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Recovery of Chinese hamster ovary host cell proteins for proteomic analysis

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Abstract

Identification and characterization of Chinese hamster ovary (CHO) host cell protein (HCP) impurities by proteomic techniques can aid bioprocess design and lead to more efficient development and improved biopharmaceutical manufacturing operations. Recovery of extracellular CHO HCP for proteomic analysis is particularly challenging due to the relatively low protein concentration and complex composition of media. We developed optimized protocols that improve proteome capture for CHO HCP. Eleven precipitation protocols were screened for protein recovery and optimized for a subset of the precipitants by a design of experiments approach. Because total protein recovery cannot describe physicochemical characteristics of proteins or detect non-protein agents, which may interfere with proteomic methods, a subset of precipitation conditions were compared by two-dimensional electrophoresis and liquid chromatography coupled with mass spectrometry, with optimized recovery shown to differ between the two proteomic methods. This work demonstrates broadly applicable methods that can be applied as initial steps to optimize sample preparation of any sample type for proteomic analysis and presents optimized precipitation protocols for extracellular CHO HCP recovery, which can vary appreciably between gel-based and shotgun proteomic methods.

Keywords

Chinese hamster ovary cells; extracellular host cell proteins; proteomics; design of experiments optimization; protein precipitation

1 Introduction

Therapeutic proteins, produced in genetically modified host cells, represent a growing class of treatments within the biopharmaceutical industry, with an estimated global market of

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\$113 billion [1]. Chinese hamster ovary (CHO) cells are the primary expression system for therapeutic proteins, which are typically secreted into the extracellular medium along with endogenous host cell protein (HCP) impurities that must be removed from the product for patient safety [2]. Identification and characterization of these extracellular CHO HCPs by proteomic techniques can aid bioprocess development, resulting in robust biopharmaceutical manufacturing operations [3]. Developing optimized sample preparation protocols that improve extracellular CHO HCP capture is fundamental to maximizing the utility of these proteomic methods.

Proteomic techniques have recently been applied to study extracellular CHO HCPs under a variety of relevant bioprocess conditions. For example, the extracellular CHO HCP composition has been shown to vary minimally with changes in a variety of cell culture conditions, such as composition of media and temperature shift [4], and extracellular CHO autocrine growth factors have been identified to optimize the composition of serum-free media [5]. Cell culture viability has been demonstrated to impact significantly the extracellular proteome by introduction of intracellular CHO HCPs at decreased viability [4, 6, 7]; however, even with viability greater than 90%, extracellular proteome changes were observed over the initial six days of cell culture [8]. In downstream purification, proteomic techniques have been used to demonstrate the impact of primary recovery operations on the extracellular CHO HCP profile [9], show impurity clearance across protein A and ion-exchange chromatography operations [6], and define operating conditions for a variety of mixed-mode chromatography resins [10]. Additionally, CHO HCPs are highly conserved across different cell lines [4, 6, 11], and non-recombinant protein producing (null) CHO cells have demonstrated CHO HCP compositions equivalent to those of cell lines producing therapeutic proteins [4, 6, 7].

Proteomic methods can be divided into two main workflows, namely gel-based and shotgun techniques. Gel-based methods such as Two-dimensional electrophoresis (2-DE) generally refer to methods to separate and quantify proteins whereas shotgun methods generally begin with a proteolytic digestion of proteins and therefore refer to methods that separate and quantify peptides. Both methods rely on tandem mass spectrometry (MS/MS) to identify proteins or peptides of interest. Limitations of 2-DE include a relatively poor ability to reliably measure low-abundance proteins as well as those proteins that are very hydrophobic or of extreme molecular weight or isoelectric point. Shotgun methods are necessarily limited in their ability to monitor protein-level changes because of the initial digestion step.

Both gel-based and shotgun workflows require complex sample preparation protocols to remove interfering agents and exchange the proteins into a favorable matrix for analysis. In a typical sample preparation protocol for extracellular HCPs, cells are first removed by centrifugation, and proteins are concentrated and separated from interfering agents by precipitation. The protein pellet is recovered by centrifugation and resolubilized in a solution that depends on the proteomic method, because each proteomic workflow has different resolubilization solution constraints. For example, 2-DE is incompatible with charged species and shotgun methods are incompatible with chaotropes and detergents. Protein recovery during sample preparation is paramount as loss limits protein detection and subsequently decreases the utility of the proteomics analysis. The value of sample

preparation optimization has previously been demonstrated across various sample types [12], and recovery of extracellular CHO HCPs is particularly challenging given the relatively low protein concentration and the variety of components in media that must be removed prior to analysis. Despite this importance, no reported studies have optimized precipitation conditions to maximize extracellular CHO HCP recovery for proteomic analysis.

This work applies design of experiments (DOE) methodologies to study the impact of various precipitation parameters on extracellular CHO HCP recovery for both gel-based and shotgun proteomics. Total protein recovery, measured by Bradford assay, was initially used to optimize precipitant type, concentration, and incubation time, with optimized methods subsequently demonstrated by proteomic techniques. This research demonstrates relatively high-throughput methodologies to optimize sample preparation prior to demonstration by proteomic techniques and presents optimized precipitation protocols for extracellular CHO HCP recovery, which vary appreciably between gel-based and shotgun proteomic methods.

2 Materials and Methods

2.1 CHO cell culture

A CHO-K1 cell line (ATCC, Manassas, VA) that does not produce recombinant protein was adapted to serum-free, suspension culture in 125 mL shake flasks containing 30 mL SFM4CHO medium (Hyclone Laboratories Inc., Logan, UT). Cultures were seeded at $5 \cdot 10^4$ cells/mL and incubated with orbital agitation for 5 days in a 37 °C cell culture incubator with 5% CO₂ and 80% relative humidity until the final cell density reached $5 - 7 \cdot 10^6$ cells/mL at 97 – 99% viability. The extracellular CHO HCPs were harvested and separated from the residual cells by centrifugation (180 g, 10 min) and stored at –20 °C until further use.

2.2 Precipitant screening

Extracellular CHO HCPs were concentrated by centrifugal filtration (10 kDa nominal molecular weight cut-off, Millipore, Bedford, MA) to 1 mg/mL as measured by Bradford assay (Thermo Fisher Scientific Inc., Rockford, IL) and divided into 100 µg aliquots, which were independently precipitated overnight in technical duplicate experiments by the following 11 methods: 2D clean-up kit (GE Healthcare, Chalfont St. Giles, United Kingdom), trichloroacetic acid (TCA, Fisher Scientific, Fair Lawn, NJ), deoxycholate (Sigma-Aldrich Chemical Co., St. Louis, MO) followed by TCA, TCA and acetone (Sigma-Aldrich Chemical Co.), acetone, acetone containing dithiothreitol (DTT, Bio-Rad Laboratories, Hercules, CA), ethanol (Decon Labs Inc., King of Prussia, PA), methanol (Mallinckrodt Chemicals, Phillipsburg, NJ), acetone and methanol, acetonitrile (Mallinckrodt Chemicals), and ammonium sulfate (Sigma-Aldrich Chemical Co.). Organic solvent precipitations were performed with absolute acetone, ethanol, methanol, and acetonitrile. Concentrated stocks of TCA, sodium deoxycholate, DTT, and ammonium sulfate were prepared without pH adjustment by vortexing with water until the solution appeared visibly clear.

Precipitation by the commercially available 2D clean-up kit was performed according to manufacturer protocol as described previously [6].

TCA precipitation was performed by addition of concentrated TCA (59%) solution to CHO HCP to a final TCA concentration of 10%. Samples were incubated overnight on ice, and precipitate was recovered by centrifugation (14 000 *g*, 10 min, 4 °C) and washed with ice-cold acetone.

Precipitation by deoxycholate followed by TCA was performed by addition of 1% aqueous sodium deoxycholate solution to CHO HCP to a final deoxycholate concentration of 0.2%, followed by 30 min incubation on ice. Concentrated TCA solution was then added to a final TCA concentration of 10%, and incubated overnight on ice. Precipitate was recovered by centrifugation (14 000 *g*, 10 min, 4 °C) and washed with ice-cold acetone.

TCA and acetone precipitation was performed by addition of four volumes of ice-cold acetone to one volume of CHO HCP, followed by immediate addition of concentrated TCA solution to reach a final TCA concentration of 10%. Samples were incubated overnight at –20 °C.

Organic solvent precipitations were performed by addition of 4 volumes of ice-cold organic solvent to one volume of CHO HCP, followed by overnight incubation at –20 °C. The organic solvents screened included acetone, acetone containing 20 mM DTT, ethanol, methanol, and acetonitrile.

Acetone and methanol precipitation was performed by addition of 12 volumes of acetone and 1.5 volumes of methanol to 1 volume of CHO HCP, followed by overnight incubation at –20 °C.

Ammonium sulfate precipitation was performed by addition of concentrated (4.3 M) ammonium sulfate to CHO HCP to obtain a final ammonium sulfate concentration of 3.4 M. Samples were incubated overnight on ice.

Following precipitation (and wash steps where applicable), protein pellets were recovered by centrifugation (14 000 *g*, 10 min, 4 °C) and resolubilized in both a representative 2-DE solution comprising 8 mM tris-hydroxymethylaminomethane (tris, Bio-Rad Laboratories), 8 M urea (Bio-Rad Laboratories), 15 mM DTT, and 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Sigma-Aldrich Chemical Co.), and a representative shotgun solution composed of 20 mM triethylammonium bicarbonate buffer (TEAB, Sigma-Aldrich Chemical Co.) by vortexing for at least 1 hr at room temperature. Samples were diluted five-fold and analyzed for total protein concentration by Bradford assay. The final protein concentration was normalized to the initial protein concentration in the concentrated extracellular CHO HCP feed. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with constant volume loading using Ready Gel 12% Tris-HCl gels at a constant 150 V until the dye front migrated to the bottom of the gel.

2.3 Precipitant optimization

Three precipitants (TCA, methanol, acetone) were selected for further optimization given their favorable recovery during precipitant screening and their prevalence in the literature. Prior to precipitation, extracellular CHO HCPs were concentrated to 1 mg/mL by centrifugal filtration (10 kDa, 4000 g) and divided into 100 µg aliquots. Precipitant concentration and incubation time were optimized by a DOE approach with parameters varied according to a two-factor central composite design with five levels for each factor (Table 1). TCA incubations were performed on ice, while methanol and acetone incubations were performed at −20 °C. Following precipitation, protein pellets were recovered, resolubilized in both 2-DE and shotgun resolubilization solutions, and analyzed for total protein concentration as described in Section 2.2. Recovery data were fit to the two-factor model

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \varepsilon \quad (1)$$

where Y represents the precipitation recovery and x_1 and x_2 correspond to the experimental factors of incubation time and precipitant concentration, respectively. The constant parameters (β) were determined from multiple regression of coded factors (Table 1). Main effect coefficients (β_1 , β_2) describe the impact of each individual factor, while the cross-interaction coefficient (β_{12}) indicates synergistic effects between factors, and self-interaction coefficients (β_{11} , β_{22}) describe the non-linear response for each factor. Statistical design and analysis were performed with JMP Pro 9 (SAS Institute Inc., Cary, NC) with coefficient estimates determined by ANOVA and statistical significance determined by t-test.

2.4 Optimized method comparison

2.4.1 Concentration recovery—Extracellular CHO HCP aliquots initially containing 50 µg/mL protein in culture supernatant were concentrated by centrifugal filtration (10 kDa, 4000 g) to 0.6 mg/mL, 1.1 mg/mL, and 2.4 mg/mL by varying centrifugation time from 28 – 77 min. Protein concentration was determined by Bradford assay and concentrated products were used for optimized precipitant comparison.

2.4.2 Precipitation dependence on protein concentration—Aliquots containing 100 µg extracellular CHO HCP at varied protein concentration were precipitated by 2D clean-up kit, TCA, methanol and acetone. Precipitations by TCA, methanol, and acetone were performed by addition of the optimized precipitant volume, as determined by precipitation optimization experiments (Section 2.3), followed by at least 1 hr incubation at the appropriate temperature. Precipitation by 2D clean-up kit was performed according to manufacturer protocol. All precipitated products from the four methods were recovered, resolubilized, and analyzed for total protein concentration as described in Section 2.2.

2.4.3 Precipitant salt dependence—Extracellular CHO HCPs were concentrated by centrifugal filtration (10 kDa, 4000 g) to 1 mg/mL and buffer exchanged into deionized water. CHO HCP samples with varied salt concentration were generated by addition of tris to a final concentration of 20 mM and sodium chloride (Fisher Scientific) to 25 – 120 mM.

Precipitation by TCA, methanol, and acetone was performed by addition of the optimized precipitant volume, as determined by precipitation optimization experiments (Section 2.3), followed by at least 1 hr incubation at the appropriate temperature. Precipitated products were recovered, resolubilized in 2-DE solution only, and analyzed for total protein concentration as described in Section 2.2.

2.5 Proteomic application of optimized precipitation methods

2.5.1 2-DE Proteomics—2-DE was performed as described previously [12] using 300 µg extracellular CHO HCP precipitated by 2D clean-up kit and optimized TCA, methanol, and acetone methods (determined from methods in Section 2.3). Briefly, precipitated proteins were resolubilized in rehydration solution comprising 8 mM tris, 8 M urea, 30 mM DTT, 2% CHAPS, 0.4% BioLytes (Bio-Rad Laboratories) and trace bromophenol blue (Bio-Rad Laboratories) and were used to rehydrate 18 cm, pH 3–10 nonlinear Immobiline DryStrips (GE Healthcare). Isoelectric focusing (IEF) was performed using a PROTEAN IEF Cell for 100,000 Vh, after which IEF gels were sequentially equilibrated with DTT and iodoacetamide (Sigma-Aldrich Chemical Co.). SDS-PAGE was performed using 13% T, 2.6% C polyacrylamide slab gels measuring 18 cm × 16 cm × 1.5 mm. Gels were stained with SYPRO Ruby (Molecular Probes, Eugene, OR) and imaged on an FLA-3000 Fluorescent Image Analyzer (Fujifilm Corp., Tokyo, Japan). Gel images were analyzed and compared using ImageMaster 2D Platinum Software v5.0 (GE Healthcare). Spots were detected using the auto-detect feature and manually edited to remove artifacts. Protein identifications were determined by matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF/TOF) mass spectrometry (MS) as described previously [13] on an ABSciex 4800 MALDI-TOF/TOF Analyzer (Framingham, MA). Data were acquired in positive ion MS reflector mode and MS/MS, and then submitted for Mascot v2.2 (Matrix Science Ltd., London, UK) database searches through GPS Explorer software v3.6 (ABSciex). Spectra were searched against translations of the CHO genome [14] with oxidation of methionine and carbamidomethylation of cysteines allowed as variable modifications, and up to 100 ppm mass tolerance. Identifications with 95% confidence or greater were accepted.

2.5.2 Shotgun Proteomics by Liquid Chromatography (LC)-LC/MALDI-TOF/TOF

—Samples containing 100 µg extracellular CHO HCP were precipitated by 2D clean-up kit and optimized TCA, methanol, and acetone methods (determined from Section 2.3), and resolubilized in shotgun solution. Prior to digestion, proteins were diluted ten-fold with 25 mM ammonium bicarbonate, incubated with 100 mM DTT for 25 min at 95 °C, and incubated with 150 mM iodoacetamide for 30 min. Trypsin digestion was performed overnight at 37 °C.

High pH reversed phase high performance liquid chromatography (RP-HPLC) was performed on an Agilent 1100 (Agilent Technologies, Santa Clara, CA). Digested CHO HCPs were loaded onto a 0.5 mL Varian PLRP-S column (Agilent Technologies) and eluted by an 18 column volume (45 min) linear gradient from 2 – 40% acetonitrile at 2.5 min residence time. Product fractions were collected at 2 min intervals and pooled into six fractions based on absorbance at 214 nm.

Low pH RP-HPLC was performed on a Tempo LC-MALDI spotter (Eksigent, Dublin, CA). Each fraction was loaded onto a 1.2 μ L CapRod RP-18E capillary column (Merck KGaA, Darmstadt, Germany), washed with 10 column volumes of 2% acetonitrile, and eluted by a 36 column volume (75 min) gradient to 65% acetonitrile. Both mobile phases also contained 0.1% TFA (Avantor, Center Valley, PA), and all operations were performed at 2 min residence time. Eluate was spotted onto target plates with α -cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemical Co.) matrix at ten second intervals.

Peptide identifications were determined as previously described [15] with minor modifications. Briefly, MS spectra were acquired in positive ion reflector mode on an ABSciex 5800 MALDI-TOF/TOF Analyzer with 800 laser shots per spot. MS data over a mass range of 920 – 4000 m/z were processed with default calibration to select a maximum of five precursors, each with a signal/noise of at least 25, for MS/MS per spot. Fragmentation was induced with $1.2 \cdot 10^{-6}$ torr of air and 2 kV collision energy. MS/MS spectra were acquired with 800 laser shots and a 200 resolution mass window per MS precursor, processed with default calibration, and submitted for Mascot v2.2 (Matrix Science Ltd., London, UK) database searches through ProteinPilot software v3.0 (ABSciex). Spectra were searched against translations of the CHO genome [14] with oxidation of methionine and carbamidomethylation of cysteines allowed as variable modifications, and 100 ppm mass tolerance. Peptide identifications with 95% confidence or greater and protein identifications containing at least one significant unique peptide were accepted.

3 Results

3.1 Precipitant screening

Eleven protein precipitation protocols were evaluated for total extracellular CHO HCP recovery following resolubilization in representative solutions used for 2-DE and shotgun proteomics (Figure 1A). The 2-DE solution composition (8 mM tris, 8 M urea, 15 mM DTT, and 4% CHAPS) was selected to maximize protein solubility while maintaining IEF compatibility by limiting the solution conductivity [16]. For shotgun proteomics, a 20 mM TEAB solution was selected to maximize solution compatibility with the majority of shotgun proteomic methods. Maintaining solution compatibility is particularly challenging for multiplexed quantitation by methods such as isobaric tagging with iTRAQ reagents, because the labeling and digestion efficiencies can be impaired by a variety of substances including primary amines, thiols, denaturants, and detergents [17].

To maximize the number of precipitation conditions that could be evaluated, it was necessary to select a higher-throughput response than is possible using 2-DE or shotgun proteomics. Total extracellular CHO HCP recovery was selected as the response for screening and optimization studies because recovery provides high-level information on the overall precipitation efficiency and can be obtained from a relatively high-throughput protein concentration assay. Total protein recovery has important limitations as a response variable, including incapacity to describe physicochemical characteristics of the recovered proteins, some of which could be elucidated at lower throughput from single-dimension SDS-PAGE or IEF gels. Recovery is also limited by the inability to detect the presence of non-protein agents, which may interfere with proteomic methods; however, other assays could be used in

parallel to detect many biological components such as nucleic acids, carbohydrates, and lipids.

The precipitation mechanism in the different protocols can be categorized into three well characterized classes: (1) TCA precipitation by hydrophobic interactions [18–20], (2) organic solvent precipitation by solution permittivity reduction [21], and (3) ammonium sulfate precipitation by salting out behavior [21]. TCA precipitation exhibited a reasonably high recovery following 2-DE resolubilization, but recovery decreased substantially in shotgun solution. Organic solvent precipitation generally demonstrated the greatest recovery for both resolubilization solutions, with the exceptions of acetonitrile and acetone containing DTT, which both showed decreased recovery in shotgun solution. Following ammonium sulfate precipitation, less than 50% of the initial HCP was recovered, regardless of which resolubilization solution was used. Resolubilized products were also analyzed by SDS-PAGE to confirm total protein concentration assay results, and recovery was shown to correlate well between the two analytical methods for both shotgun (Figure 1B) and 2-DE (Supplementary Figure 1) resolubilization.

3.2 Precipitant optimization

Three precipitants were selected for further optimization by a DOE approach. TCA was selected because it exhibited similar precipitation performance to the 2D clean-up kit, which has been previously used for extracellular CHO HCP precipitation [4, 6, 7, 9]. Methanol and acetone were also chosen as they demonstrated high precipitation recoveries in both resolubilization solutions. Because acetone has a lower dielectric constant (21.1 at 20 °C) than methanol (33.6 at 20 °C), selection of these two organic solvents allowed investigation of a relatively broad range of solvent permittivity encompassing the dielectric constant of ethanol (25.2 at 20 °C) [22]. The impacts of precipitant concentration and incubation time on total protein recovery were optimized for each precipitant by a two-factor central composite design (Table 1). The same range of incubation time (0–7 hrs) was used for all three precipitants and the precipitant concentration range varied by precipitant class. TCA concentrations are reported as the final percentage of TCA in the sample, while organic solvents are characterized by the added volume of precipitant normalized to the initial sample volume (organic solvent volume : volume extracellular CHO HCP).

Regression coefficients of coded variables were determined by fitting precipitation recovery data to the polynomial model (Equation 1) for TCA (Figure 2A), methanol (Figure 2B), and acetone (Figure 2C). The magnitude of each coefficient describes the relative impact of the factor (or combination of factors) on protein recovery, with positive coefficients describing improved recovery and negative coefficients describing impaired recovery. Self-interaction coefficients (β_{11} , β_{22}) describe second-order behavior, with positive coefficients indicating a response that is graphically concave upward in nature, and negative coefficients indicating a graphically concave downward response. All three coefficients describing incubation time (β_1 , β_{11} , β_{12}) were statistically insignificant ($p > 0.2$) for all conditions investigated, signifying that incubation time does not impact protein recovery (Figure 2A–C). Further increasing the incubation time to more than 12 hours had no additional effect on recovery (data not shown).

Precipitant concentration demonstrated a positive impact on protein recovery for all conditions examined, as the main effect coefficients (β_2) are all statistically significant positive values. The self-interaction coefficient (β_{22}) for TCA precipitation followed by 2-DE resolubilization exhibits a statistically significant negative value, while all other conditions yield statistically significant positive self-interaction coefficients (Figure 2A–C).

After removal of the insignificant incubation time factor, the total extracellular CHO HCP recovery data are shown as a function of only precipitant concentration for TCA (Figure 2D), methanol (Figure 2E), and acetone (Figure 2F) precipitations. All six combinations of precipitants and resolubilization solutions demonstrate a positive, non-linear relationship between recovery and precipitant concentration, as described by the regression coefficients. The TCA recovery response was dependent on the resolubilization solution, with maximum recovery at 15 – 17.1% TCA for 2-DE resolubilization and statistically insignificant recovery changes between 10 – 17.1% TCA following shotgun resolubilization. Further increasing the TCA concentration beyond 17.1% rapidly reduced recovery, with only 11% recovery observed in 20% TCA (Supplementary Figure 2), therefore, 15% TCA was selected as the optimized concentration to ensure a stable operating range. The two organic solvents demonstrated relatively similar responses, independent of resolubilization solution, with poor recovery observed at the lowest solvent concentration (0.5 volumes organic solvent : volume extracellular CHO HCP) and optimized recovery under all other conditions explored. The optimized organic solvent concentration of at least 1.5 times the initial sample volume (1.5 volumes organic solvent : volume extracellular CHO HCP) was selected, as further increasing the solvent concentration negligibly increased protein recovery.

3.3 Comparison of optimized methods

The three optimized precipitation methods were compared to the 2D clean-up kit by total protein recovery at varied extracellular HCP concentrations.

3.3.1 Concentration recovery—Solutions of varied protein concentration were generated by differentially concentrating extracellular CHO HCPs by centrifugal filtration. Concentration recovery was shown to decrease roughly exponentially with increasing concentration, and nearly half of the protein was lost at 85-fold concentration (Figure 3A). This loss was shown to be independent of filter loading across a range of relevant operating conditions (0.6 – 2 g/m²). Following concentration, it is necessary to perform a precipitation operation because media used for suspension CHO culture contain relatively high concentrations of shear-protecting agents that cannot be removed by 10 kDa nominal molecular weight cut-off filtration, while filtration with increased filter pore size results in considerable loss of low molecular weight proteins to the filtrate.

3.3.2 Precipitation dependence on protein concentration—Precipitation recovery increased with protein concentration for all methods investigated in both 2-DE and shotgun resolubilizations (Figure 3B–C). When optimized precipitation conditions were used, resolubilization in 2-DE solution yielded similar recovery for all four precipitation methods at each protein concentration (Figure 3B). In shotgun solution, precipitation by 2D clean-up

kit and TCA showed consistently decreased recovery compared to the organic solvents (Figure 3C).

Overall protein recovery (Figure 3D–E) for the entire sample preparation process was calculated from the concentration and precipitation recoveries. The maximum overall recoveries of 72% for 2-DE solution and 56% for shotgun solution did not vary appreciably with initial extracellular CHO HCP concentration. Recovery is impaired by precipitation of low initial protein concentrations, while increasing protein concentration by centrifugal filtration prior to precipitation results in protein loss during the concentration step. As centrifugal filtration did not increase overall recovery, the operation was removed to streamline proteomic analysis and increase throughput.

3.3.3 Precipitant salt dependence—The impact of salt concentration on precipitation recovery was explored because an ideal precipitant would be broadly applicable to a variety of CHO bioprocess intermediates with a range of solution conditions. Figure 3F shows recovery of extracellular CHO HCPs precipitated from solutions of different salt concentrations by the optimized conditions defined in Section 3.2. TCA precipitation recovery was shown to be independent of salt concentration, while the organic solvents both demonstrated poor recovery in the absence of salt. Acetone precipitation achieved optimum recovery in solutions containing at least 25 mM sodium chloride, while methanol precipitation showed the greatest salt dependence, requiring 100 mM sodium chloride to attain optimal recovery.

3.4 Proteomic application of optimized precipitation methods

3.4.1 2-DE proteomics—The three optimized precipitation methods (Section 3.2) were evaluated by 2-DE and results were compared to those obtained from the 2D clean-up kit. 2-DE precipitation recovery was quantified by both the number of spots and the total spot volume detected by ImageMaster 2D Platinum Software v5.0 (Figure 4A). TCA was selected as the optimized precipitation method for 2-DE as it generated the most spots with the greatest total spot volume and showed decreased variability compared to the 2D clean-up kit. Representative 2-DE images illustrate that TCA precipitation (Figure 4B) yields greater protein detection by 2-DE than methanol precipitation (Figure 4C). Although total protein recovery data predicted that all four precipitants should achieve similar recovery by 2-DE, TCA and the 2D clean-up kit generated an average of 77% more protein spots with 150% greater spot volume than the organic solvents. Discrepancies between total protein recovery and 2-DE spot detection are discussed further in Section 4.2.3.

3.4.2 Shotgun proteomics by LC-LC/MALDI-TOF/TOF—Total protein recovery data predicted that organic solvent precipitation would show improved recovery by shotgun proteomic methods compared to TCA and 2D clean-up kit precipitations. LC-LC/MS was used to evaluate the three optimized precipitation methods (Section 3.2) and the 2D clean-up kit for protein recovery, which was quantified by both the number of unique peptides detected and the corresponding number of unique proteins identified (Figure 4B). Methanol was selected as the optimized precipitant, allowing detection of a total of 132 proteins from duplicate experiments identifying 319 – 336 peptides. TCA, the 2D clean-up kit, and

acetone precipitation all showed decreased recovery compared to methanol precipitation, with an average of 40% fewer unique peptides and 30% fewer proteins identified. The decreased LC-LC/MS recoveries for TCA and 2D clean-up kit precipitations were consistent with total protein recovery results, while the decreased LC-LC/MS recovery for acetone precipitation was not. Discrepancies between total protein recovery and peptide detection by LC/LC-MS are discussed further in Section 4.2.4.

4 Discussion

4.1 Precipitant screening and optimization

4.1.1 Precipitant screening—Organic solvent precipitation showed the greatest total protein recovery in both 2-DE and shotgun resolubilization solutions. This finding is consistent with previous results showing that urinary proteins, which are subject to the same low protein and high salt limitations as extracellular CHO HCPs, demonstrated greater precipitation in organic solvents compared to other methods [23]. Conversely, urinary proteins demonstrated optimized 2-DE detection by acetonitrile precipitation [23, 24], which demonstrated the lowest total protein recovery of all organic solvents evaluated for extracellular CHO HCP recovery. TCA, methanol and acetone precipitations all demonstrated high protein recovery in at least one resolubilization solution (Section 3.1), are relatively simple to perform, and span a range of precipitation mechanisms and precipitant properties. Consequently, the precipitant concentration and incubation time were optimized for these three precipitants by DOE methods.

4.1.2 Incubation time—Incubation time was statistically insignificant for all three precipitants, which is consistent with TCA precipitation protocols involving relatively short incubation times [18–20]. Although research has previously demonstrated high recovery with less than 1 hr organic solvent incubation [21], incubation times for organic solvent precipitation typically range from several hours [25] to overnight [12, 24]. Decreasing precipitation incubation time can increase throughput without sacrificing protein recovery.

4.1.3 TCA precipitation—The optimal TCA concentration (15%) is consistent with previous literature using 5 – 40% TCA to precipitate various proteins [18]. At intermediate TCA concentrations, precipitation has been shown to occur by structural rearrangement of proteins into a molten globule, or ‘A’ state, that exposes hydrophobic residues, causing protein precipitation [18–20]. Increasing the TCA concentration beyond that required for the molten globule state results in complete protein denaturation and minimal protein precipitation. The protein recovery presented here was consistent with this TCA precipitation mechanism for 2-DE resolubilization solution, as resolubilization of the restructured, highly hydrophobic proteins was facilitated by the solubility enhancing components composing the 2-DE solution. Conversely, incomplete resolubilization was observed in shotgun solution comprising only buffering salts, and protein recovery was statistically indistinguishable between 10 – 17.1% TCA.

4.1.4 Organic solvent precipitation—The optimal organic solvent concentration was shown to be least 1.5 volumes organic solvent : volume extracellular CHO HCP (60%

organic solvent), with no recovery benefit from further increasing the organic solvent concentration. Although the optimal organic solvent concentration is less than typically used for CHO lysate precipitation [25, 26], this finding agrees with previous reports that 50% acetone is sufficient to precipitate most proteins greater than 20 kDa [21]. Conversely, Thongboonkerd *et al.* have shown that a final organic solvent concentration of 90% was required to optimally precipitate urinary proteins [23]. Organic solvent precipitation is thought to work by reducing the dielectric constant of the medium, which subsequently reduces protein solubility and induces precipitation by electrostatic and dipolar forces [21]. The dielectric constants of methanol (36.9 at 5 °C) and acetone (linearly extrapolated from available data to 22.6 at 5 °C) are considerably lower than the dielectric constant of the medium, which can be assumed to have a dielectric constant similar to that of water (87.8 at 0°C) [22]. Despite the difference in dielectric constants between methanol and acetone, both organic solvents demonstrated similar total protein recovery for each solvent concentration. At the lowest organic solvent concentration tested (0.5 volumes organic solvent : volume extracellular CHO HCP), only 33% (vol/vol assuming additive volumes) of the medium comprises solvent, and initial sample conditions dominate, resulting in minimal protein precipitation. Increasing the solvent concentration to 60% (vol/vol assuming additive volumes) of the medium (1.5 volumes organic solvent : volume extracellular CHO HCP), causes solvent conditions to dominate, resulting in precipitation and optimized protein recovery.

4.2 Optimized method comparison and proteomic application

4.2.1 Concentration and total protein recovery—The overall extracellular CHO HCP recovery was shown to be relatively path-independent because precipitation recovery depends on protein concentration and protein loss across concentrations is a function of the extent of concentration. The majority of reported 2-DE studies concentrate extracellular CHO HCPs at least five-fold by centrifugal filtration before precipitation [4, 6, 7, 9]. Although concentration is unnecessary, low levels of concentration can be performed with limited protein loss.

4.2.2 Precipitant salt dependence—While TCA precipitation recovery was independent of salt concentration, recovery from both organic solvents demonstrated salt dependence, with the amount of salt required inversely related to the dielectric constant. Recovering extracellular proteins from solutions of low ionic strength may require salt addition to facilitate organic solvent based protein precipitation.

4.2.3 2-DE proteomics—Although total protein recovery data showed equivalent recovery from all precipitation methods following 2-DE resolubilization, the optimized precipitation method for CHO HCP recovery for 2-DE analysis was determined to be TCA, as organic solvent precipitation involves electrostatic interactions and can co-precipitate a variety of charged species, which interfere with IEF, causing inferior 2-DE images. Many CHO lysate studies report direct loading [27, 28] or organic solvent precipitation for 2-DE analysis [12, 26] because the intracellular CHO HCP concentration is an order of magnitude greater than the extracellular protein concentration. Lower sample volumes, containing proportionally smaller amounts of interfering agents, are required for analysis and their co-

precipitation with CHO lysate does not significantly interfere with 2-DE. The relatively low concentration of extracellular CHO HCPs necessitates relatively large volumes of medium for proteomic analysis and the majority of extracellular CHO HCP studies report precipitation by 2D clean-up kit [4, 6, 7, 9]. While TCA precipitation was selected as the optimized method, the 2D clean-up kit was also shown to be a viable precipitation method, resulting in detection of an equivalent number of protein spots with 14 – 44% less total spot volume compared to TCA. 2-DE images of extracellular CHO HCP prepared by TCA precipitation were used to identify 19 spots (Figure 5), the majority of which are traditionally classified as extracellular (44%), membrane (33%), and cytoplasmic (11%) [29]. The locations on the 2-DE image of 78 kDa glucose regulated protein and peptidyl-prolyl cis-trans isomerase A are consistent with previous reports [7, 9], while the remaining identifications map novel locations of extracellular CHO HCPs.

4.2.4 Shotgun proteomics by LC-LC/MS—Methanol was shown to be the optimized precipitant for shotgun proteomics, yielding high total protein recovery and maximizing both peptide and protein detection by LC-LC/MS. TCA and 2D clean-up kit precipitations caused incomplete protein pellet resolubilization in shotgun solution, resulting in decreased total protein recovery and reduced peptide and protein detection. Acetone precipitation also exhibited decreased peptide and protein detection by LC-LC/MS despite yielding high total protein recovery in shotgun resolubilization solution. This discrepancy may be explained by the low dielectric constant of acetone, which may have enabled co-precipitation of additional medium components that were not precipitated by methanol. Such medium components may subsequently have interfered with both trypsin digestion and MALDI-TOF/TOF analysis, reducing the effectiveness of shotgun proteomics.

4.2.5 HCP identification by shotgun proteomics—As with 2-DE analysis, preparation of CHO lysate for shotgun proteomics is simplified by the availability of high protein concentrations in smaller volumes [25, 30, 31]. Shotgun studies of extracellular CHO HCPs report complex sample preparation such as desalting chromatography and affinity enrichment [32] or require toxic chemicals such as chloroform [5]. Combined results from all four precipitants used here (collected over eight LC-LC/MS experiments) yielded identification of 178 unique proteins (Supplementary Table 1), of which 142 (80%) were identified from methanol precipitation alone (collected over two LC-LC/MS experiments). Of the 178 proteins identified, 28% are traditionally classified as extracellular either as their sole cellular location or one of multiple possible locations [29]. The majority of remaining proteins are classified as membrane (16%), cytoplasmic (11%), and lysosomal (10%), and are likely released during cell lysis or secreted by a non-classical method. The identified proteins serve a variety of cellular functions such as biosynthesis (19%), growth and development (13%), adhesion (10%), proteolysis (8%), and stress response (6%). The magnitude and composition of these proteins identified from precipitation by a simple 1 hr methanol incubation are consistent with previous extracellular CHO HCP identifications from more complex sample preparation protocols [10, 32] and also include a number of novel extracellular CHO HCP identifications (Supplementary Table 1). Additionally, further optimization of HPLC methods could facilitate the identification of additional proteins, although that was not the focus of this study.

This work presented optimized methods for precipitation recovery of extracellular CHO HCPs for both 2-DE and shotgun proteomic analysis. A total protein concentration assay was demonstrated as a relatively high-throughput optimization method; however, a subset of optimized conditions were demonstrated by proteomic methods to detect non-protein agents that may co-precipitate with the proteins of interest and interfere with proteomic analysis. Because cell culture supernatant is the most complex background matrix involved during biopharmaceutical manufacturing, the optimized methods described here can also be applied to various common bioprocess streams such as chromatographic intermediates. The methods developed here maximize recovery of proteins spanning a large range of molecular weight and isoelectric point and should be directly applicable to sample streams containing recombinant proteins with varied physicochemical properties. Recombinant proteins are often expressed at much greater concentrations than extracellular CHO HCPs, and consequently it may be necessary to reduce the recombinant protein concentration or spike in additional HCPs to evaluate both recombinant protein and extracellular HCP in the same proteomic analysis. Although the optimized precipitation methods presented here are specific to extracellular CHO HCPs, the DOE methodologies are broadly applicable and can be applied to determine optimized precipitation conditions for proteomic analysis of any sample type with a relatively small number of experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CHO	Chinese hamster ovary
HCP	host cell protein
2-DE	Two-dimensional electrophoresis
MS/MS	tandem mass spectrometry
DOE	design of experiments
TCA	trichloroacetic acid
DTT	dithiothreitol
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
tris	tris-hydroxymethylaminomethane
TEAB	triethylammonium bicarbonate buffer

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
IEF	Isoelectric focusing
MALDI-TOF/TOF	matrix-assisted laser desorption/ionization tandem time-of-flight
MS	mass spectrometry
LC	Liquid Chromatography
RP-HPLC	reversed phase high performance liquid chromatography

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Figure 1.

(A) Total extracellular CHO HCP recovery by Bradford assay following precipitation according to various protocols and resolubilization for 2-DE and shotgun proteomics. Error bars represent the standard error of the mean for duplicate experiments. (B) SDS-PAGE comparison of protein recovery from different precipitation methods following resolubilization for shotgun proteomics.

Figure 2.

Central composite design regression coefficients of coded values (Table 1) for total extracellular CHO HCP recovery following precipitation by (A) TCA with resolubilization for 2-DE (model significance $p = 0.0033$, $R^2 = 0.689$) and shotgun proteomics (model significance $p = 0.0036$, $R^2 = 0.636$), (B) methanol with resolubilization for 2-DE (model significance $p = 0.0296$, $R^2 = 0.868$) and shotgun proteomics (model significance $p < 0.0001$, $R^2 = 0.599$), and (C) acetone with resolubilization for 2-DE (model significance $p = 0.0110$, $R^2 = 0.575$) and shotgun proteomics (model significance $p = 0.0391$, $R^2 = 0.490$). Total protein recovery from central composite design as function of (D) TCA concentration, (E) methanol concentration, and (F) acetone concentration. Error bars represent standard error of the coefficient estimate (A–C) or the spread of the data (D–F). All experiments performed in duplicate with the exception of methanol with resolubilization for 2-DE (single experiment) and shotgun (triplicate experiments) proteomics. Statistical significance determined by t -test and denoted as ** $p < 0.05$ and * $p < 0.1$.

Figure 3.

(A) Concentration recovery as a function of extent of concentration for two filter loadings. Precipitation recovery as a function of initial extracellular CHO HCP concentration following resolubilization for (B) 2-DE, and (C) shotgun proteomics. Overall protein recovery for concentration and precipitation operations, with respect to initial extracellular CHO HCP concentration, following resolubilization for (D) 2-DE, and (E) shotgun proteomics. (F) Precipitation recovery dependence on salt concentration with error bars representing the spread of the data from duplicate (TCA, acetone) or triplicate (methanol) experiments.

Figure 4.

(A) Total number of protein spots and overall spot volume detected by ImageMaster v5.0 software following 2-DE analysis of extracellular CHO HCPs precipitated by four different optimized methods. Representative 2-DE images of extracellular CHO HCPs prepared by (B) TCA and (C) methanol precipitations. Molecular weight (MW) and isoelectric point (pI) labels approximated from the location of seven proteins previously identified by MALDI-TOF/TOF. (D) Total number of peptides and proteins identified from shotgun analysis of extracellular CHO HCPs precipitated by four different optimized methods. Error bars represent standard error of the mean from duplicate experiments.

Figure 5.

Representative 2-DE image of extracellular CHO HCPs prepared by TCA precipitation including identification of proteins from 19 spots. Molecular weight (MW) and isoelectric point (pI) labels approximated from the location of seven proteins previously identified by MALDI-TOF/TOF.

Table 1

Central composite design for precipitation recovery with coded factors and experimental domains

Coded Factors				Experimental Domain			
	Time	Concentration	Time	Precipitant Concentration			
	(x ₁)	(x ₂)	(hrs)	TCA (%)	Methanol ^{a)}	Acetone ^{a)}	
1	Factorial	-1	-1	1	5	1.5	1.5
2	Factorial	-1	1	1	15	6.5	6.5
3	Factorial	1	-1	6	5	1.5	1.5
4	Factorial	1	1	6	15	6.5	6.5
5	Axial	-1.414	0	0	10	4	4
6	Axial	1.414	0	7	10	4	4
7	Axial	0	-1.414	3.5	2.9	0.5	0.5
8	Axial	0	1.414	3.5	17.1	7.5	7.5
9	Center	0	0	3.5	10	4	4
10	Center	0	0	3.5	10	4	4
11	Center	0	0	3.5	10	4	4

^{a)}Organic solvent concentrations reported as organic solvent volume : volume extracellular CHO HCP