

Characterization of a Humanized IgG4 Monoclonal Antibody's Charge Heterogeneity

Daniel McDonald, Jennifer Wang, Victor Ling

Protein Analytical Chemistry II, Genentech Inc., 1 DNA Way, South San Francisco, CA 94080

INTRODUCTION

Characterization of a monoclonal antibody's charge heterogeneity distribution is important in understanding the quality attributes of that drug substance. Charge heterogeneity analysis becomes a powerful tool over the life of a product. It is frequently used throughout product development, such as during comparability assessment, and the charge profile typically becomes part of a marketed product's control system. This poster describes how a CHO (Chinese hamster ovary cell) derived antibody's charge variants were characterized, during a NSO (a mouse myeloma cell line) to CHO cell line change. A novel approach to isolate and characterize the N-terminal glutamine variant, which rapidly converts to the cyclized pGlu variant when using a typical fractionation technique, is illustrated.

MATERIALS & METHODS

Fractionation of Charge Variants on IEC

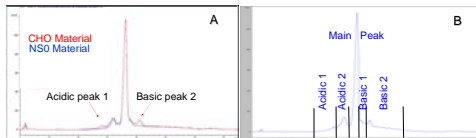


Figure 1. Cation exchange chromatograms

A) Charge profile comparison of CHO and NSO derived materials (CPB digested, on Dionex WCX-10, 2x250 column). The peaks Acidic 1 and Basic 2 represent major differences between NSO and CHO materials, and thus require in depth characterization.

B) Semi-prep fractionation (Dionex WCX-10, 9x250) of CHO material into 5 pools.

1) All five fractions were initially collected at 2-8°C, pooled and concentrated using Amicon-4, 10KD MWCO centrifugation filters at 2-8°C.

2) For basic 1 and basic 2 peaks, a secondary fractionation were performed by collecting the fractions directly into test tubes immersed in a dry ice/alcohol bath, and fractions were freeze-dried in a speed vac. The fractions were re-suspended in water or formulation buffer prior to analysis (see Table 1).

Table 1. Analytical techniques used to characterize charge variants

Sample	Charge Based Assays		N-terminal Sequencing (Edman)	Intact Mass (ABI-3000)	Reduced Mass (ABI-3000)	Potency (Antigen Binding ELISA)
	Cation IEC (w/CPB, w/ or w/out Sialidase)	Imaged cIEF (Convergent ICE200)				
Fractions						
Acidic 1	x	x		x	x	x
Acidic 2	x	x		x	x	x
Main Peak	x	x	x	x	x	x
Basic 1	x	x		x	x	x
Basic 2	x	x	x	x	x	x
Stressed						
40°C for 6 wks	x	x		x	x	x
IBHP oxidized 3hrs	x	x		x	x	x
IBHP oxidized 24hrs	x	x		x	x	x

RESULTS & DISCUSSION

1. Improved Fractionation Technique to Increase Yield of Basic 2 Fraction

- Problem: The fractionation yield of the basic fractions were only 43% (B1) and 25% (B2) in the original pools when re-analyzed (Table 2, and Figure 2, A);
- Previous work indicates that B2 fraction contained heavy chain (HC) N-terminal Gln variant. In B2 pool, the presence of main peak and the absence of B1 peak suggest that the Gln variant continues to convert to cyclized pGlu variant (Main Peak) during fractionation.
- To reduce the Gln to pGlu cyclization rate, which is temperature dependent in an Arrhenius relationship, the fractionation is frozen *in-situ* followed by freeze drying to trap Gln variant. By lowering temperature and removing water, the pGlu cyclization rate in B2 reduced, and yield improved (Table 2, and Figure 2, B).

Table 2. Charge variant composition of different fractions

Sample description	% Acidic	% Main Peak	% Basic
Starting material	14.1	77.6	8.3
Original fractionation			
Acidic 1	99.6	0.4	0.0
Acidic 2	97.3	2.7	0.0
Main peak	0.6	99.4	0.0
Basic 1	14.0	51.8	43.4
Basic 2	5.8	65.3	24.9
After using improved fractionation technique			
Basic 2	1.8	16.5	81.8

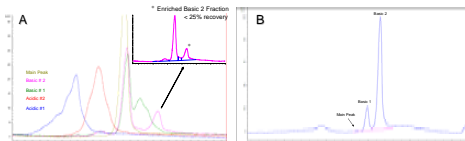


Figure 2. IEC chromatograms: A) original 5 fractions; B) fraction B2 after using improved fractionation technique.

2. N-terminal Sequence Analysis via Edman Degradation was used to support the hypothesis that Basic 2 variant contains a Gln at either HC N-terminus, while the main peak contains pGlu (blocked) at both HC N-terminus. Main Peak, Basic 2, and pyro-glutamate amino-peptidase (pGAP) treated reference material were analyzed, and the results (Table 3) confirms the hypothesis.

Table 3. N-terminal sequencing yield of LC and HC

Sample	Expected percentage of yield		Observed percentage of yield, (quantity in pmol)	
	LC	HC	LC	HC
Main Peak	100%	0%	100%, (308)	0%, (0)
Basic 2*	67%	33%	72%, (604)	28%, (240)
pGAP treated	50%	50%	53%, (720)	47%, (633)

* The 2/3 to 1/3 ratio expected for the Basic 2 variant is due to only one of the Heavy chains having Gln. The lower recovery of the observed HC (28%) can be explained by ~82% purity of the sample (33% x 82% = 27%).

3. Mass Spectrometry Analysis

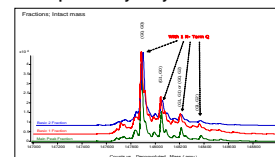


Figure 3. IEC fractions were analyzed on 2D IEC-LC-MS. Samples were first separated on an analytical IEC column, the individual fractions were trapped and washed on RP-LC columns, and then injected into a mass spectrometer (Waters Q-TOF Premier). Peaks are labeled with typical N-linked bi-antennary glycans. Intact mass of the B1 and B2 fractions showed similar profile as that of the main peak, except that B2 has a mass shift of about +17Da, corresponding to the mass difference between Gln and pGlu residue.

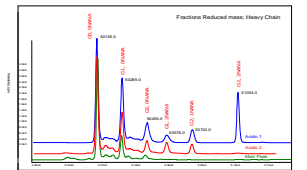


Figure 4. Reduced mass of acidic fractions and main peak was analyzed on ABI-3000 triple-quad mass spectrometer. Peaks are labeled with typical N-linked bi-antennary glycans, where NANA=N-acetyl neuraminic acid. Besides containing variants with similar masses as shown in the main peak, Acidic 2 fraction contains additional variants with one sialic acid (SA), the Acidic 1 fraction contains variants with up to 2 sialic acids per HC.

4. IEC with and without Sialidase Treatment

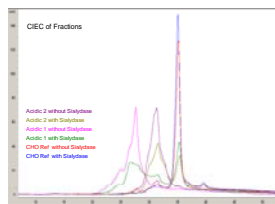


Figure 5. Overlaid IEC chromatograms with and without sialidase treatment. After treatment, acidic peak areas decreased about 1/3. Possible causes for the other 2/3 peaks include deamidation, and glycation. Further characterization work is on going. After sialidase treatment, the acidic profile of NSO and CHO derived materials are comparable (data not shown).

5. Imaged cIEF of CHO Stressed Materials

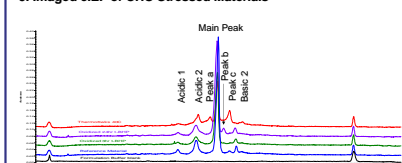


Figure 6. Imaged cIEF was used to analyze thermal stressed and oxidized materials. Under thermal stress condition, which promotes deamidation and/or isomerization, Peak a emerged from the front shoulder of the main peak, and Peak c increased significantly. When oxidized with IBHP, Peak b emerged. However in IEC, Peak b and c are not resolved (Basic 1 peak).

6. Summary of IEC Fractions of CHO Material

Peak	Peak Area (% of Total)	Possible Major Structure Differences from the Main Peak	Potency (% of Specific Activity)
Acidic 1	2.7	2 SA, 1 SA + 1 deamidation or glycation, and/or 0 SA + 2 deamidation or glycation	90
Acidic 2	13.5	1 SA, and/or 0 SA + 1 deamidation or glycation	114
Main Peak	73.8	0 SA + N-term pGlu on both HCs + no C-term Lys	98
Basic 1	5.3	Possible deamidation / isomerization, and oxidized species	95
Basic 2	4.7	1 N-term Gln and 1 pGlu on either HC	91
Starting Material			96

CONCLUSIONS

The major differences in charge profile between NSO and CHO materials are Acidic 1 and Basic 2 peaks, which are the focus of the present study. Rapid conversion from Basic 2 to Main peak posed a challenge for the typical fractionation technique. A novel fractionation strategy with frozen *in-situ* and followed by freeze drying was employed to improve the Basic 2 fraction yield. The major structure differences between the Acidic peaks and Main peak are due to the presence of sialic acid, and possible deamidation and glycation species. Basic 2 peak contains one N-terminal Gln on the HC. Stressed study suggests that Basic 1 peak may be the combination of deamidation/ isomerization, and oxidized species. The potency of these fractions are not significantly different from that of the Reference Material. The results of this charge heterogeneity analysis as part of the comparability assessment support the cell line and process change from NSO to CHO.

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