Characterization of antibody variants during process development

The tale of incomplete processing of N-terminal secretion peptide

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Abbreviations: RP-HPLC, reversed phase-HPLC; mAb, monoclonal antibody; CHO, chinese hamster ovary; LC-MS, liquid chromatography-mass spectrometry; IEX-HPLC, ion exchange chromatography; PNGaseF, peptide N-Glycosidase F

Introduction

Recombinant monoclonal antibodies (mAbs) have been successfully produced in a variety of hosts, including mouse, insect and yeast cell lines, as well as transgenic plants, mammals and algae.1-6 For the industrial production of therapeutic molecules, one of the most common cell lines is derived from Chinese hamster ovaries (CHO). This cell line offers multiple advantages such as secretion of the recombinant product in the media, high titers and productivity, and a product glycosylation pattern close to that obtained from human cell lines.7

A production cell line is typically obtained by transfection of the host cell with a plasmid carrying the open reading frames coding for the heavy and light chains of the mAb of interest. These open reading frames are inserted in a few loci on one of the 22 chromosomes of the cell line and amplified to multiply the copy number of the two genes of interest. The clone with the best stability, productivity and desirable product quality features such as low aggregation or a specific glycosylation pattern is selected and used to produce material for pharmacokinetic, pharmacodynamic and safety assessments, as well as clinical studies. Purification starts with a primary recovery step where cells and cellular debris are removed by centrifugation/filtration. This first step is followed by a capture step, typically by using Protein A affinity chromatography. Subsequent polishing steps are added to ensure both high recovery yields and product quality.8

To be considered for approval by regulatory agencies, extensive characterization of the molecule must be performed. Primary degradation pathways for the molecule and product related impurities and variants are determined.9 We report here the characterization of a variant of a IgG1 antibody product (mAb1) isolated during process development.

Results

Presence of product-related variants in the cell culture harvest and Protein A intermediate. Analytical reversed phase-high performance liquid chromatography (RP-HPLC) is our in-process control method of choice to determine mAb titer during cell culture. RP-HPLC allows high throughput capabilities and at the same time offers a first snapshot of the product quality of the mAb being generated. MAb1 was thus analyzed by RP-HPLC during clonal selection and growth condition optimization steps.
Analysis of mAb1 in the cell culture harvest showed primarily 4 peaks (Fig. 1A). The first two peaks were attributed to free heavy chain or half molecules. The third peak, which is the main peak, corresponds to the desired mAb1 product. We also detected a fourth peak, which we labeled as RP-HPLC post peak, because it eluted after the main mAb1 peak. Unlike the two early eluting peaks, the species corresponding to the RP-HPLC post peak co-eluted with our mAb product from the preparative Protein A column during purification (Fig. 1B). This observation suggested that the mAb species associated with the RP-HPLC post peak was either a locally unfolded mAb1 molecule or possibly a mAb1 variant with a modified primary sequence. To devise a purification process that could efficiently remove the species attributed to the RP-HPLC post peak, we set out to isolate and characterize this mAb1 variant by ion exchange (IEX) chromatography and by mass spectrometry (MS).

Purification of mAb1 product-related variant by IEX chromatography. To isolate fractions significantly enriched in the species responsible for the RP-HPLC post peak, we performed a series of chromatographic steps. The cell culture harvest was first passed through a standard Protein A chromatographic column followed by two polishing steps (anion and cation exchange chromatography). The mAb1 product from the cation exchange column was fractionated into three pools: a pre-main pool that was discarded, a main pool corresponding to the highly purified material used as control throughout this study, and a post-main pool significantly enriched in RP-HPLC postpeak. The fraction enriched in RP-HPLC post peak was re-chromatographed on a cation exchange column as described in the Material and Method section (Fig. S1). Elution fractions were collected, buffer exchanged and further analyzed by RP-HPLC.

We selected one fraction for MS studies because it contained the highest purity of the RP-HPLC post peak as shown in Figure 2A (87% pure). We also analyzed this fraction by size exclusion HPLC (SEC-HPLC) (Fig. 2B) and IEX-HPLC (Fig. 2C). The SEC-HPLC method revealed that the aggregation level of the mAb1 variant molecule remained lower than 5%. In addition, no mAb1 fragment was detected. The IEX-HPLC analysis of the mAb1 variant, however, was very intriguing. The species corresponding to the RP-HPLC post peak eluted as a broad and undefined peak on the basic side of the mAb1 control chromatogram with retention time at ~15 min. Because higher elution power (higher pH and higher NaCl concentration) was required to displace the mAb1 variant than the mAb1 control sample from the analytical IEX matrix, we speculated that the mAb1 variant sequence could have been altered by the addition of positive charges to the molecule. The elution position and the peak shape of the molecule could also be due to unspecific interactions of the molecule with the matrix of the IEX column. This observation was consistent with the higher hydrophobicity of the mAb1 variant, observed by the RP-HPLC analysis. To gain further insight into the nature of the molecule corresponding to the RP-HPLC post peak, we studied the mAb1 variant by MS.

RP-HPLC post peak is not due to a change in N-glycosylation pattern of mAb1. The fraction containing the mAb1 species enriched in RP-HPLC post peak and the highly purified control sample were deglycosylated by treatment with PNGaseF followed by LC/electrospray ionization (ESI)-MS analysis. The control sample showed a molecular mass of 145,180 Da, which closely corresponded to the calculated molecular mass of the deglycosylated mAb1 (theoretical molecular mass = 145,179.8 Da) (Fig. 3A). The molecular mass of the species associated with the RP-HPLC post peak was measured at 147,054 Da, which indicated a mass increase of 1,874 Da compared with the mAb1 control (Fig. 3B). The data revealed a difference in the primary amino acid sequences between the RP-HPLC post peak and the mAb1 control sample. Moreover, it showed that the difference observed in the molecular mass was not due to the N-glycans attached to the mAb1 molecule.

RP-HPLC post peak is due to a primary amino acid sequence change on the mAb1 heavy chain. The fraction containing the mAb1 species enriched in RP-HPLC post peak and the mAb1 control sample were reduced with dithiothreitol (DTT) and analyzed by LC/ESI-MS. RP-HPLC retention time and molecular masses of the light and heavy chains were measured. The light chain of the RP-HPLC post peak species eluted at the same retention time and had the same molecular mass of 23,481 Da compared with the mAb1 control (data not shown). In contrast, the heavy chain in the sample enriched in RP-HPLC post peak was detected to have two series of peaks in molecular masses (Fig. 4B). The molecules in the first series, with molecular masses of major species detected at 50,564 and 50,726 Da, matched those of the heavy chain detected in mAb1 control sample with the expected G0F and G1F glycoforms (Fig. 4A). The molecules in the second series were detected by MS to have molecular masses of 52,445 and 52,607 Da (Fig. 4B). These species were not detected in the
mAb1 control sample (Fig. 4A). Each peak in the first series had a counterpart in the second series with an increased mass of 1881 Da. This mass increase was consistent with that identified from the deglycosylated intact molecule as shown in Figure 3. The mAb1 control sample also contained species with masses of 50688 and 50850 Da, absent from the fraction enriched in RP-HPLC post peak (Fig. 4A). These species are likely to be mAb1 heavy chain with an unprocessed lysine residue at the carboxy terminus and harboring G0F or G1F N-glycan structures, a common occurrence in CHO expressed mAbs.

Our results clearly showed that the modification in the primary sequence in the RP-HPLC post peak was located on the mAb1 heavy chain. Although a precise quantitative measurement is difficult from this experiment, the data suggested a 1:1 ratio between the modified heavy chain and native heavy chain in the species associated with the RP-HPLC post peak. This information indicated that modification happened on one of the two heavy chains of the complete mAb1 antibody.

RP-HPLC post peak is due to the presence of unprocessed secretion leader on the N-terminus of mAb1 heavy chain. We further subjected the mAb1 enriched in RP-HPLC post peak and the control sample to trypsin digestion and deglycosylation followed by LC/ESI-MS/MS studies in data-dependent acquisition mode (DDA). MS/MS sequence coverage for both samples were 75.5% for the heavy chain and 68.2% for the light chain. The two peptide maps were then compared. Careful analysis showed the presence of a peak eluting at 127.04 min in the digested sample enriched with RP-HPLC post peak (Fig. 5). This peak was absent from the control sample (data not shown). ESI-MS of this peptide on Synapt HD MS showed this peak had a molecular mass of 3262.8153 Da (Fig. 6A). Matching of the acquired MS data to the predicted peptide masses of the mAb1 sequence revealed the identity of the peptide as the secretion leader sequence attached to the 12 first amino acids of mAb1 heavy chain. The leader peptide sequence missed its first methionine residue. Truncation of the N-terminal methionine is expected since eukaryotic cells are known to remove this amino acid post-translationally at the first position of the gene products. To further confirm the sequence of the peptide, we subjected the peptide (m/z 1088.26, 3+) to targeted MS/MS on Synapt HDMS (Fig. 6 and Table 1). Analysis of the b and y ions generated from the targeted MS/MS study confirmed the sequence of the peptide (Fig. 6B; Table 1). More detailed analysis of the LC/MS tryptic peptide mapping also showed the presence of two additional truncated forms of the leader sequence, starting from Cys9 or Leu10, attached to the N-terminus of the heavy chain (Fig. 5).

Discussion

Secretion of proteins through the endoplasmic reticulum (ER) in mammals was the subject of extensive investigations from the 1970s through the 1990s. The translocation step into the ER is now a fairly well understood process. MAbs produced in CHO cells utilize the secretion pathway and are subsequently released in the media. The production of IgG molecules starts with translation initiation of the light and heavy chain mRNAs in the cytosolic side of the rough endoplasmic reticulum. The nascent light and heavy chains preceded by a 20–30 amino acids secretion leader sequence exit the ribosome. The leader sequence binds to a ribonucleoprotein complex called signal recognition particle (SRP), slowing down translation. The SRP is associated with the ribosome and its leader sequence cargo is recruited by a receptor domain adjacent to the integral membrane protein conducting channel Sec61p. Sec61p binds to the leader sequence and allows for the translocation of the heavy and light chains being translated into the lumen of the ER. The final step of the process involves a five subunit signal peptidase complex that will cleave off the leader sequence and generate the native N-terminus of the mAb heavy and light chains. Once translocated in the ER lumen, the antibody heavy chains will undergo glycosylation and appropriate folding that are assisted by a series of chaperones. The oxidizing environment
of the ER together with the presence of disulfide bridge isomerases facilitate the formation of intra- and inter-chain disulfide bridges leading to the proper assembly of the mAb.  

Recent evidence points toward an important role of the leader sequence in modulating protein biogenesis. The affinities of the leader sequence to SRP and to Sec61p are believed to affect translation rate, folding and posttranslational modification and may affect the specificity and accuracy of the proteolytic cleavage. The data reported in this paper suggest that the leader sequence used for the secretion of our molecule may not be optimal. Whether the incomplete processing reflects a suboptimal interaction with SRP or Sec61p has not been established. It is relevant to note that antibody expression can reach titers as high as 5 g/L.  

No studies have been performed to determine whether the level of incomplete processing correlates with the overwhelming of the ER secretion machinery. Recent reports in the literature indicate that the presence of unprocessed secretion sequence attached to mAbs is a common occurrence. Use of leader sequence derived from the ongoing efforts of sequencing and proteomics studies of CHO may improve the secretion of therapeutic recombinant proteins in this cell line.

### Material and Methods

#### Materials

mAb1 is a fully human IgG1 with λ light chains. This Merck proprietary mAb is expressed in CHO cells. DTT, iodoacetamide and 8 M guanidine HCl were purchased from Sigma, GE Healthcare UK Limited and Thermo Scientific, Pierce Protein Research Product, respectively. Analytical grade acetoni-trile (ACN), water, sequencing grade trifluoroacetic acid (TFA) and formic acid (Sigma) were used to prepare the mobile phases for HPLC analysis.

**Methods for chromatographic separation of mAb1 enriched in RP-HPLC post peak.** A cation exchange chromatography step was performed using a 9 ml POROS HS50 column on an AKTA Explorer system (GE Healthcare). The chromatography was run at 300 cm/hr with a loading of 20 mg mAb1/ml resin. The feed was prepared in buffer A–20 mM sodium acetate and 25 mM sodium chloride. After loading, the column was washed for 5 CV with buffer A. The product was eluted from the column by using a gradient of buffer A and B (20 mM sodium acetate and 150 mM sodium chloride pH 5.5). The gradient was from 40% to 100% buffer B for 10 CV and held for 5 additional CV. Fractions (3 ml each) of the eluate were collected once the absorbance at

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**Figure 3.** LC/ESI-MS of PNGase F deglycosylated mAb1 (A) control and (B) enriched in RP-HPLC post peak.
Ion exchange HPLC. IEX-HPLC was performed on a Dionex ProPac WCX-10 column (4 × 250 mm, 3.14 ml) at ambient temperature by using an Agilent 1100 series system. Mobile phase B was 20 mM sodium phosphate and 95 mM sodium chloride, pH 8.0 and mobile A was 20 mM sodium citrate, pH 5.5. The column was first equilibrated at 20% mobile phase B at a flow rate of 1.0 mL/min for 15 min. The mAb1 protein was then eluted from the column using a gradient of mobile phase B (20% to 60.5% in 15 min). The column was then cleaned with 100% mobile phase B for 1 min and re-equilibrated for the next cycle. The absorbance at 280 nm of the eluate was monitored throughout the LC run.

Reversed phase HPLC. RP-HPLC was performed using a POROS R2/10 column (2.1 mm × 30 mm, Applied Biosystems) at 80°C using an Agilent 1200 series system. Mobile phase B consisted 0.2% trifluoroacetic acid in 90% acetonitrile (v:v) and mobile phase A consisted 0.2% trifluoroacetic acid in water (v:v). The column was first equilibrated at 25% mobile phase B for 2 min at a 2 mL/min flow rate. The elution was performed as a gradient of mobile phase B, from 25% to 60% over 5 min at 2 mL/min. The column was then cleaned with 100% mobile phase B for 1 min and re-equilibrated for the next cycle. The absorbance at 280 nm of the eluate was monitored throughout the LC run.

Size exclusion HPLC. SEC-HPLC was performed on a TSKgel G3000SW column (7.8 mm × 30 cm, TOSOH Biosciences) at ambient temperature by using an Agilent 1290 Infinity system. The separation was performed at 0.6 mL/min flow rate for 30 min by using a mobile phase containing 0.2 M l-arginine, 0.2 M sodium phosphate and 0.005% sodium azide, pH 7.0. The absorbance at 214, 230 and 280 nm of the eluate was monitored during the LC run.

Trypsin digestion and deglycosylation of mAb1. The antibody (0.55 mg/mL) was buffer exchanged to a solution containing 100 mM ammonium bicarbonate and 6 M guanidine HCl using centrifugation filters (Amicon Ultra-0.5 filter, 5000 MWCO). Disulfide bonds of the antibody were reduced by 20 mM DTT and the reaction was incubated at 37°C for 45 min.

Figure 4. LC/ESI-MS of DTT treated heavy chains of mAb1 (A) control and (B) enriched in RP-HPLC post peak.
The reduced sample was alkylated by 40 mM iodoacetamide in dark at room temperature for 45 min. The protein was then buffer exchanged to 100 mM ammonium bicarbonate using dialysis (Thermo Scientific Slide-A-Lyzer dialysis cassette, 3500 MWCO) before trypsin digestion. Using an enzyme:substrate ratio of 1:20 (w:w), the antibody was digested with sequencing grade trypsin (Promega) at 37°C for 15 h. The digested mAb1 fragments were stored at -80°C. For deglycosylation, 1 μL of PNGase F (QA-Bio) was added to 20 μL of trypsin-digested mAb1 and the mixture was incubated at 37°C for 5 h, followed by LC/MS analysis.

**Nano LC/ESI-MS of mAb1.** The antibody was analyzed by Synapt High Definition Mass Spectrometry (HDMS) system (Waters) as intact, reduced, deglycosylated, and deglycosylated and reduced protein. RP-HPLC of mAb1 was performed on a Waters nanoAcquity UPLC system consisting of a temperature-controlled autosampler. Mobile phase A was water with 0.1% formic acid (v:v) and mobile phase B was ACN with 0.1% formic acid (v:v). The antibody was loaded onto a Waters 1.7 μm BEH300 C4 column (180 μm i.d. × 20 mm) at 5 μL/min flow rate and desalted at 15% B for 5 min. The guard column was then switched online with a Waters 1.7 μm BEH300 C4 column (100 μm i.d. × 100 mm). The mAb1 was eluted using a 15–80% mobile phase B gradient for 11 min at a flow rate of 500 nL/min. The column eluent was delivered through a PicoTip emitter (20 μm i.d., TaperTip, New Objective) for the ESI-MS analysis. For protein molecular mass determination, a capillary voltage of 3 kV and a cone voltage of 40 V were

![Diagram](image-url)
applied on the Synapt HDMS. An \( m/z \) range of 700–3000 was scanned and the MS spectra were deconvoluted by MaxEnt1 software (Waters).

**Nano LC/ESI-MS of trypsin digested mAb1.** An Eksigent nano LC system consisting of a temperature-controlled autosampler and four direct flow capillary/nano LC pumps that were powered by pressurized nitrogen (100 p.s.i.) were used for separation of tryptic peptides for data-dependant acquisition (DDA). Mobile phase A consisted 0.1% formic acid and 2% acetonitrile (v:v) and mobile phase B was 0.1% formic acid in 98% acetonitrile (v:v). The peptides were loaded onto an Acclaim PepMap C18 cartridge (300 μm i.d. × 5 mm, 100 Å, LC Packings) at a flow rate of 10 μL/min and washed with 2% mobile phase A for 10 min. The cartridge was then switched online with a New Objective PicoFrit AQUASIL C18 column (75 μm i.d. × 15 cm, 15 μm tip diameter). The peptides were eluted using a 2–98% mobile phase B gradient for 124 min at a flow rate of 350 nL/min. A New Objective’s PV550 nanospray interface was used to couple the nano LC system to a Thermo Scientific LTQ OrbitrapXL mass spectrometer.

The electrospray ion source was operated in the positive ionization mode with a spray voltage of 1.9 kV. DDA was performed by acquiring one full scan mass spectrum in the Orbitrap having a resolution of 60,000, followed by data-dependant MS/MS of the top five most intensive doubly- or triply-charged ions in the ion trap with dynamic exclusion enabled. DTA files were generated from the MS/MS spectra by Bioworks software (Thermo Scientific). To ensure mass accuracy, the Orbitrap analyzer was calibrated using doubly-charged ions of a peptide mixture on the same days that the experiments were performed.

**Figure 6.** LC/ESI-MS of the leader peptide. (A) The molecular mass of the peptide is detected at 3262.8153 Da. (B) LC/ESI-MS/MS of the leader peptide (\( m/z \) 1088.26, 3+) of mAb1 enriched in RP-HPLC post peak.
Nano LC/ESI-MS/MS of selected tryptic peptides of mAb1. Targeted MS/MS of selected peptides generated from tryptic digestion were performed on nanoAcquity UPLC/Synapt HDMS for peptide sequence confirmation. Peptides generated from trypsin digested mAb1 were loaded onto a Waters 5 μm Symmetry C18 column (180 μm i.d. × 20 mm) at 5 μL/min flow and desalted at 98% A (mobile phase A: 0.1% formic acid in H2O) for 5 min prior to MS analysis. The guard column was then switched online with a Waters 1.7 μm BEH130 C18 column (100 μm i.d. × 100 mm). The peptides were eluted using a 2–98% mobile phase B gradient for 130 min at a flow rate of 500 nL/min (mobile phase B: 0.1% formic acid in ACN). The column eluent was delivered through a PicoTip emitter (20 μm i.d., TaperTip, New Objective) for the ESI-MS/MS analysis. A capillary voltage of 3 kV, a cone voltage of 35 V and collision energy of 35 eV were applied on the Synapt HDMS for MS/MS over an m/z range of 50–4000.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here: www.landesbioscience.com/journals/mabs/article/21614

Table 1. Sequence coverage of the leader peptide attached to the N-terminus of the heavy chain by MS/MS

| b | Sequence | y |
|---|---------|---|
| — | 1       | A | 30 | — |
| 171.1128 | 2 | V | 29 | 319.7207 |
| 284.1969 | 3 | L | 28 | 3092.6523 |
| 341.2183 | 4 | G | 27 | 2979.5882 |
| 454.3024 | 5 | L | 26 | 2922.5467 |
| 567.3865 | 6 | S | 25 | 2809.4627 |
| 714.4549 | 7 | F | 24 | 2696.3786 |
| 874.4855 | 8 | C(carbamidomethyl) | 23 | 2549.3102 |
| 987.5696 | 9 | L | 22 | 2389.2796 |
| 1086.6380 | 10 | V | 21 | 2276.1955 |
| 1187.6857 | 11 | T | 20 | 2177.1271 |
| 1334.7541 | 12 | F | 19 | 2076.0794 |
| 1431.8069 | 13 | P | 18 | 1929.0110 |
| 1518.8389 | 14 | S | 17 | 1831.9582 |
| 1678.8695 | 15 | C(carbamidomethyl) | 16 | 1744.9262 |
| 1777.9379 | 16 | V | 15 | 1584.8955 |
| 1891.0220 | 17 | L | 14 | 1485.8271 |
| 1978.0540 | 18 | S | 13 | 1372.7431 |
| 2106.1126 | 19 | Q | 12 | 1285.7110 |
| 2205.1810 | 20 | V | 11 | 1157.6525 |
| 2333.2396 | 21 | Q | 10 | 1058.5840 |
| 2446.3237 | 22 | L | 9 | 930.5255 |
| 2545.3921 | 23 | V | 8 | 817.4144 |
| 2673.4507 | 24 | Q | 7 | 718.3730 |
| 2760.4827 | 25 | S | 6 | 590.3144 |
| 2817.5042 | 26 | G | 5 | 503.2824 |
| 2888.5413 | 27 | A | 4 | 446.2609 |
| 3017.5839 | 28 | E | 3 | 375.2238 |
| 3116.6523 | 29 | V | 2 | 246.1812 |
| — | 30 | K | 1 | 147.1128 |

* The detected b and y ions in Figure 6 are highlighted in red or blue fonts.

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