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Investigation of degradation processes in IgG1 monoclonal antibodies by limited proteolysis coupled with weak cation-exchange HPLC

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ABSTRACT

A new cation-exchange high-performance liquid chromatography (HPLC) method that separates fragment antigen-binding (Fab) and fragment crystallizable (Fc) domains generated by the limited proteolysis of monoclonal antibodies (mAbs) was developed. This assay has proven to be suitable for studying complex degradation processes involving various immunoglobulin G1 (IgG1) molecules. Assignment of covalent degradations to specific regions of mAbs was facilitated by using Lys-C and papain to generate Fab and Fc fragments with unique, protease-dependent elution times. In particular, this method was useful for characterizing protein variants formed in the presence of salt under accelerated storage conditions. Two isoforms that accumulated during storage were readily identified as Fab-related species prior to mass-spectrometric analysis. Both showed reduced biological activity likely resulting from modifications within or in proximity of the complementarity-determining regions (CDRs). Utility of this assay was further illustrated in the work to characterize light-induced degradations in mAb formulations. In this case, a previously unknown Fab-related species which populated upon light exposure was observed. This species was well resolved from unmodified Fab, allowing for direct and high-purity fractionation. Mass-spectrometric analysis subsequently identified a histidine-related degradation product associated with the CDR2 of the heavy chain. In addition, the method was applied to assess the structural organization of a noncovalent IgG1 dimer. A new species corresponding to a Fab–Fab complex was found, implying that interactions between Fab domains were responsible for dimerization. Overall, the data presented demonstrate the suitability of this cation-exchange HPLC method for studying a wide range of covalent and noncovalent degradations in IgG1 mAbs.

1. Introduction

Monoclonal antibodies (mAbs) have become one of the most common modalities for therapeutic protein development in the field of biopharmaceutics [1,2]. Immunoglobulin G (IgG) mAbs are large and complex glycoproteins comprised of two light and two heavy chains with overall molecular weights of ~150 kDa. Pharmaceutical development of mAb-based therapeutics is challenged by a variety of degradation processes that impact product purity, efficacy, and potentially safety. In particular, protein charge variants arising from modifications of the N- and C-termini or amino acid side chains may be present in the drug substance or can accumulate upon storage. There exist a number of established methods for analyzing mAb charge variants which are based on ion-exchange and isoelectric-focusing techniques. The inherent complexity of mAbs and the diversity of degradation pathways challenge the overall resolution of these methods [3,4]. Even the use of reversed-phase chromatography coupled with mass spectrometry (RP–MS) may

Abbreviations: CDR, complementarity-determining region; CE, capillary electrophoresis; CEX, cation-exchange chromatography; CEX52, cation-exchange chromatography at pH 5.2; CEX68, cation-exchange chromatography at pH 6.8; EDTA, ethylenediaminetetraacetic acid; Fab, fragment antigen-binding; Fc, fragment crystallizable; HMW, high molecular weight; HPLC, high-performance liquid chromatography; IgG, immunoglobulin G; LC, liquid chromatography; LP, limited proteolysis; Lys-C, endoproteinase Lys-C; mAb, monoclonal antibody; MS, mass spectrometry; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; RP, reversed-phase chromatography; SCX, strong cation-exchange chromatography; SDS, sodium dodecyl sulfate; SEC, size-exclusion chromatography; TFA, trifluoroacetic acid; WCX, weak cation-exchange chromatography.

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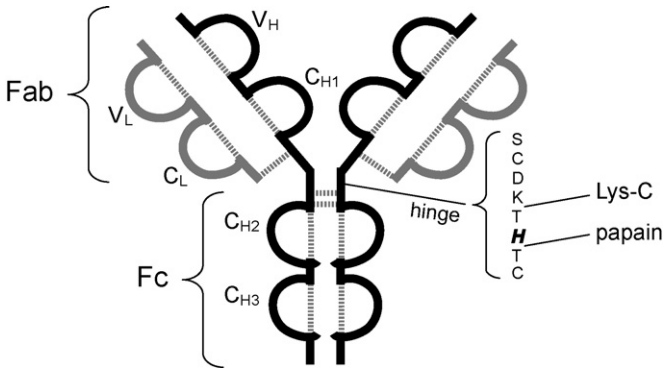

Fig. 1. An IgG1 schematic illustrating the location of the Lys-C and papain cleavage sites in the native molecule. The hinge sequence -Ser-Cys-Asp-*Lys*-Thr-*His*-Thr-Cys- is a solvent-exposed chain segment accessible to protease attack under nondenaturing conditions. Heavy chains, light chains, and intramolecular disulfide bonds are shown as black, grey, and dashed, grey lines, respectively. The hinge histidine that relocates between Fab and Fc in a protease-dependent manner is indicated in bold.

not always provide a full understanding of the charge heterogeneity due to the large size of mAbs. Therefore, it remains critical to develop approaches that facilitate assignment of chemical and structural modifications to further our understanding of antibody degradation mechanisms.

Limited proteolysis (LP) followed by high-performance liquid chromatography (HPLC) is one such approach that turns analytical characterization of these proteins into a more straightforward analysis of smaller fragment antigen-binding (Fab) and fragment crystallizable (Fc) domains. This idea has been illustrated with the application of RP–MS techniques on mAbs and peptide-Fc fusion proteins [5–9]. Development of a charge-based assay to analyze heterogeneity of mAbs after proteolytic fragmentation was also described [10]. The latter method utilized papain and demonstrated an ability to resolve charge heterogeneity associated with Fab and Fc. Under native conditions, papain is a thiol protease that can generate one Fc and two identical Fab fragments by attacking the hinge of IgG1 molecules. In the hinge sequence -Ser-Cys-Asp-*Lys*-Thr-*His*-Thr-Cys-, papain predominantly cleaves the heavy chain at the C-terminus of the histidine residue (in italics) (Fig. 1) [9–11]. Similarly, Lys-C is a serine protease that selectively attacks the hinge when incubated with IgG1 antibodies for a short time. It, however, produces Fc and Fab fragments by cleaving the solvent-exposed peptide bond at the C-terminus of the lysine residue (in italics) [5]. This protease-dependent digestion determines on which Fab and Fc fragments the two hinge histidines reside (Fig. 1), and the impact from the translocation of these amino acids was duly utilized in the current study.

Here, we describe the development of a cation-exchange chromatography (CEX) that uses similar principles but has advantages over the other methods mentioned above. Although RP–MS approaches are very powerful [12,13], it is often difficult to correlate changes in the polarity of protein isoforms with ion-exchange-based separation. Therefore, limited proteolysis coupled with cation-exchange chromatography (LP-CEX) is required to fill the gaps in mAb degradation analysis. Our new assay differs from previous LP-CEX efforts [10] in two major aspects: the use of weak cation-exchange chromatography (WCX), a column chemistry that is more conventional in mAb biopharmaceutical development; the buffering of mobile phase at pH 5.2, a value that is more common to mAb formulations and minimizes the potential for covalent modifications and physical changes in proteins [14–17]. We also make use of Lys-C and papain to assign Fab- and Fc-related species based on the elution time changes that stem from the different cleavage sites. In this work, the method was applied to characterize protein variants formed in the presence of salt under accelerated storage conditions. Its utility was further illustrated by analyzing light-induced degradation products in mAb formulations. While the effects of photodegradation on biopharmaceuticals are not well known, light-induced modifications can lead to changes in protein structure which may influence long-term stability, bioactivity, and immunogenicity [18]. In all cases, the method proved useful in separating and identifying distinct species in mAbs.

Applications of LP-CEX, however, go well beyond chemical characterization and include degradation processes associated with protein oligomerization and aggregation. These processes, which involve misfolding and other conformational changes, are poorly understood and pose a significant challenge to biopharmaceutical development [19–22]. Noncovalent dimers often represent the predominant manifestation of antibody aggregation pathways [23–26]. Consequently, great interest lies in the understanding of IgG1 dimerization mechanisms, but finding a simple yet informative assay capable of investigating noncovalent complexes is difficult. The use of RP–MS or SDS-gel techniques is generally inappropriate because of their denaturing character. Hydrogen–deuterium exchange in conjunction with NMR or MS is time consuming and technically challenging considering the large size of antibodies. As a native assay that analyzes constituent Fab and Fc fragments rather than the entire molecule, our LP-CEX method overcomes these obstacles and demonstrates excellent utility in assessing the structural organization of noncovalent dimers.

2. Materials and methods

2.1. Reagents and chemicals

All reagents and chemicals used in the following experiments were of analytical grade or better. Water was purified using a Milli-Q Ultrapure Water Purification System (Millipore SAS, Molsheim, France).

2.2. mAb samples

Salt-stressed mAb-1 material used in the fractionation of CEX isoforms was purified at 15 mg/mL in a high-salt buffer (100 mM acetate, 200 mM sodium phosphate, pH 5.0) and filter sterilized before incubation at 25 °C for 35 days.

In the photodegradation study, mAb-1 samples were formulated at 29 mg/mL in 30G52ST buffer (30 mM sodium glutamate, 4.25% sorbitol, 0.006% polysorbate 20, pH 5.2) and filter sterilized before vialing.

Material used in the purification of mAb-1 dimer was enriched in high molecular weight (HMW) species to ~12% at 5 mg/mL in 100 mM acetate, 300 mM sodium phosphate, pH 5.0 buffer.

All mAbs used in these studies were manufactured at Amgen (Thousand Oaks, CA) and purified using standard manufacturing procedures.

2.3. Photodegradation study of mAb-1

Photodegradation was carried out at 25 °C on mAb-1 material aliquoted into five separate glass vials. The vials were placed sideways on a rotating platform to maximize light exposure and continuously subjected to bright, fluorescent light (20.7 W/m^2) for 1-, 2-, 3-, and 4-week durations. At the end of every week, one vial was wrapped in aluminum foil and maintained with the others. Total light exposure of the 4-week vial approached 3.6 million lx h. One vial was designated as a dark control and remained covered with foil throughout the study. The samples were stored in a Caron 6540 Series Photostability Test Chamber (Model 6540A; Caron,

Marietta, OH) for the duration of the experiment, ~20 cm away from the light source.

2.4. Limited proteolysis (LP)

mAb samples were subjected to limited proteolysis with endoproteinase Lys-C (Roche Diagnostics, Indianapolis, IN) at a protein:enzyme weight ratio of 200:1 in 100 mM Tris–HCl, pH 7.5 buffer. The reaction mixture was incubated at 37 °C for 10 min and quenched with 150 mM ammonium acetate, pH 4.7 buffer. This procedure is similar to the Lys-C protocol previously used for RP characterization of IgG1 molecules [5].

mAb samples were subjected to limited proteolysis with papain (Roche Diagnostics) at a protein:enzyme weight ratio of 100:1 in 100 mM Tris–HCl, 4 mM EDTA, 5 mM cysteine, pH 7.6 buffer. The reaction mixture was incubated at 37 °C for 2 h and quenched with 150 mM ammonium acetate, pH 4.7 buffer. A variation of this protocol has been previously described [9].

2.5. Cation-exchange chromatography (CEX)

Cation-exchange chromatography was performed on a ProPac WCX-10 analytical column (weak cation-exchange, 4 mm × 250 mm; Dionex, Sunnyvale, CA) preceded by a ProPac WCX-10G guard column (weak cation-exchange, 4 mm × 50 mm; Dionex) at 25 °C. Fifty micrograms of protein sample was loaded onto the column and analyzed at a flow rate of 0.7 mL/min. The column was equilibrated with Buffer A, and protein was eluted with a linear gradient of Buffer B from 0 to 100% over 35 min. Following elution, the column was washed with Buffer C for 5 min and re-equilibrated with Buffer A for 16 min. Separations were carried out on Agilent 1100 Series (Agilent Technologies, Santa Clara, CA) or UltiMate 3000 Series (Dionex) HPLC systems. Absorbance was measured at 215 and 280 nm.

CEX at pH 5.2 (CEX52) used the following mobile phases: Buffer A (20 mM sodium acetate, pH 5.2), Buffer B (20 mM sodium acetate, 300 mM sodium chloride, pH 5.2), and Buffer C (20 mM sodium acetate, 1 M sodium chloride, pH 5.2).

CEX at pH 6.8 (CEX68) used the following mobile phases: Buffer A (20 mM sodium phosphate, pH 6.8), Buffer B (20 mM sodium phosphate, 100 mM sodium chloride, pH 6.8), and Buffer C (20 mM sodium phosphate, 1 M sodium chloride, pH 6.8).

2.6. Size-exclusion chromatography (SEC)

Size-exclusion chromatography was performed on two in-tandem TSKgel G3000SW_{XL} analytical columns (7.8 mm × 300 mm; Tosoh Bioscience LLC, Montgomeryville, PA) preceded by a TSKgel SW_{XL} guard column (6 mm × 40 mm; Tosoh Bioscience LLC) at 25 °C. Twenty micrograms of protein sample was loaded onto the columns and analyzed at a flow rate of 0.5 mL/min. Protein was eluted isocratically with 50 mM sodium phosphate, 300 mM sodium chloride, pH 6.8 buffer over 60 min. Separations were carried out on Agilent 1100 and 1200 Series HPLC systems (Agilent Technologies), and absorbance was measured at 215 and 280 nm.

2.7. Capillary electrophoresis with sodium dodecyl sulfate (CE-SDS)

Samples were diluted to a final protein concentration of 1 mg/mL in SDS sample buffer (ProteomeLab SDS-Gel MW Analysis Chemistries; Beckman Coulter, Fullerton, CA), reduced with β-mercaptoethanol, and incubated at 70 °C for 5 min. Samples were injected electrokinetically at −10 kV for 20 s onto a bare fused-silica capillary (50 μm, 20.2 cm effective length, 30.2 cm total length) and separated at −15 kV for 30 min. Analyses were performed on a ProteomeLab PA 800 Protein Characterization System (Beckman Coulter). Peaks were detected by UV absorbance at 220 nm. Peak areas in the electropherogram were used to quantify the relative amounts of light chain, heavy chain, nonglycosylated heavy chain, and various molecular weight species.

2.8. Fractionation of mAb-1 variants by CEX

Fractionation of basic isoforms from salt-stressed mAb-1 material was carried out in two steps. First, preparative purification was performed to remove the majority of acidic and unmodified species. A CEX column was manually packed with Fractogel SO_3 resin (EMD Chemicals, Gibbstown, NJ) to a final column volume of 200 mL. Degraded mAb-1 was diluted with water to reduce conductivity and loaded onto the column. The column was equilibrated with CEX68 Buffer A (Section 2.5), and protein was eluted with a linear gradient of CEX68 Buffer B (Section 2.5) from 0 to 100% over 10 column volumes. Separation was achieved at ambient temperature with a flow rate of 24 mL/min, and absorbance was measured at 280 nm. Multiple fractionation runs were performed to attain target protein yields. Resulting fractions with the highest total content of basic variants as determined by CEX68 (Section 2.5) analysis were pooled and concentrated. Second, semi-preparative purification was carried out to isolate each isoform from the fraction pool. Sample enriched in mAb-1 basic species was loaded onto a ProPac WCX-10 semi-preparative column (weak cation-exchange, 9 mm × 250 mm; Dionex). The column was equilibrated with CEX68 Buffer A, and protein was eluted with a linear gradient of CEX68 Buffer B from 0 to 100% over 60 min. Following elution, the column was washed with CEX68 Buffer C (Section 2.5) for 5 min and re-equilibrated with CEX68 Buffer A for 16 min. Separation was achieved at ambient temperature with a flow rate of 1 mL/min, and absorbance was measured at 280 nm. Multiple fractionation runs were performed to attain target protein yields. Resulting fractions containing the same species were pooled, concentrated, and subsequently analyzed by CEX68. Purity above 90% was achieved for each isoform. An ÄKTAexplorer system with a Frac-950 fraction collector (GE Healthcare Life Sciences, Piscataway, NJ) was used to execute both steps of the purification.

Fractionation of pre-Fab and Fab species from photodegraded mAb-1 material was carried out using the LP-CEX52 method described above (Sections 2.4 and 2.5). The 4-week sample was subjected to limited proteolysis by Lys-C and loaded onto the column. Multiple fractionation runs were performed to attain target protein yields. Fractions consisting of the same species were pooled, concentrated, and subsequently analyzed by CEX52 (Section 2.5). Purity above 95% was achieved in both cases. An Agilent 1200 Series HPLC system with a 12/13 SelValve external valve (Agilent Technologies) was used to execute the separation and purification.

2.9. Fractionation of mAb-1 dimer by SEC

mAb-1 dimer was purified using the SEC method described above (Section 2.6). Material enriched in HMW species was loaded onto the column, and multiple fractionation runs were performed to attain target protein yields. Fractions containing only dimer were pooled and concentrated. Subsequent analysis by SEC determined that purity greater than 99% was achieved. An Agilent 1200 Series HPLC system with a 12/13 SelValve external valve (Agilent Technologies) was used to execute the fractionation.

2.10. Bioassay

mAb-1 samples and control were prepared at a final protein concentration of 400 ng/mL in growth medium containing Protein G (Thermo Fisher Scientific Inc., Waltham, MA) at 1 μg/mL.

Fifty-five microliters of each sample preparation was added to a 96-well assay plate and serially diluted 1:1.8 in assay medium. COLO 205 cells (American Type Culture Collection, Manassas, VA) were added to all plate wells containing sample dilutions and to blank wells containing media at a concentration of 8×10^5 cells/mL. Assay plates were incubated for 2–3 h at 37 °C/5% CO_2 followed by equilibration at ambient temperature. Fifty microliters of Caspase-Glo 3/7 substrate reagent (Promega, Madison, WI) was added to all sample wells. Assay plates were incubated with shaking at 25 °C for 1–1.5 h and measured on an EnVision 2101 Multilabel Reader (PerkinElmer, Waltham, MA). Data were analyzed using StatLIA statistical software (Brendan Technologies, Carlsbad, CA). Biological activity was determined by comparing test sample to reference standard response.

2.11. Analytical peptide mapping

One hundred micrograms of protein sample was denatured in 6 M guanidine hydrochloride, 0.1 M Tris–HCl, pH 8.0 buffer, and the disulfide bonds were reduced by incubating with 12 mM dithiothreitol at 55 °C for 30 min. Resulting free cysteines were alkylated by incubating with 26 mM iodoacetic acid for 15 min at ambient temperature. Prior to digestion, the samples were buffer exchanged into 50 mM Tris–HCl, pH 8.0 buffer using Bio-Gel P-6 Tris columns (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Proteomics grade trypsin (Roche Diagnostics) was added to the samples at a 1:10 ratio (w/w), and the samples were incubated for 4 h at 37 °C. The digests were quenched with the addition of 10% trifluoroacetic acid (TFA).

Resulting tryptic peptides were separated on a reversed-phase Polaris C18-Ether column (2 mm × 250 mm, 3 μm particle size; Varian, Palo Alto, CA) maintained at 60 °C. Mobile phases consisted of 0.1% TFA (Buffer A) and 90% acetonitrile, 0.1% TFA (Buffer B). Peptides were eluted with a linear gradient of Buffer B from 0 to 65% over 165 min at a flow rate of 0.2 mL/min. Separations were performed on an Agilent 1200 HPLC system (Agilent Technologies).

All mass-spectrometric analyses were carried out using an LTQ ion-trap instrument (Thermo Fisher Scientific Inc.) equipped with an electrospray ionization source connected to an Agilent 1200 HPLC pump (Agilent Technologies). The MS capillary temperature was maintained at 225 °C, and analyses were performed in positive-ion mode with a spray voltage of 5.0 kV. The instrument was calibrated in an *m*/*z* range from 500 to 2000 using a mixture of caffeine, MRFA (Met-Arg-Phe-Ala) peptide, and Ultramark 1621 (Sigma–Aldrich). Spectral data for the protein digests were acquired online in an *m*/*z* range from 200 to 2000. MS/MS analyses were performed in data-dependent mode. Collision data were obtained using a relative collision energy of 40%.

3. Results and discussion

3.1. LP-CEX52 method development

In a preliminary experiment, a mAb-1 sample was subjected to limited proteolysis with Lys-C and analyzed by an established WCX method (CEX68) (Section 2.5) used for the intact molecule (Fig. 2a). The data demonstrated that the standard method was unable to adequately separate the resulting Fab and Fc fragments. Although Fab was well resolved, Fc generated a broad peak shape and eluted adjacent to Fab. Consequently, the assay was modified to improve analysis of digested mAb samples. This development involved screening the mobile-phase pH from 4.8 to 7.8 and assessing elution conditions with salt concentrations between 0.05 and 1 M. Linear and multi-step gradients for both pH and salt concentration were also evaluated. Fig. 2b shows the results of the optimized WCX method (CEX52) (Section 2.5) derived from these experiments. mAb-1 analyzed by the LP-CEX52 assay produced two major peaks: the first corresponding to Fab, and the second to Fc. Of the different conditions tested, the CEX52 method provided the greatest separation between main peaks and resolved the largest number of minor species. The extent of separation between native Fab and Fc fragments allowed tentative assignment of some minor variants as Fab or Fc related based on their proximity to the major peaks.

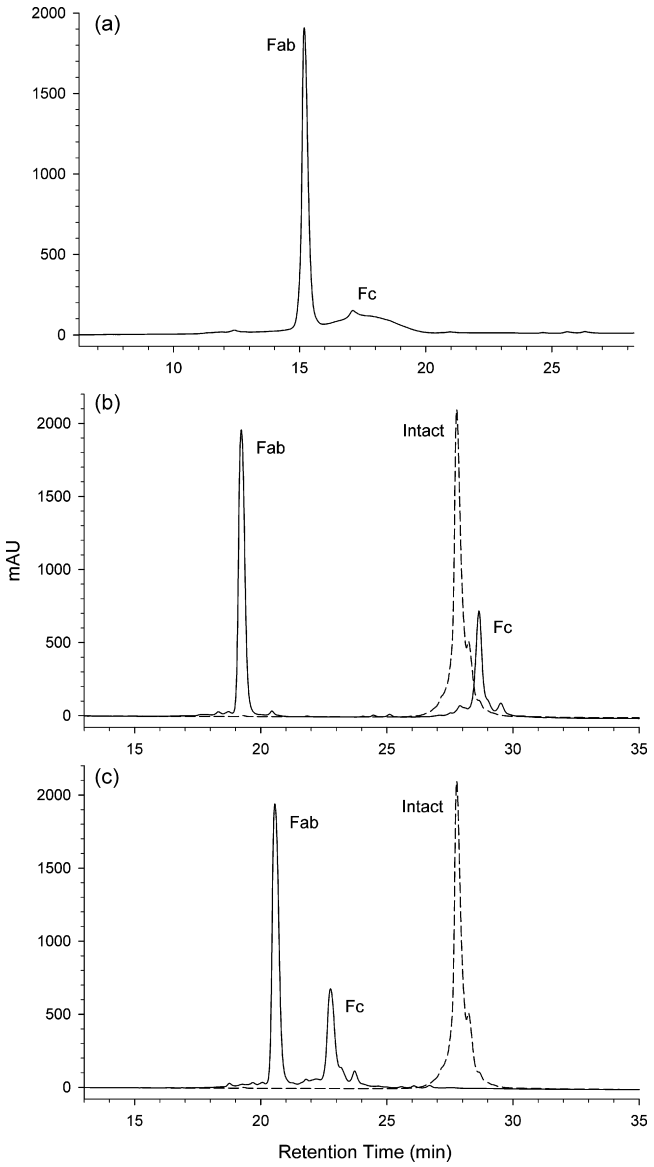

Fig. 2. Comparison of LP-CEX chromatograms generated at two mobile-phase pH values using different proteases: (a) pH 6.8 analysis, Lys-C digestion; (b) pH 5.2 analysis, Lys-C digestion; (c) pH 5.2 analysis, papain digestion. The resolving power of the CEX52 method is clearly illustrated in (b) and (c). Undigested (intact) mAb-1 is included as a reference (dashed line).

Although Lys-C was effective in fragmenting unmodified mAb-1, degraded samples exhibited some resistance to the enzyme. Papain digestion (Section 2.4), however, proved much less susceptible to mAb degradation and was used as an alternative for analyzing heavily stressed material. A typical chromatogram for papain-digested mAb-1 analyzed with CEX52 is displayed in Fig. 2c. The overall papain and Lys-C profiles appeared similar, but a large shift in elution time was observed for the Fc peak despite a difference of only two residues between the cleavage sites. A slight shift was also seen

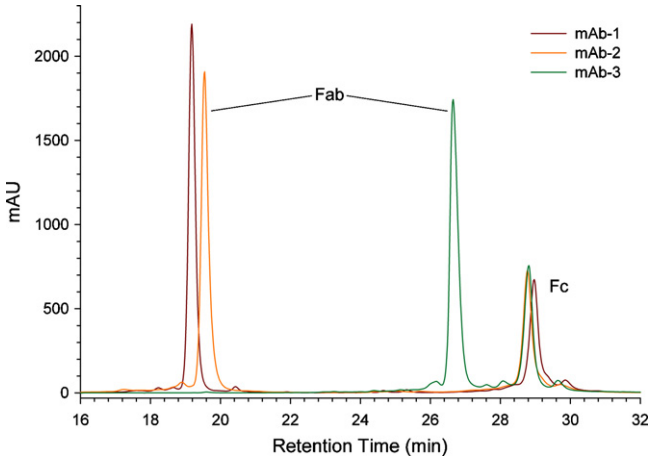

Fig. 3. LP-CEX52 (Lys-C) profiles for three different IgG1 mAbs.

for the Fab peak. This phenomenon was explained by the relocation of the two hinge histidines, which are attached to Fc rather than Fab when mAb-1 is digested with papain (Fig. 1). The imidazoles of the solvent-exposed histidines are protonated under the mildly acidic analytical conditions, and the addition or removal of the positive charges is responsible for the changing elution of Fab and Fc. This difference between Lys-C and papain fragmentation facilitated assignment of minor peaks since species representing Fab or Fc variants should demonstrate the same elution time behavior as the unmodified fragments to which they are related.

Major peaks detected in LP-CEX52 were confirmed as Fab and Fc by intact-mass and peptide mapping analysis (data not shown). We also noted that the elution order of Fab and Fc in WCX was opposite from that in strong cation-exchange chromatography (SCX) [10]. This disparity likely resulted from protonation state variations in the fragments arising from the different mobile phases used or specifics of protein-column interactions inherent to WCX and SCX.

3.2. Applicability of the LP-CEX52 method to various IgG1 mAbs

In order to test the applicability of the LP-CEX52 assay to other mAbs, two additional IgG1 antibodies, mAb-2 and mAb-3, were subjected to limited Lys-C proteolysis and analyzed (Fig. 3). As anticipated, two major peaks corresponding to Fab and Fc were observed for all three mAb samples. The Fc profiles appeared almost identical, which was not unexpected since the Fc regions of these mAbs are very similar. Conversely, the elution times for Fab varied noticeably due to the differences in primary structure of the variable domains. Taking into account the number and distribution of minor Fab isoforms, this experiment demonstrated the utility of the method in characterizing and identifying different IgG1 molecules.

3.3. Characterization of mAb-1 CEX isoforms

The potential of LP-CEX52 for studying chemical modifications of mAbs was illustrated in the characterization work performed on basic CEX68 species of mAb-1. Historical data suggested that mAb-1 stored in buffer with high-salt content accumulates a significant amount of basic isoforms. In order to enrich these species for subsequent isolation, mAb-1 was incubated in a high-salt buffer at 25 °C for 35 days (Fig. 4a, Section 2.2). Among the most abundant basic species present in the starting material, the B2 isoform increased only slightly, whereas B1 and B3 showed rapid and steady growth during incubation.

Following semi-preparative purification (Section 2.8), these mAb-1 variants were analyzed by LP-CEX52. Fig. 4b shows that

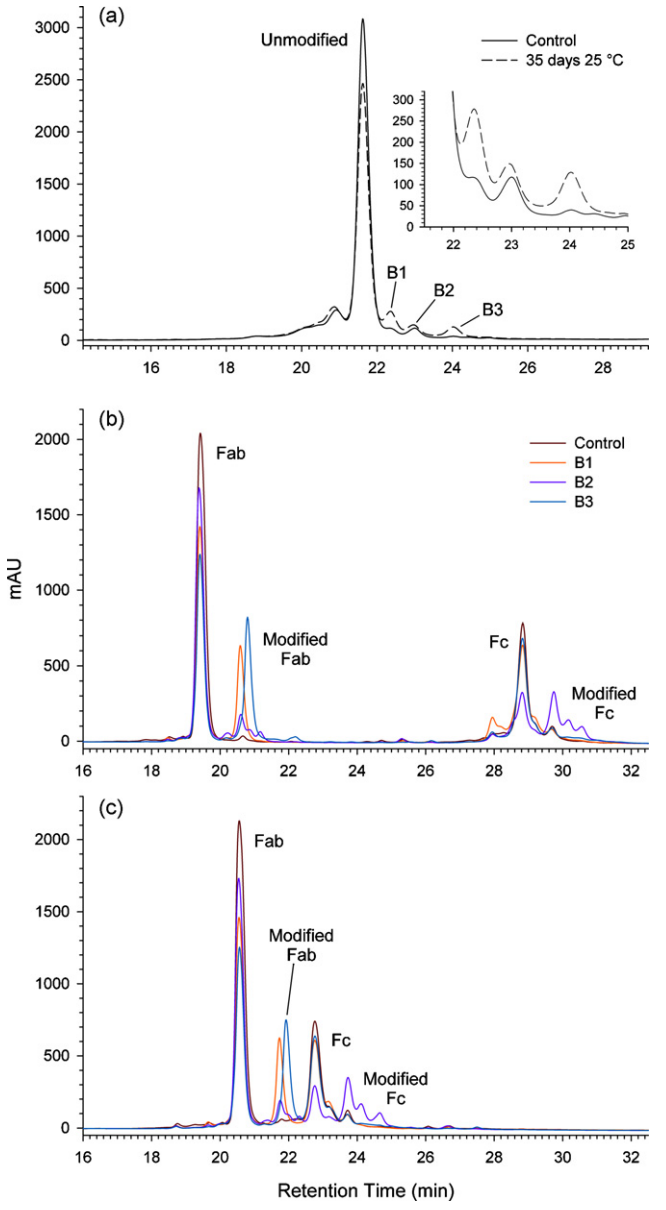

Fig. 4. (a) CEX68 profile of intact and degraded mAb-1 samples incubated in a high-salt buffer at 25 °C for 35 days. LP-CEX52 profiles for purified B1, B2, and B3 species are shown in (b) (Lys-C) and (c) (papain). The color coding in (b) and (c) is identical. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Lys-C-digested B1 differed from the control sample primarily in the Fab region, as indicated by the reduction in unmodified Fab and the corresponding appearance of a single peak at 20.6 min. B3 produced a similar profile but contained a distinct post-Fab species that eluted after the B1 peak, at 20.8 min. Proteolysis with papain induced an identical retention time shift of 1.2 min in the native and post-Fab peaks (Fig. 4c). Intact-mass analysis for B1 and B3 indicated the possibility of N-terminal cyclization of the light-chain glutamic acid residue to pyroglutamate or deamidation of a Fab asparagine residue to succinimide. Peptide mapping (Section 2.11) was largely inconclusive due to the low abundance of modified peptides, although preliminary results verified the presence of light-chain pyroglutamate in both isoforms (data not shown). This finding correlated with the LP-CEX52 data since the conversion of an acidic glutamate to a neutral pyroglutamate residue would raise the p*I* of the Fab fragment and delay its elution. Further work to

Table 1
Bioactivity data for basic CEX isoforms purified from a degraded mAb-1 sample.

Sample	% Relative potency
Control	98.2 ± 2.9
B1	42.0 ± 2.1
B2	74.2 ± 2.0
B3	23.9 ± 0.3

provide more definitive assignments for these species is needed, but in general the data confirmed that B1 and B3 originated from Fab-related changes.

In the case of Lys-C-fragmented B2, differences were mainly observed in the area surrounding Fc, as illustrated by the decrease in native Fc and the emergence of new peaks eluting between 29.4 and 30.8 min (Fig. 4b). These post-Fc species mirrored the 6.1-min shift in elution time of native Fc when papain was used (Fig. 4c). Both intact-mass and peptide mapping analysis verified unprocessed C-terminal lysines as the predominant modifications (data not shown). Again, the MS and LP-CEX52 results were in agreement, and B2 was identified primarily as an Fc variant.

The B1, B2, and B3 isoforms were also assessed for bioactivity (Section 2.10), and the results are summarized in Table 1. B1 and B3 showed greatly diminished potency while B2 only exhibited a moderate decrease. In fact, the reduced activity of B2 may largely be attributed to contamination by B1 and B3 during the purification process (Fig. 4b and c). These observations correlated well with the other data because modifications in the Fab domain are expected to have a greater impact on potency than those in the Fc region. Consequently, it indicates that LP-CEX52 may be predictive of loss in mAb-1 bioactivity depending on the degree of Fab degradation.

3.4. Characterization of photodegraded mAb-1

The utility of the LP-CEX52 method was also tested on mAb-1 material subjected to prolonged light exposure. A photodegradation study was performed by exposing a series of samples to bright, fluorescent light for up to 4 weeks (Sections 2.2 and 2.3). Upon completion of the study, the protein solutions were analyzed by SEC (Section 2.6), CEX68, LP-CEX52, CE-SDS (Section 2.7), and an *in vivo* bioassay. Fig. 5a illustrates the effect of light on the size distribution of the samples. It was observed that ~20% of mAb-1 molecules formed aggregates and fragments (Table 2). The aggregated species were predominantly dimeric, and they accumulated in a stress-dependent manner from 0.3 to 9.1%. Fragments also grew as a function of light exposure, and their combined area reached 9.3%. The two peaks eluting at approximately 35 and 40 min corresponded to molecules consisting of one Fab linked to Fc (Fab-Fc) and individual Fab/Fc fragments originating from the intact antibody. Results of CE-SDS analysis under reducing conditions showed that the aggregates detected by SEC were covalently cross-linked *via* nondisulfide bonds. Peak area for the light chain remained constant, whereas the heavy-chain peak area decreased by 15% (Table 2). Taken together with ~4% growth of the fragmented species, it was found that degradations in the heavy chain were responsible for cross-linking and fragmentation of ~15% of the material.

The bioassay data were in marked contrast to the SEC results as they revealed a dramatic change in protein activity. Loss of activity was linear and approached ~80% after 4 weeks of light exposure (Table 2). In order to obtain more information, the samples were analyzed with CEX68 (Fig. 5b). A significant reduction in main peak area occurred after only 1–2 weeks of storage (Table 2). Further light exposure led to additional

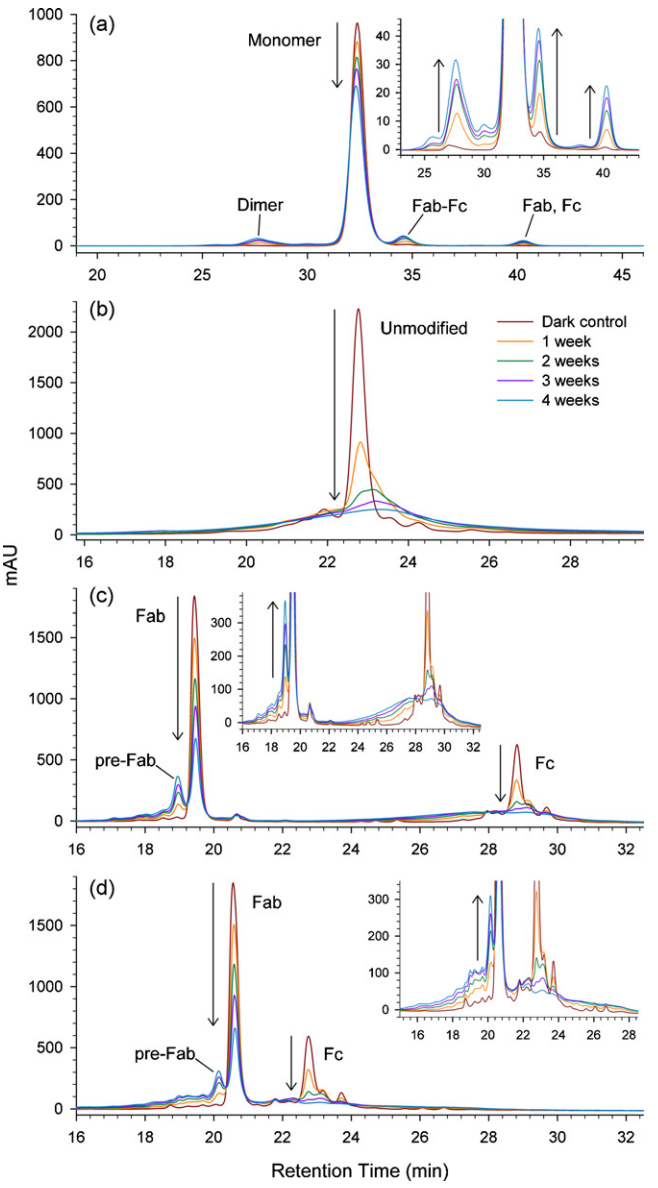

Fig. 5. Chromatograms of degraded mAb-1 samples exposed to fluorescent light for up to 4 weeks. Analyses include: (a) SEC, (b) CEX68, (c) LP-CEX52 (Lys-C), (d) LP-CEX52 (papain). Growing and diminishing species are indicated by arrows. The color coding in all four panels is identical. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

changes which generated a broad and undefined peak profile. These changes precluded quantitative analysis of the data but suggested higher sensitivity of the charge-based method over SEC and CE-SDS.

Fig. 5c and d compares the results of samples analyzed with the CEX52 assay following Lys-C and papain digestion. In contrast to the CEX68 chromatograms shown in Fig. 5b, separation of the Fab and Fc fragments resulted in higher-resolution data. The Fab peak remained sharp and well resolved, even after 4 weeks of light exposure. Resolution of the Fc peak, however, decayed rapidly, resembling the changes seen in Fig. 5b. Therefore, it was concluded that light-induced changes in the Fc portion dominated the CEX profile for the undigested antibody (cf. Fig. 5b–d). The Lys-C and papain results agreed with the chromatographic data presented in Fig. 2 as they showed similar elution time shifts for the Fab and Fc fragments. It was also observed that the Fc-related species changed quickly and dramatically within the first 2 weeks of light exposure,

Table 2
Summary of analytical data for a mAb-1 photodegradation study.

Method	Fluorescent light exposure				
	Dark control	1 week	2 weeks	3 weeks	4 weeks
Bioactivity					
% Relative potency[a]	99.1 ± 0.9	81.0 ± 2.2	57.4 ± 3.5	40.9 ± 0.2	21.0 ± 0.8
SEC					
% Dimer/aggregates	0.3	2.8	5.5	6.5	9.1
% Monomer	98.6	93.6	88.6	85.8	81.6
% Fragments	1.1	3.6	6.0	7.7	9.3
CE-SDS					
% HMW[b]	0.4	3.8	6.4	8.0	10.2
% Fragments	0.8	1.9	2.9	3.3	4.7
% Intact HC	65.6	61.1	57.0	54.3	50.6
% NG-HC[c]	0.7	0.8	0.8	0.9	1.0
% Intact LC	32.6	32.4	32.9	33.5	33.5
CEX68					
% Unmodified	66.3	27.9	n/a	n/a	n/a
LP-CEX52 (Lys-C)					
% Pre-Fab	1.2	4.7	8.3	10.8	14.1
% Fab	53.7	43.9	34.7	28.3	21.4
LP-CEX52 (papain)					
% Pre-Fab	1.2	4.2	8.0	10.6	14.2
% Fab	54.4	46.2	38.7	34.2	27.6

[a] Percent relative potency is calculated as the ratio in bioactivity between a sample and an experimental control.
[b] HMW – high molecular weight species, primarily dimeric/aggregated and covalently bound molecules.
[c] NG-HC – nonglycosylated heavy chain.

whereas the Fab species changed in a steady and more linear fashion over the entire 4-week duration (Table 2). This suggested that the linear loss in bioactivity was likely associated with degradations in the Fab region of mAb-1.

As seen in Fig. 5c and d, light exposure resulted in the appearance of a new peak that elutes prior to native Fab. This pre-Fab species was isolated along with the unmodified Fab fragment for subsequent characterization (Section 2.8). Results from the CEX52 and SEC analysis following purification are shown in Fig. 6a and b. Both sets of data demonstrated that each fraction consisted of highly homogeneous material. RP–MS analysis was applied to identify the regions of degradation. Fig. 6c compares a segment of the tryptic peptide map between the pre-Fab and Fab species, highlighting the area of greatest change. The tryptic map of the Fab sample generated the expected peptides and masses based on the known amino acid sequence of mAb-1. In contrast, the tryptic map of the pre-Fab material revealed a dramatic reduction in the H3 heavy-chain peptide and contained several new variants of H3 that elute between 86.5 and 91.0 min. RP–MS analysis of these peaks showed mass additions of either 16, 32, 33, or 50 Da. One of the H3+32 species was further characterized in an attempt to localize the modification. MS/MS spectra for a short sequence of the native and modified H3 peptide are shown in Fig. 7. When the y-ion series were compared, a mass increase of 15 Da was observed for y13 but not y12. The b-ion series showed the same mass difference for b10 but not b8. The b9 ion was not observed in the H3+32 spectrum. Despite the poor recovery of some ions, it was reasonable to conclude that the site of modification corresponds to the second histidine residue in the H3 peptide of the heavy chain. This modified residue is adjacent to a stable and solvent-exposed succinimide in the CDR2 that has been previously described for the same molecule [27]. Since the succinimide caused decreased biological activity of mAb-1, the same might be expected for degradations occurring at neighboring residues. This is the first time a modification in mAb-1 has been localized to this histidine, illustrating

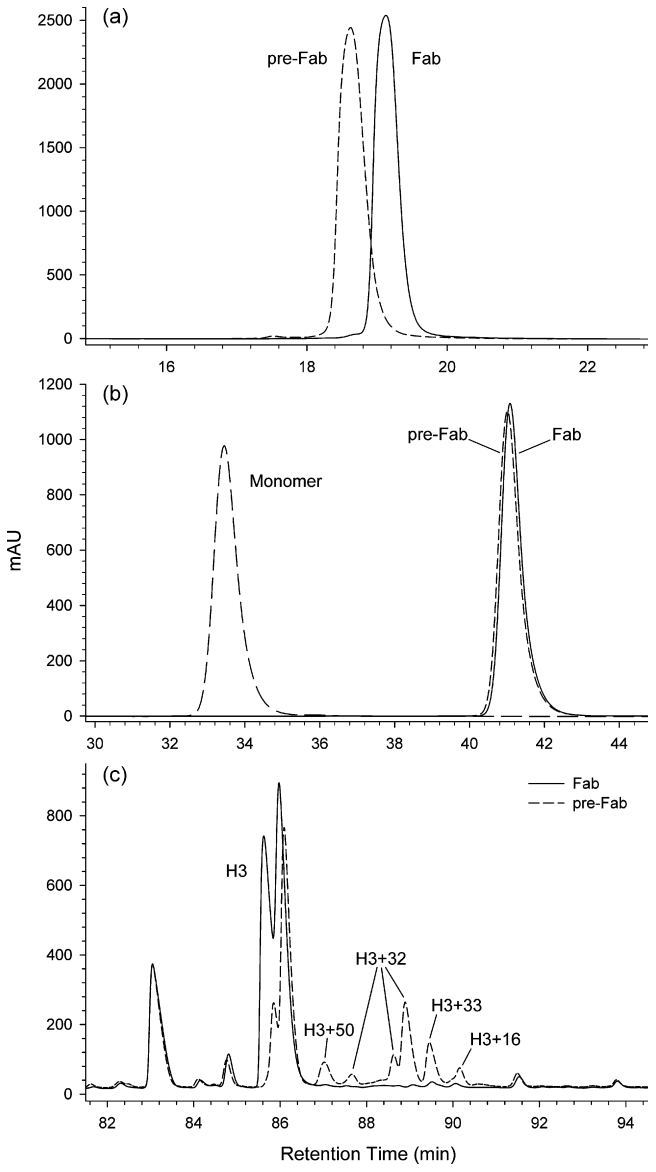

Fig. 6. (a) CEX52 and (b) SEC analysis of pre-Fab and Fab species isolated from photodegraded mAb-1 samples. Intact mAb-1 (monomer) is shown in (b) as a reference. (c) Peptide map comparison of the pre-Fab and Fab species. Recovery of the tryptic H3 peptide was greatly reduced in the pre-Fab peptide map, and new H3 peaks with net additions of 16, 32, 33, or 50 Da were observed.

an alternative protein degradation mechanism resulting from light exposure.

Although RP–MS/MS analysis identified all of the new peaks in the pre-Fab tryptic map as H3 variants, it did not corroborate the precursor mass additions derived from the RP–MS data. The fragmentation patterns generated from each of these species closely resembled the H3+32 spectrum displayed in Fig. 7 and similarly revealed only a 15-Da increase for all modified ions (data not shown). We cannot account for the discrepancies in mass or explain the elution time differences between the modified H3 peptides. Despite an incomplete understanding of the chemistry behind this labile modification, we have confidence in the site identification based upon the MS/MS data. Even though histidine modifications are unusual and none exist that are readily explained by a 15-Da increase, oxidation of histidines has been previously observed [28,29].

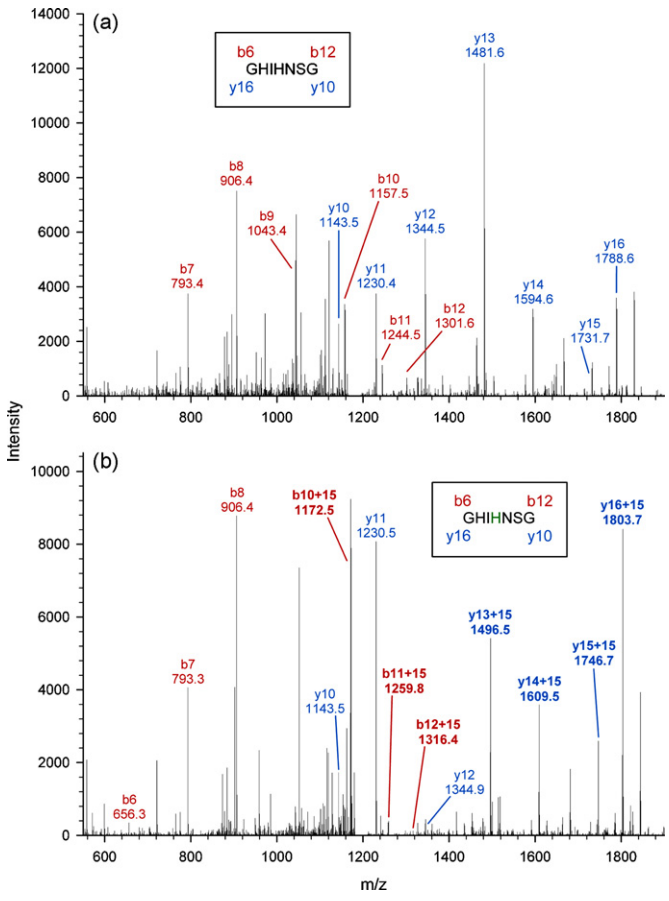

Fig. 7. (a) The MS/MS spectrum obtained from the unmodified H3 $[M+3H]^{3+}$ precursor with an m/z of 796.4. The resulting b- and y-ion series confirms the peptide sequence of unmodified H3. (b) The MS/MS spectrum obtained from the modified H3 (H3+32) $[M+3H]^{3+}$ precursor with an m/z of 807.6 resulted in a 15-Da mass increase on y13, which corresponds to the second histidine in the tryptic H3 peptide (highlighted in green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

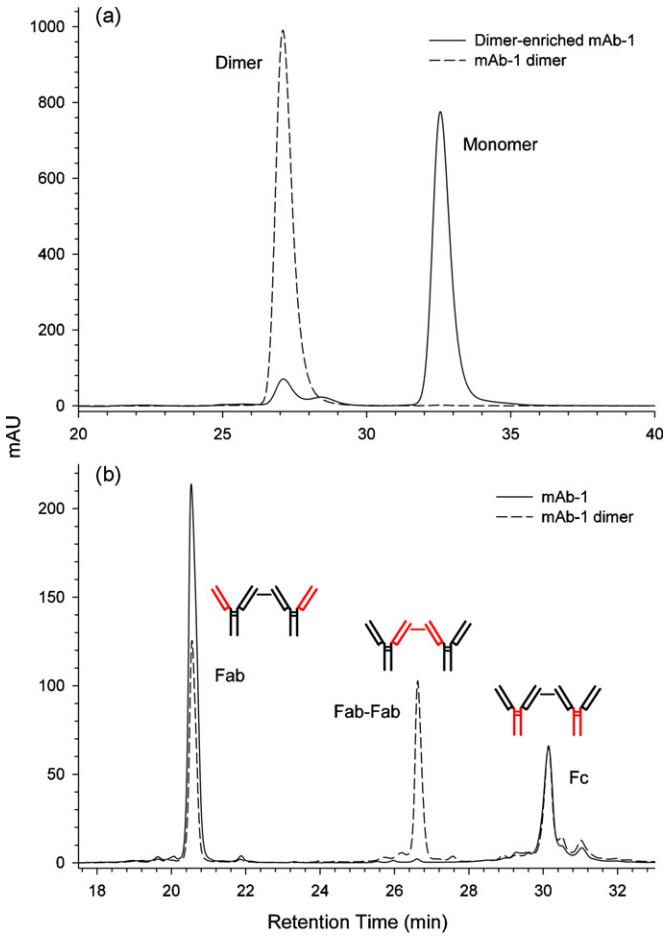

Fig. 8. (a) SEC profiles for dimer-enriched material and purified dimer from mAb-1. (b) LP-CEX52 chromatograms generated from Lys-C-digested mAb-1 and mAb-1 dimer. Antibody diagrams depict the likely scenario for dimer formation through noncovalent interaction between Fab domains. Portions highlighted in red represent the Lys-C fragments from purified dimer that produce the associated peak in the chromatogram. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 3
LP-CEX52 peak area comparison between Lys-C-digested mAb-1 and mAb-1 dimer.

Sample	% Peak area		
	Fab peaks	Intermediate peaks	Fc peaks
mAb-1	63.8	2.4	33.8
mAb-1 dimer	32.4	30.6	37.0

3.5. Characterization of a noncovalent mAb-1 dimer

mAb-1 dimer was purified to near homogeneity under native SEC conditions (Sections 2.2 and 2.9). Fig. 8a shows that the dimer did not immediately dissociate into monomers after isolation and appeared to be relatively stable. The noncovalent nature of the dimer was confirmed by SDS-PAGE analysis under both reducing and nonreducing conditions (data not shown). The dimer and unstressed mAb-1 control consisting of ~99.5% monomer were subjected to limited proteolysis with Lys-C and subsequently analyzed by CEX52, revealing striking differences (Fig. 8b). In comparison to the monomer, the Fab peak area for the dimer was reduced by ~50%, whereas the recovery of the Fc species remained relatively unaffected (Table 3). Additionally, a new intermediate peak was observed at ~26.5 min for the dimer but not the monomer. Its relative area matched the reduction in the native Fab peak, suggesting that this intermediate species originated from Fab fragments. These results were sufficient to decipher the structural organization of the mAb-1 dimer. The role of Fc was dismissed based on its full recovery, and the possibility of a dimeric form stabilized by all four Fab arms (one antibody can provide two Fabs for intermolecular binding) was inconsistent with the presence of monomeric Fab. The only scenario which was fully consistent with the LP-CEX52 data is depicted in Fig. 8b. It is evident that dimerization of mAb-1 proceeds through the formation of a stable and noncovalent intermolecular interaction between two Fab arms. The identity of the intermediate species was thus deduced as a Fab–Fab complex of ~100 kDa and subsequently confirmed by MS analysis (data not shown). Covalently bound antibody dimers formed *via* Fab–Fab interactions have been previously observed and characterized [30]. With respect to noncovalent complexes, several observations have suggested that they may involve interactions between variable regions [23,26]. While our data corroborate the results from these previous studies, the novelty of our approach lies in applying a relatively simple and nondenaturing separation technique to investigate the structural organization of weakly associated oligomers.

4. Conclusions

The option of two enzymes for limited proteolysis lends flexibility and versatility to the LP-CEX52 method. Lys-C generates Fab

and Fc fragments that can be well separated by CEX, but papain is more useful for analysis of heavily degraded samples. In particular, comparison of the Lys-C and papain data has improved our understanding of mAb behavior with WCX and facilitated the assignment of degradations specific to Fab or Fc domains. LP-CEX52 also reduces the inherent complexity of mAbs and makes subsequent characterization by mass spectrometry more robust and efficient. Additionally, it can be correlated with bioassay analysis and may therefore predict loss of biological activity in IgG1 molecules. It should be noted that applications of LP-CEX52 are not limited to analysis of covalent modifications and can be extended to explore noncovalent protein complexes. Noncovalent oligomerization may be linked to mAb aggregation, and a better understanding of these processes is crucial for developing safe and stable biopharmaceutical formulations. As the work presented here demonstrates, LP-CEX52 is a fast, simple, and high-resolution assay that offers substantial advantages over traditional nonnative approaches and can be applied to study a variety of antibody degradation mechanisms.

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