

The Effects of Media Formulations on the Biochemical Profile of IgG Expressed in Sp2/0 Cells as Measured by Cation Exchange HPLC

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ESACT 2007 Poster#1115

Introduction

One of the major concerns for the biopharmaceutical industry is maintaining the product quality parameters of recombinant proteins such as humanized monoclonal antibodies (MAB's) when process changes occur during product development. Changes in the biochemical profile of a protein product could affect its therapeutic or pharmacokinetic properties and require repeating preclinical or clinical studies¹⁻³. Since charge heterogeneity is commonly observed with recombinant MAB's, one of the assays typically used to analyze product quality is cation exchange (CEX) HPLC^{4,5}. Charge heterogeneity can result from multiple causes such as deamidation, oxidation, amino acid changes, sialic acid addition to glycoforms, N-terminal pyroglutamic acid, and C-terminal lysine clipping⁴⁻⁷.

In a recent media development project for a recombinant IgG expressed in Sp2/0 cells, our goal was to maintain a specific CEX profile while achieving target productivity levels. We used a combination of media screening and mixing experiments in shake flask cultures to identify formulations that maximized cell growth and productivity while maintaining the desired product quality parameters as measured by CEX HPLC. Further productivity and product quality improvements were achieved by optimizing feed supplements. The final media formulation and fed-batch process identified by these studies exceeded the target productivity of 800 mg/L and closely matched the reference CEX HPLC profile for product quality.

Methods

Cell Culture

Sp2/0 cells were grown as suspension cultures in shake flasks at 37 °C, 5% CO₂, using a shaking incubator at a speed of 115 rpm. Cells were seeded at an initial density of 2 × 10⁵ viable cells/ml (as measured by a Cedex counter) and passaged every 2–3 days. For growth curve studies, cells were adapted to each test media for a minimum of 3 passages. Cultures of various test media were then seeded with 2 × 10⁵ viable cells/ml and grown as described above. Samples were taken daily to measure viable cell density and at the end of the study to evaluate product quality by CEX HPLC. Growth curve studies were terminated when cell viability dropped below 40% (approximately 7 days for non-fed cultures). For fed-batch studies, cultures were initially fed with a variety of supplements when cell densities reached 2 × 10⁶ and 4 × 10⁶ cells/ml, and then every other day following the second feed for the duration of the experiment. Glucose and L-glutamine were controlled to maintain 2–4 g/L and 2–4 mM, respectively based on NOVA analysis. The pH was monitored daily and adjusted as needed with sodium carbonate.

IgG Sample Preparation and Enzyme Digestion

MAB was concentrated from spent cell culture media with a 30 kD Amicon Ultra-15 Centrifugal Filter Unit (Millipore, MA, USA) using a swing bucket rotor at 4300 rpm for 50 minutes. After concentrating, the MAB was purified with Protein A resin (G.E. Healthcare, UK) in a 96 well filter plate (Innovative Microplate, MA, USA). Prior to purification, 200 µL of absolute ethanol was used to wet the filters and the excess removed by centrifugation at 1600 rpm for 3 minutes. 100 µL of Protein A slurry was pipetted into each well of the filter plate and the storage buffer was removed by centrifugation. The Protein A was washed twice by adding 200 µL of Wash Buffer (20 mM Citrate, 150 mM NaCl, pH 7.0±0.2), shaking for 1 min., and removing the buffer by centrifugation. 300 µL of the concentrated spent cell culture media was then added to each well containing Protein A resin and the filter plate shaken for 10 min. The plates were centrifuged to remove the media and washed twice as described above. 200 µL of Elution Buffer (25 mM Citrate, pH 3.0±0.2) was then added and the filter plate shaken for 1 min. The filter plate was centrifuged and the eluent containing the MAB was collected.

MAB concentration was measured by A280 spectrophotometry and samples were diluted to a standard concentration of 1 mg/ml in Elution Buffer. For samples that required enzyme treatment to study post-translation modification of the MAB, 200 µL of purified MAB was buffer exchanged by diluting in 4 ml of Mobile Phase A and concentrating back to 1 mg/ml with a centrifugal filter unit as described above. To determine terminal lysine charge heterogeneity, 2 µL of Carboxypeptidase B (5 mg/ml; Roche, IN, USA) was added to 200 µL of purified MAB and incubated at 37 °C for 2 hours. To determine the amount of post-translational modification due to sialic acid, 2 µL of sialidase (50,000 U/ml; New England Biolabs, MA, USA) was added to 200 µL purified MAB and incubated at 37 °C overnight.

CEX HPLC

25 µg of MAB charge variants were separated by HPLC using a 4.6 mm × 250 mm ProPac WCX-10 weak cation-exchange column (Dionex, CA, USA) connected to an Alliance HPLC with PDA detection (Waters, MA, USA). Mobile phase A consisted of 10 mM sodium phosphate (pH 7.5±0.2). Mobile phase B consisted of 10 mM sodium phosphate and 100 mM sodium chloride (pH 7.5±0.2). Separation and detection were performed at 25 °C and 220 nm using the following operating parameters:

Time (min)	% A	% B	Flow Rate (mL/min)
0	95	5	0.8
2	95	5	0.8
31	60	40	0.8
31.5	0	100	1
35.1	0	100	1
35.3	95	5	1
51	95	5	1

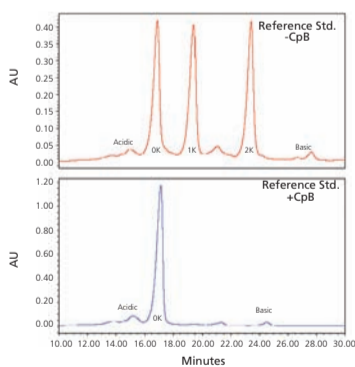


Figure 1: CEX HPLC chromatogram of recombinant MAB reference material. The dominant peaks on the chromatogram result from the proteolytic clipping of the heavy chain's C-terminal lysine resulting in antibody molecules containing 0, 1, or 2 terminal lysines (OK, 1K, and 2K peaks). Treatment of the IgG with carboxypeptidase B (CpB) removes the terminal lysines, leaving a single dominant OK peak (bottom chromatogram). Charged species that elute from the CEX column prior to the OK peak are defined as the acidic fraction and species eluting after the 2K peak as the basic fraction.

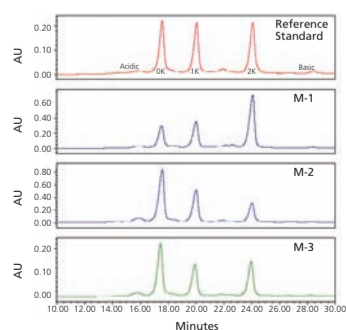


Figure 2: Preliminary media screening demonstrated that producing the MAB in cultures containing different formulations could result in CEX profiles with dramatic changes in the ratios of the OK and 2K peaks. The OK:2K peaks ratios varied from 1:2 (M-1) to 2:1 (M-2) with various test formulations. Formulations (e.g. M-3) were also identified that produced IgG with a ratio similar to the reference protein (1:1).

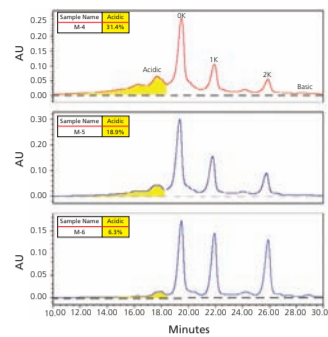


Figure 3: Preliminary media screening also demonstrated that producing the MAB with different formulations could result in CEX profiles with dramatic changes in the % of protein eluting prior to the OK peak (acidic fraction). Values for the acidic fraction protein ranged from >30% to <10%. The desired target range of the acidic fraction was <10% for this project.

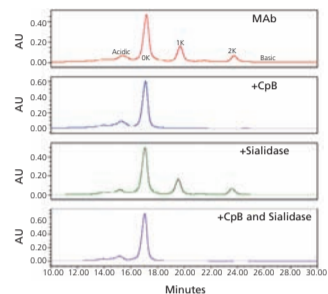


Figure 4: A sample of MAB (#258) containing a relatively high acidic fraction was treated with CpB and/or sialidase to evaluate the post-translational modifications causing the changes in % acidic fraction. Treatment of the MAB with CpB removed the C-terminal lysines, shifting protein from the 1K and 2K peaks into the OK or acidic peaks. Treatment of the MAB with sialidase removed the terminal sialic acids on the MAB's oligosaccharide structures, shifting the protein from the acidic fraction to the OK, 1K, or 2K peaks. Treating the MAB with both CpB and sialidase resulted in theoretically "uncharged" protein (OK peak) or protein remaining in the acidic fraction due to other post-translational modifications such as deamidation or pyroglutamic acid formation. Values for the % acidic fraction following these various treatments are shown in Table 1.

Treatment	% Acidic Fraction in CEX Profile	
	Reference	#258 (See Fig. 4)
None	8.3	22.2
Sialidase	6.0	17.5
CpB	17.7	33.2
CpB + sialidase	13.9	24.2

Table 1: Quantitation of IgG found in the acidic fraction of CEX profiles following treatment with sialidase and/or carboxypeptidase (CpB). In untreated samples, the reference IgG contained a small acidic fraction compared to sample #258. Treatment of samples with CpB removed the charge heterogeneity caused by C-terminal lysines, allowing a more accurate evaluation of the acidic fraction. Comparing samples treated with CpB to those treated with CpB + sialidase indicated a minimal presence of sialic acid on the oligosaccharides structures of the reference sample IgG. A similar comparison of sample #258 indicated a larger sialic acid content with approximately 11% of the IgG molecules containing one or more sialic acids on their glycoforms compared to approximately 4% of the IgG molecules in the reference sample. The post-translational modifications causing the remaining increased acidic fraction in sample #258 compared to the reference standard were not determined, but could be related to factors such as increased deamidation or pyroglutamic acid formation.

Formulation	Description
C-1	Original Growth Medium
C-2	Original Production Medium
C-3	Current Growth Medium
C-4	Current Production Medium
S-1	Lean CD Medium
S-2	Moderately Rich CD Medium
S-3	Modified Version of S-1
S-4	Rich CD Medium
S-5	Moderately Rich CD Medium
S-6	Moderately Rich CD Medium
H-1	Moderately Rich Medium Containing Soy Hydrolysate and Protein
H-2	H-1 + Lipid Complex
H-3	H-1 + Yeastolate
H-4	H-1 + Lipid Complex + Yeastolate

Table 2: Formulation chart for media screening experiment. A media screening experiment was set up to evaluate the growth, productivity, and CEX profile of MAB produced using a variety of different media formulations. Control media (yellow) represent the customer's original and current media for growth and production of the IgG secreting Sp2/0 cell line. Screening media (green) were chosen from the SAFC Biosciences media library and represented a diverse set of formulations. Cells were adapted to each media by multiple passages prior to the screening experiment.

Screening 1-Cell Growth

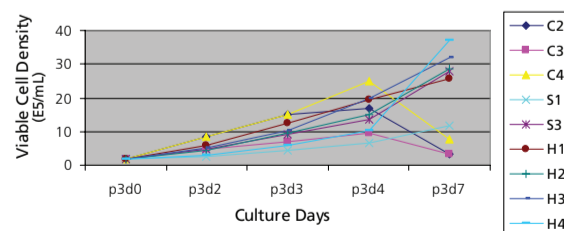


Figure 5: Cells were seeded into shake flasks containing media formulations described in Table 2 and cultured in a shaking CO₂ incubator at 37 °C for 7 days. Periodic samples were taken to measure cell growth and viability. Some formulations shown in Table 2 did not support acceptable growth and are not shown in this figure. Several screening formulations were identified that supported high viable cell densities for a greater period of time compared to the control media.

Medium	Cell Growth		CEX Profile	
	Peak VCD (x 10 ⁶)	IgG Titer (µg/ml)	% Acidic Fraction	% OK Peak
C-2	--	--	6.8	37
C-3	0.97	95	10	42
C-4	2.5	164	16	36
S-1	1.2	180	10	33
S-3	2.8	245	14	45
H-1	2.6	95	4.7	20
H-2	2.8	185	5.6	23
H-3	3.2	314	9.2	41
H-4	3.7	226	10	42

Table 3: Summary of data from media screening experiment. The chemically defined (CD) formulation S-3 exhibited excellent cell growth and productivity, however, the acidic fraction was higher with this formulation than allowed by target criteria (<10%). The formulation H-1 containing soy hydrolysate and recombinant protein, produced IgG with a very low % acidic fraction. However, despite good cell growth, H-1 exhibited poor IgG production. While additional supplements (H-2, H-3, H-4) improved the productivity of H-1, they also increased the % acidic fraction.

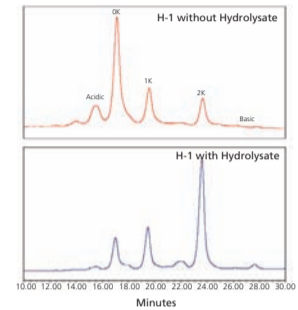


Figure 7: CEX HPLC chromatograms of IgG produced in H-1 media that had been prepared with or without hydrolysate supplementation. Since the H-1 formulation used in the previous screening experiment contained hydrolysate, we conducted an additional experiment to determine if the desired low % acidic fraction was due to the hydrolysate or the basal formulation in H-1. The dramatic increase in % acidic fraction from 3.3% in the presence of hydrolysate to 16.1% in its absence clearly demonstrates that the desired low acidic fraction resulted from the hydrolysate supplement rather than the basal media in the H-1 formulation. Comparison of these chromatograms also demonstrates that addition of hydrolysates can dramatically alter the ratio of the OK and 2K peaks.

Sample	Feeding Supplements			Peak VCD (x 10 ⁶ /ml)	IgG Titer (µg/ml)	% Acidic Fraction
	Hydrolysate	Vitamins	AA			
C1A/C1B	--	--	--	5.3/5.1	499/502	11.1/11.2
C2A/C2B	Std	Std	Std	7.9/7.8	692/629	11.3/11.5
11/16	1X	0X	0X	4.1/3.6	479/481	11.9/11.1
2/9	1X	1X	0X	3.8/3.4	465/479	11.2/10.8
3/23	1X	2X	0X	4.0/3.4	466/449	11.6/11.7
12/15	1X	0X	1X	5.8/5.4	925/935	8.4/8.5
30/36	1X	1X	1X	5.8/4.5	820/612	9.1/11.1
8/21	1X	2X	1X	5.7/5.4	785/778	9.9/9.9

Table 4: Summary of fed batch experiment using S-3 + hydrolysate as the basal media. Based upon the observed effect of hydrolysate on reducing the % acidic fraction, we evaluated fed batch conditions that could optimize the % acidic fraction for our high producing basal media, S-3. Shake flasks were seeded with the Sp2/0 cells at a density of 2 × 10⁵ cells/ml. Cultures were initially fed with a variety of supplements when cell densities reached 2 × 10⁶ and 4 × 10⁶ cells/ml, and then every other day following the second feed for the duration of the experiment. All conditions were run in duplicate and data is shown for each culture. Control cultures consisted of no feeds (C1) and a standard feeding supplement (C2). The optimal feed supplement in this study was a mixture of hydrolysate and amino acids. This combination resulted in high viable cell densities and exceeded the target productivity (>800 µg/ml). This feed supplement also resulted in the desired level of acidic fraction (<10%) with an overall CEX HPLC profile that closely matched the reference material.

Summary

Our studies have demonstrated that changes in media formulations can have dramatic effects on the charge heterogeneity of a recombinant MAB expressed in Sp2/0 cells. Using a CEX HPLC assay, we detected significant changes in the ratio of the OK, 1K, and 2K peaks when the MAB was produced in different media formulations. In addition, the amount of IgG eluting within the acidic fraction could vary dramatically. Changes in the % acidic fraction were due to several post-translational modifications including differences in the amount of terminal sialic acid on the oligosaccharide structures of the IgG molecules. By using the CEX HPLC assay throughout the media optimization project, we were able to achieve target productivity levels while maintaining the desired CEX HPLC profile. This approach required relatively high throughput Protein A purification of culture supernatants so that approximately 100 samples could be processed and loaded onto the HPLC in a single run. Our results clearly illustrate the need for careful monitoring of product quality during process development.

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