

Investigation of CHO Secretome: Potential Way to Improve Recombinant Protein Production from Bioprocess

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Abstract

The demand for recombinant protein therapeutics is increasing worldwide and hence improvements in the overall yield of such products from bioprocess are of great interest to make them affordable. Chinese Hamster Ovary (CHO) cells are the most commonly used cell lines for large scale production of such high-quality (human-like) recombinant protein products. Typically, product is released, along other secretory proteins, by the cell into the culture media. These secreted proteins may have significant impact on cell growth, product quality & quantity during the production culture and the designing of strategies for efficient product purification. However, only few efforts have been made to date to explore these secreted proteins (the "secretome"), although significant technological advancements have been witnessed in the field of proteomics during the last two decades. Even from these, the majority of studies have identified a high proportion of intracellular- and non-secretory proteins in culture supernatants which could be possibly due to the unavailability of well-defined methodologies for sample collection & preparation for mass-spectrometry and/or followed data analysis using databases that contain less number of secretory proteins. As a result, secreted CHO proteins and their potential in regulation of recombinant protein production remain unexplored.

Therefore, the goal of this article is to provide an overview of the importance of secreted proteins in improving recombinant protein production from bioprocesses and to outline potential ways for their efficient investigation using proteomic approaches. This knowledge could help in increasing the overall yield from production processes.

Keywords: Chinese hamster ovary cells; Extracellular microvesicles; Exosomes; Proteomics; Secretome; Secreted-proteins

Introduction

Chinese Hamster Ovary (CHO) cells are the preferred host cell line for the production of recombinant protein therapeutics and monoclonal antibodies, accounting for more than 70% of all current therapeutics with over \$99 billion in market value [1,2]. This is mainly because of the capabilities of CHO cells to perform human like/compatible post-translation modifications which are required to attain the bioactivity of these recombinant proteins and also because of its adaptability to different culture conditions (adherent, suspension, serum/protein-free and scalable from microliter to thousands of liters etc.) in order to achieve higher yield [3]. Though CHO cells have been successfully scaled up to produce 5-10 g/L biologics [3], further improvements in the production capabilities of bioactive recombinant proteins are of eminent importance to meet the global demand at affordable cost. This goal, with the available cell culture platforms, could be achieved mainly by: 1- improving production capability of cells in culture (improved cell specific productivity, cell density and culture longevity), 2- minimizing product degradation and heterogeneity; 3- improving product purification process. The secreted proteins are known to impact all of these parameters; however only a few CHO studies have been performed to-date to reveal their secretome and hence an important area of the proteome, the cellular secretome, largely remained unexplored [4,5]. Therefore in this article, we have discussed the potential of secreted proteins to improve yield from production culture as well as factors that may impact the investigation of secreted proteins in culture media since knowledge of these proteins is critical for developing rationale cell engineering and media formulation approaches to maximize yield and minimize product degradation.

CHO Secretome

The secretome generally refers to the collection of proteins that are secreted and/or released from the cell during the different phases of culture (lag, log, stationary and decline/death). It may contain numerous substances: (1) -peptides/proteins that regulate cell-to-cell and cell-to-extracellular matrix interactions and could affect cell growth and productivity; (2) - proteolytic enzymes which may contribute to degradation of the secreted recombinant protein product in the culture; and also, (3) - host cell proteins whose knowledge could play important role in developing strategies for efficient purification of the product from culture media. The impact of each are as discussed below-

Impact of secreted proteins on CHO growth and productivity

It was known for a long time, but never systematically studied, that the addition of condition medium to cells increases survival and clonal growth. Recently, eight growth factors, including Fibroblast Growth Factor (FGF), Hepatocyte Growth Factor (HGF), Leukemia Inhibitory

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Factor (LIF) and Vascular Endothelial Growth Factor C (VEGF-C), were identified to be secreted by CHO cells into the media [4]. Supplementation of these factors into the culture media was observed to stimulate growth even at low cell density significantly (~48%). Colony Stimulating Factor-1 (CSF1), which is involved in regulation of cell proliferation, has also been identified in the conditioned media from antibody-producing CHOK1SV cells [6]. Many reports have shown secretion of transferrin from actively growing primary or established cell lines [7,8]. Transferrin alone does not promote survival or proliferation of CHO cells; however, in the presence of IGF-1, it does [9]. Exogenous transferrin was also found to improve the growth and survival of CHO DG44 in another study [10]. The knowledge of such growth-regulatory proteins is of great importance as it may help in rationale cell engineering and media formulation to improve performance of the process since higher numbers of viable producers in culture leads to higher productivity.

Impact of secreted proteins on product quantity and quality

An anonymous assortment of proteolytic enzymes is expected to occur amongst the vast milieu of Host Cell Proteins (HCPs) present during the production culture and to be harvested along with the product in the cell culture media which could lead to undesirable proteolytic-degradation of product reducing the overall yield [11]. MMP3, MMP10, MMP12 and cathepsin-B have also been reported in the culture media during production culture [5,11]. A secretory protein, fibronectin, which induces secretion of Matrix Metalloproteinase (MMP-9), has been identified in the conditioned media [12]. The presence of these enzymes in the conditioned media could pose risk for proteolytic-degradation of the product. Although during product-purification steps, typically HCP content (along with proteolytic enzymes) is reduced to acceptable levels, it is quite possible that certain proteolytic enzymes/HCPs may bind to and co-elute with the product across individual purification steps and could finally be there with the product to impact its quality and/or quantity. A significant decrease in the quantity (~60%) of a therapeutic monoclonal-antibody produced in CHO has been observed due to the residual proteolytic enzyme activity in purified product (final drug substance) over only 20-days of monitoring [13]. However, case studies showing that active proteolytic enzymes can carry through an entire purification process to reside in the final product and impact product stability are scarce. Thus, the knowledge of the proteolytic enzymes present in culture media is of great value for developing and/or implementing use of respective inhibitors to minimize potential degradation of the product and developing efficient bioprocess for higher yield. Alternatively, with enhanced tools of targeted cell engineering now available (e.g., CRISPR or ZN-fingers), the respective enzyme genes could be knocked out to generate a degradation resistant CHO cell line.

Moreover, secreted peptides/proteins also contribute to the accumulation of waste products in culture and could alter pH and osmolality, especially during the later stage of culture to ultimately initiate apoptosis [14,15]. This could impact the micro-environment in culture and could adversely affect culture growth and product quality (by altering post-translational modifications) during the production process [16-18].

Host cell proteins (HCP) and product purification

Secreted peptides/proteins are also important component of HCPs. They can pose potential safety risks for patients, including immune reactions and adjuvant activity, if not removed from the final product during down-stream processing [13,19-21]. The purification of the

final product is the most costly part of biopharmaceutical production (it may cost up to 80% of the total cost). The most common method for HCP removal is affinity chromatography and the purified product is assessed for purity using immunoassays [22-24]. The antibodies used in immunoassays are raised against a total cell protein mixture of non-product-expressing cells and hence measure the population of non-product proteins. However, it is important to question if HCP can be appropriately monitored using the same immunoassay (which is generated against the HCP expressed in a specific phase of growth of a specific cell line/clone) for monitoring HCP in culture harvested at different phase and/or from different CHO cell lines [22,25]. This is because the affinity of the antibody to HCP may vary following change in nature and composition of HCP at different phases of growth (e.g. logarithmic or stationary) and hence could under/over-qualify the product [23]. The composition of HCP between DG44 and CHO-S has already been shown to be significantly different; suggesting the need of cell-line specific modification in the purification protocols [5]. Moreover, secreted proteins are typically under-represented in the immunogen since antibodies for such assays are generated using cell lysate (intracellular proteins). Recently, fragments of secretory and also intracellular proteins were observed to be co-purified with the end-product indicating the inefficiency of the immunoassays in assessing HCP in the final product [19,26,27]. Moreover, these assays have no capacity to identify specific proteins, and because individual HCP could carry different perceived risks leaving the room for achieving 'Quality by Chance (QbC)', not by design (QbD). This is mainly because the majority of HCP components are still un-identified and hence efficient assays for evaluating the residual content of HCP in the purified-product also remain unavailable.

Thus improving our understanding of the CHO secretome is of great importance as in order to design improved up-stream as well as down-stream processes to achieve higher yield. More efforts should be made to explore the CHO cell secretome as it may help to identify component(s) that could enable further improvement in improving cell growth and protein production.

Roadblocks and Way-out for Investigating CHO Secretome

To date, the majority of the studies investigating the CHO secretome have identified a large number of intracellular and non-secretory proteins (upto ~88%) in culture supernatant, even with cultures maintaining significantly high viability (>95%) [4,28]. Since no published data are available, it could be presumed that the percentage of these intracellular and non-secretory proteins identified in the secretome might be derived from on-going cell death in the culture. Hence the percentage of Proteins-Potentially-Contributed-by-Cell-Death (PPCD) in culture medium can be calculated by multiplying per-cell-protein content with the number of dead cells in the culture. A comparison of PPCD with the total amount of protein in the culture supernatant at any given time point may enable the early prediction of the percentage of such proteins among secreted proteins. However, in our laboratory we observed that the calculated concentration of PPCD is significantly higher than the total protein in the culture media at all-time points investigated (Figure 1). This suggests that high proportion of PPCD is being degraded by the biomolecules released by the cells and/or consumed by the viable cells present in the culture media, besides concurrent secretion of certain secretory proteins [9,11]. Although if PPCD are degraded and/or consumed selectively and contribute to the identification of high number of intracellular proteins in the secretome is unclear, yet it is known that the composition of secretome modulates

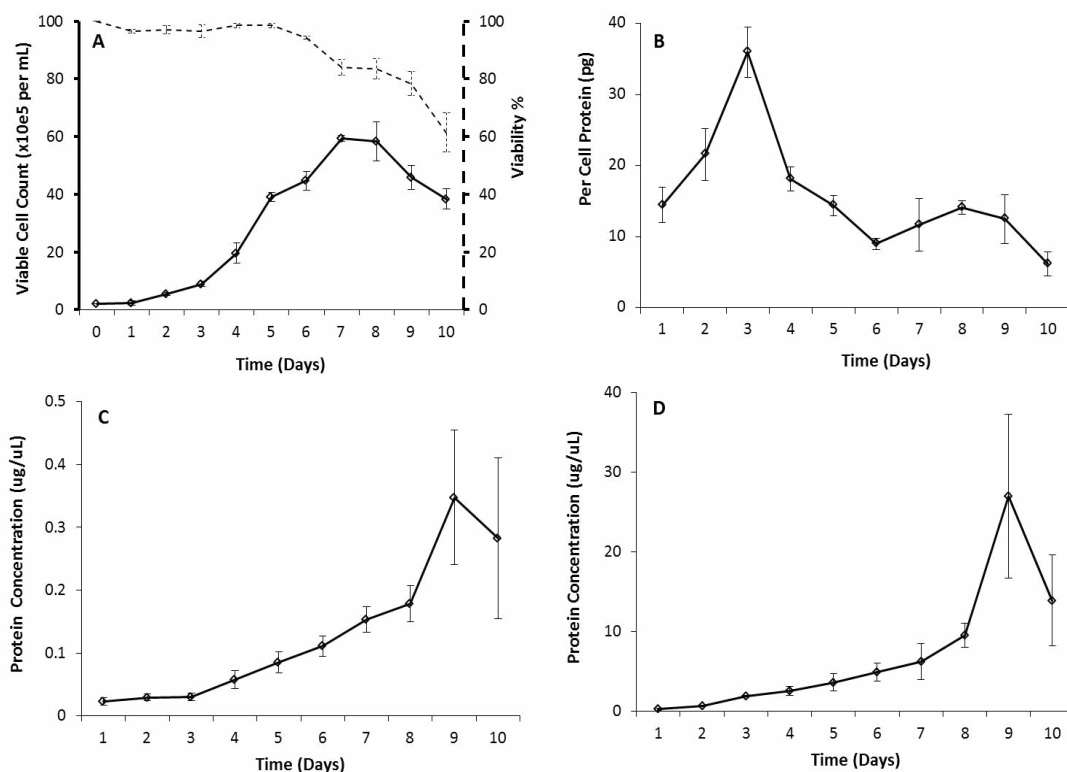


Figure 1: Estimation of protein-potentially-contributed-by-cell-death (PPCD) in culture medium. For this, CHO-K1 cells were grown in suspension culture in CD-CHO media (serum-free and chemically defined) and cell counts were performed everyday using trypan-blue dye exclusion method. Pre-decided amount of culture media was collected and centrifuged at 1000 rpm at 4°C for 20 min. The supernatant was concentrated to known volume using 5 kDa molecular weight cut-off centrifugation filters to enable the calculation of protein content per milliliter (mL) in original sample. Known number of cells was sampled and lysed using urea-based lysis buffer. Protein concentration was estimated in concentrated media and cell-lysate using Bradford protein estimation method. Per-cell-protein content was calculated by dividing the total amount of protein with total number of cells in the sample. The concentration of PPCD was calculated by multiplying the number of dead cells/mL with per-cell-protein content in the sample at that respective time-point. A: cell growth; B: per-cell-protein content, C: protein concentration detected in spent-media and D: concentration of PPCD. Error bars represents standard deviation among three biological replicates.

dynamically over time and affect cell growth & recombinant protein production in culture [29]. However, the knowledge of genuinely secreted CHO proteins is very limited which could be possibly due to the unavailability of well-defined methodologies for sample collection & preparation for mass-spectrometry and/or followed data analysis using databases that contain less number of secretory protein and hence the potential of secreted proteins in regulation of recombinant protein production from CHO cultures largely remained unexplored. Therefore in this communication, we have discussed the issues which generally complicate the investigation of secretome and tried to present potential strategies to address some of them.

Secretory-proteins could be masked and contaminated by high-abundant cytosolic proteins released following cell-lysis and -death

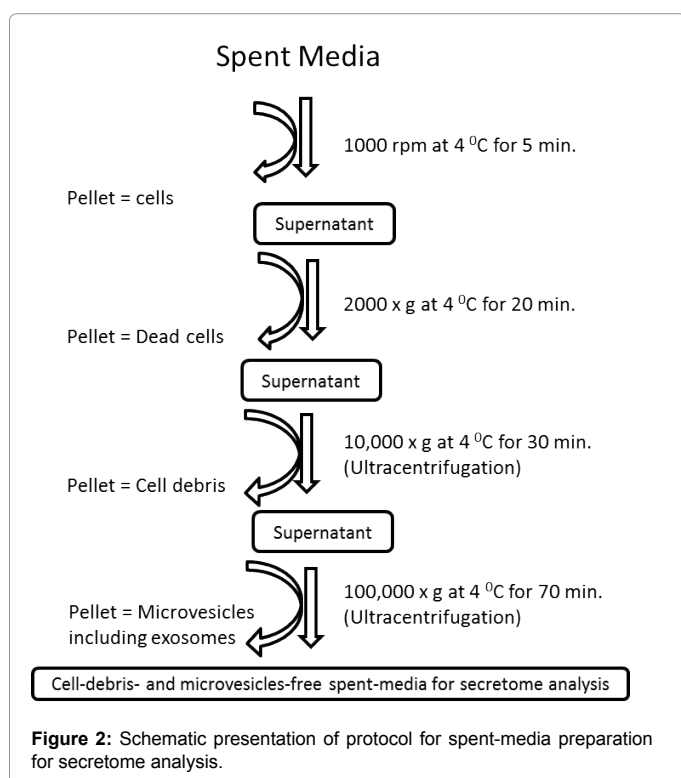
Cell death is unavoidable even under highly optimized conditions and irony is that even a very small percentage of dead cells are able to release protein amounts by far exceeding that of actually secreted proteins. Thus the contamination from cellular proteins could only be minimized by optimizing the culture conditions and sample collection, processing & storage procedures.

Cell culture conditions: Typically, for secretome analysis, cells

are incubated in serum-deprived medium once culture has achieved certain density in serum-supplemented growth medium to avoid contamination and masking of secreted-proteins by serum proteins [30]. However, it is important to consider that serum-starvation acts as a metabolic stress to the cells; under these conditions cells may reduce their proliferation rate and simultaneously activate apoptotic pathways or survival mechanisms to cope with the condition [31]. Activation of survival mechanisms will have less impact on the composition of secretome compared to the impact due to initiation of cell death. Thus prior to any proteomics analysis, preliminary studies investigating the effect of serum starvation onto the cell proliferation and death should be carefully performed to identify optimal culture conditions and time-point (where cell are least affected (specifically minimal cell death) but provide sufficient sample for analysis). Alternatively, cells could be adapted to grow in chemically-defined, serum- and protein-free media. Grow cells up to the desired cell density in chemically-defined, serum- and protein-free media in adherent culture and collecting the secretome sample by replacing spent- with fresh-media under pre-optimized conditions could be better compared to growing cells in suspension culture as this will enable removal of proteins accumulated due to cell death in the culture overtime and minimize identification of intracellular and non-secretory proteins. On the contrary, the suspension cultures, which are more closer to industrial production

process, have higher shear-forces compared to adherent culture causing higher damage to the cells and release of intracellular content into the media; and since removal of spent-media is also complicated in suspension culture, chances of contamination from non-secretory intracellular proteins will also be higher. Thus, the culture-type should be opted based on the objective of the study; i.e. investigation of CHO secretory proteins and/or identification of proteins present in the culture (due to secretion and/or cell death) along with product.

Sample collection, processing and storage: After collection, the conditioned media should be immediately centrifuged appropriately to remove cells (viable and dead) and their-debris (Figure 2). Small extracellular microvesicles, like apoptotic bodies, exosomes etc., should also be removed appropriately from the culture media since they have also been reported to contain proteins of intracellular origin [32]. However, it is quite possible that the proteins packed in the microvesicles (including growth factors and/or proteases etc.) might not be in direct contact with the product secreted into the media and hence may have limited impact on it; but they may have role in regulation of cellular physiologies (cell proliferation and death etc.) required to improve yield from culture. Filtering collected spent-media using filters with porosity 0.2 μM or above alone is also incapable of removing such microvesicles. Storing media at low temperature (-20°C or -80°C) or adding detergents / organic solvents prior appropriate processing may also cause leakage/ bursting of the cells and/or microvesicles present in the media raising the risk for potential contamination from non-secretory cellular proteins. Approximately 88% intracellular proteins were identified in the secretome of CHO cells when conditioned media was centrifuged at 1000 rpm for 15 minutes (min), stored at -80°C , filter-purified with 0.22 μM filter and precipitated using methanol/chloroform method, although the viability of culture under investigation was above 95% [4]. Similarly observations ($\sim 78\%$ of intracellular proteins in the CHO secretome) were reported by Valente et al., in suspension culture [28].



This could possibly be due to the presence of extracellular microvesicles and un-avoidable accumulation of leaked non-secretory proteins from the cells (due to even small amount of cell death overtime in bioreactor) in the conditioned media. Exosomes have already been reported to be enriched with intracellular and non-secretory proteins [33]. Besides in our laboratory, spent-media samples prepared using an ultracentrifuge (centrifuged at 1,000 rpm at 4°C for 5 min, 2,000 x g for 20 min and 10,000 x g for 30 min, ultracentrifuged at 100,000 g for 70 min, stored at -80°C , acetone precipitated and re-suspended in Tris-HCl (pH 6.8) buffer) were observed to have $\sim 20\%$ lower amount of protein compared to spent-media samples prepared without ultracentrifuge (centrifuged at 1000 rpm for 15 min, stored at -80°C , acetone precipitated and re-suspended in Tris-HCl (pH 6.8) buffer), while the viability of the culture was $\sim 98\%$.

Secreted-proteins are at relatively low concentrations due to their high dilution in cell culture media

Selective labelling and enrichment of the secreted-proteins could be one of the options for identification of these proteins. The metabolic labelling of proteins with N-azido-galactosamine (GalNAz) by culturing CHO cells in GalNAz supplemented growth medium and their enrichment using affinity chromatography has resulted in identification of only secreted proteins [5]. GalNAz is an azide analogue of the sugar residue N-acetylgalactosamine (GalNAc), which is predominantly present at the hydroxyl group of either *threonine* or *serine* side chains on the cell surface and secreted proteins with mucin-type O-glycosylations [34]. However, a limitation of selective labelling approach is that non-labelled secretory-proteins, which may contribute a higher proportion of the secretome, might remain unidentified and hence more robust and generalized approaches need to be developed and utilized to investigate secretory proteins. Alternatively, a differential labelling approach coupled with gel-electrophoresis could be utilized where cellular proteins are metabolically labeled with one label and then cells are allowed to secrete proteins into the fresh-medium under pre-optimized conditions. The total proteins in the culture media are then resolved with 2-dimensional gel electrophoresis and labelled with another label. The overlapping of images from different labels should enable to differentiate between secreted or non-secreted peptides/proteins. A similar approach has been utilized to investigate secreted proteins from cancerous cell lines where proteins were first metabolically labelled with ^{35}S -labelled methionine and cysteine and then with ruthenium (a fluorescent dye) [35]. The spots detected by autoradiography on the 2D-gels were mainly from the genuinely secretory proteins, whereas fluorescent staining showed the intracellular and non-secretory spots/proteins as well. However, optimization of culture conditions and protocols could be challenging for such investigations as cell-death is always on-going even at very tightly controlled conditions and could contaminate the secretome.

Proteins secreted at a specific phase of culture could be masked by the proteins secreted at other phases of culture

The composition of secretome depends upon several factors, including the growth media and the number of cells, viability & duration of the culture, amongst others, and modulates dynamically over time. This is because cells keep on consuming nutrients from media causing a gradual decrease in the level of nutrient proteins/peptides and releasing metabolic waste/cell debris into the media resulting in gradual increase in its level. Phase-specific proteins/peptides are also secreted by the cells which may (or may not) be utilized by the cells for growth or could be degraded by the proteolytic enzymes secreted by

the cells or released due to cell death in culture [4,29]. The cell number and viability of the culture also keeps changing over-time [36,37]. However, the viability of culture could be expected to impact the secretome more intensively than other parameters. Recently, a number of low-molecular-weight proteins/peptides has also been reported to vary significantly over 6-days of culture even when cultures maintained high viability (>90%) confirming dynamic modulation of secretome over time [29]. Henceforth, evaluation of secretome by collecting sample at only one time point might not reflect the overall secretome of cells; investigations should be targeted to cover the entire period of culture to improve secretome coverage [4,6,30].

Databases of secreted-proteins are less populated compared to intracellular and non-secretory proteins

Databases for CHO proteins are significantly less populated compared to the databases for human and mouse. CHO protein database has only ~25,000 entries compared to ~250,000 for human and ~84,000 for mouse (~10% compared to human and ~25% compared to mouse), although the estimated genome size of Chinese hamster, human and mouse are similar [4]. Even of these CHO proteins, only ~1.5% of the entries are non-redundant and manually curated with experimental data [4]. Secreted proteins (even for human and mouse) have also been under-studied compared to the intracellular and non-secretory and due to which, the database for secretory CHO proteins are negligibly populated indicating the major bottleneck in the work-flow for investigation the CHO secretome [38]. Moreover, the majority of the knowledge we have today for secreted proteins is based on proteins which are secreted using the classical secretion pathway [38,39]. These proteins contain a signal peptide at the N-terminus and the knowledge of signal peptide is utilized to predict the extracellular and/or secreted nature of a protein [40]. However, proteins can also be secreted to the extracellular space via non-classical secretion pathways and these proteins do not contain signal peptides and could be released by a variety of known and unknown processes [41,42]. Hence investigation of secreted proteins using currently available bioinformatics tools

whose algorithms heavily rely on the information available for proteins secreted via classical secretion pathway would not categorize them as secretory [43]. Besides, if a protein, currently known to be non-secretory, could also be secretory under specific circumstances (such as due to a specific PTMs etc.), such circumstances have not yet been explored for the majority of proteins [44]. Therefore, more thorough efforts are required towards populating the secretory protein databases, especially for revealing CHO secretome.

Conclusion

Secreted-proteins may impact the regulation of cell growth, product quality & quantity, and should be taken into consideration during designing of efficient strategies for product purification from culture media. Instead of being so important, only limited efforts have been made to date to explore the CHO secretome possibly due to difficulties associated with sample collection and preparation and also data analysis using less-populated secreted-protein databases. The majority of studies performed to date have identified significant number of intracellular proteins among the identified secreted-proteins using spent culture media which could possibly be due to ongoing cell death in the culture and/or incomplete removal of viable/dead cells, cell-debris and extracellular microvesicles from the sample before using proteomic approaches (Table 1). Therefore, preliminary studies should be performed to optimize culture conditions and sample collection time-point(s), processing procedures & storage conditions before using proteomics approaches to investigate secreted-proteins. The available methods for selective enrichment of secreted-proteins should also be considered. This may improve the proficiency of identifying genuinely secretory proteins among the secretome and the knowledge generated may enable development of strategies for improved recombinant protein production from the bioprocess and contribute to improve secretome analysis capabilities by populating secretory-protein databases and enabling development of secretory-protein prediction algorithms.

Cell Type	Culture conditions	Time-point for sample collection	Viable cell count and viability at the time of sample collection	Sample processing and storage	Number of total proteins identified	Known as secreted among all identified proteins (%)	Potential factor(s) affecting outcome.
CHO-K1 [4]	Cells were grown in in-house serum- and animal component-free chemically-defined media in bioreactor.	2-, 3-, 4- and 5-days of culture	Viable cell counts: ~ 1.8, 3.8, 7.4 and 9.5×10^6 respectively Viability: >95%.	Supernatant was collected, centrifuged at 1000 rpm for 15 min and stored at -80°C. Spent media was then filter-purified (0.22 µM) and proteins were concentrated using methanol/chloroform precipitation.	2512	11.54%	High proportion of intracellular- and non-secretory proteins (~ 88.46%) among secretome possibly due to on-going cell death during harvest or mechanical damage by the bioreactor impellers and presence of cell-debris and micro vesicles in the samples.
CHO-S and DG44 [5]	Cells were grown in chemically defined and serum-free CD-FortiCHO and CD-DG44 medium respectively in suspension culture.	3-days of culture	Viable Cell Counts: 100×10^6 cells/ml Viability: 98%	- Spent-media was filter-purified (0.2 µM). - Concentrated using 5 kDa molecular weight cut-offs by ultracentrifugation at 4°C. Protein sample were stored at -80°C.	325	100%	Selective enrichment of secretory proteins using metabolic labeling resulted in improved efficiency for identifying secreted-proteins only.
CHOK1SV [6]	Cells were grown in serum-free CD-CHO media in suspension culture (fed-batch mini-bioreactors).	7-, 11- and 15-days of culture	Two bioreactors with ~ 72% viability and two with ~ 57%	Spent-media were clarified by centrifugation at 900 rpm for 5 min and stored at -80°C.	84	No information in the article	High proportion of intracellular- and non-secretory proteins could be expected among secretome due to on-going cell-death and presence of cell-debris and micro vesicles in the samples.

CHO-K1 [28]	Cells were grown in serum-free SFM4CHO media in suspension culture (shake flasks).	5 th -day of culture	Viable cell count: 5-7 × 10 ⁶ cells/mL Viability: 97-99%	Spent media was centrifuged at 180 × g for 10 min, and stored at -80°C. Media was concentrated using 10 kDa molecular weight cut-offs.	178	28%	High proportion of intracellular- and non-secretory proteins (72%) among secretome possibly due to on-going cell death and presence of cell-debris and micro vesicles in the samples.
CHO-K1 [30]	Cells were grown in serum-supplemented F-12K media till confluence and then shifted to serum-free F-12K media for 12hrs after washing cells with sterile PBS.	80% confluency	No information in the article	- No information regarding centrifugation for removal of cell debris. - Spent-media was collected and concentrated using 10 kDa molecular weight cut-offs.	1977	No information in the article	12 fractions were generated from the sample to increase secretome coverage. However, high proportion of intracellular- and non-secretory proteins could be expected among secretome due to high cell-death and presence of cell-debris and microvesicles in the samples.

Table 1: Summary of recently published literature revealing CHO cell secretome.

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