

Proteomic Investigation of Recombinant CHO cells in High Density Culture

J.R. Cresswell, J.M. Zobrist, J. Wildsmith, N. Lin, R. Valdes-Camin, and M. Caple

Abstract

Bioreactor optimization is a complex task, involving both media and process development. This task is further complicated by variations in recombinant CHO cell lines. The intracellular effects of different media or reactor conditions are largely undetermined. Study of intracellular responses could provide information to improve recombinant cell performance in two ways. First, accumulated data could help predict requirements of specific cell lines, increasing the efficiency of media and process optimization. Also, such investigation may identify targets of cell engineering to increase performance of recombinant lines.

A proteomics approach was used to study the protein expression changes in a recombinant IgG-producing CHO cell line throughout a fed-batch culture. Growth, productivity and metabolism were monitored, and protein expression profiles of growth and production phases were analyzed by 2-dimensional gel electrophoresis. Differentially expressed proteins were selected using Phoretix™ 2D Expression software and identified using in-gel tryptic digest and MALDI-MS. This study provides insight into the intracellular processes affecting growth and productivity of recombinant CHO cell lines.

Materials and Methods

Bioreactor Culture

Stock culture of a recombinant IgG producing cell line was maintained in animal-component-free suspension culture under G418 selection. A 5-L bioreactor (B. Braun) was seeded at 7.5×10^4 cells/mL and maintained as fed-batch cultures until viability dropped below 80%. The culture was sampled daily and monitored for growth, viability, IgG productivity, and metabolic profile. Glucose and glutamine were fed to 3 g/L and 4 mM respectively when needed. A concentrated CHO feed (C1615) was delivered every other day beginning on day 5. Production of IgG was determined by an HPLC Protein G affinity assay.

Sample preparation

Cells (5×10^7) were extracted in 1 mL of extraction solution. The extraction solution was cellular and organelle membrane solubilizing reagent (C0356) with the addition of 10 mM spermine and 5 mM TCEP. The solutions were incubated for 1 hour with mixing at room temperature. Samples were centrifuged for 30 minutes at 16,000 RCF. Samples were reduced with tributylphosphine and alkylated with iodoacetamide using the ProteoPrep Reduction and Alkylation Kit (PROT-RA). Total protein concentration was determined using Bradford reagent (B6916). Samples were TCA precipitated using the ProteoPrep Protein Precipitation Kit (PROT-PR). Pellets were re-suspended in the extraction solution.

Separation by 2-DE

IPG strips (11 cm, pH 3–10, I3406) were rehydrated with the samples and were focused at 8,000 Volts for 85,000 Volt hours. The IPG strips were equilibrated for 20 minutes with IPG equilibration buffer (I7281) and SDS-PAGE was performed using 4–20% Tris-HCl precast gels (BioRad). The gels were electrophoresed for 10 minutes at 80 Volts followed by 70 minutes at 170 Volts. Protein bands were visualized in the gels using EZBlue gel stain (G1041). The gels were imaged using a Fluor-S™ Multimager (BioRad) and analysis of the gels was performed using Phoretix™ 2D Expression Software (Nonlinear Dynamics).

In-gel digestion

In-gel digestion of protein spots was performed using Trypsin Profile IGD Kit (PP0100). The tryptic digest was dried and the sample was brought up in a solution of 10 mg/mL α -cyano-4-hydroxycinnamic acid in 70% acetonitrile, 0.03% trifluoroacetic acid.

MALDI-MS analysis

Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) data was acquired in positive ion reflectron mode using an Axima-CFR+ mass spectrometer (Shimadzu Biotech).

Process Overview



- Recombinant IgG-producing cells cultured in bioreactor
- Cells harvested at selected timepoints
- Total protein samples extracted, reduced, and alkylated
- 250 µg total protein aliquots were TCA precipitated and resuspended



- 2-D gel electrophoresis
- Gels analyzed for changes in protein expression



- In-gel digestion of selected spots
- MALDI-MS of peptide fragments
- MASCOT search to identify proteins

Figure 1

Results and Discussion

Bioreactor Culture

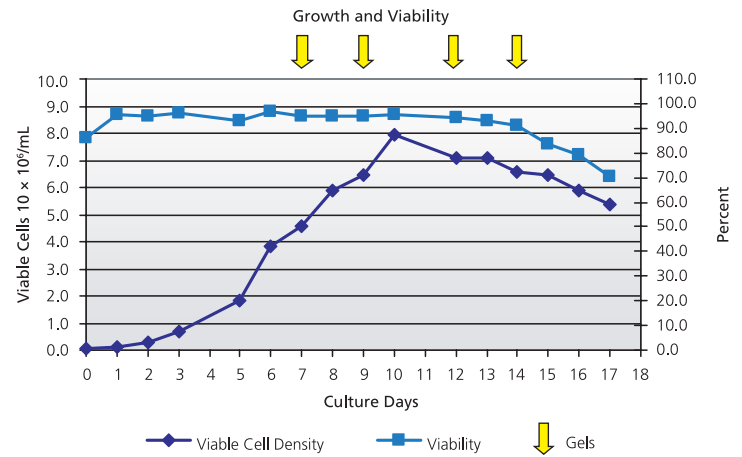


Figure 2: The bioreactor culture reached peak cell density of 8.0×10^6 cells/mL on day 10. Percent of viable cells remained above 90% through day 14. Culture was ended on day 18 when viability dropped below 70%. Harvested cells from days 7, 9, 12, and 14 were analyzed.

Growth and Production

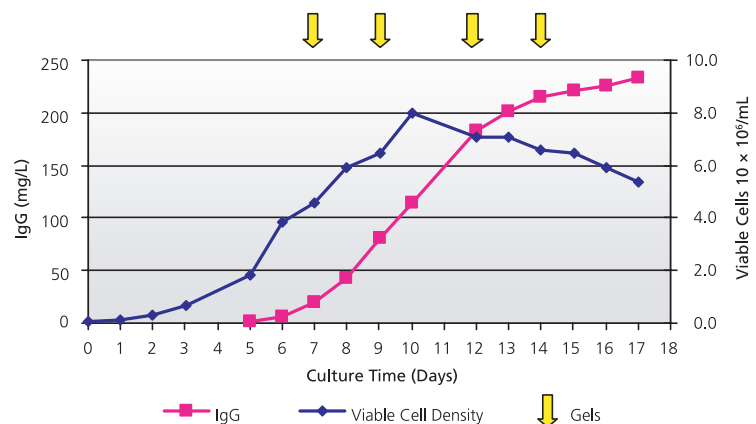


Figure 3: Recombinant IgG production became quantifiable around day 5 and increased rapidly through day 12. The rate of production then decreased sharply, although the culture maintained stationary phase for several more days. The days chosen for 2-DE analysis represent beginning, middle and end of IgG production for this fed-batch culture.

2-D Electrophoresis and Gel Analysis

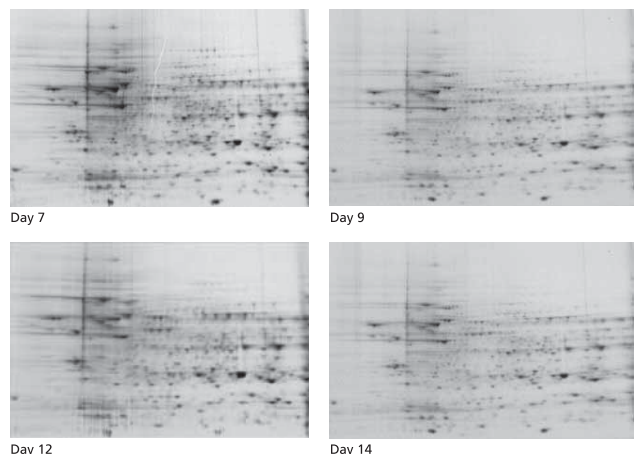


Figure 4: Processed total protein extracts from harvested cells were loaded onto 11-cm IPG strips (pH 3–10) for focusing. The focused strips were then run in the second dimension on 4–20% Tris-HCl gels. Two sets of gels were run for each time point in the culture.

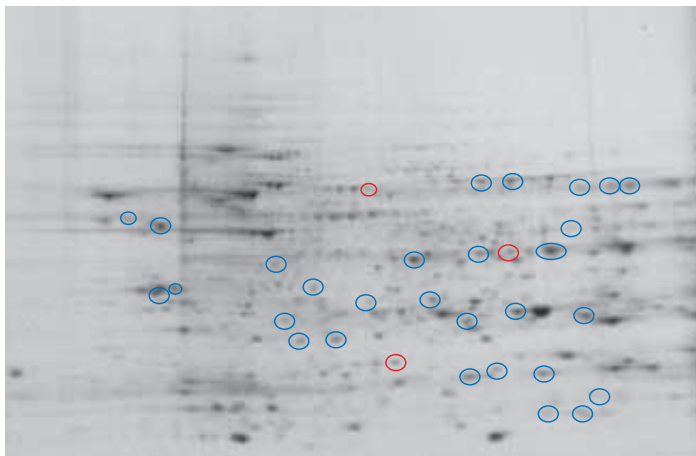


Figure 5. Stained gels were analyzed using the Phoretix™ 2D Expression Software. Protein spots with normalized volume change of 1.8 fold or greater were identified as up or down regulated. Approximately forty spots were chosen as potential candidates for identification and further analysis. Protein spots circled in blue showed significant expression changes and were submitted for identification by MALDI-MS analysis. Peptide mass fingerprint identification has been completed on the three spots circled in red.

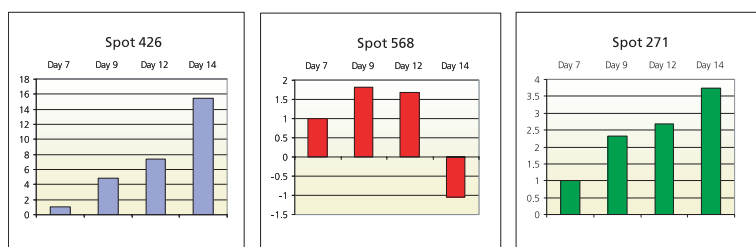


Figure 6. The expression profiles for three protein spots are shown in Figure 6. Spot volumes are normalized to day 7 to demonstrate the relative change over the course of the culture.

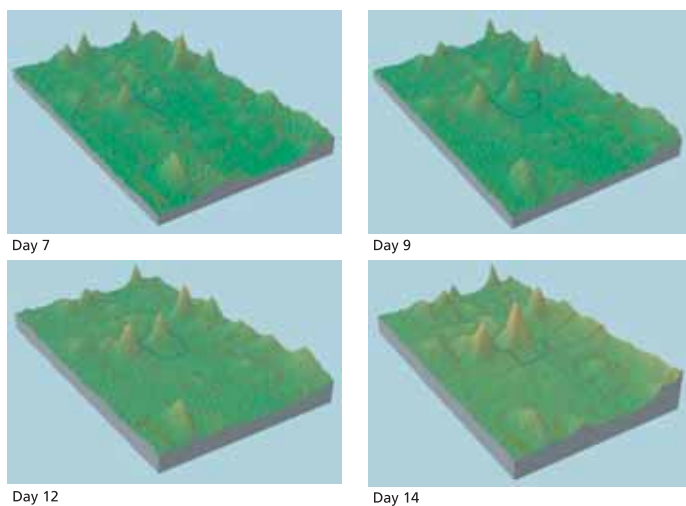


Figure 7. Contour images of spot 426 at days 7, 9, 12, and 14 demonstrate the progressive increase in protein expression relative to surrounding protein spots in each gel.

MALDI-MS Analysis

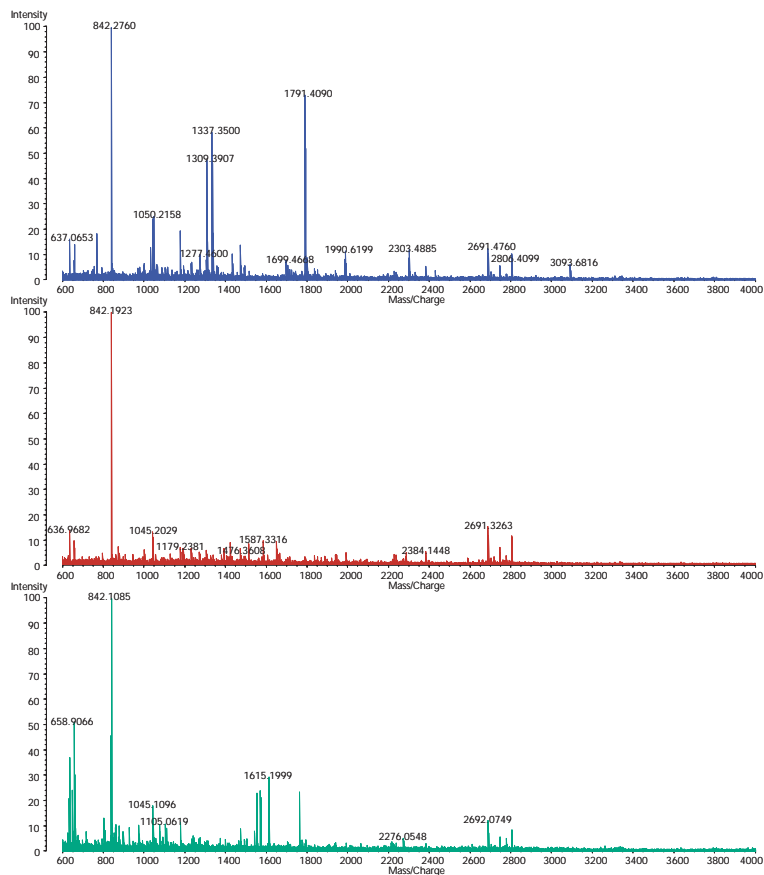


Figure 8: Gel spots of interest were excised from the 2-DE gels and digested with trypsin. The digested samples were analyzed by MALDI-MS to generate peptide mass fingerprints.

| Spot Number | Protein Identification | Mascot Score | Sequence Coverage |
|-------------|------------------------|--------------|-------------------|
| 426 | Cofilin | 71 | 77% |
| 568 | ERP57 | 79 | 49% |
| 271 | Aldose Reductase | 103 | 43% |

Figure 9: Peptide mass fingerprints from MALDI-MS analysis were submitted to MASCOT database for protein identification. Protein spots were identified with a significance threshold of $p < 0.05$.

Conclusions

- Proteomic analysis was successfully utilized to study changes in protein expression during a recombinant CHO bioreactor culture.
- Three proteins with altered expression levels were identified:
 - Cofilin**
 - ADF family protein.
 - Depolymerizes actin filaments
 - Upregulated throughout the culture
 - ERP57**
 - Also called GRP58. Endoplasmic reticulum chaperone protein.
 - Upregulated during log phase and early stationary phase.
 - Downregulated during late stationary phase. Coincides with decreased IgG production rate and decreased viability.
 - Aldose reductase**
 - Converts glucose to sorbitol in polyol pathway
 - Upregulated throughout the culture

Ongoing Studies

- MALDI-MS analysis of additional up and down-regulated proteins from 2-DE
- Compare protein expression profiles of high and low producing subclones
- Confirm identification and expression of proteins of interest with Western blots