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Analysis of human antibody IgG2 domains by reversed-phase liquid chromatography and mass spectrometry

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ABSTRACT

It has been well documented that papain cleaves an IgG1 molecule to release Fab and Fc domains; however, papain was found unable to release such domains from an IgG2. Here we present a new combinatory strategy to analyze the heterogeneity of the light chain (LC), single chain Fc (sFc), and Fab portion of the heavy chain (Fd) of an IgG2 molecule released by papain cleavage under mild reducing conditions. These domains were well separated on reversed-phase high performance liquid chromatography (RP-HPLC) and analyzed by in-line liquid chromatography time-of-flight mass spectrometry (LC–TOF/MS). In addition, some modifications of these domains were revealed by in-line mass spectrometry, and confirmed by the peptide mapping on LC–MS/MS analysis. This same strategy was proven suitable for IgG1 molecules as well. This procedure provides a simplified approach for the characterization of antibody biomolecules by facilitating the detection of low-level modifications in a domain. In addition, the technique offers a new strategy as an identification assay to distinguish IgG2 molecules on RP-HPLC, by which highly conserved Fc domains remain at a constant retention time (RT) unique to its subisotype, while varying RTs of the light chain and the Fd distinguish the monoclonal antibody from other molecules of the same isotype based on the underlying characteristics of each antibody.

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1. Introduction

With his original work performed in 1959, Porter demonstrated that limited digestion of an IgG1 molecule by papain produces biologically active fragments, which were later called Fab and Fc fragments [1]. Since then, these domains have been investigated in great detail, and it has been found that they can be purified by gel filtration, ion exchange, or affinity chromatography [2–7]. Separation of these domains has facilitated investigation of the micro-heterogeneity of human monoclonal antibodies. Several recent publications described the analysis of IgG1 Fab and Fc domains using hydrophobic interaction chromatography (HIC), RP-HPLC, and LC–TOF mass spectrometry, which facilitated the confirmation of chemical and post-translational modifications (PTM) such as N-terminal cyclization, oxidation, deamidation, and C-terminal processed lysine residues [8–10]. These efforts, in addition to structure and sequence analysis of therapeutic IgGs, have dramatically increased our understanding of the relationship between the antibody structure and its biological function [11–13].

In spite of these progresses, the use of papain cleavage is still very limited for analysis of therapeutic monoclonal IgG2 antibodies due to some significant heterogeneous fragments produced by papain cleavage. The reason for these undesirable results was not clear until it was found very recently that the IgG2 subclass exists as structural isoforms (IgG2A, IgG2A/B, and IgG2B), which differ by the disulfide connectivity in the hinge region [14,15]. These structural constraints prevent the specific papain cleavage of the hinge region in an IgG2, and consequently provide an explanation for the great complexity of the papain cleavage pattern in the IgG2 subclass.

Disulfide heterogeneity presented a technical challenge for a domain based analysis of an IgG2 molecule, and required a novel strategy to overcome the barrier. The fact that redox treatments were able to enrich the IgG2 isoforms that exist in an IgG2 control material suggests the possibility that a homogeneous structure can be introduced by some mild redox treatments [14]. Given the fact that a cleavage of the inter-heavy chain disulfide in IgG Fc does not lead to any large structural change in the antibody [16], and that without the disulfide bond, the LC and HC could still associate together strongly with the association constant of $\sim 10^{10}\ M^{-1}$ [17,18], we reason that it is possible for papain to specifically cleave an IgG2 hinge if the disulfide bond isomers can be removed. Therefore, we explored a number of strategies to break the heterogeneous

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disulfide connections in the hinge region, and found that mild DTT treatment caused a reduction of the disulfide bonds that allowed a specific cleavage by papain at the glutamic acid–cysteine (EC) bond in the hinge sequence "KCCVECPPCPAPP", generating the LC, sFc, and Fd domains. This strategy not only allows analysis of IgG2 subclass antibodies, but also provides a chromatographic assay for an identity test of IgG2 subclass molecules. The highly conserved Fc fragment serves as a unique identifier for the subisotype of the monoclonal antibody (mAb), while the variability of both the light chain and the Fab portion of the heavy chain (Fd) provide a clear distinction for each mAb when compared with other molecules of the same subisotype.

2. Experimental

2.1. Materials

The recombinant human monoclonal IgG2 and IgG1 antibodies analyzed in this study were manufactured at Amgen and purified using standard manufacturing procedures. A total of eight IgG2 and three IgG1 antibodies were used in this study, each with either a kappa or lambda light chain subtype. All chemicals used in this study were of the highest grade available.

2.2. Generation of domains by papain cleavage

Papain (Roche applied science) cleavage was performed according to the procedure described previously [9] with some modifications. Briefly, the cleavage was carried out by incubating the IgG2 at a final concentration of 1 mg/mL in 0.1 M Tris–HCl (pH 7.6), 4 mM EDTA, 5 mM cysteine, and 10 mM of either dithiothreitol (DTT, Sigma), or 2-mercaptoethanol (BME, Sigma), or Tris(2-carboxyethyl) phosphine hydrochloride (TCEP, Pierce). The digestion was initiated by the addition of papain (diluted to 1 mg/mL with water) to give a final protein to enzyme ratio of 100:1 (w/w). The digestion was carried out for 2 h at 37 °C, then quenched with 5% formic acid and chilled on ice for 15 min.

2.3. Reversed-phase high performance liquid chromatography (RP-HPLC) and time-of-flight mass spectrometry (TOF/MS)

Two RP-HPLC methods were employed in this study. For an identification assay, a MAC-MOD Analytical HALO C8 column (MAC-MOD Analytical Inc.) with 2.7 μm particle size, 90 Å pore size, 150 mm × 4.6 mm on Agilent 1100 HPLC systems was used. The column eluant was monitored by UV signals at 215 nm. The mobile phases included water with 0.1% trifluoroacetic acid (TFA, Pierce) and 10% isopropyl alcohol (IPA) as solvent A and acetonitrile (Burdick Jackson) with 0.1% TFA and 10% IPA as solvent B. The initial condition was set at 21% B, then a gradient from 21 to 41% B in 25 min. The procedure was operated at 75 °C with a flow rate of 0.5 mL/min.

For the characterization assay, RP-HPLC was performed on Agilent 1200 HPLC systems. Agilent Zorbax Stable Bond SB300 C8 column with 300 Å pore size, 150 mm × 2.1 mm with a 3.5 μm particle size, was used for the RP-HPLC and LC–TOF/MS analysis. The column eluants were analyzed at 215 nm and directed to an in-line TOF/MS spectrometer. The mobile phases included water with 0.11% trifluoroacetic acid (TFA, J.T. Baker) as solvent A and acetonitrile (Burdick Jackson) with 0.09% TFA as solvent B. The initial condition used 32% B for 5 min, then a gradient from 32 to 45% B in 35 min. The procedure was operated at 75 °C with a flow rate 0.6 mL/min. Some oxidation-containing fractions were collected manually, and neutralized immediately using 10% (v/v) of 0.5 M ammonia acetate. After concentrating by speed vacuum to a small volume, typically around 100 μL, Lys-C peptide mapping was performed following the procedure described below. Peak areas were calculated by using ChemStation software (Agilent) with the tangent skimming technique for incompletely resolved peaks.

Electrospray ionization time-of-flight (ESI-TOF) mass spectrometry was performed with an in-line Agilent 6210 LC–TOF mass spectrometer equipped with an Agilent 1100 HPLC, as described previously [9].

2.4. Reduction, alkylation and Lys-C protease digestion

Reduction and alkylation were performed under denaturing conditions as described previously [9]. Briefly, antibody was diluted to 1 mg/mL with a buffer of 50 mM Tris–HCl, pH 8.8, containing 4 M guanidine hydrochloride (final concentration). An aliquot of 0.5 M DTT (Sigma) stock solution was added to obtain a 5 mM concentration and the reaction mixture was placed at 75 °C for 5 min. The protein solution was then cooled to room temperature and an aliquot of 0.5 M sodium iodoacete (IAA, Sigma) stock solution was added to reach a final concentration of 10 mM IAA, and the alkylation was performed at room temperature for 40 min in the dark. To quench the alkylation reaction, the 0.5 M DTT stock solution was added to obtain a final concentration of 15 mm DTT. The reduced and alkylated samples were buffer-exchanged into 50 mM Tris–HCl (pH 7.5) by using Bio-Spin columns (Bio-Rad), following manufacturer instructions. Lys-C digestion was performed for 5 h at 37 °C using an enzyme to protein ratio of 1:10 (w/w) in the presence of 10% acetonitrile. The digest was either placed immediately in the autosampler maintained at 4 °C for LC–MS/MS analysis or frozen at −80 °C for future analysis.

2.5. RP-HPLC separation of Lys-C peptides

All peptides generated by Lys-C were separated using an Agilent 1200 HPLC equipped with a UV detector, autosampler, flow cell and temperature-controlled column compartment. A Phenomenyx C5 column, 150 mm × 2.1 mm, packed with 3 μm particle size, 300 Å pore size resin was used for peptide separation. Solvent A contained 0.11% trifluoroacetic acid (TFA) in water, and solvent B contained 0.09% TFA in acetonitrile. A linear gradient from 2 to 60% B was run over 205 min. The column flow rate was 200 μL/min and the column temperature was maintained at 60 °C. The column eluant was analyzed by UV detection at 215 nm and then directed to an in-line ion trap mass spectrometer.

2.6. Peptide mapping by LC–MS/MS

A Thermo Finnigan LTQ ion trap mass spectrometer was used in-line with the HPLC system to identify peptides. Mass analyzer software developed in the laboratory was used for peptide identification [19].

3. Results and discussion

3.1. Human recombinant antibody

The antibodies used in this study were human recombinant antibodies of the IgG2 and IgG1 subclasses. They were expressed in Chinese hamster ovary (CHO) cells and purified by successive affinity and conventional chromatography steps.

3.2. Papain cleavage created an identification assay for IgG2 antibodies

Each IgG2 molecule contains four disulfide bonds in the hinge region of the heavy chain. Among the five cysteines in the light chain, one of them – located at or near C-terminus – is involved in

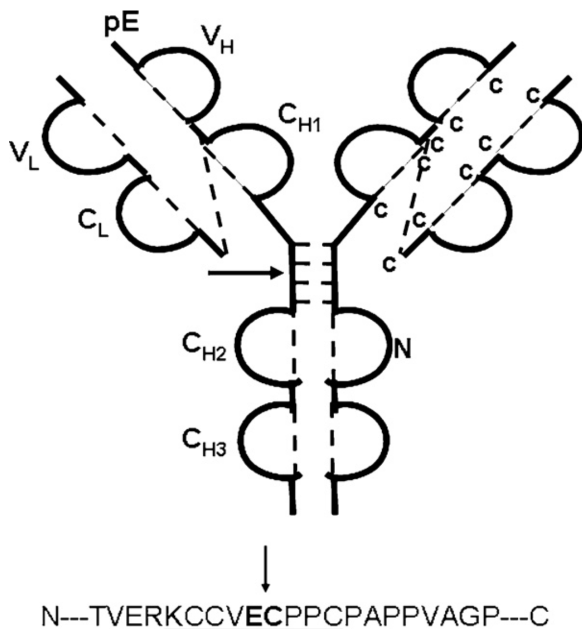

Fig. 1. Structure model for an IgG2. The cysteine residues connected in intra-disulfide bonds and inter-chain disulfide bonds represented by those dished lines. V_L and V_H represent variable domains, and C_L and C_H represent constant domains, each of the light and heavy chain, respectively. There are three constant domains in each heavy chain (C_H1, C_H2, and C_H3). The C_H2 domains are glycosylated. Between the CH1 and CH2 domains are short stretches of peptide referred to as the hinge regions in this paper. The N-glycosylation site in the C_H2 domain is indicated at N. The papain cleavage site between four disulfide bonds in the hinge region is located with an arrow.

an inter-chain disulfide bond with the cysteine located between the constant and variable region of the heavy chain Fab domain (Fig. 1). It has been demonstrated that in monoclonal human IgGs, the inter-heavy and heavy–light chain disulfide bonds are equally sensitive to reduction [20]. Therefore, we examined the effectiveness of various reduction reagents and concentration ranges (10–100 mM DTT, 10–100 mM TCEP, and 10–100 mM BME) in reducing the disulfide heterogeneities in the hinge region. DTT was found to be the most efficient of the three reducing agents (the details not shown). Papain cleaves the EC bond of the hinge sequence "KCCVECPPCPAPP" in the presence of DTT, and thus generates three domains, the light chain (LC), single Fc (sFc) and Fab portion of the heavy chain (Fd). The cleavage is specific, and the single point of cleavage is highly reproducible, confirmed by RP-HPLC–TOF/MS analysis (not shown). Over the range of DTT concentrations explored (10–100 mM), identical UV profiles of the papain digested samples on RP-HPLC were observed (the details not shown). A DTT concentration of 10 mM was chosen for the procedure reported here.

In this study, eight human IgG2 antibodies were analyzed to compare their profiles on RP-HPLC using a 30 min run, as described in the procedure above. The three subdomains – LC, sFc and Fd – provide three signature fragments for each antibody and are well resolved by RP-HPLC. The single Fc subdomains from all eight IgG2 antibodies displayed a consistent RT by RP-HPLC, while each of the LC and Fd subdomains varied in RT. Two IgG2 molecules, indicated by traces "f" and "h" in Fig. 2, had the same RT for their Fd domains, but different RT for their LC domains. These results suggest that the Fc single chain serves as an identifier for the subisotype of the IgG molecules, and that the method could be used as an identification assay to distinguish antibodies.

We observed that for three of the molecules, the LC domain eluted earlier than the sFc domain; however, we did not observe a correlation between the LC isotype and its RT. Among the eight IgG2 molecules analyzed in this study – three with kappa type LC

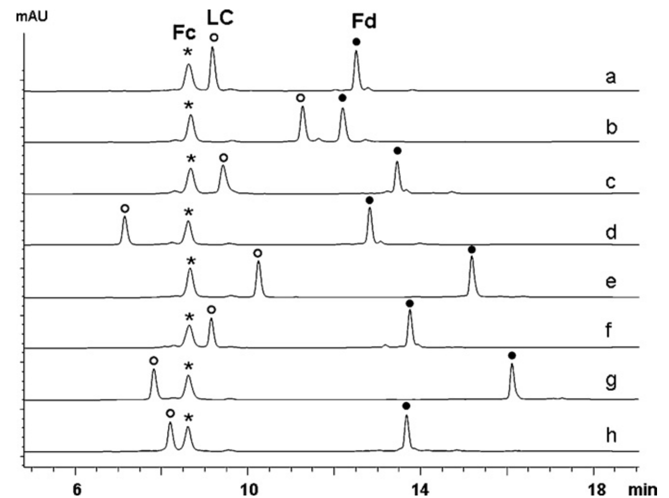

Fig. 2. Reversed-phase chromatogram overlay of papain digested IgG2 monoclonal antibodies (a–h). The Fc peak (*) elutes at the same retention time (RT) for all IgG2 molecules due to the shared sequence homology of the Fc domain. The LC (◦) and Fd (●) peaks contain the variable domains of the antibody and elute at different RT for each molecule.

and five with lambda type – no significant impact of the LC isotype on the RT was found. Of the three LCs that had an earlier RT than their corresponding sFc domains, two of them had kappa type LC and one had lambda type. These results indicated that the LC isotype has no impact on the RT of the LC domain.

3.3. Experimental conditions for analysis of domains generated by papain cleavage

For characterization purposes, one of the IgG2 molecules was chosen for RP-HPLC–TOF/MS analysis. The UV profiles of its domains were monitored at 215 nm (Fig. 3). LC–TOF/MS is capable of detecting molecular mass of a protein with high accuracy. For example, upon reduction and alkylation in the presence of 4 M guanidine hydrochloride (GdnCl), LC–TOF/MS analysis indicated a molecular mass of 22994.3 Da for the LC and 51223.4 Da for the HC, which contains a G0 glycan moiety, complete cyclization at the N-terminus, and completely processed C-terminal lysine. These data are in excellent agreement with the theoretical masses of 22994.5 Da and 51223.7 Da, respectively (details not shown). With

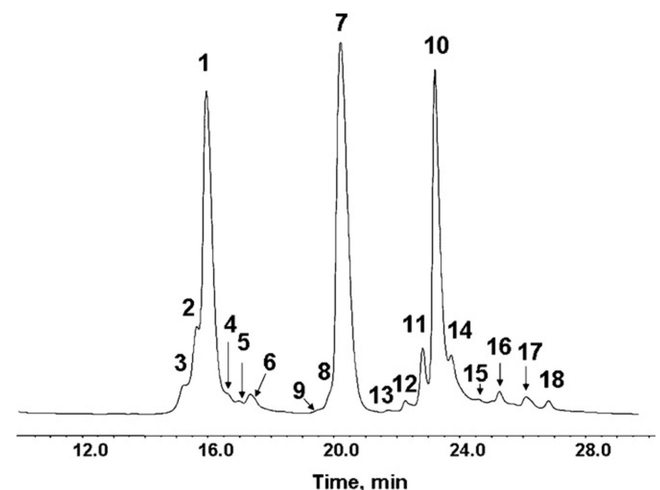

Fig. 3. Reversed-phase chromatographic profile at 215 nm of the domains of an IgG2 generated by papain cleavage. The sample is analyzed by a Zorbax SB C8 column, 2.1 mm × 15 cm, with a 3.5 μm particle size. The peaks are labeled by numbers.

our RP-HPLC conditions, there is ∼3% of the Asp–Pro (D–P) bond cleavage product of the HC when analyzing the LC and HC, which was due to acid hydrolysis of an acid-labile D–P bond in the heavy chain under the low pH and high temperature environment of the column.

When the papain-generated domains were analyzed on RP-HPLC–TOF/MS, the molecular mass of each domain was found to be ∼4 Da less than its corresponding theoretical mass. Although DTT reduction likely breaks all inter-chain disulfide bonds, it is possible that the intra-chain disulfide bonds remained intact. Therefore, the −4 Da deficits could be attributed to the existence of the intra-chain disulfide bonds, as discussed in more detail in the following section. We explored the possibility of alkylating free –SH groups generated by DTT reduction, without introducing the mispairs or other side reactions that may compromise the separation efficiency. We attempted to quench the digestion by either adding iodoacetic acid (IAA) to a 50 mM final concentration in the presence or absence of GdnCl, or by lowering the pH with trifluoroacetic acid (TFA) and then adding *N*-ethylmaleimide (NEM) to a 50 mM final concentration. Unfortunately, all of these efforts resulted in a very complicated UV profile with multiple additional peaks.

Papain is a cysteine protease and requires the presence of free cysteine (Cys) in the reaction solution for its activation. The alkylation with IAA following papain digestion inadvertently reduced the proteolytic activity of the enzyme by depleting free Cys residue in the reaction solution. Quenching with TFA decreased the pH to ∼2, which minimized the reactivity of a thiol group, and the following NEM alkylation depleted DTT and Cys, causing a complicated profile. On the other hand, quenching by GdnCl could denature the antibody, and consequently lead to non-specific cleavages. For these reasons, the reducing only condition was employed for subsequent analyses in this study.

3.4. LC–TOF/MS analysis of Fc and its variants

Fc and its variants (peaks 1–6) in Fig. 3 were analyzed and the results are shown in Fig. 4. Peak 1 was identified as the sFc domain based on the glycosylation profiles that include glycoforms G0, G1 and G2 [9,21] (Fig. 4A). The molecular mass of 26167.6 Da corresponds to an Fc monomer with a G0 glycan moiety, but is 4.1 Da less than the theoretical mass of 26171.7 Da for a completely reduced form. The other two domains (LC and Fd) were shown to also have a ∼4 Da mass deficit compared to the theoretical mass (see details in the following sections). An explanation for this discrepancy is that the intra-chain disulfide bonds in the LC and HC were not reduced, and it is also possible that some inter-disulfide bonds was not reduced completely, as some Fd dimmers were observed (see below). Thus, we propose that the ∼4 Da deficits are due to the existence of the intra-disulfide bonds. Although the CPPC peptide is capable of forming a disulfide bond in the hinge region [22], the fact that higher torsion and angle bending energies are required for such a bond formation implies that the ∼4 Da difference was more likely due to disulfide bond formations in the C_H2 and C_H3 intra-domains.

Fig. 4B and C contain the deconvoluted spectra of peaks 2 and 3, respectively, which exhibit a similar distribution of the glycoforms compared to the main peak (Fig. 4A). The G0 glycoform has the masses of 26185.77 Da and 26185.47 Da, respectively, indicating the presence of an approximately +18 Da modification on both of them. The two components eluted earlier than Fc main peak on

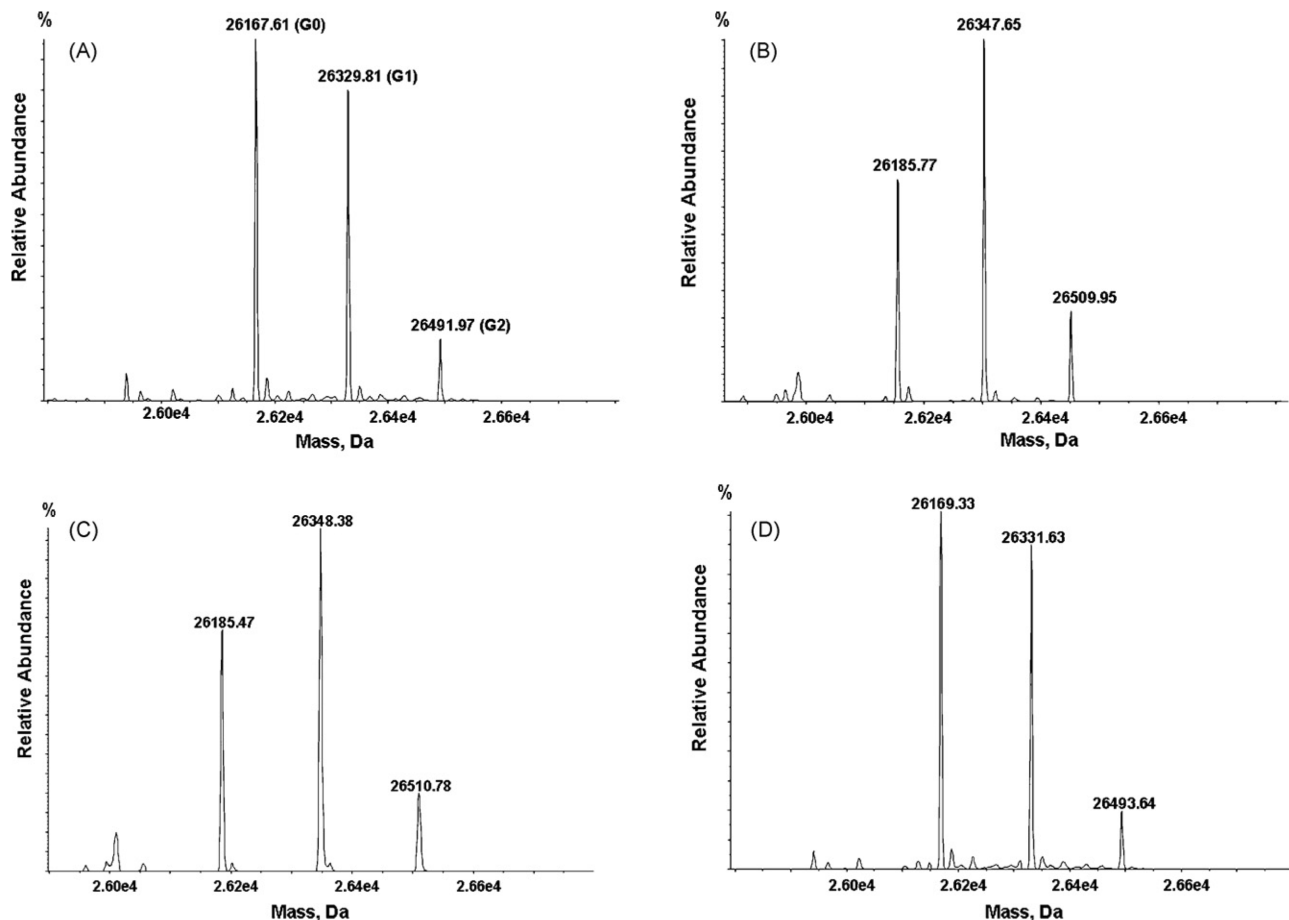

Fig. 4. The deconvoluted mass spectra of the Fc and its variants. Each peak was labeled with molecular mass and the corresponding glycan structure. Panel A: peak 1 (Fc main peak); panel B: peak 2 (Fc with oxidation 1); panel C: peak 3 (Fc with oxidation 2); panel D: peak 4 (Fc with free –SH groups).

RP-HPLC, suggesting a more hydrophilic nature, very likely due to the oxidized Fc species. It should be pointed out that under RP-HPLC conditions employed in this study, D/P bond cleavage existed at ~3% level when analyzing the reduced and alkylated HC. Since the intra-chain disulfide bond existed for the sFc, it is likely that the D/P cleavage took place to generate a species with a +18 Da higher mass than the expected Fc mass, which co-eluted with these oxidized Fc species on RP-HPLC. However, giving the high extant of the oxidized species at ~18% (see Table 2), the majority of the peaks 2 and 3 could be still attributed to the oxidation. This conclusion was confirmed by a peptide mapping study (see below).

Peaks 4 and 5 showed the same mass and glycoform distribution as the main peak with a G0 mass of 26167.5 Da. The different retention times indicated that they are resolved isobaric forms, possibly products of deamidation (details not shown).

Fig. 4D shows an observed mass of 26169.3 Da for peak 6, 1.7 Da heavier than the theoretical mass of the Fc main peak (Fig. 4A), suggesting the presence of mispaired sFc with one unpaired intra-chain disulfide bond that resulted in an increase of +2 Da. This conclusion was supported by the observation that NEM modification of the papain digested sample resulted in the disappearance of peak 6, and an emerging peak with an earlier RT and additional +250 Da, corresponding to incorporation of two NEM molecules. These results confirmed that the species in peak 6 contains free –SH groups. Similar results were obtained from our previous study [10].

3.5. Analysis of the light chain and its variants

TOF analysis of peak 7 showed a mass of 22704.4 Da (Fig. 5A), 4.0 Da less than the theoretical mass of 22708.4 Da. This is consistent with the mass of the expected light chain with two disulfide bonds. Peak 8, with a mass of 22720.5 Da, eluted approximately 0.5 min earlier and was 16.1 Da heavier in mass than peak 7 (Fig. 5B). The earlier elution time and mass increase relative to unmodified species suggested peak 8 to be an oxidized species. The deconvoluted mass spectrum of peak 9 revealed two masses (Fig. 5C). The earlier RT for the component with a mass of 22704.20 Da implied the presence of an isomerization or deamidation product; however, the identification of the +18 Da modification with a mass of 22722.13 Da remains unclear at this time.

3.6. Analysis of the Fd and its variants.

Fig. 6 shows the deconvoluted mass spectra of Fd and its variants. The Fd species were separated into nine peaks (peaks 10–18 in Fig. 3). Peak 10, with a molecular mass of 24323.5 Da, corresponds to the Fd fragment with pE at the N-terminus of the HC (Fig. 6A). This is 3.9 Da less than the theoretical mass of 24327.4 Da, consistent with previous results.

Peaks 11 and 12 displayed more hydrophilic nature than the main peak, and had molecular masses of 24340.4 Da and 24341.1 Da, respectively (Fig. 6B and C). The fact that they were 16–17 Da heavier than the Fd main peak suggested the presence of two separate mono oxidations in the Fd. Peak 13 contains two species (Fig. 6D): one with a molecular mass of 24340.8 Da, suggesting the presence of a third oxidation site or non-cyclized Gln at the N-terminus of the HC, and the second with a molecular mass of 24358.3 Da, suggesting either di-oxidiations or one oxidation and one non-cyclized Gln. All these proposed oxidations could be explained by the presence of non-cyclized Gln at the N-terminus of the HC; however, a peptide mapping result indicated only ~0.4% of non-cyclized Gln at the N-terminus (not shown), far less than the oxidation levels in the Fd (see Table 2). Thus, these modifications should be derived from the oxidations, and confirmed by our peptide mapping studies (see below).

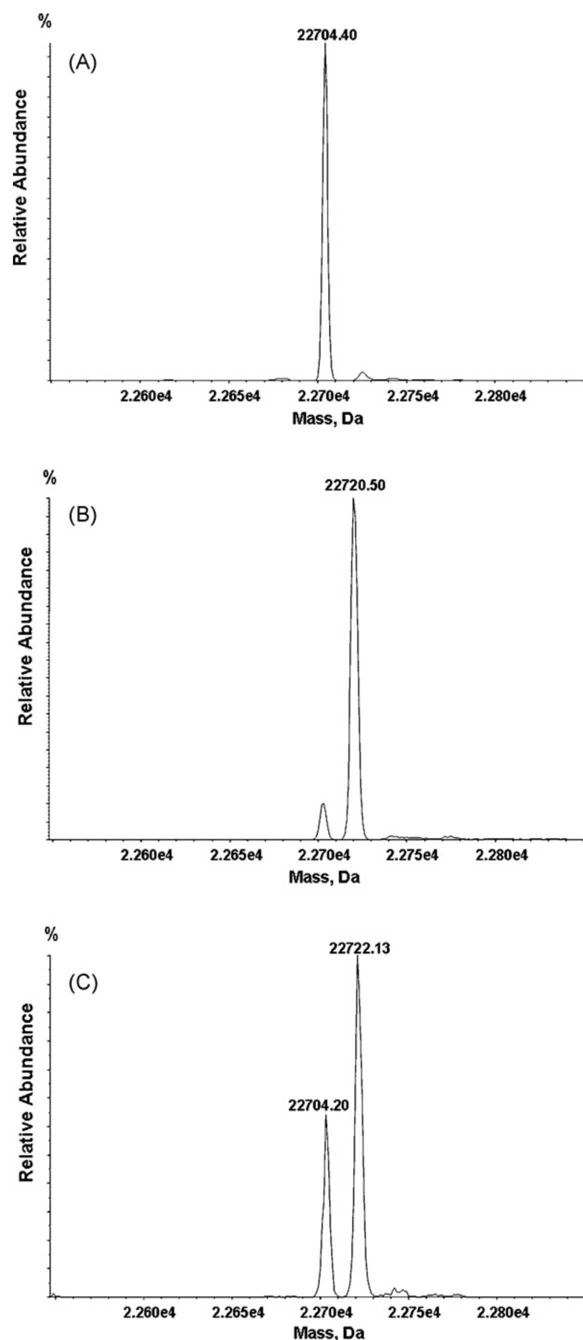

Fig. 5. The deconvoluted mass spectra of the LC and its variants. Panel A: peak 7 (LC main peak); panel B: peak 8 (LC with oxidation); panel C: peak 9 (LC with a deamidation product and an unknown modification).

Peaks 14 and 15 showed molecular masses of 24324.3 Da and 24324.0 Da, respectively, which are approximately +1 Da heavier than the main peak, suggesting the presence of deamidation (details not shown).

Mass analysis showed that peaks 16–18 all have the same mass of 48646.3 ± 0.2 Da, which agreed well with the theoretical mass for the Fd dimer (Fig. 6E). The different retention times found for these species indicated that they have different hydrophobic natures. Papain cleavage at the glutamic acid–cysteine (EC) bond of the hinge sequence "KCCVECPPCPAPP" resulted in the peptide KCCVE remaining in Fd. It is possible that the inter-disulfide bonds in the hinge was not completely reduced by the mild DTT reduction, and a partial reduction of the inter-disulfide bonds could result in

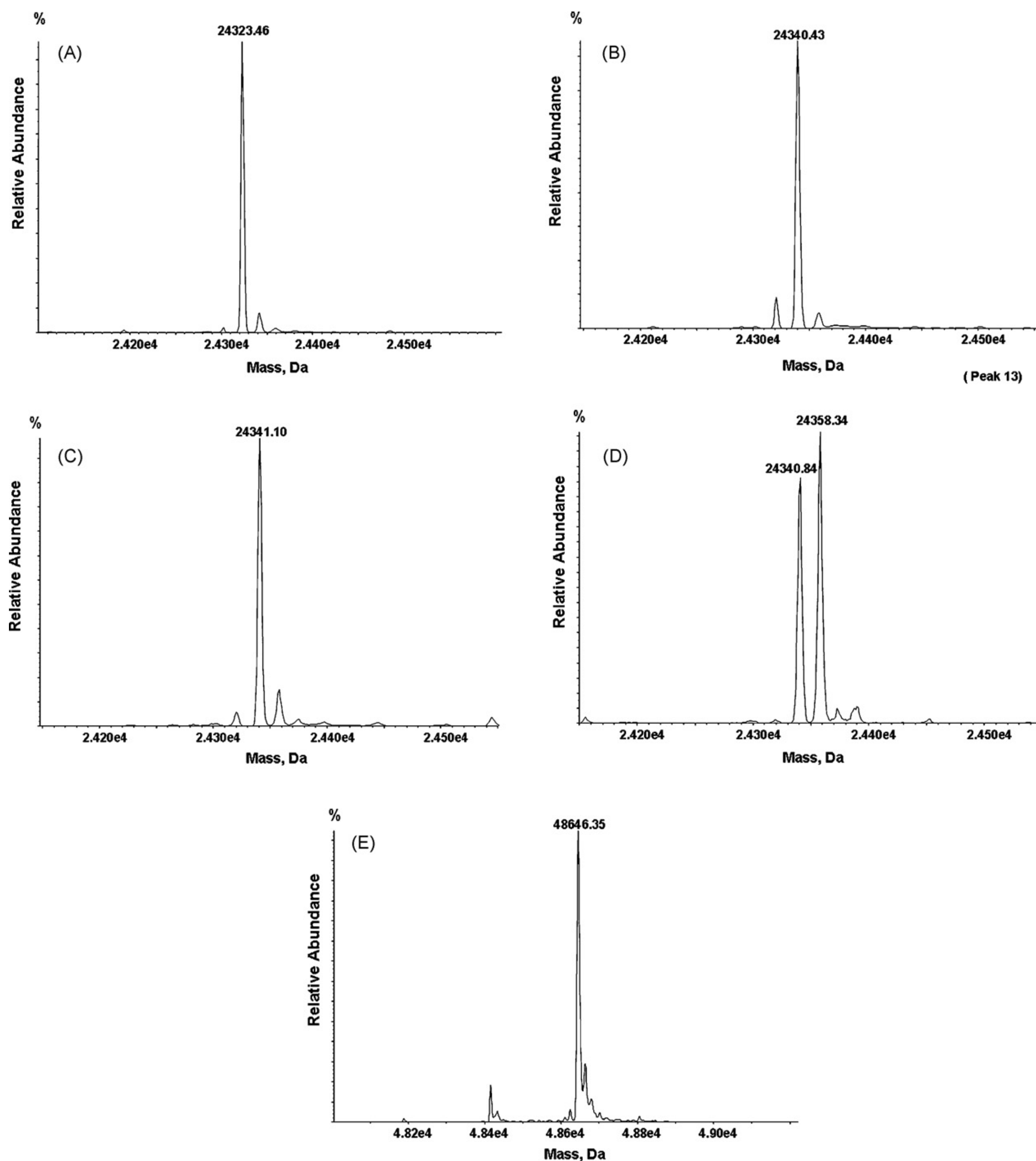

Fig. 6. The deconvoluted mass spectra of the Fd and it variants. Panel A: peak 10 (Fd main peak); panel B: peak 11 (Fd with one oxidation); panel C: peak 12 (Fd with one oxidation); panel D: peak 13 (Fd with one and two oxidations); panel E: peak 16 (Fd dimer).

one unpaired disulfide bond in the C-terminus of Fd. Thus, we suggest that these Fd dimmers were the products of a partial reduction or non-reduction of the inter-disulfide bonds. Table 1 summarizes some of the found modifications of the IgG2.

3.7. Identification of the oxidation sites by Lys-C peptide mapping

Lys-C peptide map was used to determine the oxidation sites, and to confirm the results of RP-HPLC–TOF/MS. The IgG2 used in this study contains six methionine (Met) residues in the HC (three in the Fd, and three in the Fc), and one Met residue in the LC. The six Met residues in the HC are located in peptides H1, H5 (contains two Met residues), H11, H19, and H24, while the one Met residue in the LC is in peptide L3. The results from the Lys-C peptide map showed that all these Met residues were partially oxidized (Fig. 7), as summarized in Table 2. When compared to the oxidation levels measured from RP-HPLC–TOF/MS, we found good correlation between these two procedures (see Table 2 for the details). Note that there is no Met residue in the C_H1 domain; therefore, the two oxidations detected in peak 12 of the papain-digested sample can be attributed to H5 peptide in the V_H domain, which contains two Met residues. The relatively high discrepancies between the methods in the oxidation levels at two of the sites (oxidation #4 in the LC and L3, and oxidation #5 in peak 11 and H1) could be attributed to the very late retention time of the two peptides (L3 and H1) in which they are found. There is less than perfect separation for these two

Table 1
Modifications of the IgG2 domains proposed by RP-HPLC–TOF/MS.

Peak	Modifications	Domain	Theoretical mass (Da)[a]	Observed mass (Da)
1	sFc with G0 glycan	sFc	26171.7	26167.6
2	Oxidation	sFc	26171.7	26185.8
3	Oxidation	sFc	26171.7	26185.5
4	Deamidation	sFc	26171.7	26167.5
5	Deamidation	sFc	26171.7	26167.5
6	Free SH group	sFc	26171.7	26169.3
7	Intact LC	LC	22708.4	22704.4
8	Oxidation	LC	22708.4	22720.5
9	Deamidation	LC	22708.4	22704.2
	Unknown	LC	22708.4	22722.1
10	Intact Fd	Fd	24327.4	24323.5
11	Oxidation	Fd	24327.4	24340.4
12	Oxidation	Fd	24327.4	24341.1
13	Oxidation/non-cyclization	Fd	24327.4	24340.8
	Unknown	Fd	24327.4	24358.3
14	Deamidation	Fd	24327.4	24324.3
15	Deamidation	Fd	24327.4	24324.0
16	Dimer	Fd	48652.8[b]	48646.3
17	Dimer	Fd	48652.8	48646.1
18	Dimer	Fd	48652.8	48646.5

[a] Theoretical mass is a fully reduced mass; for Fc, the mass listed here is the glycosylated G0 form.
[b] Dimer mass is based on one pair of disulfide bonded dimer.

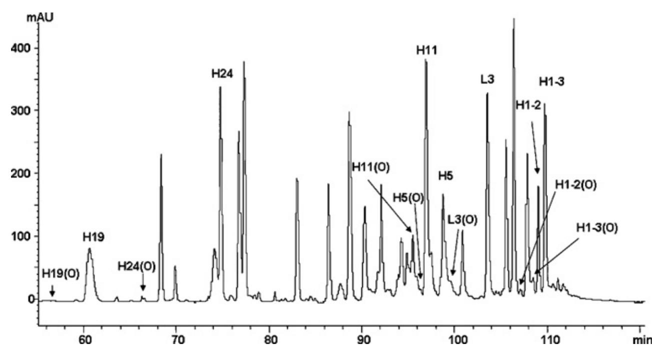

Fig. 7. Lys-C peptide map to determine oxidation level in an IgG2 molecule. All peptides generated by Lys-C digestion were separated on a Phenominex C5 column (4.8 mm × 250 mm) with Agilent 1200 system connected with LTQ mass spectrometry. Only those peptides containing Met residue(s) were labeled by the peptide numbers in the map, and oxidized peptides were labeled with (O). The H1 peptide (containing one Met residue) was not completely digested, and was quantified by integrating peptides H1-2 and H1-3. Quantitation was performed by peak integration.

peptides (Fig. 7), which compromised the resolution and resulted in inconsistencies in quantitation.

To validate the correlations in the amounts of oxidation measured between LC–TOF/MS and peptide map, the oxidized species

Table 2
Quantitation of oxidations in the IgG2 by UV peak area.

Oxidation	Papain digest		Lys-C digest	
	Peak (domain)	%	Peptide (domain)	%
1	Peak 2 (Fc)	12.3	H11 (C_H2)	11.8
2 and 3	Peak 3 (Fc)	5.1	H19 (C_H3)	2.0
			H24 (C_H3)	3.3
4	Peak 8 (L)	3.5	L3 (V_L)	7.5
5	Peak 11 (Fd)	15.6	H1 (V_H)	12.8
6	Peak 12 (Fd)	3.6	H5 (V_H)	4.0

Note: quantitative assessment for different modifications was done after the papain cleavage. There is no Met residue in the C_H1 domain of the Fab portion of the heavy chain. Quantitation of H1 peptide was performed by using both H1-2 and H1-3 peptides. Due to the existence of the D/P cleavage product in the peaks 2 and 3 (the maximal level is at ~3%), the estimate of the oxidation for peaks 2 and 3 could be less than what listed in the table.

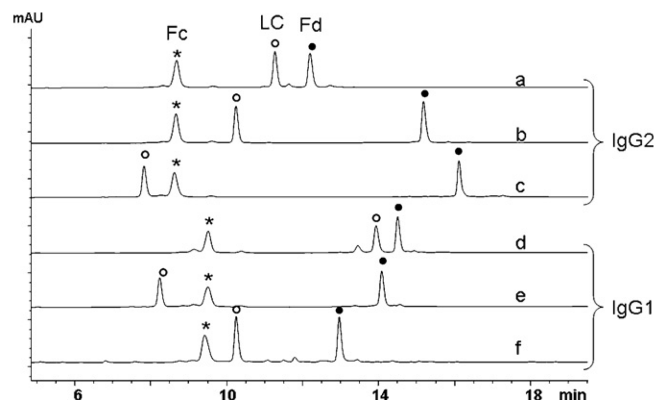

Fig. 8. Comparison of RP-HPLC profiles at 215 nm of the IgG1 and IgG2 domains generated by papain cleavage. The digestion conditions for IgG1 molecules were the same as described in the method section, except that digestion time was shortened to 30 min. For clarity, only three IgG1 and three IgG2 molecules are shown, and Fc (*), LC (◦) and Fd (●) peaks are labeled.

found by RP-HPLC–TOF/MS were further characterized. Fc peaks 1–3, and Fd peaks 10–12 from RP-HPLC (Fig. 3) were manually collected, neutralized immediately with 10% (v/v) of 0.5 M NH_4Ac, and then concentrated to a small volume by speed vacuum. After adjusting pH with 100 mM Tris–HCl, the samples were digested by Lys-C, and analyzed by LC–MS/MS. Comparison of the peptide maps of the three Fc peaks (1–3) illustrated the following: H24 and H19 peptides (each containing a Met residue) from peak 3 were found heavily oxidized, H11 from peak 2 was heavily oxidized, and there was no oxidation observed in peak 1. In addition, from the peptide maps of the Fd peaks, H1 peptide from peak 11 and H5 peptide from peak 12 were each determined to contain oxidized Met residues (details not shown). These results confirmed that the oxidations observed from RP-HPLC–TOF/MS are oxidized Met residues.

3.8. Papain cleaves IgG1 in the presence of DTT

The IgG2 procedure, following a slight modification to shorten the digestion time to 30 min, was also found to work well for IgG1 molecules. Papain cleaves an IgG1 molecule at the H–T bond of the hinge sequence DKTHTCPPC. As for IgG2 molecules, in the presence of DTT the cleavage releases the sFc, LC, and Fd domains. However, some distinguishable differences were revealed when IgG1 and IgG2 RP-HPLC profiles were compared, as shown in Fig. 8. It was seen that all Fc domains in IgG1 molecules appear to elute at 9.4 min, later than the 8.6 min RT of IgG2 Fc domains. This difference of approximately 0.8 min in RT could be used as an indicative maker to distinguish the two IgG subisotypes on RP-HPLC. Characterization of IgG1 heterogeneity by RP-HPLC–TOF/MS was able to reveal some modifications within the molecule domains, similar to that observed by analysis of the IgG2 molecule (details not shown).

4. Conclusion

The work presented in this paper illustrates a new combinatory strategy, which allows one to distinguish monoclonal antibodies (mAbs) in short analysis times for more efficient analytical throughput. The procedure presents a potential identification assay for distinguishing mAbs via release of the sFc, LC, and Fd domains by papain digestion, and subsequent separation of the domains on RP-HPLC. The Fc fragment showed a constant RT on RP-HPLC, while the LC and the Fd domains showed varying RTs that are specific and unique to each molecule due to the underlying characteristics in the variable domain. This suggests that the RP-HPLC method can be used to both determine the subisotype of a mAb

based on the elution position of the Fc, and to differentiate mAbs of the same subisotype based on the inherent differences in the variable domain. In addition, the method provides a simplified procedure for the characterization of mAbs by dissecting these complex biomolecules into smaller, more manageable sub-units. The existence of low-level modifications can be directly measured by accurate mass measurement of smaller fragments, including the assessment of various post-translational modifications such as Met oxidation. Lastly, the procedure works well for both IgG1 and IgG2, demonstrating the feasibility of domain based analysis of IgG molecules as a routine application.

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