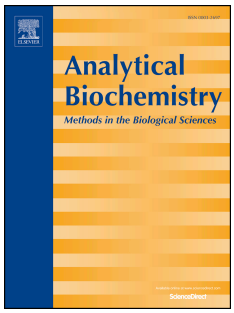
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Characterization of Recombinant Monoclonal Antibody Charge Variants Using WCX Chromatography, icIEF, and Mass Spectrometry

Hailong Liu[1], Weicheng Ren[1], Li Zong[1], Jinliang Zhang[1], Yingwu Wang[1, 2, *]

[1] School of Life Science, Jilin University, Changchun, Jilin, China

[2] Key Laboratory for Molecular Enzymology and Engineering of the Ministry of Education, Jilin University, Changchun, Jilin, China

[*] Corresponding author: Dr. Yingwu Wang, Professor, School of Life Science, Jilin University, 2699 Qianjin Street, Changchun 130012, China.

E-mail address: wyw@jlu.edu.cn (Y.-W Wang).

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Characterization of Recombinant Monoclonal Antibody Charge Variants Using WCX Chromatography, icIEF and LC-MS/MS

Keywords:

Monoclonal antibody, charge variant, weak cation-exchange chromatography, mass spectrometry, imaged capillary isoelectric focusing

Abstract

Charge heterogeneity is an important aspect of research into the development of monoclonal antibody drugs. In the present study, charge variants were separated into four fractions using weak cation exchange chromatography and were thoroughly analyzed using liquid chromatography–mass spectrometry at multiple levels. Molecular weight analysis of intact antibody and subunits confirmed the presence of heavy-chain leader sequences, light-chain leader sequences, dehydration, and cysteinylation. Peptide mapping of the fractions using different enzymes further localized the modified sites. Modified proportions identified at peptide level were compared with the purity detected by imaged capillary isoelectric focusing, the results showed that basic variant 1 consisted of cysteinylation and dehydration of asparagine, and basic variant 2 fully accounted for the N-terminal leader sequence of the heavy chain. About 14.8% of the acidic variant can be explained by N-terminal leader sequences in the light chain, and 18% of the acidic variant was demonstrated to be deamidation of asparagine in the heavy chain. There was approximately 54.2% of the acidic variant still cannot be explained. It was hypothesized that those acidic variants that have not yet been identified are an ensemble of molecules with slight molecular weight differences or the same molecular weight but different structures.

1. Introduction

Recombinant monoclonal antibodies (mAbs) are an important class of therapeutic proteins, which are heterogeneous in their biochemical and biophysical properties owing to multiple posttranslational modification and degradation events. These result in changes to molecular weight [1, 2], hydrophobicity [3-5] and structure [6], which have an impact on efficacy [7],

circulation half-life [8-10] and safety [11-14]. These changes can also affect the local distribution of charged residues or modify the acid dissociation constant (pKa), thus changing the overall surface-charge distribution of the antibody [15, 16]. Each type of charge variant is generally not a single component [17, 18] and may even differ in its primary structure. These differences are generally due to fragmentation, truncation [19-23], and incomplete processing of the signal peptide [24-27]. Similar to oxidation [28], isomerization [29] and deamidation, cysteinylation [30] is another possible modification, especially of the free cysteine residues in the antigen-binding fragment (Fab) region, which may lead to antibody inactivation [31]. Compared with the major constituents, species with a lower apparent isoelectric point (pI) are referred to as acidic species whereas those with a higher pI are classified as basic species. Normally, the presence of C-terminal lysine [32], N-terminal glutamine [33, 34], C-terminal amidation [35] and the formation of succinimide [36] from isomerization of aspartate residues are observed modifications that form basic variants. Methylglyoxal of arginine residues [37], deamidation of asparagine residues [38, 39], glycation, or the presence of sialic acid [40] and trisulfide bonds [41] may lead to acidic variants. Charge variants contribute different importance levels of critical quality attributes, which depend upon their effect on safety, immunogenicity, and efficacy. Normally, charge variants only observed in recombinant mAb but not in endogenous IgG molecules are considered to pose a higher risk of immunogenicity in patients [42, 43]. Therefore, both acidic and basic variants need to be characterized in detail, especially when they are unique to recombinant mAbs.

The main types of chromatographic and electrophoretic techniques commonly used to analyze charge variants are isoelectric focusing (IEF) gel electrophoresis, capillary zone electrophoresis (CZE) [44, 45], capillary isoelectric focusing electrophoresis (cIEF), imaged capillary isoelectric focusing (icIEF) [46, 47], free-flow electrophoresis (FFE) [48], and ion exchange chromatography (IEC) [49-51]. Good separation and intact molecular weight can usually be obtained using IEF, cIEF [52, 53], and especially two-dimensional capillary zone electrophoresis (2D CZE) coupled with a mass spectrometry detector [54], but it is difficult to collect sufficient material for detailed characterization. FFE is suitable for sample collection but separation cannot be monitored in real time. Weak cation-exchange chromatography (WCX) has become one of the most widely used methods as it allows for simultaneous separation, analysis, and sample preparation. In addition, high-resolution mass spectrometry coupled with ultra-

performance liquid chromatography (UPLC) can be used to identify the detailed structures of charge variants.

In this research, the acidic and basic species of a recombinant monoclonal antibody were collected from a WCX column and analyzed at multiple levels using icIEF, liquid chromatography–mass spectrometry (LC-MS), and liquid chromatography–tandem mass spectrometry (LC-MS/MS). N-terminal leader sequence in the heavy chain, cysteinylation, and dehydration of asparagine were the major modifications identified in the basic fractions. Asparagine deamidation and partial leader sequences in the light chain N-terminal were identified in the acidic variant. The modified proportions identified at peptide level and the purity detected by icIEF were compared, to determine how many modifications were identified in each variant.

2. Material and methods

2.1. Protein and reagents

Recombinant monoclonal antibody was produced in a stably transfected Chinese hamster ovary (CHO) cell line and purified using an Äkta avant system (GE Healthcare, Chicago, IL, USA). Trifluoroacetic acid (TFA), citric acid monohydrate, and Lys-C were purchased from Wako Chemicals (Richmond, VA, USA). Endoproteinase Glu-C, trypsin, and tris(hydroxymethyl)aminomethane (Tris) were purchased from Roche (Indianapolis, IN, USA). Formic acid, hydrochloric acid, dithiothreitol (DTT), sodium hydroxide, ammonium bicarbonate, iodoacetamide, ethylenediaminetetraacetic acid (EDTA), guanidine hydrochloride, urea, and 2-(N-Morpholino)ethanesulfonic acid (MES) anhydrous were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (LC-MS grade) and water (LC-MS grade) were purchased from Thermo Fisher Scientific (San Jose, CA, USA). PNGase F was obtained from New England Biolabs (Ipswich, MA, USA). Ultrafiltration centrifugal tubes with a molecular weight cutoff of 5 kDa were purchased from Sartorius (Goettingen, Germany).

2.2. PNGase F treatment and DTT reduction

A total 150 μg of each mAb was concentrated to greater than 15 mg/mL using ultrafiltration centrifugal tubes. The mAbs were then adjusted to a concentration of 1 mg/mL with 0.05 M

ammonium bicarbonate, pH 8. To release the N-glycans, 3 μL of PNGase F (75,000 units) was added to 150 μL of each sample, and the mixture was incubated for 6 h at 37°C to obtain the deglycosylated samples. After PNGase F treatment, 50 μL of each sample was removed and reduced using 1 μL of 1M DTT at 37°C for 60 min, for subunit molecular weight analysis.

2.3. Reduced Glu-C and Lys-C digestion

A total 200 μg of each variant in formulation buffer was mixed with a denaturing buffer consisting of 4.5 M guanidine hydrochloride, 0.05 M Tris, 0.005 M EDTA, 0.02 M DTT, pH 8.0, to a volume of 200 μL. The mixture was then incubated at 56°C for 1 h. After incubation, alkylation was performed using a final concentration 0.05 M IAM at 37°C for 1 h, protected from light. The samples were buffer exchanged into 1 M Tris, 1 M urea, pH 7.0, using ultrafiltration centrifuge tubes with a molecular weight cutoff of 5 KD. The samples were digested with Glu-C and Lys-C separately at 1:40 (w/w, enzyme:antibody) and incubated at 37°C for 16 h. After digestion, the samples were kept at −20°C for 1 h to stop the reaction.

2.4. Non-reduced Glu-C digestion and trypsin plus Lys-C digestion

A total 200 μg of each variant was buffer exchanged with 0.05 M citric acid, pH 6.0, and the sample solution was controlled to a final volume of 200 μL. For Glu-C digestion, the sample was then mixed with Glu-C at 1:40 (w/w, enzyme:antibody) and incubated at 37°C for 16 h. For trypsin plus Lys-C digestion, 2.5 μL of Lys-C was added to the sample solution, and the mixture was incubated at 37°C for 8 h; then, 2.5 μL of trypsin was added to the sample solution, which was then incubated at 37°C for another 8 h. Glu-C, trypsin, and Lys-C were all diluted to a concentration of 1.0 mg/mL in 0.05 M citric acid, pH 6.0. After digestion, the samples were kept at −20°C for 1 h to stop the reaction.

2.5. Weak cation-exchange chromatography

A Waters Alliance HPLC System (Waters Corporation, Milford, MA, USA) and a WCX column (WCX-10, 4.6 × 250 mm; Thermo Fisher Scientific, Sunnyvale, CA, USA) were used for starting material detection and variant preparation. Mobile phase A contained 20 mM MES, pH 5.6. Mobile phase B contained 20 mM MES and 0.5 M sodium chloride, pH 5.6. The antibody in low-salt buffer was loaded onto the column at 90% mobile phase A and 10% mobile phase B. The percentage of mobile phase B was increased to 20% over 30 min. The column was washed

using 100% mobile phase B for 9 minutes and then equilibrated using 10% mobile phase B for 20 minutes. Proteins eluted off the column were monitored with ultraviolet (UV) absorbance at 280 nm. The column was operated at 30°C with a flow rate of 1 mL/min. Fraction collection was guided by UV absorption. The collected fractions were concentrated using ultrafiltration centrifugal tubes with a molecular weight cutoff of 5 KD.

2.6. Imaged capillary isoelectric focusing

A Protein Simple iCE3 system (San Jose, CA) was used to analyze the mAb and WCX fractions. The samples were diluted to 0.25 mg/mL using IEF buffer containing methylcellulose (0.3%), 3-10 ampholytes (0.1%), and pI markers (0.15%). About 50 μL of the diluted samples were loaded into a capillary cartridge and prefocused for 1 min at 1500 V, followed by a hold at 3000 V for 6 min. The anolyte and catholyte electrolytic tanks at each end of the capillary cartridge were filled with 80 mM phosphoric acid in 0.1% methyl cellulose and 100 mM sodium hydroxide in 0.1% methyl cellulose, respectively. All of the above reagents are obtained from Protein Simple (Santa Clara, CA, USA). The main component and charge variants were focused to their respective pI values and then acquired using a whole-column UV detector at 280 nm.

2.7. LC-MS analysis of intact mAb, deglycosylated mAb, and subunits

An Evion LC 30AD system coupled with a TripleTOF 6600 mass spectrometer (AB Sciex, Framingham, MA, USA) and a Waters ACQUITY UPLC Protein BEH C4 (300 Å, 1.7 μm, 2.1 mm × 150 mm) column were used to measure the molecular weight of the antibody. Throughout the analysis, the flow rate was set at 0.3 mL/min and the column temperature was set at 70°C. For intact molecular weight analysis, 5 μL of each sample was injected into the column at 97% mobile phase A (0.1% FA in LC-MS grade water) and 3% mobile phase B (0.1% FA in acetonitrile). After running 3% mobile phase B for 4 min, mobile phase B was increased to 90% for 5 min, the column was washed using 90% mobile phase B for 0.5 min, and then equilibrated for 3 min using the initial condition.

The mass spectrometer was operated in positive mode with a scan range of m/z 350−4000. Both ion source gas 1 and ion source gas 2 were set to 50 psi, the curtain gas was set to 35, the ion spray voltage floating was set to 5500 V, declustering potential was set to 150 V, and the

temperature of the heater gas was set to 550°C. Spectra consisting of multiple charged protein ions were deconvoluted using PeakView 2.2 software.

2.8. LC-MS analysis of peptides

The TripleTOF 6600 mass spectrometer and Evion LC 30AD system coupled with a Waters ACQUITY UPLC Peptide BEH C18 (300 Å, 1.7 μm, 2.1 mm × 150 mm) column was used to analyze the charge variant digestions. The column was operated at 40°C with a flow rate of 0.25 mL/min. The mass spectrometer was operated in positive mode with a full scan range of m/z 350−4000 and an MS/MS scan range of m/z 50−1600. Both ion source gas 1 and ion source gas 2 were set to 50 psi, the curtain gas was set to 35, the ionspray voltage floating was set to 5500 V, declustering potential was set to 80 V, and the temperature of the heater gas was set to 550°C.

All fraction digests were analyzed with ultrapure water containing 0.05% TFA as solvent A and acetonitrile containing 0.05% TFA as solvent B. All the reduced Lys-C digests and unreduced trypsin plus Lys-C digests were separated using the following gradient: 0 min, 0% B; 2 min, 0% B; 72 min, 37% B; 72.5 min, 90% B; 74 min, 90% B; 74.5 min, 0% B; 80 min, 0% B. The reduced and non-reduced Glu-C digests were separated using the following gradient: 0 min, 1% B; 5 min, 1% B; 50 min, 16% B; 75 min, 30% B; 90 min, 55% B; 91 min, 90% B; 95 min, 90% B; 95.5 min, 1% B; 100 min, 1% B.

3. Results

3.1. Purity analysis of fractions using icIEF

The workflow of the study is shown in Fig. 1.

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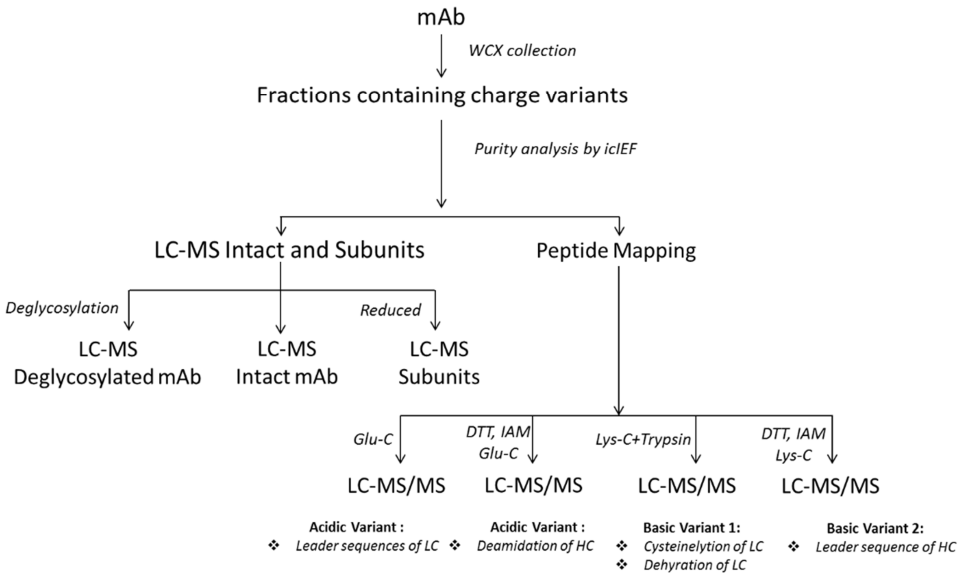


Fig. 1. Workflow of the experiments.

Charge variants of the mAb were separated into four fractions using WCX chromatography. These fractions were collected and designated as fractions A, B, C, and D, which corresponded to the acidic variant, main component, basic variant 1, and basic variant 2, respectively. The purity of each variant in the fractions was measured by icIEF (as shown in Fig. 2).

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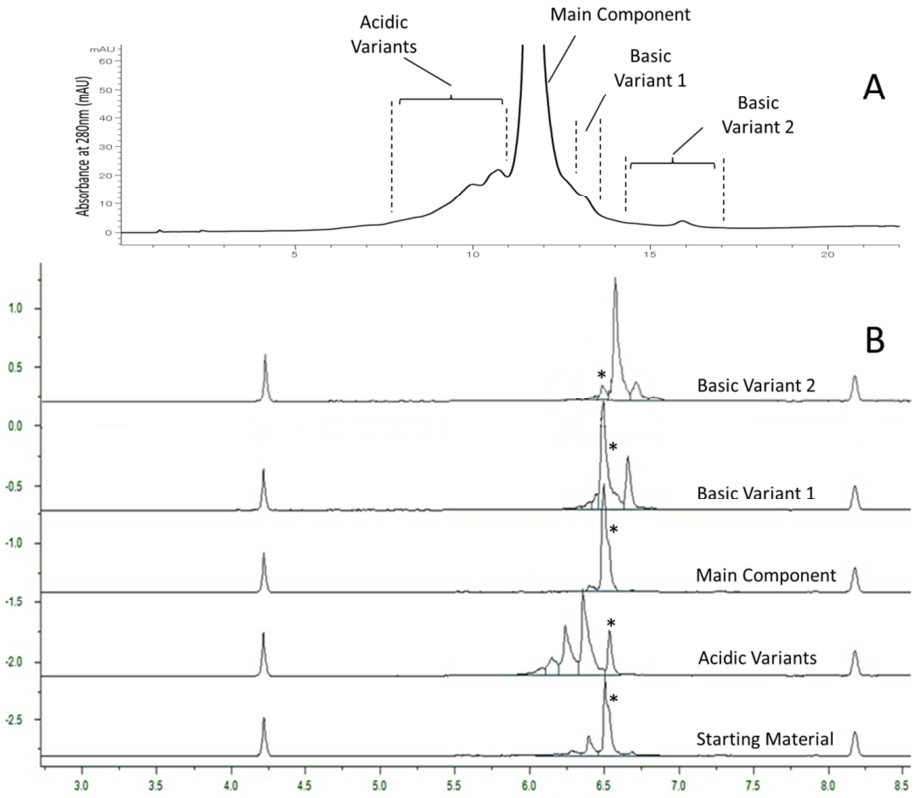


Fig. 2. WCX chromatogram of starting material and icIEF analysis of fractions. A: WCX chromatogram of starting material and isolated fractions. The chromatogram was obtained by injection of 100 μg of mAb. Fraction collection was guided by UV absorption, as labeled in the figure. B: icIEF analysis of starting material and WCX fractions. The main components in the figure are marked with asterisks.

In agreement with the WCX chromatography, fraction B appeared as a major peak on icIEF analysis of the starting material. Fraction A was classified as an acidic variant based on the pI values; fractions C and D were classified as basic variant 1 and basic variant 2, respectively. There were multiple peaks detected in fraction A, and the purity of the acidic species was about 87%. The purity of the main compound in fraction B was estimated to be 93%, and 6% of acidic species were included due to incomplete separation. The main compound was the major component of fraction C (about 59%), and the peak area percentage of basic variant 1 was about 41%. The purity of basic variant 2 in fraction D was estimated to be 70%. Good separation was obtained between the main compound and basic variant 2 on WCX-10 chromatography (Fig. 2A), but their pIs on icIEF analysis were very similar (Fig. 2B); the likely reason is that the distribution of charge affects the interaction of mAbs with column resins.

3.2. Molecular weight analysis of intact mAbs and deglycosylated mAbs

Intact molecular weights of the antibody in each fraction were analyzed using LC-MS to detect modifications with substantial molecular weight differences, including cysteineylation of unpaired cysteine, dehydration of aspartic acid [55], and the presence of a portion of the leader sequence. As shown in Fig. 3, the main peak molecular weight from analysis of the starting material is 147789.2 Da, which corresponds to antibody with G0F on both heavy chains. The peak with molecular weight 147643.3 Da corresponds to the same species, but with G0 instead of G0F. Molecular weight 147951.4 Da, approximately 162 Da higher than the main peak, corresponds to antibody with one heavy chain with G0F and the other heavy chain with G1F. The peak with molecular weight 148115.4 Da, approximately 326 Da higher than the main peak, corresponds to antibody with one heavy chain with G0F and the other heavy chain with G2F, or with G1F on both heavy chains. The peak with molecular weight 148071.5 Da is approximately 282 Da higher than the main peak and corresponds to antibody with partial leader sequence 1 (the amino acid sequence is SEA). Signal with molecular weight 148570.2 Da is approximately 781 Da higher than the main peak, which corresponds to antibody with partial leader sequence 2 (the amino acid sequence is LCTGSEA), and the cysteine residues of leader sequence 2 are cysteinylated. Molecular weight 147906.3 Da, approximately 119 Da higher than the main peak, corresponds to antibody with cysteinylation modification.

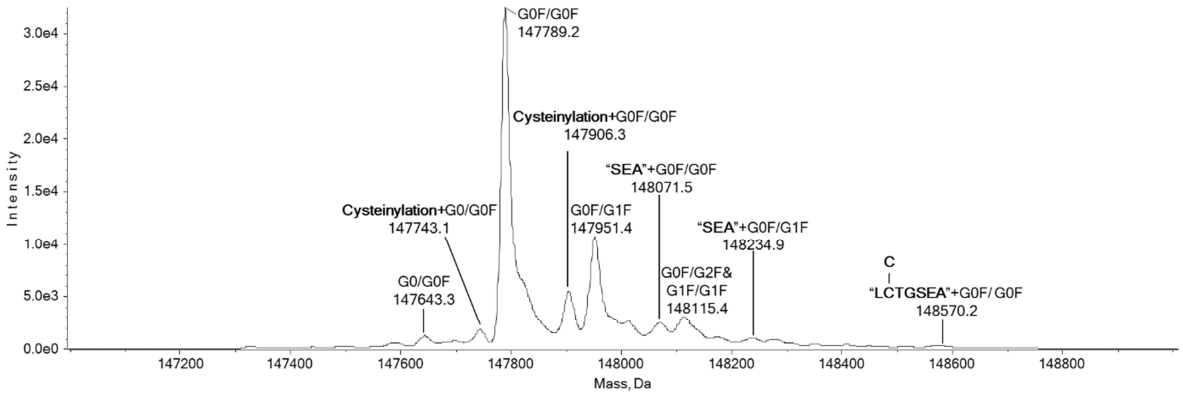


Fig. 3. Deconvoluted mass spectra from analysis of the antibody starting material.

Mass spectra from analysis of the intact antibody molecular weights are shown in Fig. 4. Peak assignments are based on the common modifications of recombinant mAbs reported in the literature and the observed molecular weights of each peak. Comparison of the molecular

weights of the intact mAbs and deglycosylated mAbs was used to find the differences in several charge variants, and to judge whether the differences resulted from glycosylation.

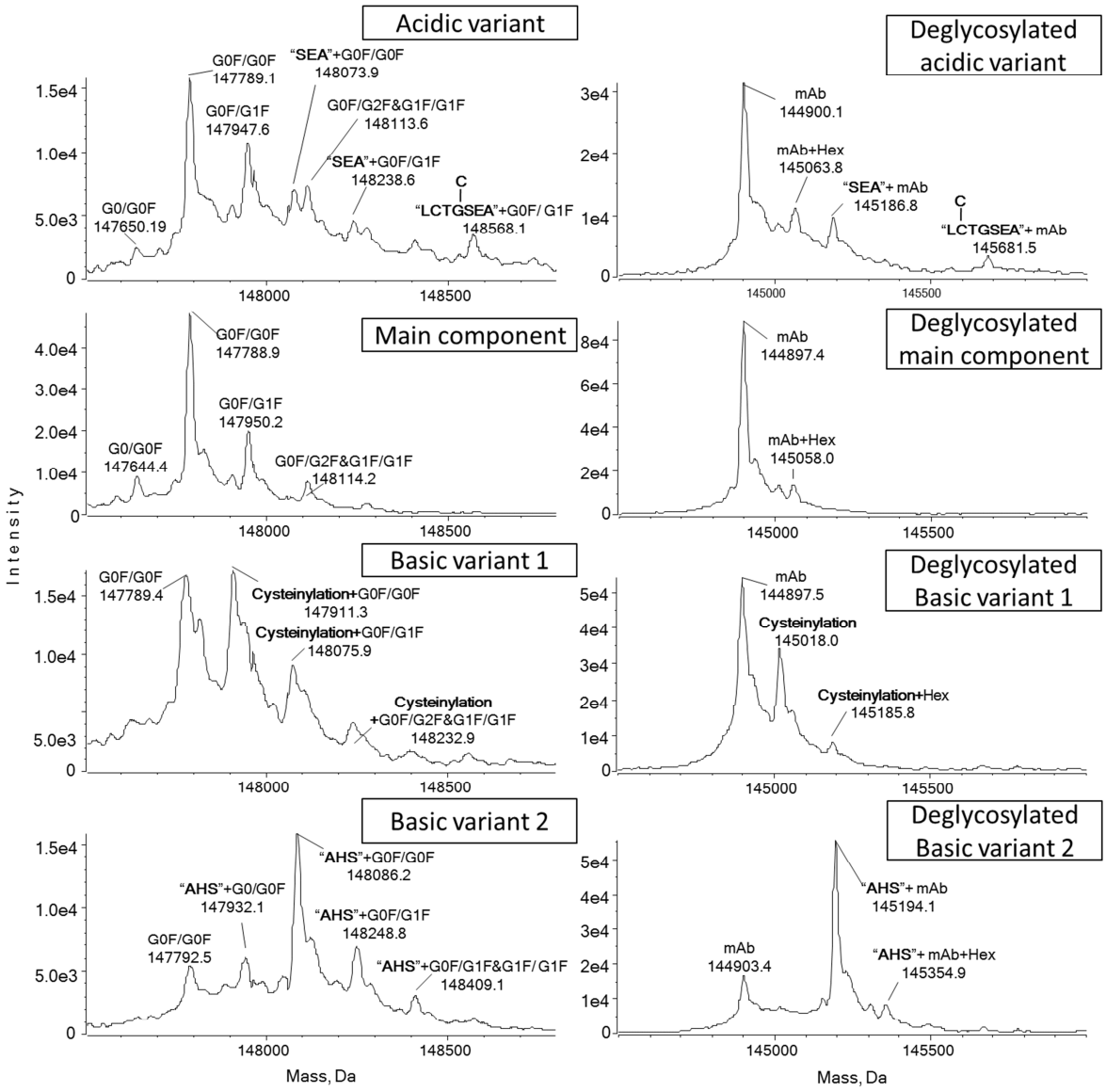


Fig. 4. Deconvoluted mass spectra from analysis of the variants (main component) and deglycosylated variants (main component).

The main peak molecular weight from analysis of the main compound is 147788.9 Da, which corresponds to antibody with G0F on both heavy chains. The peak with molecular weight 147644.4 Da, approximately 145 Da lower than the main peak, corresponds to the same species as the main peak but with G0 on one of the heavy chains (G0F lacking a fucose residue). The

molecular weights of 147950.2 Da and 148 114.2 Da correspond to the main component with different glycosylations. After deglycosylation, all molecular weights of the main component with different glycosylations changed to 144897.4 Da.

Similar to the molecular weights shown in the deconvoluted mass spectra of the acidic variant, signals of 147789.1 Da, 147947.6 Da, and 148113.6 Da of fraction A correspond to the molecular weights of the main peak with different glycosylations. The peak with molecular weight 148073.9 Da is approximately 285 Da higher than the main peak and corresponds to the antibody with partial leader sequence 1 (amino acid sequence SEA). Signals of 148398.0 Da and 148233.6 Da correspond to antibody with different glycosylations. Another obvious signal, with molecular weight 148567.5 Da, which is 778.4 Da higher than the main peak, corresponds to antibody with partial leader sequence 2 (LCTGSEA) on one light chain, and the cysteine residues of leader sequence 2 are cysteinylated. The molecular weight measurement of deglycosylated charge variants showed clearer signal as 145186.8 Da (+289 Da, SEA) and 145681.5 Da (+783 Da, LCTGSEA, cysteinylated).

Similar to the molecular weight measurement shown in the deconvoluted mass spectra of basic variant 1, signal of 147789.4 Da corresponds to the molecular weight of the main peak. Molecular weight 147911.3 Da, which is approximately 121 Da higher than the main peak, corresponds to antibody with cysteinylation of a free sulfhydryl. Correspondingly, signals of 148080.6 Da and 148398.0 Da are considered to be cysteinylated antibody with different glycosylations. Mass spectra of deglycosylated basic variant 1 also display a peak of 145018.0 Da, which is approximately 119 Da higher than the main peak (144897.5 Da), also providing strong evidence for the existence of cysteinylation.

The molecular weight measurement of basic variant 2 shown in Fig. 4 has a signal of 148086.2 Da, which is approximately 297 Da higher than the main peak, corresponding to leader sequence 3 (amino acid sequence AHS). After deglycosylation with PNGase F, a significant signal of 145194.1 Da was observed, which confirmed the previous speculation. This observation was expected because antibody variants with leader sequence 3 are more basic (due to the existence of histidine) and should be enriched in basic fractions.

3.3. Molecular weight analysis of subunits

Deconvoluted mass spectra of the subunits with molecular weights of the main component and variants are shown in Fig. 5.

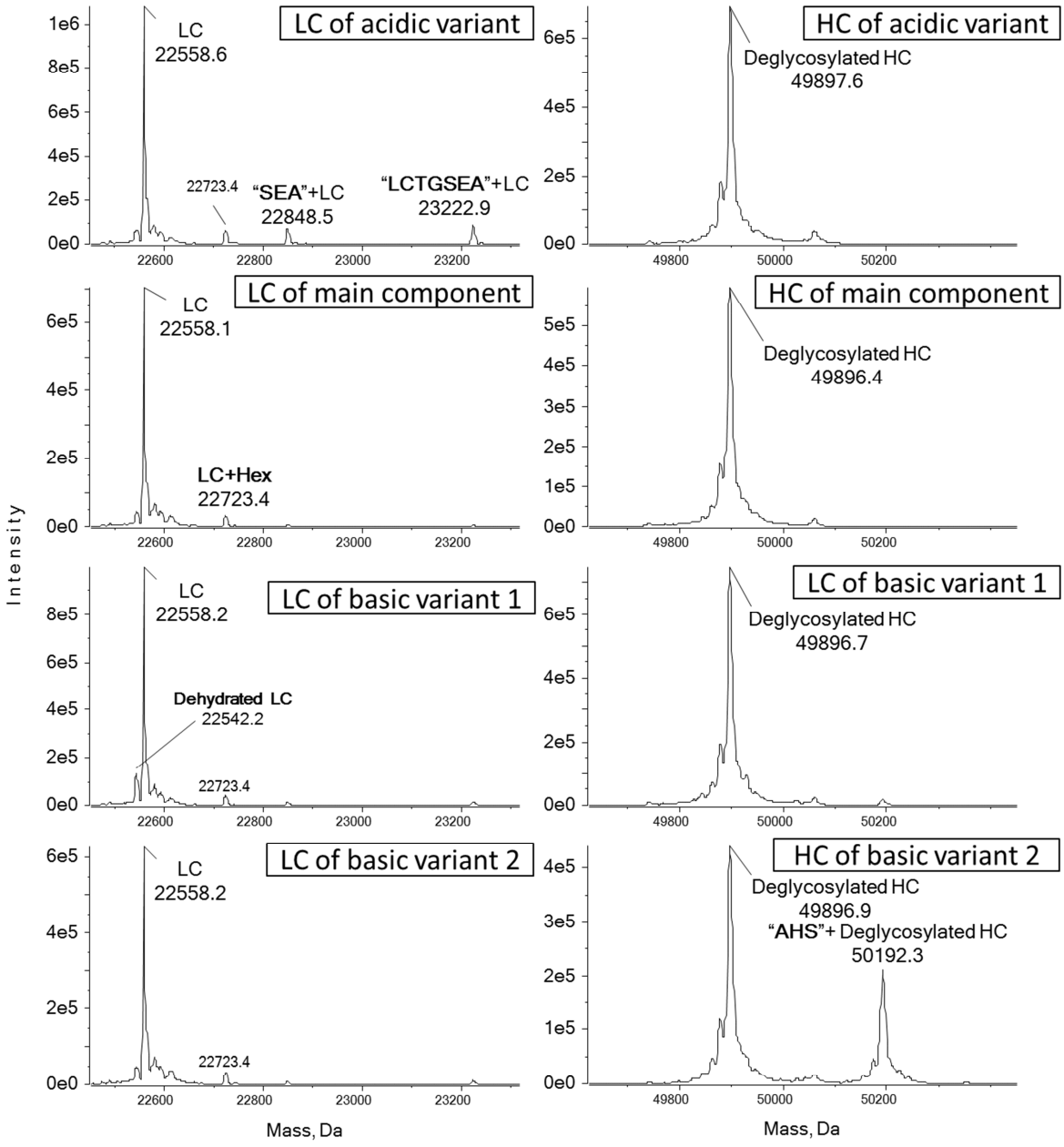


Fig. 5. Deconvoluted mass spectra from analysis of the deglycosylated subunits of variants and main component. Analysis of the subunits is indicated in each spectrum.

Several peaks with the same molecular weights were observed in all fractions. For the main component, molecular weight 22558.1 Da corresponds to the light chain, and 49896.4 Da

corresponds to the deglycosylated heavy chain. For the acidic variant, after treatment with DTT, the light chain shows obvious signals at 22848.5 Da and 23222.9 Da; the former corresponds to the light chain with leader sequence 1 (+287.7 Da, SEA), and the latter corresponds to the light chain with leader sequence 2 (+664.2 Da, LCTGSEA). It is worth noting that the molecular weight difference of leader sequence 2 is no longer 783 Da because the cysteinylation of leader sequence 2 was reduced by DTT. Obviously, leader sequence 1 and leader sequence 2 of the acidic variant all exist in the light chain.

In the mass spectra of basic variant 1, as expected, the signal of cysteinylation (molecular weight difference of 119 Da) no longer existed in basic variant 1 due to DTT treatment. Also, the light chain-related signal of 22542.2 Da increased slightly, showing a 16-Da decrease in molecular weight compared with the main component. This observation suggests that some types of modifications, such as dehydration of asparagine, may be present [54] and shift the mAbs to the basic variant formation.

For basic variant 2, the mass spectra showed a very obvious signal of 50192.3 Da, which corresponds to leader sequence 3 (with amino acid sequence AHS). This observation also localized leader sequence 3 to the heavy chain.

The glycation product of the light chain was also found in all four fractions by molecular weight analysis of the subunits. Because the proportion of glycation in the four fractions was nearly the same (about 1.5%, data not shown), this suggests that glycation is ubiquitous in the antibody. Therefore, glycation is not considered the main reason for charge heterogeneity of the antibody.

3.4. Peptide mapping

The collected fractions were further analyzed by LC-MS peptide mapping with and without reduction, to identify the modifications in greater detail. Suitable enzymes and conditions were selected to analyze the corresponding modifications in the fractions.

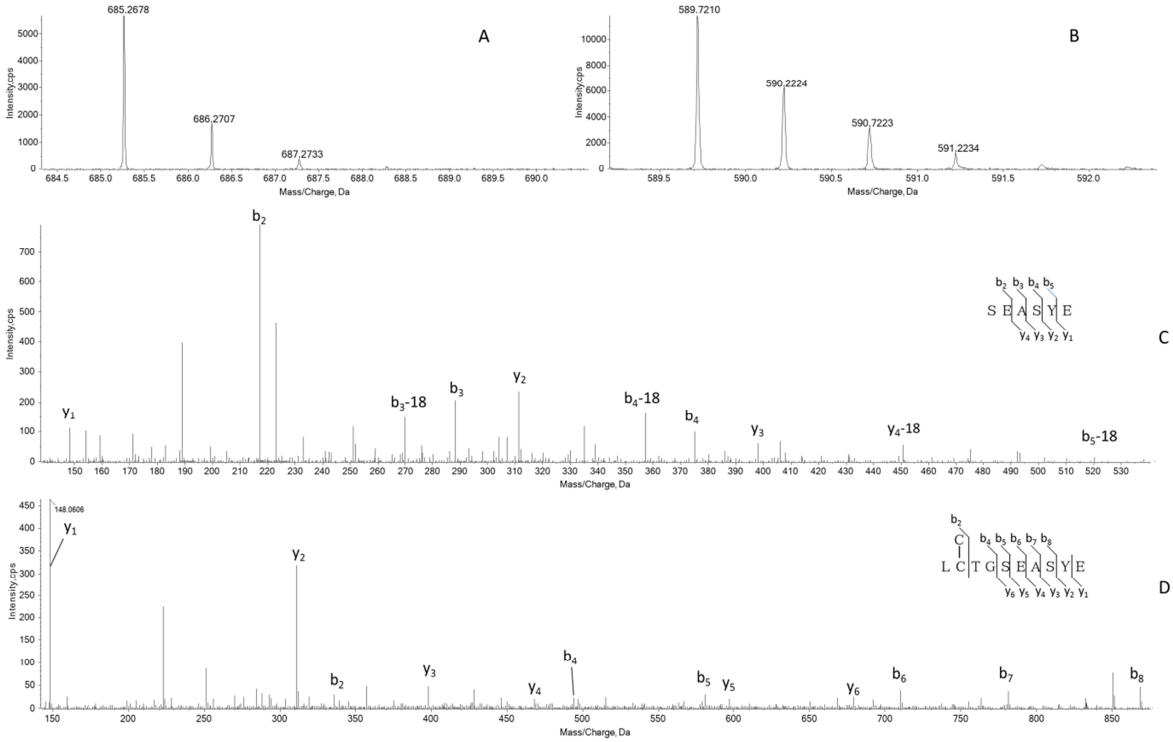


Fig. 6. MS and MS/MS spectra of the peptides containing light chain leader sequences. The amino acid sequences of the two peptides are shown on each MS/MS spectrum. (A) Full scan mass spectrum of the singly charged peptide containing N-terminal leader sequence 1 "SEA". (B) Full scan mass spectrum of the doubly charged peptide containing N-terminal leader sequence 2 "LCTGSEA" (cysteinylated). (C) MS/MS spectrum of the peptide containing N-terminal leader sequence 1. (D) MS/MS spectrum of the peptide containing N-terminal leader sequence 2. The y and b ions from the amino acid sequence are labeled in each MS/MS spectrum. Matching of the major fragment ions confirmed the identity of the peptides.

In the earlier investigation of the acidic variant described above, the leader sequences were confirmed to be associated with the N-terminal of the light chain. In non-reduced Glu-C peptide mapping, the two leader sequences were found. As shown in Fig. 6A, an N-terminal peptide of the light chain with amino acid sequence SEASYE (with a single miscleavage at the first glutamate) was identified. This peptide is associated with leader sequence 1 and has a single charged precursor ion of m/z 685.2678. The MS/MS data of this precursor are in good agreement with the theoretical values (as shown in Fig. 6C). The N-terminal peptide associated with leader sequence 2 shows a doubly charged precursor ion of m/z 589.7210 (Fig. 6B), corresponding to amino acid sequence LCTGSEASYE. It is worth mentioning that this peptide is cysteinylated at

the cysteine of leader sequence 2, the b2, and b4 to b8 ions of the peptide (refer to Fig. 6D) confirmed this conclusion. Glutamic acid is included in both leader sequences in the acidic variant that shifted the antibody to the acidic region of the WCX chromatogram.

In addition to leader sequences, deamidation of asparagine was also identified in the acidic species, in reduced Glu-C peptide mapping. As shown in Fig. 7, the Glu-C peptide with amino acid sequence ALHNHYTQKSLSLSLG was found to be deamided at the 442th amino acid of the heavy chain. The observed doubly charged precursor ions of m/z 885.4676 (Fig. 7A) and m/z 884.9802 (Fig. 7B) are in good agreement with the theoretical Glu-C peptide monoisotopic molecular weights of 1768.9181 Da for the peptide with deamidation and 1767.9341 Da for the peptide without deamidation. The observed MS/MS ions of the peptides also match well with the theoretical values, as shown in Fig. 7C and 7D.

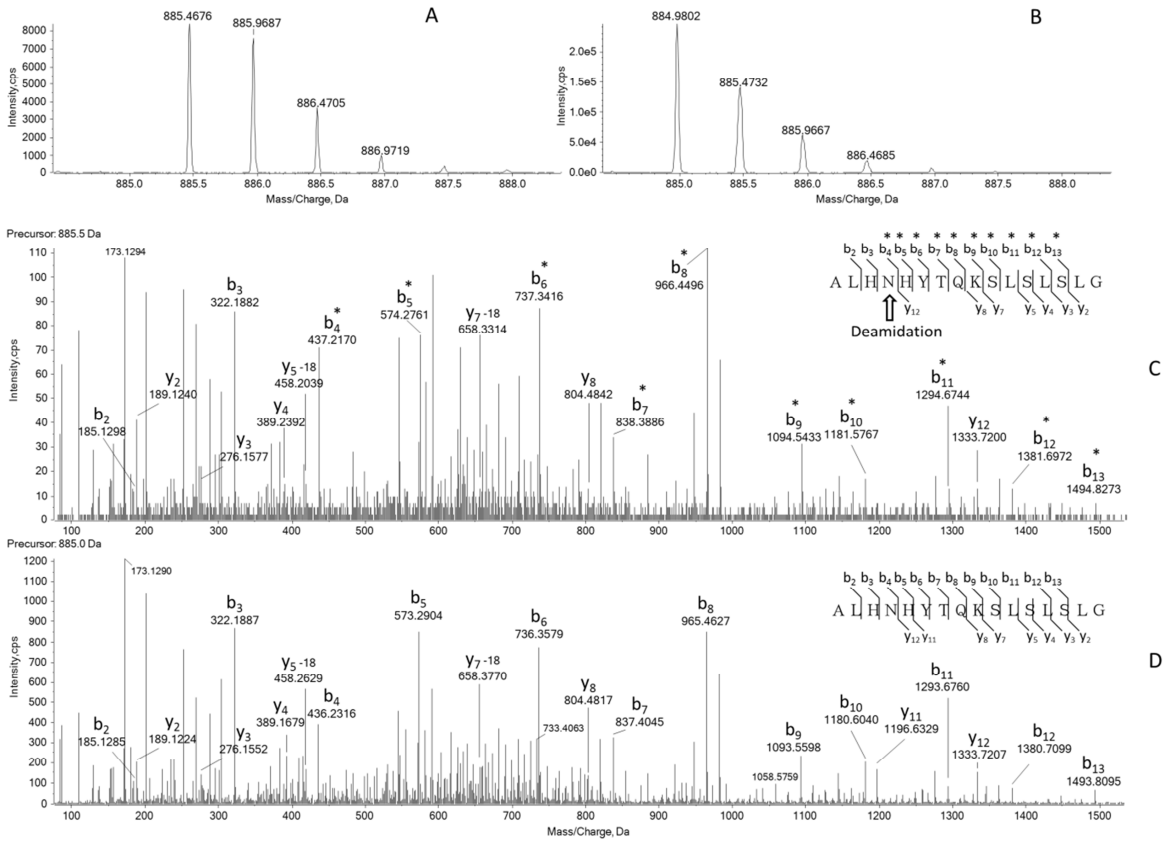


Fig. 7. MS and MS/MS spectra of the peptide of HC439-454 and the corresponding deamidated peptide. The amino acid sequences are shown on each MS/MS spectrum. (A) Full scan mass spectrum of the doubly charged deamidated peptide HC439-454. (B) Full scan mass spectrum of the doubly charged

peptide HC439-454. (C) MS/MS spectrum of the peptide HC439-454, which is deamidated at asparagine 442. (D) MS/MS spectrum of the peptide without deamidation. The y and b ions from the amino acid sequence were labeled in each MS/MS spectrum. Fragment ions containing deamided sites are marked with asterisks. Matching of the major fragment ions confirmed the deamided sites of the peptide.

Most of fraction B was demonstrated to be the main component without any modifications. A small amount of N-terminal leader sequence of the light chain and deamidation of the heavy chain were detected in the main component, which is owing to the existence of the acidic variant.

Basic variant 1 was confirmed to be associated with cysteinylation of the unpaired cysteine in the light chain; the evidence was found on non-reduced trypsin/Lys-C peptide mapping. The cysteinylated peptide was 31-52 of the light chain with amino acid sequence FACWYQQKPGQSPVLVIYQDTK; the modified site was the only cysteine at amino acid serial 33 of the light chain, as shown in Fig. 8.

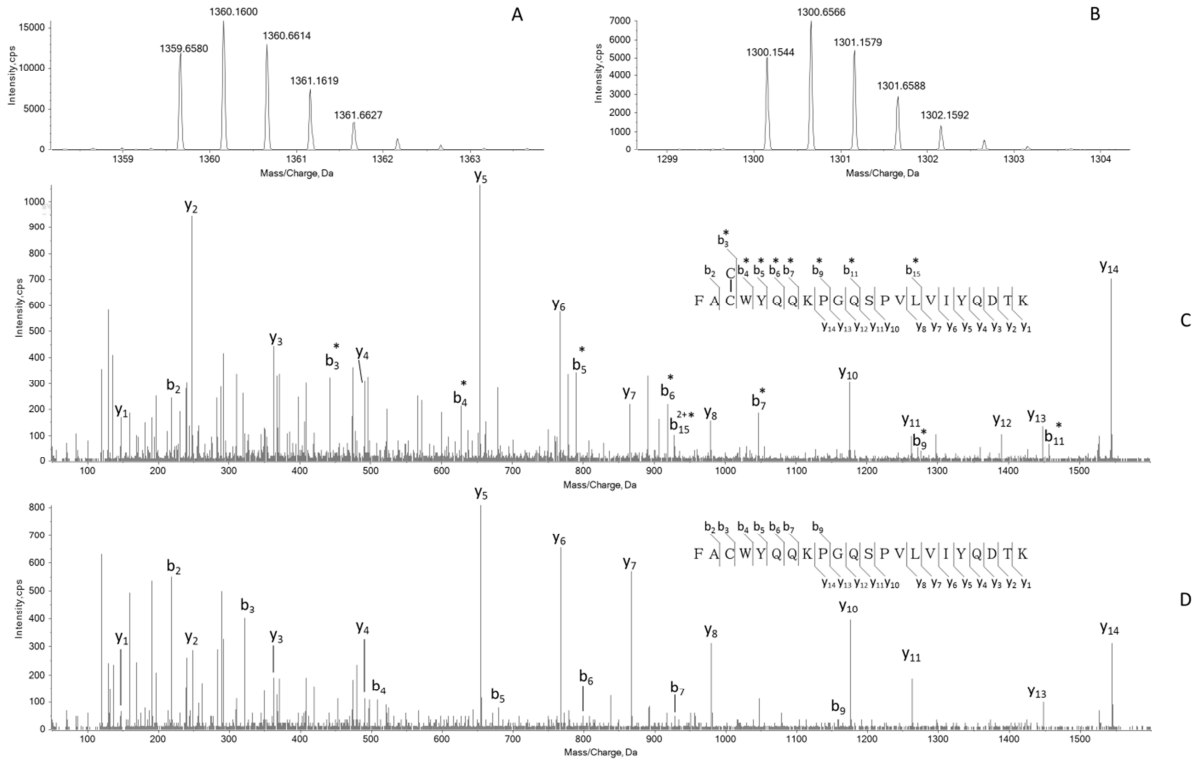


Fig. 8. MS and MS/MS spectra of the peptide LC 31-52 and the corresponding cysteinylated peptide. The amino acid sequences are shown on each MS/MS spectrum. (A) Full scan mass spectrum of the doubly charged cysteinylated peptide LC 31-52. (B) Full scan mass spectrum of the doubly charged peptide LC 31-52. (C) MS/MS spectrum of the peptide LC 31-52, which is cysteinylated at cysteine 33. (D) MS/MS

spectrum of the peptide without cysteinylation. The y and b ions from the amino acid sequence are labeled in each MS/MS spectrum. Fragment ions containing cysteinylated sites are marked with asterisks. Matching of the major fragment ions confirmed the cysteinylated sites of the peptide.

The observed doubly charged precursor ions of m/z 1359.6580 Da (Fig. 8A) and m/z 1300.1544 Da (Fig. 8B) are in good agreement with the theoretical molecular weights of 2717.2982 Da for the peptide with cysteinylation [29] and 2598.2941 Da for the peptide without cysteinylation. The observed MS/MS ions of the peptides also match well with the theoretical values, as shown in Fig. 8C (cysteinylated) and Fig. 8D (non-cysteinylated). The b ions with a "*" marker in the figure represent ions with cysteinylation.

In addition to cysteinylation, aspartic acid dehydration of the light chain was found in basic variant 1. As shown in Fig. 9.

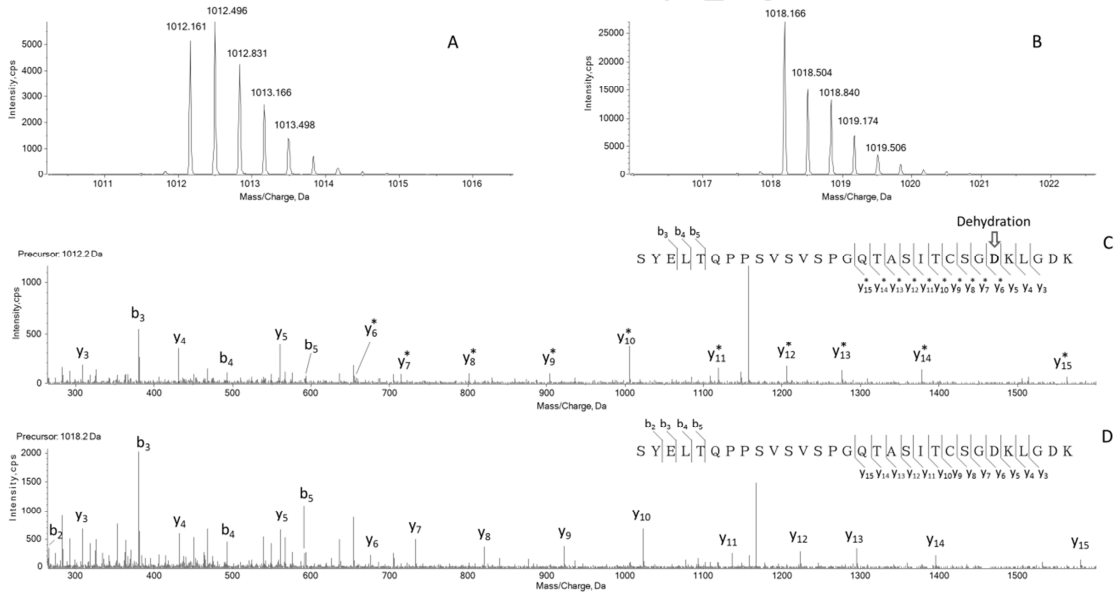


Fig. 9. MS and MS/MS spectra of the peptide LC 1-30 and the corresponding dehydrated peptide. The amino acid sequences are shown on each MS/MS spectrum. (A) Full scan mass spectrum of the triply charged dehydrated peptide LC 1-30. (B) Full scan mass spectrum of the triply charged peptide LC 1-30. (C) MS/MS spectrum of the peptide LC 1-30, which is dehydrated at aspartic acid 25. (D) MS/MS spectrum of the peptide without dehydration. The y and b ions from the amino acid sequence are labeled in each MS/MS spectrum. Fragment ions containing dehydrated sites are marked with asterisks. Matching of the major fragment ions confirmed the dehydrated sites of the peptide.

The observed triply charged precursor ions of m/z 1012.1612 Da (Fig. 9A) and m/z 1018.1661 Da (Fig. 9B) are in good agreement with the theoretical molecular weights of 3033.4836 Da for the peptide LC 1-30 (amino acid sequence SYELTQPPSVSVSPGQTASITCSGDKLGDK) without dehydration, and 3051.4983 Da for the peptide with dehydration at aspartic acid 25.

Reduced Lys-C peptide mapping of basic variant 2 (fraction D) showed obvious signal as a doubly charged precursor ion of m/z 805.4350 (as shown in Fig. 10A). The observed molecular weight of 1608.8700 Da, calculated based on the doubly charged ion, is in good agreement with the theoretical molecular weight of 1608.8544 Da. This peptide is approximately 295 Da higher than the N-terminal peptide of the heavy chain and corresponds to the N-terminal peptide with leader sequence 3 (amino acid sequence AHSEVQLVESGGGLVK). The correct sequence was also confirmed by MS/MS experiments (Fig. 10B), in which multiple b and y ions were observed, corresponding to that are predicted from the amino acid sequence. The presence of histidine in leader sequence 3 contributed to the basic variants of the mAb.

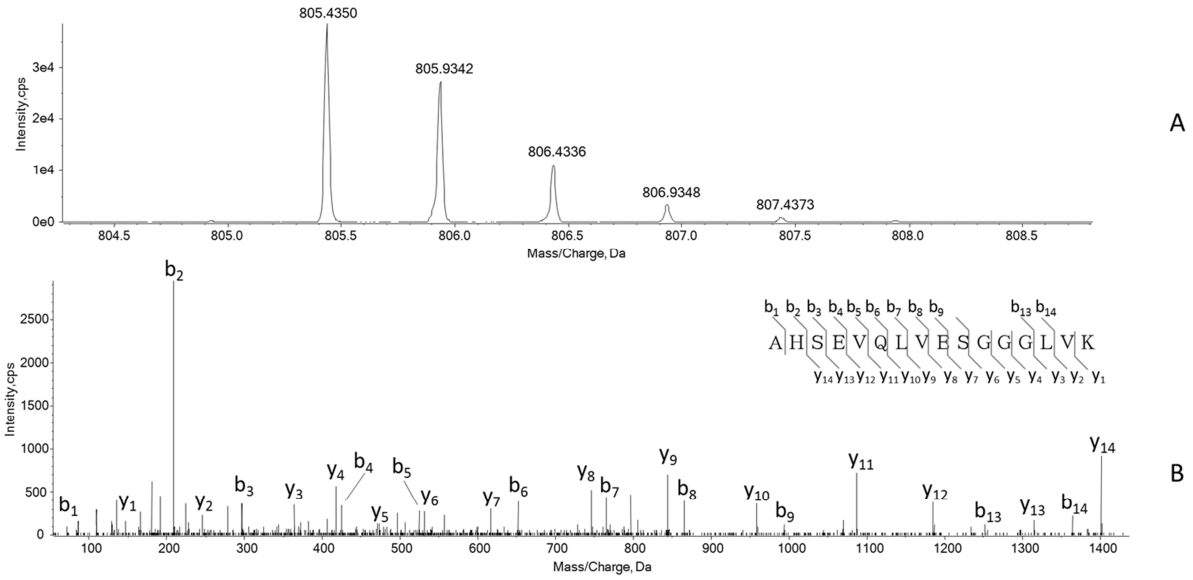


Fig. 10. MS and MS/MS spectra of the peptide containing a portion of leader sequence 3. The amino acid sequence of this peptide is shown on the MS/MS spectra. (A) Full scan mass spectrum of the doubly charged peptide. (B) MS/MS spectrum. Matching of the major fragment ions in the spectrum to the predicted y and b ions from the amino acid sequence confirmed the identity of this peptide.

Proportions of the modified component were calculated based on the peak areas of each modified and unmodified peptide; the results are summarized in Table 1.

Table 1. Identities of WCX fractions.

Fraction/Variant	Purity analysis by icIEF	Modification type	Modified site	Modified proportion[§]
Fraction A (Acidic variant)	87.0%	Leader sequence 1[a]	N-terminal of LC[*]	6.4%
		Leader sequence 2[b]	N-terminal of LC[*]	8.4%
		Deamidation	HC[#]442(Asn)	18.0%
Fraction B (Main component)	93%	Leader sequence 1[a]	N-terminal of LC[*]	0.2%
		Leader sequence 2[b]	N-terminal of LC[*]	0.3%
		Deamidation	HC[#]442(Asn)	5.1%
Fraction C (Basic variant 1)	41%	Cysteinylation	LC[*]33 (Cys)	25.6%
		Dehydration	LC[*]25(Asp)	17.8%
Fraction D (Basic variant 2)	70%	Leader sequence 3[c]	N-terminal of HC[#]	67.3%

*: LC = light chain; #: HC = heavy chain.

a: leader sequence 1 = N-terminal leader sequence of the light chain with amino acid sequence "SEA".

b: leader sequence 2 = N-terminal leader sequence of the light chain with amino acid sequence "LCTGSEA"; the cysteine in this sequence was cysteinylated.

c: leader sequence 3 = N-terminal leader sequence of the heavy chain with amino acid sequence "AHS".

§: Because the modifications identified were all singly modified and antibody molecules consist of two heavy chains and two light chains, the identified proportion in Table 1 is twice as large as the one calculated in the peptide mapping.

As shown in Table 1, basic variant 1 and basic variant 2 fully accounted for the cysteinylation, dehydration, and N-terminal leader sequence of the heavy chain. A portion of the acidic variant can be explained by N-terminal leader sequences of the light chain and deamidation of asparagine in the heavy chain. Approximately 54.2% of the acidic variant still cannot be explained.

An experiment involving 2-AB labeled N-glycans was also carried out and the analysis showed no difference among the fractions (data not shown). The study findings indicate that charge variants of the mAbs do not result from glycosylation.

4. Discussion

Charge variants of mAbs were first separated by WCX chromatography and then desalted and enriched using ultrafiltration centrifugal tubes. The purity of each charge variant was measured by icIEF. From the molecular weight analysis of intact mAbs and subunits, large differences were found between the charge variants and main peaks. These differences suggest that there are leader sequences in the light chain of the acidic variant and the heavy chain of basic variant 2. The presence of N-terminal leader sequences in the light chain were further demonstrated by peptide mapping. Compared with several known leader sequences [4, 16, 26], the N-terminal leader sequence with the amino acid sequence LCTGSEA is a rare signal peptide that consists of both acidic amino acid and cysteinylation. Whether the presence of this signal peptide in the light chain affects the safety of recombinant mAb needs to be further researched. The N-terminal leader sequence of the heavy chain with the amino acid sequence AHS was the only reason for formulation of basic variant 2; a similar situation has been reported by Neill et al. [16]. Moreover, deamidation of the acidic variant, dehydration, and cysteinylation of basic variant 1 were also identified by peptide mapping. As reported in the literature, a low level of cysteinylation is common when free cysteine is present in the cell culture media [56]. Cation exchange chromatograms of cysteinylated and de-cysteinylated mAb were compared in a previous study [31], and the results showed that the cysteinylated mAbs were eluted in the acidic region. In the present work, cysteinylated mAb was enriched in basic variant. An alternative explanation is that cysteinylation induced structural change and changed the surface charge of the mAb; this change has a certain degree of randomness, so it shifts to the acidic region in some cases and to basic region in other cases.

The percentages of the modified component were calculated based on the peak area of the extracted ion chromatogram of each modified peptide. As a result, basic variant 1 and basic variant 2 were almost fully accounted for. The main component was demonstrated to be without any leader sequence or other modifications. We think it is owing to the existence of acidic variant that a small amount of N-terminal leader sequence and deamidation were detected in the main component. The icIEF analysis shows that the acidic variant consists of at least three components with different pI values. We were unable to obtain each component of the acidic variant due to the low content of acidic variant in the starting material and the limitations of the separation methods. For these reasons, about 54% of the acidic variant still cannot be explained. We hypothesize that acidic variant is an ensemble of molecules with minimal or no molecular

weight differences, such as molecules with disulfide-bond scrambling, unpaired cysteine, or other multiple sources of modification.

5. Conclusions

In this work, WCX chromatography, icIEF, and mass spectrometry were used to characterize charge variants at different levels. The main component was demonstrated to be mAb without any leader sequence or other modifications. Leader sequences of light chain and aspartic acid deamidation of the heavy chain constitute about 32.8% of the acid variant. Basic variant 1 was fully accounted for by cysteinylation and dehydration of aspartic acid. Basic variant 2 was identified as molecular with basic leader sequence at the N-terminal of the heavy chain.

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Highlights:

Leader sequences were found to cause acidic variant of monoclonal antibody.

Cysteinylation was identified in basic variant of monoclonal antibody.

The combination of multiple methods helps to analyze charge variants.

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