Antibody Affinity Extraction™ (AAE™) Empowers HCP Identification by Mass Spectrometry

AAE as an enrichment method to enable detection of low-level HCPs in the presence of drug substance

Summary

Host cell proteins (HCPs) co-purify with biological drug substances (DS) and pose potential risks for both patients and drug manufacturers. Regulatory authorities require that biopharmaceutical companies perform comprehensive assay qualification to demonstrate the suitability of HCP ELISAs prior to proceeding to clinical trials. ELISA is the gold standard method for monitoring HCP levels but it is limited with respect to the information it provides regarding which HCPs are present. Identification and quantification of HCPs by mass spectrometry (MS) is a powerful complementary method to ELISA, however drug substances often mask HCPs. In this study, CHO HCPs were identified by MS before and after antibody affinity extraction in a DS sample. We show that AAE followed by MS is a powerful HCP enrichment method that can identify unknown HCPs and enrich them by as much as 240-fold. ELISA and MS are orthogonal methods that provide data throughout biopharmaceutical drug purification processes to inform manufacturers about how to modify their manufacturing processes and DS purity.

Contents

Introduction

Host cell proteins constitute a major group of process-related impurities of biological drugs produced using cell culture technology. HCPs are produced inadvertently during expression of recombinant biopharmaceuticals as secreted from host cells, from aberrant cellular trafficking due to cell stress, and due to cell lysis over the course of bioprocess manufacturing. More than 70% of biological drug substance are produced using Chinese hamster ovary cells (CHO, 1), but *Escherichia coli*, *Pichia pastoris*, and HEK 293 cells are also common expression platforms. HCPs constitute a major process-related impurity no matter what cell type is used.

HCPs can co-purify with biological DS as “hitch-hiker” proteins due to their affinity to the drug substance and pose patient risk as a process-related impurity. When present in the administered product even at low levels, HCPs can induce an undesired immune response or interfere with drug efficacy. HCPs can potentially impact drug pharmacokinetics, stability, and immunogenicity, and must be adequately removed in the downstream purification process. Documented CHO HCPs include Phospholipase B Like 2, Monocyte Chemoattractant Protein 1, Serpin B6, Plasminogen Activator, HTRA1 Serine Peptidase 1, and TGF beta 1 (1-4). All HCPs that are enzymes, such as *proteases*, *proteinases*, peptidases, *phospholipases*, *cytokines*, and growth factors, can also be problematic. In addition to using publicly available lists (1-4), Cygnus Technologies has applied years of experience with CHO HCP analysis to curate a proprietary database of CHO HCPs to better inform biopharmaceutical manufacturers.

Regulatory authorities have provided recommendations for biopharmaceutical companies on the removal of HCPs in the guidance documents by the International Conference of Harmonization in ICH Q11 in 2012, the FDA in 21CFR610.13 in 2016, and the US government in 42USC262 in 2011. Biopharmaceutical companies are encouraged to report the level of HCPs present in their DS within certain self-defined acceptance criteria, and biopharmaceutical manufacturers should monitor HCPs to demonstrate reproducibility of their purification process to increase their confidence during clinical trials. ELISA is the biopharmaceutical standard method to obtain a certificate of quality analysis, but it is limited in detecting only HCPs that are immunogenic. Orthogonal methods such as MS, AAE, two dimensional SDS-PAGE (2D-PAGE), and two-dimensional differential gel electrophoresis (2D-DIGE) are used to determine total drug substance purity.

MS is emerging as a valuable tool in HCP analytics, and over 1000 individual HCPs can be identified when it is coupled to AAE or two-dimensional liquid chromatography. Due to the advances in the resolution and scanning speed of MS instrumentation, biopharmaceutical companies can monitor which HCPs are enriched or are persisting through a purification process, and this facilitates purification process development and process validation. MS also provides a second level of coverage analysis in addition to 2D-PAGE or 2D-DIGE. MS detects, identifies, and quantifies individual HCPs and is an orthogonal method to ELISA, providing in depth data about specific HCPs. For process purification, ELISA can provide the total host cell protein to DS ratio, while MS supplements these data with the identity and quantity of individual problematic HCPs. This information is needed to determine HCP isoelectric points to guide optimization of chromatography conditions to eliminate targeted HCPs in DS.

Here we demonstrate how AAE empowers MS identification of CHO HCPs in the presence of DS. Total protein and HCP concentrations were determined, and AAE was performed on a DS sample containing CHO HCPs. AAE enriched the CHO HCPs and depleted the DS resulting in as much as 240-fold enrichment of CHO HCPs. The total protein concentration was used to quantify CHO HCPs in ppm relative to DS, and AAE enrichment enabled quantification of CHO HCPs that were undetectable due to the presence of DS. Using the identity of these CHO proteins, their isoelectric points and molecular weight (MW) were calculated.

Materials and Methods

Sample Preparation

A recombinant murine humanized IgG1κ DS was diluted to 2 mg/mL and spiked with 300 µg/mL CHO-K1 cell line HCPs from a clarified cell culture fluid of null-transfected CHO cells.

Quantification of HCPs

Total protein was quantified pre- and post-AAE using an extinction coefficient of 1.4 for IgG and UV absorbance at 280 nm and the Coomassie Plus/Bradford method (Thermo Fisher Scientific) at 595 nm. Total HCPs were quantified pre- and post-AAE with CHO 3G ELISA (Cygnus Technologies) at 450/650 nm. All absorbances were acquired using a SpectraMax* M3 Multi-Mode Microplate Reader (Molecular Devices).

AAE

CHO 3G polyclonal antibodies (Cygnus Technologies) were covalently immobilized on a chromatography support. The column was conditioned to prevent significant leaching of the antibody and to minimize non-specific binding. The HCP containing sample was passed over the column for binding and elution, and fractions were collected using a BioLogic LP chromatography system (Bio-Rad Laboratories). All HCP elution fractions were neutralized, pooled, buffer exchanged, and concentrated.

LC-MS

100 µg of protein was digested with MS-grade trypsin according to manufacturer's protocol (Thermo Fisher Scientific).
1 µg of peptides from digested proteins were injected directly with an in-line 2 µm × 75 µm × 20 mm C18 trap (Thermo Fisher Scientific) and EASY-Spray (Thermo Fisher Scientific) C18 Reversed-Phase LC 2 µm × 75 µm × 250 mm column (in 98:2:0.1 Water:Acetonitrile:Formic Acid using a nano EASY 1200 HPLC into a Q Exactive HF (QE-HF) hybrid quadrupole-Orbitrap MS (Thermo Fisher Scientific). Data were acquired in 60-minute gradients in discovery mode with data independent acquisition with the following settings: IT= 100 ms, AGC=1e6, 120,000 resolution, and a mass range of 400-2000 m/z.

Bioinformatics

Raw mass spectra were searched using the Byos* HCP Workflow (Protein Metrics) against Cygnus Technologies' CHO proteome database. Peptides were identified using a precursor mass tolerance of +/- 40 ppm and a fragment mass tolerance of +/- 70 ppm with a false discovery rate of 0.1%. Data were graphed in GraphPad Prism 8 (GraphPad).

Results

The total protein concentration was determined to be 2.97 mg/mL by UV-Vis at 280 nm, 2.68 mg/mL by Coomassie Plus at 595 nm, and 0.509 mg/mL by Cygnus CHO 3G ELISA at 450/650 nm (Figure 1). AAE was performed on the DS sample and the concentration was determined to be 0.63 mg/mL by UV-Vis at 280 nm, 0.421 mg/mL by Coomassie Plus at 595 nm, and 0.638 mg/mL by Cygnus CHO 3G ELISA at 450/650 nm (Figure 1). These results show that DS was depleted by AAE and the HCP content remained similar before and after AAE. Total identified proteins were identified before and after AAE by MS and data were analyzed using the Byos HCP workflow (Figure 2), and the relative percentage of HCPs and DS according to their extracted ion chromatograms in each sample were both normalized to one.

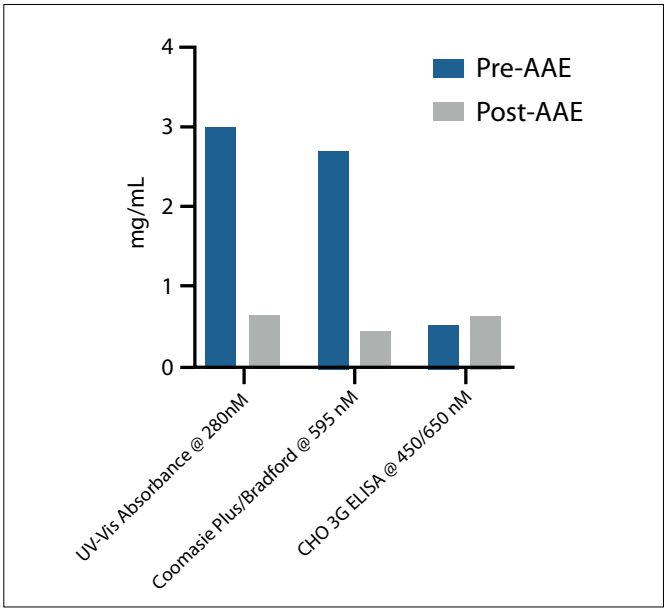

Figure 1. Total Protein and HCP Concentration Pre- and Post-AAE.
The total protein and HCP concentrations in the DS sample were determined of the DS sample Pre- and Post-AAE by UV-Vis at 280 nm, Coomassie Plus/Bradford at 595 nm, and CHO 3G ELISA at 450/650 nm.

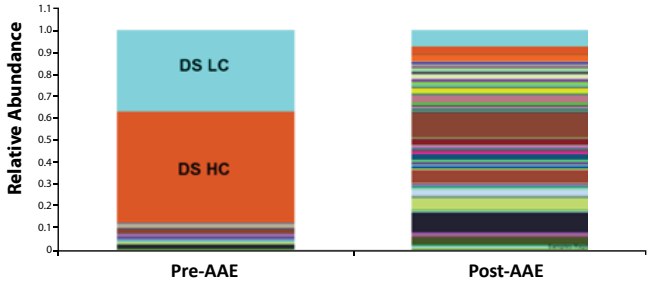

Figure 2. AAE is Highly Effective at Enriching HCPs and Depleting DS.
The relative abundance of DS and HCPs according to their extracted ion chromatograms were normalized to one and graphed in a stacked bar chart. Pre-AAE (left) shows the drug substance heavy chain (DS HC) in orange, the drug substance light chain (DS LC) in light blue and HCPs in assorted colors beneath. Post-AAE (right) shows that the relative abundance of the HCPs have been increased after AAE, and that of the DS HC and LC has decreased.

The sample on the left is the pre-AAE sample which shows the heavy chain in orange and the light chain in light blue of the drug substance. The identified HCPs are beneath the drug substance boxes and represented by various colors. The figure shows that most peptides identified by MS in the sample before AAE belong to the DS at the expense of the HCPs. Following AAE enrichment, however, the relative abundance of the HCPs dramatically increased and that of the DS decreased. Analysis of the top 20 enriched CHO HCPs in the post-AAE sample showed that Annexin A5 was the most enriched CHO HCP (240-fold enrichment), followed by Calcium-dependent Serine Proteinase (65-fold enrichment, Figure 3).

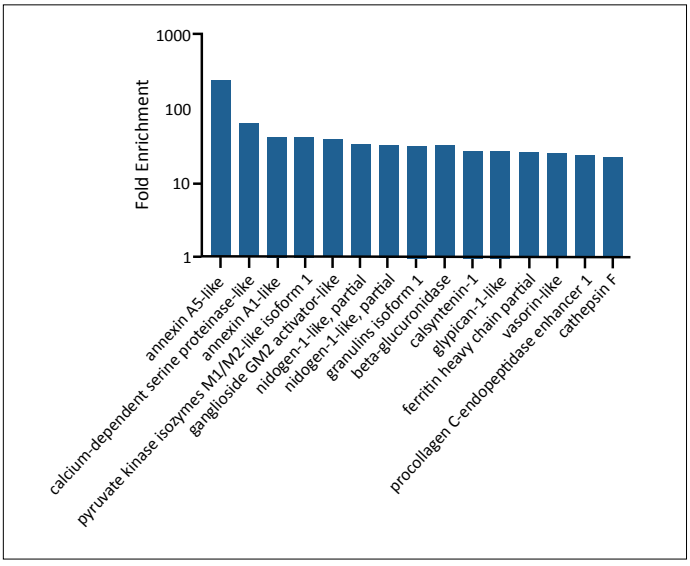

Figure 3. AAE Enrichment of Total CHO HCPs.
The top 20 enriched CHO HCPs of the total HCP profile of the DS Post-AAE show that Annexin A5 is the most enriched CHO HCP by 240-fold, followed by Calcium-dependent Serine Proteinase at 65-fold.

Further analysis of this dataset using Cygnus Technologies' curated database of CHO HCPs showed quantification in ppm of the top 20 potentially problematic CHO HCPs relative to the DS in this upstream process sample (Figure 4). CHO chemokines, cathepsins, proteinases, and peptidases were all present and would need to be removed to minimize potential immunogenicity and increase DS stability and efficacy.

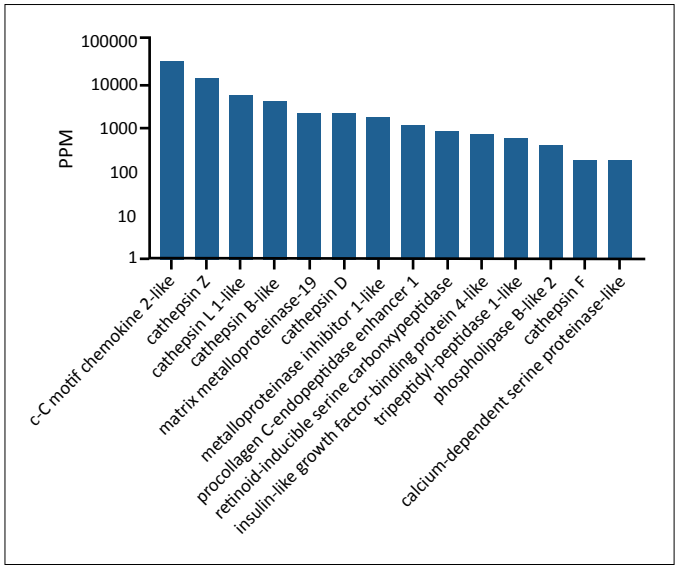

Figure 4. Detection of Potentially Problematic HCPs.
The top 20 potentially problematic CHO HCPs were identified in the Post-AAE DS sample using Cygnus Technologies' CHO HCP curated database. For this sample, the concentration of the DS was known in mg/mL, so the relative concentration of CHO chemokines, cathepsins, proteinases, and peptidases were calculated in parts per million (PPM).

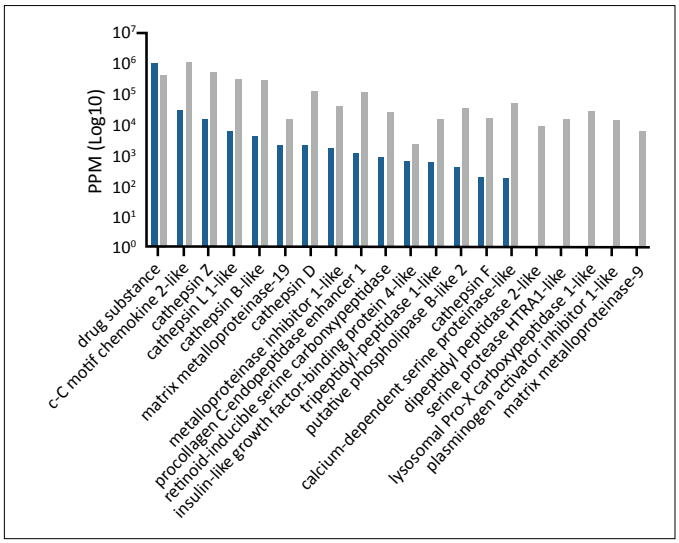

Figure 5. AAE Enriches Known Problematic CHO HCPs.
The DS was analyzed Pre- and Post-AAE by MS, and PPM quantification was calculated relative to the most abundant protein in each sample. For the pre-AAE sample, known problematic CHO HCPs were quantified relative to the DS. For the post-AAE sample, CHO HCPs were quantified relative to the most abundant protein, C-C motif chemokine 2.

The DS, both with and without AAE, was then analyzed by MS (Figure 5), and quantification was performed relative to the most abundant protein present in each sample. for the pre-AAE sample, known problematic CHO HCPs were quantified relative to the DS and for the post-AAE sample, CHO HCPs were quantified relative to C-C motif chemokine 2. The pre- and post-AAE data show that previously undetected, known problematic CHO HCPs such as dipeptidyl peptidase 2, serine protease HTRA1, and plasminogen activator inhibitor 1 were quantified. In addition, there was no stepwise decrease in the amounts of each CHO HCP, implying their abundance had changed following AAE, possibly due to the DS obscuring their detection. Finally, this MS-based identification enabled the calculation of theoretical isoelectric points and molecular weight for unknown protein sequences such as the CHO proteome. After MS identification, the PPM, mg/mL relative to DS, isoelectric point and molecular weight of CHO HCPs were calculated (Table 1).

Table 1.MS Provides PPM, IEP, and MW Data.
The PPM, mg/mL, isoelectric point, and molecular weight (MW) of CHO HCPs are shown relative to the DS. Isoelectric point and MW are predicted theoretical values.

Protein	PPM	mg/mL	IEP	MW
drug substance	1000000	2.9700	8.49	49607
c-C motif chemokine 2-like	29827	0.0886	9.32	15858
cathepsin Z	14646	0.0435	7.52	34028
cathepsin L 1-like	5712	0.0170	5.94	37298
cathepsin B-like	4116	0.0122	5.73	37504
matrix metalloproteinase-19	2179	0.0065	7.71	58942
cathepsin D	2130	0.0063	6.54	44111
metalloproteinase inhibitor 1-like	1665	0.0049	8.84	22401
procollagen C-endopeptidase enhancer 1	1111	0.0033	8.16	50446
retinoid-inducible serine carbonxypeptidase	829	0.0025	5.31	51255
insulin-like growth factor-binding protein 4-like	659	0.0020	6.77	27726
tripeptidyl-peptidase 1-like	572	0.0017	5.94	61450
phospholipase B-like 2	388	0.0012	5.90	65541
cathepsin F	176	0.0005	6.94	51641
calcium-dependent serine proteinase-like	166	0.0005	4.73	77412

Discussion

The AAE technique effectively depleted the DS while enriching CHO HCPs, as demonstrated by UV-Vis, Coomassie, ELISA, and MS quantification methods. CHO HCPs reactive with the CHO 3G polyclonal antibody were enriched from 10- to 240-fold. Without enrichment by AAE, MS alone was not sensitive enough to detect and quantify all 20 known problematic CHO HCPs in a limited dataset. The five proteins that were not detected may have been obscured due to their low abundance and orders of magnitude higher concentration of the DS. MS ion exclusion windows and ion reaction monitoring could be developed to detect the obscured proteins, but AAE overcame the DS saturation of the MS.

These data demonstrate that AAE and MS are complementary, orthogonal methods for the identification of individual HCPs present in a DS. The combination of these two methods aids in the decision of whether an immunoassay is fit for purpose in process monitoring and product lot release. HCPs are more than a check off box on an IND or BLA submission. Knowledge of what HCPs are present can inform purification strategies to reduce their levels in DS.

Conclusion

Enrichment of HCPs by AAE and detection by MS is a powerful orthogonal method to ELISA. ELISA is the gold standard for total quantification of immunoreactive HCPs, but biopharmaceutical companies that integrate MS with ELISA data can provide comprehensive quality control data for regulatory agencies by identifying and quantifying confidently the HCPs present in their bioprocess and DS.

References

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