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An omics approach to rational feed Enhancing growth in CHO cultures with NMR metabolomics and 2D-DIGE proteomics

Eric J.M. Blondeel[a], Raymond Ho[b], Steffen Schulze[a,c], Stanislav Sokolenko[a], Simon R. Guillemette[a], Igor Slivac[d], Yves Durocher[d], J. Guy Guillemette[e], Brendan J. McConkey[b], David Chang[f], Marc G. Aucoin[a,*]

[a] Waterloo Institute for Nanotechnology, Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario, Canada
[b] Department of Biology, University of Waterloo, Waterloo, Ontario, Canada
[c] Institute of Applied Biotechnology, University of Applied Sciences Biberach, Biberach, Germany
[d] Biotechnology Research Institute, National Research Council of Canada, Montreal, QC, Canada
[e] Department of Chemistry, University of Waterloo, Waterloo, Ontario, Canada
[f] Chenomx Inc., Edmonton, Alberta, Canada

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ABSTRACT

Expression of recombinant proteins exerts stress on cell culture systems, affecting the expression of endogenous proteins, and contributing to the depletion of nutrients and accumulation of waste metabolites. In this work, 2D-DIGE proteomics was employed to analyze differential expression of proteins following stable transfection of a Chinese Hamster Ovary (CHO) cell line to constitutively express a heavy-chain monoclonal antibody. Thirty-four proteins of significant differential expression were identified and cross-referenced with cellular functions and metabolic pathways to identify points of cell stress. Subsequently, 1D-^{1}H NMR metabolomics experiments analyzed cultures to observe nutrient depletion and waste metabolite accumulations to further examine these cell stresses and pathways. From among fifty metabolites tracked in time-course, eight were observed to be completely depleted from the production media, including: glucose, glutamine, proline, serine, cystine, asparagine, choline, and hypoxanthine, while twenty-three excreted metabolites were also observed to accumulate. The differentially expressed proteins, as well as the nutrient depletion and accumulation of these metabolites corresponded with upregulated pathways and cell systems related to anaplerotic TCA-replenishment, NADH/NADPH replenishment, tetrahydrofolate cycle C1 cofactor conversions, limitations to lipid synthesis, and redox modulation. A nutrient cocktail was assembled to improve the growth medium and alleviate these cell stresses to achieve a ~75% improvement to peak cell densities.

1. Introduction

The majority of recombinant therapeutic glycosylated biologics are produced in Chinese Hamster Ovary (CHO) cell lines (Ghaderi et al., 2012). Expression of these recombinant proteins draws energy and resources away from the endogenous cellular systems and infrastructure. Strategies to mitigate these stresses and maximize cell growth and productivity include optimization of growth medium and nutrient feeding (Kim et al., 2012, 2005; Kim and Lee, 2009; Lee et al., 1999; Ma et al., 2009; Sellick et al., 2011; Xing et al., 2011).

Growth medium, while historically relying on 'complex' components with somewhat unknown content such as serum and hydrolysates, has trended towards 'fully chemically defined' formulations. Switching to fully chemically defined growth medium (CDM) was motivated by high component costs, batch-to-batch ingredient variation, and most notably the potential for contamination from mycoplasma, prions, and viruses from fetal bovine serum (as reviewed by Butler, 2013). Non-animal derived complex components, such as hydrolysates derived from soy, wheat, gluten and yeast, while removing many of these concerns, remain 'black boxes' for process optimization. Approximately 450 commercial 'off the shelf' serum-free growth media are available on the market for var-

* Corresponding author at: Department of Chemical Engineering, University of Waterloo, Waterloo, ON, Canada.
E-mail address: marc.aucoin@uwaterloo.ca (M.G. Aucoin).

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ious animal cell types (as reviewed by van der Valk et al., 2010). However, commercial growth medium can be very expensive at production scale, and for any given bioprocess, a host of factors including but not limited to: the choice of cell-line and cell-line derivative, clone selection, gene-amplification strategy, the recombinant protein expressed, and production scale will impact cellular metabolism and how successful an 'off the shelf' growth medium will be, necessitating some level of tailored optimization.

Process and growth medium optimization can often be accomplished empirically in a 'blind' manner with multivariate methods, or by attempting to utilize simulation. However, omics methods such as proteomics and metabolomics provide an exciting opportunity to look under-the-hood of a bioprocess and make real determinations of cell stresses and system limitations for cells expressing recombinant proteins.

Omics methods have been well utilized to enhance culture growth by investigating a broad spectrum of nutrients and excreted metabolites through classical analytical chemistry methods, including liquid chromatography mass spectrometry (LC–MS) (Chong et al., 2009, 2010b; Ma et al., 2009; Selvarasu et al., 2012) and/or gas chromatography mass spectrometry (GC–MS) (Dietmair et al., 2012; Ma et al., 2009; Schaub et al., 2012; Sellick et al., 2011) as well as NMR-based methods (Aranibar et al., 2011; Bradley et al., 2010; Carinhas et al., 2013; Goudar et al., 2010; Read et al., 2013; Sokolenko et al., 2014). NMR possesses several advantages such as ease of sampling, which is rapid and non-destructive (as reviewed by Sokolenko et al., 2014). Further, NMR is useful for detecting volatile organic acid metabolites, which are not observable by some conventional HPLC and MS methods, and have only recently been reported in CHO cells by metabolomics researchers employing NMR (Aranibar et al., 2011; Bradley et al., 2010; Carinhas et al., 2013; Sokolenko et al., 2014).

In this work, a proteomics analysis by two dimensional in-gel electrophoresis (2D-DIGE) was conducted between a parental CHO cell line and a stably transfected derivative cell line expressing a single-domain chimeric heavy chain camelid-derived antibody (EG2-hFc) (Agrawal et al., 2012) to measure differential expression of endogenous cellular proteins. Subsequently, a metabolomics analysis of cell culture supernatant from cells stably producing EG2-hFc grown in CDM, by targeted profiling of 1D-^{1}H NMR spectra, was conducted to measure depleted nutrients and the accumulation of excreted metabolites. Together, these omics methods were utilized to evaluate the system stresses of the CHO cells and to tailor production growth medium to maximize the cell growth of the cultures. The production growth medium was supplemented with nutrients corresponding to four reported metabolic systems in CHO cells that were up-regulated after gaining the ability to produce EG2-hFc. These pathways related to anaplerotic TCA-replenishment, NADH/NADPH replenishment, tetrahydrofolate cycle C1 cofactor conversions, limitations to lipid synthesis, and redox modulation.

2. Materials & methods

2.1. Cell line

DXB11-derived CHO^{BRI}-1A7 cells, negative for dihydrofolate reductase (DHFR-), were transfected with pTT44-EG2hFc1 to stably express the 80 kDa single-domain chimeric heavy-chain monoclonal antibody EG2-hFc (Agrawal et al., 2012). Briefly, EG2-hFc cDNA was cloned into pTT44, a modified pTT5 vector (Durocher et al., 2002) containing a puromycin expression cassette, under the control of a cytomegalovirus (CMV) promoter. The parental strain of CHO^{BRI} cells was transfected with the linearized construct pTT44-EG2hFc1 using PEImax in 2 mL of CD DG44 media (Invitrogen, ON, Canada), and puromycin selection was applied (10 μg/mL) until the pool of resistant transfectants finally reached ~80 +% viability. Single cell dilution in 96-well plates was applied to isolate the best-producing clone, called CHO^{BRI}-1A7.

2.2. 2D-DIGE proteomics experiments

2.2.1. Cell culture

Culture supernatant samples from parental CHO^{BRI} cells and the newly established CHO^{BRI}-1A7 cell line that constitutively produces EG2-hFc were harvested after 8 days of batch cultivation. Six parallel cultures were carried out in 250 mL polycarbonate shaker flasks (Corning, NY, USA) for both cell lines with PowerCHO-2CD growth medium (Lonza, NY, USA). The shaker flasks, each containing 60 mL, were grown at 37 °C with 5% CO2, with an agitation speed of 120 rpm for eight days. Both cell lines and their replicates were grown under the same culture conditions. On day 8, culture supernatant samples were harvested and filtered via 0.45 μm membrane. Cell pellets were collected and kept frozen until analysis.

2.2.2. Cell lysis & protein extraction

Approximately 2×10^{7} cells were harvested from cultures after 8 days of batch cultivation and pelleted at 1000 rpm (~250 rcf) for 5 min and stored at −80 °C. Cell pellets were subsequently resuspended and washed three times with sterile ice cold 1X PBS solution. Following washing, cell pellets were resuspended in 500 μL of lysis buffer at 4 °C, containing 7 M urea, 2 M thiourea, 50 mM Tris pH 8.0, and 4% CHAPS (Fisher, ON, Canada). The lysis buffer also included 15 μL protease inhibitor cocktail (Sigma, ON, Canada). The cells in lysis buffer were subsequently vortexed and sonicated twice for 30 s using a Microson Ultrasonic Cell Disruptor (Misonix, NY, USA) at a power level of 4 at 4 °C. Following sonication, the samples were incubated on a rotator at 4 °C for 3.5 h to ensure complete lysis. The samples were then centrifuged for 30 min at 14,000 rpm (22,000 rcf) at 4 °C to remove insoluble cell debris. Supernatant was recovered and any further interfering substances were removed via an Amersham 2-D Clean-Up Kit (GE Healthcare, Uppsala, Sweden). Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad Laboratoris Ltd., ON, Canada) with bovine serum albumin (BSA) (Sigma, ON, Canada) utilized as the standard. Protein samples and BSA standards were measured in triplicate at an absorbance of 595 nm with a Varian Cary 50 Bio UV–vis Spectrophotometer (Varian, QC, Canada).

2.2.3. 2D differential In-Gel electrophoresis (2D-DIGE)

Six biological replicate gels were prepared for comparative proteomic analysis between CHO^{BRI}-1A7 and parental CHO^{BRI} cell cultures. Each of six gels contained one CHO^{BRI}-1A7 and one CHO^{BRI} sample replicate. A dye swap procedure was employed, where gels 1–3 used a Cy5 label for CHO^{BRI} and a Cy3 label for CHO^{BRI}-1A7, and gels 4–6 reversed the Cy3 and Cy5 labels; CyDye DIGE Fluor minimal dye (GE Healthcare, Uppsala, Sweden). 50 μg of each protein sample was labeled with 1 μL of 200 pmol/μL CyDye DIGE Fluor minimal dye. Following incubation with 10 mM lysine stop solution, samples were combined with rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, and 20 mM dithiothreitol (DTT) (Fisher, ON, Canada), and 0.5% immobilized pH gradient buffer ampholytes (GE Healthcare, Uppsala, Sweden). Protein samples in rehydration buffer were applied to Immobiline Drystrips, pH 3–10NL, 24 cm immobilized pH gradient (IPG) gel strips (GE Healthcare, Uppsala, Sweden) with a total volume of 450 μL per strip and incubated overnight.

Isoelectric focusing (IEF) for first-dimension separation was performed using an *Ettan IPGphor II system* (GE Healthcare, Uppsala, Sweden) at 20 °C for 24 h with the following voltage program: 100 V (1 h), step increase to 500 V (2 h), gradient to 1000 V (2 h), gradient