Introduction

The majority of recombinant monoclonal antibody (mAbs) products contain heterogeneous variants. These variants are commonly the result of modifications that occur during cell culture production due to enzymatic processes or spontaneous degradation, and can accumulate during production, purification, formulation and storage. Heterogeneity in mAbs is represented by charge variants, typically caused by deamidation, isomerization, succinimide formation, oxidation, sialylation, N-terminal pyroglutamic acid or C-terminal lysine (Lys) clipping. In addition to these variants, ‘species’ of unknown origin may also exist, and these species must be characterized to ensure the safety and efficacy of the products. Characterization or comparability data must be generated in order to demonstrate the consistency in product quality for regulatory filings.

C-terminal α-amidation is a modification recently identified in mAbs. C-terminal proline amidation (pro-amidation) was first identified and characterized in 2007. In spite of its relatively widespread occurrence in bioactive proteins and short polypeptides from invertebrates and vertebrates, including human, the exact biological impact of proline amidation remains to be fully understood. In higher organisms, the amidation reaction is catalyzed by peptidylglycine α-hydroxylating monooxygenase (PAM). Human PAM expressed in Chinese hamster ovary (CHO) cells has been previously characterized in reference 20, and copper was shown to be critical for the catalytic function of the PAM. In addition, the copper also plays an important role in the structure and molecular trafficking of the PAM. However, exactly how pro-amidation is mediated by certain ions remains unclear.

During the development of a new chemically defined medium (CDM) platform cell culture process, it was found that supplementing copper in the production medium above the original levels in the historical medium formulation helped maintain cell viability and improve mAb titers. Here, we present a case study demonstrating the impact of copper concentration in the production media on the charge profiles of an IgG1. In a copper titration study, the relative abundance of basic variants detected by imaged capillary isoelectric focusing (ICIEF) was found to correlate directly with the copper concentration in the basal production media. We report that the C-terminal pro-amidation exists as a basic charge variant of the IgG1. In contrast to previous observations that pro-amidation exists as a minor proportion of the basic charge variants, pro-amidation constituted the majority of the basic charge variants of this IgG1, in single and double amidation forms at the C-terminus of the heavy chains. To further characterize the pro-amidation and charge variants in the IgG1, a pH gradient cation exchange-high performance liquid chromatography (pH-IEC) was employed to isolate the basic charge variants. Analyses of the basic charge variants from different productions also indicated that the basic peak levels measured by ICIEF and pH-IEC methods correlate well with the pro-amidation level determined by peptide mapping, further supporting
the conclusion that the majority of the basic variants were due to pro-amidation.

**Results**

Observation of basic charged variants. ICIEF profiles of the IgG1 generated with and without carboxyl peptidase B (CpB) treatment are shown in Figure 1. Compared to non-CpB treated sample with a basic peak at 8.1%, the similar basic level at 7.8% with the CpB treatment indicated that very few C-Lys containing variants were present. Consistent with previous observations, including the molecule presented here, higher basal media copper concentrations are correlated with lower lactate accumulation during the production process. However, since higher copper results in higher levels of basic charge variants, a small scale (2 L bioreactors) study was conducted with variable copper concentrations to determine a target concentration for large scale production that would permit a well-controlled upstream process without compromising product quality. The purified antibodies were analyzed by ICIEF, and the resulting chromatograms are shown in Figure 2. While the acidic charge variant profile remained unchanged for all copper concentrations, the relative abundance of certain basic peaks increased with increasing copper concentration over the range tested (inset in Fig. 2B), suggesting an involvement of copper in mediating the basic charge profile of the IgG1.

Characterization of the basic charged variants. Examination of the basic charge variant profiles of the samples from different production lots revealed consistent peak profiles, suggesting that no new species were generated as a result of the varied copper concentrations. To determine the nature of the basic peaks that appeared sensitive to copper concentration, three separate productions were conducted with different copper concentrations to supply material for characterization. A pH-IEC method was used to isolate the basic charge variants; the pH-IEC profile is shown in Figure 3A. The three major basic peaks (B1–B3 in Fig. 3A) were purified, and these fractionated samples were analyzed again by ICIEF again to assess the purity of each fraction. As shown in Figure 3B, each of the fractions behaved as expected based on the ICIEF profile; the purity was estimated at 28% for B1, 76% for B2 and 79% for B3, respectively.

To confirm that basic peaks B2 and B3 were derived from pro-amidation, tryptic peptide maps were conducted to compare the three fractions. Figure 5 shows areas in the resultant maps containing differences among the samples, particularly amongst the C-terminal heavy chain peptides. Compared to the C-terminal peptide of SLSLPG eluting at 51.5 min, the B3 fraction showed the highest level of the C-terminal amidated peptide of SLSLP eluting at ~50 min, while the B2 fraction indicated pro-amidation on roughly half of the C-terminal peptides. The relatively high purity of these fractions and the agreement with the RP-HPLC-TOF/MS data gave further confidence to the conclusion that the
Tryptic peptide maps were also employed to analyze samples from the three production lots to determine the level of pro-amidation. Due to coelution of some species in the UV signals, levels of the pro-amidation were measured by using extracted ion chromatograms (EIC), which correlated well with the basic peak levels (Fig. 6). In contrast to pH-IEC or ICIEF, which detect the basic charge variants at the whole molecule level, tryptic peptide mapping detects pro-amidation at a peptide level under reducing conditions, which may explain the difference in the observed results from two methods. Because the B2 fraction represents about half of the basic charge variants and it bears pro-amidation on only one heavy chain, the observation that percent values of the basic charge variants from pH-IEC is roughly twice the percent value of the pro-amidation suggests that pH-IEC, rather than ICIEF, seems to be capable of measuring the unique modification more accurately.

A plot of basic charged variant levels versus copper ion concentrations from 12 lot productions at various scales using process conditions similar to those described for the copper titration study is shown in Figure 7. Compared with an $R^2$ value of 0.93

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**Figure 2.** Effect of copper concentrations on the basic peak levels detected by imaged capillary isoelectric focusing. The IgG1 was cultured in the presence of varying concentrations of Cu$^{2+}$ ions and purified IgG1 samples were analyzed by ICIEF; Inset: Correlation between the Cu concentration and basic peak levels.

B3 fraction possessed pro-amidation at both C-termini, while the B2 fraction contained one amidated proline at the C-terminus.

Additionally, a number of other minor differences were observed, indicated by numbers shown in Figure 5. Most of the peaks were found to be truncated peptides, which were found at a much higher level in fraction B1 than B2 and B3, as summarized in Table 1. Peak No. 2 mainly contained a number of fragments; interestingly, one species is identified as an elongated N-terminal peptide containing the Val-His-Ser (VHS) sequence of the signal peptide. Such N-terminal extensions have been observed previously in reference 10. It should be noted that peak No. 3 is likely due to non-specific cleavages derived from chymotrypsin activity in the trypsin used for the peptide maps. Another dominant peptide in the B1 is peak No. 5, which coelutes with another peptide at ~95 min (not shown), and identified as a cyclized N-terminal peptide from the heavy chain. It was found at a much higher level in the B1 fraction (7%), than in the B2 and B3 fractions (1%). It is thus reasonable to believe that cyclized pE, VHS-containing species and a few clip fragments constitute the major components in the B1 peak.

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from the small scale study in Figure 2, the R² value of 0.54 in Figure 7 implies that there are other factors that affect levels of pro-amidation besides copper concentration. However, the general relationship between copper concentration and pro-amidation level observed in the small scale experiments (Fig. 2) appears to hold true across production scales.

Discussion

The amidation reaction is catalyzed sequentially by two domains of the PAM: peptidylglycine-hydroxylating monooxygenase (PHM) and peptidyl-hydroxglycine-amidating lyase (PAL). The PAM is localized in subcellular compartments, and exists as soluble and membrane-bound forms. This raised an important question if the pro-amidation takes place in the cell culture media or by the membrane bound form of the PAM or both. Since it is believed that C-terminal Lys processing occurs in the cell culture media and proline is adjacent to a C-terminal Lys, more investigations are needed for a complete understanding of the mechanism for the pro-amidation in mAbs.

Copper has been shown to affect the performance of CHO cells. At concentrations under 1 μM in the cell culture media, copper has been able to reduce lactate accumulation, increase cell density and improve titer; however, at higher concentrations, copper in the basal media may reduce protein aggregation. However, adding large amounts of copper to the media can be undesirable because it can facilitate toxic free-radical reactions and affect PAM trafficking. Our results highlighted the potential of the copper concentrations in the culture media to affect levels of the pro-amidation. Production scale, operational conditions, and other unknown factors may play a role, making it difficult to control pro-amidation levels with copper alone. Therefore, it would be appropriate to implement analytical methods into quality control programs to monitor charge variants, in particular, the pro-amidation in mAbs development and manufacturing.

As demonstrated by Khawli et al. deamidation/isomerization in the Phe-Glu-Asn-Asn-Tyr sequence of Fc domain and C-terminal Lys variants tend to be benign and have yet to demonstrate effects on safety or efficacy. With its location at the C-terminus of the heavy chain and a charge profile similar to the well-characterized C-terminal lysine variants, pro-amidation is not expected to affect biological activity. However, variable levels of pro-amidation that are sensitive to the copper concentration suggest that more experimental results and knowledge are needed to understand this unique modification and its effects on the process and product quality consistence, which may provide more information on how to better control this modification for a more consistent process in mAb production. Further investigation and characterization could enhance understanding of any potential effects of this modification to mAbs, and facilitate cost-effective production, with the ultimate goal of putting well-characterized therapeutics into the clinic.

Materials and Methods

Antibodies. The recombinant mAb used in this study was expressed in Chinese Hamster Ovary cells and purified using conventional manufacturing process steps, including a three-step purification process of Protein A, cation exchange and anion exchange chromatography.

Copper titration study. The CHO cell line used in this study was derived from a glutamine synthetase-minus (GS-) CHO-K1 host, and genetically engineered to secrete the IgG1 recombinant protein of interest using a glutamine synthetase/methionine sulfonimine selection method. Cells were maintained for 12 days in proprietary, chemically-defined medium in 2 L bioreactors equipped with calibrated dissolved oxygen, pH and temperature probes. Temperature control was achieved via a heating blanket. Dissolved oxygen was controlled on-line through sparging with air or oxygen, and pH was controlled through additions of CO₂ or 1 M Na₂CO₃. Target copper concentrations of 400, 550, 750 and 1,000 nM were achieved via direct addition of 1 mM copper sulfate stock solution to media from a single basal media.
further characterization, according to the procedure described previously in reference 26, with minor modifications. To enrich the basic peaks of interest, 500 µg of sample was injected onto a Dionex ProPac WCX column (4 x 250 mm) kept at 25°C. Loading buffer (Buffer A) consisted of 2.4 mM Tris, 1.5 mM imidazole and 11.6 piperazine at pH 6.0. The flow rate was set at 1.0 mL/min. Elution buffer (Buffer B) consisted of Buffer A titrated with NaOH to pH 9.3. The elution time Table was as follows starting with 100% Buffer A at 1 mL/min: 0–3 min to 20% Buffer B, 3.0–33.0 minutes to 80% Buffer B, 33.0–35.0 minutes to 100% Buffer B, 35.0–35.1 minutes to 0% Buffer B and 35.1–53.0 minutes at 100% Buffer A. Detection was by UV at 280 nm. Fraction collection was accomplished using an Agilent fraction collector set to a time-based collect mode. Fractions were pooled, concentrated and buffer exchanged into 1x PBS buffer pH 7.4.

Imaged capillary isoelectric focusing. The distribution of charge variants was assessed by ICIEF using an iCE280 analyzer (Convergent Bioscience) with a fluorocarbon coated capillary cartridge (100 µm x 5 cm). The ampholyte solution consisted of a mixture of 0.35% methyl cellulose (MC), 2.2% 8–10.5 carrier ampholytes, 0.9% 5–8 carrier ampholytes and 2 M Urea in preparation. Actual copper concentrations were confirmed by inductively coupled plasma-optical emission spectroscopy to be within 6% of target value for all 4 concentrations tested.

pH gradient ion-exchange chromatography method. pH gradient ion-exchange chromatography method (pH IEC) was used to separate and isolate charged isoforms of basic nature for Figure 4. Deconvoluted spectra of the heavy chains from the fractions 1–3 on reversed phase high performance liquid chromatography with in-line time of flight mass spectrometry. (A) Control sample, (B) Fraction B1, (C) Fraction B2 and (D) Fraction B3.

Figure 5. Tryptic peptide maps of the B1–B3 fractions. Differences in the C-terminal peptides between them are indicated by arrows. Other minor differences are indicated by arrows and numbers (1–4), except for peak No.5, eluting at ~95 min (not shown).
performed under denaturing conditions to generate the free heavy and light chains for analytical characterization. Samples were diluted to 1 mg/ml with a buffer containing 3 M guanidine hydrochloride (final concentration; Mallinckrodt), 50 mM Tri-HCl (final concentration; Sigma), pH 8.3. A 0.5 M DTT (Sigma) stock solution was added to obtain 50 mM final concentration and the reaction mixture was placed at 75°C for 5 min.

Reversed-phase high performance liquid chromatography (RP-HPLC) was performed on an Agilent 1200 HPLC system. The mobile phases consisted of water with 0.1% formic acid and 0.025% trifluoroacetic acid as solvent A and acetonitrile (Honeywell Burdick & Jackson) with 0.1% formic acid and 0.025% trifluoroacetic acid as solvent B. A Varian PLRP-S (Varian, Inc., Palo Alto, CA), 4.6 x 50 mm, 8 μm particle size, 1,000 Å pore size column was used for the RP-HPLC time-of-flight (TOF) mass spectrometric (MS) analysis. The column eluent was analyzed by UV detection at 215 nm and then directed in-line to a TOF mass spectrometer. The initial mobile phase was 25% solvent B for 5 min, and then a two-stage gradient was applied: 2% solvent B per min from 25–30% solvent B, followed by 0.3% solvent B per min from 30–42% solvent B. The separation was performed at 75°C at a flow rate of 0.5 ml/min. Electrospray ionization TOF/MS was performed on Applied Biosystems QSTAR Elite XL mass spectrometer equipped with an Agilent 1200 HPLC system. The electrospray ionization mass spectra were analyzed using BioAnalyst protein deconvolution software (Applied Biosystems).

**Table 1. Summary of peptide fragments detected in basic variant fractions 1, 2 and 3**

| Peak No. | Sequence | Fractions | % UV signals |
|---------|----------|-----------|-------------|
| 1       | ISPANGNTYA (DSVK) | B1 | 5.9 | 2.5 | 0.9 |
| 2A      | VHSDIQMTOSPSSLSSASYGDR | B2 | 2.9 | 1.4 | 0.2* |
| B       | DQMTOSPSLSASYG (DR) | B3 | 1.1 | 0.5 | 0.1* |
| C       | (GFYPS) DIAVEWESNGQPENNYK | B3 | 0.8 | 0.3 | 0.1* |
| 3       | (ELYIMD) WQGQTLTVSSASTK | B1 | 2.4 | 0.4 | 0.0 |
| 4       | (ELYIM) DYWGQGLTVSSASTK | B3 | 13.1 | 5.5 | 0.5 |
| 5       | pEVOLVEGGGLVQPGGSLR | B2 | 6.9 | 0.7 | 0.8* |

Note: Determined peptides are underlined, missing residues from a tryptic peptide are included by bracket for a reference purpose. Quantitation was performed according to UV peak area %, and the values represent an estimate due to the calculation made using a fragment versus a full length of tryptic peptide. *quantitation was made using extract ion chromatogram signals from peptide maps, as co-elution made UV signal measurement impractical for a reliable value.

Figure 6. Correlation between percent C-terminal proline amidation at the peptide level in three productions by tryptic peptide maps and percent basic variants by imaged capillary isoelectric focusing and pH gradient ion-exchange chromatography. Quantitation of the proline amidation level was calculated by integration of extracted ion chromatogram signals.

Protease digestion and peptide maps. Peptide mapping was performed according to a previously described procedure with some minor modifications.27 Briefly, samples were buffer-exchanged into 50 mM Tris-HCl, pH 8.3, using Bio-Spin 6 columns (Bio-Rad) according to the manufacturer’s instructions. Recombinant sequencing grade trypsin (Roche Applied Science) was added to samples at an enzyme to protein ratio of 1:10 (w/w). Digestion occurred for 4 h at 37°C. Analytical peptide maps consisted of loading 50 μg of the digest onto a Phenomenex Jupiter C18 column (Phenomenex, Torrance, CA), 2.0 x 250 mm, 5 μm particle size, 300 Å pore size column, heated to a temperature of 55°C. The separation was performed by gradient elution on an Agilent 1200 HPLC system. The column was held at the initial condition of 100% solvent A (0.1% trifluoroacetic acid in water) at a flow rate of 0.25 ml/min for 1 min, followed by a potential of 3,000 V for 10 min. For the CpB treated samples, CpB was added to each sample at an enzyme to substrate ratio of 1:100 (w/w) followed by incubation at 37°C for 30 min.

Reversed phase high performance liquid chromatography time of flight mass spectrometry. Reduction of the IgG1 was purified water. The anolyte was 80 mM phosphoric acid, and the catholyte was 100 mM sodium hydroxide, both in 0.1% MC. Samples at 1.0 mg/mL were mixed with the ampholyte solution, and then focused by introducing a potential of 1,500 V for 1 min, followed by a potential of 3,000 V for 10 min. For the CpB treated samples, CpB was added to each sample at an enzyme to substrate ratio of 1:100 (w/w) followed by incubation at 37°C for 30 min.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 7. A plot for Cu²⁺ ion concentration and basic peak % from 12 different productions in a scale range from 2–2,000 liters.

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