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Structure-Function Assessment and High-Throughput Quantification of Site-Specific Aspartate Isomerization in Monoclonal Antibody Using a Novel Analytical Tool Kit

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ABSTRACT

Isomerization of surface-exposed aspartic acid (Asp) in the complementarity-determining regions of therapeutic proteins could potentially impact their target binding affinity because of the sensitive location, and often requires complex analytical tactics to understand its effect on structure-function and stability. Inaccurate quantitation of Asp-isomerized variants, especially the succinimide intermediate, presents major challenge in understanding Asp degradation kinetics, its stability, and consequently establishing a robust control strategy. As a practical solution to this problem, a comprehensive analytical tool kit has been developed, which provides a solution to fully characterize and accurately quantify the Asp-related product variants. The toolkit offers a combination of 2 steps, an ion-exchange chromatography method to separate and enrich the isomerized variants in the folded structure for structure-function evaluation and a novel focused peptide mapping method to quantify the individual complementarity-determining region isomerization components including the unmodified Asp, succinimide, and isoaspartate. This novel procedure allowed an accurate quantification of each Asp-related variant and a comprehensive assessment of the functional impact of Asp isomerization, which ultimately helped to establish an appropriate control strategy for this critical quality attribute.

Introduction

Since human insulin was launched as the first recombinant protein therapeutic in the 1980s, protein therapeutics have emerged as an important segment in the biomedical and health care industry.[1] Recent FDA approvals of antibodies targeting immune checkpoints such as cytotoxic T-lymphocyte antigen 4 and programmed cell-death protein 1, and their breakthrough outcomes in cancer treatment further boosted the development of protein therapeutic drugs.[2,3] Compared to small molecules, development of these biological macromolecules brings unique challenges in the chemistry, manufacturing, and control space, where biochemical and biophysical liabilities of these large molecules are often complex due to their large size and high degree of heterogeneity.[4] Therefore, comprehensive characterization and appropriate control of these biomolecules during manufacturing, storage, transportation, and administration are critical to ensure consistent safety and efficacy profiles.

Among many potential post-translational modifications (PTMs) that may alter protein's structure and function,[5-7] isomerization of the aspartic acid (Asp) residue can be a significant concern, especially when the modification occurs in the complementarity-determining regions (CDRs) of antibody molecules that are directly involved in antigen-binding. The modification not only may lead to loss of potency[8-12] but may also trigger immune response.[13,14] The Asp/isoAsp isomerization process is nonenzymatic, which involves Asp converting to cyclic imide intermediate (Asu), followed by maleimide ring opening to revert to Asp or to generate its isoform isoAsp (Fig. 1). The intrinsic properties of protein, such as primary amino acid sequence, are a key factor in isomerization.[15-17] For example, it has been shown that the Asp-Gly (DG) motif is highly prone to isomerization.[8,18,19] Other factors including solvent dielectric constants, pH, and temperature have been demonstrated

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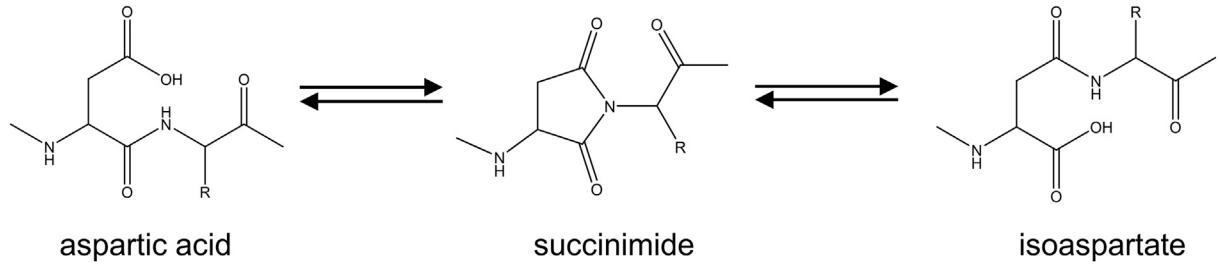

Figure 1. A schematic of spontaneous conversion of peptide sequence containing aspartic acid to isoaspartate through the formation of a cyclic amide intermediate (succinimide, Asu).

to influence the isomerization kinetics, presumably due to their effect on protein conformation as well as local environment related to the specific Asp residue.[18,20-24]

To ensure consistent efficacy and safety profiles of protein therapeutics during manufacturing and storage, the structure-function relationships and the degradation pathways of the protein need to be fully understood and appropriate control strategy must be established. For characterization, mass spectrometry (MS)-based multiattribute methods[25-28] and 2-dimensional liquid chromatography (2DLC)[29,30] with MS detection[31,32] may find more utility due to their efficiency in obtaining more information with a smaller set of analytical methods. However, orthogonal traditional approaches are still required in delivering the requisite data during characterization and release testing in quality control (QC) settings, which simultaneously deliver a more complete picture of the degradation pathways. Oftentimes, several analytical methods are necessary in these development studies, although fewer are used in QC settings such as Good Manufacturing Practice release and stability testing. It is not uncommon that the routinely used separation-based methods do not meet the requirements for enrichment of protein degradation products while preserving its folded protein structure. This issue arises due to the method protocol requirements of denaturation and major modifications made to the protein structure, such as the conditions often used in isoelectric focusing or imaged isoelectric focusing. Ion-exchange chromatography and hydrophobic interaction chromatography (HIC) are 2 commonly used approaches for separation and enrichment of protein variants in the folded state.[33-35] These methods, however, have challenges of its own. For example, ion-exchange chromatography may not be able to separate Asp and isoAsp species which share the same net charge and mass at the whole antibody level. HIC, on the other hand, may be able to separate isomerized antibodies from their native forms, albeit often with poor resolution of intact protein variants. To achieve better resolution and more accurate quantitation, molecules can be broken into their subunits before separation, such as cleaving antibodies into $(Fab)_2$ and Fc.[36,37] This subunit approach nevertheless is molecule-specific and often requires extensive method development and optimization to achieve desired outcome, adding significant burden to analytical development. An alternative approach to these methods is tryptic peptide mapping (TPM) by LC coupled with MS that can be extremely powerful in probing protein PTMs as it provides comprehensive information on deamidation, oxidation, isomerization, glycan profiles, etc.[4,38,39] The drawback of the comprehensive TPM method is the long sample preparation and data analysis time, thus limiting its application in process and formulation development in which high-throughput is highly desirable. In addition, obtaining a comprehensive data set on protein PTMs also requires use of a powerful (and expensive) mass spectrometer. The sample preparation conditions for peptide mapping can also be concerning as alkaline pH and high temperature could potentially generate method-induced artifacts such as destabilizing Asu and accelerating its conversion to Asp/isoAsp, therefore underestimating the actual Asu concentration in the sample.[40-42]

To address the challenges discussed previously in characterizing Asp-related product variants, a practical approach was created using a model monoclonal antibody (mAb1) which contains an isomerization-prone Asp in its CDR. Orthogonal analytical methods were developed and used in this approach to fully characterize critical degradation pathways and structure-function relationships. To alleviate the concerns of throughput of analytical methods used in process and formulation development, the newly developed method was amenable to high-throughput operations. Implementation of this approach resulted in detection and quantification of the native and isoAsp forms of mAb1, as well as a moderate level of Asu intermediate which in general is unstable, thus observed only at low level for many protein molecules.[15,16,22,43] We demonstrate in the research work presented here that an in-depth characterization of mAb1 site-specific isomerization products was possible by development of a comprehensive analytical tool kit.

Experimental

Materials and Methods

Chemicals and Reagents

Sodium chloride, calcium chloride, urea, L-methionine, guanidine hydrochloride, iodoacetamide, dithiothreitol (DTT), tris base, acetonitrile, trifluoroacetic acid (TFA), and formic acid were purchased from Sigma Aldrich (St. Louis, MO). Hydrochloric acid was purchased from Amresco (Solon, OH). Sequencing grade modified trypsin was from Promega (Madison, WI). NAP-5 desalting columns were purchased from GE Healthcare (Marlborough, MA). Water used in this study was purified by a Millipore Milli-Q system from EMD Millipore (Billerica, MA).

Monoclonal Antibodies

The IgG monoclonal antibody (mAb1) used in this study was recombinantly expressed in Chinese hamster ovary cells and purified with conventional chromatography steps. Antibody samples were stored in their original formulation buffers. The sample with high Asu content was generated by adjusting the buffer pH to 5.5 followed by storage at 25°C for extended period.

Conventional Trypsin Digestion Condition

A total of 500 μg of IgG sample was mixed with 400 μL of denaturant (8M guanidine hydrochloride, 50 mM Tris, pH 7.6). Water was then added to a total volume of 500 μL. After adding 35 μL of 100 mM DTT solution, the sample solution was vortexed and kept at 37°C for 20 min. Subsequently, 35 μL of 200 mM iodoacetamide solution was added followed by another 20 min of incubation at room temperature in dark. The obtained solution was desalted with a NAP-5 column, and antibody was eluted from the

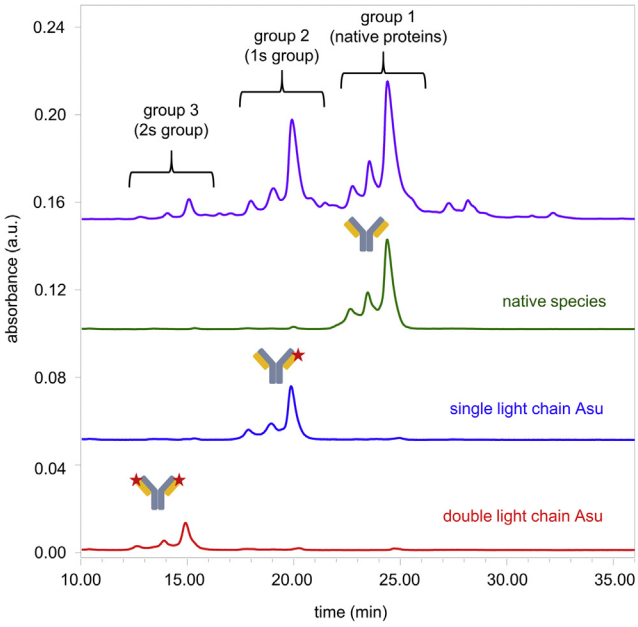

Figure 2. AEX chromatograms of mAb 1 stressed at pH 5.5, 25°C, for 1 mo, and reinjections of the isolated groups 1, 2, and 3 corresponding to native proteins, proteins with Asu modification in one of the light chains (1s group), and proteins with Asu modification in both of the light chains (2s group).

column with 1 mL of digestion buffer (50 mM Tris, 10 mM $CaCl_2$, 10 mM methionine, 2 M urea at pH 7.6). The samples were then digested by trypsin at a 1:50 trypsin: antibody (w/w) ratio at 37°C for 150 min. After quenching the reaction with 12 μL of 1 M hydrochloride acid, the digested peptides were stored at −80°C freezer before analysis.

Rapid Trypsin Digestion Condition

A total of 500 μg of IgG sample was mixed with a denaturation/reduction buffer (8M urea, 10 mM DTT, 50 mM Tris, pH 7.2) to a total volume of 200 μL. After incubating at 70°C for 5 min, the sample solution was diluted with 800 μL of 50 mM Tris buffer at pH 7.2, followed by addition of trypsin at the same ratio as in the conventional digestion method. The mixture solution was kept at 37°C for 5 min, and 100 μL of 10% TFA was used to stop the digestion. The obtained peptide sample was stored at −80°C freezer until analysis. During method development, the buffer pH, digestion time, and reduction temperature were all optimized.

Chromatographic Condition for the Peptide Mapping Method

Tryptic peptides were analyzed using a Waters (Waltham, MA) Acquity ultra-performance liquid chromatography system coupled with a photodiode array detector, a fluorescence detector, and a QDa mass detector. The LC column used for peptide separation is an Acquity ultra-performance liquid chromatography Peptide BEH C18 column (2.1 mm × 150 mm) from Waters. The mobile phases consisted of 0.1% formic acid in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The separation was performed using a gradient from 17% to 18.5% solvent B in 5 min at a flow rate of 300 μL/min. Column temperature was set at 50°C, and UV detection was performed at the wavelength of 280 nm. For fluorescence detection, peptides were monitored using an excitation wavelength of 290 nm and an emission wavelength of 372 nm. The QDa mass range was set from m/z 100 to 1250.

Anion-Exchange Chromatography Condition

Anion-exchange chromatography (AEX) was performed using ProPac SAX-10, 4 × 250 mm, 10 μm, analytical column using 10 mM MES, pH 6.5 as solvent A and 10 mM MES, 0.5 M NaCl, pH 5.5 as solvent B. The flow rate was set at 1.0 mL/min, column temperature at 40 ± 5°C, sample temperature at 5 ± 3°C, and signal detection at 280 nm. The samples were prediluted to 1.0 mg/mL with solvent A and the injection volume was 25 μL. The following gradient was used: 0%-10% solvent B from 0 to 3 min, 10%-45% solvent B from 3 to 45 min, 100% solvent B from 45.1 to 48 min, and then 0% solvent B from 48.1 to 60 min.

Results and Discussion

Enrichment of Isomerized Antibody Variants for Bioactivity Evaluation

Establishing structure-function relationship for the identified post-translationally modified product variants is one of the key activities in biologics chemistry, manufacturing, and control development. The knowledge derived from the structure-function studies directly feeds into the development strategy so that proper controls are implemented to ascertain drug efficacy and safety. For accurate assessment of bioactivity, purified (enriched) antibody variants with a specific PTM must be generated. The enrichment is typically done by collecting and concentrating peaks of interest using salt-based chromatography methods to preserve protein native structures. For mAb1 isomerization, HIC-based methods were initially attempted to separate all Asp variants—Asp, Asu, and isoAsp species—but with limited success (data not shown). AEX in contrary provided baseline separation of Asu species. As shown in Figure 2, molecules containing native Asp species, isomerization at either light chain (1s group), and isomerization at both light chains (2s group) were all baseline-separated in an mAb1 sample stressed at pH 5.5 at 25°C for extended period (1 month). The selected condition for mAb1 ensures that isomerization is the main degradation product which is known to be favored at lower pH formulations, and restricts the formation of other degradation products driven by elevated pH and temperature.[44] Peaks in the chromatogram were identified through specific enzyme digestion as well as by other biochemical means such as high pH treatment (data not shown). The baseline separation for the Asu form by AEX is likely contributed by strong interaction between the Asu forms and the column stationary phase that enabled straightforward separation of all 3 groups of peaks related to Asp variants. TPM analysis confirmed that the purified group 2 and 3 peaks contained 38.1% and 94.4% Asu species, respectively. Although AEX method was not able to resolve isoAsp species, TPM analysis revealed that group 1 peaks were composed of primarily

Table 1
Relative Potency for Asp Isomerization Fractions Determined by Cell-based Bioassay (Refer to Fig. 2 for Peak Identification)

Sample	isoAsp (TPM, %)	Asu (TPM, %)	Asp (TPM, %)	Relative Potency (%)
Group 3 (2s group)	1.7	94.4	3.9	3
Group 2 (1s group)	8.4	38.1	53.5	27
Group 1 (Native proteins, isoAsp enriched)	58.4	3.0	38.6	99

Table 2
Comparison of Theoretical Retention Time Predicted by DryLab and Experimental Retention Time for the Asp Isomerization Peaks

Peak	Theoretical RT (min)	Experimental RT (min)	Accuracy (%)
IsoAsp	3.90	3.81	97.7
Asp	4.17	4.10	98.3
Asu	4.60	4.48	97.4

isoAsp species at 58.4%. In addition, TPM analysis also confirmed that isomerization was the primary modification in these purified samples, and they are suitable for assessment of the impact of Asu and isoAsp components on biological activity by cell-based potency assay. As listed in Table 1, relative potency of 27% for group 2 peaks where Asu formed in one of the light chains clearly demonstrated a major drop in biological activity as a result of the modification. The impact was even more dramatic for group 3 peaks (3% activity), where both light chains were isomerized to Asu. By contrast, group 1 peaks with 58.4% of isoAsp (and very little Asu) showed comparable potency (99%) to the reference standard, suggesting no impact due to isoAsp formation. These data indicate that risk of isoAsp adversely impacting mAb1 product quality is low, whereas Asu is obviously a critical quality attribute for mAb1, which should be controlled during manufacturing and storage. It should be noted that the effect of Asp-related variants on biological activity is likely to be protein-dependent, specifically the location of the Asp residue and structural consequences due to isomerization. Yan et al reported impaired antigen-binding and associated structural changes caused by isoAsp formation.

Focused Peptide Mapping Method for Site-Specific Isomerization

Chromatographic Separation of the Target Peptides

High-performance liquid chromatography separation of the peptides for a typical peptide mapping sample of monoclonal antibody usually takes several hours to achieve adequate resolution for comprehensive monitoring of most, if not all of the PTMs. The duration of the separation step in the method can be significantly reduced if the interest is specifically focused on the isomerized CDR peptides. The comprehensive peptide mapping method was 2-h long, where the native (Asp unmodified), Asu, and isoAsp species were identified by high-end MS. The high-performance liquid chromatography modeling software, DryLab® (Molnár Institute, Berlin, Germany) was then applied to optimize separation parameters including column temperature, gradient slope, and flow rate to focus on shortening the separation time while maintaining adequate resolution. Briefly, several scouting runs were performed with varying column temperatures and gradient slopes, and a retention model was built to establish optimal separation parameters. As shown in Table 2, the actual separation profile is in excellent agreement with the prediction, and the total separation time was significantly reduced from 120 to 8 min with less than 1 min separation window for all 3 isomerization related peptides. The peak identity was simultaneously confirmed by MS (Waters QDa Detector) as shown in Figure 3, where the target peptide mass was assigned to each of the corresponding peaks.

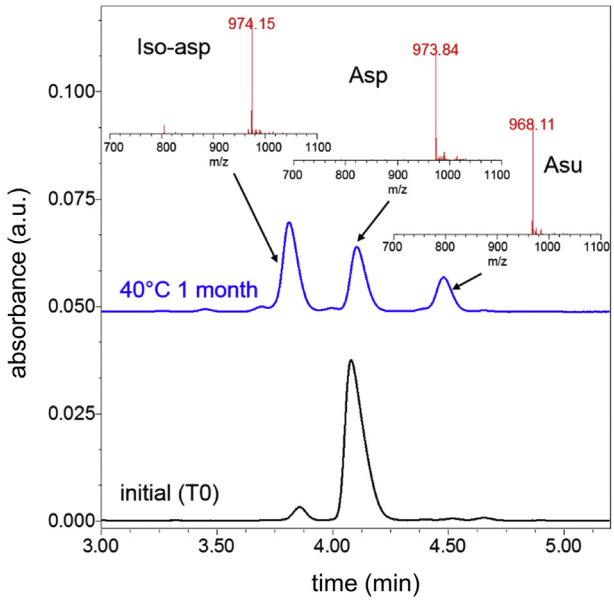

Figure 3. TPM chromatograms of mAb 1 at initial (T0) condition and after 1 month storage at 40°C stressed condition. Increase in isoAsp and Asu and decrease in Asp on storage at the stressed condition were clearly captured by the 8-min chromatographic separation. The peak identity was verified by mass spectrum at 3.81 min (isoAsp), 4.10 min (Asp), and 4.48 min (Asu).

Rapid Sample Preparation and Its Digestion Efficiency

Separation of the peptides of interest in less than 10 min is very encouraging; however, the overall analysis turnaround time is also affected by the duration of sample preparation. A protocol for comprehensive TPM sample preparation typically consists of denaturation, alkylation, desalting, and digestion steps which could add up to 24 h. The lengthy sample preparation stands obviously as a bottle neck for developmental activities where high-throughput analysis is highly desirable for screening of process and formulation conditions. Recently, Li et al[45] evaluated an ultrafast tryptic digestion approach for monitoring site-specific oxidation in antibody. However, accurate measurement of Asp-related isomerization variants in a high-throughput fashion remains a challenge because of instability of succinimide under conventional sample preparation conditions. Such challenge was carefully evaluated and addressed by the development of an isomerization-focused TPM method. Compared with the conventional digestion approach, the focused method uses elevated temperature to accelerate the denaturing/reducing process. Because the target peptide does not contain cysteine residue and is free from disulfide bond reformation, the alkylation step was eliminated. In addition, a simple dilution step was used to decrease urea and DTT concentrations to replace the lengthy desalting step. Finally, the trypsin digestion time was reduced from hours to minutes. To evaluate the effect of digestion time on digestion efficiency and method sensitivity, a comparative digestion study was performed at pH 7.2, 37°C, from 3 to 150 min. Data showed that the digestion is a relatively quick process: the total peak area (Asp, isoAsp, and Asu) from 5-min digestion was approximately 80% of that at 150-min digestion, when the signal reached a steady state indicating complete digestion (Fig. 4a). The observed high efficiency of digestion is most likely due to the fact that the CDR peptides are surface-exposed with easy access to solvent and enzyme molecules. In addition, relative percentage of the Asu peak area at each digestion duration remained consistent (Fig. 5a), confirming that quantification of the isomerization components do not require complete digestion of the target peptides. Besides digestion time, pH of the reaction mixture is another critical parameter that has been shown to affect digestion efficiency. Figure 4b compares again the total peak area at pH 7.0, 7.2, 7.6, and 8.0, with 5 min of digestion at 37°C. Peak area was the highest at pH 7.6, indicating the highest digestion efficiency. At pH 7.0 and 7.2, peak areas were approximately 29% and 81% of that at pH 7.6, respectively. Although trypsin activity is expected to be higher at pH 8.0, lower peak area was observed instead. As 2 asparagine (Asn) residues are present in the target peptides, a fraction of the peptides that undergoes accelerated deamidation at

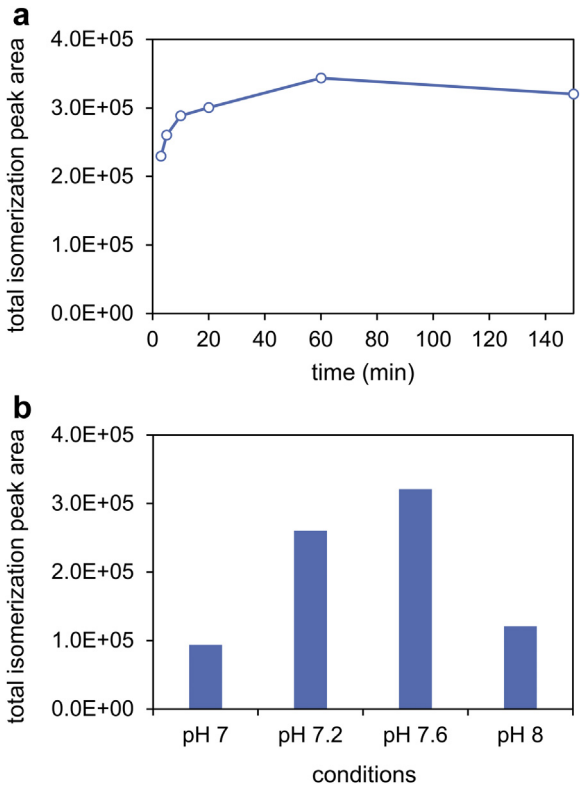

Figure 4. (a) Effect of time on digestion efficiency. Digestion was carried out in a pH 7.2 buffer at 37°C. Total isomerization peak area (Asp, Asu, and isoAsp) was measured at 3, 5, 10, 20, 60, and 150-min digestion time points. (b) Effect of pH on digestion efficiency. Total isomerization peak area (Asp, Asu, and isoAsp) was measured for different digestion pH - at 7.0, 7.2, 7.6, and 8.0. The digestion time was 10 min and temperature was 37°C.

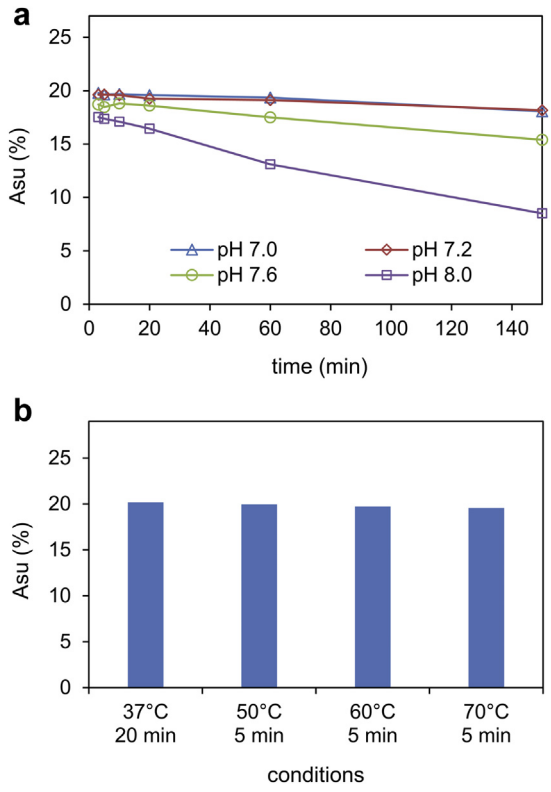

Figure 5. (a) The effect of buffer pH on method-induced Asu degradation. Samples were denatured and reduced at 70°C for 5 min, followed by digestion at 37°C for different durations—at 3, 5, 10, 20, 60, and 150 min. (b) The effect of denaturing/reducing temperature on method-induced Asu degradation. Samples were denatured at different temperature/duration including 37°C for 20 min, and 50°C, 60°C, 70°C, for 5 min each. Samples were subsequently digested at 37°C for 10 min.

this pH condition[46] will result in the loss of unmodified target peptides. At pH 7.2, the deamidation rate was expected to be lower, yet prolonged incubation could still lead to a slight decrease in the total peak area, as seen in Figure 4a.

Method-Induced Succinimide Hydrolysis

In addition to methionine oxidation and asparagine deamidation as a result of elevated temperature and alkaline pH condition used in peptide mapping methods,[44,47] the tryptic digestion process is particularly unfavorable in terms of preserving Asu species as they are in general unstable at alkaline pH and can hydrolyze into Asp and isoAsp. Such method-induced degradation of Asu needs to be minimized to allow for accurate quantification of Asu in a protein product and evaluation of its impact on binding activity. Our strategy for reducing the artifact was to decrease the digestion pH and duration while maintaining sufficient trypsin activity and digestion efficiency. To achieve an optimal digestion condition, buffer pH, duration of digestion, and denaturing/reducing temperature were evaluated. mAb1 formulations were first denatured and reduced at 70°C for 5 min; subsequently, the denatured molecules were digested at 37°C, and reaction was quenched at different time points from 3 to 150 min. As shown in Figure 5a, measured relative percentage of Asu is clearly dependent on both pH and duration of digestion. With a 3-min digestion reaction, Asu level at pH 8.0 and 7.6 were approximately 2.2% and 1.1% lower, respectively, compared with the measured Asu level of 19.8% at pH 7.0. The differences at these pH conditions increased further with progressing digestion time, and reached 9.7% and 2.8%, respectively, at the 150-min time point. At the lower end of the pH range, Asu was noticeably more resistant to method-induced degradation; no meaningful difference in Asu level was observed between pH 7.0 and 7.2 regardless of digestion time, and the level only decreased by 1.5% during the 150-min digestion. While the pH 7.6 condition did offer higher digestion efficiency (Fig. 4b), pH 7.2 seems to be a balanced choice between digestion efficiency and preservation of Asu content. To further shorten the sample preparation time, the denaturing and reducing process was also accelerated. Elevated temperatures at 50°C, 60°C, and 70°C for a 5-min denaturing/reducing step was evaluated using Asu level as a performance indicator. As shown in Figure 5b, the Asu levels ranged from 20.0% to 19.6%, suggesting that the denaturing/reducing temperature between 50°C and 70°C has no obvious impact on method-induced Asu degradation within the short period of the denaturing/reducing step. Ultimately, buffer pH of 7.2 with a 5-min denaturing/reducing time at 50°C followed by another 5-min digestion at 37°C was selected as the optimal method condition to minimize method-induced Asu degradation while maintaining adequate method sensitivity.

Impact of Rapid Digestion on Peak Purity

One of the key factors affecting the applicability of the focused TPM method in process and formulation development is quantitation by UV signal instead of MS readout. To ensure that the UV detection provides reliable and accurate measurements, the peak of interest must be free of interference such as coeluting peptides. While QDa mass detector offered straightforward approach for peak identification, a more sophisticated linear trap quadrupole mass spectrometer was used to explore possible interfering peaks

Table 3
Percentages of isoAsp, Asp, and Succinimide of mAb 1 Sample Measured by Focused TPM Method With UV Detection (280 nm) and Fluorescence Detection (Excitation 290 nm and Emission 372 nm)

Peak	LC-UV	LC-Fluorescence
IsoAsp	25.0%	25.2%
Asp	55.1%	55.3%
Asu	19.9%	19.5%

under both conventional and focused TPM preparation conditions. Data revealed that the focused TPM preparation condition resulted in a low abundance, missed cleavage peptide—which coelutes with the Asp peak; while under the conventional preparation condition, the isomerization components are free of interference. The Asp peptide contains 3 tyrosine (Tyr) and one tryptophan (Trp) residues, whereas the identified coeluting peptide contains neither of these amino acid residues which are the major contributors in UV absorbance at 280 nm. As a result, this missed cleavage peak is not expected to have an impact on the quantitation of the Asp component. This is further supported by comparing quantitation results by UV absorbance at 280 nm and fluorescence with excitation at 290 nm and emission at 372 nm, as fluorescence signal is dominated by Trp which is present in the target peptide but absent from the coeluting peptide. The difference in the percentages of the 3 isomerization peptides was less than 0.5% as measured by both methods (Table 3), confirming that the coeluting peptide signal is negligible at 280 nm and ensured that isomerization products are accurately measured.

Comparison of AEX, Conventional and Focused TPM Methods

Asu species as a critical quality attribute for mAb1 determined by bioactivity assessment can be monitored by both AEX and TPM methods. Herein, we performed detailed comparison among these methods. From the quantification perspective, because AEX and TPM measure overall Asu and individual Asu on each light chain, respectively, the following equation can be used to compare values measured by these 2 methods, where %Asu, %1s group, and %2s group are the relative percentage peak area measured by AEX for these peaks:

$$\%\,Asu\ (in\ equivalence\ to\ TPM) = (\%1s\ group + 2 * \%2s\ group) / 2$$

Results in Figure 6 demonstrated that AEX and both conventional and focused TPM measurements of Asu were in good agreement in the 40°C stressed stability samples, in which Asu reached its maximum level around 2 week time point before leveling off. Between the 2 TPM methods, Asu level measured by the focused TPM were consistently higher in all data points due to the optimized experimental condition which minimized method-induced Asu conversion as discussed earlier. Although the AEX method did not carry such issue (method-induced Asu degradation), the separation profile/resolution is greatly affected by sample condition (i.e., content of Asp-related variants and charge variants). For example, AEX at the initial condition showed higher Asu level compared to the focused TPM result. This is because AEX was unable to fully resolve the Asu peaks (1s group, Fig. 2) from the native C-terminus lysine variants of mAb1, which accounts for approximately 2%-3% of the total protein. These coeluting native basic variants, although at relatively low level, attributed to over-quantification of the Asu species, and is more pronounced when the Asu level was low. Furthermore, when proteins are stressed for extended period, in addition to isomerization, other chemical and physical changes are expected to occur. These changes can lead to

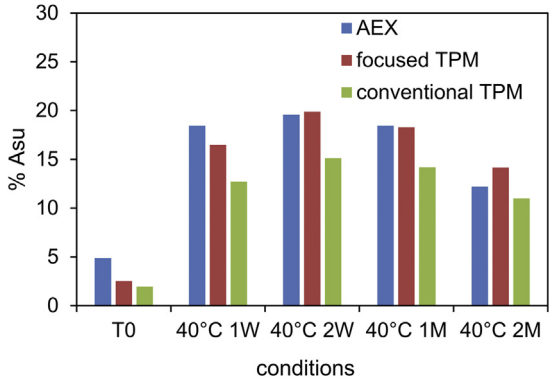

Figure 6. Comparison of Asu percentage measured by AEX, focused TPM, and conventional TPM method for mAb1 samples stored at 40°C for a 2-mo period.

significant undesired alteration in the separation profile and consequently compromise integration/quantification accuracy by AEX. Focused TPM in contrary measures isomerization through digested peptides which included all isomerization components as compared to AEX which only measures Asu. In addition, separation resolution of these peptides is much higher in focused TPM, and consequently, the quantification is free from interference regardless of the stress conditions used in the formulation screening studies. Taken together, these results clearly demonstrated that the focused TPM method provides most accurate and reliable quantification of Asp-related variants among all methods studied here. From the method applicability perspective, reducing the sample preparation and separation time to a total of 20 min, the focused TPM method overcomes the major drawback of the conventional TPM method and even exceeds the throughput of the AEX method for mAb1 in which the chromatography step is as long as an hour. In addition, quantification by UV absorbance simplifies the TPM method by eliminating the need for an MS detector and makes the method more user-friendly in QC space. AEX as an orthogonal method to focused TPM filled the gap in the characterization space where TPM is incapable of enriching intact Asp-isomerization species. With the analytical toolkit reported here that combines multiple methods, a comprehensive data package on Asp isomerization is generated with high level of confidence.

Conclusion

Post-translational modifications that can potentially impact safety and efficacy of protein therapeutics require thorough characterization and monitoring if necessary. Although it is desirable to develop a single method for characterization, process/formulation development, and QC purposes, complex chemical degradation processes such as Asp isomerization often require several orthogonal analytical techniques to generate a complete picture of the degradation pathways. The presented analytical tool kit utilized AEX for overall isomerization and enrichment of isoforms for subsequent structure-function relationship studies, and focused TPM to quantify site-specific Asp-isomerization with greatly enhanced throughput. A major short-coming of the conventional TPM method is the time consuming steps of sample handling and analysis. Such disadvantage makes the method less ideal to support process and formulation development which typically generate a large number of samples screening many conditions and require fast turnaround time. The complexity of the conventional TPM method and the MS-based detection also renders the method semiquantitative and less QC-friendly for Good Manufacturing Practice testing laboratory. These problems were addressed by

focusing on the site-specific isomerization, which as a result, allows significant reduction in both sample preparation and chromatography steps. Method-induced Asu conversion was minimized by developing a fast sample preparation step as well as fine-tuning the conditions. Combined with quantification by UV rather than mass signal, the method is tremendously simplified and the accuracy is greatly improved making it ideal for process and formulation development, as well as for QC purposes. The missing capability of the focused TPM method to enrich isomerized intact proteins was effectively bridged by implementation of the AEX method. The bioactivity characterization together with TPM quantification allows critical assessment of the Asp modifications from both qualitative and quantitative perspective. The analytical tool kit developed here allowed comprehensive assessment of the biological impact of the CDR isomerization in mAb1 with each Asp-related variant accurately quantified. The collective data from this approach ultimately helped to establish an appropriate control for this critical quality attribute of mAb1. The knowledge generated laid the foundation for establishing effective analytical control strategy such that the product quality can be maintained throughout its life cycle.

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