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Proteomic Profiling of Secreted Proteins from CHO Cells Using Surface-Enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry

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Chinese hamster ovary (CHO) cells are the most commonly used host cell line for the production of recombinant biopharmaceuticals. These biopharmaceuticals are typically secreted from CHO cells and purified from harvested cell culture media. The purpose of this study was to investigate changes in the secreted proteome of CHO cells over the various stages of the growth cycle using Surface Enhanced Laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS). Conditioned media samples were collected each day over a 6 day growth period from CHO-K1 cells grown in low serum (0.5% FBS) conditions in monolayer culture. Samples were profiled on a number of ProteinChip arrays with different chromatographic surfaces. From this study, 24 proteins were found to be differentially regulated at different phases of the growth cycle in CHO-K1 cells, when profiled on two chromatographic surfaces, Q10 (anionic) and IMAC30 (metal affinity) ProteinChip arrays.

Introduction

Chinese hamster ovary cells are the most commonly used host cell line for the production of protein therapeutics. The application of global expression profiling methods such as proteomic analysis to mammalian cell culture processes is an important new tool for process and cell line development (*1*). Protein profiles generated from both intracellular and extracellular samples from bioreactors may give insights for genetic intervention to possibly create better host cell lines, or even to provide clues to more rational strategies for process development (e.g., media design). For these reasons, secreted host cell proteins potentially contain a rich source of information that may be exploited to improve process performance.

Depending on the nature of the product and culture process, crude recombinant proteins are typically harvested in the late exponential or the early stationary phase of growth and are subjected to a multistep purification process to purify the recombinant product from the culture medium, which also contains contaminants such as host cell proteins and nucleic acids (*2*). During the developmental process, removal of CHO cell-derived proteins from the recombinant product is monitored using multiproduct immunoassays. Such immunoassays are developed by raising antibodies to a single CHO protein preparation, usually from nonproducing CHO cell lines (*3, 4*).

Most of the proteomic studies to date have concentrated on profiling cell lysates from high-producing CHO and other producer cell lines such as NS0 to gain an understanding of the biology of these cells to improve productivity (*5−9*). Few studies to date, however, have explored the proteome of extracellularly secreted proteins from mammalian cell lines. Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) is a high-throughput proteomic method that uses chromatographic surfaces (e.g., cation/anion exchange, metal affinity, hydrophobic) to retain proteins on ProteinChip arrays based on their physicochemical characteristics. Retained proteins and peptides are then analyzed using a ProteinChip Reader to record the time-of-flight and calculate the accurate molecular weight of proteins/peptides in the form of a spectral map containing mass-to-charge ratios (*m/z*) and intensities corresponding to each bound proteins/peptides. Biomarker wizard software analyzes the spectral map and detects differentially expressed protein/peptides with statistical significance. This technique is ideal for the analysis of small sample volumes and allows screening of low molecular weight proteins that are often difficult to separate on 2D gels (<20 kDa), and as such has been applied to profile biological fluids such as serum for biomarker discovery (review *10*). The technique has been successfully used to demonstrate that protein profiles are useful for the early detection of breast, prostate, and bladder cancers (*11−13*). The use of SELDI-TOF MS in mammalian cell culture processes has the potential to assess markers from very small cell-free samples, causing minimal perturbation of the fermentation process.

In this study, conditioned media samples from CHO-K1 cells cultured in low serum-containing medium (0.5% FBS) were investigated over time, representing the different phases of the growth cycle, i.e., lag, log, and stationary phase, using SELDI-TOF MS to monitor the differential expression of secreted host cell proteins. Proteins and peptides that can modulate cell growth in culture including endogenous growth factors and waste

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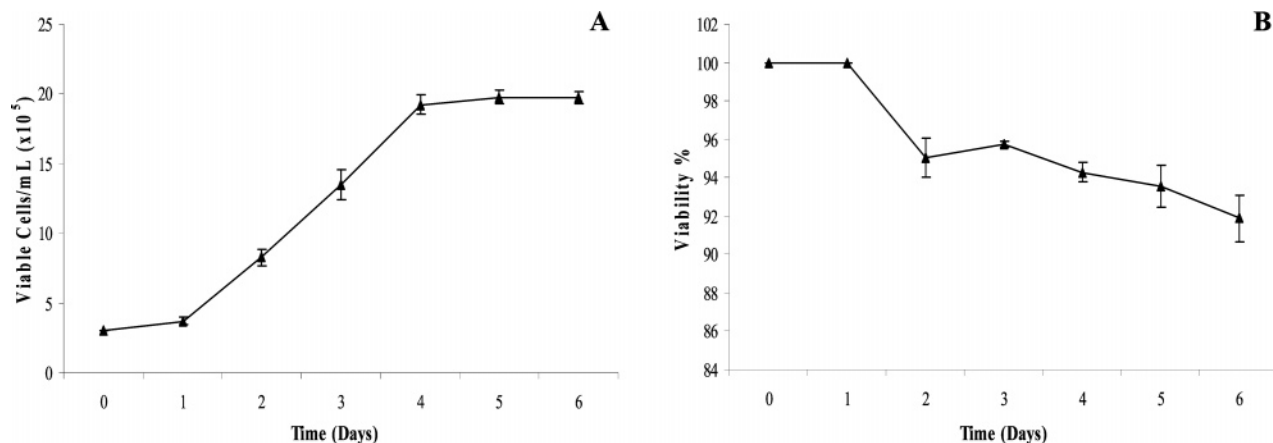
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Figure 1. Growth profiles of CHO-K1 cells adapted to growth in low serum (0.5%) medium in monolayer culture: (A) viable cell counts and (B) percentage viability percentage. Error bars represent the standard deviation calculated from data obtained from the experiment ($n = 4$).

products are also known to be excreted by the cells, therefore representing a valuable resource for use in potentially designing better culture processes and media. This technique may also be a useful way of monitoring the performance of a culture in a reproducible manner.

Materials and Methods

Chemicals. All culture media, serum, and chemicals used in the maintenance of the cell lines were obtained from Sigma Aldrich (Dublin, Ireland). IMAC30, CM10, H50, Q10 Protein-Chip arrays, all-in-one protein standard, and sinapinic acid were obtained from Ciphergen Biosystems (CA) and Bio-Rad (Alpha Technologies, Dublin, Ireland).

Cell Culture. The CHO-K1 cell line was obtained from the American Type Culture Collection (ATCC). Cells were routinely maintained at 37 °C using DMEM/Ham's F-12 (1:1) medium supplemented with 2 mM L-glutamine (Invitrogen), sodium pyruvate (Invitrogen), and 10% fetal bovine serum (Sigma). For adaptation of cells to low-serum conditions, cells were seeded at 3×10^5 cells/mL in three T-75 cm^2 culture flasks in 10 mL of medium and counts were performed every second and fourth day of culture. If cells achieved $\geq 1 \times 10^6$ cells/mL within 4 days of culture, they were transferred to culture medium with a reduced serum level. (The process involved adapting cells from 10% to 5% to 2.5% to 1% and then 0.5% FBS over time.) If not, the medium was replaced in the third flask and once cells become confluent, they were seeded again as stated above. The same workflow was followed until the cells were adapted to grow in 0.5% serum-supplemented medium. Adapted cells were seeded at 3×10^5 cells/mL in T-75 cm^2 culture flasks with 10 mL working volume of media and were grown for 6 days at 37 °C. Control flasks containing cell-free culture media were also processed in parallel at each time point. Cell counts were carried out every 24 h using the trypan blue exclusion method to assess viability. Conditioned media samples from four biological replicates were collected, centrifuged at 170g for 15 min to remove cellular debris, decanted into clean tubes, and stored at -80 °C until analysis.

Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF MS) of Conditioned Media from CHO-K1 Cells. Conditioned media samples were concentrated using 5000 MW cutoff centricons (Millipore). Prior to full profiling analysis, optimization studies were performed with the day 4 sample using 10 μg and 50 μg of extracted protein from conditioned media samples on four different chromatographic surfaces to see which protein concentration and Pro-

teinChip array gave optimal results. The following ProteinChip arrays were selected, an IMAC30 (immobilized metal affinity capture) activated with copper ions, CM10 (weak cation-exchange) with 20 mM Tris pH 4.5, Q10 (strong anion-exchange) with 50 mM Tris pH 8.0, and H50 (reversed-phase hydrophobic surface) with 10% acetonitrile/0.1% trifluoroacetic acid. The IMAC30 and Q10 ProteinChip 8-spot arrays provided better profiles under these conditions in terms of number and resolution of peaks and therefore were selected for the final profiling studies. IMAC30 arrays were coated with 5 μL of 100 mM CuSO$_4$ for a total of 30 min (2 × 15 min applications) and then rinsed with high-performance liquid chromatography (HPLC) grade water. The copper ions were charged by applying 50 μL of 100 mM sodium acetate for 5 min. Each array was placed in a Bioprocessor (Ciphergen) and the IMAC-Cu^{2+} arrays were washed twice with 250 μL of 250 mM sodium chloride containing 0.1% Triton X-100, and Q10 arrays were washed twice with 50 mM Tris pH 8.0. Ten micrograms of protein from each sample was diluted in binding buffers. Next, 250 μL of diluted samples was applied to the spots of the arrays. The arrays were placed on a shaker and gently agitated for 90 min to allow for interaction of proteins with the array surface. After the samples were removed, the IMAC-Cu^{2+} arrays were washed twice with 250 μL of 250 mM sodium chloride containing 0.1% Triton X-100, and Q10 arrays with 50 mM Tris pH 8.0 for 5 min, followed by a brief HPLC-grade water wash. Aliquots (0.8 μL) of saturated sinapinic acid (SPA; dissolved in 50% acetonitrile containing 0.5% trifluoroacetic acid) were then added to each of the spots which were then allowed to dry, and the process was repeated. The IMAC-Cu^{2+} and Q10 ProteinChip arrays were then analyzed in a Ciphergen Series PBS-IIC ProteinChip System, and time-of-flight (TOF) data were generated by averaging a total of 220 laser shots collected at a laser intensity of 200, a detector sensitivity of 8, and molecular mass range from 5 to 20 000 Da for the low molecular weight range and 20 000 to 100 000 Da for the high molecular weight range.

Analysis of SELDI-TOF MS Spectra. Molecular weights were calibrated externally using an all-in-one protein standard. All data was analyzed using Biomarker Wizards Software, version 3.1 (Ciphergen Biosystems). After automatic baseline noise correction, all of the spectra were normalized together by the "total ion content" method as described by the manufacturer, i.e., with an m/z between 5 000 and 100 000. The peaks with an m/z value <5 000 were excluded, as these peaks were mainly ion noise from the matrix (sinapinic acid), and 5 kDa
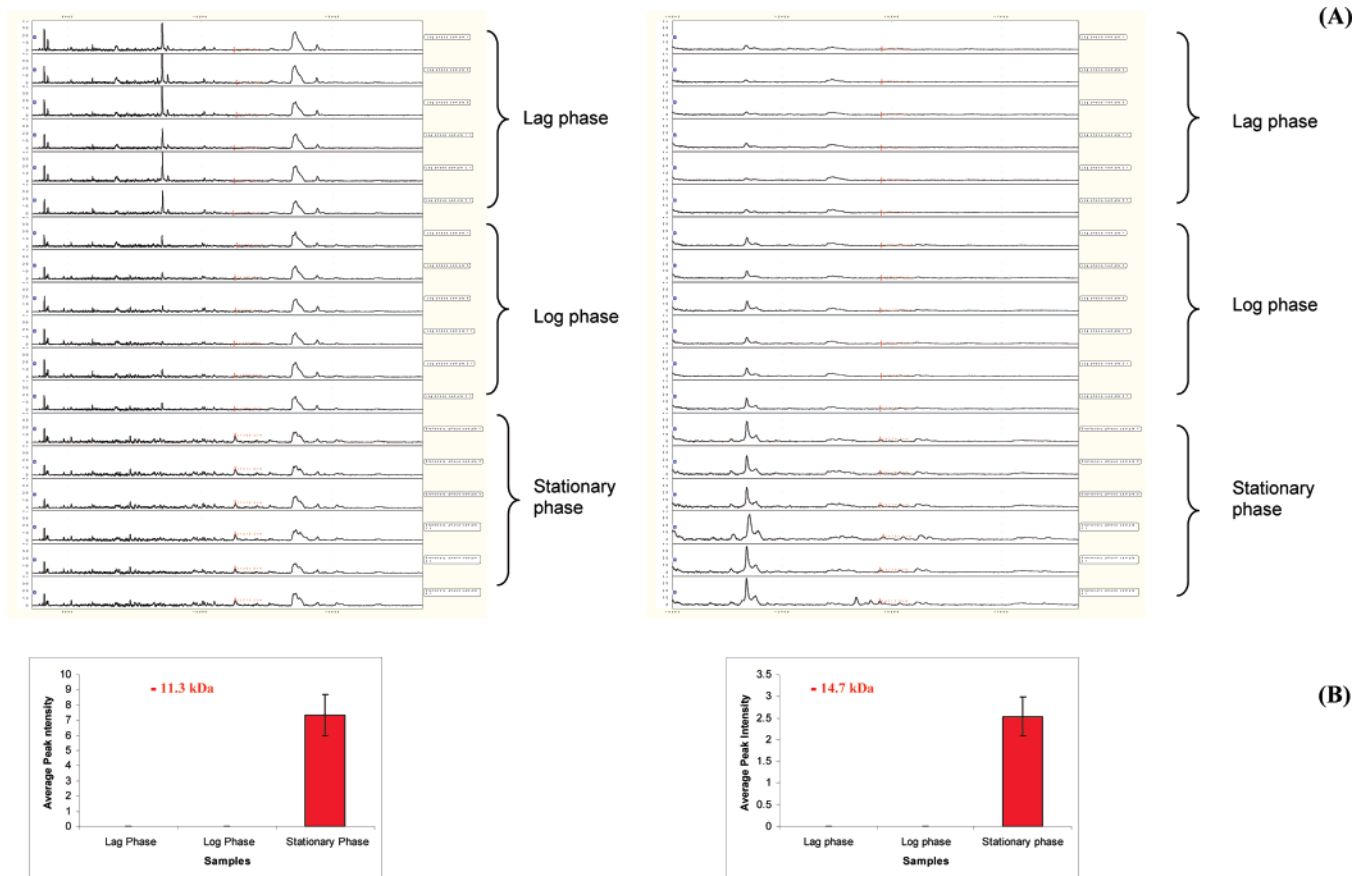
Figure 2. SELDI-TOF MS profiling of day 1 (from lag phase), day 3 (from log phase), and day 5 (from stationary phase) conditioned media from CHO-K1 cells. (A) Three biological replicate samples from each time point were profiled, along with a technical duplicate from each sample (total of 6 samples per time point) to demonstrate the reproducibility of the method for profiling conditioned media from CHO-K1 cells. (B) Graph showing the comparison of the average peak intensities for the 11.3 kDa protein on the Q10 array and the 14.7 kDa protein on the IMAC30 array.

cutoff centricons were used to concentrate the samples in this analysis. Peak clusters were generated by automatically detecting qualified mass peaks with a signal-to-noise ratio (S/N) >5 in the first pass, completed with a second-pass peak selection of S/N >3, with a 0.3% mass error for 5 000–20 000 Da, and the same for 20 000–100 000 Da. Statistically significant peaks were considered to be those with p <0.05.

Results and Discussion

Growth of CHO-K1 Cells in Low Serum Medium. CHO-K1 cells were grown in reduced serum medium (0.5% FBS) over 6 days of culture. Four biological replicate flasks were set up for each time point. Cells were counted and conditioned media samples were collected from each flask every 24 h. Control flasks containing cell-free media were also set up at each time point to account for serum proteins that may be degraded to peptides over time in culture media. A 0.5% serum background is suitable for SELDI-TOF MS profiling studies. Figure 1 shows the growth of the cells over 6 days in culture, clearly showing the different stages of growth (lag, logarithmic, and stationary phases).

Reproducibility of SELDI-TOF MS for Profiling of Conditioned Media from CHO-K1 Cells. Three biological replicate samples of conditioned media representing lag, log, and stationary phases of culture were profiled, along with a technical duplicate of each sample, to assess the reproducibility of SELDI-TOF MS for profiling conditioned media samples from CHO-K1 cells. One time point was selected from each of

the growth phases: day 1 from the lag phase, day 3 from the logarithmic phase, and day 5 from the stationary phase of growth. Figure 2A shows the SELDI-TOF MS profiles for each of these six samples at the three selected time points from different phases of the growth cycle. Figure 2B shows the increased expression of two peaks: one at m/z 11 347 (an 11.3 kDa protein) on the Q10 array and a second peak at m/z 14 735 (a 14.7 kDa protein) on the IMAC30 array. From this experiment, coefficients of variance (CV) values were calculated from the intensity readings from five randomly selected peaks from each conditioned media sample from each of the ProteinChip array surfaces. The CV values ranged from 11.42% to 20.7% for the Q10 array, and from 8.05% to 17.4% on the IMAC30 array, demonstrating the reproducibility of the technique. We also looked at interexperimental variability, and found that the average mean CV from the intensity readings from five randomly selected peaks across all experiments in the study was 22.68%. Recently SELDI-TOF MS-based serum profiling has been subject to criticism, with results published by some groups not being reproduced by others (*14–16*). However, the same level of criticism may not apply to cell culture-based analyses since multiple biological replicate samples from the same cell line are easily obtainable.

Expression Difference Matching Analysis of CHO-K1 Conditioned Media Over Time in Culture. Three biological replicate samples from each time point were profiled on each of the two surfaces, IMAC30 and Q10 ProteinChip arrays. Twenty four proteins/peptides were found to be differentially

Table 1. Protein/Peptide Peaks Differentially Expressed over Time in a Culture of CHO-K1 Cells on Q10 and IMAC30 ProteinChip Arrays

	Q10			IMAC30	
	m/z of peak	*p* value		*m/z* of peak	*p* value
1	5 753.43	0.042	1	5 109.49	0.037
2	5 913.81	0.029	2	5 261.48	0.045
3	6 191.46	0.032	3	5 864.21	0.026
4	7 346.27	0.022	4	6 065.27	0.043
5	7 542.91	0.048	5	8 118.21	0.032
6	8 492.30	0.027	6	8 591.44	0.034
7	9 746.85	0.034	7	14 735.28	0.029
8	9 882.51	0.031	8	17 975.62	0.022
9	10 883.34	0.044	9	20 343.55	0.023
10	11 347.01	0.046	10	20 853.77	0.021
11	12 168.91	0.047	11	25 798.46	0.037
12	13 593.09	0.038			
13	15 215.28	0.024			

expressed over the 6 days in culture with a *p* value <0.05. The results for these 24 peaks are shown in Table 1. Figure 3A shows the SELDI-TOF MS profiles from two of these differentially expressed peaks at *m/z* 8 118 and 14 735 from the IMAC30 chip. Figure 3B clearly shows that the 8.1 kDa protein (*m/z* 8 118 peak) is increased until day 4 of culture and then its expression is decreased over the next 3 days suggesting it is being consumed by the cells or being degraded over the latter stages of culture, possibly by proteolysis. The 14.7 kDa protein (*m/z* 14 735 peak) is accumulated over time in culture, appearing during the stationary phase, at days 5 and 6 of growth. Figure 4A shows the SELDI-TOF MS profile of two differentially

expressed peaks from the Q10 array at *m/z* 7 346 and 11 347. Figure 4B clearly shows that the 7.3 kDa protein (*m/z* 7 346 peak) is increased over time in culture from day 1 to day 6, and that the 11.3 kDa protein (*m/z* 11 347 peak) is again increasing over time in culture but accumulating from day 4 onward.

The majority of the proteins differentially expressed on the two chromatographic surfaces used in this study are increased over time in culture and are therefore most likely associated with increasing numbers of cells within the cultures, such as the 7.3 and 11.3 kDa proteins detected on the Q10 array (Figure 4). These proteins are accumulating in the media over time and are potential contaminants that may need to be purified from the recombinant protein being produced by the cells. Knowledge of these proteins may be useful for designing strategies for monitoring and removing host cell proteins from the recombinant protein product (*17*).

However, there are also a number of differentially expressed proteins that show a different type of profile that may not necessarily be due to increased expression over time in culture as a direct result of increasing cell numbers. For example, some of these proteins such as the 8.1 kDa protein differentially expressed on the IMAC30 surface are secreted at the early stages of culture, as demonstrated by the fact that it is absent in the control, cell-free flasks (Figure 3). This may represent a protein cleavage product or a secreted protein/peptide, and its expression increased as the biomass increases over time. This 8.1 kDa protein is then either consumed or degraded by the cell after
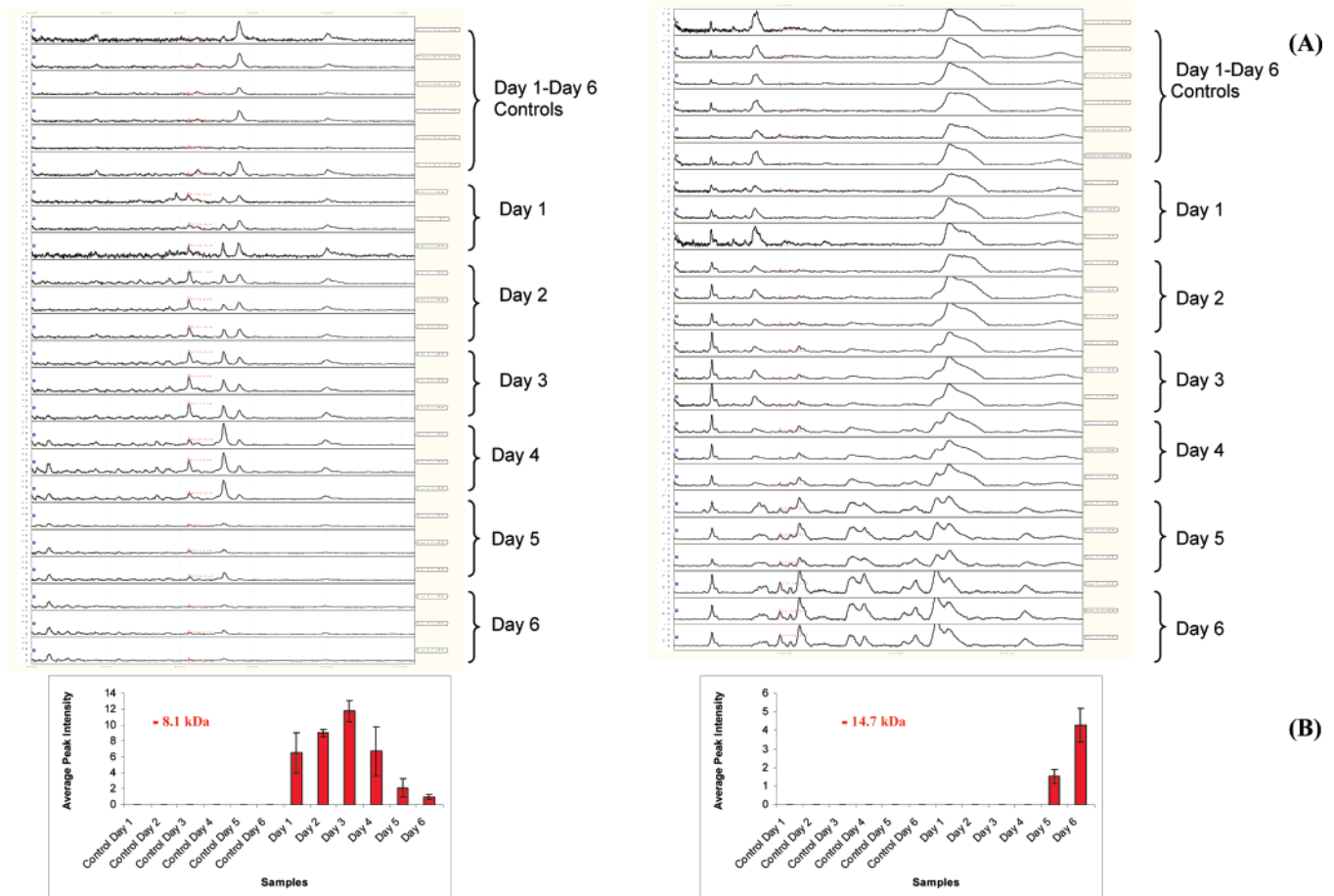

Figure 3. Differentially expressed proteins of CHO-K1 cells over time in culture. (A) Protein profiles on the IMAC-Cu^{2+} array of three biological replicate samples from each time point. Profiles from control, cell-free flasks at each time point are also shown, i.e., one profile from each of days 1−6. The differentially expressed proteins at 8.1 and 14.7 kDa in each of the samples from day 1 to 6, including the controls at each time point, are highlighted in red. (B) Graph of the comparison of the average peak intensities for 8.1 and 14.7 kDa protein/peptides over time in culture.
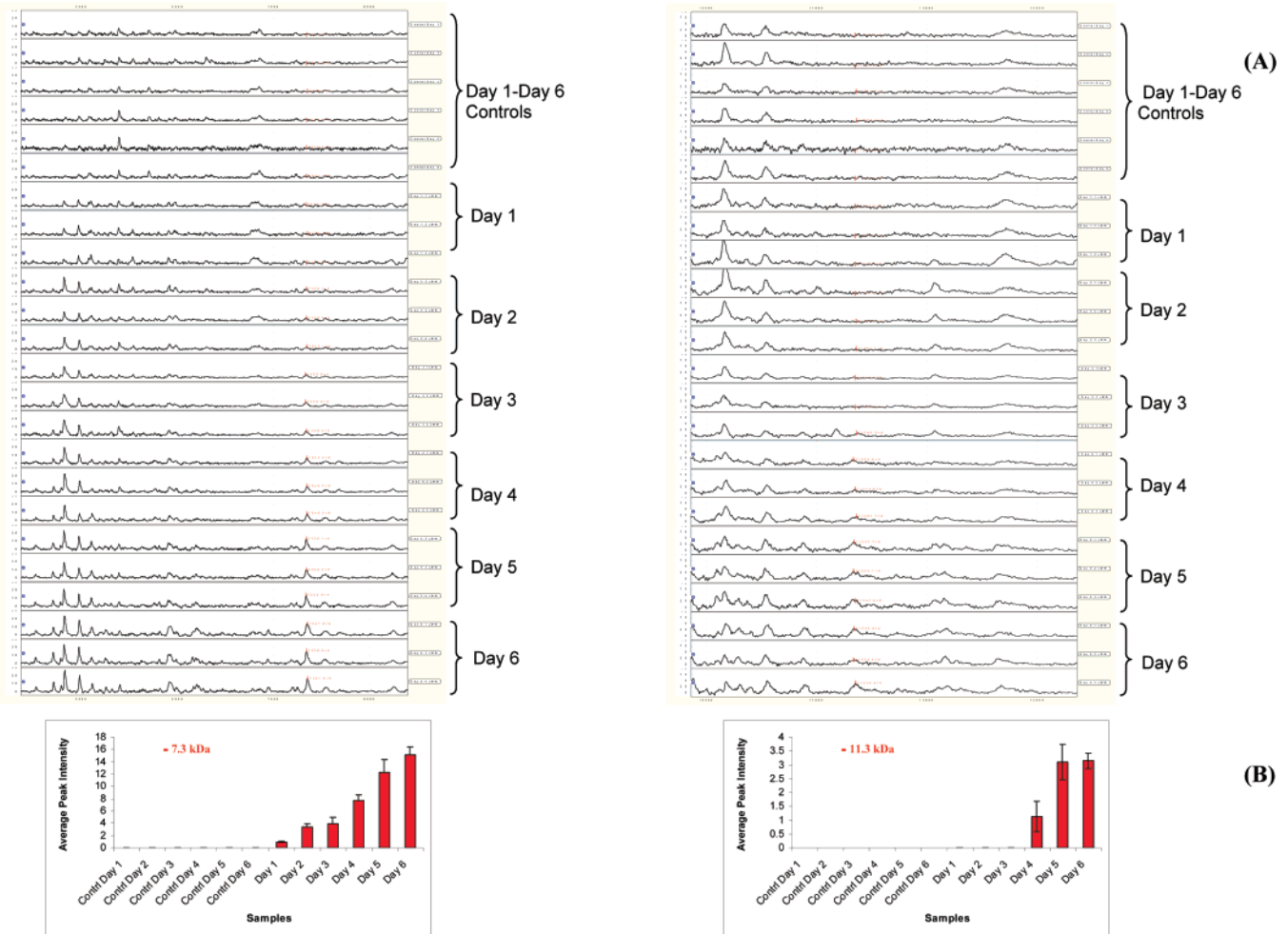
Figure 4. Differentially expressed proteins of CHO-K1 cells over time in culture. (A) Protein profiles on Q10 array of three biological replicate samples from each time point. Profiles from control, cell-free flasks at each time point are also shown, i.e., one profile from each of days 1−6. The differentially expressed proteins at 7.3 and 11.3 kDa in each of the samples from day 1 to 6, including the controls at each time point, are highlighted in red. (B) Graph showing the comparison of the average peak intensities for 7.3 and 11.3 kDa protein/peptides over time in culture.

day 4 of culture as the biomass levels increase with decreased levels over days 5 and 6 of culture, either through consumption as nutrients are used up or via the accumulation of proteases over time. In manufacturing recombinant proteins, the use of serum-free media for cell cultivation is preferred in order to meet quality and regulatory requirements but this may increase the exposure of the expressed product to proteolytic attacks that can result in product heterogeneity, denaturation, and loss of protein function. For example, an MMP pro-enzyme has been found to be released from CHO cells during the production phase of recombinant factor VIII, and as a result of autoproteolysis, a number of smaller, less specific MMPs were also detected (*18*). In another study, pro-MMP9 (gelatinase B) was identified from conditioned media of CHO-K1 cells grown in serum-free conditions (*19*). These studies and others (*20*) demonstrate that knowledge of proteolytic enzymes secreted by CHO cells is important for optimal bioprocess conditions.

This study has also demonstrated that SELDI-TOF MS can identify proteins that are secreted into the media and specifically detected during the latter stages of culture, such as the profile observed for the 14.7 kDa protein on the IMAC30 chip (Figure 3). In this case, the 14.7 kDa protein is first detected at day 5 and then increased on day 6, with no corresponding increase in cell numbers (Figure 1A). Over the same period, however, the cellular viability is beginning to decline (Figure 1B). The 14.7 kDa protein might be linked to cell death and are therefore released by the cells as the viability begins to decrease, as waste

products begin to accumulate in the media. This knowledge may be useful in designing cell-engineering strategies using antiapoptotic mechanisms to increase viability over time, especially for suspension cultures which are subjected to harsher conditions compared to cells grown in monolayer (*21*).

The next steps are to identify these 24 proteins/peptides using various enrichment techniques followed by mass spectrometry, and also to apply the technique to more bioprocess-relevant conditions such as serum-free suspension culture. Identification of protein markers by SELDI TOF MS remains challenging due to the fact that it is difficult to elute proteins/peptides of interest from ProteinChip arrays for direct mass spectrometry identification, as compared to 2D gel-based methods. Identification can involve fractionation and enrichment of proteins/peptides of interest using spin columns with the same chemistry as the ProteinChip array surfaces, followed by separation on 1D gels and identification by mass spectrometry (*22, 23*). However, despite these challenges, the use of SELDI-TOF MS to monitor culture progress for process optimization is attractive because of the small sample size and the rapid assay time, compared to other possible monitoring methods. Proteomic patterns (or protein "fingerprints") may also be very useful for distinguishing or predicting a "good" or "bad" culture with high sensitivity and specificity, and this approach is being used in discovering protein profiles that distinguish disease and disease-free states (*24*).

Conclusions

This proof of concept study to evaluate the potential use of SELDI TOF MS to discover process-related markers has yielded 24 differentially expressed proteins over time in the culture of CHO-K1 cells grown in monolayer in the low molecular weight range, <20 kDa. These proteins/peptides appear to have a number of distinct profiles over the course of the culture. These results suggest that secreted host cell proteins potentially contain a rich source of information that may be exploited to improve process performance, and a greater understanding of these secreted proteins is required in order to potentially achieve these aims.

Acknowledgment

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