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Metabolic analysis of antibody producing Chinese hamster ovary cell culture under different stresses conditions

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Chinese hamster ovary (CHO) cells are commonly used as the host cell lines concerning their ability to produce therapeutic proteins with complex post-translational modifications. In this study, we have investigated the time course extra- and intracellular metabolome data of the CHO−K1 cell line, under a control and stress conditions. The addition of NaCl and trehalose greatly suppressed cell growth, where the maximum viable cell density of NaCl and trehalose cultures were 2.2-fold and 2.8-fold less than that of a control culture. Contrariwise, the antibody production of both the NaCl and trehalose cultures was sustained for a longer time to surpass that of the control culture. The NaCl and trehalose cultures showed relatively similar dynamics of cell growth, antibody production, and substrate/product concentrations, while they indicated different dynamics from the control culture. The principal component analysis of extra- and intracellular metabolome dynamics indicated that their dynamic behaviors were consistent with biological functions. The qualitative pattern matching classification and hierarchical clustering analyses for the intracellular metabolome identified the metabolite clusters whose dynamic behaviors depend on NaCl and trehalose. The volcano plot revealed several reporter metabolites whose dynamics greatly change between in the NaCl and trehalose cultures. The elastic net identified some critical, intracellular metabolites that are distinct between the NaCl and trehalose. While a relatively small number of intracellular metabolites related to the cell growth, glucose, glutamine, lactate and ammonium ion concentrations, the mechanism of antibody production was suggested to be very complicated or not to be explained by elastic net regression analysis.

[**Key words:** Metabolomics; Biotechnology; Chinese hamster ovary cells; NaCl and trehalose culture; Cell growth; Antibody production; Statistical analysis]

Based on the technological development of the last decade, metabolomics has become an essential tool for exploring the frontiers of emerging biotechnological fields, including progress with respect to sample preparations, analytical techniques, and informatics for data processing. Metabolomics has been used not only in plant science (1), yeasts-related research (2), nutrition research (3), food processing (4) but also applied to the mammalian system in relation disease diagnosis and therapeutic efficacy, pharmacology and pre-clinical drug safety assessment (5). Metabolomics has already been established to identify and quantify many metabolites in an organism sample under specified living conditions and has been applied to the analysis of different types of human diseases (e.g., cancer) with drug development (6–12) and recombinant therapeutic proteins (antibody) production (13–18).

Recombinant therapeutic proteins are engineered in the laboratory and have significantly changed the scenario of modern medical treatment of a variety of refractory illnesses of human diseases, including many types of cancer and orphan diseases (19,20). Therefore, exceedingly increasing the demand for production of recombinant protein therapeutics has imposed significant pressure on the biopharmaceutical industry for biological and medical research with a huge global market values. Through the last 25 years, mammalian cells are used premier host cells for the commercial production of therapeutic proteins that require complex post-translational modifications and assembly (21). One of the shortcomings for recombinant protein production by using mammalian cells is low specific productivity (22), but this problem can be overcome using Chinese hamster ovary (CHO) cells with gene amplification. Among many cell lines, including human embryonic kidney 293, human retina-derived PerC6, baby hamster kidney, mouse myeloma-derived NS0, mouse fibroblast (23–27), CHO cells are most frequently used as the host cell lines concerning their ability to produce therapeutic proteins with complex post-translational modifications and assembly, which are indispensable to biological functionality and safety (28–31).

Metabolic profiling is useful for an understanding of molecular mechanisms of how cells grow and produce recombinant proteins

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and for exploring production-limiting factors (32). A metabolite profiling approach enables to identify the metabolites which are depleted or accumulated during the culture and to develop strategies to increase the viable cell density and protein production (33,34). CHO cell cultures have various growth phases, including an exponential growth phase where the cell growth and lactate production rates are maximum, a low growth or stationary phase where the cell growth slows or stops and protein production increases, and a death phase where the cell viability declines (35). Metabolic profiles were used to characterize the dynamics of extra- and intracellular metabolites during the antibody production of CHO cell line in both growth and non-growth phases (35–37). The culture medium represents a suitable platform to analyze the extracellular metabolite profiles (38). It is well known that the glucose and glutamine are consumed by CHO cells, lactate is used as both a growth substrate and waste, and ammonium ion is secreted as waste (39–41). During the manufacturing process of therapeutic proteins, induction of the unfolded protein response genes is used for an effective strategy for preventing aggregation and enhancing the production of therapeutic proteins (42–44). Culture conditions are typically optimized for cell growth and protein production rather than for suppressed protein aggregation. On the other hand, it is of great interest to suppress protein aggregations for enhanced production. Trehalose was used as a chemical chaperone that provides protection against denaturations of several commercially available therapeutic antibodies and proteins (45). While the addition of trehalose increased the osmotic pressure in the medium and suppressed cell growth, it enhanced antibody production (46–49). It is reported that high osmolarity or addition of NaCl decreases cell growth, increasing antibody production in many hybridoma and CHO cell lines (50–58). At present, an important thing is to explore some differences in mechanism between trehalose and NaCl cultures.

In this paper, we measured the time course of the extra- and intracellular metabolome of the CHO–K1 cells in a batch culture to analyze how the addition of NaCl or trehalose affects phenotypes, such as cell growth, antibody production, and substrate uptake (product) rate. We identified several reporter metabolites that show distinct changes between the NaCl and trehalose cultures and some critical metabolites that affect the phenotypes. These metabolites can be key to reveal some mechanisms of cell growth and antibody production by CHO cells.

MATERIALS AND METHODS

Cell culture and sample analysis The CHO–K1 derived CHO HcD6 cell line, which produces recombinant humanized IgG, was used in this study (59). The CHO cells were cultured in serum-free TOp2 medium (Irvine Scientific, Santa Ana, CA, USA) containing 8 mM L-glutamine and 15 μg/mL puromycin in a CO_2 incubator shaker (Climo-shaker ISF1-X, Kühner AG, Birsfelden, Switzerland) at 37 °C under 5% CO_2 and 80% humidity. The rotation rate was 80 rpm (rotary shaking). Trehalose-containing cell culture was performed using TOp2 medium with 100 mM (Osmolality was 509 ± 1 mOsm/kg). Sodium chloride containing cell culture was also performed using TOp2 medium with 68 mM NaCl (Osmolality was 509 ± 1 mOsm/kg). Trehalose was kindly supplied by Hayashibara Co. Ltd. (Okayama, Japan) and osmolality also kindly measured by Hayashibara Co. Ltd. using osmometer (Micro-Osmometer Type13/13 DR-Autocal, CAMLAB, Cambridge, UK). Viable and total cell concentrations were determined using the Vi-cell XR (Beckman Coulter, Fullerton, CA, USA). Antibody concentrations in the cultivation medium were determined by a chemiluminescent assay based on AlphaLISA technology using Human IgG immunoassay kit (PerkinElmer, Wellesley, MA, USA). Glucose, glutamine, lactate and ammonia concentrations in the medium were determined by BioProfile400 biochemical analyzer (Nova Biomedical, Newton, MA). Analysis of intracellular metabolites were kindly performed by C-SCOPE analysis service (Human Metabolome Technologies (HMT) Inc., Tsuruoka, Japan). In brief, cell culture medium containing 4×10^6 cells was centrifuged, and the cell pellet was washed using 5% (w/w) mannitol solution. After centrifugation, the cell pellet was treated by 0.8 mL methanol, and internal standard solution (HMT) was added. After centrifugation, metabolites were extracted by removing protein using centrifugal filter devices (HMT). Extracted metabolites were analyzed by capillary electrophoresis (CE)-mass spectrometry (MS).

Data processing The raw datasets are provided by Table S1 in the supplementary file 1. We used unit unification for the all extra- and intracellular metabolites are molar concentration (mM) for the control, NaCl and trehalose cultures. In the control culture, extracellular metabolites were measured on days 0–10. In the NaCl and trehalose cultures, they were measured on days 0–14. Intracellular metabolites were measured on day 3, 5, 7, and 9 in the control culture. In the NaCl and trehalose cultures, they were measured on day 5, 7, 9, 11, and 13. Two independent measurements were performed. We removed the time-course series of metabolites that have non-detected values and missing concentrations. The resultant data including 69 metabolome time course data are defined as the matrixes as follows:

$$\mathbf{Xm_k} = (xm_k(i,j)) \tag{1}$$

$$\mathbf{Xc_k} = (xc_k(i,j)) \tag{2}$$

$$\mathbf{Ym_k} = (ym_k(i,j)) \tag{3}$$

$\mathbf{Xm_k}$ is the matrix of the concentrations of cell growth, antibody production, glucose, glutamine, lactate and ammonium ion in the medium, where a subscript of k indicates a culture condition (c: control (normal), s: NaCl, t: trehalose), i is the index of VCD (cell growth), antibody production, glucose, glutamine, lactate and ammonium ion, and j is the index of the measured time. $xm_k(i,j)$ is the concentration in the medium under a culture condition of k at an element of i and j. $\mathbf{Xc_k}$ is the matrix, whose element $xc_k(i,j)$ is the intracellular concentrations under a culture condition of k. $\mathbf{Ym_k}$ is the matrix of the specific rates for cell growth, antibody production, glucose, glutamine, lactate and ammonium ion in the medium under a culture condition of k. A specific rate $ym_k(i,j)$ is given by:

$$ym_k(i,j) = \frac{xm_k(i,j+1) - xm_k(i,j)}{(day(j+1) - day(j)) \times xm_k(i,j)} \tag{4}$$

Principal component analysis Principal component analysis (PCA) is an unsupervised modeling of an orthogonal linear transformation to convert a set of possibly correlated variables (metabolites) into a smaller set of uncorrelated variables called principal component's (PC's). The PCA variance weights are visualized by the Pareto chart that contains both bars (individual values in descending order) and a line graph (cumulative total). The greatest variance within the data by any projection is explained on the first coordinate (PC1) and the least variance is explained by subsequent PC's (PC2, PC3, and so on). The similarities among metabolite dynamics are displayed by a biplot. In the overview, PCA is used to identify the dominant patterns in data, such as groups, outliers, and trends. In this analysis, the PCA was performed in extra- and intracellular metabolites by using ($\mathbf{Xm_c}$,$\mathbf{Xm_s}$,$\mathbf{Xm_t}$) and ($\mathbf{Xc_c}$,$\mathbf{Xc_s}$,$\mathbf{Xc_t}$), respectively.

Classification and hierarchical clustering The dynamics for the intracellular metabolome ($\mathbf{Xc_c}$,$\mathbf{Xc_s}$,$\mathbf{Xc_t}$) in the three different (control, NaCl, trehalose) cultures was classified by the pattern matching method and hierarchical clustering (HC) analyses. In the pattern matching, the dynamics of intracellular metabolites was divided two phases: the increase phase (I) and decrease phase (D). The similarities of the I and D patterns were calculated among all metabolite dynamics. The HC analysis is best to explain the groups of the data over a variety of scales, which creates the cluster or dendrogram. In this analysis, the complete linkage agglomerative HC was performed for ($\mathbf{Xm_c}$,$\mathbf{Xm_s}$,$\mathbf{Xm_t}$) and ($\mathbf{Xc_c}$,$\mathbf{Xc_s}$,$\mathbf{Xc_t}$).

Volcano plot A volcano plot is a type of scatter plot that is used to identify most-meaningful changes of metabolites that are considered both statistically significant (above the p-value line) and differentially expressed (outside of the fold change lines). It makes clear the relationship between the p-values of a statistical test and the magnitude of the difference in expression values of the samples in the groups. The log fold change is plotted on the x-axis and the negative log10 p-value is plotted on the y-axis. Therefore, the volcano plot measures the magnitude of the change, which enables quick visual identification of those data-points (metabolites) that display large-magnitude changes by the corresponding p-values (at the 5 % level of significance, i.e., having $p < 0.05$). In this analysis, the volcano plot was performed for the intracellular metabolomes ($\mathbf{Xc_c}$,$\mathbf{Xc_s}$,$\mathbf{Xc_t}$). In this study, statistically significant and differentially expressed metabolites are named reporter metabolites.

Least absolute shrinkage and selection operator and elastic net Variable selection by a regression model is extremely important and has to select a parsimonious set for the efficient prediction of a response variable from a large collection of possible covariates. Least absolute shrinkage and selection operator (LASSO) is an innovative variable selection method for regression and estimate a smaller model, with fewer predictors than ordinary least-squares and ridge regression. LASSO was used to identify for critical metabolites (factors) that affect cell growth, antibody production, glucose, glutamine, lactate, and ammonium ion concentrations. Elastic net is a related technique of LASSO and outperforms when the data are highly correlated. Elastic net technique solves the general regularization problem given as follows: