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Exploring Packaged Microvesicle Proteome Composition of Chinese Hamster Ovary Secretome

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Abstract

Background: Chinese Hamster Ovary cells (CHO) are the most preferred host cells to meet the increasing demand for high quality 'human-like' complex biologics production, but is faced with the challenge of achieving high yield at an affordable price. Secreted proteins critically impact cell growth and product quality and quantity and an integral part of secretome is the packaged microvesicles. In spite of numerous efforts to characterize spent-media proteome, none have identified specific contribution of microvesicles, necessitating further differential analysis of these defined fractions of spent-media proteome, specifically packaged microvesicles.

Methods: We have investigated proteome of microvesicles isolated from lag, log, stationary and death phase of CHO batch culture using LC-MS/MS based-proteomic approach to identify proteins that may be involved in regulation of cell growth, viability and productivity in culture.

Results: A total of 89 unique proteins were identified in the microvesicles isolated from lag, log, stationary and death-phase of culture; of these only 8.9% were categorized as secretory proteins leaving ~91% proteins of intracellular and non-secretory nature. Microvesicles were observed to contain a number of culture phase-specific proteins which included cell-signaling molecules, transcription and translation regulators and molecular chaperons; many of which are known growth regulators, indicating the potency of microvesicles in regulating culture health.

Conclusions: This is the first report of CHO microvesicular proteome and this knowledge is critical in developing rationale design of perfusion process, downstream purification process for rendering improved product stability and also novel cell engineering approaches to maximize growth and improved media formulations to maximize yield and minimize product degradation.

Keywords: CHO; Proteome; Microvesicles; Secretome; Recombinant protein production

Abbreviations: LC-MS/MS: Liquid Chromatography Mass Spectrometry; CHO: Chinese Hamster Ovary.

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]. Though CHO cells have been successfully scaled up to produce 5-10 g/L biologics [3], further improvements in the production are of eminent importance to meet the global demand at affordable cost. This could be achieved mainly by three ways; 1- improve production capability of cells in culture (improved cell specific productivity, cell density and culture longevity), 2- minimize product degradation and heterogeneity, 3- improve product purification process.

It is known for a long time that proteins/peptides present in the spent-media could regulate various bioprocess-related phenotypes including cell growth, quality and quantity of recombinant protein product. For example, a number of growth-regulating factors (such as Fibroblast Growth Factor (FGF), Hepatocyte Growth Factor (HGF), Leukemia Inhibitory Factor (LIF), Vascular Endothelial Growth Factor C (VEGF-C) and transferrin) have been identified in spent-culture media of CHO cells, whose supplementation in the culture media enhances the cell growth in culture (~48%) even at low cell density and improves the performance of production culture [4]. On the other hand, a number of proteolytic enzymes (matrix metallopeptidase 3 (MMP3), MMP10, MMP12 and cathepsin-B) have been identified in culture media which may pose risk for proteolytic-degradation of the product leading to low yield from production batches [5,6]. Besides, efficient removal of these peptides/proteins along with other Host Cell Proteins (HCPs) from the final product during down-stream processing is mandatory for product safety and/or longer shelf-life. Majorly there are three sources for these type of peptides/proteins in the spent-media, a) proteins/peptides directly secreted by the healthy cells into the culture media, b) proteins/peptides packed into microvesicles (like exosomes (30-100 nm), microvesicles (100-1000 nm)) for transducing signal to other cells in the culture and/or c) proteins/peptides leaked into the culture media from damaged/dead cells. To date, a number of studies have been performed to identify spent-media proteome [4,6,7], however they were never designed to identify and differentiate proteins directly secreted/leaked from the cells from packed in microvesicles. Microvesicles have already been shown to increase or reduce cell

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proliferation and hence have capabilities to regulate bioprocess-related cellular phenotypes (like cell growth, yield and product stabilization) in production culture [8-10]. They may be enriched or deprived for specific proteins, based on their functional purpose and their cellular origin and this could be responsible for its dynamic effect on cells. The microvesicles purified from the supernatant of T24 bladder cancer cells were enriched for B-cell lymphoma 2 (Bcl-2) and cyclin D1 (anti-apoptotic proteins), but deprived for BCL2-Associated X Protein (Bax) and caspase-3 proteins (pro-apoptotic proteins) contributing towards immunity against programmed cell-death by cancerous cells [11]. Moreover, the amount of secreted microvesicles has also been correlated with the expression of phosphorylated protein kinase B (Ak) t and extracellular signal-regulated protein kinases (ERKs) which are crucial elements regulating apoptosis and cell differentiation processes. The microvesicles have also been shown to be enriched for multiple proteolytic enzymes, like MMP-2 and MMP-14 [12,13]. Though these enzymes are important for various cellular processes, they could also impact the quality and quantity of the product in culture. Besides, microvesicular proteins also contribute to HCPs which need to be removed from product during product purification steps.

Therefore in this investigation, we aimed to reveal the proteome of microvesicles specifically since they are also an integral part of the secretome. For this, CHO cells were grown in chemically-defined protein-free culture medium in shake flask and microvesicles were isolated using ultracentrifugation method over lag, log, stationary and death phase of batch culture. The purified microvesicles were investigated using gel-free proteomics tools (LC-LTQ Orbitrap MS) in order to identify microvesicular proteins that may have impact on cell growth, viability and/or productivity and also better define HCP.

Materials and Methods

Cell culture

Suspension-adapted CHO-K1 cells were grown in chemically-defined, protein-free medium, CD-CHO media (Gibco, Life Technologies) supplemented with 6mM glutamine. Cells were seeded at 0.2×10^6 viable cells/mL in 250 mL shake-flask (Erlenmeyer flask, Corning) with working volume of 100 mL and maintained at 120 rpm at 37°C in CO_2 incubator. The cell counts and viability assays were performed at every 24 hours (hrs.) interval using trypan-blue (Amresco) dye exclusion method with hematocytometer and data was utilized to plot the growth curve.

Microvesicles isolation and sample preparation

Conditioned media was collected after day 2, 4, 6 and 8 reflecting lag, log, stationary and death phase of culture. The microvesicles were isolated using ultracentrifugation methods. Briefly, media was centrifuged at 1000 rpm for 5 minutes to remove cells from sample. The supernatant was collected in fresh 50 mL tubes and was again centrifuged at 10000 rpm for 20 minutes at 4°C to remove dead cells and cellular debris. The supernatant was collected and then finally centrifuged (Beckmann Coulter SW40T) at 100,000 g for 60 minutes. The pellet (microvesicles) was re-suspended in 7 M urea based lysis buffer (7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS, 5 mM magnesium acetate, pH 8.5) after washing twice with ice-cold PBS by ultracentrifugation at 100,000 g for 60 minutes. The gradual centrifugation is of importance to minimize breakage of cellular debris due to high centrifugation forces during ultracentrifugation and potential contamination of debris-associated proteins to microvesicular fraction. Protein concentration was determined using the thiourea-compatible Bradford (Amresco Pure Protein Grade) protein assay (Bio-Rad XMark Microplate Spectrophotometer). Approximately 50 µg microvesicular protein was achieved from 300 mL of conditioned media collected after 4 days of culture.

Microvesicle analysis

Western blot analysis: Protein (5 µg) was separated using 10% SDS polyacrylamide gels and transferred onto nitrocellulose membranes (PAL Corporation Biotrace NT) using the semi-dry transfer method. Membranes were blocked with 5% Marvel-PBS for 1 hr at room temperature and probed overnight at 4°C with rabbit anti-CD63 antibodies (Santa-cruz, SC-15363). Blots were then washed and incubated with peroxidase-labelled anti-rabbit secondary antibodies (anti-rabbit IgG-HRP, Cell Signalling, 70745). After washing, bands specific for microvesicular marker were confirmed by the Enhanced Chemiluminescence (ECL) method (GE Healthcare) with *Gel Doc*™ XR (Bio-Rad). Coomasie stained gel was used as a loading control.

Particle size determination: Microvesicular fraction was evaluated for particle size by Dynamic Light Scattering (DLS) techniques using Zeta-sizer, (Nano-ZS-90, Malvern Instruments, USA) and analyzed by DTS Nano software. Ultra-pure water was used as a dispersant medium. Microvesicular samples isolated using ultracentrifugation method for biological triplicates were diluted with aqueous Phosphate Buffer Saline (PBS) solution and were confirmed for particle size.

In-solution protein digestion

Protein samples were dissolved in 50 mM ammonium bicarbonate (NH_4HCO_3) solution to a final concentration of 0.1 µg/µl. Lyophilized trypsin was dissolved in ice cold 1 mM acetonitrile (ACN) to achieve final working concentration of 13 ng/µl and pH of ~8.5 was adjusted using 50 mM NH_4HCO_3. The trypsin was then added to result in a protein-to-trypsin ratio of 1:30, and enzymatic digestion was carried out at 37°C for overnight. For LC-MS/MS analysis, the resulting peptide mixtures were diluted in 5% Formic acid (v/v in water) to 0.067 µg/µl.

Nano LC-MS/MS and data analysis

For LC-LTQ Orbitrap MS analysis, samples were re-solubilized in 2% [v/v] acetonitrile, 0.1% [v/v] formic acid in water and injected onto the trap column at a flow rate of 20 µl/min subsequently peptides were separated on Zorbax 300SB-C18 (Agilent, Santa Clara, CA, USA) by a gradient developed from 2% [v/v] acetonitrile, 0.1% [v/v] formic acid to 80% [v/v] acetonitrile, 0.1% [v/v] formic acid in water over 180 min at a flow rate of 300 nl/min onto an Agilent 1200 (Agilent, Santa Clara, CA, USA) nano-flow LC-System that was in-line coupled to the nano-electrospray source of a LTQ-Orbitrap discovery hybrid mass spectrometer (Thermo Scientific, San Jose, CA, USA). Full MS in a mass range between m/z 300-2,000 was performed in the Orbitrap mass analyzer with a resolution of 30,000 at m/z 400 and an AGC target of 2×10^5. The strongest five signals were selected for CID (collision induced dissociation)-MS/MS in the LTQ ion trap at a normalized collision energy of 35% using an AGC target of 1×10^5 and two micro scans. Dynamic exclusion was enabled with one repeat counts during 45 s and an exclusion period of 180 s. Peptide identification was performed by CID-based MS/MS of the selected precursors. For protein/peptide identification, MS/MS data were searched against the *Cricetulus griseus* amino acid sequence database using an in-house Mascot server (version 2.4) through the Proteome Discoverer 1.4 software. The search was set up for full tryptic peptides with a maximum of three missed cleavage sites. Carbamidomethyl on cysteine, and oxidized methionine were included as variable modifications. The precursor mass tolerance threshold was 10 ppm, and the maximum fragment mass error was

0.8 Da. The confident identifications were screened using following criteria; (i) proteins with ≥ 2 peptides matched, and (ii) a MASCOT score ≥ 30 and (iii) identified in all three biological replicates.

Cellular localization analysis

Protein sequences were fetched from NCBI database for all identified proteins and were used for evaluating protein localization using web-based freely available tools for protein localization, CELLO (http://cello.life.nctu.edu.tw/; version-2.5), NgLOc (http://genome.unmc.edu/ngLOC/index.html; version-1.0), SignalP (http://www.cbs.dtu.dk/services/SignalP/; version 4.1). The data obtained was used for comparative analysis for protein localization.

Results

Cell growth

Suspension-adapted CHO-K1 cells were seeded at 0.2×10^6 cells/mL in chemically-defined, protein-free medium, CD-CHO medium in shake flask and maintained at 37°C over the batch culture. The cell counts and viability assays were performed every day and data for three biological replicates was utilized to plot the growth curve. Different phase of growth (lag, log, stationary and decline phase) were clearly reflect during the culture in growth curve (Figure 1). Highest viable cell concentration was achieved at day-6 of the culture ($6.5 \pm 0.6 \times 10^6$ viable cells/mL) with maintained high culture viability (95.2 ± 1.3%). The cell-free conditioned media was harvested from all three biological replicate flasks after day 2, 4, 6 and 8 reflecting lag, log, stationary and death phase of culture and utilized to harvest micro vesicle using gradual centrifugation approach.

Quality analysis of microvesicles

Western blot analysis was performed using proteins from microvesicular fraction using anti-CD63 antibody to ensure the quality of microvesicular samples before proceeding for mass-spectrometry for proteome identification. CD63 is a know marker for microvesicles [15]. Western blot analysis clearly indicated that CD63 was present in all three biological replicate samples suggesting the presence of microvesicular proteins in the microvesicle protein fraction (Figure 2). Besides during size analysis, only single peak (avaraged at 255.87 ± 6.12 nm) was observed in all biological replicates suggesting microsomal enrichment in the sample and hence supporting the quality of the microsomal sample for further proteomic analysis (Table 1).

In-solution protein digestion

Protein samples were dissolved in 50 mM NH_4HCO_3 solution to a final concentration of 0.1 µg/µl. DTT was added (final concentration 1 mM) and incubated at 60°C for 15 min followed by incubation whilst protected from light in the presence of 5 mM iodoacetamide at 4°C. Lyophilized trypsin was dissolved in ice cold 1 mM HCl to achieve final working concentration of 13 ng/µl and pH of ~8.5 was adjusted using 50 mM ammonium bicarbonate. The trypsin was then added to result in a protein-to-trypsin ratio of 1:30, and enzymatic digestion was carried out at 37°C for overnight. For LC-MS/MS analysis, the resulting peptide mixtures were diluted in 5% Formic acid (v/v in water) to 0.067 µg/µl.

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Microvesicular protein identification

A total of 31 proteins from lag-phase, 80 from log-phase, 48 from stationary-phase and 60 from death-phase were identified by following criteria of mascot search score ≥ 30, at least ≥ 2 peptides used for identification and identified in all three biological replicate samples (Table 2). This cumulatively resulted into identification of 89 unique proteins in the microvesicles isolated from lag, log, stationary and death-phase of culture (Table 3).

The proteins identified in microvesicles collected from lag, log, stationary and death-phase were also overlapped in order to identify phase-specific and process-related proteins (Figure 4). The analysis revealed that 10 proteins were only identified in lag-phase, 16 in log-phase, 2 in stationary-phase and 5 in death-phase. A total of 15 proteins were identified in all the culture-phase (lag, log, stationary and death-phase) indicating them to be process-related.

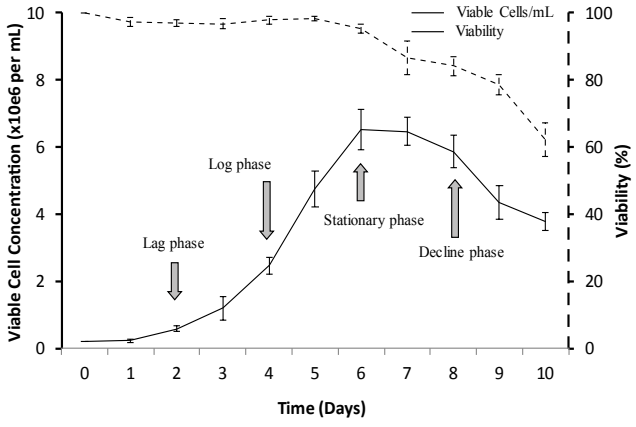


Figure 1: Growth curve of suspension-adapted CHO-K1 cells cultured in serum-free and chemically defined media. Error bars represent the standard deviation calculated using three biological replicate batch cultures.

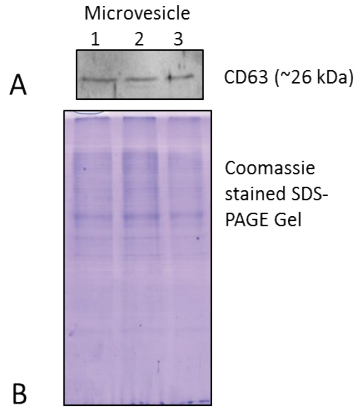


Figure 2: Western blot analysis of proteins samples from microvesicles; A: Western blot analysis with anti-CD63 antibody for microvesicular fraction after ultracentrifugation (biological triplicates). B: Coomasie staining of microvesicular fraction in SDS-PAGE (biological triplicates).

Parameter	Biological Replicate-1	Biological Replicate-1	Biological Replicate-1	Average (Standard Deviation)
Size (d.nm)	254	262.7	250.9	255.87 (6.12)
Width (d.nm)	47.71	45.88	45.17	46.25 (1.31)
Intensity (%)	100	100	100	100

A Particle size D (90%) represents number distribution pattern.

Values reported are mean ± S.D. (n=3)

Table 1: Particle size analysis of microvesicles.

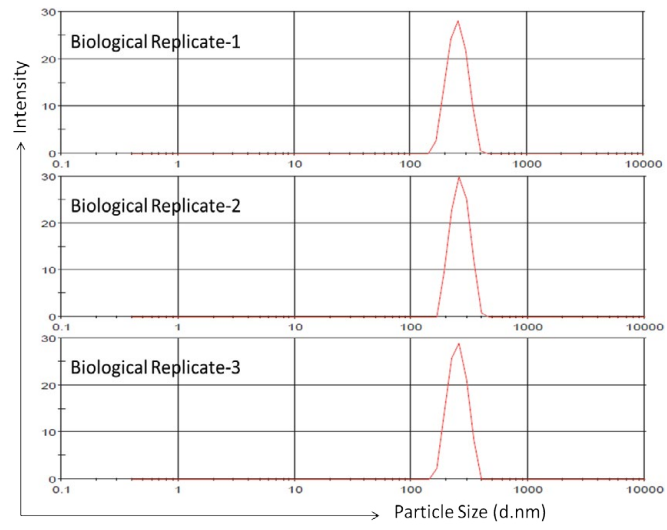


Figure 3: Particle size analysis of microvesicular fractions (biological triplicates).

Microvesicular protein localization analysis

All 89 unique proteins were extensively evaluated to categorize them into secretory and/or non-secretory groups using published literature and bioinformatics tools. The protein predicted to be secretory by all three algorithm used for the purpose was considered as secretory protein. Interestingly, based on the criteria, only 8 proteins (8.9%) were identified as secretory by all three algorithms suggesting that >91% proteins packed in microvesicles were intracellular and non-secretory in nature (Table 4 and Figure 5).

Functional analysis of microvesicular proteins

Identified proteins were further analyzed for its functional categorization and found to be majorly of the following types: structural proteins (28%), metabolic proteins (16%), cell signaling proteins (17%), transcription factors (5%) and translation factors (4%) and molecular chaperons (7%) (Figure 6). Other functional categories included stress response, proteasomal degradation complex etc.

Discussion

Chinese Hamster Ovary (CHO) cells are the most commonly used host cell line for the production of recombinant protein therapeutics and antibodies [1,2]. These products are typically expensive because of high production cost. Though efforts are being made constantly to improve the yield from CHO production cultures to meet increasing demand of high-quality and affordable products, we are still far away in terms of achieving production capability of natural producer cells (plasma cells). This is mainly because we still know very little about CHO cells and their behavior in production cultures and hence are able to utilize just a fraction of their potential for the purpose. Thus there is a need to focus more on understanding CHO cells, the components/biomolecules present in the microenvironment of production culture and their dynamic interaction to be enabled in developing rationale cell engineering approaches and media formulation strategies to improve performance of the production process.

To date majority of studies executed on CHO cells have focused on understanding the cellular physiology in respect to recombinant protein production capabilities [16-19]. The proteins/peptides present in surroundings (spent-media) have largely remained unknown yet, though they have already been known to impact bioprocess-related phenotypes including cell growth and quality and quantity of recombinant protein products. The spent-media may have proteins/peptides which are directly secreted into the culture media by the cells, packed into the microvesicles (like exosomes) for transducing signal to other cells in the culture and/or leaked into the culture media from damaged or dying cells. The proteins directly secreted or leaked into culture media have direct contact with the cells and product in the culture and hence could be expected to have direct impact on cell growth behavior and quality and quantity of the product. Whereas, proteins packed in the microvesicles could regulate cell number by controlling cell proliferation or death in production culture and hence could also impact overall yield. Hence knowledge of both, packed or non-packed proteins is of great importance for efficient process control and improving overall yield.

A few efforts have been made to reveal the proteome of CHO spent-media [4,6,7]; however they have never been focused to identify and differentiate packed vs. non-packed proteins though their purpose of origin and mechanism of action towards impacting cell growth and recombinant protein production could be immensely distinct. Besides, differentiation between proteins secreted from healthy cells vs. leaked from damaged or dead cells from packed in microvesicles is also extremely important in designing downstream processing strategies and ultimately to better understand secretome.

Therefore in this investigation we aimed to reveal the proteome of CHO microvesicles using gel-free proteomics tools (LC-LTQ

Phase of Culture				Number of identified Unique Proteins
Lag	Log	Stationary	Death	
31	80	48	60	89

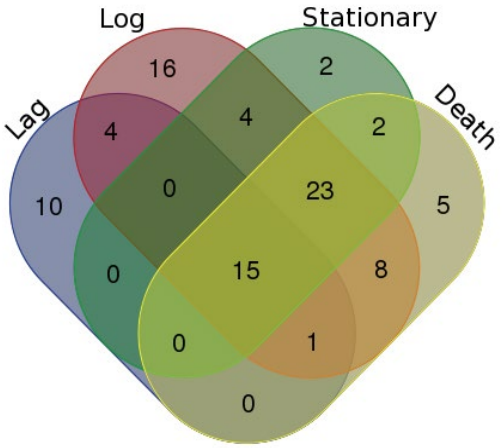
Table 2: Number of proteins identified in microvesicles isolated from lag, log, stationary and death-phase of CHO culture.

S No.	Accession	Description	Gene Symbol	Score	Coverage	Peptides	AAs	MW [kDa]	calc. pI
	Structural								
1	347360906	Actin, cytoplasmic 1	ACTB	149.59	17.6	6	375	41.7	5.39
2	354492323	Moesin	MSN	54.27	3	2	566	66.5	6.28
3	354482483	Vimentin	VIM	283.18	14.16	8	466	53.7	5.12
4	354503280	Annexin A2-like, partial	ANXA2	220.87	39.65	8	227	25.4	5.97
5	354501786	Cofilin-1-like	CFL1	106.65	30.72	3	166	18.5	8.09
6	354489212	Myosin-Ic-like isoform 1	MYO1C	163.56	7.49	5	1028	118.1	9.38
7	345842430	Tubulin alpha-1B chain	TUBA1B	129.5	12.64	4	451	50.1	5.06
8	354472141	Alpha-actinin-1	ACTN1	79.43	2.58	2	931	107.5	5.39
9	354504022	Myosin-9	MYH9	434.54	7.5	11	1880	217.6	5.64
10	354496812	Annexin A1-like	ANXA1	202.12	21.1	6	346	38.8	7.02
11	354482221	Annexin A11	ANXA11	81.65	4.57	2	503	54.1	7.65
12	354491741	Annexin A4-like isoform 1	ANXA4	78.41	13.17	3	319	35.8	5.57
13	354497282	Actin, cytoplasmic 2-like, partial	ACTG1	149.59	18.97	6	348	38.5	5.36
14	354490960	Ezrin	EZR	437.42	14.89	9	638	74.9	6.89
15	354481554	Prelamin-A/C-like isoform 1	LMNA	143.58	6.63	4	664	73.9	7.81
16	354481232	Radixin	RDX	54.27	2.92	2	583	68.5	6.27
17	354483018	Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	173.12	2.06	7	4412	471.6	6.54
18	354473830	Tropomyosin alpha-1 chain-like	TPM1	377.6	35.21	10	284	32.8	4.75
19	354478924	Tropomyosin alpha-3 chain-like isoform 3	TPM3	405.59	35.08	10	248	28.9	4.75
20	354500481	Filamin-A	FLNA	448.02	8.83	13	2538	268.8	5.9
21	354481182	Filamin-B isoform 2	FLNB	168.01	2.73	5	2603	277.7	5.74
22	354494381	Fibronectin isoform 1	FN1	183.15	3.59	7	2480	272.7	5.63
23	354472895	Histone H4-like	HIST1H4A	80.76	29.13	3	103	11.4	11.36
24	354483672	Spectrin beta chain, brain 1 isoform 1	SPTBN1	137.69	2.37	4	2363	274	5.59
25	354501005	Lactadherin-like, partial	MFGE8	195.72	37.93	5	174	19.9	8.65
	Metabolism								
1	354496013	Fatty acid-binding protein, adipocyte-like	FABP4	124.41	24.24	4	132	14.7	7.93
2	354473486	Pyruvate kinase isozymes M1/M2-like isoform 1	PKM	182.09	17.14	6	531	57.9	7.27
3	354499945	Alpha-enolase	ENO1	245.84	16.78	5	429	46.7	6.16
4	349501082	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	171.5	13.51	3	333	35.7	8.34
5	354474551	Acyl-CoA-binding protein-like	DBI	190.54	28.74	3	87	10	8.82
6	354472325	Tubulointerstitial nephritis antigen	TINAGL1	53.31	5.59	2	465	52.5	7.08
7	354476904	Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	134.37	5.65	4	1116	123	5.53
8	354468711	Cathepsin Z	CTSZ	118.16	13.4	3	306	34	7.58
9	354478439	Nucleoside diphosphate kinase B-like	NME2	162.48	35.53	5	152	17.3	7.99
10	350537635	4F2 cell-surface antigen heavy chain	SLC3A2	142.27	15.01	4	533	58.9	5.47
11	346227178	L-lactate dehydrogenase A chain	LDHA	124.31	14.16	4	332	36.5	7.42
12	354472752	Cytochrome c, somatic-like	CYCS	71.43	20.95	2	105	11.7	9.51
13	354482641	Delta-aminolevulinic acid dehydratase	ALAD	183.01	17.58	5	330	36.1	6.98
14	354497685	Pantetheinase	VNN1	73.08	5.47	2	512	56.7	5.63
	Cell Signaling								
1	354469007	Rho GDP-dissociation inhibitor 1-like	ARHGDIA	237.74	31.86	4	204	23.4	5.2
2	354496917	Calmodulin-like	CALM1	984.41	22.26	6	265	29.1	4.75
3	354466994	Phosphatidylethanolamine-binding protein 1-like	PEBP1	214.21	49.2	5	187	20.9	7.53
4	354478483	Caltractin-like	CETN2	984.41	18.79	6	314	34.8	5.82
5	354478978	Protein S100-A6-like	S100A6	467.41	28.09	2	89	10	5.48

6	354484391	14-3-3 protein zeta/delta-like	YWHAZ	165.18	22.86	4	245	27.7	4.79
7	354476375	Transforming protein RhoA-like	RHOA	65.91	13.47	2	193	21.8	6.1
8	354501872	Rho-related GTP-binding protein RhoC-like	RHOB	65.91	13.47	2	193	22	6.58
9	354487034	Macrophage-capping protein-like	CAPG	111.01	10.03	2	349	38.7	6.73
10	354471813	EH domain-containing protein 4, partial	EHD4	57.66	3.69	2	515	58.6	6.58
11	350539723	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	GNB1	166.79	13.82	4	340	37.4	6
12	354508126	14-3-3 protein epsilon-like, partial	YWHAE	103.43	33.33	3	105	11.9	5.83
13	354494229	SH3 domain-binding glutamic acid-rich-like protein-like	SH3BGRL	144.62	74.56	5	114	12.7	5.07
14	354495613	Thrombomodulin-like	THBD	68.15	6.24	2	577	61.9	4.69
15	354482553	Thrombospondin-1	THBS1	268.51	8.89	8	1170	129.6	4.93
	Transcription								
1	354475571	NSFL1 cofactor p47-like isoform 1	NSFL1C	105.33	12.1	3	372	41	5.2
2	537153542	Bromodomain adjacent to zinc finger domain protein 1A	BAZ1A	89.58	2.39	3	2011	228.4	6.09
3	537191106	Nuclear factor related to kappa-B-binding protein	NFRKB	34.07	0.6	1	2505	275.9	7.68
4	354502621	Nuclease-sensitive element-binding protein 1-like	YBX1	231.37	21.36	4	337	38.3	10.15
	Translation								
1	346227155	Elongation factor 2	EEF2	110.3	3.61	3	858	95.3	6.83
2	354486063	Clusterin-like	CLU	67.54	7.42	2	445	51.5	5.85
3	346986359	Elongation factor 1-alpha 1	EEF1A1	95.09	8.44	2	462	50.1	9.01
4	354495391	60S acidic ribosomal protein P2-like	RPLP2	60.2	24.35	2	115	11.7	4.54
	Proteosomal Degradation Complex								
1	350540096	Lysosome-associated membrane glycoprotein 1	LAMP1	146.08	9.34	3	407	43.8	6.71
2	354494438	Sulfated glycoprotein 1	PSAP	126.27	7.53	3	598	65.7	5.49
3	354468731	Proteasome subunit alpha type-7-like	PSMA7	112.06	7.59	2	382	41.7	9.61
4	346986300	Polyubiquitin	UBB	64.28	31.31	2	1038	116.6	8.38
5	354473967	Ubiquitin-60S ribosomal protein L40-like isoform 1	UBA52	64.28	19.53	2	128	14.7	9.83
6	354483686	Ubiquitin-40S ribosomal protein S27a-like	RPS27A	64.28	16.03	2	156	17.9	9.6
7	537136494	Ubiquitin carboxyl-terminal hydrolase 5 isoform 2	USP5	41.67	2.47	2	1051	116.4	5.12
	Stress Response								
1	354466320	Superoxide dismutase [Cu-Zn]-like	SOD1	114.63	14.88	2	242	25.9	9.55
2	350537945	Peroxiredoxin-1	PRDX1	60.01	12.56	3	199	22.2	8.05
3	354487462	Thioredoxin reductase 1, cytoplasmic	TXNRD1	431.06	24.88	10	611	67.2	7.8
4	354470385	Catalase	CAT	121.73	6.83	3	527	60	7.68
5	354507545	Glutathione S-transferase Mu 1-like	GSTM1	76.36	10.09	2	218	25.5	6.37
6	354506476	Glutathione S-transferase Mu 7-like	GSTM7	76.36	10.09	2	218	25.9	7.37
7	350537543	Glutathione S-transferase P 1	GSTP1	107.27	12.38	2	210	23.6	7.8
	Cell adhesion								
1	354496408	Galectin-1-like	LGALS1	67.81	24.44	2	135	14.8	5.8
2	350539683	Intercellular adhesion molecule 1	ICAM1	119.38	9.3	4	527	57.6	7.24
3	354473282	Galectin-3-binding protein-like	LGALS3BP	130.62	10.8	4	574	63.8	5.19
4	537201836	Hyaluronan and proteoglycan link protein 3	HAPLN3	356.88	18.05	13	881	97.9	7.62
	Protein folding (molecular chaperones)								
1	354483044	Peptidyl-prolyl cis-trans isomerase A-like	PPIA	145.54	22.56	3	164	17.9	8.28
2	354474350	Peptidyl-prolyl cis-trans isomerase B-like	PPIB	80.95	12.5	2	216	23.6	9.58
3	350537423	78 kDa glucose-regulated protein	HSPA5	344.8	22.48	12	654	72.3	5.16
4	350539823	Heat shock cognate 71 kDa protein	HSPA8	146.65	12.07	5	646	70.8	5.36

5	350540064	Heat shock protein HSP 90-alpha	HSP90AA1	85.04	5.59	3	733	84.8	5.03
6	354479029	Heat shock protein HSP 90-beta-like	HSP90AB1	140.59	7.04	4	724	83.1	5.02
	Miscellaneous								
1	354480912	Basigin	BSG	245.79	13.45	4	409	44.6	6.2
2	354501595	Complement regulatory protein Crry-like	CD59	88.75	11.16	2	421	46.6	7.64
3	354485425	Platelet glycoprotein 4-like	CD36	63.15	7.42	2	472	52.9	8.56

Table 3: List of identified unique proteins in microvesicles isolated from CHO batch culture.



Culture Phase	Total Proteins	Identity of Protein(s)
Lag	10	RHOB, RHOA, ACTN1, GSTM1, EHD4, GSTM7, TUBA1B, HSP90AA1, CD59 and VNN1
Log	16	USP5, LAMP1, HAPLN3, CFL1, CD36, EEF2, NFRKB, TINAGL1, UBA52, RPS27A, FN1, UBB, ATP1A1, BAZ1A, YWHAE and RPLP2
Stationary	2	CTSZ and PSMA7
Death	5	SH3BGRL, MYH9, LMNA, ALAD and CAT
Lag, Log	4	SLC3A2, GSTP1, EEF1A1 and MYO1C
Log, Stationary	4	TPM3, FABP4, THBS1 and EZR
Log, Death	8	HSP90AB1, LDHA, NME2, CAPG, SPTBN1, SOD1, LGALS1 and FLNB
Stationary, Death	2	YBX1 and PPIB
Lag, Log, Death	1	ANXA1
Log, Stationary, Death	23	CETN2, CYCS, ENO1, DBI, FLNA, HSPA5, PEBP1, PPIA, MYH9, GAPDH, ANXA11, S100A6, NSFL1C, HIST1H4A, BSG, TPM1, GNB1, TXNRD1, VIM, YWHAZ, CALM1, ARHGDIA and PSAP,
Lag, Log, Stationary, Death	15	ACTB, ANXA2, HSPG2, MFGE8, HSPA8, CLU, RDX, ICAM1, ANXA4, MSN, PKM, THBD, ACTG1, LGALS3BP and PRDX1

Figure 4: Overlapping of proteins identified in microvesicles isolated from lag, log, stationary and death-phase of CHO Batch Culture.

S No	Accession	Description	Gene Symbol
1	537201836	Hyaluronan and proteoglycan link protein 3	HAPLN3
2	354496408	Galectin-1-like	LGALS1
3	354494381	Fibronectin isoform 1	FN1
4	354473282	Galectin-3-binding protein-like	LGALS3BP
5	354482553	Thrombospondin-1	THBS1
6	354468711	Cathepsin Z	CTSZ
7	354497685	Pantetheinase	VNN1
8	354472325	Tubulointerstitial nephritis antigen	TINAGL1

Table 4: List of microvesicular proteins predicted to be secretory in nature.

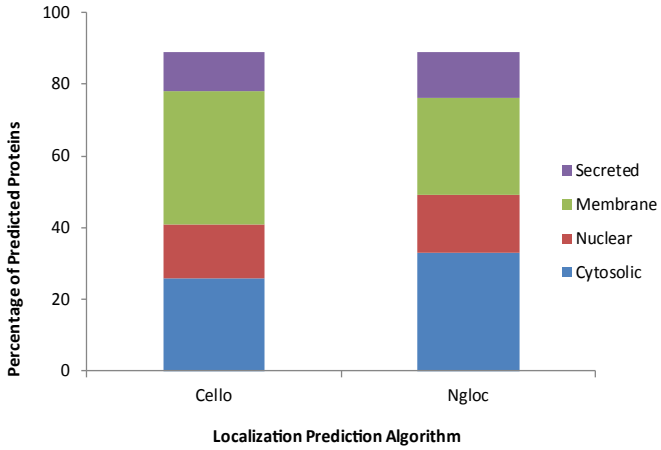


Figure 5: Prediction of cellular Localization of microvesicular proteins by predictive bioinformatics tools.

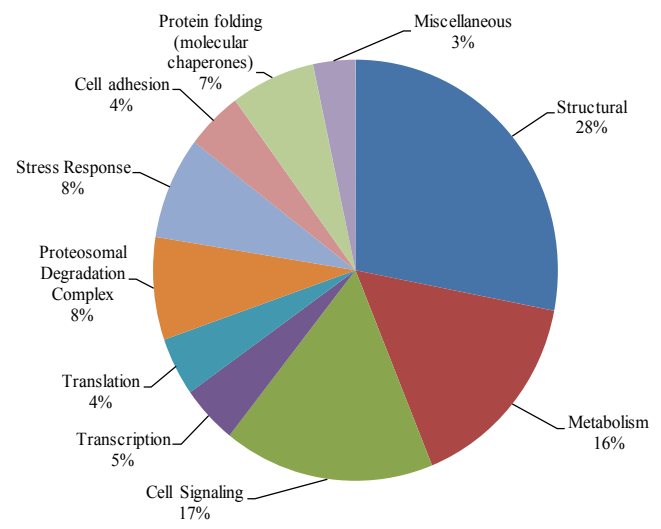


Figure 6: Functional categories of microvesicular proteins.

Orbitrap MS). To the best of our knowledge, this is the first report of the microvesicular proteome of CHO cells. A total of 89 proteins were identified in the CHO microvesicles collected from the exponentially growing cells. Though higher number of proteins have been identified in microvesicles from human samples, smaller number has also been identified in other cell lines; 41 in microvesicles from SKBR3, 43 proteins in microvesicles from BT474 and 59 in microvesicles from MCF7 [20,21] suggesting that the number of identified protein may vary from cell line to cell line. All the identified were also mapped with ExoCarta (www.exocarta.org), a database for microvesicular proteins [22]. All the proteins identified were observed to be present in ExoCarta database suggesting that the microvesicular proteins are highly conserved along mammalian species.

Cellular localization analysis of microvesicular proteins

Only 8 proteins (8.9%) were categorized as secretory proteins by all three different bioinformatics tools used, leaving ~91% of microvesicular proteins intracellular and non-secretory nature in microvesicles (Table 4 and Figure 5). This is in-line with published literature reporting ~90% of the identified proteins in microvesicles to be non-secretory in nature [20,23]. This suggests that the protein which cannot be secreted through classical cell secretion pathway but are required to serve any specific purpose by donor/receiving cell could be packed into the intracellular membranous vesicles, like exosomes, and transferred from one to another and hence is one of the method used for cell-to-cell communication.

Besides, identification of proteome of microvesicles is important because significant numbers of intracellular and non-secretory proteins are being identified in spent-media and considered part of secretome, though are non-secretory in nature. For example, ~88% of the identified proteins in suspension culture in the spent-media of CHO cells were categorized as intracellular and non-secretory by Lim et al. and ~78% by Valente et al., although the viability of culture under investigation was above 95% [4,24]. This is mainly because majority of the studies designed to identify secreted proteins in media have been able to remove only large sized microvesicles (centrifuge sample ≤10000 rpm or filter with 0.45 µm filter) leaving the high proportion of small microvesicles (~200 nm), including exosomes, in the culture media. These microvesicles carry intracellular cargo, including intracellular and non-secretory proteins, which lead towards identification of significant number of intracellular and non-secretory proteins along with truly secreted proteins in the sample. Therefore, identification of microvesicular proteome of CHO cells could be of great help in better differentiation of secretory proteins from the intracellular and non-secretory proteins packed in microvesicles.

Functional analysis of microvesicular proteins

Like cells, structural proteins (actin, vimentin, tubulins, annexins, cofilins, and filamins) were observed to be abundant (28%) in microvesicular proteome which could be expected to maintain mechanical coupling between cytoskeletal microtubules and actin microfilaments which ultimately could be preserving shape, size and integrity of microvesicles (Table 3 and Figure 6) [25]. Adhesion molecules, such as galectin-1 (LGALS1) like, intracellular adhesion molecule 1 (ICAM1) and hyaluronan and proteoglycan link protein 3 (HAPLN3), were also observed in the microvesicles. These proteins may be associated in adhesion and efficient delivery of their cargo of microvesicles to recipient cells [26,27]. Hence knowledge of structural proteins and adhesion molecules of microvesicles is of immense importance to understand microvesicles, their mechanism to deliver intracellular cargo to recipient cell(s) and impact biological process effectively.

Besides, the microvesicles were observed to contain proteins involved and/or associated with regulation of wide variety of cellular process, such as transcription, translation, cellular stress and protein folding which, upon delivery of microvesicular content to recipient cells, could enable to regulate various biological process including folding and secretion of recombinant protein product, cell proliferation and cell death in the culture (Table 3 and Figure 6). For example, nuclease-sensitive element-binding protein 1 (YBX1)-like protein was identified in microvesicular fraction. YBX1 is known to be involved in many cellular functions including transcription/translation, alternative splicing, mRNA degradation/processing in P-bodies including small RNA processing and function [28,29]. YBX1 has been shown to induce the expression of pro-proliferative genes (such as epidermal growth factor receptor, *cyclin A*, and *cyclin B) and hence induce cell proliferation in the culture [30]*. Superoxide dismutase 1 (SOD1), a cellular stress regulator, catalyzes the conversion of superoxide ion (O_2^-) into H_2O_2 and O_2 to maintain low levels of Reactive Oxygen Species (ROS). Overexpression of SOD1 has been reported to induce cell proliferation

without affecting cell cycle progression and lower the apoptosis of lung carcinoma cell lines (H358 and H1975 cells) which promotes growth by increasing survival [31]. Regulation of cellular process, such as cell proliferation and cell death, is of imminence importance in designing of efficient bioprocess and hence identification of microvesicular proteome is of obvious importance.

The overlapping of proteins identified in microvesicles isolated from lag, log, stationary and death phase of cultured enabled identification of 10 lag-phase specific proteins, 16 log-phase specific, 2 stationary phase specific and 5 death-phase specific proteins (Figure 4). The presence of phase-specific protein suggests that microvesicles may contain specific proteins based on physiological state of cells at the time of microvesicle origin and their functional purpose; and this could be responsible for its dynamic effect on cells. The lag-phase specific proteins mainly contained cell signaling proteins (Ras homolog gene family, member A (RHOA), Ras homolog gene family, member B (RHOB) and EH domain-containing protein 4 (EHD4)) followed by structural proteins (Alpha-actinin-1 (ACTN1) and Tubulin alpha-1B (TUBA1B)) and stress response proteins (Glutathione S-transferase Mu 1 (GSTM1) and Glutathione S-transferase Mu 7 (GSTM7)). RHOA and RHOB are known to regulate cell proliferation [32,33]. The structural proteins also plays crucial role in cell proliferation [34]. Hence, the presence of cell-proliferation regulation-associated cell signaling and structural proteins could be linked with the preparation of cells for higher rate of proliferation in the upcoming log-phase. Cells in lag phase have been reported to high intercellular stress level compared to cells in log, stationary and death-phase [35]. The stress proteins present in the microvesicles could be expected to be associated with regulation of intracellular stress in lag-phase of culture. Microvesicles from log phase were mainly enriched with proteins involved in proteosomal protein degradation pathway (Polyubiquitin (UBB), Ubiquitin-60S ribosomal protein L40 (UBA52), Ubiquitin-40S ribosomal protein S27a (RPS27A), Ubiquitin carboxyl-terminal hydrolase 5 (USP5), Lysosome-associated membrane glycoprotein 1 (LAMP1)). Ubiquitin (UBB) has a major role in targeting cellular proteins for degradation by the 26S proteasome, but it is also involved in the maintenance of chromatin structure, regulation of gene expression, and stress response. The overexpression of UBB has been observed to induce cell proliferation and henceforth inhibitors of UBB are being explored as potential therapy for various cancers [36]. It could also fuse with ribosomal proteins S27a (RPS27A) and L40 (UBA52). Both (UBA80 and UBA52) were also observed to be packed in microvesicles. Ubiquitin acts as a chaperone for its fusion partners and hence its presence is critical for proper ribosome biogenesis and thereof mRNA translation [37]. This is in line with the observation where exogenous expression of RPS27A has already been shown to induce cell proliferation whereas inhibition reduced the cell viability, induced cell cycle arrest at S and G2/M phases and increased cell apoptosis in the culture [38]. Whereas, inhibition of UBA52 didn't showed similar effects on cellular health as expression of only ~7% mRNAs was observed to be altered suggesting transcript-specific translation by the UBA52 [39]. This could be important for recombinant protein production as low temperature has been believed to cause transcript-specific translation and hence improve overall yield from the cultures [18,39,40]. USP5 is involved in disassembly of conjugated ubiquitin to maintain proteasome function and its availability for other functions [41]. Besides, two transcription factor (Nuclear factor related to kappa-B-binding protein (NFRKB) and Bromodomain adjacent to zinc finger domain protein 1A (BAZ1A)) and two translational factors (Elongation factor 2 (EEF2) and 60S acidic ribosomal protein P2 (RPLP2)) were also identified in microvesicles isolated from log-phase of CHO culture further suggesting potential impact of microvesicles on receiver's translational status. Microvesicles from stationary-phase has only two unique proteins, Cathepsin Z (CTSZ; metabolic protein) and Proteasome subunit alpha type-7 (PSMA7; proteosomal protein degradation pathway associated protein), whereas death-phase microvesicles had two structural (Myosin-9 (MYH9) and Prelamin-A/C (LMNA)) [42-44], one metabolic (Delta-aminolevulinic acid dehydratase (ALAD)), one cell signaling (SH3 domain-binding glutamic acid-rich-like protein (SH3BGRL)) and one stress response (Catalase (CAT)) protein unique [45].

A total of 15 proteins were identified in microvesicles isolated from all phases of culture, lag, log, stationary and death-phase. Besides being potentially integral part of the microvesicles in terms of structure, function, delivery and/or their genesis, these proteins could be significantly contributing to the host cell proteins which need to be removed during down-stream processing. Of the 15, 8 proteins were structural proteins (Actin cytoplasmic 1 (ACTB), Annexin A2 (ANXA2), heparan sulfate proteoglycan (HSPG2), Lactadherin (MFGE8), Radixin (RDX), Annexin A4 (ANXA4), Moesin (MSN) and Actin cytoplasmic 2 (ACTG1)); potentially required for maintenance of microvesicular shape, size and integrity [46-48]. Two adhesion molecules (Intercellular adhesion molecule 1 (ICAM1) and Galectin-3-binding protein (LGALS3BP)) were also present which could be associated with delivery of the microvesicular content into the recipient cells.

Hence the knowledge of microvesicular proteome is of great importance in order to identify proteins that may regulate cell growth and recombinant protein product in culture as well as the proteins that need to be removed during down-stream processing. This knowledge would enable development of rationale cell engineering and media formulation approaches to improve performance of the CHO based bioprocess.

Conclusion

CHO cells are the most commonly used cell lines for production of recombinant protein therapeutics. Microvesicles are small membranous vesicles that are released by various cell types, including CHO, into the culture as integral part of spent-media proteome. The microvesicles transfer biomolecular cargo from one cell to another and hence could regulate various cellular phenotypes, including cell growth, cell-death and recombinant protein production; efficient regulation of all of these phenotypes is of utmost importance for achieving higher yield from production culture. To date, a number of efforts have been made to identify spent-media proteome of CHO cells; however microvesicles specifically and exclusively have not been explored though recent publications have proven the importance of microvesicles in regulation of various bioprocess-related phenotypes. Therefore in this investigation, we have explored the proteome of CHO microvesicles collected from the lag, log, stationary and death phase of CHO batch culture. Microvesicles were observed to contain a number of phase-specific proteins suggesting the dynamic nature and effects of microvesicles on bioprocess. The microvesicles from log-phase have a number of proteins, phase-specific proteins (proteosomal protein degradation pathway associated proteins (UBB, UBA52, RPS27A, USP5 and LAMP1) and transcription (NFRKB, BAZ1A) and translation regulators (EEF2, RPLP2)) which are already known to be associated with regulation of cell growth in culture. Whereas, lag-phase specific proteins mainly contained cell signaling proteins (RHOA, RHOB and EHD4) followed by structural proteins (ACTN1 and TUBA1B) and stress response proteins (GSTM1 and GSTM7). These could be linked with

the preparation of cells for higher rate of proliferation in the upcoming log-phase. A number of proteins were identified in microvesicles isolated from all phases of culture, lag, log, stationary and death-phase. Besides being potentially integral part of the microvesicles in terms of structure, function, delivery and/or their genesis, these proteins could be significantly contributing to the host cell proteins which need to be removed during down-stream processing.

Henceforth the investigation of microvesicular proteome may help to identify proteins that may enable improved regulation of cell growth and productivity in bioprocess and development of efficient downstream processing approaches.

Competing Interest Statement

The authors declare no financial or commercial conflict of interest regarding this work.

Authors' Contribution

NK, SBh and SC designed, supervised and executed the entire study. NK and SC contributed to writing of the manuscript. SK and PM carried out the mass spec analysis, data compilation and database identification of proteins and ratios. SBa, JP and BM carried out the quality analysis of exosomes. NK and DGG carried out all cell culture and fractionation work. SBh, AT and SH contributed intellectually to many aspects of the study. All authors contributed to revising and approving the final manuscript.

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