used for the non-reduced samples, RP HPLC/MS of the reduced and alkylated samples was done on an Agilent Zorbax stable-bond SB300 C8 column that was 50×1 mm, with 3.5 μm particle size and 300 Å pore size. Elution of the heavy and light chains was done with the column temperature at 80°C and a flow rate of 50 μl/min. The eluate was analyzed by the UV detector and then directed to an on-line mass spectrometer. The reduced antibody chains were eluted using a linear gradient from 21 to 28% B over 42 min.

### Electrospray Ionization Time-of-Flight (ESI-TOF) Mass Spectrometry of the Intact and Reduced Antibody

On-line with RP HPLC, electrospray ionization time of flight (ESI-TOF) mass spectrometry was performed using a Waters/Micromass LCT orthogonal TOF mass spectrometer equipped with an ESI atmosphere-vacuum interface. The mass spectrometer was operated in a positive ion mode with capillary voltage at 2,900 V, sample cone at 125 V, m/z in the range of 1,000–4,000, and analyzer in V-mode with mass resolution of 5,000. The instrument was calibrated externally in a m/z range of 1,500–4,000 using multiply charged ions of a standard, fully characterized, and purified, recombinant monoclonal antibody with a MW value of 148,251.2 Da. Deconvolution of the ESI mass spectra was performed using a MaxEnt1 algorithm, which is a part of the MassLynx software from Micromass. More details about the accurate mass measurement and deconvolution procedure for intact antibodies can be found in reference (28).

### Tryptic Digestion

For peptide mapping, the antibody was first reduced and alkylated as described above. However, the reduced and alkylated protein solution was exchanged with a digestion buffer containing 0.1 M TRIS, pH 7.5, instead of 10 mM sodium acetate, pH 5. The NAP-5 gel filtration column was equilibrated with 10 ml of 0.1 M Tris-HCl pH 7.5 buffer, and then 0.5 ml of sample was loaded onto the column. The reduced and alkylated antibody was eluted with 1 ml aliquot of 0.1 M Tris-HCl pH 7.5 buffer to a final protein concentration of approximately 1 mg/ml. A stock trypsin solution was prepared by dissolving lyophilized sequencing grade trypsin (Worthington Biochemical Corporation, Lakewood, NJ, USA) in water to a final concentration of 0.5 mg/ml. Tryptic digestion was performed for 2 h under the described denaturing conditions at 37°C using an enzyme: protein ratio of 1:50 (w:w). The same amount of trypsin was added a second time, making the final enzyme: protein ratio 1:25 (w:w). The digestion continued for another 2 h; then the digests were frozen at −20°C until use. More details of the peptide mapping can be found in (29).

### Reversed-phase HPLC of Tryptic Peptides

Tryptic peptides were separated by RP HPLC using a Polaris Ether column that was 250×2 mm and packed with a C18 resin of 3 μm particle size and 300 Å pore size (Varian, Torrance, CA, USA). The mobile phase was 0.1% (v/v) TFA in water in solvent A, and 90 : 9.015 : 0.085% (v/v) of ACN : water : TFA in solvent B. Peptides were eluted using a linear gradient from 0 to 50% B over 205 min. The column flow rate was 200 μl/min, and its temperature was 50°C. Per analysis, 20 μg of protein digest was injected onto the column. The eluate was analyzed by an UV detector, and then directed to an on-line ion trap mass spectrometer.

### Ion Trap Mass Spectrometry for Peptide Maps

A Thermo Finnigan ion trap mass spectrometer LCQ DECA was used on-line with the RP HPLC system to identify the digested products. Masses of the peptides and their fragments were obtained using a triple play method, which included full scan, followed by zoom and MS/MS scans. A standard off-axis ESI source was used as the atmospheric vacuum interface. The instrument was tuned with the doubly charged ions from a synthetic peptide at m/z 842 Da. The sequence algorithm from the Thermo Finnigan BioWorks 3.1 software and a mass analyzer software developed in house (30,31) were used for peptide identifications.

### Succinimide Hydrolysis in $H_2{}^{18}O$, Followed by Tryptic Digestion in Regular ($H_2{}^{16}O$) Water

$^{18}O$ water was purchased from Cambridge Isotope Laboratories, Inc (97% enriched, lot no. WP-05-36). The antibody sample was dried under vacuum, using a SpeedVac, and resolubilized in $^{18}O$ water to remove residual regular water in the samples. This process was repeated twice. Subsequently, the dried sample was reduced and alkylated under denaturing conditions similar to the reduction and alkylation procedure described above, but using only solutions that were prepared with $^{18}O$ water. The preparation was as follows. The dried samples were dissolved in 0.5 ml of 8 GndHCl with 0.1 M ammonium bicarbonate (Sigma), pH 7.5. An aliquot of a 0.5 M stock solution of the reducing agent dithiothreitol (DTT, Sigma) was added to the protein solution to obtain a final concentration of 5 mM DTT. Subsequently, the overall reaction mixture was incubated at 37°C for 60 min, and then cooled to room temperature. Alkylation of the reduced antibody was done by adding an aliquot of a 0.5 M iodoacetic (IAA, Sigma) stock solution, making the final concentration of the IAA in the protein solution 12 mM. The protein solution was then stored at room temperature in the dark for an additional 30 min A second addition of DTT (7 mM) was used to quench any unused IAA. After approximately 2 h of the antibody reduction and alkylation in the $H_2{}^{18}O$ denaturing solution, the reduced and alkylated antibody was buffer exchanged with 0.1 M Tris-HCl that was prepared in regular ($^{16}O$) water, pH 7.5, using a NAP-5 gel filtration column packed with Sephadex G-25 resin (Amersham Bioscience, Uppsala, Sweden). Then, tryptic digestion was performed according to the protocol described above. Once digestion was completed, the samples were analyzed immediately by RP HPLC/MS/MS.

## RESULTS AND DISCUSSION

### CEX Analysis of the Antibody Aged at Different pH Values

A CEX chromatogram of the intact monoclonal IgG2 stored at −70°C showed multiple Main Peaks, labeled 1 to 4, and low traces of acidic and basic variants (Fig. 1, *broken line*). When stored at elevated temperatures such as 37 and 45°C for prolonged periods of time in mildly acidic buffers, the protein exhibited a different CEX profile, in which there was a loss of the Main Peaks, as observed by decreased absorbance, and a noticeable increase of the basic variants (Fig. 1, *solid line*). There was negligible increase in the acidic variants. The basic variants were separated further into two distinct groups, CEX Basic 1 and Basic 2. In the antibody samples that had been stored at 37°C for a three-month period, the levels of CEX Basic 1 and 2 (Fig. 2a and b, respectively) steadily increased over time and were greatest

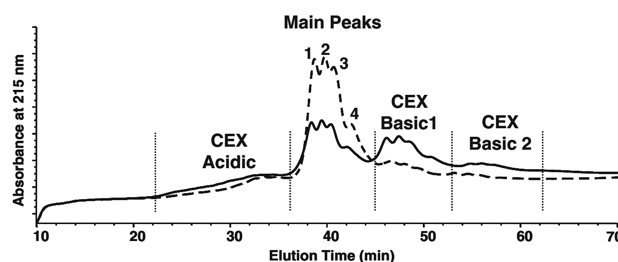


**Fig. 1.** CEX chromatograms of the monoclonal human IgG2 antibody stored at −70°C (*broken line*) and after 2 weeks at 45°C (*solid line*) in a pH 5 buffer. Four groups of peaks are resolved, and are labeled CEX Acidic, Main (Peaks 1–4), and CEX Basic 1 and Basic 2. The multiple CEX Main peaks was attributed to disulfide heterogeneity found in IgG2 antibodies by reversed-phase HPLC (26) and will not be discussed here.

at pH 4.1 and lowest at pH 6.2. Increase of the percentage of total CEX Basic variants (CEX Basic 1 plus CEX Basic 2, Fig. 2c) correlated well with the loss of the Main Peaks (Fig. 2d). For the same storage period but at 4°C, the increase in CEX Basic 1 and Basic 2 was minimal, less than 1% (data not shown), thus indicating that the increase of these two species were dependent on the pH and the storage temperature. Note that the CEX chromatograms of the frozen control sample contained approximately 12% of the basic peaks due mainly to the remaining lysine residues on the C-termini of the heavy chains. Peptide mapping showed that the percentage of the CEX basic peaks closely correlated with the percentage of the remaining C-terminal lysines. Although genetically coded, these residues are almost completely cleaved off by enzymes when antibodies are produced in mammalian cells.

Fractions of the CEX Basic 1, Basic 2, as well as Main Peaks 1 to 4 were collected and analyzed by RP HPLC/MS to determine their masses and other differences between these charged variants. The MW values of the three sugar isoforms with none, one, and two terminal galactose residues (0G, 1G and 2G, respectively) were measured using the deconvoluted ESI mass spectra shown in Fig. 3. From the deconvoluted ESI mass spectra of the different CEX fractions, the 0G masses of CEX Main Peaks 1, 2, 3, and 4 were determined to be 147,797.7; 147,796.7; 147,797.4 and 147,794.6 Da, respectively (Table I). Altogether these values produced an average MW of 147,796.6 Da with a standard deviation of 1.4 Da, which was within the precision of the TOF mass spectrometer (28). The closeness in MW between Main Peaks 1 to 4 demonstrated that they were isoforms with identical mass but slightly different apparent surface charge properties. This polymorphism was attributed to disulfide heterogeneity found in IgG2 antibodies by reversed-phase HPLC (26) and will not be discussed here.

### RP HPLC/MS Analysis of the Intact and Reduced Antibody, and Peptide Maps of the CEX Fractions

The MW of the CEX Basic 1 fraction was 16.7±1.4 Da less than the average mass of Main Peaks 1 to 4, whereas the MW value of the CEX Basic 2 fraction was 32±5 Da less than the Main Peaks 1 to 4 (Table I). The precision of the TOF mass spectrometer is typically ±1.4 Da for an injection of 5 μg of the intact antibody. The CEX Basic 2 fraction was available in a lower amount, leading to a lower ion signal. As a result, the error increased to ±5 Da for this CEX fraction. The mass differences between CEX Basic 1 and the

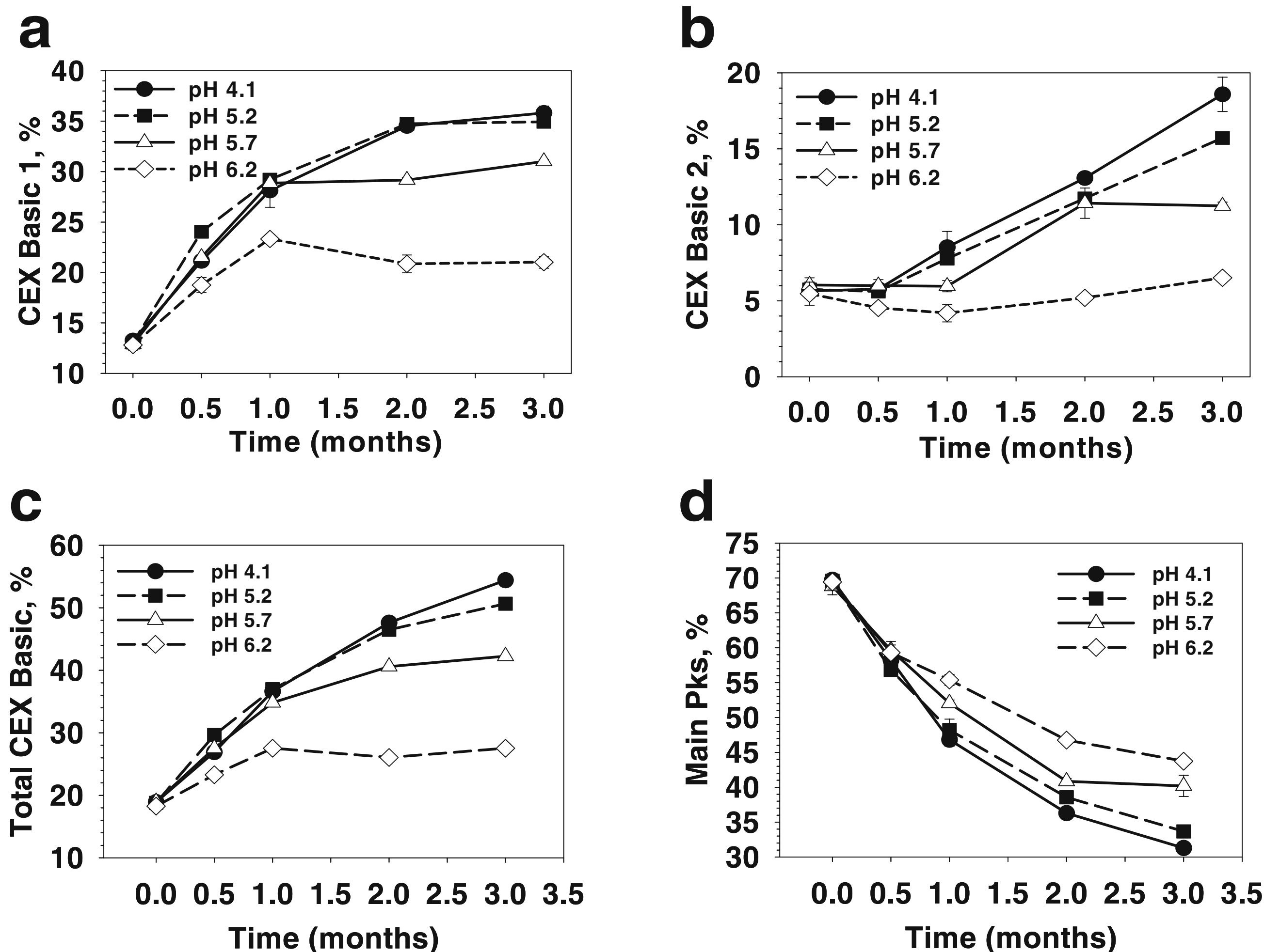


**Fig. 2.** Percentages of CEX **a** Basic 1, **b** Basic 2, **c** total basic variants, and **d** Main Peaks after the IgG2 was aged at 37°C for up to 3 months in pH 4.1 to 6.2 buffers. The percentages are obtained by dividing the area under the specific CEX peak by the sum of the areas for all CEX peaks.

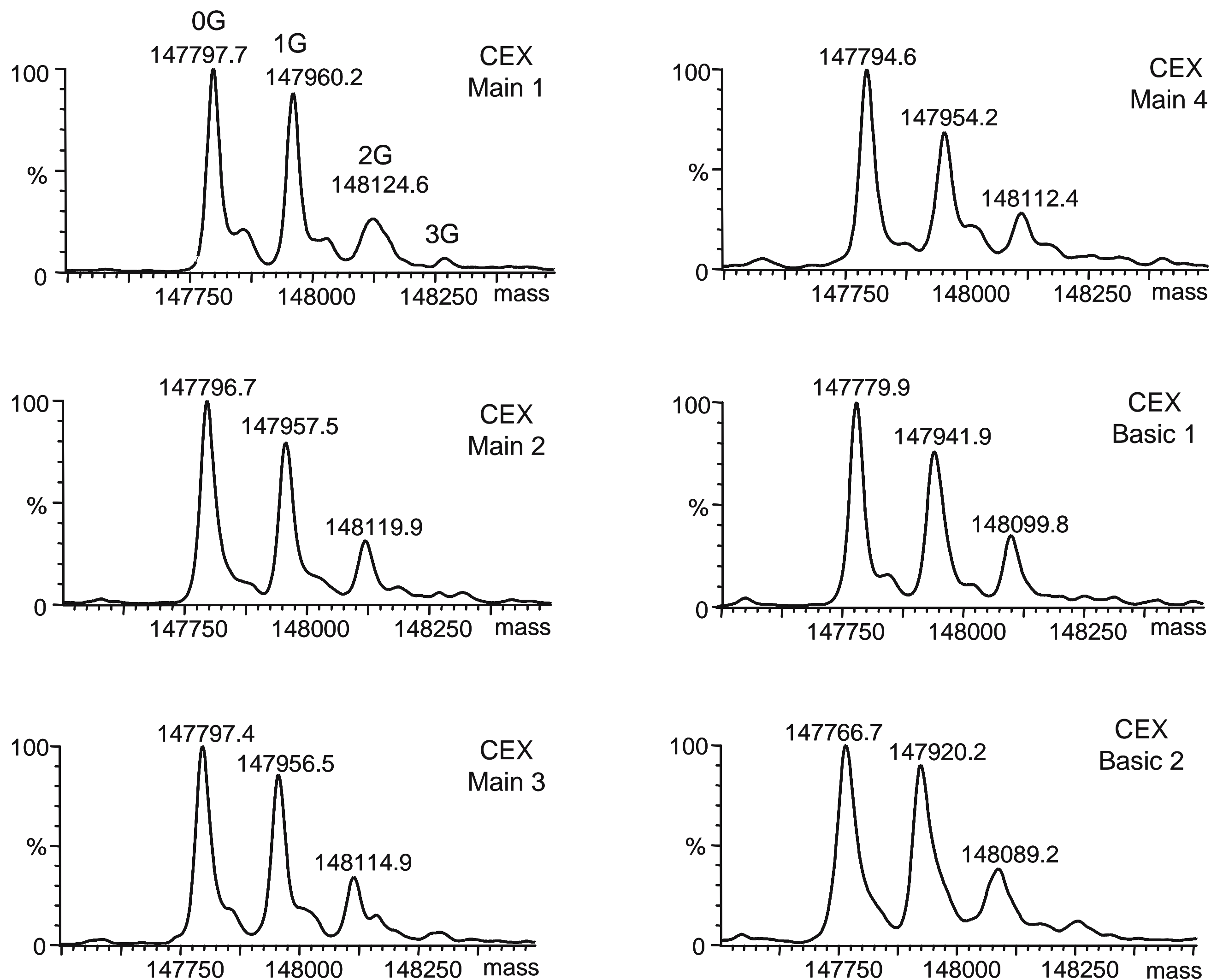


**Fig. 3.** Deconvoluted ESI mass spectra of the CEX fractions from the aged antibody separated in the CEX chromatogram in Fig. 1. Each mass spectrum contains multiple peaks, each differing by 162 Da, because of the heterogeneity in the terminal galactose residues (G) in the two sugar moieties.

Main Peaks indicated that the former had undergone a chemical modification that resulted in a mass loss of 16.7±1.4 Da. Since IgG molecules are homodimers, the nearly double mass loss in the CEX Basic 2 fraction suggests that the modifications in this set of basic variants have occurred on both chains instead of one chain of the antibody, which would be the case in the CEX Basic 1 fraction. Also, similar to the CEX Main Peak, the CEX elution profiles of what we have grouped as CEX Basic 1 and Basic 2 (Fig. 1, *solid line*) showed heterogeneity, as evident by the multiple peaks within each group. In short, CEX Basic 1 and 2 were indicative of an isoform that had undergone a chemical modification of 16.7±1.4 Da in one or both chains, respectively.

Searches through the Delta Mass database of post-translational modifications http://www.abrf.org/index.cfm/dm.home?AvgMass=all) found in proteins propose several modifications that can lead to a mass loss in the range from 15 to 18 Da (16.7±1.4 Da). These modifications include

**Table I.** Measured Masses of Intact Antibodies From Different CEX Fractions Separated in Fig. 2

| | CEX Main 1 | CEX Main 2 | CEX Main 3 | CEX Main 4 | Ave Main | SD | CEX Basic1 | CEX Basic1 Minus Ave | CEX Basic2 | CEX Basic2 Minus Ave |
|---|---|---|---|---|---|---|---|---|---|---|
| 0G | 147,797.7 | 147,796.7 | 147,797.4 | 147,794.6 | 147,796.6 | 1.4 | 147,779.9 | −16.7 | 147,766.7 | −29.9 |
| 1G | 147,960.2 | 147,957.5 | 147,956.5 | 147,954.2 | 147,957.1 | 2.5 | 147,941.9 | −15.2 | 147,920.2 | −36.9 |
| 2G | 148,124.6 | 148,119.9 | 148,114.9 | 149,112.4 | 148,118.0 | 5.4 | 148,099.8 | −18.2 | 148,089.2 | −28.8 |
| | | | | | | | | −16.7 | | −31.8 |

The masses were obtained from the deconvoluted ESI mass spectra shown in Fig. 3 after RP HPLC/MS of the fractions. Three of the most abundant antibody sugar isoforms with a total of zero (0G), one (1G), and two (2G) galactose residues were used for the mass calculations.

pyroglutamate formation at the N-terminal glutamic acid (E→pE) (32) and succinimide formation from an aspartic acid residue (D→Su). Schematics of these two modifications are illustrated in Fig. 4. Pyroglutamate formation from glutamate theoretically should be an electrically neutral modification under CEX conditions at pH 5. Nevertheless, we decided to verify this experimentally. If the theory is correct and N-terminal cyclization of glutamic acid does not change the net charge, each of the CEX fractions should contain pyroglutamate (pE).

We have shown previously that the heavy and light chains of an antibody containing N-terminal pyroglutamate could be separated by reversed-phase chromatography from the native chains that contained N-terminal glutamine and glutamic acid residues (27,32). To determine if the CEX fractions contained pyroglutamate, RP HPLC/MS analyses were performed on the following reduced and alkylated samples: the unfractionated protein that had been stored at 37°C in pH 5 for three months; the Acidic, Main Peaks, Basic 1, and Basic 2 CEX fractions from this aged sample. The RP chromatograms for all samples were similar and had two dominant RP peaks (Fig. 5). The peak eluting at ~ 14.5 min was identified by MS to have a MW of 23,801 Da, indicating it was a light chain (LC). The peak eluting at ~ 26 min had a MW of 51,152 Da, correlating to a heavy chain (HC). Also detected in the unfractionated aged sample as well as the CEX fractions was a minor peak eluting slightly later than the LC at ~ 16 min. Further MS analyses identified its mass to be 23,783 Da, 18 Da less than the LC. Tryptic peptide maps and "top-down" fragmentation of the entire chains (data not shown) identified this post-LC peak to be a LC variant with pE in place of the N-terminal glutamic acid, similar to the case reported previously (32). Quantitation of the percentage of pE by the area under the curve for this post-LC peak at 16 min showed it was about 7±2% in the unfractionated sample and also in each of the CEX-separated fractions. Thus, pE formation was excluded as the modification that lead to the CEX Basic peaks shown in Fig. 1. Measured masses of the light and heavy chains of the CEX Acidic fraction (Fig. 5b) were similar to the CEX Main fraction within the measurement error of ± 1 Da, and there were no extra peaks in the chromatograms of the reduced CEX fractions and the unfractionated sample. This result came as a surprise taking into account that the same CEX Basic 1 and Basic 2 intact fractions exhibited a significant mass decrease of 16.7±1.4 Da and 32±5 Da, respectively. The logical explanation was that the CEX Basic peaks were due to the formation of succinimide from an aspartic acid residue (−18 Da), which rapidly hydrolyzed to isoaspartic and aspartic acid residues during a reduction and alkylation procedure under denaturing conditions at pH 7.5. The chains with aspartic and isoaspartic acid residues would have the same mass, and their elution time could be the same due to the poor separation of these large protein variants.

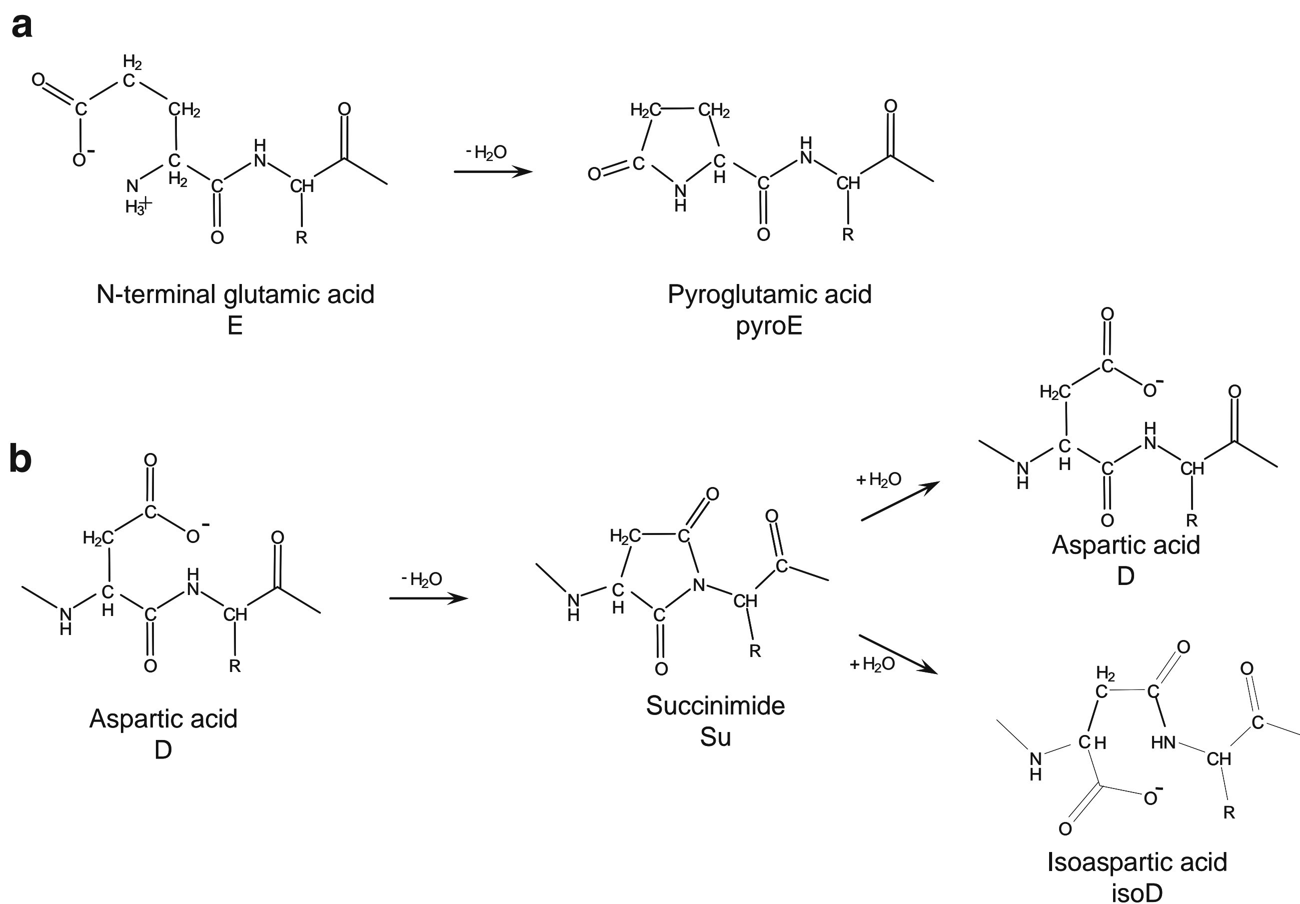


**Fig. 4.** Schematic of two modifications found in IgG molecules leading to 18-Da mass decrease: **a** formation of pyroglutamate from N-terminal glutamate, and **b** conversion of aspartic acid to succinimide and its hydrolysis to aspartate and isoaspartate.

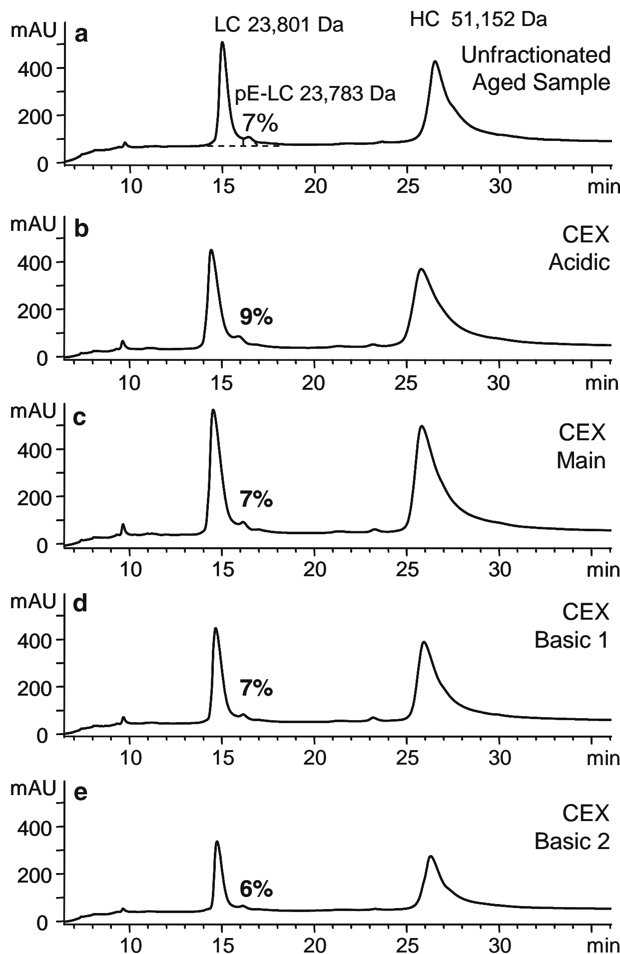


**Fig. 5.** Reversed-phase chromatograms of the reduced and alkylated antibody samples with UV detection at 215 nm: **a** unfractionated sample aged in a pH 5 buffer at 37°C for 3 months; **b** CEX acidic fraction; **c** CEX Main; **d** CEX Basic 1; and **e** CEX Basic 2 collected from the unfractionated aged sample. The masses for the light and heavy chains are labeled. The light chain containing N-terminal pyroglutamate (pE-LC) and its overall abundance in each CEX fraction are indicated.

Peptide mapping was employed as the next step in order to detect the possible differences in the CEX fractions. A comparison of the reconstructed ion chromatograms indicated that all the fractions had a peak eluting at 100 minutes (Fig. 6). However, the key difference between the CEX fractions was the peak at 97 minutes and its relative amount in each of the CEX fractions (Fig. 6). The doubly charged precursor ion for this peak at 97 minutes had m/z of 749.2 Da, indicating that the peak had a mass of 1498.4 Da. Peptide maps of protein samples stored at the same pH of 5 but at 4°C did not contain this 97 min peak. Both of the peaks at 97 and 100 minutes had similar fragmentation mass spectra, and were identified to contain the same tryptic peptide of the light chain, A*25*SQSVDSNLAWYR*37* (A25-R37). The logical explanation for a similar mass but different elution time was that these two peaks were isomers of the same peptide. Isoaspartate-containing peptides had been reported to have an earlier elution time on RP chromatogram and a mass identical to the native peptide (4). Because the relative amount in the peak at 97 minutes varied between the different CEX fractions, this peak was deduced to be an isomer of the peptide A25-R37 (iso A25-R37). As shown in Fig. 6, the basic variants had the highest level of iso A25-R37, with the CEX Basic 2 fraction having the greatest, followed by the CEX Basic 1 fraction. In contrast, only 2–3% iso A25-R37 were detected in the Main and acidic CEX fractions.

### Identification and Quantitation of Succinimide by $^{18}$O Labeling

Since the A25-R37 peptide contained only one aspartic acid residue, we deduced that D30 had been converted to succinimide and then hydrolyzed to isoD30. The hydrolysis of D30 occurred rapidly during the reduction and alkylation step, which was performed under denaturing conditions at pH 7.5. The use of $H_2{}^{18}O$ has been applied previously to label the hydrolysis products of succinimide in peptides in combination with HPLC/MS/MS (24,25) and NMR (33). To confirm the site of isomerization, the aged antibody sample was reduced and alkylated under denaturing conditions in $^{18}$O water at pH 7.5 for 2 h (see "MATERIALS AND METHODS"). After buffer exchange, the $H_2{}^{18}O$ -treated protein sample was digested with trypsin in regular water ($H_2{}^{16}O$ ) followed by the RP HPLC/MS/MS analysis of the tryptic peptide. The hydrolysis of succinimide in $H_2{}^{18}O$ would lead to the appearance of $^{18}$O-isoaspartate and $^{18}$O-aspartate with a 2-Da mass increase on the hydrolyzed residue. A comparison of the fragmentation mass spectra of the iso A25-R37 eluting at 97 min after reduction and alkylation under denaturing condition in $H_2{}^{18}O$ (Fig. 7a) and $H_2{}^{16}O$ (Fig. 7b) identified aspartic acid residue 30 as the site of isomerization. The C-terminal containing fragment ions of the peptide, *y8*, *y9* and *y10*, all of which contained residue 30 were 2 Da heavier in the sample prepared in $H_2{}^{18}O$ (Fig. 7a), as compared to the same y-ions of the peptide from the sample prepared in regular ($H_2{}^{16}O$ ) water (Fig. 7b). At

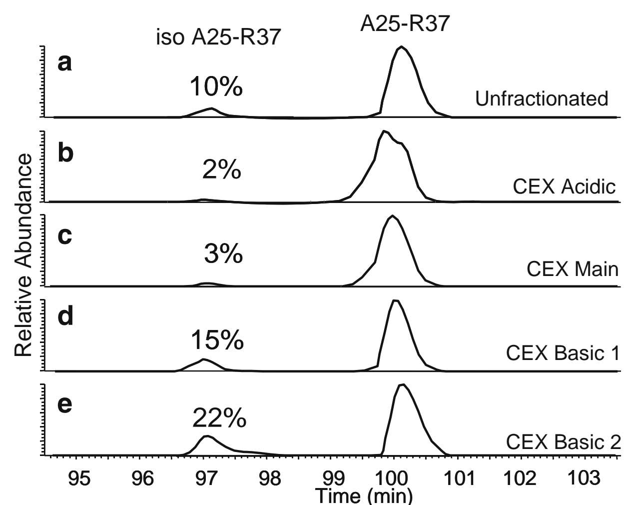


**Fig. 6.** Reconstructed ion chromatograms of the peptide maps of collected CEX fractions resolved in Fig. 1. The m/z 749 of the doubly-charged tryptic peptide A25-R37 from the light chain of the antibody was used to reconstruct the chromatograms. The MS/MS fragmentation pattern is the same for the peaks eluting at 97 and 100 min, suggesting that they are isomers of the same peptide.

the same time, the *y*-ions not containing residue 30, *y2*–*y7*, had the same mass. The same result was obtained when considering the *b*-ion series. In short, ions containing residue D30 showed an increase of 2 Da in mass, whereas ions without residue D30 did not show a mass increase (Fig. 7). The incorporation of $H_2{}^{18}O$ in aspartate 30 of the peptide could be explained only by the presence of succinimide at this residue before hydrolysis, hence unambiguously identifying aspartic acid 30 as the site of isomerization in the LC of the aged antibody.

To estimate the amount of $^{18}O$ incorporated in the isoD30 and D30, on-line high-resolution mass measurements were performed using ESI-TOF mass spectrometer with resolution of approximately 5,000. Fig. 8a and b show ESI mass spectra of doubly charged ions of the A25-R37 and iso A25-R37 peptides after hydrolysis in $H_2{}^{18}O$, respectively. Each peptide exhibits several peaks, each of which is approximately 1 Da apart from the other. The heavier peaks are due to the small percentages of rare isotopes, containing one or several extra neutrons, of the elements $^{2}H$, $^{13}C$, $^{18}O$, $^{15}N$, $^{34}S$, etc. The observed difference between the measured (Fig. 8, solid line) and calculated (Fig. 8, dotted line) isotopic distribution is due to the incorporation of $^{18}O$ into the peptide molecules after succinimide hydrolysis in $H_2{}^{18}O$. The peak areas between the solid and dotted lines represent the abundances of $^{18}O$-labeled peptides in A25-R37 and iso A25-R37 (Fig. 8a and b, respectively). Approximately 80% of the molecules in iso A25-R37 have incorporated $^{18}O$ and

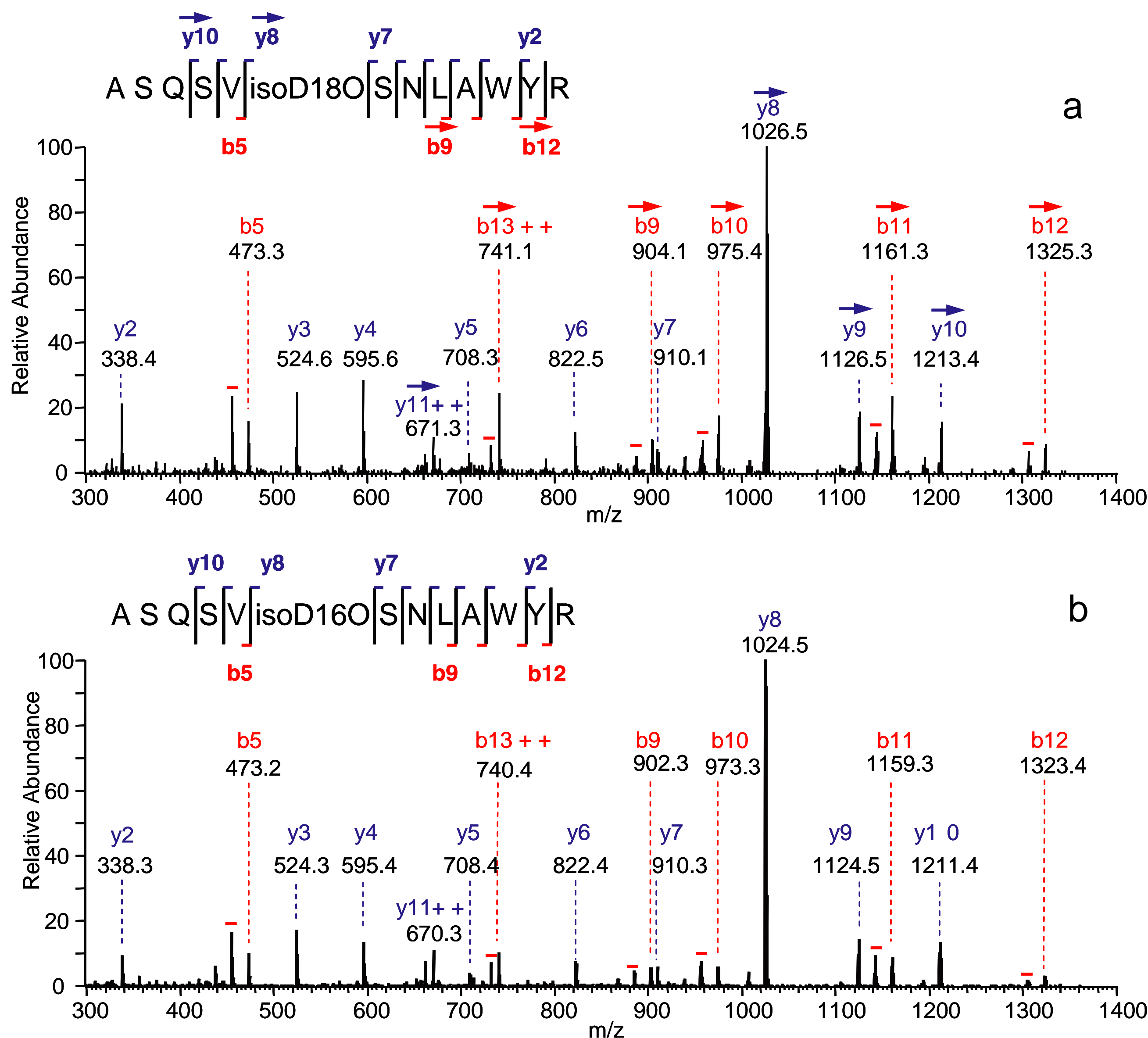


**Fig. 7.** Tandem mass spectra of the ASQSVisoD30SNLAWYR tryptic peptide of the aged IgG2 antibody eluting at 97 min in Fig. 6. Data were obtained by collision induced dissociation of the $(M+2H)^{2+}$ precursor ions. The isoaspartic acid 30 (isoD30) was created by **a** succinimide hydrolysis during unfolding, reduction and alkylation in 7.5M GdnHCl at pH 7.5 for 2 h in $H_2^{18}O$, followed by tryptic digestion in regular water $H_2^{16}O$; and **b** performing both, the succinimide hydrolysis and digestion in regular water ($H_2^{16}O$). *Minus signs* (−) label ions created by loss of water from b-ions, probably from N-terminal serine residues. The mass spectra unambiguously identify isoaspartate 30 as the site of the succinimide hydrolysis and incorporation of $^{18}O$.

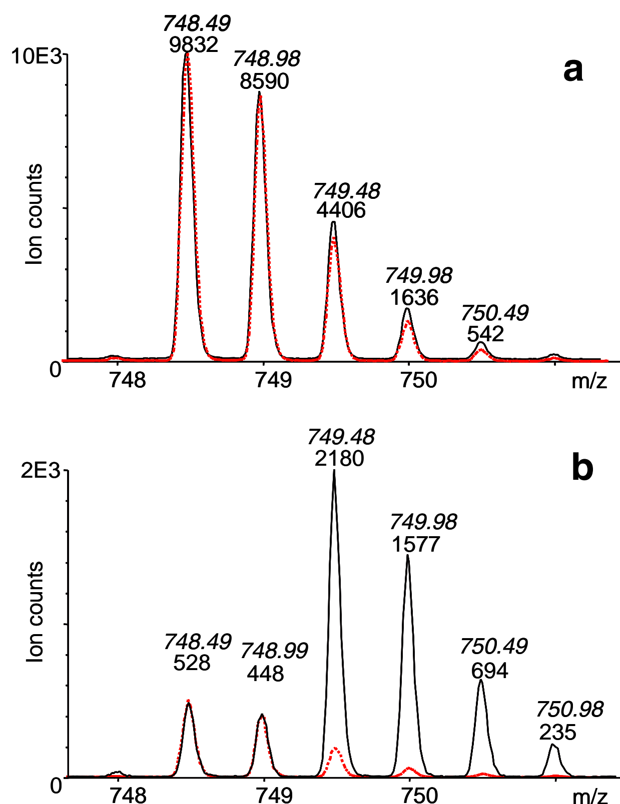


**Fig. 8.** Mass spectra of doubly-charged peptide variants **a** A25SQSVD30SNLAWYR37 and **b** A25SQSVisoD30SNLAWYR37 eluting at 100 and 97 min, respectively, after hydrolysis of succinimide 30 in $H_2^{18}O$. The *dotted line* represents the theoretical isotopic distribution of the peptide based on the natural isotopic abundances of the elements H, C, O, N and S. The *solid line* is the experimentally measured isotopic distribution. The numbers above each peak indicate the ion count (*non-italicized*) and its mass (*italicized*).

the remaining 20% of isoD incorporated $^{16}$O (Fig. 8b). There could be at least three possible sources of $^{16}$O in the isoD: 1) hydrolysis of succinimide in regular $^{16}$O water prior to the exchange to $^{18}$O; 2) 3% of $^{16}$O water in $^{18}$O water purchased from the manufacturer; and 3) some amount of $^{16}$O water, which may be carried with the protein during the $^{16}$O to $^{18}$O buffer exchanges after drying. Because the source of the $^{16}$O in 20% of isoD is not known at this time, the error of the method should be specified as 20%. The same rationale can be applied to aspartate created from succinimide.

The area between the dotted and solid lines in Fig. 8a and b represents the number of $^{18}$O-containing ions including residue 30, which was initially succinimide in the mildly acidic buffer and then hydrolyzed to isoaspartate and aspartate. This area divided by the total area under the solid lines (representing the total number of measured ions) gives the percentage of succinimide 30 in the mildly acidic buffer. Based on the $^{18}$O measurements, the aged protein sample contained 20% of Su30 and 80% of D30. Taking into account that the antibody has two independent light chains, each of which may contain Su30 or D30, the statistical probabilities for zero, one, or two succinimide residues in each intact antibody molecule can be derived from the following expression: $(\%D30 + \%Su30)^2 = (0.80 + 0.20)^2 = 0.64(zero) + 0.32(one) + 0.04(two)$. CEX showed that the same sample that had been analyzed by the $^{18}$O method contained 40% of CEX Basic 1, from the initial 12% (an increase of 28%), and a 7% increase for CEX Basic 2. These CEX percentages, 65%, 28%, and 7%, correlate to molecules with zero, one and two succinimide residues, respectively. These values were in good agreement with those from the $^{18}$O method: 64%, 32% and 4%. The near-total incorporation of $^{18}$O in isoD30 indicated that almost all isoD30 was generated during the $^{18}$O hydrolysis and was not originally present in the sample that was aged in a mildly acidic buffer.

In the non-isomerized A25-R37 peptide (Fig. 8a), the detected abundance of $^{18}$O was much smaller because the majority of D30 was present in the sample in its native form and was not a result of hydrolysis of succinimide in the sample. However, an accurate measurement of the areas between the solid and dotted lines indicated that the isoD:D ratio of the succinimide hydrolysis was approximately 3.5:1 (Fig. 8b: Fig. 8a), which is in agreement with previous studies that used short, unstructured peptides to characterize succinimide hydrolysis (4).

## Kinetics of Succinimide Formation and Hydrolysis at Different pH Values, as Monitored by CEX

The $^{18}$O results provided direct evidence that the isoaspartate in the iso A25-R37 peptide originated from a succinimide in the sample and that hydrolysis of this succinimide occurred during reduction and alkylation of the antibody, in which a denaturant and neutral/alkaline pH were used. This explained the reason for peptide mapping being able to detect only isoD30 and D30 but not succinimide. Also, reversed-phase HPLC/MS of the reduced and alkylated samples (Fig. 5) did not detect succinimide for the same reason. The CEX method described in this study was found to be useful for monitoring and quantifying succinimide formation in the intact IgG2 antibody during aging under different pH values. Our CEX method, which utilized a mobile phase pH of 5.7, could resolve the succinimide intermediate through detection of the basic variants CEX Basic 1 and Basic 2, and enabled the calculation of the percentage of succinimide that had accumulated over time via the percentages of these basic species (Fig. 2). Having identified the CEX Basic 1 and Basic 2 peaks, we used the three-month aging data plotted in Fig. 2 to determine the kinetics of succinimide formation after incubation in the pH 4.1, 5.2, 5.7, and 6.2 buffers at 37°C. The half-life of the Main Peak species was 2.2 months at pH 4.1; 2.6 months at pH 5.2; and 10 months at pH 5.7 based on extrapolation (Fig. 2d).

Assuming pseudo first-order reaction kinetics, the observed rate constant values for the increase of the total CEX Basic species due to succinimide formation ($k_{Su}$) were computed from the plot in Fig. 2c as a function of time for a given pH. Fig. 9a shows the observed rate constants for the formation of the succinimide-containing total CEX Basic species for buffers with different pH values. The rate of succinimide formation was fastest at pH 4.1 but increasingly slower at pH 5.2, followed by pH 5.7. At pH 6.2, the rate was 10-fold less than that at pH 4.1. No succinimide intermediate was observed as the buffer pH became more neutral (data not shown). This indicated that the succinimide intermediate

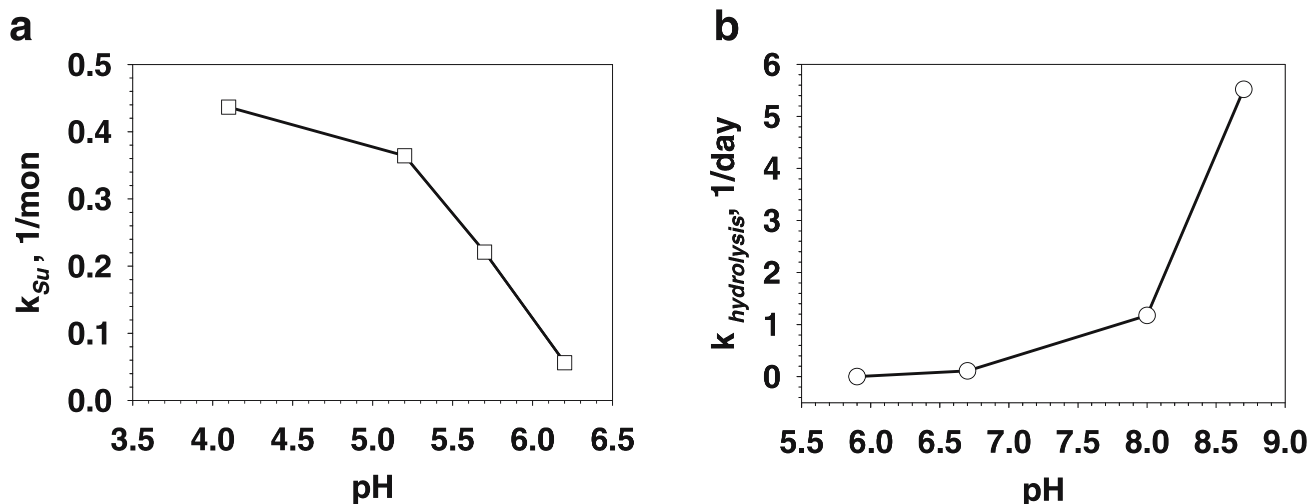


**Fig. 9. a** Observed rate constants for succinimide formation in the natively folded monoclonal human IgG2 antibody stored in pH 4.1–6.2 and at 37°C. The constants are calculated from the percentage increase of the total CEX basic peaks during the 3-month aging presented in Fig. 2c. **b** Observed rate constants for the decrease of the total CEX Basic peaks in the natively folded antibody incubated in pH 5.9, 6.7, 8.0, and 8.7 buffers at 37°C. The constants are calculated from the percentage decrease of the total CEX basic peaks during the 22-day incubation presented in Fig. 10. The decrease of the total CEX Basic peaks is attributed to predominantly the hydrolysis of succinimide 30 and also to deamidation of asparagines of the antibody (see text for details).

in our aged IgG2 was stable as long as the pH remained mildly acidic.

The succinimide that is formed is susceptible to hydrolysis at neutral and alkaline pH values (4). In this study, we used aged samples that contained high levels (up to 52%) of total CEX Basic variants to characterize succinimide hydrolysis. The hydrolysis in $^{18}O$ water followed by peptide mapping of these aged samples detected the percentage of succinimide 30 that was in agreement with the percentage of the CEX Basic peaks, thereby confirming that the increase of CEX Basic variants was caused by succinimide 30-containing molecules. The rate of succinimide hydrolysis in our intact natively folded antibody was determined at the pH values of 5.9, 6.7, 8.0, and 8.7. To prepare protein solutions at these pH values, a protein sample that was aged in pH 4.1 and had accumulated approximately 52% of succinimide was diluted in a pH 5.9 succinate buffer; pH 7.0 sodium phosphate; and pH 8.0 and 8.7 Tris-HCl. The diluted samples were incubated in the autosampler of the Agilent HPLC system with the autosampler set to 37°C. Successive injections of each sample were taken and characterized by CEX at various time intervals. Another set of the diluted samples was stored at 4°C to compare the effects of temperature on succinimide hydrolysis.

The percentage of the total CEX Basic variants in the diluted samples decreased as pH increased at 37°C (Fig. 10). The CEX Basic variants in samples diluted with the 8.7 buffer decreased from 52 to 26% after a few hours at 37°C. Decrease of the total CEX Basic variants at pH 6.7 also progressed, but was slower than that at pH 8.0 and 8.7. In contrast, the samples prepared in pH 5.9 showed only a minor decrease in total CEX Basic (Fig. 10). There was only a minor change in the level of total CEX Basic for any of the samples stored at 4°C after 22 days (data not shown). The calculated rate constants of the decrease of the total CEX basic, $k_{\mathrm{hydrolysis}}$, in the folded native antibody in the different buffers, as summarized in Fig. 9b, was fastest at pH 8.7 followed by 8.0 and 6.7. At pH 5.9, the rate constant was significantly slower than that at pH 8.7. The half-life of the total CEX basic variants, which we defined to be the time required for the level of the total CEX Basic to be half of that at time zero, was calculated to be 3 h at pH 8.7, 14 h at pH 8.0, and 150 h at pH 6.7. The rate of succinimide hydrolysis in the intact folded protein increased rapidly with increasing pH. The succinimide which took several months to accumulate at acidic pH hydrolyzed readily and rapidly in a few days at neutral and alkaline pH. Capasso *et al.* (11) determined the rate of succinimide hydrolysis as a function of pH for a synthetic peptide Ac-G-G-Su-G-G-NHMe. Based on the data reported in (11), the half-life values of the succinimide in that peptide were calculated to be 0.6 h at pH 8.7, 3 h at pH 8.0 and 30 h at pH 6.7. On average, succinimide hydrolysis was fivefold faster in the unstructured peptide Ac-G-G-Su-G-G-NHMe (11) as compared to our folded anti-

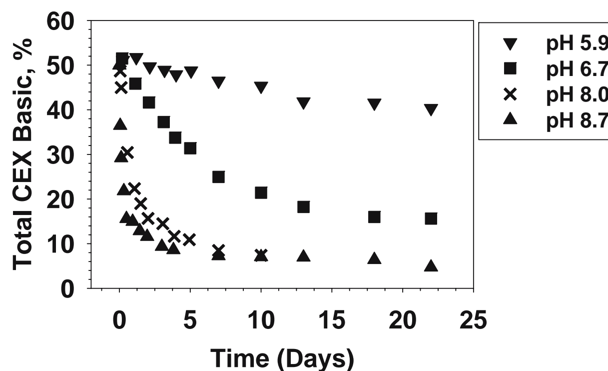


**Fig. 10.** Comparison of the decrease of the total CEX Basic peaks at pH 5.9, 6.7, 8.0, and 8.7 as a function of incubation time at 37°C. The percentage of total CEX basic variants was obtained from CEX chromatograms similar to those in Fig. 1. Four samples were prepared from a sample aged in a pH 4.1 buffer for 3 months at 37°C, which had accumulated 52% of succinimide. The decrease of the total CEX Basic peaks in the samples was monitored by CEX HPLC at different incubation time points for up to 22 days.

body. In this study, succinimide hydrolysis in the denatured antibody at pH 7.5 was completed in less than 2 h, as described earlier for the reduced and alkylated aged sample. Preliminary data from our new study that uses fluorescence spectroscopy to monitor antibody unfolding show that the duration of succinimide hydrolysis is in the order of a few seconds after complete denaturation in 8M guanidine-HCl. An entire report summarizing the data will be published elsewhere.

Concurrently with the decrease of the total CEX Basic peaks, we observed an increase in the Main CEX peaks and a smaller increase in the CEX Acidic variant. Further incubation at the neutral and alkaline pH resulted in a decrease in the Main CEX peaks and continued increase in the CEX Acidic variant (data not shown). This indicated that deamidation, the cause for the CEX Acidic variant, would be the dominant modification after prolonged incubation in neutral and alkaline pH at 37°C, which is in agreement with previous reports (29). This also indicated that the decrease of the total CEX Basic peaks was caused by two reactions: the hydrolysis of succinimide and the deamidation of labile asparagines in the antibody. The rate of deamidation was estimated by measuring the percentage of the CEX Acidic variant with time. We found that for the same periods (3 h at pH 8.7; 14 h at pH 8.0; and 150 h at pH 6.7) the CEX Acidic variant increased by only 11%, two-fold less than the 23% decrease of the total CEX basic variants. (The 23% value is half of the measured difference between 52% and 6% of the total CEX Basic peaks.) These calculations allowed us to conclude that, although the rate of hydrolysis of succinimide 30 was faster than the rate of deamidation of asparagines in the antibody, the latter reaction should be taken into account when using CEX to determine the rate of the succinimide hydrolysis. Although CEX has provided useful approximation of the succinimide hydrolysis rate, hydrolysis in $H_2{}^{18}O$ followed by peptide mapping should be used for a more accurate quantitative measurement of the hydrolysis of succinimide 30 on a peptide level. This work is underway and will be presented elsewhere.

The accumulation of molecules containing succinimide 30 and isoD30 has raised the question of their effect on the activity of the antibody. Evaluation of the binding affinity and potency of the CEX Basic 1 and 2 fractions showed a 10–20% difference from the Main isoforms (data not shown). This difference was within the deviations of assay variability. Thus, the activity of the succinimide-containing protein samples was observed to be comparable to those without succinimide, suggesting that succinimide formation and any possible structural changes that might occur in the complementarity determining region (CDR), if it takes place at all, do not compromise the overall antibody activity.

## CONCLUSIONS

In this paper, we described the accumulation of succinimide in the light chain of a monoclonal human IgG2 antibody that had been aged in mildly acidic buffers at elevated temperatures. CEX of the aged antibody separated Main Peak and basic variants of high abundance. Identification of the CEX basic variants presented an analytical challenge and required several complementary techniques. Four different reversed-phase (RP) HPLC/MS methods were used to analyze the two basic CEX fractions and to identify that they were caused by succinimide 30 in one or both light chains of the antibody. Of the four methods, intact mass analysis of the CEX basic fractions by RP HPLC/MS proved to be a powerful detection tool, because it pinpointed succinimide as the major modification. The accurately measured mass loss of the basic peaks agreed well with the theoretical mass loss during succinimide formation in one (−18 Da) and two chains (−36 Da). In contrast, analysis of the reduced antibody samples failed to detect succinimide, because it was hydrolyzed to isoaspartate and aspartate in less than 2 h in the process of reduction and alkylation under denaturing conditions. Intact isomerized light chain was not detected because it had the same elution time and mass as the native light chain. Further analysis by peptide map separated and identified the isomerized tryptic peptide A25-R37 as having a slightly earlier elution time, and the same mass and fragmentation pattern (MS/MS). Finally, succinimide hydrolysis in $H_2{}^{18}O$ followed by tryptic digestion in regular water labeled and identified the site of cyclization as aspartic acid 30. RP HPLC/MS of reduced antibody and peptide were also useful in detecting minor percentages of pyroglutamate, which was present in equal amounts in all the CEX fractions. This indicated that the conversion of glutamate to pyroglutamate, an electrically neutral modification, was not the cause for the CEX basic peaks.

The levels of succinimide can be inferred by its hydrolysis in $H_2{}^{18}O$ followed by peptide map. However, the CEX method described in this study was more useful for monitoring succinimide formation in the intact IgG2 antibody during aging at different acidic pH values, because it is faster in terms of sample preparation and data processing. Using CEX, we were able to obtain the rate of succinimide formation and hydrolysis in the natively folded protein as a function of pH. Kinetic measurements of succinimide formation in the natively folded IgG2 protein as a function of pH at elevated temperatures showed that it was 10-fold faster at the mildly acidic pH 4.1 than at pH 6.2. No succinimide was observed as the aging buffer pH became more neutral. The accumulated succinimide in our aged IgG2 was highly susceptible to hydrolysis at neutral pH in both the natively folded form and after the protein had been reduced and alkylated in a denaturant. The half-life of the succinimide was approximately six days in the folded antibody when stored in a pH 6.7 buffer at 37°C, but less than 2 h in 7.5 M GdnHCl, pH 7.5. These observations suggest that protein conformation affects the rate of succinimide hydrolysis. Additional experiments indicated that the rate of succinimide hydrolysis in intact folded protein increased rapidly with increasing pH. The half-life of succinimide in the intact folded protein was pH-dependent: 50 times shorter at pH 8.7 than at pH 6.7. Despite the accumulation of succinimide, the bioactivity of the succinimide-containing CEX-separated species was comparable to the Main isoforms. This suggests that succinimide formation at D30 of the light chain in our IgG2 antibody does not inhibit antigen binding.

## ACKNOWLEDGEMENTS

We would like to thank Thomas Dillon for help with the RP HPLC of the intact IgG2 antibody; Alexis Lueras for her

assistance with preparation of some of the aged protein samples; Barbara Norwood for her assistance in bioactivity measurements; Tiansheng Li for his support and David Brems for his comments and suggestions.

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